

# **1 Advanced wastewater treatment for algal removal: literature review and general thesis introduction**

## **1.1 Background**

The importance of water as a global resource for human life is irrefutable. It follows then that the need to manage and protect this resource has been recognised for centuries, such that it is now a conservation priority the world over. Advancements in the efficiency, convenience and sanitation of human society have owed directly to the development and distribution of large-scale dependable supplies of high-quality potable water (Oswald, 1988b). Unfortunately, these same developments have also allowed for the convenient aqueous disposal of objectionable, infectious and toxic wastes away from their points of origin and, most commonly, into the nearest natural body of water (Oswald, 1988b; Shiny *et al.*, 2005). It is this aqueous waste, or ‘wastewater’, and the processes involved with its remediation that form the basis of this thesis.

A prominent threat to global water quality in general is its contamination with human-derived wastes of residential, industrial and commercial origins. This is particularly the case for freshwater resources, where human-derived wastewaters are one of the major sources of contamination and pollution (Craggs *et al.*, 1996). In recent times, a general decline in environmental water quality—a consequence of anthropogenic interactions—has given rise to significant environmental problems and public health concerns (Hoffmann, 1998). These pollution-associated issues have, therefore, justifiably received increasing levels of attention, to the extent that they are nowadays of major concern to modern society (de la Noüe *et al.*, 1992).

Previously, occasional monitoring of final effluent quality from wastewater treatment operations was often all that was required (Hurse and Connor, 2000). More recently, the application and enforcement of environmental laws governing wastewater and its discharge has become increasingly more stringent (Hurse and Connor, 2000) due to heightened public pressure as well as inputs from concerned governing bodies and agencies (Middlebrooks *et al.*, 1974; de la Noüe *et al.*, 1992). This increased regulatory

pressure has served as the historical driving force behind initial changes to wastewater treatment technologies and indeed general waste treatment philosophy (Middlebrooks *et al.*, 1974) and will no doubt continue to drive process and technological advancements into the future, or as long as the pollution-associated problems remain.

Methods for wastewater treatment used earlier last century simply relied on the self-purification mechanisms of natural waterways for the renovation, dispersion and redistribution of low-concentration wastes (Craggs *et al.*, 1996). Whilst these natural mechanisms might have historically provided adequate treatment, current effluent discharge volumes and concentrations now exceed effective treatment thresholds of these natural ecosystems (Harlin and Darley, 1988). This is highlighted in the fact that many conventional treatment plants discharge in excess of  $10^6$  L of wastewater per day, with nutrient levels in this discharged effluent being up to three orders of magnitude more concentrated than in the receiving waters (de la Noüe *et al.*, 1992; Hoffmann, 1998). It is not surprising then that municipal wastewater is recognised as one of the main contributors to freshwater pollution and the subsequent eutrophication of receiving water bodies (Craggs *et al.*, 1996). Among other things, this pollution-induced heightened eutrophic state can lead to a reduction in the natural species diversity of the receiving waterway—destroying the ecosystem's natural heterogeneity and subsequently decreasing its self-purification capacity (Brix and Schierup, 1989). Without proper attenuation of wastewater-borne bioavailable substrates prior to discharge, this 'positive-feedback' cycle could result in the eventual destruction of the very aquatic ecosystems so heavily relied upon for safe disposal.

Current global industries surrounding wastewater and its treatment technologies are both extensive and wide-spread. Not only do these treatment options vary extensively in terms of process and technical design, but also with respect to the associated costs and level of operator expertise necessary for efficient operation. One of the most basic and pioneering wastewater treatment techniques that is now recognised as being a 'staple' treatment alternative is the Waste Stabilisation Pond (WSP). WSPs (sometimes also referred to as oxidation ponds, redox ponds or sewage lagoons) in their simplest form are defined as shallow earthen basins containing wastewater of some description (Metcalf and Eddy, 1991). Historically, WSPs are said to have been employed,

particularly in Asia, for the treatment of wastewater for thousands of years (Uhlmann, 1980); with the first recorded construction of a modern pond system being in the United States, San Antonio, Texas in 1901 (Reed *et al.*, 1988). Ponds were initially used in the US simply as containment basins for preventing wastewaters from entering into unwanted locations (Oswald, 1988a). Prior to 1950, however, this form of treatment was actively discouraged in the US (O'Brien *et al.*, 1973) and it was not until post World War II that WSPs were more thoroughly investigated for their potential role in wastewater treatment (Oswald, 1988a).

## **1.2 Waste Stabilisation Ponds**

WSPs represent an extremely robust, low maintenance, low-energy treatment system well suited for use especially (but not exclusively) in rural areas (Cooke and Matsuura, 1969; Mara *et al.*, 1998). WSPs are inherently associated with user benefits such as: low capital establishment costs (Mitchell, 1980; Polprasert and Bhattarai, 1985); simple management practices (Mezrioui and Oudra, 1998); minimum maintenance and operational inputs (McGarry and Tongkakame, 1971); zero energy requirements (Ellis, 1983; Alexiou and Mara, 2003); and the ability to withstand both organic and hydraulic 'shock-loadings' (Truax and Shindala, 1994; Naméche and Vassel, 1998). Because there are no additional energy requirements for aeration and circulation, and due to their reliance on solar power for biological waste conversion, treatment is simple and inexpensive. Given the current global 'energy climate', and taking into account the political emphasis now placed upon *green* technologies, WSPs may indeed find themselves the subject of renewed interest from carbon-conscious governing agencies; thereby ensuring their technological relevance well into the future.

WSPs exploit natural biological phenomena for the reduction of organic material, removal of dissolved nutrients and metals, and for the attenuation of pathogenic microorganisms in wastewaters (Mitchell, 1980; Pedahzur *et al.*, 1993; Hoffmann, 1998). They are complex and dynamic biotic systems with a recognised and high capacity for 'self-regulation' (Uhlmann, 1980; Hosetti and Frost, 1998). As a result of this functional autonomy, treatment is generally achieved in an efficient, ecologically safe and financially favourable manner (de la Noüe *et al.*, 1992; Hoffmann, 1998).

According to Oswald (1995), WSPs are by far the most cost-effective treatment reactors available for the effective capture of solar energy and treatment of liquid wastes. In fact, periodic desludging of accumulated benthic materials constitutes the single major operational maintenance requirement of the technology.

WSPs can range in size from just a few hundred kilolitres up to several gigalitres, with hydraulic residence times in the order of hours to months (Sweeney, 2004). Their relative operational flexibility means that WSPs can function at the primary treatment stage of a wastewater treatment train or can just as effectively be operated as tertiary-level treatment systems. WSPs are most generally classified according to their loading characteristics and the nature of the biological processes occurring within (Ganapati, 1975; Polprasert and Bhattarai, 1985; Reed *et al.*, 1988). Several major classes of WSP are recognised, each with distinct design and operational parameters, and each serving to perform a discrete treatment function. According to both Ramalho (1988) and Metcalf and Eddy (1991), there are four general ‘classes’ of WSP: anaerobic; aerobic–anaerobic (facultative); aerobic (maturation); and aerated or ‘high-rate’; with facultative ponds being recognised as the most widely used pond type.

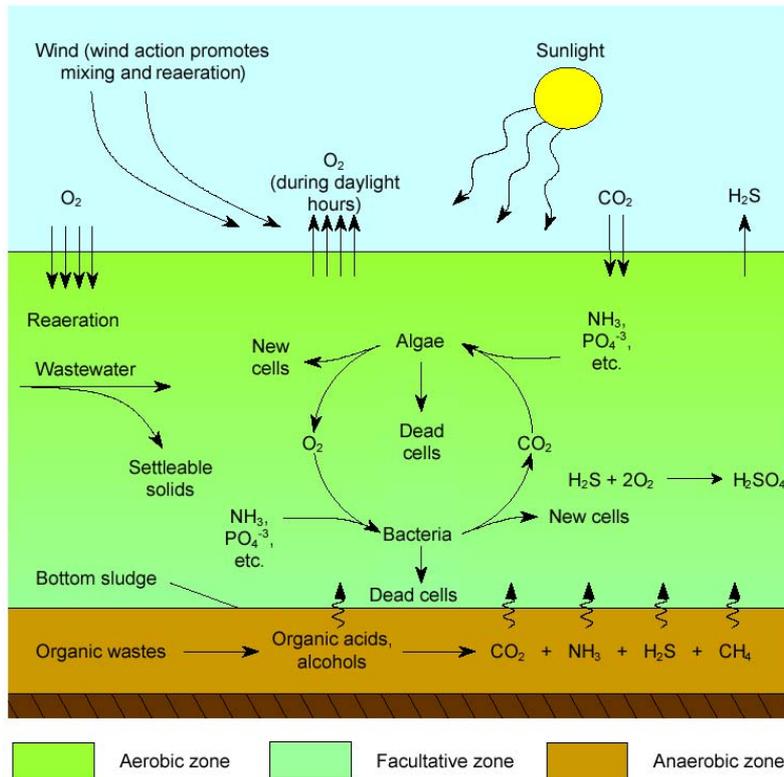
WSPs are commonly arranged and operated in series, with an anaerobic pond preceding a facultative pond which then feeds into one or more maturation ponds (Mara *et al.*, 1992). Because they possess a greater relevance to the current work, a brief discussion of the operational role of both facultative and maturation ponds will be given in Sections 1.2.1 and 1.2.2, followed by a more thorough description of WSP biology—as it relates to wastewater treatment—in later sections.

### **1.2.1 Facultative WSPs**

Nowadays, WSPs are often regarded as the ‘method of choice’ for many wastewater treatment applications around the world; something owing to their more cost-effective, flexible and sometimes more efficient operation (Cauchie *et al.*, 2000a; Mara *et al.*, 2001; Mara, 2004). In the United States for example, there are over 7,000 operational facultative ponds (USEPA, 2002b); with extensive installations also throughout Europe, and in excess of 10,000 in both rural and urban China (Zhao and Wang, 1996). Earthen

WSPs are typically 1.2 to 2.4m deep and are not mechanically mixed or aerated. Individual ponds can vary greatly in size, from small-serving rural installations to an approximate maximum surface area in excess of 100 hectares (Mitchell, 1980). Hydraulic retention times (10–180 days) and organic loading rates ( $50\text{--}400\text{kg BOD}_5 \text{ha}^{-1} \text{d}^{-1}$ ) vary substantially within the literature according to factors such as geographical location, pond geometry and operational configuration, and also the specific nature of the influent wastewater.

WSPs evolved conceptually according to existing and naturally occurring biological self-purification or ‘stabilisation’ processes (Brücker *et al.*, 1998), such that pond operation is viewed simply an intensification of these natural treatment processes within a self-contained reactor vessel (Bartsch, 1961; Tschörtner, 1968; Pearson, 1990; Tharavathi and Hosetti, 2003). Natural biological treatment phenomena within these WSP systems include complex interactions between heterotrophic microbes, algae, protists and metazoans (Cauchie *et al.*, 2000b); with these complex and highly productive trophic interactions leading to an accelerated biological stabilisation of the inflowing wastewater. Since the pioneering work of Ludwig *et al.* (1951) and Oswald *et al.* (1953a; 1953b), it has been recognised that the core treatment processes within a WSP are centered around inherent biological interactions—specifically those between heterotrophic microbes and algae. This functional synergism between photosynthetic algal oxygenation and aerobic microbial oxidation is the driving force behind effective WSP operation, and is depicted in Figure 1.1 below. This cyclic ‘algal–bacterial’ mutualism is necessarily relevant to the current work and will be discussed in more detail within Section 1.2.3.



**Figure 1.1.** Schematic representation of daytime WSP operation (Metcalf and Eddy, 1991).

Facultative WSPs generally have discernible layers through the water column depth. The lower anoxic pond layer contains sludge deposits and supports anaerobic microorganisms. The intermediate layer, termed the *facultative zone*, ranges from anaerobic at the bottom to aerobic toward the upper region. Finally, the remaining section of the water column at the pond surface contains heightened levels of dissolved oxygen (DO) and is termed the *aerobic zone*. Oxygenation of the water column is provided to a limited extent by surface re-aeration (including wave action), with the vast majority evolving from oxygenic algal photosynthesis (Maynard *et al.*, 1999). Such is the importance of algal re-aeration in the overall oxygen budget of a WSP, that DO concentration in un-aerated ponds varies almost directly according to the level of photosynthetic activity within the pond (Reed *et al.*, 1988). This biological re-aeration is an integral part of facultative pond operation, with the presence of algae in the aerobic and facultative zones considered as no less than essential to the successful performance of a WSP (USEPA, 2002a). During daylight hours, and with favourable conditions, DO concentration in the aerobic zone can exceed saturation point. Conversely, large quantities of respiring microbial and algal biomass can deplete oxygen supplies at night

and, in bloom situations, the very heavy respiratory oxygen demand of resident biota can be such that the pond may become completely anoxic soon after sunset (Ellis, 1983).

### **1.2.2 Aerobic ‘maturation’ WSPs**

Aerobic WSPs (also known as maturation or polishing ponds) are primarily used for the treatment of soluble organic wastes and for final ‘polishing’ of effluents from up-stream facultative ponds or other secondary wastewater treatment processes (Polprasert and Bhattarai, 1985). They are operated at relatively shallow depths (1–1.5m) in order to facilitate maximal sunlight and UV penetration, and as a result, they can often remain aerobic throughout the entire pond depth during daylight hours (Hartley and Weiss, 1970; Pearson, 1990). This tertiary-level final polishing stage is primarily concerned with pathogen removal and, to a lesser degree, the sequestration of any remaining dissolved nutrients (Maynard *et al.*, 1999). Effective pathogen removal is thought to result from a combination of factors, namely: hydraulic retention time; wind action and the subsequent sedimentation rate; UV disinfection; microbiological attack (lytic bacteria and phage); grazing and predation (protozoan and metazoan); nutrient limitation and competition; algal population structure; photosynthetically-elevated pH; humic substances together with high DO leading to photo-oxidation; and also from elevated temperature (Pretorius, 1962; Oswald, 1973; Moeller and Calkins, 1980; Lijklema *et al.*, 1987; Pearson *et al.*, 1987d; Sarikaya and Saatçi, 1987; Curtis *et al.*, 1992; Patil *et al.*, 1993; Ceballos *et al.*, 1995; Soler *et al.*, 1995; Davies-Colley *et al.*, 1999; Maynard *et al.*, 1999; Brissaud *et al.*, 2003); although the relative contributions of each factor toward overall pathogen die-off remains the subject of continued debate. Because maturation ponds are a tertiary-level intervention, organic loadings are commonly low. As a result of this reduced eutrophic state and increased photic depth, highly diverse populations of plankton (both algal and zooplankton) can often develop in high densities (Pearson *et al.*, 1987c; Pearson, 1990); with these organisms contributing significantly to final maturation pond effluent biochemical oxygen demand (BOD<sub>5</sub>) and suspended solids (SS).

### **1.2.2.1 WSP technology and treatment performance**

WSPs are undeniably a more simplistic treatment technology in the face of other more recently conceived and more sophisticated treatment processes, yet in spite of their relatively primitive nature, they have remained a popular and widely adopted wastewater treatment technology. Their modest requirements for establishment and operational inputs, along with their high efficiency for pathogen removal (Ceballos *et al.*, 1995), have ensured that WSPs remain an attractive treatment alternative for both developing countries and smaller developed communities (Cooke and Matsuura, 1969; Mitchell, 1980) where land is cheap and more sophisticated wastewater treatment systems may not be a viable option. WSP systems are, however, not restricted in application simply to lesser-developed regions; with Kilani and Ogunrombi (1984) revealing that even the most developed nations were resorting to the use of WSPs wherever feasible—a trend still observed today (Mara, 2004; Mara, 2006). This is especially the case with respect to the local situation, where in South Australia, for example, there exists over 425ha of WSPs treating domestic wastewater from a population of in excess of 820,000 people (Mitchell, 1980; Palmer *et al.*, 1999; Buisine and Oemcke, 2003).

WSP performance efficiency is measured according to a number of parameters. Along with the traditional core water quality parameters such as the relative oxygen requirement of the effluent (measured as BOD<sub>5</sub>) and SS, water quality analyses commonly involve quantification of nutrients (nitrogen (N) and phosphorous (P) species), metals and indicator microorganisms (e.g. faecal coliforms; FC). Generally speaking, average BOD<sub>5</sub> removal is reported to be in the range of 60–90%, with Ellis (1983) suggesting that 98–99% removals are often achievable. This yields a BOD<sub>5</sub> treatment efficiency for WSPs that encompasses a range of other alternative treatment processes, such as trickling filters (70–75%) and activated sludge (80–90%; Kilani and Ogunrombi, 1984), whilst at the same time having a very low or zero-energy requirement (Ellis, 1983; Pearson, 1996; Alexiou and Mara, 2003). WSP treatment not only ensures that costly process and/or chemical additions are not required, but also produces a relatively ‘chemical-free’ and therefore useable sludge by-product. For example, a common tertiary treatment process for the removal of phosphorous can lead to increased levels of aluminium in the final sludge—creating problems for safe sludge disposal (Hoffmann, 1998).



Effective SS removal is achieved during WSP treatment through physical sedimentation of suspended particulates and subsequent anaerobic digestion (for organic solids) or sludge accumulation (for colloidal or refractory solids). Performance figures for SS removal are highly variable within the literature, and depend greatly on site-specific factors such as organic and hydraulic loading regime, pond size and depth, and geographical location. Generally speaking, SS removals are normally to a level that complies with regulatory discharge limits for WSPs (Truax and Shindala, 1994). This effective physical solids removal also results directly in efficient BOD<sub>5</sub> removal, due to the large proportion of total domestic wastewater BOD<sub>5</sub> being particulate in nature (40–60%; Alexiou and Mara, 2003). Indicator organism and pathogen removals are also high in WSP systems, with up to 4–6-log<sub>10</sub> unit removals for FC, 3–4 log<sub>10</sub> unit removals for faecal viruses, and 100% removals possible for protozoan cysts and helminth eggs (Mara *et al.*, 1992).

Alongside their evident advantages, there are some operational requirements and drawbacks associated with this technology. WSPs are only feasible where large land areas are available at a low cost, and where a reliably high-quality final effluent is not required (Ramalho, 1983). This sometimes ‘compromised’ final effluent quality is an inherent feature of WSPs in many instances, and one that is directly owing to the nature of the treatment process itself. Specific issues relating to WSP effluent quality and the further upgrading of final pond effluents will be discussed in the coming sections.

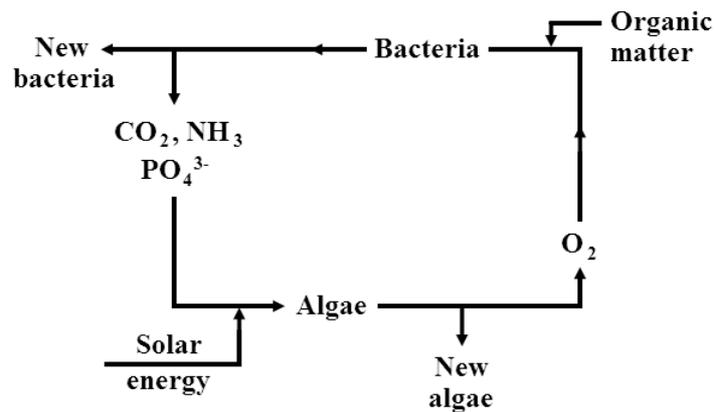
### **1.2.3 Heterotrophic microbes and algae—the backbone of effective WSP treatment**

Algae are recognised to play a central role in the natural self-purification of contaminated waters (Oswald, 1988a; Mezrioui and Oudra, 1998; Schumacher and Sekoulov, 2002). This capacity for natural algal treatment is, however, not without biological association. Early pioneering work (Ludwig *et al.*, 1951; Oswald *et al.*, 1953a; Oswald *et al.*, 1953b; Ganapati, 1975) has established and defined the interrelationship or mutualistic ‘symbiosis’ between algae and bacteria in WSPs. The interactions between microbes and algae in many aquatic ecosystems have since been widely researched; with this natural ‘co-occurrence’ ranging from bacterial endosymbiosis in some algae, to synergistic co-metabolism, right through to

competitive, antagonistic and even inhibitory phenomena (Mitchell, 1980; Cole, 1982). However loose or tight these associations, the co-existence of these particular microorganisms is synonymous with aquatic environments.

Numerically, bacteria and algae are by far the most dominant organisms amongst the planktonic biota of lakes and oceans; with their combined metabolism largely controlling pelagic energy flow and nutrient cycling in aquatic environments (Cole, 1982; Prézelin *et al.*, 1991; Falkowski, 1994). The treatment of more concentrated wastes in a WSP relies upon these same basic microbiological processes to achieve treatment—albeit at a somewhat intensified rate. In essence, a WSP can be considered simply as a reactor in which waste concentrations are intensified, leading to an accelerated rate of ‘naturally occurring’ treatment and purification processes (Tharavathi and Hosetti, 2003). At the centre of this natural biological stabilisation process is the cyclic synergistic relationship between algae and heterotrophic bacteria (Oswald *et al.*, 1953b; Patil *et al.*, 1975; Ramalho, 1983). It should be noted also that certain fungi are recognised to play a quantifiable role in this algal–microbial stabilisation of organic wastes within WSPs (Cooke and Matsuura, 1969) such that the metabolic aspects of fungi, bacteria and algae are seen as interrelated (Patil *et al.*, 1993).

The role of algae in the treatment of wastewater has been investigated and discussed within the literature since the early 1950’s (Ludwig *et al.*, 1951; Oswald *et al.*, 1953a; Bartsch and Allum, 1957), with the biochemistry of waste treatment now firmly established. The central algal–bacterial interrelationship is represented schematically in Figure 1.2, and a concise process overview—as described by Mitchell (1980) and Mezrioui and Oudra (1998)—is given below.



**Figure 1.2.** Cyclic ‘symbiosis’ between algae and bacteria within a WSP environment (Ramalho, 1983).

Heterotrophic microbial mineralisation of inflowing organic materials produces stable, oxidised, inorganic by-products such as carbon dioxide ( $\text{CO}_2$ ), ammonia-nitrogen ( $\text{NH}_3\text{-N}$ ), phosphates ( $\text{PO}_4^{3-}\text{-P}$ ) and essential vitamins. These synthesised products of bacterial metabolism are then utilised by autotrophic algae for their own development and growth via photosynthesis. Splitting of water molecules during the course of algal photosynthesis (Ganapati, 1975) in turn supplies aerobic microbes with the necessary oxygen for the oxidative decomposition of wastewater organics (Pearson *et al.*, 1987c; de la Noüe *et al.*, 1992) and so the process is allowed to continue in this ‘positive-feedback’ cycle. The aquatic chemistry depicted in Figure 1.2 also accounts for the diurnal shifts in both dissolved oxygen concentration (photosynthesis and respiration) and pH (carbonate–bicarbonate equilibrium) commonly observed within WSPs. Of the incoming organic carbon, approximately one third is oxidised to carbon dioxide (which is then available for algal uptake) while the remaining two thirds is assimilated into new bacterial biomass (McKinney, 1976).

Given the importance of this synergistic relationship, it follows that the interactions between algae and bacteria, and their subsequent role in the stabilisation of organic material, have been by far the most thoroughly investigated aspect of WSP biology (Oswald *et al.*, 1953a; Oswald *et al.*, 1953b; Bartsch and Allum, 1957; Wiedeman, 1965; Ganapati, 1975; Pearson *et al.*, 1987c; Oswald, 1988a; Oswald, 1988b; Hoffmann, 1998). Such is the recognised importance of this relationship, that WSPs are sometimes described simply as reactors for housing algal–bacteria ‘symbioses’ (Ganapati, 1975; El

Ouarghi *et al.*, 2002). Although this synergistic relationship is strictly speaking not *symbiotic* in the true microbiological sense, and whilst this may indeed be seen as a somewhat over-simplified description of ‘overall’ treatment function, WSPs do indeed rely extensively upon this interactive relationship in order to drive the biological stabilisation process.

Aside from their paramount role in driving the bacterial oxidation of organic materials, algae can directly influence treatment processes in other ways, including: the sequestration of heavy metals (Duggan *et al.*, 1992); direct assimilation of organic matter and nutrients (Pearson *et al.*, 1987c; Reed *et al.*, 1988; Banat *et al.*, 1990; Mezrioui and Oudra, 1998); provision of a useful protein source for other pond biota (Patil *et al.*, 1993); and beneficial bio-concentration of xenobiotics (de la Noüe *et al.*, 1992). Additionally, algae also play a definable role in the promotion of pathogen die-off (see Section 1.2.1 for more information). Photosynthetically-elevated pH in WSPs during daylight hours occurs as a result of algal scavenging of dissolved CO<sub>2</sub> and subsequent shifts in the carbonate–bicarbonate equilibrium (Boyd, 1990), resulting in aqueous pH exceeding optimum values for enteric indicator organisms such as faecal coliforms (7–8), streptococci (6–9; Sinton *et al.*, 1993; Naméche *et al.*, 2000) and presumably also that of most pathogenic bacteria (Green *et al.*, 1996). Extreme algal-derived diurnal fluctuations in pond pH can therefore have a disinfecting effect on resident pathogenic microorganisms (Ramalho, 1983; de la Noüe *et al.*, 1992; El Ouarghi *et al.*, 2002) thereby enhancing their die-off. Algae can also play a direct role in WSP disinfection via an increase in water temperatures caused by the conversion of light energy to heat during photosynthesis (Banat *et al.*, 1990).

#### **1.2.4 Waste stabilisation: a state change from liquid to solids**

The effective ‘stabilisation’ of unstable, readily bio-available organic and inorganic waste substrates is the primary treatment outcome of most wastewater treatment methods. This biological stabilisation process converts a large percentage of the incoming wastewater substrates into living biomass—said to be in the order of 50% for aerobic systems (Hobson and Wheathey, 1993). Whilst a reasonable portion of the incoming wastes are removed through ‘physical’ processes (although these physical

mechanisms have predominantly biological end-points), the remaining soluble fraction relies on aerobic oxidation by heterotrophic microbes. The carbon dioxide produced during this process is then either lost to the atmosphere, or is re-incorporated into new algal biomass, thereby creating an effective shift from wastewater BOD into algal and bacterial BOD.

The conversion of burdensome waste-derived substrates into algal biomass not only has immediate benefits for safe waste disposal, but the effective mass cultivation of algae within WSPs can significantly contribute to the management of down-stream effluent-receiving aquatic ecosystems. Algal-based systems offer a more environmentally sound approach toward reducing the eutrophication potential of point source wastewater inputs than is achieved by alternate modern-day treatment practices (Hoffmann, 1998). Whilst this conversion of labile nutrients into a more stable living biomass embodies the fundamental treatment process within a WSP, it does create significant quantities of new—particularly algal—biomass, which can then be problematic in terms of its presence in final pond effluents. Whilst this algal biomass is absolutely essential for effective in-pond treatment, its removal from final WSP effluent prior to discharge constitutes a significant and serious problem for pond operators and represents the single major drawback of the treatment technology. It is a problem that has received much prior research interest and hence forms the topic of discussion for the following section.

### **1.2.5 Algae and WSPs: a ‘love–hate’ relationship**

WSP performance is all but dictated by localised conditions such as wind velocity, light intensity, temperature and also influent wastewater quality (Uhlmann, 1980; Hine, 1988; Tharavathi and Hosetti, 2003). The resulting stochastic short-term variations in WSP hydrodynamic conditions, as well as the fact that bio- and ecological processes are in a near-permanent transient state (Uhlmann, 1980), often leads to highly variable and often poor effluent water quality—especially with respect to algal populations. Although the necessity of algae for effective WSP operation is well documented and widely accepted, their presence in these systems is not without complication. The primary disadvantage of algae in the WSP treatment process is their residual presence in final pond effluents, where occasionally large quantities ( $40\text{--}100\text{mg SS L}^{-1}$ ) of algal biomass in final pond

effluents have been reported (Swanson and Williamson, 1980; Reed *et al.*, 1988; Middlebrooks, 1995). Whilst algal populations are desirable, if not essential, for adequate oxygenation of the pond environment, their presence in final WSP effluents represents one of the most serious performance problems associated with the technology (Dinges, 1978; Reed *et al.*, 1988).

It is well established that the major reason behind many WSP systems failing to meet discharge water quality guidelines is the presence of large concentrations of algal-derived SS and BOD<sub>5</sub> in their effluent (Stutz-McDonald and Williamson, 1979; Harrelson and Cravens, 1982). Algal biomass contributions to these water quality parameters vary considerably with operational parameters as well local climate and season, but can be high. For example, algal contributions to WSP effluent BOD<sub>5</sub> can be anywhere between 50–90% of the total figure, especially during summer (Harrelson and Cravens, 1982; Mara *et al.*, 1992; Ceballos *et al.*, 1995; Schumacher and Sekoulov, 2002; Kayombo *et al.*, 2006). In addition, ponds yielding acceptably low SS content in effluents (i.e.  $\leq 30\text{mg L}^{-1}$ ) can have spikes in excess of  $100\text{--}200\text{mg L}^{-1}$  as a result of unpredictable algal blooms (Ellis, 1983). Final effluent SS can also commonly exceed that of the influent (Mitchell, 1980; Hine, 1988), with total effluent SS reportedly able to exceed 250% that of the influent during peak algal productivity (Bartsch and Allum, 1957). It follows that the adequate recovery of high concentrations of algal biomass from WSP effluents is a significant problem, and one that is further complicated by the small size (3–30 $\mu\text{m}$ ) of algal species common to WSP environments (i.e. *Chlorella*, *Euglena*, *Chlamydomonas* and *Scenedesmus*). Only through the removal of this algal biomass prior to discharge can pond effluent quality be sufficiently improved such that WSPs can be incorporated into viable low-cost wastewater treatment systems (Friedman *et al.*, 1977). These issues relating to unwanted algal biomass in WSP effluents and its subsequent removal, form the basis for this thesis and will be discussed in more detail within later sections.

As discussed above, algae can contribute significantly to effluent SS and BOD<sub>5</sub>. Discharged algal biomass may lead to oxygen depletion and the production of anaerobic gases (especially in slow moving waterways) or can settle to the bottom, creating a blanket of anaerobic sludge that smothers native benthic biota (Hirse Korn, 1974). Early

research has shown that approximately 1–1.2mg of O<sub>2</sub> is required for the oxidative destruction of 1mg of algal biomass SS (Friedman *et al.*, 1977; Harrelson and Cravens, 1982); this is in addition to any respiratory oxygen demand of living cells. This algal ‘carryover’ from pond effluents ultimately imposes a significant and undesirable oxygen demand on receiving water bodies and can lead to the promotion of anoxic conditions (Friedman *et al.*, 1977; Metcalf and Eddy, 1991). Algae can also play an indirect role in heightening the eutrophication potential of the effluent by representing a large and labile ‘store’ of nitrogen and phosphorous, available for release upon discharge through algal cell death, cell lysis and subsequent microbial degradation (Mitchell, 1980; Sutherland, 1981; Cosser, 1982).

Conversely, the viewpoint of Kryutčhkova (1968) was that any conversion of unstable organics into stable living biomass—no matter what the scale—should not be considered as ‘pollution’ *per se* (or perhaps a less noxious form in the very least). This viewpoint was echoed later by Mara (1996) through the suggestion that any algae present in WSP effluents may be rapidly consumed by zooplankton populations in the receiving waters; a notion supported by the earlier work of Bain *et al.* (1970, cited in Cosser, 1982). This concept has actually been recognised by some international environmental agencies (e.g. the USEPA and the Council of European Communities) in that algal SS, and hence algal BOD<sub>5</sub>, are different in nature to organic wastewater solids and BOD<sub>5</sub> and should therefore be considered as being less environmentally damaging (Pearson, 1996). Furthermore, Mara (1996), following on from the earlier hypotheses of Bartsch (1961) and later of Cosser (1982), suggested that algal populations might actually continue to produce oxygen in receiving environments during daylight hours, and will therefore have little chance to exert their BOD<sub>5</sub> on the receiving watercourse; although it is now the general consensus that algae will not survive for any great period in receiving waterways (Bain *et al.*, 1970; Sutherland, 1981; Cosser, 1982; Mitchell and Williams, 1982a; Hickey *et al.*, 1989). Some authors have even suggested that the discharge of highly nutritious algal-laden effluent might actually be beneficial in terms of enhancing the biological productivity of nutrient-limited ‘oligotrophic’ waterways (e.g. Mara *et al.*, 1992); although this would no doubt only be seen as beneficial under exceptional circumstances.

In addition to their adverse effects on receiving waters, large quantities of algae can impose serious constraints on the reuse potential of WSP effluents, representing a particular concern for water-scarce regions (Saidam *et al.*, 1995). For example, large amounts of suspended particulates and algal cells in pond effluents destined for agricultural reuse applications can impose significant problems for irrigation infrastructure networks; particularly low-flow drip-irrigation systems, where physical blockages can result (Teltsch *et al.*, 1992; Saidam *et al.*, 1995; Taylor *et al.*, 1995; Ravina *et al.*, 1997; Zimmo *et al.*, 2002). High levels of algal SS in WSP effluents also have the potential to adversely heighten the treatment load placed on post-pond quaternary-level processes, resulting not only in compromised process performance, but also in increased process costs. This situation has again been realised at the local Bolivar Wastewater Treatment Plant (WWTP) and will be referenced in the coming sections.

### **1.2.6 WSP effluent compliance—a complex problem for a simple technology**

In 1972, the US Clean Water Act passed to provide both funding for new wastewater treatment methods as well as setting effluent discharge and primary water treatment standards. In 1977, amendments to the Act stipulated a requirement for minimum monitoring of effluent BOD<sub>5</sub> and SS prior to all municipal wastewater discharge. Generally speaking, and according to the 1977 amendments to the Act, WSP effluents are considered to effectively comply with secondary treatment requirements for discharge into effluent-limited water bodies (Truax and Shindala, 1994). In the case of water quality limited waterways, however, effluent discharge standards are inevitably more stringent, such that WSP treatment alone is inadequate. This directly affects the vast majority of smaller communities, where pond effluents are often discharged into streams with very small or intermittent flows and strict water quality standards are in place (Truax and Shindala, 1994). With respect to the local situation, South Australian marine discharge guideline values for BOD<sub>5</sub> and SS are somewhat more stringent than the classical '20/30' standard (see Table 1.1) and whilst these discharge limits are not actively enforced, the issue of effluent quality compliance is one that has particular relevance to local WSP operators.



The above algal-associated effluent problems, along with growing public awareness of pollution-associated issues (de la Noüe *et al.*, 1992) and both the introduction and more rigorous enforcement of discharge water quality guidelines (Stutz-McDonald and Williamson, 1979; Gonçalves and de Oliveira, 1996; Hurse and Connor, 2000), has meant that once effective WSP treatment systems now require upgrading in order to comply with more stringent effluent quality requirements (see Table 1.1). In order to satisfy discharge water quality limits, there are two obvious alternatives: (1) replace the WSP system with more expensive and complex treatment processes; or (2) upgrade existing ponds such that they are capable of delivering a suitable quality effluent.

**Table 1.1.** Regional WSP effluent quality upper limits for discharge with respect to BOD<sub>5</sub> and SS. Data sourced from Meiring and Oellermann (1995), Mara (1996), and SAEPA (2003).

<b>Geographical region</b>	<b>Effluent BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Effluent SS (mg L<sup>-1</sup>)</b>
UK (1912)*	20 <sup>†</sup>	30
Kenya (1970)	50 <sup>‡</sup>	n.s.
France (1980)	40 <sup>‡</sup>	< 120
European Union (1991)	25 <sup>‡</sup>	n.s.
South Africa (1995)	n.s.	< 25
South Australia (2003)	10 <sup>†§</sup>	10 <sup>§</sup>

\* Most commonly adopted 'generic' standard

<sup>†</sup> Based on raw, unfiltered effluent BOD<sub>5</sub>

<sup>‡</sup> Based on non-algal, filtered effluent BOD<sub>5</sub>

<sup>§</sup> Guideline values for marine discharge only; limits not enforced by regulatory bodies

n.s. Not specified

Given that WSPs are most commonly the treatment method of choice for smaller or rural municipalities (USEPA, 2002b), replacing them with other more involved and more costly treatment procedures can place a large economic burden on many of these smaller communities in which they are installed (Hirse Korn, 1974; Truax and Shindala, 1994). At the same time, one can appreciate that it would effectively nullify the inherent advantages of existing WSP systems if they were to be upgraded with more intensive, higher-cost, higher-maintenance treatment process add-ons. This scenario has particular local relevance to South Australia, where there are currently in excess of 180 small-scale decentralised WSPs (so-called Community Waste Management (CWM) schemes) treating wastewater from a regional population in excess of 120,000 people. The solution then, is to upgrade these WSPs with affordable and operationally manageable

processes capable of satisfying more stringent effluent quality requirements, whilst at the same time retaining the underlying advantages of the original installation. This viewpoint has previously been echoed by several authors in the field (O'Brien *et al.*, 1973; Middlebrooks *et al.*, 1974; Gloyna and Tischler, 1980; Swanson and Williamson, 1980; Ellis, 1983; Bonomo *et al.*, 1997; Neder *et al.*, 2002).

### **1.2.7 The upgrading of WSP effluents**

The need for removal of algal solids from WSP effluent prior to final disposal has been recognised now for some 40 years (Golueke and Oswald, 1965; Van Vuuren and Van Duuren, 1965). As highlighted in previous sections, the effective removal of algal-derived SS and associated BOD<sub>5</sub> from final WSP effluents poses a significant logistical and financial problem for pond operators. Effluent upgrade methods and costs associated with algal removal assume a position of great importance, particularly when excessively high concentrations of planktonic algae must be removed from WSP effluent prior to discharge (Golueke and Oswald, 1965). It is no surprise then, that the volume and variety of research investigating how best to improve the quality of WSP effluents has been significant (McGarry and Tongkakame, 1971; Folkman and Wachs, 1973; O'Brien *et al.*, 1973; Middlebrooks *et al.*, 1974; Friedman *et al.*, 1977; Harrelson and Cravens, 1982; Ayoub *et al.*, 1986; Truax and Shindala, 1994; Yahi *et al.*, 1994; Middlebrooks, 1995; Saidam *et al.*, 1995; Elmaleh *et al.*, 1996; Pearson, 1996; Gonçalves and de Oliveira, 1996; Kim *et al.*, 2001; Alfafara *et al.*, 2002; Hansen *et al.*, 2004). The problem with removing algal cells from pond effluents relates to a combination of their often small size (3–30 $\mu\text{m}$ ), low sinking velocity (in the order of 0.1–0.3m day<sup>-1</sup> for common WSP species; Stutz-McDonald and Williamson, 1979) and low specific gravity—factors that prevent the adoption of routine settling or sedimentation procedures (Golueke and Oswald, 1965). This extreme process difficulty regarding ‘solids–liquids’ separation, means that most processes are either ineffective or require huge amounts of energy (Oswald, 1978).

Given the scale and complexity of the problem, considerable research effort has gone toward developing and testing a number of techniques for removing algal biomass from WSP effluents. These upgrade technologies are in the form of both *in-pond* and *out-of-*

*pond* treatment processes, with some of the reported techniques including: dissolved air flotation/filtration; coagulation–flocculation; intermittent sand filtration; microstraining; centrifugation; autoflocculation; aquatic macrophytes (both floating and emergent); and rock filtration (Middlebrooks *et al.*, 1974; Friedman *et al.*, 1977; Stutz-McDonald and Williamson, 1979; Harrelson and Cravens, 1982; Truax and Shindala, 1994; Middlebrooks, 1995). These upgrade technologies are all associated with varying costs and degrees of success, with some of the more commonly adopted techniques discussed in more detail below.

### **1.2.8 Advanced techniques for upgrading WSPs**

Firstly, the term *advanced*—introduced to the field by Oswald and co-workers in the early 1990’s (Oswald, 1991)—is used to describe these WSP upgrade techniques because the pond design now incorporates some ‘advancement’ over and above that of a conventional WSP (Green *et al.*, 1996). An early review by Middlebrooks *et al.* (1974) on the topic of advanced wastewater treatment upgrades for the removal of algae from WSP effluents, suggested that this objective may be accomplished by many methods and that each technique, under certain conditions, may prove to be sufficiently economical and operationally viable. Middlebrooks (1995, p. 368) in a more recent review of WSP upgrade technologies also stated that of the above-listed techniques, coagulation–flocculation, dissolved air flotation/filtration (DAF/F), centrifugation and autoflocculation were “used infrequently” although their potentials were alluded to.

Centrifugation, whilst it has yielded promising performance data with regard to algal removal, has been largely found to be energy-expensive, prohibitively sophisticated and requiring considerable operator skill and time, hence it is deemed impractical for small-scale upgrade applications (Middlebrooks *et al.*, 1974; Truax and Shindala, 1994). Coagulation–flocculation and DAF/F, whilst they too have delivered excellent results for the upgrading of WSP effluents, they have been both operationally challenging and capital expensive due to advanced engineering requirements and also the necessity for chemical flocculants (Harrelson and Cravens, 1982). In addition to these issues, techniques such as centrifugation and coagulation–flocculation have been shown to concentrate pathogenic wastewater-borne microbes and viruses (Cooper, 1962); creating

additional complications with respect to processing of the collected algal biomass. Procedures such as DAF/F also create alum-contaminated process by-products (sludge) which are likely to cause additional operational problems for smaller communities in terms of safe disposal (Middlebrooks *et al.*, 1974; Truax and Shindala, 1994) and further increase overall process costs.

Autoflocculation describes the process whereby a spontaneous flocculation and precipitation of algal cells (with other algal cells as well as organic and colloidal materials) occurs at a highly elevated pH and also in the presence of divalent cations such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  (Ives, 1959; Middlebrooks *et al.*, 1974; Ayoub *et al.*, 1986; Elmaleh *et al.*, 1996; García *et al.*, 2000). This process has also been suggested as a potential mechanism for the harvesting of algal biomass in high-rate ponds (Hoffman, 1998). Despite its future potential, the actual mechanisms involved in autoflocculation and conditions surrounding its occurrence are both poorly understood and difficult to control in full-scale outdoor operations, resulting in significant fluctuations in process efficiency and subsequent effluent quality (USEPA, 1983; Nurdogan and Oswald, 1996; Hoffmann, 1998; García *et al.*, 2000). These factors, therefore, make it unlikely as a viable option for the upgrading of WSP effluents in the short-term.

Microstraining has demonstrated some success for algal removal (Harrelson and Cravens, 1982; Reed *et al.*, 1988), but with high capital establishment and ongoing operational costs, as well as skilled operator requirements, it has largely been viewed as having “little if any economic application to upgrading lagoon effluents (Truax and Shindala, 1994)”. Intermittent sand filtration, with proper design and operation, has been demonstrated to be an effective and economical process for the upgrading of WSP effluents to meet discharge requirements (USEPA, 1983; Truax and Shindala, 1994; Middlebrooks, 1995). Being an *out-of-pond* technique, as are many of the abovementioned technologies, sand filtration has the additional requirement of regular operational management, maintenance and skilled supervision, as well as the inherent possibility of pumping costs associated with moving effluent wastewater from the pond, to (and also through) the sand filters.

It should also be noted that some of the above out-of-pond technologies for algal removal may have the additional capacity to recover valuable algal biomass as a potential means of off-setting part of the higher associated capital and maintenance costs. This harvested algal biomass may offer economic returns from its potential use as a dietary protein and vitamin supplement in animal feeds, as well as from inorganic nutrient recovery and biofuel applications (Cooper, 1962; Ellis, 1983; Oswald, 1995; Pearson, 1996; Scragg *et al.*, 2003). Additionally, it could be argued that the algal biomass within WSPs may represent an undervalued resource with respect to their CO<sub>2</sub> sequestering potential; in fact there have been numerous papers within the academic journal *Energy Conversion and Management* reporting on investigations into exactly this topic. It is beyond the scope of this review, however, to enter into any further or more detailed discussion of this concept.

There has been a limited volume of prior work investigating the use of hydraulic 'baffles' for the upgrading of WSPs based on the concept of improving *in situ* flow patterns and subsequently enhancing treatment performance (Kilani and Ogunrombi, 1984; Pedahzur *et al.*, 1993; Pearson *et al.*, 1995; Muttamara and Puetpaiboon, 1996; Muttamara and Puetpaiboon, 1997; Lloyd *et al.*, 2003; Hansen *et al.*, 2004). Although results have shown promise with respect to improved flow hydraulics (i.e. more plug flow), BOD<sub>5</sub>, SS, and nutrient removal, the technology appears to have not been widely accepted as a viable pond upgrade alternative. In a similar vein, the importance of inlet and outlet design and configuration on WSP performance has been emphasized by numerous authors (Pearson *et al.*, 1995; Naméche and Vasel, 1998; Shilton, 2001). Following this realisation, and recognising that water column depth can have a marked influence on effluent quality in terms of algal SS (Pearson *et al.*, 1987a; Pearson *et al.*, 1987b), manipulation of pond outlet off-take depth was proposed by Herdianto (2003) as a means of actively reducing concentrations of algal solids in final WSP effluent. Results from the work of Herdianto, however, suggested that little improvement in effluent quality would be expected in full-scale WSPs through active control of outlet off-take depth.

There have also been several more recently conceived and in some cases *novel* approaches to algal control in WSP effluents. The direct chemical control of

phytoplankton through the use of aquatic herbicides like Hydrothol 191 (Ruzycki *et al.*, 1998) has been proposed for algal SS abatement in WSPs; although the potential for ecotoxicological and regulatory concerns are obvious. Similarly, the manipulation of water chemistry for enhanced algal flocculation—commonly via lime application—has also been investigated (Golueke and Oswald, 1965; Folkman and Wachs, 1973). Other relatively new algal removal technologies such as the PETRO hybrid trickling filter system (Meiring and Oellermann, 1995; Shipin *et al.*, 1998; Shipin *et al.*, 1999a; Shipin *et al.*, 1999b) and the similar SFDT system of Kaya *et al.* (2006) are still awaiting wider performance evaluation, and so they are not discussed in any detail. Some research has even focused on physical segregation of the two entities by effectively keeping the algae ‘out of the pond’ via immobilised algal systems or shallow algal streams (Hemens and Mason, 1968; Ozaki *et al.*, 1991; Travieso *et al.*, 1992; Craggs *et al.*, 1996; Tam and Wong, 2000; Schumacher and Sekoulov, 2002); however, these are not discussed here. Finally, the use of emergent macrophytes (i.e. in wetland arrangements), whilst it has demonstrated significant performance success, is an extensive subject area in its own right and so will not be reviewed. The reader is instead directed to a limited number of more recent publications on the subject (Karpiscak *et al.*, 1996; Bays *et al.*, 2001; Baldizón *et al.*, 2002; Tanner *et al.*, 2005; Kayombo *et al.*, 2006; Mara, 2006).

#### **1.2.8.1 In-pond vs. out-of-pond upgrades**

Considering the inherent nature of the WSP treatment process, and as was emphasized above, any technique chosen for the advanced upgrading of a WSP system must abide by the same basic principles (i.e. low operational inputs and simplistic methodology) that makes pond treatment the method of choice in that instance. Because many communities using WSP systems are small, it can be appreciated that upgrading them with more sophisticated and costly process ‘add-ons’ would place undue economic burden upon them, such that the upgrade itself would no longer be a viable solution. Thus, the obvious choice is to upgrade WSPs with a comparatively simple and economical process capable of satisfying the discharge standards for effluent quality with regard to algal SS.

From this, selection criteria for candidate processes must incorporate operational simplicity, minimum maintenance and cost, performance consistency as well as process efficiency (Middlebrooks *et al.*, 1974; Truax and Shindala, 1994). So-called ‘natural treatment systems’ for the upgrading of WSP effluents with respect to algal removal have been evaluated by Neder *et al.* (2002) as being the most capable of satisfying the necessary selection criteria, in terms of being simple, low-cost, easily implemented and delivering a good overall treatment efficiency. These so-called ‘innovative’ and ‘alternative’ natural treatment methodologies exploit naturally occurring physical, chemical and biological processes within the WSP “ecosystem reactor” in order to facilitate the desired treatment outcomes (Metcalf and Eddy, 1991; Neder *et al.*, 2002); with specific focus on energy efficiency and simplistic operation (Zhao and Wang, 1996).

Due to the fundamental requirement of *effluent relocation* associated with any ‘out-of-pond’ technology, in-pond methods for algal removal are—purely on a cost basis—generally viewed as being more economically viable for WSP systems (Mitchell, 1980; Truax and Shindala, 1994; Middlebrooks, 1995). Indeed Mitchell (1982b) sighted the high degree of difficulty and associated cost of final algal removal as justification of the need for additional research into in-pond algal control. Despite the range of abovementioned obstacles surrounding some in-pond removal technologies, there has been and continues to be a significant body of research effort devoted to assessing the efficacy of various in-pond WSP upgrades for algal removal. Two advanced techniques for the upgrading of WSP effluents that fall under the umbrella of both ‘in-pond’ and ‘natural’ treatment technologies are floating aquatic macrophytes and rock filtration. These two advanced in-pond upgrades share similarities in terms of their low requirements for initial capital input, low maintenance demands, and significant potential for process performance. Following initial pre-selection, these two pond upgrade methodologies were adopted as suitable candidate technologies for investigation as part of this research and will therefore be discussed in more detail within the following sections.

### **1.2.8.2 Upgrading WSPs with aquatic macrophytes**

Shallow, eutrophic, aquatic ecosystems dominated by aquatic macrophytes are among the most productive in the world, and the considerable “self-purification” capacity of these environments is well recognised (Brix and Schierup, 1989). Aquatic plants possess an outstanding ability for the assimilation of dissolved nutrients, whilst at the same time creating favorable conditions for microbial decomposition of organic materials (Brix and Schierup, 1989). The concept of using aquatic plants as a cost-effective and energy-efficient means of treating municipal effluent has been under investigation for many decades; with initial research questions gaining momentum during the early to mid 1960s (Cillie, 1962; Ehrlich, 1966) followed by a more extensive research effort during the 1970s (Culley Jr. and Epps, 1973; Harvey and Fox, 1973; Sutton and Ornes, 1975; Sutton and Ornes, 1977; Dinges, 1978; Wolverson and McDonald, 1979). Even more recently, macrophyte systems have again attracted attention as a potential treatment alternative for decentralised wastewater treatment (Nhapi *et al.*, 2003).

Since most wastewater treatment systems are viewed simply as an intensification or extension of natural eutrophic ecosystems, the incorporation of ‘ecological solutions’ within a wastewater treatment train has the potential to contribute significantly to treatment systems that have historically been dominated by sanitary engineers (Hillman and Culley Jr., 1978). The design of so-called ‘living technologies’ for wastewater treatment has been the focus of a significant volume of research interest over the past two decades (Gordon *et al.*, 1982; Tarifeno-Silva *et al.*, 1982; Smith, 1985; Rao, 1986; Guterstam and Todd, 1990; Smith, 1993; Todd and Josephson, 1996; Kumar and Sierp, 2003); especially since the inauguration of the journal *Ecological Engineering* in 1992. Macrophyte-based wastewater treatment systems possess several potential advantages over conventional treatment systems, making them of particular interest to small and medium-sized communities. Some of these potential benefits are: lower operating costs; lower energy requirements; lower requirements for operator skill; enhanced operational flexibility with less susceptibility to shock loading; and a key advantage of being able to construct these ‘low-tech’ installations at the site where the wastewater is produced (Brix and Schierup, 1989). Since macrophyte wastewater treatment systems rely on solar radiation to drive the treatment process, they inherently have reduced energy



requirements compared with other more conventional methods of secondary or tertiary-level waste treatment (Sutton and Ornes, 1975).

Following even the most cursory survey of the relevant literature, it soon becomes apparent that in recent times there has been a renewed worldwide interest in the use of macrophyte-based wastewater treatment systems; particularly for use in smaller communities. This growing chorus of interest toward the use of aquatic plants—in particular duckweed—for wastewater treatment, has come from the recognised demand for adequate treatment systems to serve the needs of smaller and decentralised communities in a cost-effective way (Erol Nalbur *et al.*, 2003). Considering the advantages of such systems, it is perhaps unsurprising that there has been such a rekindling of interest in macrophyte-based wastewater treatment technologies. This realisation serves as additional basis for the incorporation of aquatic macrophytes into this research.

Regarding WSPs and free-floating aquatic macrophytes, the literature base is extensive. Upon assessment of this published work, it is immediately apparent that a select number of species have been the focus of the vast majority of this research; with by far the most commonly investigated floating aquatic plants in wastewater treatment being species of water hyacinth and duckweed (USEPA, 1988). Prior research has already described and demonstrated the efficacy of floating macrophyte pond systems for wastewater treatment. In effect, the algal community—with its aqueous suspended biomass and fast turnover rate—is replaced by a rapidly growing macrophyte that continuously converts dissolved organics and inorganic nutrients into a ‘standing biomass’ which, if harvested, is not constantly recycled and hence does not contribute to the total organic carbon (TOC) of the system (Wolverton and McDonald, 1979). This means that the plant biomass, unlike suspended algal biomass, is retained within the pond system and is not present in the effluent BOD<sub>5</sub> and SS fractions; with these assimilated substrates able to be effectively and easily removed from the system by harvesting of the plant biomass. The potential for resource recovery through harvesting and utilising this plant material as an energy source, compost, or as animal fodder, has been emphasized by some authors (Culley Jr. and Epps, 1973; Brix and Schierup, 1989; Edwards *et al.*, 1992); with economic off-set benefits the ultimate goal of such practices.

### **1.2.8.3 Water hyacinths**

Existing research has demonstrated the significant potential for water hyacinth to effectively reduce the levels of nitrogen, phosphorous, BOD<sub>5</sub>, SS (Cornwell *et al.*, 1977; Wolverton and McDonald, 1979; McDonald and Wolverton, 1980; Reddy and De Busk, 1985b; Orth and Sapkota, 1988; Mandi, 1994; Ouazzani *et al.*, 1995; Costa *et al.*, 2000; Kim and Kim, 2000), faecal coliforms and heavy metals (Dinges, 1978) in WSP effluents; with treatment efficiency for organics and solids removal capable of reaching levels above and beyond that of standard facultative ponds (Orth and Sapkota, 1988; Ouazzani *et al.*, 1995). BOD<sub>5</sub> and SS removals have been reported as high as 97 and 95% respectively, yielding effluent values for these water quality parameters of less than 10mgL<sup>-1</sup> in some instances (Dinges, 1978).

These floating plants have both a well-developed and finely-structured root system extending anywhere from 30cm to 1m below the water surface and allowing for the direct uptake and assimilation of both dissolved nutrients (N and P) and organics from the surrounding water (Wolverton and McDonald, 1979; Reddy and De Busk, 1985b; Reed *et al.*, 1995). This feature also allows water hyacinths to grow at a phenomenal rate, with reported production rates in the order of 15% of their surface area per day (Wolverton and McDonald, 1979). The fibrous root structure of water hyacinths is also known to serve as a suitable substrate and microenvironment for many aquatic species. Dinges (1978) reported that species of bacteria, fungi, protozoa, ciliates, rotifers and snails can reside within the plant root zone; with copepods, cladocerans, insect larvae and nematodes also present in the aqueous environment beneath the plant cover.

Although water hyacinth has a larger net biomass productivity than duckweed and a comparatively attractive C:N:P ratio, the carbon is present primarily as hard fibre—ultimately decreasing its usefulness (e.g. for animal feed) and making it more difficult to manage (Alaerts *et al.*, 1996). In addition, processing and transport costs associated with aquatic plants in general can be high, and the comparatively bulky water hyacinth must be handled with heavy equipment as well as having to be chopped up to facilitate handling and processing (Hillman and Culley Jr., 1978). This is in contrast to other aquatic species such as duckweed, which are small enough to be pumped through pipes as a slurry and do not have to be chopped prior to processing (Hillman and Culley Jr.,

1978). Finally, the susceptibility of water hyacinth to cold conditions is well recognised (Dinges, 1978; McDonald and Wolverton, 1980). During winter, plant biomass can die and unless removed from the pond, can contribute a considerable organic load back to the pond system (Wolverton and McDonald, 1979) resulting in increased effluent BOD<sub>5</sub> and SS during this period. During winter operation, McDonald and Wolverton (1980) suggested that proper management of a balanced pond system could involve removing the water hyacinth and substituting it with more cold-tolerant plants—quoting duckweed as an appropriate substitute.

Whilst water hyacinth has been shown to be a viable WSP upgrade technology in other regions, the same plant (*Eichhornia crassipes*) has been declared a highly invasive noxious species according to the Government of South Australia (Department of Water, Land and Biodiversity Conservation; [http://www.dwlbc.sa.gov.au/biodiversity/pests/weeds/plants\\_list.html](http://www.dwlbc.sa.gov.au/biodiversity/pests/weeds/plants_list.html)). This precluded it from further consideration as part of the current research.

#### **1.2.8.4 Duckweed**

The second type of aquatic macrophyte commonly associated with use in wastewater treatment applications are the duckweeds. Duckweed is a small aquatic macrophyte with a world-wide cosmopolitan distribution spanning 4 genera (*Lemna*, *Spirodela*, *Wolffia* and *Wolffiella*) and around 40 species (Rahman *et al.*, 2001). Duckweeds, unlike other aquatic plants, have no distinct leaves and stems; instead, the plant body represents a fusion of both within a single ‘frond’. Ranging in frond size from 1.5cm (*Spirodela* spp.) to a mere 1–2mm frond diameter for species of *Wolffia* (Hillman, 1976), this worldwide family of floating aquatic monocotyledons (Lemnaceae) constitute the smallest and simplest form of all flowering plants (Hillman and Culley Jr., 1978).

As a result of this morphological simplicity, duckweeds are among the most vigorously growing plants on earth and are capable of very high rates of vegetative growth; believed to be in the order of 30% faster than the water hyacinth (USEPA, 1988). These high growth capabilities are largely a consequence of the fact that nutrient uptake occurs not only through the root (as in other higher plants) but throughout the entire plant frond

(Bonomo *et al.*, 1997) and also because the duckweed frond consists of predominantly metabolically active non-structural tissue, allowing for the allocation of minimal photosynthetic energy toward structural biomass requirements (Hillman and Culley Jr., 1978). For the same reasons, duckweeds contain at least twice as much crude protein ( $\approx 37\%$  dry weight), fat, nitrogen and phosphorous as hyacinth, whilst having half the cellulose content and also a lower fibre content than that of water hyacinth—giving them a very high nutritional value (Wolverton and McDonald, 1981; Oron *et al.*, 1984; USEPA, 1988; Edwards *et al.*, 1992).

Duckweeds are most commonly observed growing in thick, ‘blanket-like’ surface mats on still or slow flowing nutrient-rich waters (Leng, 1996). Duckweed fronds comprising this biological cover are capable of growth in very dense colonies many fronds deep and to a mat thickness in the order of 2cm (Zirschky and Reed, 1988). This dense floating plant mat (for *Lemna* species) may reduce incident light penetration into the underlying water by a factor in excess of 99.5% (Short *et al.*, 2007), thereby suppressing the growth of both submerged macrophytes and also planktonic algae (Janes *et al.*, 1996; Parr *et al.*, 2002) and enhancing the quiescent sedimentation and decay of SS and algal biomass (Reed *et al.*, 1988; Mara *et al.*, 1992; van Donk and van de Bund, 2002). In addition to the inhibition of sunlight, *Lemna* are said to fiercely compete for aqueous nutrient resources, resulting in out-competition and the rapid elimination of various algal species under conditions of high nutrient concentrations (Ngo, 1987; Zirschky and Reed, 1988; Roijackers *et al.*, 2004) and even in waters low in total nitrogen (Leng, 1996). In addition to their uptake of inorganic materials, *Lemna* have demonstrated an ability to directly assimilate complex organic molecules such as carbohydrates and amino acids from the aqueous phase for use in heterotrophic nutrition (Hillman, 1976; Frick, 1994).

#### **1.2.8.5 Duckweed as advanced WSP treatment**

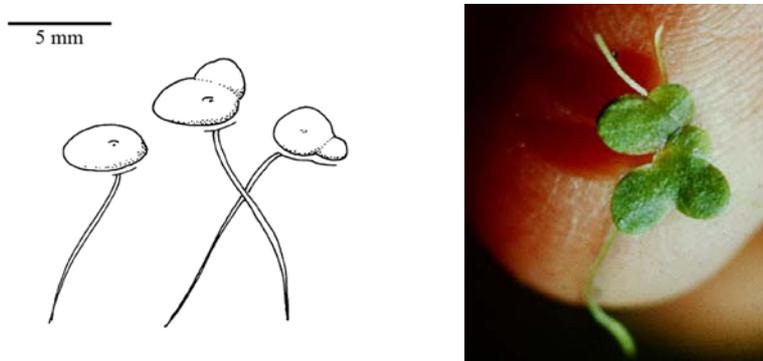
The potential of duckweed for use in wastewater treatment was first realised in Asia during the 1950s (Dalu, 2003). A duckweed WSP is essentially the same as a conventional WSP, the fundamental difference being that it is covered by a floating duckweed ‘mat’ (Erol Nalbur *et al.*, 2003). These rapidly growing plants can serve as an effective nutrient sink in wastewater applications, absorbing primarily N, P, Ca, Na, Mg,

C, and  $\text{Cl}^-$  from wastewater, which can then be permanently removed from the system by biomass harvesting (Leng, 1996). Although there are many species of duckweed, the majority of previous research into the area of wastewater treatment has most commonly involved the use of *Lemna* species. This has no doubt been owing to the fact that early research already demonstrated that species of *Lemna* are often the superior organism for use in wastewater treatment applications (Harvey and Fox, 1973; Hillman and Culley Jr., 1978; Reddy and De Busk, 1985b; Reddy and De Busk, 1985a; Oron *et al.*, 1986) and also because *Lemna* physiology and biochemistry has been widely researched (Hillman, 1976; McLay, 1976; Wedge and Burris, 1982; Ullrich *et al.*, 1984; Filbin and Hough, 1985; Frick, 1991; Monselise and Kost, 1993; Frick, 1994). Following this, a native species of *Lemna* was investigated during this research.

Maximal duckweed (*Lemna*) growth rates have reportedly seen doubling times in the order of 24 hours under optimal culture conditions (Datko *et al.*, 1980). *Lemna* grown in secondary effluent has reportedly yielded biomass doubling times of 4 days under controlled laboratory conditions (Harvey and Fox, 1973) and between 4 and 5 days in the field (Ellis, 1983; Ngo, 1987); with each frond capable of reproducing at least 10–20 times during its approximate 35 day life cycle (Hossell and Baker, 1979; USEPA, 1988). Hillman and Culley Jr. (1978) reported biomass doubling times for *Spirodela* species grown outdoors on cattle wastewater of 1.5 to 3 days, concluding that duckweeds can grow at least twice as fast as other higher plants. Although high growth rates are achievable, duckweed growth does depend on, and is altered by, variations in light intensity, temperature (Wedge and Burris, 1982), pH, wind speed, duckweed mat density, mixing, ammonia-nitrogen concentration (Al-Nozaily and Alaerts, 2002) and phosphorous availability (Sutton and Ornes, 1975; Hillman and Culley Jr., 1978). This means that duckweed can be expected to respond differently based on the specific nature of the wastewater in question and also according to localised climatological factors.

Although effective wastewater treatment can be achieved in duckweed ponds, it is generally at a lesser degree of efficiency with regard to nutrient assimilation compared to other macrophyte pond systems such as that of water hyacinth (Ellis, 1983). This lower rate of uptake of dissolved nutrients (N and P) in duckweeds compared with water hyacinth (Reddy and De Busk, 1985) relates to a lack of extensive duckweed rootstock

and indeed the associated reduction in amount of attached periphyton (Reed *et al.*, 1988; Bonomo *et al.*, 1997). Unlike water hyacinths, duckweeds—particularly *Lemna* species—have a ‘no frills’ root system as a result of a progressive evolutionary reduction of all structures non-essential to life in quiescent fresh water environments (Hillman and Culley Jr., 1978). This simple, un-branched root structure—usually less than 10mm in length—represents only a small fraction of the overall plant biomass (Dalu, 2003) and can be seen in Figure 1.3 below. As a result of this, most of the biological activity in a duckweed pond—as for a conventional un-covered WSPs—is considered to come from heterotrophic microbes and other microorganisms within the water column (Zirschky and Reed, 1988; Bonomo *et al.*, 1997).



**Figure 1.3.** Schematic (left) and photographic (right) depictions of the Australian native duckweed *Lemna disperma* Hegelm.

Some species of *Lemna* are known to have a wide-ranging temperature tolerance. *Lemna gibba*, for example, is capable of vegetative growth at water temperatures as low as 5°C and up to 30°C (Oron *et al.*, 1984) and air temperatures down to 1–3°C (Harvey and Fox, 1973); although its optimum growth temperature is in the range of 25–30°C (Wedge and Burris, 1982; Al-Nozaily and Alaerts, 2002). Many duckweed species are well equipped to cope with low temperatures. This resilience to low temperatures is aided by their ability to form a ‘turion’, followed by sinking of the plant to the pond bottom where it remains dormant until the return of warmer water promotes the resumption of normal growth (Leng, 1996). This broad temperature tolerance has promoted *Lemna* species in particular as more attractive candidates for use in wastewater treatment applications over a much broader geographical range than the cold-sensitive water hyacinth (Edwards *et al.*, 1992).

#### **1.2.8.5.1 BOD<sub>5</sub>, SS, nutrient and pathogen removal in duckweed ponds**

Generically speaking, the effectiveness of a given wastewater treatment system is measured by its ability to reduce the levels of corresponding BOD<sub>5</sub>, SS, as well as inorganic micronutrients such as N and P (Wolverton and McDonald, 1979). In wastewater applications, duckweed systems have demonstrated a capacity for effective wastewater treatment in terms of nutrients (N and P), BOD<sub>5</sub> and SS removal (Harvey and Fox, 1973; Sutton and Ornes, 1975; Reddy and De Busk, 1985b; Alaerts *et al.*, 1996; Zimmo *et al.*, 2002). The specifics of duckweed pond system performance do, however, vary according to differences in local environmental parameters, pond volume and depth, system loading and hydraulic retention time, duckweed biomass density and harvesting regime, algal competition, as well as the particular nature of the wastewater in question (O'Brien, 1981; Edwards *et al.*, 1992; Oron, 1994; Alaerts *et al.*, 1996; Szabó *et al.*, 1998; 1999).

A significant volume of prior research has demonstrated the treatment efficacy of duckweed ponds for BOD<sub>5</sub> and SS removal. BOD<sub>5</sub> removal processes in duckweed ponds are similar to that of a standard conventional WSP, with the plants themselves *directly* contributing very little to the overall removal of BOD<sub>5</sub> (Reed *et al.*, 1995). According to Ngo (1987), duckweed ponds can actually allow for an enhanced BOD<sub>5</sub> removal capacity in comparison with standard WSPs due to the maintenance of anaerobic conditions which allows for constant anaerobic digestion of inflowing organics—similar to that of a standard anaerobic WSP. As described above, the reported performance of duckweed ponds can vary considerably based on a number of factors, although BOD<sub>5</sub> removal efficiencies are commonly in the range of 60–80% at organic loading rates in the order of 15–30g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup> (USEPA, 1988; Alaerts *et al.*, 1996; Karpiscak *et al.*, 1996; Bonomo *et al.*, 1997; van der Steen *et al.*, 2000; Baldizón *et al.*, 2002; Zimmo *et al.*, 2002; Ran *et al.*, 2004).

SS removal in duckweed ponds is largely considered to be more effective than that achievable in conventional WSPs—a consequence of increased algal senescence due to shading and improved hydraulic quiescence under the surface mat (Zirschky and Reed, 1988). According to Smith and Moelyowati (2001), duckweed ponds are thought to

remove SS primarily via: (1) physical sedimentation to pond sludge; (2) biodegradation of organic materials; (3) adsorption or entrapment of a minor fraction by the duckweed roots; (4) and through the inhibition of algal proliferation; although the sludge accumulation data of Oron *et al.* (1987b) suggests predominantly physical removal. Whilst the promotion of quiescent conditions under a duckweed mat is likely to enhance the sedimentation and subsequent decomposition of SS including algal cells (Zirschky and Reed, 1988; Mara *et al.*, 1992), at the same time, it can also reduce the potential for natural surface re-aeration and wind-induced mixing within the pond environment—factors usually considered as desirable for normal pond operation (Ellis, 1983; El Ouarghi *et al.*, 2002). Reported performance data for SS removal efficiency in duckweed ponds has been largely variable (as for BOD<sub>5</sub> removal above), with mean SS removal efficiencies most commonly reported in the range of 50–75% (USEPA, 1988; Zirschky and Reed, 1988; Bonomo *et al.*, 1997; van der Steen *et al.*, 2000; Baldizón *et al.*, 2002; Ran *et al.*, 2004; Zimmo *et al.*, 2002).

According to Van der Steen *et al.* (1998) Körner *et al.* (2003) and Zimmo *et al.* (2004a; 2004b) duckweed systems remove N from the underlying wastewater by several processes. Nitrogen removal is principally achieved via: ammonification; nitrification–denitrification by attached and suspended microbes; direct plant uptake (preferentially ammonium-N); sedimentation of particulate N; and by ammonia volatilisation; although system interactions dictating nutrient removal are both highly interrelated and very involved. Data from Körner *et al.* (2003) suggest that N removal resulting directly from duckweed-associated microbial processes accounts for between 35–46% of the total system removal. Oxidic–anoxic gradients in and around macrophyte beds may also lead to enhanced denitrification—further restricting the availability of inorganic nitrogen for phytoplankton populations (van Donk and van de Bund, 2002).

There is also some evidence to suggest a potential for N inputs in duckweed systems through fixation by naturally occurring cyanobacterial associations, with reported fixation rates ranging from 1–2mg (Duong and Tiedje, 1985) up to 12.5mg N m<sup>-2</sup> d<sup>-1</sup> (Zuberer, 1982) or roughly 2–20% of the plant's daily growth requirements. Despite this, Körner *et al.* (2003) deemed N inputs from these associations to be an unimportant component of the nitrogen balance in duckweed WSPs; a conclusion that seems



ecologically feasible given the universally hypereutrophic status of WSPs. Direct plant uptake of dissolved N reportedly contributes to 16–75% of the total system removal (Reddy and De Busk, 1985; Alaerts *et al.*, 1996; Körner and Vermaat, 1998; Körner *et al.*, 2003), however, this figure varies according to factors such as plant species, plant biomass density, substrate N concentration and temperature (Reed *et al.*, 1988). Regardless of specific uptake values, the direct utilisation of ammonia by duckweed is an anabolic, sunlight-driven process, in contrast to the catabolic, energy-consuming microbial nitrification performed during the activated sludge treatment process (Porath and Pollock, 1982). This *ecological* remediation process, whilst less rate-intensive than activated sludge, makes nutrient removal by duckweed systems also less energy-intensive and therefore generally less expensive than other conventional secondary and tertiary treatment processes (Sutton and Ornes, 1975; Rao, 1986; Craggs *et al.*, 1996).

In macrophyte-based ponds, *Lemna* has been reported to out-perform other floating macrophyte species, such as water fern (*Salvinia*) and water lettuce (*Pistia*), in terms of its capacity for N removal (particularly during winter months). *Lemna* has also been reportedly capable of out-performing even the veracious water hyacinth in terms of its winter phosphorous removal capacity (Tripathi *et al.*, 1991); although *Lemna* was ranked 3<sup>rd</sup> by the same authors in the *overall* ‘nutrient removal’ stakes. This trend for winter dominance by duckweed species has been reported elsewhere, where *Lemna* generally out-competes other aquatic plant species during cooler periods (Sutton and Ornes, 1975; Sutton and Ornes, 1977; Wolverson and McDonald, 1979).

Phosphorous removal in duckweed ponds is generally seen as being limited and of secondary importance to other wastewater parameters, due to its largely variable and transitory treatment performance (Bonomo *et al.*, 1997). Direct P uptake in duckweed ponds reportedly accounts for approximately 12–73% of the total P removed in summer and only 9–35% during winter (Reddy and De Busk, 1985), but once again the reported performance range varies significantly (Alaerts *et al.*, 1996; Leng, 1996; Körner and Vermaat, 1998; Vermaat and Hanif, 1998; Körner *et al.*, 2003). Data from Körner *et al.* (2003) suggests that a significant proportion of P removal in duckweed systems—somewhere in the range of 31–71% of the total—is attributable to duckweed-associated microbial biofilms. Whilst some studies have reported very high P removal rates in

pilot-scale duckweed ponds (up to 89%; Ngo *et al.*, 1988), macrophyte WSPs are not generally the ‘method of choice’ for phosphorous removal; with removal rates commonly orders of magnitude less than that achievable in parallel algal-based systems (Reddy, 1983; Valderrama *et al.*, 2002; Roijackers *et al.*, 2004).

Attenuation rates as high as 30–70% for P have been achieved for duckweed grown on domestic wastewater (Körner and Vermaat, 1998); however, these high removal rates often come from small-scale laboratory incubations and so would not be reasonably expected to be achievable *in situ*. Furthermore, continuous harvesting of plant biomass is required in order to achieve consistently high nutrient removals of both N and P in duckweed WSPs in order to prevent the re-release of biomass-sequestered nutrients following inevitable plant death and decay (Zirschky and Reed, 1988; Öbek and Hasar, 2002; Nhapi *et al.*, 2003). It is possible that the limited capacity of duckweed for reliable P removal *in situ* might also be influenced by the potential for anaerobic re-release of sediment-bound P reserves, although it has so far not been reported.

The capacity for duckweed pond systems to attenuate pathogens has also been investigated by a number of researchers. Generally speaking, the removal of indicator organisms—namely faecal coliforms (FC) and *Escherichia coli* (*E. coli*)—in duckweed systems, is commonly similar or slightly less effective than is achieved by conventional WSPs. Mean reported removal efficiencies have ranged from as low as <0.5 to 1- $\log_{10}$  unit removals (Falabi *et al.*, 2002; Zimmo *et al.*, 2002) up to 3- $\log_{10}$  unit organism removals (van der Steen *et al.*, 1999; van der Steen *et al.*, 2000), with some authors even reporting zero FC removals in duckweed-covered systems (Dewedar and Bahgat, 1995). It should be noted, however, that specific organismal removal magnitudes reported in the literature vary largely as a direct consequence of experimental reactor depth and volume, hydraulic loading regime, and also according to the prevailing environmental conditions.

#### **1.2.8.5.2 Advantages and disadvantages of duckweed ponds**

Whilst their less advanced root system may lead to duckweeds having a lower affinity for aqueous nutrients, at the same time, the additional costs associated with harvesting and processing generated plant biomass are viewed as a major deterrent toward the

integration of aquatic crops into WSP systems (Culley Jr. and Epps, 1973; Ward, 1987). Routine plant biomass harvesting is necessary in order to ensure optimal pond performance and reduce sludge accumulation from senescent and decaying plants; with harvesting rate suggested to be approximately 20% of standing biomass (Reed *et al.*, 1988).

Al-Nozaily and Alaerts (2002) stated that “the use of duckweed has been promoted” over other aquatic plants in macrophyte-based wastewater treatment systems. This preferential usage of duckweed no doubt relates—to some extent—to their relative ease of harvest compared to other aquatic species such as water hyacinth (Edwards *et al.*, 1992). For a smaller scale duckweed pond, partial harvest can be achieved simply by netting or dragging a baffle across the pond surface and removing the collected plant biomass. For larger-scale applications, duckweed harvest can be easily implemented via the use of a floating ‘skimmer’ system (Culley Jr. and Epps, 1973; Hillman and Culley Jr., 1978). This sort of floating system has the added advantage of less disruption to the benthic sludge layer than that required to harvest submerged or emergent macrophytes. This feature would no doubt be considered advantageous by pond operators, in that the resuspension potential for previously settled materials (SS and particulate BOD<sub>5</sub>) within the pond is minimised. It should be noted that there are in fact commercially available floating systems specifically for the mechanical harvesting of duckweed from WSPs; however, regular harvesting does increase the capital and operational input requirements of the technology. There is some evidence to suggest a potential for resource recovery through on-selling of the duckweed as animal feed (Culley *et al.*, 1981; Oron, 1994) or through further processing of the harvested plant biomass for energy production (Wolverton and McDonald, 1981; Smith and Moelyowati, 2001). The large-scale practical viability of these options, however, has been the subject of past debate (Ward, 1987) and remains under contention (Nhapi *et al.*, 2003).

One major disadvantage of the fine physical structure of duckweed, is the increased susceptibility of duckweed to wind-dispersion (Zimmels *et al.*, 2004). This is of greater concern in larger ponds, where duckweed mats are recognised as being very prone to wind fetch and are blown easily to the pond edges (Hillman and Culley Jr., 1978; Edwards *et al.*, 1992) resulting in sub-optimal pond surface coverage and reduced pond

performance (USEPA, 1988). This problem can be easily overcome in small installations by planting a border of vegetative wind-breaks (Hillman and Culley Jr., 1978; Dalu, 2003) or, for larger systems, by installing a floating containment barrier network (Dalu, 2003). This system effectively divides the pond surface into discrete ‘cells’ designed to contain the duckweed—preventing their wind dispersion. Such a floating grid system can be seen below (Plate 1.1) in operation at a local agricultural site in Virginia, South Australia. Their fine morphological structure means that duckweed is also highly susceptible to accidental discharge from ponds with surface outlets; a problem easily overcome through the use of surface baffles in front of effluent weirs, or by increasing the effluent take-off depth (Rich, 2003).



**Plate 1.1.** Photograph of an established duckweed surface mat being contained by a floating containment grid network.

The inherently minimalist morphology possessed by duckweed is also advantageous for wastewater treatment applications, in that it results in a standing crop density that is much lower than that of other aquatic plants—giving duckweed operational versatility and high specific productivity (Alaerts *et al.*, 1996). This simple physiology would be considered especially appealing in situations where the floating plants simply serve as a pond upgrade for the suppression of algal growth. In this instance, the floating duckweed mat may serve purely as an inexpensive ‘biological cover’ for the pond, with any participation in biological *treatment* an added bonus.

Heavy duckweed growth can render the underlying water anaerobic (Lewis and Bender, 1961); with oxygen transport through the duckweed mat, and the subsequent re-aeration rate of the underlying water, a linear function of surface mat thickness (Morris and Barker, 1977). This reduced dissolved concentration can lead to both a reduction in

biological activity in the underlying water as well as the production of malodours (Reed *et al.*, 1988) and may also lead to post-aeration being required prior to discharge in some cases (Zirschky and Reed, 1988; Reed *et al.*, 1988). The development of anaerobic conditions beneath the duckweed surface cover may, however, be considered a desirable feature in some instances, given the recognised treatment efficacy of anaerobic ponds as a pre-treatment step in some WSP configurations (Almasi and Pescod, 1996; Pescod, 1996; DeGarie *et al.*, 2000; Alexiou and Mara, 2003). Anoxia in duckweed ponds might also promote microbial denitrification, thereby maximising nitrate-nitrogen ( $\text{NO}_3^-$ -N) removal (Reddy, 1983; Brix and Schierup, 1989) and further restricting the availability of inorganic N for algal growth (van Donk and van de Bund, 2002).

Additionally, there is evidence to suggest some degree of photosynthetic re-oxygenation of the underlying water directly surrounding the root zone of the floating duckweed surface mat (Zirschky and Reed, 1988). This narrow aerobic zone near the water surface might also contribute to the oxidation of rising gases, such as reduced products of anaerobic organic fermentation like  $\text{H}_2\text{S}$ , by providing a favourable environment for aerobic sulphide oxidising microbes and ultimately preventing the release of malodours (Bonomo *et al.*, 1997; van der Steen *et al.*, 2003). According to Ngo (1987), duckweed ponds can actually prevent the release of malodours such as  $\text{CH}_4$  and  $\text{H}_2\text{S}$  by not only maintaining an aerobic zone near the surface (as above), but also through the promotion of quiescent conditions at the water surface; thereby minimising the liberation of undesirable odours from the water column as a result of surface agitation and wave action. Other researchers have taken the view that a thick surface duckweed mat might be able to suppress the release of both malodours (e.g.  $\text{H}_2\text{S}$ ) and greenhouse gases (e.g.  $\text{CH}_4$  and nitrous oxide) from underlying waters by acting as a physical barrier that entraps rising gas bubbles and stifles normal mass transfer processes at the water surface interface (Hammouda *et al.*, 1995; van der Steen *et al.*, 2003). An executive summary of these, along with some of the more commonly reported advantages and disadvantages of duckweed when used as an advanced WSP upgrade, are presented in Table 1.2.

**Table 1.2.** Summary of the most commonly reported advantages and disadvantages of duckweed for the upgrading of WSP effluent (Lewis and Bender, 1961; Culley Jr. and Epps, 1973; Dale and Gillespie, 1976; Reddy, 1983; Zirschky and Reed, 1988; Brix and Schierup, 1989; Edwards *et al.*, 1992; Reed *et al.*, 1995; Bonomo *et al.*, 1997; van der Steen *et al.*, 2003).

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### Advantages

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- increased SS and particulate BOD<sub>5</sub> removal through enhanced quiescent sedimentation and algal shading
- accelerated nutrient (N and P) removal under a sustained biomass harvesting regime
- increased thermal stability and reduced stratification potential of underlying water
- potential generation of large quantities of useful biomass for composting, animal feed or biogas production
- reduced evaporation rates, and prevention of malodor / greenhouse gas release due to floating plant 'blanket'
- thick surface mat can help maintain anaerobic conditions for enhanced N removal (microbial denitrification)
- reduced mosquito breeding from an inability to penetrate the surface mat as well as an anaerobic water column

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### Disadvantages

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- possibility of anaerobiosis under high BOD<sub>5</sub> loading – can lead to post-aeration requirements
  - susceptibility to wind dispersion necessitating a floating containment network in medium to large-scale systems
  - reduced organic loading and removal capabilities due to lower dissolved oxygen levels
  - can experience problems maintaining year-round surface coverage (due to weather, competition and infection)
  - reduced pathogen removal efficiency as a result of physical shading and stifled photosynthetic pH fluctuations
  - lack of positive control with respect to effluent quality (decaying biomass may also contribute to final SS / BOD<sub>5</sub>)
  - capital inputs required for continuous biomass harvesting, and possibility of sludge resuspension during harvest
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#### 1.2.8.5.3 Duckweed as an advanced in-pond upgrade for algal solids removal

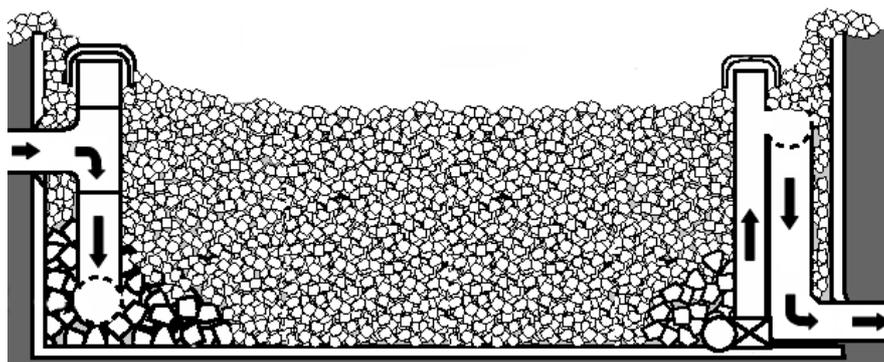
The upgrading of WSPs with aquatic plants has opened avenues for the cost-effective and efficient conversion of aqueous BOD<sub>5</sub>, to a tangible, more controllable and readily removable plant biomass (Rao, 1986). The majority of prior research involving duckweed grown on wastewater has been focused on: their nutrient removal efficiency (Reddy and De Busk, 1985b; Öbek and Hasar, 2002); their production as a useable proteinaceous biomass (Culley Jr. and Epps, 1973; Oron *et al.*, 1987a; Oron and Willers, 1989; Oron, 1994); their usefulness for composting and biogas (methane) production (Wolverton and McDonald, 1981); their potential role in the sequestration of heavy metals (Hammouda *et al.*, 1995; Boniardi *et al.*, 1999); and/or their role in pathogen removal (van der Steen *et al.*, 2000; Awuah *et al.*, 2001; Awuah *et al.*, 2002; Falabi *et al.*, 2002). In contrast to this, very little research effort has been concerned with assessing the potential of macrophyte systems as an advanced treatment technology for the upgrading of WSP effluents, purely with the goal of enhancing algal solids removal.

SS (and hence particulate BOD<sub>5</sub>) removal in duckweed ponds is largely considered to be more effective than that achievable in conventional WSPs; something thought to be a consequence of increased algal senescence due to shading and improved hydraulic quiescence under the surface mat (Zirschky and Reed, 1988; Körner *et al.*, 2003). As discussed previously (Section 1.2.8.5.1), duckweed ponds are thought to remove SS primarily via: (1) physical sedimentation to pond sludge; (2) biodegradation of organic materials; (3) adsorption or entrapment of a minor fraction by the duckweed roots; (4) and through the inhibition of algal proliferation. In addition to these processes, there is also evidence to suggest that other factors may contribute to an accelerated algal removal in macrophyte systems, such as antagonistic allelopathy and zooplankton grazer effects (Ehrlich, 1966; Fitzgerald, 1969; Hutchinson, 1975; Hillman and Culley Jr., 1978; Gopal and Goel, 1993; van Donk and van de Bund, 2002).

While many authors have reported on the SS and BOD<sub>5</sub> removal efficacies of duckweed pond systems, few have specifically reported on *algal* removal potential. Although there have indeed been a small number of authors reporting on algal removal potential in duckweed-covered ponds (Oron *et al.*, 1987b; Ngo *et al.*, 1988; Zirschky and Reed, 1988; Hammouda *et al.*, 1995; Bonomo *et al.*, 1997; Valderrama *et al.*, 2002), of these studies, almost all have been qualitative observations or inferences surrounding algal removal; with Valderrama *et al.* (2002) and Zimmo *et al.* (2002) the only authors formally offering quantitative data (in the form of either direct algal counts or chlorophyll *a* concentration). Given the overbearing influence of algal biomass on BOD<sub>5</sub> and SS in particular (see Section 1.2.5), one of the aims of the current research was—in addition to assessing the gross SS and BOD<sub>5</sub> removal potential of duckweed ponds—to attempt to quantify *algal* removal potential in duckweed ponds. In order to achieve this, algal biomass (chlorophyll *a*) levels were quantified, in addition to directly investigating phytoplankton community dynamics in duckweed-covered ponds compared with uncovered controls. Zooplankton populations were also monitored alongside phytoplankton to look for potential differences in grazer populations between uncovered and duckweed-covered ponds; populations that may contribute toward enhancing the algal removal capacity of duckweed systems.

### 1.2.8.6 Rock filtration as an advanced WSP upgrade

A rock filter consists of a submerged bed of typically coarse aggregate rock media through which the inflowing wastewater percolates horizontally under gravity flow (Figure 1.4). Although some rock filter systems have inlet and outlet configurations to allow for vertical ‘bottom-up’ flow, the most common configuration for rock filtration is the horizontal-flow pattern (USEPA, 2002a). Additionally, because these vertical-flow rock filters are likely to be less suitable as *post hoc* in-pond upgrades (due to difficulties associated with attaining vertical hydraulic flow patterns *in situ*) they will not be discussed in any further detail. It should also be noted that rock filter operational principles and treatment mechanisms remain essentially the same regardless of the particulars of their hydraulic flow regime, and so no further distinction will be made between vertical and horizontal-flow systems.



**Figure 1.4.** Schematic cross-section of a rock filter bed showing the typical horizontal-flow configuration (modified from Powell *et al.*, 1998).

The concept of the rock filter was originally developed in Kansas, USA in the early 1970s (Martin, 1970; O’Brien *et al.*, 1973; Hirsekorn, 1974). According to the USEPA (2002a) there are currently around 20 operational rock filter systems in the United States, with most having been constructed between 1970 and 1985. The origins of the rock filter most likely come from vertical-flow trickling filters, and in essence, a rock filter can be seen simply as a completely fluidised trickling filter bed operated under horizontal- rather than vertical-flow conditions. Particle size distribution of the rock media is most commonly relatively coarse—in the order of 5–15cm (Middlebrooks, 1995). Some work has been carried out using rock filters with a somewhat smaller rock aggregate size  $\leq 13\text{mm}$  (Saidam *et al.*, 1995; Johnson and Mara, 2002) although this has



been associated with: a reduced rock filter void space volume and hydraulic retention time (Mara *et al.*, 2001); accelerated filter head loss (Archer and Donaldson, 2003); and is generally thought to result in reduced filter performance (Middlebrooks, 1988). Furthermore, both Saidam *et al.* (1995) and Johnson and Mara (2002) both reported no performance benefits associated with the use of these smaller size rock aggregates.

#### **1.2.8.6.1 Rock filters for final effluent polishing: nutrients; BOD<sub>5</sub>; and SS abatement**

Generally speaking, and according to Liao and Ødergaard (2002), wastewater treatment is to a very large extent a ‘simple’ matter of particle separation. This is largely a consequence of the fact that the majority of wastewater pollutants exist in particulate or colloidal form (Liao and Ødergaard, 2002) and also because most microorganisms in suspension within biological wastewater treatment reactors (such as WSPs) are present in aggregate flocs rather than as discrete entities (Li *et al.*, 2003). This implies a predominantly *physical* treatment modality for most wastewater treatment processes, of which rock filtration is no exception. Infiltrating particulates, including both organic and inorganic solids, settle out of suspension and become entrapped by or attached to the surfaces of the rock media, such that treatment within the body of the rock filter is said to be achieved primarily through physical means (Martin, 1970; Hirsekorn, 1974; Stutz-McDonald and Williamson, 1979; Swanson and Williamson, 1980; Rich, 1988); although settled organic materials must ultimately be *biologically* degraded. There is some evidence to suggest that solids removal is assisted somewhat by attached biofilms within the rock filter (O’Brien *et al.*, 1973; Hirsekorn, 1974; Swanson and Williamson, 1980); however, the overall consensus from critical assessment of rock filter treatment mechanisms is that physical sedimentation facilitates the bulk of treatment performance.

Because of their overwhelmingly physical nature in terms of treatment delivery, rock filters are most commonly designed for final WSP effluent SS and associated particulate BOD<sub>5</sub> removal. Whilst rock filter treatment is predominantly physical, the rock media does also provide a significant amount of supplemental inert surface area ( $\approx 45\text{m}^2\text{ m}^{-3}$ ; Metcalf and Eddy, 2002) for additional biological treatment processes such as nitrification and denitrification (Martin, 1970; Johnson and Mara, 2002; Archer and Donaldson, 2003) as well as habitat for invertebrate (protozoan and metazoan) grazing

activities (Shelef and Azov, 2000). In this sense, a rock filter functions as both an *in situ* physical ‘strainer’ as well as a coarse media biofilter to achieve a combination of both physical and biological wastewater treatment processes.

Because of the high percentage of ‘dead’ rock media volume within a rock filter (commonly 50–60%), hydraulic loadings are typically low to allow for the sufficiently low hydraulic flow velocities required for effective sedimentation of suspended particulates. Operational rock filter hydraulic loadings within the relevant literature are most commonly reported to be within the range of 0.15–0.8 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> and most commonly ≤0.5 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> (Hirse Korn, 1978; Swanson and Williamson, 1980; USEPA, 1983; Middlebrooks, 1988; Saidam *et al.*, 1995; Mara, 2003; von Sperling and de Andrada, 2006; Johnson *et al.*, 2007); with a typical linear reduction in performance efficiency reported with increased hydraulic loading (USEPA, 1983; Swanson and Williamson, 1980; Tanner *et al.*, 2005). Mara *et al.* (2001), for example, operated experimental rock filters at hydraulic loadings of 1.0–2.0 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> but reported a significant reduction in overall rock filter performance (with respect to BOD<sub>5</sub>, chlorophyll *a*, SS removal) when operated at higher hydraulic loadings. Similarly, USEPA (2002a) reported rock filter hydraulic loadings of up to 1.2 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>, with inconsistent effluent quality a common symptom under high volumetric loading regimes. Whilst their effectiveness at removing suspended particulates is well documented, rock filters are largely recognised as being inefficient for the removal of dissolved nutrients such as NH<sub>3</sub>-N and PO<sub>4</sub><sup>3-</sup>-P. A limited capacity for phosphorous removal has been reported within the literature (Saidam *et al.*, 1995; Johnson and Mara, 2002); however, removals are widely variable and commonly negligible, such that phosphorous removal is not generally considered as part of normal rock filter treatment outcomes.

Whilst there have also been a limited number of reports of NH<sub>3</sub>-N removal following rock filtration (Martin, 1970; O’Brien *et al.*, 1973; Mara *et al.*, 1992; Johnson and Mara, 2002; Archer and Donaldson, 2003), they are not designed nor installed for achieving N removal. This is primarily due to the fact that rock filters are commonly hypoxic or anoxic in operation; conditions that favour the anaerobic remineralisation of digested organics, whilst at the same time restricting aerobic microbial processes such as nitrification. Some authors have actually reported an increase in effluent NH<sub>3</sub>-N

concentration (Mara *et al.*, 2001), but in spite of this, their low cost and simple operation make rock filters attractive for small installations that are not subject to ammonia discharge limits (USEPA, 2002a). Furthermore, under anaerobic conditions, undesirable compounds such as H<sub>2</sub>S and various organic acids may also be generated (Stutz-McDonald and Williamson, 1979)—causing additional problems for treatment plant operators. There has been a small volume of more recent work involving the use of aerated rock filters for the promotion of aerobic operation to combat these problems associated with filter anoxia (Johnson and Mara, 2005; Mara and Johnson, 2006; Mara and Johnson, 2007); however, this research is not discussed as part of the current review. A summary of the most commonly reported advantages and disadvantages of rock filtration for the upgrading of WSP effluents is provided in Table 1.3 below.

**Table 1.3.** Listing of the most commonly reported advantages and disadvantages of rock filtration for the upgrading of WSP effluent (USEPA, 2002a; Middlebrooks, 1995).

<b>Advantages</b>
<ul style="list-style-type: none"> <li>• demonstrated capacity for reliable and consistent SS (and associated particulate BOD<sub>5</sub>/COD) removal</li> <li>• relatively low associated capital establishment and operational costs</li> <li>• highly simplistic mode of operation</li> <li>• potential for additional nitrogen removal under conditions of low organic loading (e.g. maturation pond effluent)</li> <li>• high rate of annual capital recovery of the technology compared to alternative algal removal technologies</li> </ul>
<b>Disadvantages</b>
<ul style="list-style-type: none"> <li>• possibility of malodors and the potential requirement for post-treatment effluent aeration due to anaerobic operation</li> <li>• possibility of exceeding ammonia discharge limits due to anaerobic remineralization of settled organic nitrogen</li> <li>• operational lifespan is dependent on individual loading criteria, and so is variable (10–25 years within the literature)</li> <li>• absence of rigorously established filter cleaning protocols</li> <li>• lack of positive control with respect to effluent quality (i.e. treatment relies solely on passive remedial processes)</li> </ul>

As discussed earlier, increasingly stringent wastewater discharge guidelines throughout the world are making it necessary to upgrade final WSP effluents to ensure that they comply with these water quality criteria. It has also been highlighted above, that it is often difficult for conventional WSP systems to meet such effluent quality requirements (especially with respect to SS and BOD<sub>5</sub>) and so final WSP effluents must undergo additional ‘polishing’ prior to final disposal. Middlebrooks (1995)—following a review of a range of technologies for final polishing of maturation pond effluents—concluded that rock filters were especially suitable for this task, and that they also offered “dramatic” cost advantages over other upgrade technologies. Similarly, and in spite of

the technology itself being recognised as old, Brissaud (2008, p. 7) concluded that rock filters still had the capacity to “*lead to the considerable enhancement of (stabilisation) pond system performance.*” Rock filtration was, therefore, included in this research as a potential means of achieving cost-effective final WSP effluent polishing.

#### **1.2.8.7 Artificial attached-growth media**

Ødegaard *et al.* (1994) highlighted the fact that there had been a trend of ever-increasing interest in biofilm processes for both municipal and industrial wastewater treatment. This interest has since led to an increase in the development of new so-called ‘biofilm reactors’ for wastewater treatment. Conventional WSPs are, by nature, predominantly ‘suspended-growth’ biological treatment systems (ignoring the fact that suspended microbes might actually be attached to substrate particulates in suspension). The concept of attached-growth media (AGM) systems in a WSP context implies, in its simplest form, the addition of some kind of physical substrate to a pond environment in order to support further biological growth. Beyond this, it could be most adequately described as the addition of a synthetic, low density, high surface area media to a WSP system in order to facilitate treatment.

The initial concept of adding AGM to WSPs to form what is known as an attached-growth WSP (AGWSP), was first conceived by Shin and Polprasert (1987) as a means of intensifying the biological activity within a pond and therefore potentially reducing the extensive land area requirements normally associated with the technology. The authors observed densities of heterotrophic microbes in laboratory-scale AGWSP systems to be an order of magnitude (some 60%) greater than in conventional WSP reactors—adding weight to the initial concept of an intensified pond biology. Since that time, and since the discussion paper of Parker (1988) highlighted their “*considerable promise for achieving space and cost reductions*” in wastewater treatment applications, there has been an ongoing recognition of the potential for the use of AGM systems in general wastewater treatment applications. Lessel (1991) highlighted the potential of using submerged biofilm reactors as a means of combining the traditional contact oxidation process (like that of a trickling filter) with the suspended biomass process (like that of activated sludge or WSPs)—the aim being an intensification of overall biological

treatment processes. This is essentially the theory behind AGWSP systems, where it is thought that both physical contact and suspended biomass oxidation processes can be exploited as a way of enhancing conventional WSP treatment.

AGWSPs represent a new approach to increasing the functional biomass of traditional WSPs with the desired outcome of accelerating and improving overall treatment efficiency (Pearson, 1996). Early research has demonstrated the elevated treatment efficiency of AGWSPs for the removal of organics, ammonia and some heavy metal compounds (Shin and Polprasert, 1988; Polprasert and Charnpratheep, 1989; Polprasert and Sookhanich, 1995). In spite of these promising findings, there has been only a very limited amount of research focusing on the use of *in situ* artificial media in WSP environments. Prior work has most commonly involved the use of PVA or PVC ‘strings’ or ‘fibrous carriers’ as a way of bolstering the available surface area for enhanced in-pond treatment (Shin and Polprasert, 1987; Polprasert and Charnpratheep, 1989; Peishi *et al.*, 1993; Polprasert and Sookhanich, 1995; Zhao and Wang, 1996; Lapolli *et al.*, 2006). Other work has involved the use of polyethylene ‘plate’ type AGM (McLean, 1999; McLean *et al.*, 2000) and there have also been a limited number of other miscellaneous media substrates that have been investigated as *in situ* biofilm intensification or pond upgrade techniques (Kilani and Ogunrombi, 1984; Lessel, 1991; Nambu *et al.*, 1991).

#### **1.2.8.7.1 Microorganisms and biofilm processes in AGWSPs**

Although the small surface area-to-volume ratio of classical WSPs precludes surface biofilms from playing a major role in the wastewater treatment process (particularly in very large WSPs), prior work has suggested that biofilms attached to pond surfaces—such as baffles, side walls and pond bottom—can play a significant role in waste substrate utilisation and the overall stabilisation process in conventional WSPs (Reynolds *et al.*, 1975; Baskaran *et al.*, 1992; Polprasert and Agarwalla, 1994; Polprasert and Agarwalla, 1995). Polprasert and Agarwalla (1995), for example, found that 46–49% of the total BOD<sub>5</sub> removal in pilot-scale facultative WSPs could be attributed to the treatment activity of biofilms attached to the side walls and pond bottom. Muttamara and Puetpaiboon (1997) made reference to this also, offering the possible enhancement of

attached biofilm biomass within baffled WSPs as an explanation for the observed increase in treatment performance. It is therefore feasible that the overall treatment processes might be accelerated or enhanced by supplying large amounts of additional surface area for biofilm development. This is fundamental basis for the use of attached-growth media in WSPs.

Lessel (1991) and Nambu *et al.* (1991)—both reporting on the use of AGM in the activated sludge process—discussed how the presence of additional physical substrate promoted the development of both a higher density and also a wider array of microorganisms (including higher organisms such as nematodes) which could feasibly be consuming particulate organics at the same time as the suspended processes are occurring. Zhao and Wang (1996) also reported a significant abundance of protozoan and also higher metazoan populations (such as round worms, rotifers and *Daphnia*) associated with the AGM in their system—organisms that can play an important role in the wastewater stabilisation process (Mitchell, 1980). This amplified biomass density in AGM systems was said to also further increase the relative buffering capacity of the treatment process against fluctuations in influent quality and quantity.

Whilst an increase in the quantity of active *in situ* biomass may promote accelerated treatment within a WSP, at the same time, the large biofilm surface area-to-volume ratios of AGWSPs means that the system is also inherently subjected to increased ‘mass transfer’ limitations for general biological processes. At very high biomass densities, the overall rate of treatment may therefore be limited; either by the concentration of waste products, or just as easily by the competition-induced reduction in oxygen availability within the system. In this sense, the biomass density will most likely be self-limiting according to the physicochemical environment within the AGWSP, but it can be appreciated also that any surplus AGM added to the system will not be biologically utilised and may even be detrimental to overall pond performance (in terms of flow hydraulics and HRT) or in the very least an unnecessary waste of resources.

Another consequence of the large biomass surface area-to-volume ratio—as alluded to above—is the issue of maintaining an adequate oxygen supply. Large quantities of attached heterotrophic biomass could easily exert a significant and labile source of

respiratory BOD within the system, potentially leading to the development of anaerobic conditions during periods of low DO (e.g. nighttime). In spite of this being a logical and no doubt prominent concern for such systems, only a handful of researchers have made note of DO concentrations within their AGWSPs (e.g. Zhao and Wang, 1996; McLean *et al.*, 2000) and only one of those accounts was quantitative (i.e. McLean *et al.*, 2000). In this sense, it is likely that AGM systems used for the upgrading of WSPs may be susceptible to similar oxygen supply limitations as those already discussed for rock filters (Section 1.2.8.6.1). DO levels were, therefore, monitored as part of this research in order to investigate the issue more closely. A summary of the potential disadvantages and advantages of AGM as an advanced WSP upgrade is shown in Table 1.4.

**Table 1.4.** Summary of the most commonly reported advantages and disadvantages of using attached-growth media for the upgrading of WSP effluent (Shin and Polprasert, 1987; Lessel, 1991; Nambu *et al.*, 1991; Polprasert and Sookhanich, 1995; Zhao and Wang, 1996; McLean, 1999).

<b>Advantages</b>
<ul style="list-style-type: none"> <li>• significant increase in physical sites for SS and particulate BOD<sub>5</sub> removal through entrapment and/or sedimentation</li> <li>• evidence of a capacity for reliably producing consistently higher quality effluent (NH<sub>4</sub><sup>+</sup>-N, SS, BOD<sub>5</sub>/COD)</li> <li>• evidence to suggest good removal of soluble organics and nutrients – not just particulates as for rock filtration</li> <li>• significantly enhanced biofilm density, and hence potentially accelerated rate of treatment performance</li> <li>• very high specific surface area compared with rock filter media (1 order of magnitude greater or more)</li> <li>• significant reduction in 'dead' volume compared with a traditional rock filter (90–95% voids compared with 40–50%)</li> </ul>
<b>Disadvantages</b>
<ul style="list-style-type: none"> <li>• large quantities of attached biomass could exert a significant respiratory BOD, possibly leading to anaerobiosis under high organic loading or during periods of low dissolved oxygen (e.g. nighttime)</li> <li>• lack of positive control with respect to effluent quality (as for rock filtration)</li> <li>• lack of long-term large-scale performance data due to it being a relatively novel and under-researched technology</li> <li>• absence of rigorously established design criteria (for reasons as above)</li> <li>• inherently increased susceptibility to mass transfer limitations at high surface area to volume ratios</li> </ul>

#### **1.2.8.7.2 AGM for final effluent polishing: SS; BOD<sub>5</sub>; and nutrients abatement**

The majority of work involving AGM and WSPs has focused on the removal of dissolved nutrients or heavy metals, with only a limited number of studies reporting on SS and/or BOD<sub>5</sub> removal in AGWSPs (Shin and Polprasert, 1987; Peishi *et al.*, 1993; Zhao and Wang, 1996; McLean, 1999; McLean *et al.*, 2000) and even less specifically investigating algal solids removal (Shin and Polprasert, 1987; McLean *et al.*, 2000). Prior research has shown the significant capacity of AGM to improve conventional WSP

treatment. Whilst specific performance data is both scarce and also largely variable within the literature (according to differences in AGM specific surface area, media packing density, wastewater strength and volumetric loading regime), AGWSP systems always perform at least as well as, and in most cases better than, conventional control ponds. Specific performance data for SS removal efficiency in AGWSPs varies according to the operational criteria, but SS concentrations in AGWSP effluent are most commonly in the order of 5–45% lower than for conventional controls. Despite this relatively low volume of research effort into attached-growth systems, Pearson (1996, p. 3) concluded that there appears to be “*little doubt of its potential*” for enhancing WSP treatment efficiency. The current research therefore has a particular focus on SS and BOD<sub>5</sub> abatement in AGM systems when used in final WSP effluent polishing applications.

#### **1.2.8.7.3 Fixed-bed horizontal-flow AGM**

Because of the relatively concise body of research on the application of AGM to WSP systems, the technology is far from being fully established and well understood. In addition to being under-researched, much of the prior work has focused on non-fixed-bed ‘passive flow’ AGM, whereby discrete arrangements of non-rigid ‘strings’ of AGM are suspended within the WSP environment around which the water then flows in a random fashion. The only exception to this media arrangement has been the work of McLean (1999) and McLean *et al.* (2000). In this work, the AGM was indeed installed in a ‘fixed-bed’ arrangement (polypropylene sheets oriented vertically and longitudinally); however, this AGM extended only part of the pond depth and there was also no significant *horizontal* component to the added media. An obvious drawback to having a non-fixed-bed type media, is the requirement for additional support structures in order to maintain the AGM in suspension—a problem afflicting all prior research.

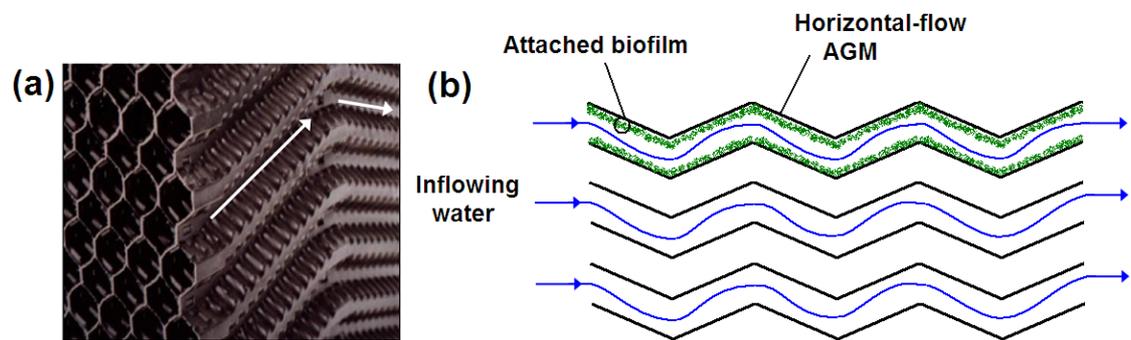
Following on from these realisations, an entirely novel form of AGM was investigated during this research for the upgrading of WSP effluent. The novel media selected for investigation during the current research was chosen based on it satisfying a number of preliminary selection criteria:

- low density and high void volume;



- rigid and self-supporting nature;
- high specific surface area for biological growth;
- horizontally- as well as vertically-oriented media planes.

The particular type of media chosen for advanced WSP treatment application as part of this research project is shown in Figure 1.5. It consists of a rigid polypropylene ‘honeycomb’ design and has traditionally been applied used in trickling biofilters rather than WSPs.



**Figure 1.5.** (a) Close-up view of a novel, horizontal-flow attached-growth media for use in WSP applications, and (b) schematic representation of the biofilm attachment and *in situ* horizontal-flow regime.

It was thought that this so-called ‘horizontal-flow’ AGM, despite having traditional application in non-submerged vertical-flow trickling filters, would be able to function under a fully submerged and horizontally-oriented arrangement within a WSP environment. In a similar way to other classical sedimentation devices such as ‘plate’ or ‘tube settlers’, horizontal-flow AGM systems might allow for an increase in sedimentation effectiveness by significantly increasing the relative settling area per unit volume (Korkut, 2003). In such systems, SS removal can also be assisted by the growth of biofilms on the media surfaces that can then assist in the attraction and retention of small particles (Characklis, 1990), as well as the potential for electrical charge interactions between infiltrating particulates and the media surface further encouraging particulate attachment (McDowell-Boyer *et al.*, 1986; Stevik *et al.*, 2004). Under this configuration, it was anticipated that the horizontal-flow AGM might therefore serve not only as supplemental physical support media for enhanced biological activity, but would also significantly increase the number of horizontal ‘sedimentation planes’ for enhanced

physical removal of SS particulate BOD<sub>5</sub>. In this sense, the horizontal-flow AGM, as well as being an active biofilter, may achieve effective solids retention in a similar way to that of a rock filter. Unlike a rock filter, however, the horizontal-flow AGM might offer the added advantages of a much greater specific surface area ( $150\text{m}^2\text{ m}^{-3}$  as opposed to around  $40\text{m}^2\text{ m}^{-3}$ ) and an approximate 100% increase in void space volume; resulting in a reduced dead volume and a subsequent increase in HRT for more effective treatment. To the author's knowledge, this is an entirely novel application for this type of biofilter media.

### **1.3 Local WSP systems**

Waste stabilisation ponds are used extensively throughout Australia (Mitchell, 1980). WSPs in South Australia in particular cover a total area of around 425ha (Mitchell, 1980), including over 180 so-called community waste management (CWM) schemes, and serve in total over 820,000 people (Palmer *et al.*, 1999; Buisine and Oemcke, 2003). Australia is particularly well suited to the adoption of WSP technology as a result of the high annual sunlight levels and also the relative abundance of expansive and inexpensive land—particularly in rural areas. Far and away the largest of these installations is located at the Bolivar WWTP 18km north of Adelaide, South Australia. Here the climate is Mediterranean, with hot dry summers (29°C/16°C) and temperate winters (16°C/7°C; Sweeney *et al.*, 2003).

#### **1.3.1 Bolivar WSPs**

Commissioned in three stages from 1964 to 1969, the Bolivar WWTP (Plate 1.2) treats effluent from a population equivalent of 1.3 million people, comprising domestic effluent from in excess of 700,000 people and industrial effluent representing a population equivalent of about 600,000 (Hine, 1988; Buisine and Oemcke, 2003). The treatment train incorporates a preliminary mechanical screening step followed by pre-aeration, grit removal and primary sedimentation. Effluent then undergoes activated sludge treatment, secondary-level clarification, and final tertiary-level treatment; with the wastewater then flowing into the largest WSP system in the Southern hemisphere. Spanning an area of 346 ha, the two parallel sets of three lagoons have a nominal operational depth of approximately 1.3m, allowing for a 30–35 day hydraulic residence

time at an average flow rate of around 145ML day<sup>-1</sup> (Hine, 1988; Buisine and Oemcke, 2003; Sweeney *et al.*, 2005a). Detail on the geometry and hydraulic configuration of the largest and most extensively studied pond in the Bolivar network (Pond 1) can be found in Sweeney *et al.* (2003) and additional information on Pond 1 can also be found in a number of publications by the same author (Sweeney *et al.*, 2005a; 2005b; 2007).



**Plate 1.2.** An aerial view of the expansive Bolivar WSP system, located north of Adelaide, South Australia (photograph courtesy of Keremane and McKay, 2006).

Australia is recognised as being one of the driest continents on Earth. Anderson (1996) highlighted the need for more diligent water management of both Australia's natural water allocation and also the requirement for better use, and reuse, of the precious resource—citing reclaimed wastewater initiatives among other potential future strategies for water conservation. Prior to 1999, 100% of the final WSP effluent from the Bolivar WWTP was discharged into the adjacent St. Vincent Gulf via a 13km long open ocean outfall channel. Since then, and following a commitment from the South Australian Water Corporation and local authorities to an Environmental Improvement Programme, the Bolivar WWTP was supplemented with a 150 ML d<sup>-1</sup> quaternary-level DAF/F plant. The AU\$55 million scheme draws tertiary effluent from the Bolivar WSPs and delivers high quality reclaimed water for use within the local horticulture industry and throughout the neighboring agricultural belt north of Adelaide. The Bolivar DAF/F plant, as part of Australia's largest high-quality reclaimed water reuse operation, delivers

high quality treated effluent to over 250 growers from approximately 200km<sup>2</sup> of surrounding agricultural land through the Virginia Pipeline Scheme (Huijbregsen *et al.*, 1999).

As part of an ongoing investigative research collaboration (between the South Australian Water Corporation, United Water International and numerous external collaborators), work by Boshier *et al.* (1998), Buisine and Oemcke (2003) and Martyn *et al.* (2004) has identified particular WSP phyto- and zooplankton species as having an adverse impact on DAF/F plant operation and performance (namely algal species of *Euglena*, *Chlorella* and *Chlamydomonas* as well as copepod and cladoceran zooplankton); with the presence of these organisms in the DAF/F influent resulting in enhanced and undesirable turbidity breakthrough, premature filter-bed clogging and advanced headloss accumulation. The presence of large numbers of these plankton populations has also necessitated some adjustments to DAF/F plant chemical dosing regimes in order to meet the relevant treated water quality criteria. These alterations to DAF/F treatment protocols have come in the form of an increase in the levels of chemical coagulant/polymer required to achieve the same level of treatment performance.

At the time of initial assessment, Buisine and Oemcke (2003, p. 363) deemed that such process alterations had “*proven efficient in treating the raw water to turbidity standards, but at a considerable chemical cost and to the sacrifice of the treated water pH requirement*”. Buisine and Oemcke (2003, p. 362) also suggested that in order “*to save on chemical use, and to ensure compliance with pH and aluminium levels as well as turbidity, we need to develop alternatives*”. Consequently, plankton community dynamics following treatment by the selected advanced in-pond treatment upgrades was monitored during the course of this research in order to assess the likely ecological effects of the various WSP upgrades and how they may in turn impact on down-stream DAF/F process performance.

Large quantities of suspended particulates and algal cells in pond effluents destined for agricultural reuse applications can impose significant problems for irrigation infrastructure networks; particularly low-flow drip-irrigation systems, where physical blockages can result (Teltsch *et al.*, 1992; Taylor *et al.*, 1995; Ravina *et al.*, 1997). In

addition to physical blockages of irrigation networks, high levels of algal biomass in reuse water increases the potential for re-growth in storage basins as well as increasing the required disinfection dosage and reducing disinfection residuals (Heidenreich *et al.*, 2004); something which can then lead to the undesirable growth of biofilms in distribution pipelines (Bosher *et al.*, 1998). Given that the Bolivar WSP effluent is typically highly variable in terms of both numeration and speciation of its algal populations (Heidenreich *et al.*, 2004), it can be appreciated that any WSP upgrade capable of stabilising the quantity and/or quality of planktonic biota in the final effluent would be of considerable value to on-site DAF/F plant operation.

### **1.3.1.1 Bolivar WSP plankton ecology**

Currently, phytoplankton population dynamics (i.e. total counts and species composition) in the Bolivar WSPs are unpredictable and have exhibited no discernible relationship to seasonal parameters (Heidenreich *et al.*, 2004; Martyn *et al.*, 2004). This is despite the fact that seasonal parameters, such as temperature and solar irradiance, are recognised to have a prominent and influential role in temporally regulating the seasonal periodicity of algal populations in the natural environment (Pearson, 1990; Tharavathi and Hosetti, 2003; Reynolds, 2006). The largely variable and unpredictable Bolivar WSP algal ecology was thought to have developed as a consequence of the relatively recent commissioning of an up-stream activated sludge plant in 2001 (Cromar *et al.*, 2005; Sweeney *et al.*, 2005). The activated sludge plant installation has resulted in a dramatic improvement in the overall treatment train efficiency and has significantly lessened the nutrient loading on the Bolivar WSPs. Whilst this resulted in an overall improvement in WSP performance and reduced the concentration of algal SS in final effluents, it has at the same time led to a more variable algal ecology, which in turn has led to unpredictable and often undesirable process ramifications for down-stream DAF/F treatment performance. A summary of some of the more commonly observed algal species within the Bolivar WSP network is given in Table 1.5.

**Table 1.5.** Typical phytoplankton species found in the Bolivar WSPs (modified from Buisine and Oemcke, 2003; Herdianto, 2003; and Martyn *et al.*, 2004).

<b>Taxonomic Phyla</b>	<b>Morphology</b>	<b>Motility</b>	<b>Gas vacuoles</b>
<b>Chlorophyta (green algae)</b>			
<i>Actinastrum</i>	Colony	Non-motile	No
<i>Ankistrodesmus</i>	Single cell	Non-motile	No
<i>Chlamydomonas</i>	Single cell	Motile	No
<i>Chlorella</i>	Single cell	Non-motile	No
<i>Closterium</i>	Single cell	Non-motile	No
<i>Coelastrum</i>	Colony	Non-motile	No
<i>Dictosphaerium</i>	Colony	Non-motile	No
<i>Micratinium</i>	Colony	Non-motile	No
<i>Oocystis</i>	Colony	Non-motile	No
<i>Scenedesmus</i>	Colony	Non-motile	No
<b>Euglenophyta</b>			
<i>Euglena</i>	Single cell	Motile	No
<b>Cyanobacteria</b>			
<i>Arthrospira</i>	Filamentous	Non-motile	Yes
<i>Lyngbya</i>	Filamentous	Non-motile	No
<i>Microcystis flos-aquae</i>	Colony	Non-motile	Possible
<i>Phormidium</i>	Filamentous	Non-motile	No
<i>Planktothrix</i>	Filamentous	Non-motile	Yes
<i>Pseudanabaena</i>	Filamentous	Non-motile	No
<b>Chrysophyta (golden algae)</b>			
<i>Mallomonas</i>	Single cell	Motile	No
<b>Chryptophyta</b>			
<i>Chroomonas</i>	Single cell	Motile	No
<i>Cryptomonas</i>	Single cell	Motile	No
<b>Bacillariophyta (diatoms)</b>			
<i>Cyclotella</i>	Single cell	Non-motile	No
<i>Navicula</i>	Single cell	Non-motile	No

This natural variability and unpredictability in the *in situ* plankton community structure has made it difficult to develop and optimise DAF/F plant treatment regime in terms of the optimal chemical dosing strategy required for a given influent quality (Heidenreich *et al.*, 2004). In an attempt to manage this algal problem, actively combatant strategies need to be developed and implemented within the WSP environment. In order to achieve this dynamic management approach, there is firstly a need for fusion of the two treatment processes. Indeed it has been suggested that “*if we treat the WSPs as the first stage of the DAF/F plant, it is likely that we can manipulate them to alter the species composition of the lagoons*” (Buisine and Oemcke, 2003; p. 362). One can appreciate that when these treatment stages are treated and operated independently, there is little scope for interactive operational modifications; since each treatment step is seen simply to function as a discrete process. What is required instead, is more active consultation

between DAF/F plant and WSP operators to allow for performance feedback as to “what works” in terms of WSP effluent and DAF/F treatment performance, and just as importantly “why?”

The heightened algal variability within the Bolivar WSP network is probably also a consequence of the reduced trophic state within the WSPs, whereby they have gone from a consistently nutrient-rich ‘hypereutrophic’ pre-activated sludge plant state, to a situation where they are now commonly situated at the lower bounds of the ‘eutrophic’ classification (Carlson, 1977). In other shallow freshwater environments, for example, a reduction in nutrient abundance and corresponding trophic state (from hypereutrophic downward) is commonly accompanied by an increase in phytoplankton species richness and diversity (Watson *et al.*, 1997; Olding *et al.*, 2000; Romanuk *et al.*, 2006) such that this could go partly toward explaining some of the increased variability in resident algal populations. In actuality, the installation of the activated sludge plant has been so effective at reducing the nutrient load on the WSP network, there is evidence to suggest that the Bolivar WSPs at times are nutrient (N)-limited (Cromar *et al.*, 2005).

Considering the above operational issues surrounding the Bolivar WSP network, one might be justified in asking the question “*Why not bypass the pond system and avoid the problem of undesirable algal and zooplankton growth all together?*” The answer as to why the WSPs remain a desirable inclusion in the Bolivar WWTP is that they provide invaluable and advanced pathogen disinfection, whilst at the same time serving as a large water storage reservoir (4 gigalitres) for the maintenance of an hydraulic buffer upstream of the DAF/F plant during summer periods of peak water demand (Huijbregsen *et al.*, 1999; Sweeney *et al.*, 2005a).

### **1.3.1.2 Active management strategies for the Bolivar WSPs**

As discussed above, it is theoretically possible that there may be potential capacity for ‘active control’ of the WSP environment in order to bring about a more favourable final effluent quality. Several interventionist strategies were outlined by Buisine and Oemcke (2003) as possibilities for achieving this prospective manipulation of WSP effluent (and

hence DAF/F influent) quality and bringing about positive-control in terms of the plankton community structure present in final WSP effluents. These included:

1. variable depth effluent off-takes to manage problem algae that are motile and can preferentially ‘float’ at or near the surface (such as *Euglena* species), so as to avoid taking them into the DAF/F plant in high numbers;
2. alternating between WSP outlets to suit prevailing wind conditions (as certain wind conditions may concentrate these organisms around particular outlets of the WSP);
3. changes in WSP habitat, such as increasing the available surfaces for grazing protozoa and zooplankton;
4. shading with a synthetic structure or with floating macrophytes;
5. mixing the whole lagoon, or mixing near the outlet; or,
6. depth-profiling of the WSP.

Option 1 above has been investigated previously by Herdianto (2003) and was found not to be a viable management strategy for the reduction of effluent algal solids in the Bolivar WSPs. Options 3 and 4 in the above list have also been underlined because they encompass the particular areas of interest for this research (i.e. rock filtration, attached-growth media addition and duckweed surface coverage).

Since duckweed ponds are generally recognised to produce effluents with low concentrations of SS (Körner *et al.*, 2003), it is anticipated that the technology could offer significant potential for reducing the treatment load on the Bolivar DAF/F plant. In fact, the use of duckweed for tertiary-level post-treatment is a recognised technology in the United States (Alaerts *et al.*, 1996), with the U.S. Environmental Protection Agency approving it as an innovative/alternative wastewater treatment technology (Ngo, 1987). Following an extensive literature search, it was discovered that there exists very little local work on the use of duckweed in wastewater treatment applications. This is in spite of the concept having been proposed some 20 years ago by Hine (1988) as a means of controlling high concentrations of algal SS in the Bolivar WSP effluent.

Leng (1996)—reporting on pilot investigations performed in the Hunter Valley of New South Wales—concluded that duckweed (*Spirodela* and *Lemna* species) displayed a



significant capability for final wastewater polishing with respect to nutrient removal. The author also concluded that since their work was conducted during the summer and autumn months, more work was required in order to evaluate how duckweed performs *in situ* during the Australian winter. Kumar and Sierp (2003) investigated the potential role of *Lemna* for nutrient (N and P) removal and also looked at the effect of duckweed on suspended algal populations. Although this research was conducted locally, results of the short-term (15 day) batch reactor studies were mixed and somewhat dubious, and overall the duckweed ponds showed no significant advantage over standard control ponds in terms of both their nutrient removal potential and capacity for algal attenuation. The work of Kumar and Sierp (2003) is, therefore, not expected to be an adequate predictor of the prospective *in situ* performance of advanced duckweed pond upgrades at Bolivar.

It should be noted also that Option 4 in the above list, in addition to floating plants, includes the concept of a synthetic structure for shading the WSPs. Obviously, an opaque surface coverage of any type (artificial or natural) will restrict the growth of algae in WSPs. It should be stated that there are indeed commercially available synthetic systems for covering WSPs of varying sizes (e.g. the 4 ha floating ‘geomembrane’ cover of DeGarie *et al.* (2000) and the 0.3 ha suspended shade-cloth coverage of Hunter (2002). Whilst these artificial covers could be seen as offering a highly practical solution for managing algal populations in smaller WSPs, it could also be argued that synthetic options, such as those above, would be less practical and possibly even prohibitively expensive for very large-scale WSP systems (such as the 346 ha Bolivar WSP network). In addition to this, the two-dimensional, inert, synthetic surface coverage would not be expected to play as big a role in supplemental biological treatment as would a living duckweed cover; although, this could actually be considered advantageous in instances where the routine harvest of plant biomass represents an undesirable operational burden. Synthetic surface covers were, therefore, not tested as part of this research.

To the author’s knowledge, there have been no prior local investigations involving the use of either rock filters or AGM for the upgrading of final WSP effluent. It should be noted that both floating macrophyte systems and rock filtration have actually been assessed side-by-side elsewhere (on paper) for their potential application as *post hoc* upgrades of final WSP effluent with specific emphasis on algal solids removal (Neder *et*

*al.*, 2002) and were found to be the top ranking, or most preferable methods from a number of candidate upgrades (i.e. rock filtration, sand filtration, floating macrophytes, constructed wetlands and overland flow). The addition of a rock filter or AGM to the Bolivar WSP environment would fall under ‘Option 3’ in the above list of prospective pond alterations. It is anticipated that both of these upgrade methodologies would constitute a manipulation of physical WSP habitat, with potential flow-on biological alterations through the provision of significant additional substrate for colonisation by grazing protozoa and metazoa. This sort of instigated ‘biomanipulation’ (a term coined originally by Shapiro *et al.*, 1975) of resident pond biota has in fact been cited by Sweeney *et al.* (2005) as a potential management strategy for the optimisation of Bolivar WSPs and hence DAF/F performance.

Bayley *et al.* (2001) reported on the potential success for active biomanipulation of algal population ecology within a water supply reservoir (through the promotion of zooplankton grazing) as a means of managing nuisance algal blooms and limiting the occurrence of excessive and expensive blockages during sand filtration. It is possible that a similar biomanipulation of phyto- and zooplankton populations could be achieved through modification of the physical environment within the Bolivar WSPs, with the ultimate goal being a more favourable DAF/F influent quality for optimal process performance. It is likely that the installation of a duckweed cover, or similarly the addition of AGM or a rock filter, will result in some shift in plankton community structure. The real question lies in whether this manipulated effluent ecology will be *favourable* or *antagonistic* with respect to DAF/F efficiency. Despite the recognised problematic nature of some zooplankton taxa (crustacean copepods and cladocerans) to DAF/F plant process performance, there has so far been no prior assessment of zooplankton population ecology within the Bolivar WSPs (Buisine and Oemcke, 2003). This is despite Martyn *et al.* (2004) highlighting the importance of zooplankton grazing in terms of the negative pressure they exert upon algal populations at Bolivar. Algal and zooplankton population dynamics were therefore monitored during this research as part of routine performance assessments of investigated Bolivar WSP upgrades.

Finally, since WSPs are to a large extent biological treatment reactors, any ecologically-minded in-pond interventions aimed at ultimately managing the pond’s ecology are also

expected to be more cost-effective in the long term (Rao, 1986; Craggs *et al.*, 1996; Buisine and Oemcke, 2003). The use of macrophyte treatment systems in particular, is thought to offer significant operational cost-benefits; with Hofman and Harusi (2001, cited in Zimmels *et al.*, 2004) citing the cost of macrophyte systems for wastewater treatment in Israel at approximately half that of other quaternary-level treatment processes. This also agrees with the work of Oswald (1988a) who suggested that for each additional stage in the wastewater treatment process (i.e. primary, secondary, tertiary, etc.), the relative cost of each subsequent treatment step approximately doubles. Considering this, it makes economic sense to direct any process alterations toward the front end treatment stages (i.e. WSPs) rather than those further along the treatment chain (i.e. DAF/F). This, therefore, serves as additional economic grounds for these investigations into up-stream in-pond process upgrades at the Bolivar WWTP.

### **1.3.2 Local community waste management (CWM) schemes**

In addition to large-scale centralised wastewater treatment operations, South Australia also has a significant number (>180) of smaller decentralised wastewater treatment facilities serving the needs of regional communities. Historically, these country townships were served exclusively by on-site septic tank soakage trench systems. However, since 1962, and following the inadequacy of these systems to cope with increasing wastewater loads as well as the growing public health concern, centralised CWM schemes have become increasingly popular, to the point where they now service almost all South Australian towns (Palmer *et al.*, 1999). Wastewater treatment in these CWM schemes is achieved primarily via secondary-level WSPs; however, there have also been a number of more recently developed CWM reuse schemes, some of which recycle 100% of the treated effluent for purposes such as irrigation and wetland development (SAEPA, 2003).

In these systems, the issue of upgrading effluent prior to discharge into a receiving water body is of less relevance due to an ever-increasing volume of wastewater being demanded for reuse applications. In this instance, the removal of SS, BOD<sub>5</sub> and also nutrients is considered to be of lesser importance (in terms of its eutrophication potential) since the effluent is most likely destined for land application. In fact, the

presence of SS, particulate organic BOD<sub>5</sub> and nutrients might actually be seen as desirable—serving as soil conditioners in an agricultural setting. Instead, the necessity for upgrading of final WSP effluent (with respect to the above parameters) gains relevance from the negative effects they can have on the final disinfection process, especially for the high-grade ‘Class A’ and ‘Class B’ recycled effluents (SAEPA, 1999). In this sense, the upgrading of small-scale CWM WSPs could offer potential cost benefits in terms of both a reduction in the expenses associated with effective solids removal and disinfection, and also from increased revenue as a result of the production of a higher grade recycled effluent.

It is possible, therefore, that potentially viable WSP upgrade methodologies trialed at the Bolivar WWTP may find future relevance and application to these local CWM systems, particularly for the final upgrading of WSP effluent prior to reuse scenarios. It can be appreciated that any supplemental in-pond upgrading (primarily SS removal) of final WSP effluent prior to tertiary-level treatment and reuse, will have the potential to offer cost savings through a reduced solids load on these tertiary-level treatment procedures. Following reporting on the experimental performance of the selected in-pond upgrades, discussion of results will also include a discussion of any prospective applications of the advanced technologies for the upgrading of local CWM schemes.

## **1.4 Thesis questions, objectives and research design**

It has been firmly established throughout the course of this introductory chapter that significant quantities of algal biomass in the final effluents of WSPs are a major problem, not only for treatment plant operators, but also for the receiving aquatic waterways and their ecosystems. Numerous and varied methodologies have been trialed in order to try and circumvent or remedy this operational drawback, with varying degrees of success and costs associated with each technology. Based on a thorough literature survey, and considering the constraints imposed particularly with respect to the use of exotic macrophyte species, this thesis aims to assess and characterise the performance of three advanced WSP upgrade technologies at the local Bolivar WWTP: rock filtration; duckweed; and attached-growth media; by comparing them in parallel to what is essentially a non-interventionist ‘control’ on a pilot scale. These three in-pond

removal technologies have been selected because they represented relatively low-tech and low-cost solutions to the operational problem at hand.

To the best knowledge of the author, this thesis represents the first direct comparison between two previously well-researched WSP upgrades (rock filtration and duckweed surface coverage). The proposed research will also provide the first assessment of rock filters, attached-growth media and duckweed ponds for the upgrading of tertiary-level maturation WSP effluent, with specific emphasis on the capacity of each effluent upgrade system for algal and zooplankton solids removal. Work presented in this thesis will also include the first known performance assessment and characterisation of a novel *in situ* horizontal cross-flow AGM for the upgrading of a WSP effluent.

In addition to experimental monitoring and performance assessment of pilot-scale WSP upgrade methodologies, another branch of the research project was conceived in order to investigate some additional research questions regarding the fate of algal cells within these advanced in-pond upgrade environments. A series of laboratory experiments were designed in an attempt to recreate *in situ* conditions (in terms of light and oxygen availability) that might exist within a rock filter or under a duckweed surface mat for example. Using culture isolates of common WSP algal species, long-term monitoring of the physiological status of algal cells during dark-exposure under conditions of reduced oxygen availability will be performed in order to quantify the likely effect(s) of these particular environmental conditions on *in situ* survival. This part of the overall thesis work will be introduced in Chapter 6, followed by an overview of experimental methods (Chapter 7) and finally presentation and discussion of these results in Chapters 8 and 9.

### 1.4.1 Thesis questions:

1. What are the relative treatment efficacies of a duckweed surface coverage, rock filtration and attached-growth media—compared with each other as well as a non-interventional open ‘control’—for the final upgrading of the Bolivar WSP effluent in respect of algal and zooplankton solids removal?
2. Are there likely to be any additional treatment outcomes, such as enhanced organics/BOD<sub>5</sub>, turbidity, nutrient, and/or pathogen removal, as result of advanced in-pond upgrade treatment with duckweed coverage, rock filtration or attached-growth media systems?
3. What are the likely physiological effects of advanced in-pond upgrade treatment on algal viability and dark survivorship *in situ*? Furthermore, what are the practical implications of experimental algal dark-survival for the advanced WSP upgrade systems, and in particular, what is the likely duration of treatment exposure necessary for effective attenuation of suspended algal populations?
4. What are the likely ecological effects of the investigated advanced in-pond upgrade technologies, in terms of their direct influence on post-treatment phyto- and zooplankton community structure? Additionally, are these changes in WSP effluent plankton ecology likely to be advantageous or antagonistic with respect to Bolivar DAF/F plant performance efficiency?

## **2 Experimental pilot plant construction, characterisation, operation and performance monitoring**

This methods chapter provides details of the experimental pilot plant, as well as outlining the general analytical methodology used for pilot plant performance monitoring throughout this research. Details of pilot plant construction and configuration, hydraulic operation and characterisation, water quality analyses, and the relevant statistical analyses are provided.

### ***2.1 Pilot plant design and characterisation***

An experimental pilot plant was constructed at the Bolivar WWTP (34°45'28"S 138°34'15"E) situated approximately 18km north of Adelaide, South Australia at around 10m above sea level. For a comprehensive description of the Bolivar WWTP, refer to Section 1.3. The pilot plant was located 50m directly adjacent to the Bolivar DAF/F plant at the final discharge end of the WSP network (see Plate 2.1). This location was initially selected to ensure that pilot plant influent would be as close as possible to that of the DAF/F plant. This allowed for some use of routine DAF/F influent water quality data, as well as providing the capacity for direct comparisons between pilot and DAF/F plant performance.

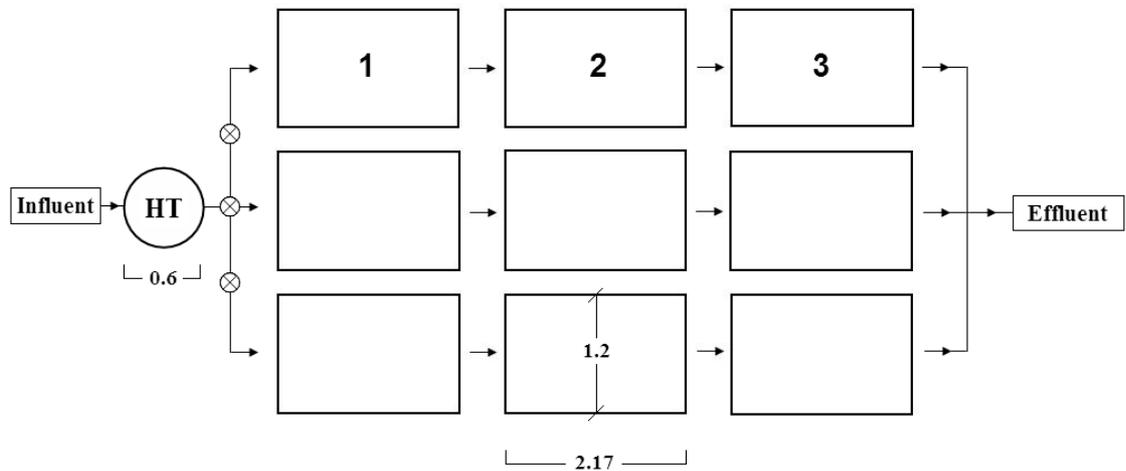


**Plate 2.1.** Aerial view of the Bolivar WWTP (top left) showing the pilot plant location, and inset, an up-close aerial view of the Bolivar DAF/F plant, inlet sump and pilot plant location (photographs courtesy of United Water International and Google Earth; <http://earth.google.com>).

The pilot plant consisted of nine ponds arranged in three parallel series (represented schematically in Figure 2.1). Individual pilot ponds consisted of open, above-ground, rectangular, high-density polyethylene (HDPE) vessels (Bushman Tanks, South Australia) encaged within a steel support frame. Individual pond dimensions were 2.17m long by 1.2m wide by 1.1m high, with an effective gross volume of approximately 2.8m<sup>3</sup>. Ponds were operated to a hydraulic depth of 1m, giving them an operational

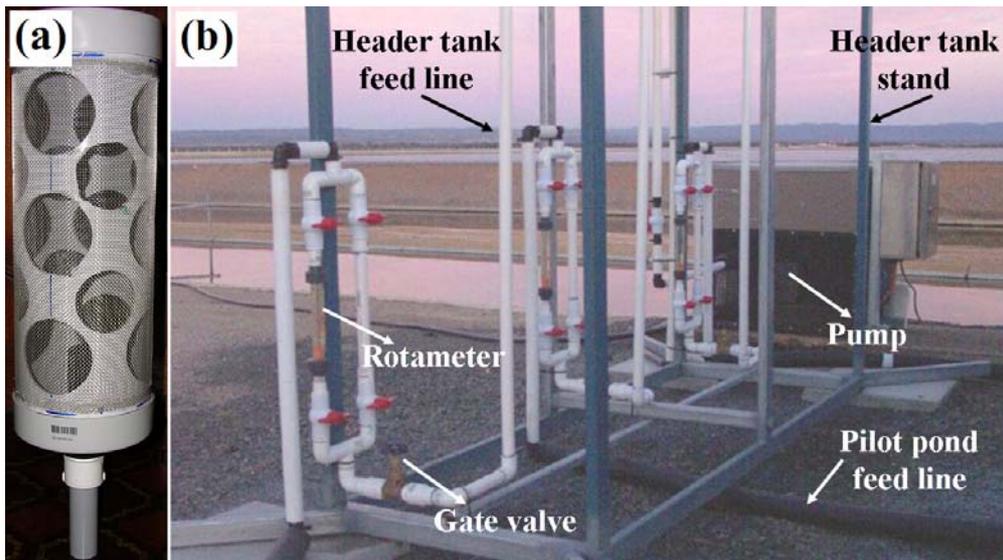


hydraulic volume in the order of  $2.56\text{m}^3$  (accounting for small volumetric losses from curved internal corners and molded supporting ridges).



**Figure 2.1.** Schematic of the experimental pilot plant system, showing: the header tank (HT); multiple pond layout with down-the-line pond numbering format; and hydraulic configuration (dimensions given in metres).

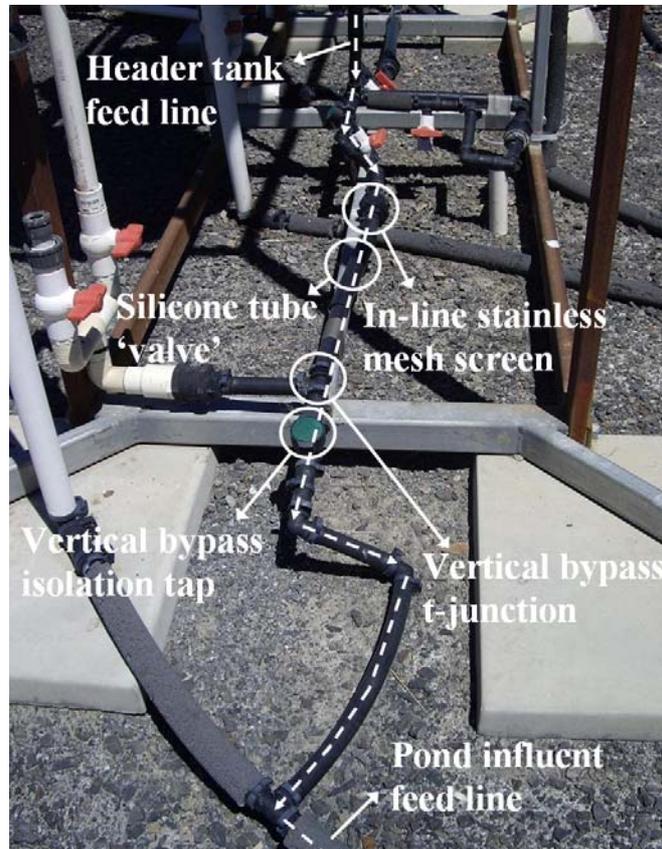
Water was drawn from the DAF/F inlet pumping sump (refer Plate 2.1) and pumped to a small ( $\approx 60\text{L}$ ) pilot plant header tank via a 70m length of underground pipe (25mm diameter) and with the aid of a Bredel<sup>®</sup> SP/25 peristaltic hose pump connected to an SEW-Eurodrive gearmotor and variable-speed controller. From the header tank, influent wastewater flowed under gravity through a 2mm passive inlet screen located within the header tank (Plate 2.2(a)) and then to each of the three parallel treatment pond series through a network of 25mm diameter polyvinyl chloride (PVC) and low-density polyethylene piping. The initial ‘Phase 1’ plumbing configuration between the header tank and first pond in each parallel treatment series is shown in Plate 2.2(b). Under this design, flow rate to each of the treatment series was controlled using a small-bore gate valve and an inline variable-area (rotameter) flow meter (SK71, George Fischer).



**Plate 2.2.** (a) 2mm stainless steel passive influent screen, and (b) detail of the pilot plant influent feed piping under the initial ‘Phase 1’ configuration.

Under Phase 1 configuration, significant and ongoing interruptions to treatment pond inflow were experienced during pilot plant commissioning and startup. This was largely due to physical blockages (predominantly by small snails) of the narrow aperture within the flow regulating gate valve (see Plate 2.2(b)). Troubleshooting of the problem led to a change in the initial Phase 1 piping design and configuration. The 2mm passive header tank inlet screen was added in order to reduce the number of larger objects taken into the influent feed lines. Also, the sharp-edged gate valve was replaced with an in-line 10cm length of narrow bore silicone tubing around which a small hose clamp was fastened. This ‘Phase 2’ valve design allowed for a more uniform and circular valve aperture (not unlike the operation of a camera aperture) through which the flow rate could be readily and accurately adjusted by tightening or loosening the hose clamp. Additionally, a smaller 25mm bore size stainless steel mesh screen (2mm aperture) was inserted just prior to in-line silicone tube ‘valves’ so as to prevent any dislodged snails (which may have been growing within the  $\approx 2\text{m}$  length of piping between it and header tank) from blocking the silicone valves. This Phase 2 influent configuration (Plate 2.3) all but alleviated the problem of valve blockages experienced under the Phase 1 setup. As a precautionary measure, however, flow controlling valves were regularly opened to maximum flow rates in order to achieve flushing of inlet plumbing and avoid the unwanted accumulation of large particulates and spent snail shells within the influent feed piping. Phase 2 configuration also included a vertical flow rotameter bypass in

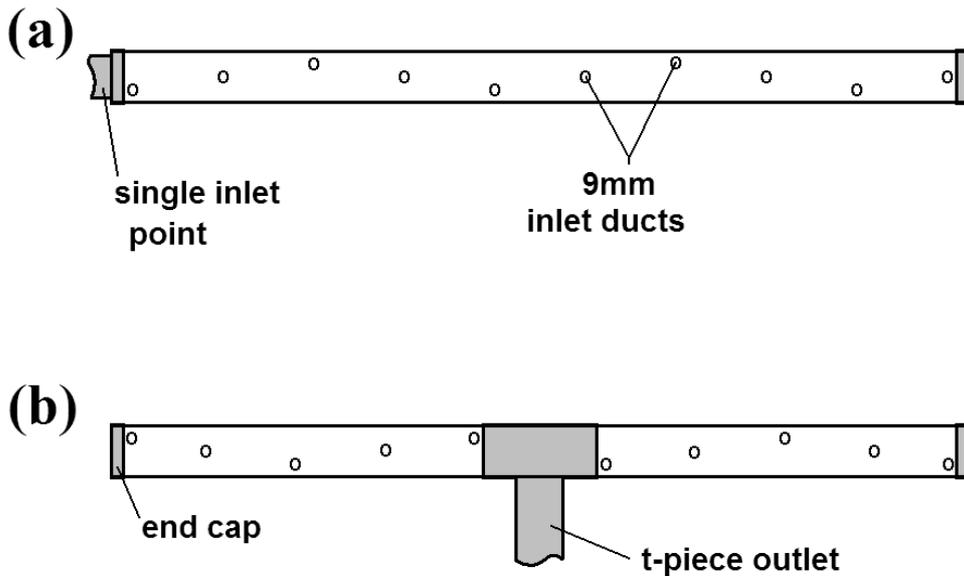
order to shorten the flow path length from the header tank to the first pond in each series. This flow bypass could again be redirected via the rotameter during periodic flow rate checks, by simply switching the bypass isolation tap to the ‘off’ position.



**Plate 2.3.** Detail of the pilot plant influent feed piping under the modified ‘Phase 2’ configuration (broken arrows show the direction of flow).

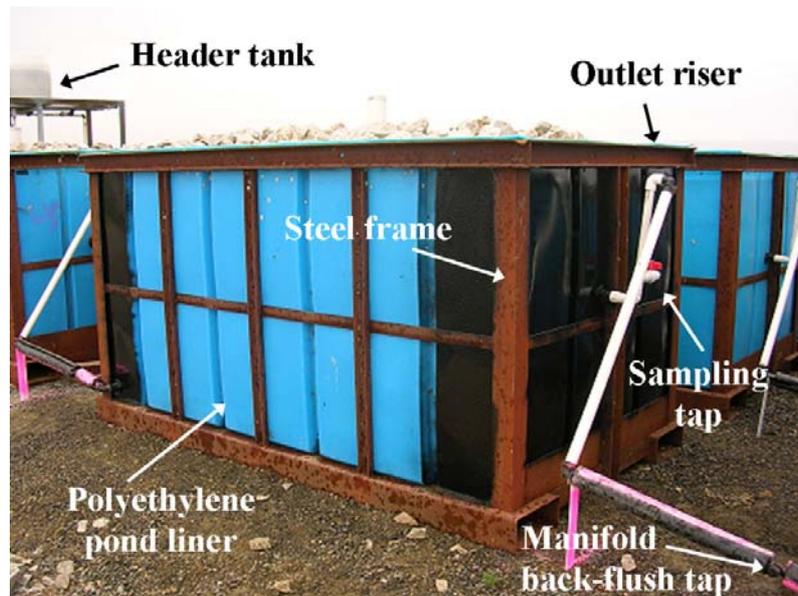
Individual pond influent flowed into each pilot pond through a horizontal influent manifold, shown schematically in Figure 2.2(a) below. Individual pond inlet and outlet manifolds consisted of horizontal 1m lengths of 25mm diameter PVC pipe containing 10 equidistant 9mm diameter hydraulic ‘ducts’ running the entire pond width. Inlet manifolds had one hydraulic input and were located on the pond bottom as close as possible to the anterior pond wall. Outlet manifolds were identical to inlet manifolds except they had a single central ‘t-piece’ hydraulic output and were located on the most posterior wall and ‘mid-pond’ at a depth of 40cm below the water surface (Figure 2.2(b)). They also had 9mm ducts drilled into both the top and bottom of the outlet manifold piping such that each outlet manifold contained 20 hydraulic ducts as opposed

to 10 for inlet manifolds. This inlet–outlet design was chosen in accordance with the hydraulic design recommendations of both Pearson (1990) and Metcalf and Eddy (1991). Ponds in each of the three parallel series were operated by gravity feed from one pond to the next, such that the effluent from the first pond in each series served as the influent for the second, and so on.



**Figure 2.2.** Schematic representation of an individual pond inlet (a) and outlet (b) manifolds showing arrangement of the inlet and outlet hydraulic ducts.

Water depth in each pilot pond was maintained at 1m through the use of an outlet riser pipe (Plate 2.4). Influent manifolds were periodically back-flushed via a tap in the pond influent feed line (Plate 2.4) in order to prevent manifold blockages and maintain original hydraulic flow conditions. Where appropriate, pond effluent samples were collected from the effluent manifold sampling tap as shown in Plate 2.4. Also, when required, outlet manifolds were periodically flushed clean by opening the sampling tap to maximum flow for a short period.



**Plate 2.4.** Posterior view of a pilot pond (2<sup>nd</sup> in series), showing the polyethylene pond liner, supporting steel frame, and outlet piping configuration.

### 2.1.1 Pilot plant experimental treatments

As introduced in Chapter 1, this research set out to assess four experimental treatments used for the upgrading of final Bolivar WSP effluent. These treatments were: duckweed surface coverage (DW); quiescent impoundment in an ‘open pond’ (OP); rock filter (RF) treatment; and a novel horizontal-flow attached-growth media (AGM). Given that there were only three parallel pilot pond treatment trains available at any given time, the four experimental treatments were staggered, such that the first period of pilot plant operation (*Period 1*) was performed under DW–OP–RF configuration (Plate 2.5) followed by a second operational duration (*Period 2*) AGM–OP–RF (Plate 2.6). These two staggered operational periods occurred over an approximate one year period from July of 2005 until August of 2006 and encompassed two discrete influent flow rates and hydraulic loadings; shown below in Table 2.1. There was a one month period of operational downtime during January of 2006, during which time the AGM treatment was phased in to replace the DW pond series. No pilot plant monitoring occurred during this time.



**Plate 2.5.** Elevated view of the experimental pilot plant operating under experimental *Period 1* (from left to right): Duckweed, Open Pond, and Rock Filter treatment configuration.



**Plate 2.6.** Elevated view of the experimental pilot plant operating under experimental *Period 2* (from left to right): Attached-Growth Media, Open Pond, and Rock Filter treatment configuration (picture taken during a filamentous algal bloom in the Open Pond series).

**Table 2.1.** Pilot plant operational calendar for monitoring *Period 1* and 2 for all four experimental treatments: Duckweed (DW); Rock Filters (RF); Open Pond (OP); and Attached-Growth Media (AGM). Shading indicates treatment configuration during each monitoring period.

		Monitoring <i>Period 1</i> – 2005						Monitoring <i>Period 2</i> – 2006							
		Hydraulic loading – $0.73\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$						Hydraulic loading – $1.03\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$							
		July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.
Experimental treatment	DW														
	RF														
	OP														
	AGM														

### 2.1.1.1 Duckweed treatment

The DW treatment comprised a series of three pilot ponds through which Bolivar WSP effluent passed under a dense floating ‘mat’ of the native duckweed *Lemna disperma* Hegelm (AKA *Lemna minor* L. prior to 1983; Plate 2.7). Each DW pond was initially inoculated with a similar wet weight of plant biomass from a culture stock, after which the duckweed was allowed to acclimate and multiply. Experimental monitoring was not commenced until complete surface coverage was established (in the order of 1–2 weeks) and duckweed biomass was never harvested at any time during the study duration.



**Plate 2.7.** Photograph of the established *L. disperma* surface mat on a pilot duckweed pond, and inset, a more detailed view of the floating duckweed mat structure.

### **2.1.1.2 Open Pond treatment**

The Open Pond treatment train consisted of an identical three-pond series to that of the DW system, except that the pilot ponds remained un-covered. It was envisaged that the operation of the OP series would most closely reflect the functional performance of a classical ‘algal-based’ pond system. Whilst this OP train was not an experimental control in the true sense, it allowed for effective ‘control’ or quantitation of the relative treatment performance of a pilot pond system with no direct experimental intervention *per se*. In other words, the OP series served principally as a means of controlling for the effects of ‘quiescent impoundment’ of the inflowing wastewater *without* any additional treatment intervention. As a result, it was assumed that any additional performance achieved by the other three treatments (above and beyond that of the parallel OP series) could reasonably be attributed to the implementation of that particular treatment intervention. Consequently, treatment performance of the pilot DW, RF and AGM upgrade systems is discussed in Chapters 3 and 4 relative to that of the OP ‘control’ series.

### **2.1.1.3 Rock filter treatment**

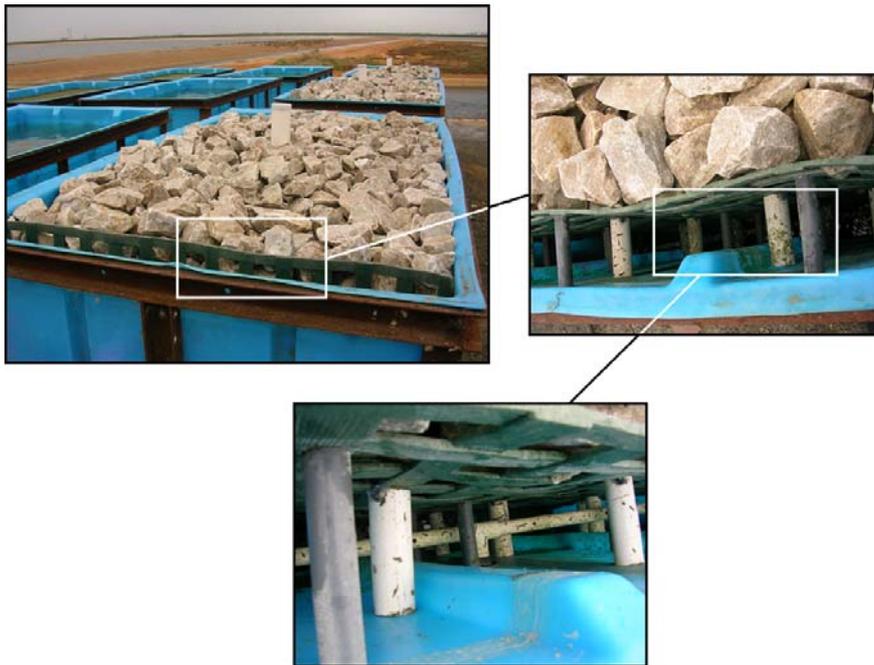
The RF treatment series consisted again of three parallel pilot ponds, each filled with a coarse aggregate ( $\approx 10$ cm diameter) quartz rock media to a total bed depth of  $\approx 1.15$ m. The hydraulic operating depth of 1m allowed for a 15cm ‘dry zone’ above the water surface within the rock filters. This surface dry zone is recommended as a means of discouraging filamentous algal and/or cyanobacterial colonisation of the exposed rock surfaces (Swanson and Williamson, 1980; Ellis, 1983). The particular rock media size distribution used in our rock filters was similar to that used most commonly in the United States (Middlebrooks, 1988). It should be stated, however, that although some of the more recent rock filter research has adopted a considerably smaller rock aggregate size distribution ( $\leq 5$ cm; Mara *et al.*, 2001; Johnson and Mara, 2002), a larger aggregate size rock media was utilised in the current research due to it being readily available at the Bolivar WWTP as a result of full-scale trickling filter decommissioning.

According to Metcalf and Eddy (2002), the specific surface area of ‘river rocks’ with a size distribution and void volume similar to those used in the current work (7–15cm rock diameter and 60% voids) is  $45\text{m}^2\text{ m}^{-3}$ . Also, based on the data of Saidam *et al.* (1995),



the corresponding specific surface area for rock aggregate with an approximate size distribution of 10cm is in the order of  $32\text{m}^2 \text{m}^{-3}$ . Since no attempts were made to quantify the specific surface area of the actual rock media used in this research, an average of these two figures was taken; yielding a final specific surface area of approximately  $39\text{m}^2 \text{m}^{-3}$ . This figure was, therefore, adopted as the approximate specific surface area of the pilot rock filters in this research.

The hydraulic design of the rock filters in the current work differed slightly from that of previous studies. Previous researchers have used rock filters with single inlet and outlet ducts, commonly located at the bottom and top of the filter bed respectively (Johnson *et al.*, 2007), and covered completely by the rock media. Since others have commented on performance problems associated with poor hydraulic flow patterns and short-circuiting in some rock filters (Hirse Korn, 1974; Swanson and Williamson, 1980; Middlebrooks, 1988), it was thought desirable to include inlet and outlet ‘mixing chambers’ in the current filter design as a way of minimising inlet jetting and promoting more optimal flow hydraulics; especially given the short flow path length in each filter unit. These mixing chambers were created by installing vertically positioned HDPE lattice sheets, to which cylindrical PVC supports were attached in order to effectively retain the rock media away from the pond ends. These mixing chambers effectively excluded the rock media from  $\approx 15\text{cm}$  of the pond length at both ends of the filter, and can be seen below in Plate 2.8. It was envisaged that these mixing chambers would promote a more uniform distribution of the influent wastewater throughout the entire filter bed depth than would be achieved with a single hydraulic input at the base of a solid rock media bed. It can be appreciated that this was a somewhat *permanent* design inclusion and no effort was made to test rock filter flow hydraulics with and without the mixing chambers in place.



**Plate 2.8.** Detail of the rock filter mixing chamber design, showing the placement of the retaining lattice and PVC supports.

Exposed ends of each rock filter were coated with black paint (refer Plate 2.4) in an attempt to minimise the extent of illumination in inlet and outlet mixing chambers through the partially translucent HDPE pond liner. Mixing chambers were, however, left uncovered, such that they were periodically exposed to a limited amount of incident sunlight from above only. Each of the three RFs were fitted with a single, central, vertically positioned, perforated cross-flow sampling port to allow for *in situ* water quality analysis. Opaque PVC caps were installed over the top of the *in situ* sampling ports to prevent any unwanted obstructions or filamentous algal growth from occurring. Positioning and design of the *in situ* sampling ports can be seen in Figure 2.3.



**Figure 2.3.** Three-pond rock filter series layout showing *in situ* sampling port location with PVC covers in place, and inset, schematic detail of the *in situ* perforated sampling port design and dimensions.

In order to later calculate the ‘void space’ volume (i.e. water volume) within the RFs, a displacement test was carried out on the rock media. To do this, a  $0.1\text{m}^3$  plastic bin was filled to the top with rock media and then filled completely with tap water. The rocks were then carefully removed and the volume of water accurately measured with a graduated measuring cylinder; a slight correction factor for water remaining on the rocks was also applied. The void volume of the pilot RFs was then calculated by extrapolating this volume of water found within  $0.1\text{m}^3$  of rock media. Given that the rock media did not occupy the inlet and outlet mixing chambers, a correction factor was again applied in order to give the total rock filter void volume. Final void volume was then expressed as a percentage of the gross hydraulic operating volume (see Table 2.2).

#### **2.1.1.4 Fixed-bed horizontal-flow attached-growth media**

The attached-growth media treatment train consisted of an identical three-pond series to that of the OP system, except that the pilot pond reactors were almost entirely filled with horizontal-flow AGM. The specific type of AGM used was ‘TKP-319’ polypropylene fill media (2H Plastics, Victoria Australia). The AGM had a 19mm channel width and a specific surface area of  $150\text{m}^2\text{ m}^{-3}$  (Plate 2.9), giving each AGM pond an available media surface area in the order of  $340\text{m}^2$ . The media consists of numerous layers of 0.3mm thick corrugated polypropylene sheets, each welded together to form a

‘honeycomb-like’ matrix of flow-through channels. The media itself is lightweight, rigid and self-supporting; with a very high void space volume (95% v:v).



**Plate 2.9.** Up-close and structural views of the TKP-319 horizontal-flow attached-growth media (pictures courtesy of 2H plastics; <http://www.2h.com.au>).

The AGM reactors were similar to RFs in terms of their internal ‘packing’. AGM reactors had identical 15cm long inlet and outlet mixing chambers at either end; although no retaining lattice was required in the AGM reactors due to the self-supporting structure of the artificial media. AGM reactors also had centrally located sampling ports cut into the media. These *in situ* sampling ports—as for the RFs—extended the entire 1.1m media bed depth of the pilot pond reactors, and were again kept covered when not in use. Since the specifications supplied by the manufacturers already included the media void volume of 95%, displacement tests were not performed. Taking into account the mixing chamber volume unoccupied by the media, the overall void volume of each AGM reactors was in the order of 95.7% voids (or a media packing density of 4.3% v:v). This AGM packing density of 4.3% adopted during the current work was similar to the optimum range of 5–10% reported by Shin and Polprasert (1987). A summary of the physical characteristics of both the RF and AGM pilot-scale upgrade systems is provided in Table 2.2.

**Table 2.2.** Physical characteristics of individual Rock Filters (RF), Attached-Growth Media reactors (AGM) and Open Ponds (OP).

<b>Treatment</b>	<b>OP</b>	<b>RF</b>	<b>AGM</b>
Pond length (m)	2.17	2.17	2.17
Pond width (m)	1.20	1.20	1.20
Gross pond depth (m)	1.10	1.10	1.10
Media layer depth (m)	n/a	1.15	1.10
Water column depth (m)	1.00	1.00	1.00
Average rock media diameter (cm)	n/a	10	n/a
Effective interstitial void space depth (cm)	100	4 <sup>†</sup>	1.9
Gross media volume (m <sup>3</sup> )	n/a	2.58	2.46
Immersed media volume (m <sup>3</sup> )	n/a	2.24	2.24
Specific surface area (m <sup>2</sup> m <sup>-3</sup> ) <sup>‡</sup>	3.6	39	150
Total pond specific surface area (m <sup>2</sup> )	9.2	97	345
Void volume (%)	100	55.86	95.7

<sup>†</sup> Based on that of Swanson and Williamson (1980) for similar rock media size

<sup>‡</sup> Including all internal pond surfaces (i.e. walls and base)

All pilot treatments were allowed to equilibrate and ‘run-in’ for a period of 2–3 weeks prior to commencement of experimental monitoring. No attempts were made to monitor experimental ‘start-up’ performance during this initial acclimation period.

### 2.1.2 Pilot plant flow hydraulics

The importance of flow hydraulics on the overall treatment performance of biological reactors in general has long been recognised. It should be emphasized, however, that it was not the aim of the current work to investigate the potential effects of hydraulic flow pattern on pond performance, rather, flow patterns within the experimental pilot ponds would simply be characterised for completeness. Flow characterisations would also allow for identification of any anomalous hydraulic flow patterns, such as a high dead-space volume or severe short-circuiting, which might then aid the identification of potentially erroneous experimental results. Additionally, characterisation of reactor hydraulics in this instance was performed *post hoc* rather than being used as a performance prediction or engineering design tool—as is most commonly the case in wastewater treatment reactor engineering (Bischoff and McCracken, 1966).

### **2.1.2.1 Hydraulic characterisation**

According to Levenspiel (1999) the operational flow pattern within the pilot plant ponds is classified as ‘steady state’ flow, meaning that the influent flow rate is deemed to be constant with time. In order to characterise the patterns of flow within the experimental ponds, hydraulic tracer experiments were performed. These tracer studies were done with the aid of the fluorescent dye rhodamine WT in conjunction with a SCUFA<sup>®</sup> submersible fluorometer/data logger (Turner Designs, Inc., Sunnyvale, CA) and associated software (SCUFASoft<sup>™</sup> v. 2.1). This dye has been developed specifically for and is therefore well suited to hydraulic tracer studies (Smart and Laidlaw, 1977). Rhodamine WT has also been the tracer of choice of various other research investigations into the hydraulics of natural waterways and WSPs alike (Replogle *et al.*, 1966; Kilpatrick, 1970; Pedahzur *et al.*, 1993; Cauchie *et al.*, 2000b; Shilton *et al.*, 2000; Barter, 2003).

Initial rhodamine WT slug injection dosage was calculated so as to allow for sufficient fluorescence peak height in the outflow according to Kilpatrick (1970). Slug injections (commonly  $\approx 30$  ml) of a  $10^{-2}$  dilution of the 20% active rhodamine WT stock solution were used for all tracer experiments. 30ml tracer slugs were further diluted to  $\approx 150$ ml and slowly injected into the influent manifold feed line (prior to several 90° elbows to further aid dye mixing) using a 60ml syringe, and the data logger simultaneously started. Rhodamine WT fluorescence during tracer assessments was then measured and recorded, commonly at 1 minute intervals, using the submersible SCUFA<sup>®</sup> fluorometer/data logger. Following the cessation of each individual tracer run, the data was then downloaded and exported directly into Microsoft<sup>®</sup> Office Excel XP (Microsoft Corporation, Washington, USA) for later analysis.

Hydraulic flow rates were recorded at the commencement and cessation of individual tracer runs, using both the rotameter flow meter readouts as well as volume-based flow measurements taken with the aid of a graduated measuring cylinder and a stopwatch. The average of these two flow rate measurements was then used for calculating the theoretical hydraulic residence time (HRT) of the pilot pond. The theoretical HRT ( $\tau_{th}$ ) for each pilot pond was calculated simply by dividing the pond’s hydraulic volume ( $V_p$ ;  $m^3$ ) by the hydraulic flow rate entering the pond ( $m^3 h^{-1}$ ). Hydraulic residence time

distribution (RTD) curves—also known as  $C$  curves—were compiled based on the fluorescence–time data from the tracer studies, and were then used to characterise the flow patterns within each pilot treatment pond. RTD curves were compiled using PRISM 4.03 (GraphPad Software, San Diego, CA, USA). Actual mean HRT ( $\tau$ ) was calculated based on the raw tracer data from the fluorescence–time RTD curves according to the method of Levenspiel (1999) as shown in Equation 2.1.

$$\tau = \frac{\sum_i t_i C_i \Delta t_i}{\sum_i C_i \Delta t_i} \quad (\text{Equation 2.1})$$

where  $\tau$  = the mean HRT (hours)

$t_i$  = the elapsed time (minutes)

$C_i$  = the tracer fluorescence at each logged time interval

$\Delta t_i$  = the elapsed time interval between each measurement of  $C_i$

The theoretical HRT can then be compared with the mean HRT as determined from the tracer data in order to provide some information about the magnitude of dead volume or the extent of short-circuiting within the pond reactor (Baléo *et al.*, 2001). The existence of dead volume is indicated by  $\tau < \tau_{th}$  whereas short-circuiting is said to occur if  $\tau > \tau_{th}$  (Cauchie *et al.*, 2000b; Baléo *et al.*, 2001). The dead volume ( $V_d$ ) and short-circuiting flow rate ( $Q_{SC}$ ) are then calculated according to Equations 2.2 and 2.3 respectively.

$$V_d = V_p \cdot \left( 1 - \left( \frac{\tau}{\tau_{th}} \right) \right) \quad (\text{Equation 2.2})$$

$$Q_{SC} = Q_{in} \cdot \left( 1 - \left( \frac{\tau_{th}}{\tau} \right) \right) \quad (\text{Equation 2.3})$$

where  $V_p$  = hydraulic volume

$Q_{in}$  = the daily influent flow rate

$\tau$  and  $\tau_{th}$  are the calculated mean and theoretical HRT respectively (as above)

Commonly, duplicate hydraulic tracer experiments were performed on individual pilot ponds in order to assess the consistency of the observed flow pattern. To allow for direct comparisons between duplicate tracer runs, RTD curves were normalised so that the areas under the curves of duplicate runs were equal to unity. This was done according to the method of Levenspiel (1999), whereby the measured tracer fluorescence at each logged time interval was divided by the area under the raw RTD curve (calculated using PRISM 4.03)—giving resultant normalised RTD curves a uniform area of 1.

### **2.1.2.2 Hydraulic balance**

The hydraulic balance within a WSP is normally governed by the influent and effluent flow rates, as well as the local rainfall and evaporation intensities (Somiya and Fujii, 1984). The effects of both rainfall dilution and evaporative concentration on the overall water balance was evaluated quantitatively with data obtained from the nearby Edinburgh Air Force Base and also from Adelaide Airport (courtesy of the Australian Government Bureau of Meteorology). Both of these monitoring locations are located within the same climatological district (i.e. Adelaide plains) as the Bolivar WWTP according to the Bureau of Meteorology and are suitably close to the Bolivar site in accordance with the recommendations of Pearson *et al* (1987a).

Based on the rainfall and evaporation data, the positive effects of rainfall and negative effects of evaporation on the overall hydraulic balance within the pilot plant were neglected. Given that average daily rainfall for the region was in the order of 1.0mm and the daily evaporation rate was in the order of 5.7mm, the net daily evaporative loss of approximately 4.7mm was deemed insignificant in comparison with the relatively high influent flow rate during the study period (1800–2640L d<sup>-1</sup>); with daily evaporative losses representing only 0.5% of daily influent volume. It was also assumed that the evaporation rate would be expected to be relatively consistent across all treatment ponds, such that any small-scale ‘concentration effects’ should be similar between treatments.

### **2.1.2.3 Hydraulic operation**

All ponds were operated at a water depth of 1m for the entire study duration. This operational depth was chosen to reflect the actual *in situ* hydraulic depth of the Bolivar WSPs ( $\approx$ 1.3m). Influent flow rate into each of the three pilot treatment series was equal under any given hydraulic loading regime and was calibrated using an inline rotameter



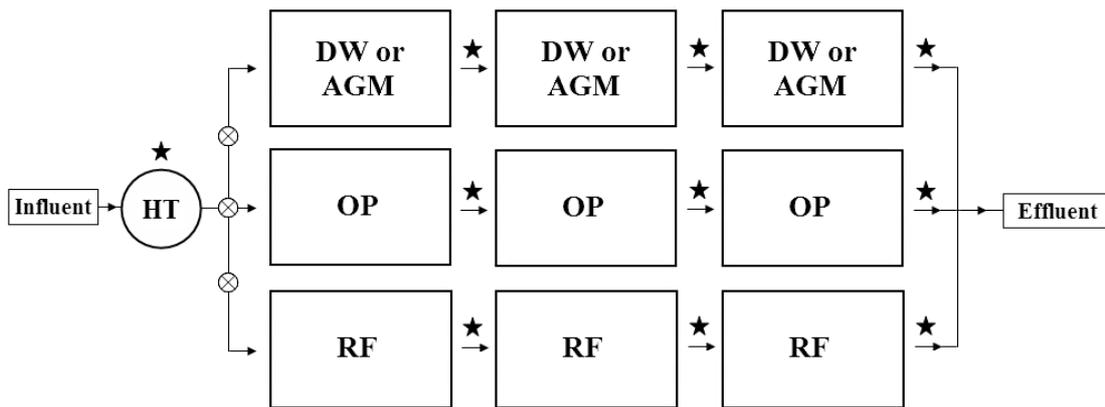
flow meter. The relatively unpredictable nature of the final Bolivar WSP effluent made it very difficult to attempt to vary the organic loading rate by simply adjusting the hydraulic flow rate. Rather than attempting to control the organic loading rate (OLR) through continual flow rate adjustments, hydraulic loading rate (HLR) was maintained at as close as possible to a constant level, whilst the OLR was allowed to vary randomly with natural up-stream variations in WSP *in situ* conditions. This also allowed for a standardisation of any potential differences in treatment performance arising from differences in hydraulic flow velocity; something especially relevant to physical treatment processes such as sedimentation.

## **2.2 Operational sampling and water quality analyses**

As described in Section 2.1.1, it was necessary to stagger experimental pilot treatments in order to assess all of the four interventions. Under the first treatment configuration (DW–OP–RF; *Period 1*), the pilot plant was operated continuously for a period of six months between July and December of 2005. Under the second configuration (AGM–OP–RF; *Period 2*), the pilot systems were operated continuously for a period of six months between February and August of 2006. During both of these operational periods, routine sampling was performed periodically according to the details below.

### **2.2.1 Experimental sampling protocols**

Pilot plant sampling was conducted, on average, at least once-weekly during the entire operational duration at 1200 ( $\pm 2$ ) hours. Daily sampling protocols involved taking a combined total of 2L grab samples either from the entire pond depth using a 1.2m long 40mm internal diameter water column sampler (DW, OP and AGM treatments) or directly from pond effluent manifold taps (RF treatment). Full water column samples could not be taken from the rock filters due to physical obstruction of the column sampler by a network of PVC supports within the effluent mixing chambers (see Plate 2.8). Samples for faecal coliform and *E. coli* analyses were taken directly from pond effluent manifolds for all four pilot treatments. In total, 10 samples were collected during each daily sampling interval (represented by filled stars in Figure 2.4).



**Figure 2.4.** Schematic representation of the experimental pilot plant system showing daily sampling locations (indicated by filled stars) for each of the treatment trains across all treatments: Duckweed Ponds (DW); Attached-Growth Media reactors (AGM); Open Ponds (OP); and Rock Filters (RF).

Since some phyto- and zooplankton species ubiquitous to WSPs (e.g. *Euglena* and *Daphnia* species) are reportedly capable of *in situ* phototaxis and daily vertical migration (Hartley and Weiss, 1970; Starkweather, 1983), OP samples for chlorophyll *a* and algal quantitation and speciation were taken from the top  $\approx 90$ cm of the 1m water column depth (as close as possible to the outlet manifold of the individual pilot ponds) using a water column sampler according to standard protocols (AHPA, 1992) and following the recommendations of Pearson *et al.* (1987b). This was also in accordance with the findings of Pearson *et al.* (1987c) who found that maximal algal concentration occurred at a depth of 20–25cm below the surface—recommending that algal samples be taken from the entire water column. Due to difficulties associated with whole water column sampling within the DW and RF treatments, samples for algal population and biomass analysis were withdrawn directly from effluent manifold sampling taps. Additionally, samples taken from the DW treatment ponds used for chlorophyll *a* analysis were coarsely filtered (2mm) prior to analysis in order to minimise the contribution of chlorophyll-containing duckweed plant tissue to daily measurements. All samples for algal population analyses were preserved using 0.7% Lugol’s iodine solution according to the recommendations of Pearson *et al.* (1987a; 1987b) and following the standard method 10200 B (APHA, 1992).

Qualitative visual observations on-site, revealed significant ‘patchiness’ (both vertically and horizontally) in zooplankton distribution within some pilot ponds—predominantly

the OP series. In order to minimise the effects of non-uniform spatial distribution and non-representative community sampling, combined sub-samples were taken from different regions of the pond, or were taken from areas that appeared visually to represent the entire pond (the entire 1m water column depth could often be clearly visualised). Water column samples (precisely 1L) collected for qualitative and quantitative zooplankton characterisations were taken in an identical manner as those for chlorophyll *a* and algal analyses above for the DW, OP and AGM treatment series. For the RF series, and due to physical obstruction of the standard column sampler within effluent mixing chambers as described above, RF samples for zooplankton analyses were withdrawn using a narrow bore column sampler (20mm internal diameter).

Following collection, all zooplankton samples were then filter-concentrated on site from 1L down to a final volume of approximately 20–30ml using a standard “Wisconsin” type 64 $\mu$ m zooplankton nylon mesh (Pace and Orcutt Jr., 1981). Whilst it is likely that this mesh aperture size may have promoted under-sampling of some smaller (<100 $\mu$ m) rotifer species (e.g. Likens and Gilbert, 1970; Bottrell *et al.*, 1975), it was deemed sufficiently small to ensure adequate sampling of the vast majority of zooplankton species in the WSP environment, including even the smallest life stages of planktonic crustacean such as copepod nauplii (Nichols and Thompson, 1991; Ghadouani *et al.*, 1998). This mesh was also identified as having the smallest practicable aperture size for filtration of the often highly particulate WSP effluent, and incidentally, was considerably finer than the 158 $\mu$ m aperture mesh used by Hamilton *et al.* (2005) for zooplankton sampling within other Australian WSPs. The 20ml filtrate was transferred to a 100ml plastic container and preserved using 0.7% Lugol’s iodine (as above) for later examination in the laboratory. Lugol’s preservative was used here instead of the more common ‘sugar–formaldehyde’ solution, because it allowed for superior optical contrast during conventional light-based microscopic examination of the highly contaminated WSP samples. Protocols adopted here for zooplankton collection and preservation were similar to those of Park and Marshall (2000).

Unless otherwise specified, all samples were collected in sterile (121°C for 15 minutes), chemically-inert 1L polyethylene vessels (Nalgene®). All field samples were stored on ice in the dark immediately after collection and during transport to the laboratory in

accordance with *Standard Methods for the Examination of Water and Wastewater* (APHA, 1992) and were always processed on the day of sample collection (except for nutrient analyses and Lugol's-preserved plankton samples). Long-term storage and preservation of samples for nutrient analyses were in accordance with *Standard Methods* (APHA, 1992). Where possible, water quality analyses were done according to the same standard methods (APHA, 1992).

## **2.2.2 Field- and laboratory-based water quality analyses**

### **2.2.2.1 *In situ* water quality monitoring**

At each of the sampling points indicated in Figure 2.4 above, temperature, pH, DO (concentration and saturation) and conductivity were measured using a YSI 600XM probe connected to a YSI 556 data-logging handset (Yellow Springs Instruments, Ohio, USA) at the same time of day as for Section 2.2.1 above. *In situ* monitoring of pilot ponds was done at a depth of 20cm below the water surface; a depth which has proven to be representative of mean water quality throughout the entire water column depth (Cauchie *et al.*, 1999). This depth was also selected in line with the findings of Pearson *et al.* (1987c), that maximum algal concentrations (and hence maximum pH and DO fluctuations) can be found at this depth between 1100 and 1400 hours. Probe heads on the YSI 600XM sonde were periodically calibrated and, where necessary, were serviced according to the manufacturer's specifications.

24 hour online DO monitoring was also performed for part of the experimental duration during monitoring *Period 2* (according to equipment availability). Dissolved oxygen concentration was recorded at 10 minute intervals for the pilot plant influent (INFL), Rock Filter 1 (RF-1), Open Pond 1 (OP-1) and Attached-Growth Media Reactor 1 (AGM-1) using Danfoss Evita<sup>®</sup> Oxy Clarke-type dissolved oxygen sensors (Danfoss Australia, Victoria, Australia) connected to a T-TEC 6-3A data logger with 4–20mA signal input (Temperature Technology, Adelaide, South Australia).

### **2.2.2.2 *Total and volatile suspended solids***

Samples were analysed for total suspended solids (SS) with volatile suspended solids (VSS) fractionation according to standard methods 2540 D and 2540 E respectively

(APHA, 1992). Briefly, duplicate aliquots of well mixed samples (commonly 250ml) were filtered through pre-washed, pre-combusted (500°C for 1 hour) and pre-weighed glass fibre filter papers (GF/C, 1.2µm nominal pore size; Whatman®, UK) using a vacuum flask and filter funnel. Papers and residue were then dried at 105°C for 24 hours and weighed (SS) and then combusted (500°C for 1 hour) and re-weighed (VSS). SS and VSS were reported as mg L<sup>-1</sup>. Variation between duplicate samples was always less than 10% of the average weight, and most commonly within 5%.

### **2.2.2.3 Turbidity**

Turbidity was quantified using a HACH Ratio/XR turbidimeter and expressed as standard nephelometric turbidity units (NTU).

### **2.2.2.4 Total five-day biochemical oxygen demand**

Five-day biochemical oxygen demand (BOD<sub>5</sub>) was measured in a temperature-controlled cabinet (20°C) using the WTW OxiTop®-C system equipped with a WTW OxiTop®-OC100 hand controller. BOD<sub>5</sub> availability kinetics data was downloaded directly from the OxiTop® hand controller. The manometric OxiTop method measures changes in headspace pressure in a closed system and as such provides both a highly accurate and precise BOD measurement; indeed the manufacturer (WTW Weilheim, Germany) quotes an accuracy of 1% the measured value (± 1 hPa) and a resolution of 0.7% measured BOD<sub>n</sub>. Despite the very low ammoniacal-nitrogen levels in the pilot system wastewater, BOD<sub>5</sub> was measured as carbonaceous BOD<sub>5</sub> (cBOD<sub>5</sub>) through the inclusion of 100µl of nitrification inhibitor (1000mg L<sup>-1</sup> allylthiourea) in all samples. For ease of discussion, measured cBOD<sub>5</sub> will be reported simply as BOD<sub>5</sub>. It should also be noted that due to an often variable equipment availability status, all 9 experimental ponds were not always analysed with respect to BOD<sub>5</sub> at every sampling interval. In these cases, the *second* pond in each three-pond series was omitted from daily BOD<sub>5</sub> analyses.

### **2.2.2.5 Total organic carbon**

Total organic carbon (TOC) and inorganic carbon (IC) concentration was measured using a Shimadzu TOC-5000A Total Organic Carbon analyser (Shimadzu Corporation, Kyoto, Japan) according to the procedure outlined in the user's manual. Standards of

anhydrous potassium biphthalate and anhydrous sodium carbonate/sodium bicarbonate were used for calibration of TOC and IC measurements respectively.

#### **2.2.2.6 Chlorophyll a**

Chlorophyll a was measured according to (the trichromatic) method 10200 H (APHA, 1992). Briefly, samples were firstly vacuum filtered (Whatman<sup>®</sup> GF/C; nominal pore size 0.45 $\mu$ m), extracted in ice-cold 90% acetone, centrifuged at 3000g for 10 minutes at 4°C (Sigma 6K15) and finally analysed spectrophotometrically at 630, 647 and 664nm. The extraction, sample preparation and analysis protocols were identical to the standard method 10200 Parts '1 and 2', with the exception that a vortex mixer was used in place of a tissue grinder for sample homogenisation. Because samples were always extracted on the day of collection and analysed within a maximum period of 2 weeks, corrections for chlorophyll degradation products (phaeopigments) were not made. Reported chlorophyll concentrations, therefore, represent the sum of chlorophyll *a* and a likely insignificant amount of phaeopigment.

#### **2.2.2.7 Ammoniacal-nitrogen**

Samples for ammoniacal-nitrogen (NH<sub>4</sub><sup>+</sup>-N) analysis were pre-filtered (Whatman<sup>®</sup> GF/C) prior to analysis as follows. NH<sub>4</sub><sup>+</sup>-N was determined using the nesslerization method 4500-NH<sub>3</sub> C (APHA, 1992). To improve colour development, EDTA was substituted with 1 drop of mineral stabiliser (Biolab, cat# 23766-26) and 1 drop of polyvinyl alcohol dispersing agent (Biolab, cat# 23765-26). Analyses were performed in triplicate, and, for quality assurance, were run parallel to daily validation standards. It should be noted that *ammoniacal-nitrogen* is expressed here in chemical notation as *ammonium-nitrogen* (NH<sub>4</sub><sup>+</sup>-N). This is due to the fact that within the common pH and temperature ranges experienced during the course of this research, the ratio of unionized NH<sub>3</sub>-N to ionized NH<sub>4</sub><sup>+</sup>-N will be <0.1, such that the vast majority (>90%) of ammoniacal-nitrogen will in fact be present as ammonium-nitrogen (Boyd, 1990). Consequently, all following reference to 'ammonia-nitrogen' will be done so using the notation NH<sub>4</sub><sup>+</sup>-N.

#### **2.2.2.8 Oxidised nitrogen (nitrate and nitrite)**

Samples to be analysed for nitrate-nitrogen (NO<sub>3</sub><sup>-</sup>-N) and nitrite-nitrogen (NO<sub>2</sub><sup>-</sup>-N) were pre-filtered as for NH<sub>4</sub><sup>+</sup>-N analysis. NO<sub>3</sub><sup>-</sup>-N and NO<sub>2</sub><sup>-</sup>-N were determined using the

hydrazine reduction method 4500-NO<sub>3</sub> G, and the colorimetric method 4500-NO<sub>2</sub> B respectively (APHA, 1992). Analyses were performed in triplicate together with daily validation standards.

#### **2.2.2.9 Soluble reactive orthophosphate**

Samples analysed for soluble reactive orthophosphate (PO<sub>4</sub><sup>3-</sup>-P) were pre-filtered as for NH<sub>4</sub><sup>+</sup>-N analysis. PO<sub>4</sub><sup>3-</sup>-P was determined according to method 4500-P D (APHA, 1992). Analyses were performed in triplicate together with daily validation standards.

#### **2.2.2.10 Indicator microorganisms**

Thermo-tolerant faecal coliforms (FC) and *Escherichia coli* were enumerated according to the Colilert<sup>®</sup>-18 defined substrate “Quanti-tray” method (IDEXX Laboratories, Maine, USA) following the manufacturer’s instructions and after a minimum of 18 hours sample incubation at 44°C. Although an incubation period of precisely 18 hours is recommended by the manufacturer, Colilert<sup>®</sup>-18 test results were found to be stable for at least up to 24 hours, and so were generally analysed within this 18–24 hour incubation period. *E. coli* were identified according to the manufacturer’s instructions under ultra-violet illumination (Vilber Lourmat, VL-215; 60W). Results were then expressed as most probable number (MPN) organisms 100ml<sup>-1</sup>.

#### **2.2.2.11 Heterotrophic microbial plate counts**

Quantitation of heterotrophic microbial density was done following a standard spread-plating method. Briefly, samples were serially-diluted where necessary (sterile 0.1% peptone water, Oxoid) then sample aliquots (100μl) were aseptically spread onto standard agar plates (R2A, Oxoid) followed by 24 hour incubation at 31°C and final examination. Heterotrophic microbial density was then expressed as colony forming units (CFU) ml<sup>-1</sup>.

#### **2.2.2.12 Light–depth profiling**

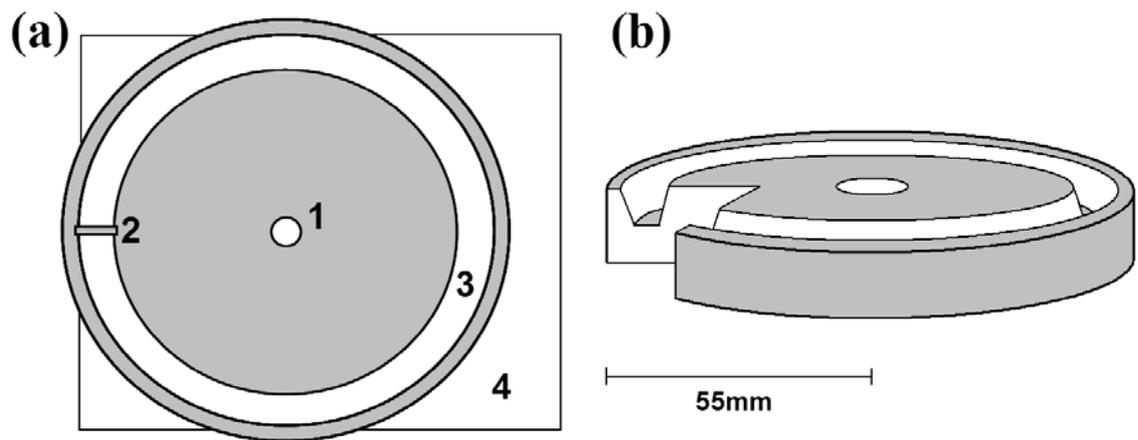
Photosynthetically available radiation (PAR; 400–700nm) depth profiling was performed using a quantum sensor (SKE-510, Skye Instruments, Wales, UK) attached to a graduated measuring pole. PAR was expressed as μmol photons m<sup>-2</sup> s<sup>-1</sup>.

### **2.2.2.13 Phyto- and zooplankton quantitation and identification**

Phytoplankton densities were determined by sedimentation of samples followed by optical inverted microscopy (Utermöhl, 1958) according to standard methods 10200 C and 10200 E respectively. All phytoplankton enumerations and taxonomic classifications were performed by qualified staff at the 'NATA' accredited Australian Water Quality Centre (Bolivar, South Australia). For zooplankton analysis, a 5ml Eppendorf pipette was used to transfer the entire 20ml of concentrated Lugol's-preserved samples from each sampling location (refer Section 2.2.1) into the zooplankton counting wheel (see below)—usually in 10ml aliquots. The disposable pipette tip was cut to make a 4–5mm diameter opening so that large crustacean zooplankton could be easily transferred (Edmondson and Winberg, 1971).

Zooplankton enumeration and gross taxonomic identifications were performed using relevant taxonomic reference material (Koste, 1979; Shiel *et al.*, 1982; Benzie, 1988; Bayly, 1992; Fernando, 2002) in conjunction with a custom-made, 15ml capacity, clear acrylic zooplankton 'counting wheel' (Figure 2.5) and a Leica MZ6 dissecting microscope at 40× magnification (Leica Microsystems). Further, more detailed taxonomic identifications preserved samples were performed by experienced and qualified staff (Russell Shiel) at the University of Adelaide, Department of Environmental Biology (Adelaide, South Australia) according to relevant taxonomic keys. Ostracod identifications were kindly performed by Stuart Halse (Bennelongia Environmental Consultants Pty. Ltd., Wembley, Western Australia). Organism photographs were recorded for the major zooplankton using a standard compound microscope (Olympus BX50) and digital camera (Q-Imaging) with associated software (Micropublisher 5.0) for small (<500µm) organisms, and for larger organisms (>500µm), photographs were taken using a Leica MZ16 dissecting microscope with inbuilt digital camera and associated software (Leica Microsystems).





**Figure 2.5.** (a) Complete acrylic zooplankton counting wheel and base, showing: central pivot point (1); start/stop point (2); circular counting well (3); and acrylic base stand (4); and (b) cross-sectional view of the counting wheel removed from the base stand.

Individual zooplankton were counted as merely ‘present’ or ‘absent’ and were recorded as single adults only for all zooplankton species, such that the numbers of ovigerous (egg-bearing) females were not recorded. Individual copepod nauplii were recorded as ‘nauplii’ up to the stage where they qualitatively began to resemble adult morphology (i.e. naupliar stages N1–N6 until around copepodite ‘C1’ stage; Dussart and Defaye, 2001). Moreover, their morphologically distinct form, by comparison to the copepodite stages, warranted the separate functional classification of nauplii (Hamilton *et al.*, 2005). From there onward, C1–C5 copepodites were all recorded as ‘adults’—as done also by Mitchell and Williams (1982a)—and were divided between calanoid and cyclopoid groups according to the daily observed ratio of identifiable C5 adult stages. Because of the complexities associated with taxonomic identification of copepod nauplii (Hawking and Smith, 1997), no attempts were made during enumerations to separate nauplii into respective ‘calanoid’ and ‘cyclopoid’ groupings. As was again done by Mitchell and Williams (1982a) during zooplankton monitoring of another South Australian WSP, no distinction was made when enumerating juvenile cladoceran stages, rather, all juvenile individuals recorded as single adults of the dominant cladoceran species (invariably *Daphnia carinata*). All plankton counts were expressed as the number of organisms  $L^{-1}$ .

Individual organism biomass values ( $\mu\text{g}$  dry wt individual $^{-1}$ ) were calculated according to pre-defined length–weight allometric equations for the same genera or species (Dumont *et al.*, 1975; Mitchell and Williams, 1982b) following the technical

recommendations of Bird and Prairie (1985). Body length measurements were performed electronically on a random selection of adult individuals from each taxon using a standard compound microscope (Olympus BX50) with digital camera (Q-Imaging) and associated software (Micropublisher 5.0) for small ( $<500\mu\text{m}$ ) zooplankton, and a Leica MZ16 dissecting microscope with inbuilt digital camera and associated software (Leica Microsystems) for larger organisms ( $>500\mu\text{m}$ ). Measurements of *Daphnia* were taken from the top of the head to the base of the carapace spine following the method of Cauchie *et al.* (2000a). Where published data was available, organismal dry weights were estimated for those species directly from published biomass values (e.g. Kobayashi *et al.*, 1996). Where data were not available for particular zooplankton species, regression equations or published dry weight values from morphologically similar species were used (e.g. *Brachionus calyciflorus* (Pallas) for *Brachionus novaezealandiae*). Ostracod biomass was estimated from the published organism dry weight values of Ikeda (1990) using a mid-range mean body length of 1.3mm. An intermediate single weight was adopted for all copepod nauplii biomass calculations based on a mean body length of  $160\mu\text{m}$  in conjunction with the length–weight equation of Dumont *et al.* (1975). This single naupliar weight estimate also reflected the early-stage (N2) cyclopoid and calanoid nauplii weights reported by Culver *et al.* (1985) and was also similar to the mean naupliar weight reported by Pedrós-Alió and Brock (1983). Actual length–weight regression equations and final dry biomass estimates for the most commonly recorded zooplankton taxa are provided in Appendix E.

Because the brood (egg) volume can represent a significant portion of total organism biomass ( $\approx 25\%$  according to Pauli (1989) for smaller organisms such as rotifers such as *Keratella* species;  $\approx 30\%$  for *Brachionus* species,  $\approx 10\%$  for *Daphnia magna* (Straus) and *Moina micrura* (Kurz),  $20\%$  for *Chydorus* species,  $20\%$  for adult cyclopoid (*Mesocyclops*) copepods,  $25\%$  for adult calanoid (*Eudiaptomus*), and up to  $50\%$  for *Bosmina* species according to Dumont *et al.* (1975)), during these calculations,  $10\%$  of all counted organisms were considered to be egg-bearing at the time. Although not directly quantified, this figure of  $10\%$  was considered to be a relatively accurate—in some cases conservative—estimate based on qualitative observations made during zooplankton enumerations, and generally increased the daily biomass total by no more than  $3\%$ . Furthermore, it was a somewhat more conservative approach than that adopted

by Kobayashi and Church (2003) who multiplied all dry weight biomass values by a factor of 1.1 to account for the proposed 10% error associated with back-transforming length–weight data. Because no distinction was made between newborn, juvenile and adult Cladocera during counting, a reduced and relatively conservative overall mean organismal length was used (1.8mm) as a way of correcting for the inclusion of smaller individuals.

Whilst length–weight relationships for zooplankton are known to vary according to *in situ* food availability (Geller and Müller, 1985; Hessen, 1990; Cauchie *et al.*, 2000c), no attempt was made to factor in organismal nutritional status during biomass conversions. Similarly, and as highlighted by Kobayashi *et al.* (1996), whilst the use of constant weights for rotifers may ignore important spatial and temporal variations in the weight distributions of individual taxa, their universally small size means that all methods for measuring the individual mass of microzooplankton are inherently subject to large errors in terms of accuracy and precision (McCauley, 1984). Therefore, constant mean weights were adopted for all individual organismal dry biomass values as described above. Total zooplankton biomass (mg dry wt L<sup>-1</sup>) for a given sample was estimated as the sum of the product of the zooplankton density and inferred organismal dry weight for each recorded taxon.

#### **2.2.2.14 Plankton community diversity**

Insights into the relative biodiversity of zooplankton communities from each of the four pilot treatments were gained by calculating the Shannon diversity index ( $H'$ ) of Shannon (1948) according to Equation 2.4 below.

$$H' = - \sum (P_i) \times (\log_e P_i) \quad \text{(Equation 2.4)}$$

where:  $P_i$  = proportion of individuals of the  $i$ th species and estimated as  $n_i/N$

$n_i$  = number of individuals of species  $i$

$N$  = total number of individuals of all species

$\log_e$  is the natural logarithm

### **2.3 Data assessment, manipulation and statistical analysis**

In accordance with the prior recommendations of Haas (1996), all ‘average’ indicator organism values will be reported as *arithmetic* means  $\pm 1$  standard deviation (SD) unless otherwise stated. Indicator organism data (FC and *E. coli*) was  $\log_{10}$  transformed prior to data analysis using the transformation  $y = \log_{10}(y + 1)$ ; where  $y$  = the number of microorganisms (expressed as MPN 100ml<sup>-1</sup>). Average percentage removal efficiencies for each parameter and across all pilot treatments were calculated according to Equation 2.5:

$$\% \text{ removal} = 100 - (C_{\text{effluent}} / C_{\text{influent}}) \times 100] \quad (\text{Equation 2.5})$$

where  $C_{\text{influent}}$  = concentration of measured parameter in the pilot plant influent

$C_{\text{effluent}}$  = concentration of measured parameter in the effluent

In order to demonstrated the long-term variability of both influent water quality and subsequent pilot treatment performance, average percentage removal efficiencies for each measured parameter are provided with their corresponding 95% confidence intervals (CI's) to allow for evaluation of treatment performance consistency.

Prior to performing any statistical testing, data set normality was firstly assessed using Kolmogorov–Smirnov or Shapiro–Wilk testing in addition to supplemental Q–Q normal probability plot analysis in some instances. Homogeneity of data variances were also checked using Bartlett's or Levene's tests (depending on the statistical software used). Where raw data satisfied the underlying assumptions of normality and homoscedasticity, parametric statistical analyses were always performed on the raw un-transformed data. Where these assumptions were not satisfied, data transformations (commonly  $\log_{10}$  or  $\sqrt{\quad}$ ) were performed in an attempt to normalise the data (except for temperature and pH), followed again by normality testing and parametric statistical analysis where appropriate. In these instances, significant performance differences between treatments were assessed via standard parametric 1-way ANOVA with Tukey's honestly significant difference multiple comparison *post hoc* testing. For all ANOVAs, the variance ratio ( $F$ ) and associated degrees of freedom between ( $A$ ) and within groups ( $B$ ) are provided along with the corresponding sample size ( $n$ ) and corresponding  $p$  value. Where data

transformations failed to normalise data distributions and/or variance heterogeneity, non-parametric statistical analyses were employed. In these instances, significant differences between treatments were assessed by way of non-parametric Kruskal–Wallis testing with Dunn’s multiple comparison *post hoc* testing. All Kruskal–Wallis test results are provided with the corresponding Chi-square approximated test statistic ( $\chi^2$ ), associated  $\alpha$  significance level ( $_{0.05}$ ), between-groups degrees of freedom ( $_A$ ) and corresponding  $p$  value.

Where appropriate, parametric, one sample  $t$ -tests were used to identify differences between average daily percent parameter removal efficiencies and a theoretical ‘zero mean’ removal efficiency. Where data were non-normal, non-parametric Wilcoxon signed-rank tests were used for the same purpose as for  $t$ -testing above. Simple correlation analysis was used to identify any significant associations between measured water quality parameters; either by way of parametric Pearson’s correlation ( $r$ ) or non-parametric Spearman rank correlation ( $r_s$ ) according to the assumptions of data normality as above. Where appropriate, differences between the slopes of multiple fitted regression lines were assessed by way of Analysis of Covariance (ANCOVA) using PRISM 4.03 and according to the methods of Zar (1996). All significance testing was performed at or below  $\alpha \leq 0.05$  level. All statistical computations were performed using either PRISM 4.03 or SPSS 15.0.0 (SPSS Inc., Chicago, Illinois, USA). Graphical data was compiled using both PRISM 4.03 and Microsoft® Office Excel 2003. Data tables were formulated using Microsoft® Office Excel 2003.

### **3 Relative performance of duckweed ponds and rock filtration for the upgrading of WSP effluent**

Parts of this chapter have been published elsewhere (see Appendix A).

#### **3.1 Introduction**

As introduced in Chapter 1, WSPs represent an extremely robust, low-maintenance, cost-efficient wastewater treatment alternative. A major issue affecting WSP performance, however, is the unpredictable and often high concentrations of algal-based SS and accompanying BOD<sub>5</sub> in their effluent. Further upgrading of WSPs is therefore required if pond effluent is to be of a reliably high quality for either final waterway discharge or quaternary treatment processing prior to reuse applications (as is the case for the Bolivar WWTP). This chapter describes the experimental performance data from pilot plant operational monitoring *Period 1* (July–December, 2005; see Table 2.1) comparing DW, OP, and RF treatments; of which in-depth reviews are provided within the relevant Sections of Chapter 1. The treatment efficacies of the three experimental interventions are detailed and discussed below, with special reference given to the discrete and relative treatment performances of each system, as well as the reliability or consistency of performance. As outlined within the thesis aims of Chapter 1, research presented in this chapter aimed to investigate in parallel the treatment efficacies of these pilot-scale WSP upgrades. Within these performance evaluations, and in line with the thesis aims, special reference will also be made to the algal removal efficacy of each pilot WSP upgrade methodology.

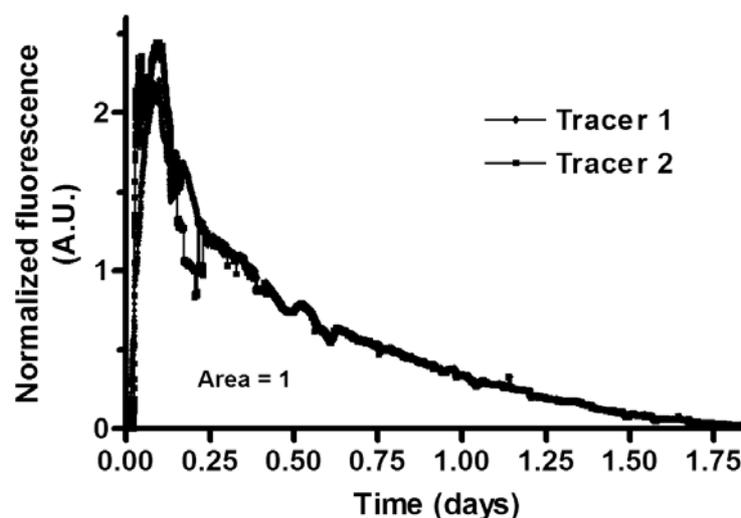
#### **3.2 Materials and Methods**

A detailed description of pilot plant construction, configuration, operation and monitoring protocols, is provided in Chapter 2.

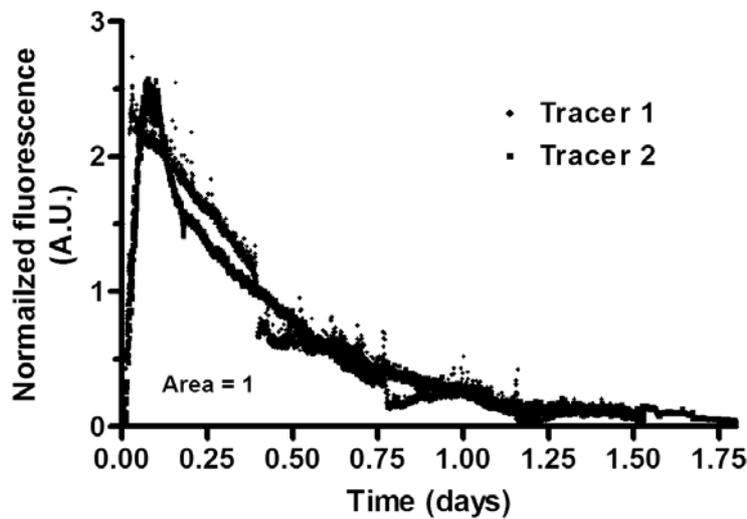
### 3.3 Results and Discussion

#### 3.3.1 Pilot plant hydraulics

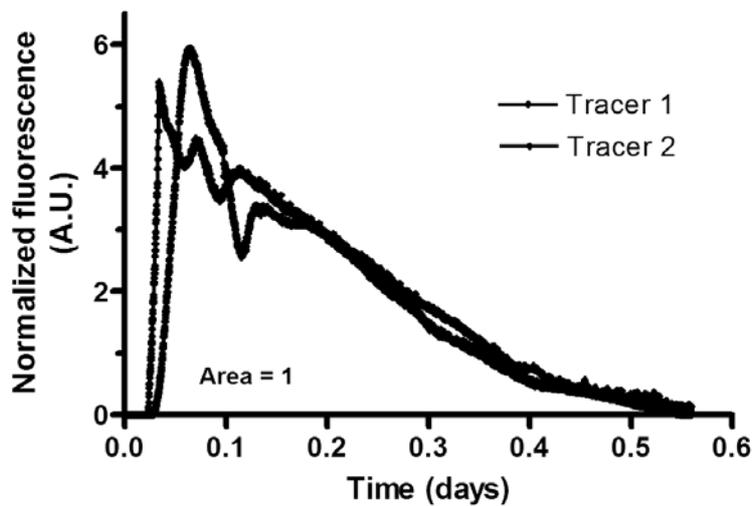
It is well understood that pond efficiency is a function of both the biochemical transformations as well as the hydraulic processes occurring within the pond (Polprasert and Bhattarai, 1985). Although BOD<sub>5</sub> attenuation is achieved predominantly through algal–bacterial interactions, hydraulic flow patterns can also have a significant bearing on WSP treatment performance (Polprasert and Bhattarai, 1985); therefore, the hydraulic flow regime within each of the pilot-scale pond systems was characterised. It was not the specific aim of this work to investigate the potential impact(s) of flow hydraulics on treatment performance, rather hydraulic conditions were assessed to aid the understanding of aspects of pilot plant performance which may have been influenced by specific hydraulic regime. Methods used to determine pilot pond hydraulic flow regime are detailed in Section 2.1.2.1. In order to allow direct comparison of multiple tracer runs performed under different flow rates, the fluorescent tracer data from Figures 3.1 to 3.3 was normalised so that the area under the curve was unity. The procedure involved dividing the raw tracer time–concentration data by the area under the non-normalised residence time distribution (RTD) curve to produce a ‘normalised RTD curve’ with an area equal to 1. RTD curves from corresponding hydraulic tracer experiments for each of the three pilot pond treatment systems are given in the Figures 3.1 to 3.3 below.



**Figure 3.1.** Duckweed treatment system: duplicate single pond normalised residence time distribution curves showing normalised rhodamine WT fluorescence (A.U). Tracer experiments performed under a standing duckweed plant biomass density of no less than 2kg m<sup>-2</sup> (wet weight).



**Figure 3.2.** Open Pond treatment: duplicate single pond normalised residence time distribution curves showing normalised rhodamine WT fluorescence (A.U.).



**Figure 3.3.** Rock filters: duplicate single pond normalised residence time distribution curves showing normalised rhodamine WT fluorescence (A.U.).

The single-pond RTD curves represented in Figures 3.1–3.3 all show a relatively well dispersed hydraulic flow pattern consistent with that of a completely mixed tank reactor; with a maximum asymmetric fluorescence peak near to the  $y$ -axis followed by a slow and steady decrease within a pronounced tail (Levenspiel, 1999). The long tails represent the tracer dye quickly becoming well mixed, and then slowly being diluted and washed out of the pond as the entire contents is gradually turned over. Maximum tracer fluorescence was observed in the pond outflow after only a fraction of the theoretical hydraulic residence time (HRT), suggesting that the flow pattern is more mixed than



plug (Naméche and Vasel, 1996; Torres *et al.*, 1999). This initial fluorescence peak seen in Figures 3.1–3.3, whilst being representative of a completely mixed tank reactor, is also indicative of a combination of short-circuiting and the existence of dead spaces within all pilot ponds (Bischoff and McCracken, 1966; Uhlmann, 1979; Levenspiel, 1999; Torres *et al.*, 1999). The presence of small-scale accessory peaks within the tail of the RTD curves is also indicative of some degree of localised recirculation within the pilot pond reactors.

Overall, the RTD curves of Figures 3.1 to 3.3 are similar to what would be expected from a relatively well-mixed pond reactor. The theoretical HRT ( $\tau_{th}$ ) for each treatment pond varied according to variations in the precise flow rate at which individual tracer experiments were conducted (refer Table 3.1). Data from within-treatment duplicate tracer runs were first averaged in order to yield one value for both  $\tau_{th}$  and actual mean HRT ( $\tau$ ). Using Equation 2.1 in conjunction with the RTD curve data from Figures 3.1–3.3,  $\tau$  for single DW, OP and RF pond reactors was then calculated for each tracer experiment. Then, using this information together with Equation 2.2, the dead volume for each pilot pond system was calculated. Quantitative analysis of the tracer data from Figures 3.1–3.3 revealed differing  $\tau$  values for each treatment based on individual treatment void volumes and tracer experiment flow rates. Mean residence times under the respective tracer experiment flow rates for individual DW, OP and RF reactors 0.96, 0.78 and 0.37 days respectively (note that for actual monitoring *Period 1* mean residence time values, see Table 3.2 below). Calculated dead volumes within each pilot pond treatment were in the order of 13.2, 13.6 and 27.9% for DW, OP and RFs respectively. A summary of these hydraulic characterisations is provided in Table 3.1.

Although there have been reports of improved hydraulic conditions under a duckweed surface cover compared with un-covered open ponds (Benjawan and Koottatep, 2007), tracer data suggested that flow patterns were very similar in both duckweed-covered and uncovered ponds; an observation in line with that of Zimmo (2003) for similar scale pilot pond systems. Flow hydraulics also appeared to be more ideal in the unoccupied DW and OP reactors, with the presence of large volumes of rock media apparently degrading the flow conditions and doubling the reactor's dead volume. Poor patterns of

flow distribution and hydraulic short-circuiting have been reported for rock filters elsewhere (Swanson and Williamson, 1980).

**Table 3.1.** Hydraulic characterisation of individual pilot ponds for the three treatment systems: Duckweed (DW); Open Pond (OP); and Rock Filter (RF). Individual parameter values represent the mean of duplicate tracer determinations.

Hydraulic parameter	<i>Experimental treatment</i>		
	DW	OP	RF
Gross reactor volume (m <sup>3</sup> )	2.56	2.56	2.56
Hydraulic flow rate (m <sup>3</sup> day <sup>-1</sup> )	2.21	2.76	2.76
Hydraulic loading rate (m <sup>3</sup> m <sup>-3</sup> day <sup>-1</sup> ) <sup>†</sup>	0.86	1.08	2.08
Void space volume (V <sub>p</sub> ; m <sup>3</sup> )	2.56	2.56	1.43
Void space volume (% total)	100	100	55.86
Theoretical residence time (τ <sub>th</sub> ; days)	1.16	0.93	0.52
Actual mean residence time (τ; days)	0.96	0.78	0.37
Dead volume (% V <sub>p</sub> )	13.16	13.63	27.86

<sup>†</sup> Based on gross reactor volume not void space volume

As can be seen from the data of Table 3.1,  $\tau < \tau_{th}$  for all treatments, indicating the existence of dead volume within each of the pilot pond series. Somewhere in the range of 13–28% of reactor void space volume was realised as ‘dead volume’ within the pilot ponds. Whilst not ideal, dead volumes recorded for the current pilot ponds were significantly lower than the approximate 60% dead spaces reported by Zimmo (2003) following hydraulic characterisations of similar pilot-scale duckweed and open control ponds. Although there is no *truly* dead space in a real system (since even in a completely non-moving region, transport of matter would eventually occur by molecular diffusion), regions of the reactor vessel with fluid retention times of 5–10 times more than that of the bulk of the fluid are for practical purposes referred to as dead, and are essentially seen as ‘wasted space’ (Bischoff and McCracken, 1966).

Dead volumes of the orders seen in Table 3.1 were likely to have had a measurable impact on the hydraulic efficiency and subsequent treatment performance of each of the pond reactors, but this was neither quantified nor corrected for here. Despite the initial design of the pilot pond systems adhering to several recognised elements of good hydraulic design, such as: the use of rectangular reactors; positioning the inlets and outlets as far from each other as possible; and the use of multiple inlet and outlet ‘ducts’

(Moreno, 1990); the observed flow conditions were far from optimal. According to Shilton *et al.* (2000), any change to the hydraulic operation (e.g. by baffling or the inlet/outlet configuration) that can effectively delay the arrival of the tracer peak at the outlet, if only for a short period, has the potential to significantly improve the overall pond performance. It was later thought that the inclusion of baffled inlet and outlet manifolds may have significantly improved the hydraulic flow regime within the pilot pond reactors, but in this instance, the *post hoc* nature of hydraulic characterisations prohibited the assessment of this. Even though flow conditions were proven to be non-ideal, data from Figures 3.1–3.3 did show good reproducibility between duplicate tracer tests; suggesting that hydraulic conditions recorded over the short duration of each tracer experiment were likely to have been relatively consistent during the extended six month experimental duration.

Although wind effects have the potential to adversely influence hydraulics flow patterns within large-scale shallow basins (e.g. Sweeney *et al.*, 2003), the small scale of the current pond reactors allowed only a very narrow wind fetch (< 3m); such that wind effects were not taken into consideration during hydraulic assessments. Additionally, the 10cm head space above the water surface in the current pilot pond vessels would have most likely served as a ‘wind buffer’—further reducing the effects of wind-induced flow alterations within the pilot-scale reactors. Similarly, and although thermal stratification is also known to be capable of influencing flow hydraulics within exposed shallow basins (Uhlmann, 1979), thermal stratification was unlikely to have played a significant role in dictating flow hydraulics within these pilot-scale reactors; based on the relatively large hydraulic throughput, and also due to the above-ground nature of the pond reactors most likely moderating and improving convective heating processes throughout the entire water column.

### **3.3.2 Pilot plant loading conditions and influent wastewater characteristics**

During operational *Period 1* (July–December, 2005), average pilot plant influent flow rate was  $78 \text{ L h}^{-1}$ , with an average daily inflow across all treatments of  $1.87 \text{ m}^3 \text{ d}^{-1}$ . This corresponded to an average daily hydraulic loading rate (HLR) for all treatments of

0.73 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> (note that in this instance, the hydraulic loading (m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) and aerial surface loading (m<sup>3</sup> m<sup>-2</sup> d<sup>-1</sup>) rates were identical due to the 1m hydraulic depth in all pilot reactors). DW and OP HLRs are given as m<sup>3</sup> of wastewater per m<sup>3</sup> of gross pond volume per day, and RF loading rates are stated in a similar way in terms of m<sup>3</sup> of wastewater per m<sup>3</sup> of gross (submerged) rock volume per day. Corresponding single pond theoretical mean HRTs under monitoring *Period 1* flow rates were 1.37 days for the OP and DW systems and 0.76 days for each RF pond (based on RF void space volume of 55.86%). It should be noted that these theoretical residence times are longer than those given in Table 3.1 because the tracer experiments were performed *post hoc* under a somewhat higher flow rate (115 L h<sup>-1</sup>). A summary of these and other operational parameters for the pilot-scale treatments are provided here in Table 3.2.

**Table 3.2.** Summary of the hydraulic and organic loading characteristics of the individual pilot-scale WSP upgrade treatment reactors during operational *Period 1*.

<b>Parameter</b>	<b>Treatment</b>		
	<b>DW</b>	<b>OP</b>	<b>RF</b>
Hydraulic flow rate (m <sup>3</sup> d <sup>-1</sup> )	1.87	1.87	1.87
Hydraulic loading rate (m <sup>3</sup> m <sup>-3</sup> d <sup>-1</sup> ) <sup>a</sup>	0.73	0.73	0.73
Aerial surface loading rate (m <sup>3</sup> m <sup>-2</sup> d <sup>-1</sup> ) <sup>a</sup>	0.73	0.73	0.73
Theoretical fluid velocity (m d <sup>-1</sup> )	1.56	1.56	2.84
Theoretical mean HRT (d)	1.37	1.37	0.76
Actual mean HRT (d) <sup>†</sup>	1.19	1.15	0.54
Influent organic strength (g BOD <sub>5</sub> m <sup>-3</sup> ) <sup>‡</sup>	5.75	5.75	5.75
Organic loading rate (g BOD <sub>5</sub> m <sup>-3</sup> d <sup>-1</sup> ) <sup>‡,a</sup>	4.20	4.20	4.20

<sup>†</sup> Implied from the ratio of  $\tau : \tau_{th}$  measured during tracer experiments

<sup>‡</sup> Based on median influent BOD<sub>5</sub> concentration during the operating period

<sup>a</sup> Based on gross reactor volume not void space volume

The hydraulic loading rate used here (0.73 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) was generally higher than those reported in the relevant literature for pilot-scale DW, OP and RF systems. The reasoning for operating the experimental pilot plant under such elevated HLRs was that the organic strength of the influent wastewater—from a tertiary level maturation WSP—was very low. According to the classifications outlined by Metcalf and Eddy (1991), the pilot plant influent feed wastewater was of ‘weak’ organic strength, but is within the reported range for tertiary maturation pond effluent with respect to BOD<sub>5</sub> and SS. A summary of

the influent loading and water quality parameters during monitoring *Period 1* is provided in Table 3.3 below.

Due to the refined nature of the pilot plant influent, and in order to achieve an organic loading rate (OLR) comparable with those reported in the relevant literature, the HLR was increased several fold compared with reported HLRs for other pilot-scale rock filter and duckweed pond research. For example, HLRs for rock filters are most commonly  $\leq 0.5 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  (see Section 1.2.8.6.1) and the guideline HLR for a rock filter treating maturation pond effluent in the United Kingdom is  $0.3 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  (Mara, 2003). The only exceptions to this appear to be the work of Mara *et al.* (2001) and von Sperling *et al.* (2007), where rock filter hydraulic loadings were in the very high range of  $1.0\text{--}2.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ . Similarly, other pilot-scale duckweed pond systems have generally been operated at HLRs in the range of  $0.1\text{--}0.23 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  (Bonomo *et al.*, 1997; Zimmo *et al.*, 2002; Ran *et al.*, 2004).

**Table 3.3.** Pilot plant loading conditions and influent water quality for the first pond reactor of each three-pond treatment series.

<b>Parameter<sup>†</sup></b>	<b>Loading range</b>	<b>Influent range</b>	<b>Median quality</b>
<b>BOD<sub>5</sub><sup>‡</sup></b>	1.5–22 g m <sup>-3</sup> d <sup>-1</sup>	2–30	5.75
<b>Chl. a</b>	4–69 mg m <sup>-3</sup> d <sup>-1</sup>	6–94 μg L <sup>-1</sup>	13 μg L <sup>-1</sup>
<b>SS<sup>‡</sup></b>	5–35 g m <sup>-3</sup> d <sup>-1</sup>	7–48	13
<b>Turbidity (NTU)</b>	4–58	4–58	8.7
<b>NH<sub>4</sub><sup>+</sup>-N<sup>‡</sup></b>	0.8–4.1 g m <sup>-3</sup> d <sup>-1</sup>	0.5–2.4	1
<b>PO<sub>4</sub><sup>3-</sup>-P<sup>‡</sup></b>	4.1–12.6 g m <sup>-3</sup> d <sup>-1</sup>	2.1–5	3.7
<b>FC<sup>a</sup></b>	4.5–6 m <sup>-3</sup> d <sup>-1</sup>	1.7–3.2	2.4
<b><i>E. coli</i><sup>a</sup></b>	4.4–5 m <sup>-3</sup> d <sup>-1</sup>	1.5–2.1	1.8

<sup>†</sup> BOD<sub>5</sub>, biochemical oxygen demand; Chl. a, chlorophyll a; SS, suspended solids;

NH<sub>4</sub><sup>+</sup>-N, ammonia nitrogen; PO<sub>4</sub><sup>3-</sup>-P soluble reactive orthophosphate; FC, faecal coliforms

<sup>‡</sup> Expressed as mg L<sup>-1</sup>

<sup>a</sup> Expressed as log<sub>10</sub> MPN 100mL<sup>-1</sup>

As can be seen in Table 3.3, the final Bolivar maturation WSP effluent is of a highly refined tertiary-level nature, therefore the influent to the experimental pilot plant was generally of much higher water quality than what is typical for most WSP effluents. In

fact so highly polished is the final Bolivar wastewater, that there is even evidence to suggest that the pond network is at times “nutrient-limited” (Cromar *et al.*, 2005).

For the performance data reported in this chapter, the daily influent for both Ponds 1 and 3 is taken as that of the pilot plant influent; as sampled from the header tank (refer Figure 2.1). Pond 2 effluent data was not used for determining Pond 3 influent loading conditions due to the lack of analytical data for Pond 2 treatments across some monitored parameters (see Section 2.2.2.4). Pond 1 effluent data was also not used to define Pond 3 influent because of the desire for a ‘down-the-line’ treatment performance assessment of each upgrade system compared to a common influent. In addition to this, the relatively high HLR and subsequently short HRT of each experimental pond meant that the pilot plant influent was considered to have been relatively stable over the course of one complete three-pond series hydraulic turnover; that is the influent water quality should have remained relatively stable over the course of the three to four day HRT of one entire three-pond treatment series. Therefore, in an attempt to provide a more consistent and concise analysis and interpretation of the presented performance data, only the data from **Ponds 1 and 3** will be discussed in text. Furthermore, the comparatively small data set for the second pond in each treatment series precluded the inclusion of Pond 2 data in some of the performance analyses.

Due to the pilot scale of the experimental pond reactors ( $\approx 2800$  L) and the relatively deep operational hydraulic depth (1m), the impact of ‘wall effects’ (resulting from high surface area-to-volume ratios) are thought not to have played a significant influential role in the observed treatment performance for the current pilot systems. Somiya and Fujii (1984) cited the potential impact of wall-attached biofilms in the overall waste treatment process from their work on similar scale pond reactors. In work presented here, however, and in the absence of any data to the contrary, it was assumed that any potential ‘wall effects’ were similar across all three treatment series due to the identical nature of the 9 pilot-scale reactors. Wall effects will therefore not be further discussed except where they may be of specific relevance.

### 3.3.3 Duckweed mat properties, and biomass density vs. light attenuation

Following inoculation, the duckweed multiplied quickly in all ponds and maintained a robust and complete surface coverage for the entire six month duration from winter (July) to summer (December). Steady-state operational duckweed surface mat thickness was in the order of 2cm, with a final mean duckweed mat density of  $8.29 (\pm 0.43) \text{ kg m}^{-2}$  fresh weight ( $\approx 0.58 \text{ kg}$  dry weight) for all three ponds in series. The full surface mat coverage was successfully established during the latter two months of winter (July–August), during which time the mean DW Pond water temperature was  $12.1^\circ\text{C} (\pm 1.6)$ . This observation of complete successful winter duckweed growth could go toward answering an earlier question posed by Leng (1996) (see Section 1.3.1.2), who—following observations of favourable *Lemna* performance during the Australian summer and autumn months—was unsure as to what the survival of duckweed would be during the Australian winter.

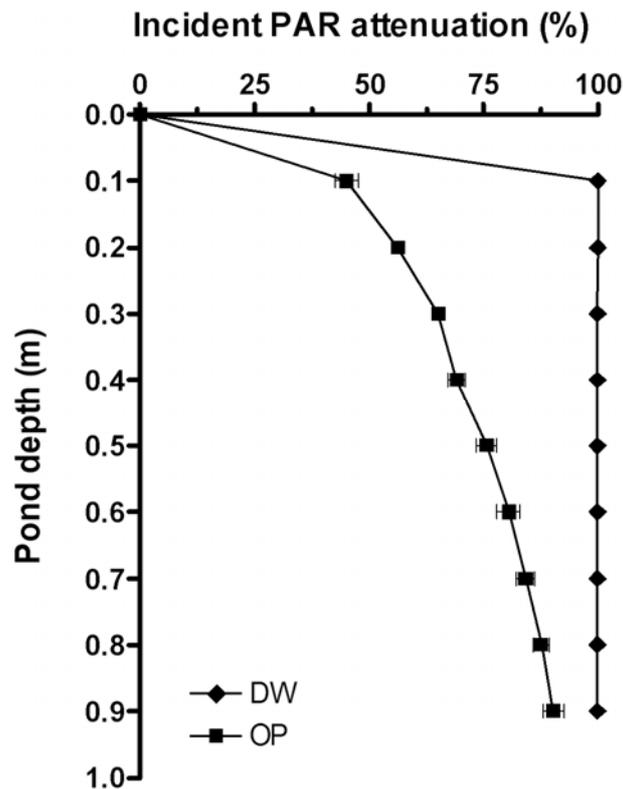
Since the operational *Lemna* surface mat was in the order of 2–3cm thick, it is likely that there was significant self-shading of duckweed fronds in the lower regions of the floating plant mat. Physical appearance and general ‘healthiness’ of the duckweed Pond surface mats was periodically assessed over the six month experimental duration. Generally, individual duckweed frond size decreased as the surface mat aged, reaching approximately half the initial frond size a few months post-inoculation. Frond colour was also observed to gradually change from a brilliant green at the outset of mat formation to a more dull green colouration over time, but remained healthy and in a vegetative state at all times. Frond root length was observed to generally increase with duckweed mat age—a likely consequence of an ever-increasing mat thickness. Some anthocyanin pigmentation (under-frond purple coloration) was observed in ageing duckweed surface mats over time—a factor attributed to S and Fe deficiencies (Leng, 1996)—but again this was not to the detriment of overall duckweed mat healthiness and structural integrity.

Another factor known to affect duckweed mat integrity is competition, mainly from filamentous cyanobacteria and green algae. Filamentous algae and cyanobacteria can become entangled in the duckweed rootstock, effectively smothering the plants

(Roijackers *et al.*, 2004) and ultimately resulting in death and disintegration of the surface mat integrity (Edwards *et al.*, 1992; Al-Nozaily and Alaerts, 2002). Underlying filamentous algae and cyanobacteria can also cause disruption of duckweed surface mats by photosynthetic gas production and subsequent flotation, causing an elevation of the duckweed above the surface of the water leading to desiccation and nutrient starvation (Leng, 1996; Özbay, 2002); however, no such observations were noted here. According to Edwards *et al.* (1992), duckweed mats can also be susceptible to insect infestation by aphids such that surface mat integrity can be compromised. Some degree of aphid infestation was observed during the current study period, although it was not severe enough to compromise the plant mat integrity. It is known that species of *Lemna* are able to utilise the organic carbon (carbohydrates) secreted by neighboring plants (Gopal and Goel, 1993) and also from the water column (Hillman, 1976; Frick, 1994) for their own heterotrophic nutrition. It is possible that such modes of nutrition could have contributed in some way to the persistent maintenance of a dense and complete duckweed surface mat during the course of the current work, although this remains purely speculative.

Depth-wise light penetration profiling of the DW and OP systems was performed. Profiling revealed that under an incident PAR of  $600\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ , in excess of 99.5% ( $\pm 0.04$ ) of the PAR light spectra was attenuated at a depth of 0.1m below the surface for the DW Ponds—a result on par with figures reported elsewhere (Giorgi and Malacalza, 1994; Parr *et al.*, 2002). This is compared to an average 45% ( $\pm 2.6$ ) PAR attenuation for the Open Ponds at the same depth (Figure 3.4). Depth-wise profiling also showed that light extinction was extremely rapid, and based on the data of Goldsborough (1993), the bulk of light attenuation was likely to have occurred within just the top 0.5cm of the plant mat. This is a well recognised advantage of duckweed pond systems, whereby the plant surface mats can dramatically reduce light penetration to the underlying water to such an extent such that algal growth is suppressed or even prevented (Zirschky and Reed, 1988; Mara *et al.*, 1992; Hammouda *et al.*, 1995).

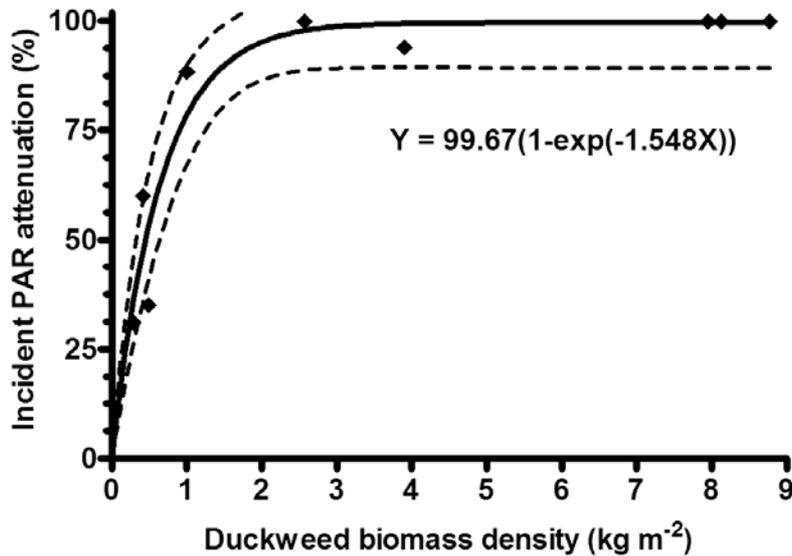




**Figure 3.4.** Irradiance–depth PAR profiles for Duckweed (DW) and Open Pond (OP) systems, showing percent attenuation at  $600\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  incident irradiation and standing duckweed plant biomass density of  $8\text{kg m}^{-2}$  (fresh weight). Individual data points represent mean determinations from three parallel treatment ponds ( $\pm 1$  S.D.)

Reduced light penetration as a result of duckweed mat formation is a phenomenon quantitatively reported by only a few authors. Ngo (1987) stated that a  $0.5\text{kg m}^{-2}$  duckweed mat will reduce incident light penetration by 35%, whilst a  $3.9\text{kg m}^{-2}$  duckweed surface mat reduces incident light penetration by 94%. Janes *et al.* (1996) also reported 88.4% incident PAR attenuation under a  $1\text{kg m}^{-2}$  *Lemna* mat. Goldsborough (1993) recorded 99.9% PAR attenuation under a  $2.6\text{kg m}^{-2}$  *Lemna* mat at a similar depth to that reported here, whilst Roijackers *et al.* (2004) observed that a surface coverage of *Lemna* at equivalent fresh biomass densities of  $0.27$  and  $0.41\text{kg m}^{-2}$  attenuated 31 and 60% of incident irradiance respectively. It should be noted that the biomass data of Roijackers *et al.* (2004) was given as dry weight, and a correction factor subsequently applied for conversion to fresh weight based on an average water content of 93% (Landolt, 1986; Leng *et al.*, 1995; Goopy and Murray, 2003). When these reported values for duckweed biomass density versus light attenuation are plotted against values recorded here, the resultant curve displays a very good single-phase exponential fit to

the data ( $r^2 = 0.90$ ;  $n = 9$ ) suggesting a probable exponential relationship between duckweed mat biomass density and incident light attenuation (Figure 3.5).



**Figure 3.5.** Exponential fit of available duckweed biomass density vs. incident light attenuation data (broken lines represent 95% confidence intervals for the fitted line).

This relationship was consistent with that of Westlake (1964), who found light extinction to be a logarithmic function of plant (*Ranunculus pseudofluitans*) biomass density. Interestingly, Westlake (1964) also noted that not only was there greater total light energy attenuation under aquatic plant cover, but also a greater portion of the PAR light spectra was absorbed; presumably through photosynthetic interception by the overlying plant biomass. This is no doubt another contributing factor as to why overlying aquatic macrophytes are renowned for reducing or minimising the potential for underlying algal growth.

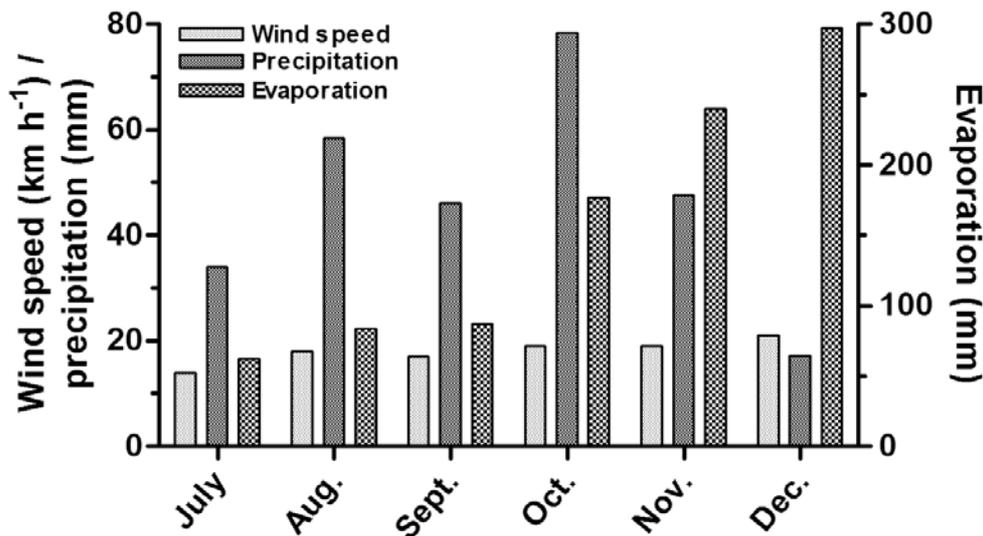
Where appropriate, duckweed biomass values from the fitted curve of Figure 3.5 could provide commercial duckweed pond operators with additional insight into optimal plant biomass harvesting regimes based on the plotted minimum biomass density coverage required for maximum light attenuation and greatest pond performance (i.e. in the order of 1–2kg m<sup>-2</sup>). This critical biomass density value could be of considerable importance, given that incomplete *Lemna* surface coverage (<50% coverage or <0.6kg m<sup>-2</sup>) is known to be an ineffective algal growth inhibitor (Leng *et al.*, 1995; Szabó *et al.*, 1998). Incidentally, this critical value (somewhere within the range of 1–2kg m<sup>-2</sup>) was similar

to the operational biomass density of  $1.3\text{kg m}^{-2}$  chosen by Bonomo *et al.* (1997) during their large-scale pilot duckweed pond trials, and also that of Alaerts *et al.* (1996) who adopted a standing biomass density of  $1.6\text{kg m}^{-2}$  (fresh weight) during their full-scale duckweed pond operations.

As highlighted in Chapter 1 (Section 1.2.8.5.2), it is a commonly associated operational requirement for duckweed ponds to have a floating containment ‘grid’ system to prevent wind-dispersion of the tiny plants—a structure that constitutes an additional capital input for duckweed WSPs. Results from the current work, however, have shown that if there is no ongoing harvesting of accumulated duckweed biomass, then the surface mat is capable of reaching very high standing biomass densities and mat thicknesses of 2–3cm. Under such circumstances, the structural integrity of the duckweed mat was found to be very robust and resilient to physical disruption; suggesting that such that a floating containment system may not be necessary in small-scale installations. This was commented on by Rich (2003), who claimed that several duckweed pond systems had been operating in the United States without floating containment systems. This may offer an additional cost benefits for duckweed systems, particularly in instances where it is used solely for algal solids removal and duckweed biomass production is not an operational objective.

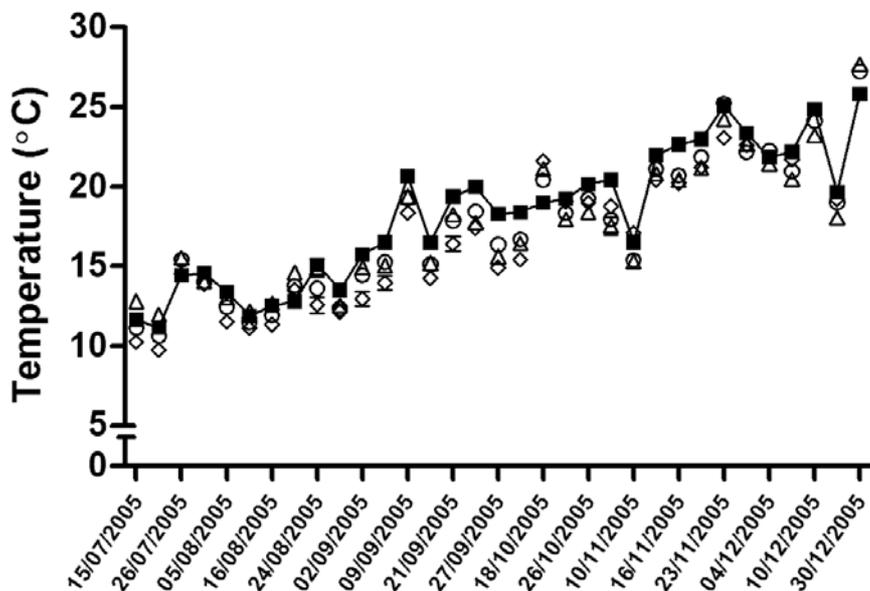
### **3.3.4 Environmental and physicochemical parameters**

The pilot plant site received a daily average of 9.8 hours of sunlight in the summer season (December–February) and 5.3 hours during winter (June–August) at an average annual daily solar irradiance of  $17\text{MJ m}^{-2}$  (data based on that recorded at the nearby Edinburgh Air Force base,  $34^{\circ}42'40''\text{S } 138^{\circ}37'20''\text{E}$ ; Australian Government Bureau of Meteorology). A summary of the prevailing weather conditions experienced at the Bolivar WWTP during monitoring *Period 1* of 2005 is provided below.

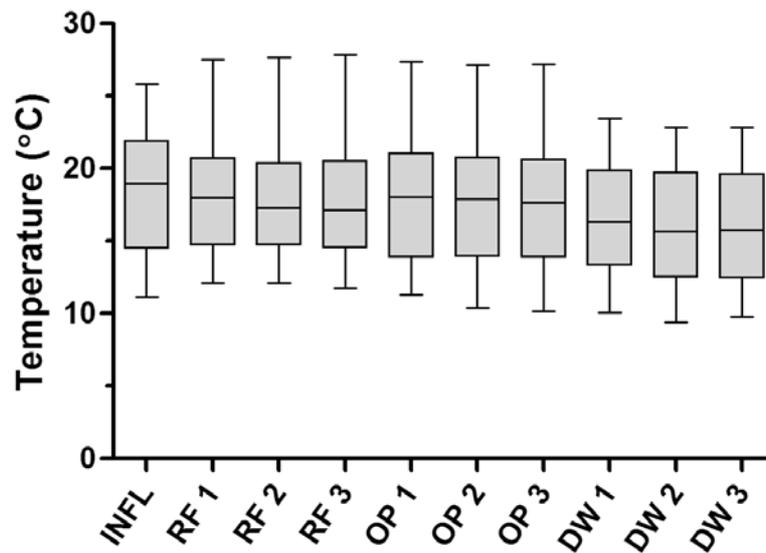


**Figure 3.6.** Selected mean monthly site weather conditions from July–December of 2005. Left y-axis shows average daily wind speed and monthly precipitation, and the right y-axis shows mean monthly evaporation (data courtesy of the Australian Government Bureau of Meteorology).

As outlined in Section 2.2.2.1, various physicochemical water quality parameters were monitored for the pilot plant influent and three experimental treatments during the 2005 monitoring *Period 1*. Results from these analyses are provided below.



**Figure 3.7.** Water temperature data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Duckweed Ponds (◇). For ease of interpretation, data points show only the mean temperature ( $\pm 1$  S.D) averaged across each three-pond treatment series, with a line fitted only to the influent data set.

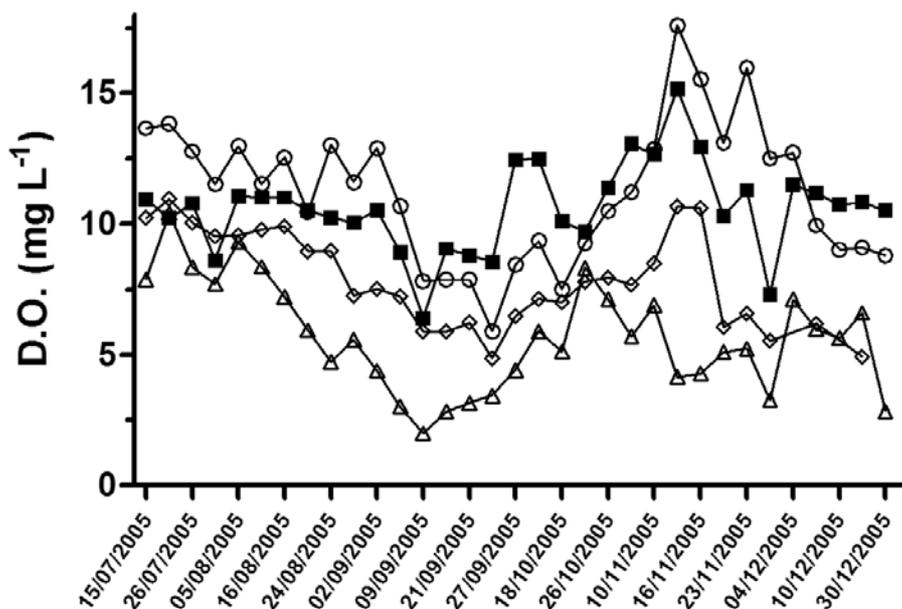


**Figure 3.8.** Water temperature box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). The shaded ‘box’ represents the interquartile data range (IQR), the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

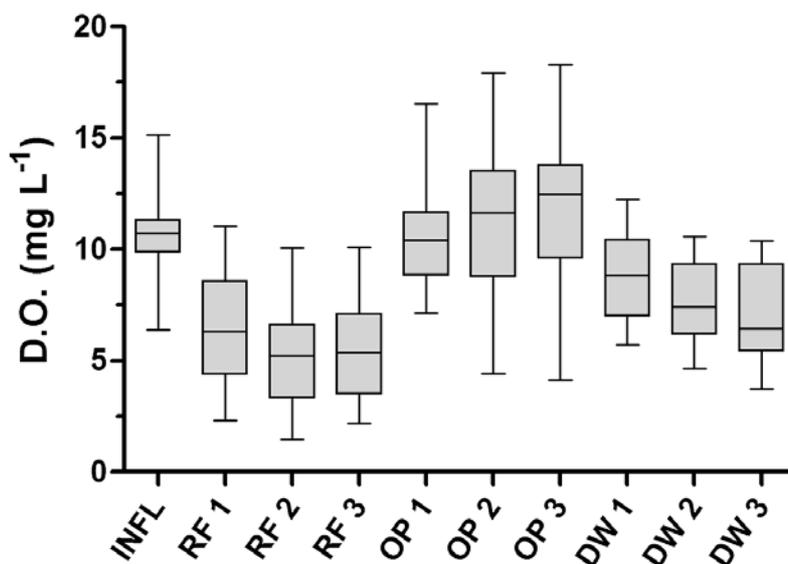
Data from water temperature monitoring is represented in Figures 3.7 and 3.8. During the course of the six month monitoring period, the pilot plant water temperature increased steadily from around 11.5°C in July 2005, to approximately 26°C by the end of December, 2005. Generally speaking, the water temperature in the RF and OP treatment systems was—compared to influent temperature—reduced on average by less than 1°C during pilot plant passage. DW treatment train water temperature was reduced by an average of 2.0°C compared with the influent temperature; although as for the RF and OP systems, this was not a significant reduction (1-way ANOVA;  $F_{(9,311)} = 0.873$ ;  $p = 0.55$ ).

Qualitatively, this slight reduction in temperature within the DW Ponds is in agreement with the reporting of others. Zimmo *et al.* (2002) reported similar magnitude temperature reductions in their pilot-scale duckweed ponds compared with uncovered ponds. Dale and Gillespie (1976) commented on an increased capacity for thermal stability under a surface cover of duckweed, whereby the plant mat reflects more and transmits less energy than open water—creating less extreme temperature fluctuations and also lower temperatures at greater depths beneath the duckweed mat. Although not significant in the pilot-scale system here, it is thought that temperature would be significantly reduced in larger-scale duckweed pond systems. Generally speaking, the

effects of long-term temporal changes in water temperature were thought to have been applied evenly across all three pilot treatments, and no attempts were made to distill the potential effects of temperature on the treatment performance of each pilot-scale WSP upgrade.



**Figure 3.9.** Dissolved oxygen data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Duckweed Ponds (◇). For ease of interpretation, data points show only the mean DO concentration ( $\pm$  1 S.D.) averaged across each three-pond treatment series.



**Figure 3.10.** Dissolved oxygen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

Data from DO monitoring is given in Figures 3.9 and 3.10. During the six month monitoring period, the pilot plant influent DO levels displayed a fluctuating pattern according to localised environmental conditions. Long term trends in pilot pond DO levels mimicked that of the influent (Figure 3.9), although DO generally: decreased slightly for DW Ponds; increased slightly for Open Ponds; and decreased substantially for RFs (Figure 3.10). Average influent DO concentration was in the order of 10.5 ( $\pm 1.7$ ) mg L<sup>-1</sup>, with three-pond mean DW, OP and RF dissolved oxygen concentrations of 7.9 ( $\pm 1.8$ ), 11.2 ( $\pm 2.9$ ), and 5.7 ( $\pm 2.2$ ) mg DO L<sup>-1</sup> respectively. DO levels generally decreased down the respective pond series for both the DW and RF treatment trains, and increased for the OP series (although not significantly in any case;  $p > 0.05$ ). In the DW treatment train, DO decreased significantly compared to influent concentrations only in Ponds 2 and 3 (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 166.6$ ;  $p \leq 0.01$ ) but not after the first pond ( $p > 0.05$ ). In the OP series, the small DO increase was not significant in any of the three ponds ( $p > 0.05$ ), and for the RFs, the average 45% reduction in the levels of inflowing DO was highly significant across all three filters ( $p < 0.001$ ), something which is clearly shown in Figure 3.10. With respect to between-treatment DO levels, the OP series had significantly elevated oxygen concentrations than did the DW treatment for Ponds 2 and 3 only ( $p \leq 0.01$ ) and significantly higher DO concentrations across all three ponds when compared to RF concentrations ( $p < 0.001$ ). Between the DW and RF treatments, oxygen concentrations were greater in both DW-1 and DW-2 ( $p < 0.01$ ) but were effectively similar for the third pond of both series ( $p > 0.05$ ).

This observed trend for a reduced DO concentration in duckweed systems has been reported elsewhere. Early work has already demonstrated the ‘smothering’ effect of a complete duckweed surface cover on the underlying aqueous oxygen concentration (Lewis and Bender, 1961; Morris and Barker, 1977); with the reduced DO concentration a result of inhibited surface re-aeration potential, impeded oxygenic algal photosynthesis, and also because duckweeds exchange the bulk of photosynthetically-evolved oxygen with the atmosphere and not the underlying water (Morris and Barker, 1977; Giorgi *et al.*, 1998; Al-Nozaily *et al.*, 2000a). Zimmo *et al.* (2000; 2002)—interpreting data from both laboratory- and pilot-scale reactors—reported that the DO concentration in duckweed ponds was consistently around half that of parallel algal-based ponds. The magnitude of DO reduction observed here was about half that of

Zimmo and co-workers, however, this was likely to be a consequence of the organic loading rate being some 10-fold lower for the Duckweed Ponds reported here.

Whilst many authors have reported on a generally severe restriction of DO levels resulting from a floating duckweed cover, it is not universally the case. As can be seen in both Figures 3.9 and 3.10 above, all DW Ponds continuously maintained fully-aerobic (i.e.  $>3\text{mg L}^{-1}$ ; Arunachalam *et al.*, 2004) conditions at an average of 80% saturation during the entire monitoring period. The higher than anticipated DO concentrations within these DW Pond systems is most likely a reflection of the refined nature of the tertiary-level Bolivar influent wastewater imparting a relatively low organic ( $\text{BOD}_5$ ) loading on the pilot system, and also a consequence of their relatively high hydraulic throughput. Interestingly, this trend for continued aerobic duckweed pond operation was also observed by Alaerts *et al.* (1996) following the four year operation of a full-scale duckweed pond system. According to the authors, aerobic conditions were able to be maintained throughout the entire pond depth at all monitoring periods; something again almost certainly a consequence of their relatively low  $\text{BOD}_5$  organic loading regime ( $4.8\text{--}6\text{g BOD}_5 \text{m}^{-2} \text{d}^{-1}$ )—a similar order of magnitude to that of the current systems. Nhapi *et al.* (2003) actually reported a significant re-aeration in duckweed ponds fed with anaerobic pond effluent, with inflowing DO concentration increasing from an average of  $1.8$  to  $6.5\text{mg L}^{-1}$  in full-scale ponds. Despite this apparent exception, it appears that in low-organic-strength wastewaters, DO conditions can remain fully aerobic even with a complete duckweed surface mat in place.

The slight but non-significant increase in DO down the pond series for the OP treatment was most likely a consequence of some additional photosynthetic re-oxygenation during the temporary impoundment of inflowing wastewater. This result was considered to be of no great interest in the context of the current work, and merely served to demonstrate an effective maintenance of *in situ* WSP conditions within the *ex situ* pilot ponds. Similar to the DW Ponds, influent DO levels were again reduced within all three RFs. This trend for a reduction in DO concentration following RF treatment is a well recognised operational disadvantage associated with the technology, whereby RF anaerobiosis can lead to undesirable  $\text{H}_2\text{S}$  production,  $\text{NH}_4^+\text{-N}$  remineralisation, and can also necessitate post-filter effluent aeration prior to discharge in many cases

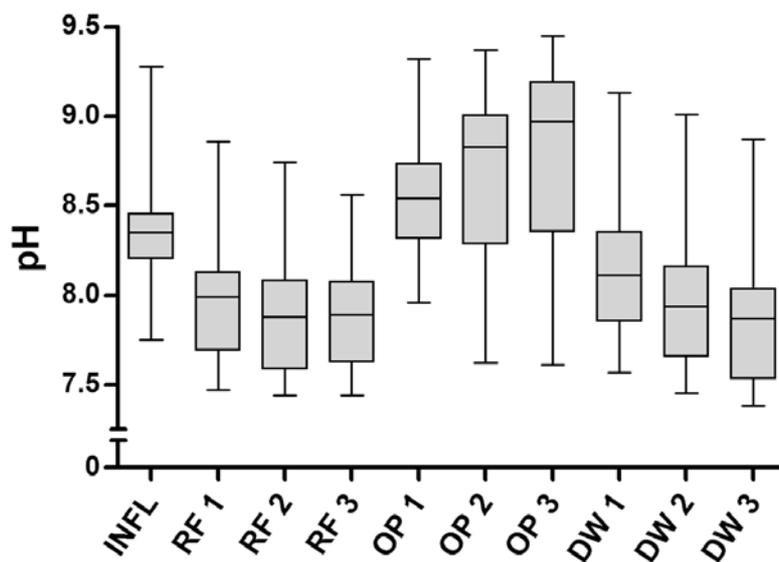


(Middlebrooks, 1988). Many studies have reported that RF effluent can be significantly de-oxygenated, commonly to  $<0.5\text{mg L}^{-1}$  and even to the point of anoxia (Hirsehorn, 1974; USEPA, 1983; Saidam *et al.*, 1995; Mara *et al.*, 2001; Tanner *et al.*, 2005); however, reductions in DO were not so extreme in the pilot-scale filters reported here.

Unlike the above reports, some authors have reported on the maintenance of aerobic conditions rock filter operation. Swanson and Williamson (1980) for example recorded a general decline in rock filter effluent DO from  $11.0$  to  $3.5\text{mg L}^{-1}$  but DO concentration was always  $>1.8\text{mg L}^{-1}$  and most commonly above  $3\text{mg L}^{-1}$ . Strang and Wareham (2005) reported a general decline in DO (commonly by  $\approx 3\text{mg L}^{-1}$ ) following rock filter passage in full-scale systems operating in New Zealand. The authors also reported that their rock filters remained aerobic during the 7 month monitoring period; although there were a few reported instances of very low ( $< 1\text{mg L}^{-1}$ ) DO concentrations. For Rock Filters here, DO was able to be maintained at a relatively high average concentration of  $5.7\text{mg L}^{-1}$ , and was always  $\geq 1.5\text{mg L}^{-1}$ .

Some authors have suggested a reduction treatment performance capacity at greatly reduced DO concentrations. Tanner *et al.* (2005) suggested that very low DO levels ( $\approx 1\text{mg L}^{-1}$ ) were likely to have reduced the rates of organic matter decomposition as well as having limited the development of microbial nitrifier populations within their pilot-scale wetland–rock filter systems. DO concentrations within the entire RF treatment train were maintained at much higher levels than those observed by Tanner *et al.* (2005), and there was no evidence to suggest that the reduced *in situ* oxygen concentration had a negative impact on Rock Filter BOD<sub>5</sub> removal performance (see Section 3.3.5) or on resident microbial nitrifier populations (see Section 3.3.7.1). Tanner *et al.* (2005) went on to suggest that in addition to its effect on the composition and activity of microbial communities, very low DO conditions were likely to have a negative impact on protozoan, zooplankton and other higher invertebrate grazer communities. Whilst such low-level DO conditions were not recorded here, it is possible nonetheless that the significantly reduced levels of oxygen—particularly within the RF treatment train—might have had an influential role on the resident zooplankton community structure. This concept will be investigated further in Chapter 5.

The higher than anticipated DO concentrations observed within both the DW and RF systems were most likely a reflection of the refined nature of the tertiary-level Bolivar WSP effluent. This highly ‘polished’ wastewater therefore imparted only a relatively low organic ( $BOD_5$ ) loading on the shaded DW and RF pond reactors, allowing DO levels to remain higher than what would be expected if they had been fed with higher organic strength wastewater. Another likely contributing factor to the relatively high *in situ* DO concentrations is that all DO measurements were taken as one-off ‘daytime’ measurements at  $1200 \pm 2$  hours. Although these daily spot measurements were not recording the peak of daily DO (and no doubt accompanying pH) fluctuations (refer to Section 4.3.3 for more information), it is likely that the 24 hour average DO concentration would have been significantly lower than these daytime measured values. In spite of the *precise* magnitude of daily mean DO concentrations, and given that all daily physicochemical monitoring was performed at the same time on any given sampling interval, it was assumed that the recorded DO and pH values for each experimental pond provided an accurate reflection of the ‘between-treatment’ differences in these water quality parameters.



**Figure 3.11.** pH box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

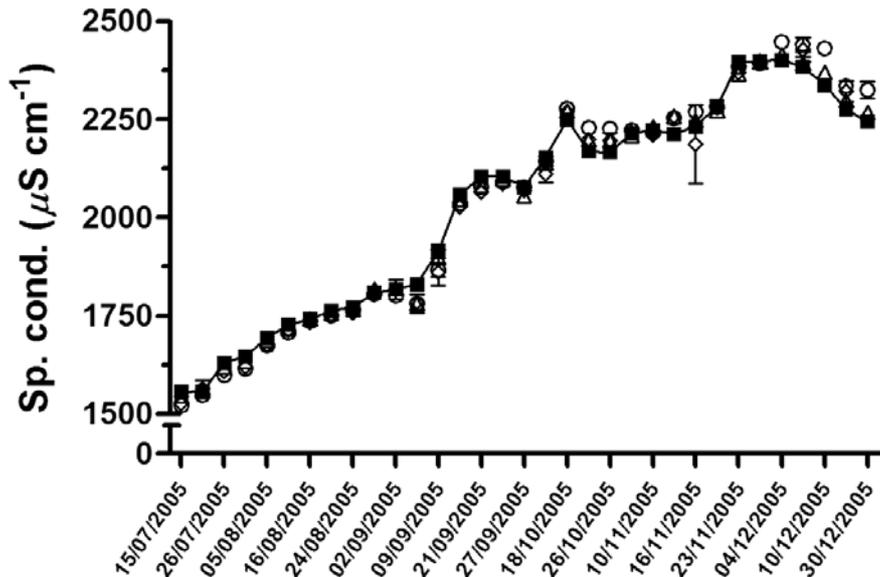
Data from the monitoring of water pH is shown above in Figure 3.11. Recorded pH was always alkaline, and was commonly  $>7.7$  across all treatments (based on lower 95% CI

of mean). Wastewater pH generally decreased slightly during both DW and RF treatment; dropping approximately 0.5 units from 8.4 to 7.9 by the final pond both series. Conversely, pH within the OP treatment train steadily increased through the pond series by roughly the same order of magnitude, from  $\approx 8.4$  up to 8.8. Statistically, this decrease in pH through the respective pond series was significant for both the DW and RF treatment trains (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 120.2$ ;  $p < 0.001$ ); however, the 0.4 unit pH increase was not significant for the OP series ( $p > 0.05$ ). Wastewater pH was also significantly elevated following passage through the OP treatment series relative to the DW and RF treatments ( $p < 0.001$ ), but overall, wastewater pH of both the DW and RF treatments was similar ( $p > 0.05$ ).

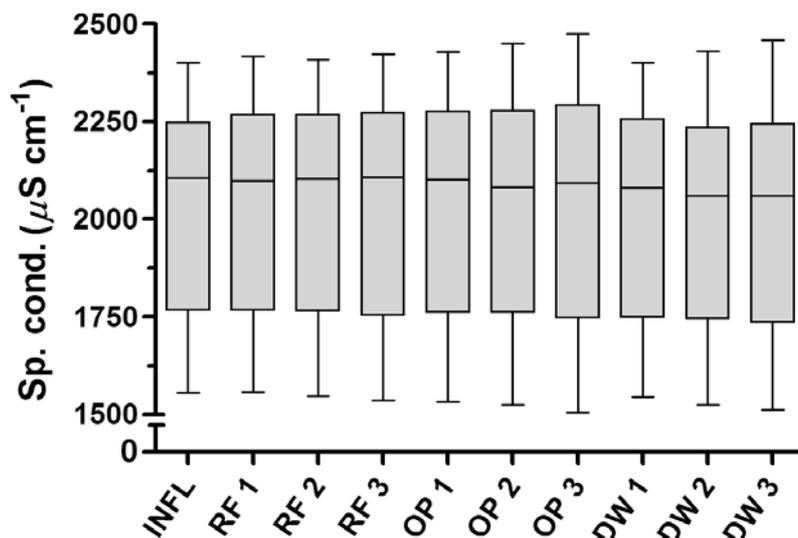
Caicedo *et al.* (2000) and Tanner *et al.* (2005) both reported on a stabilising effect on the underlying water pH (i.e. both lowering pH and making it more stable over time) from a duckweed cover. A similar trend was observed here (Figure 3.11), as evidenced by the declining median pH within the DW Pond series, as well as the stable interquartile range of the pH data for the DW Pond series compared to an increased variability in pH down the un-covered OP treatment train. This slight decrease in pH for DW Ponds was most likely due to the reduced rates of primary production and suppression of normal algal photosynthesis within the shaded environment of these ponds. This was supported by the lack of a significant relationship between DO and pH through the pond series within the DW treatment train; going from a highly significant relationship in the pilot plant influent (Pearson  $r = 0.74$ ;  $p < 0.0001$ ), to a less significant correlation within DW Pond 1 ( $r = 0.53$ ;  $p < 0.01$ ), and finally no relationship in DW Pond 3 ( $r = 0.33$ ;  $p > 0.05$ ).

The observed decrease in wastewater pH during RF passage is a trend also noted by others during long-term RF operation (Hirse Korn, 1974; Swanson and Williamson, 1980; Mara *et al.*, 2001). This pH reduction during rock filtration was thought to be a result of several factors, namely: the dark suppression of algal photosynthesis; an increase in CO<sub>2</sub> and carbonic acid production as a result of enhanced microbial respiration within the filter; and also from a small amount alkalinity consumption and H<sup>+</sup> ion production during the oxidation of a limited amount of ammonia via microbial nitrification (Heard *et al.*, 2002; see also Section 3.3.7.1). In general, a pH shift within these pilot systems in the order of  $\leq 0.5$  units was thought to be of limited consequence with respect to the

treatment efficacy of each system. The small pH increase in the OP series was far from that which is required to effect pH-induced flocculation of SS (Ayoub *et al.*, 1986; Elmaleh *et al.*, 1996) and so pH was thought not to have had any significant bearing on the overall treatment performance of any pilot pond system investigated here.



**Figure 3.12.** Specific conductivity data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Duckweed Ponds (◇). For ease of interpretation, data points show only the mean conductivity ( $\pm 1$  S.D.) averaged across each three-pond treatment series, with a line fitted only to the influent data set.

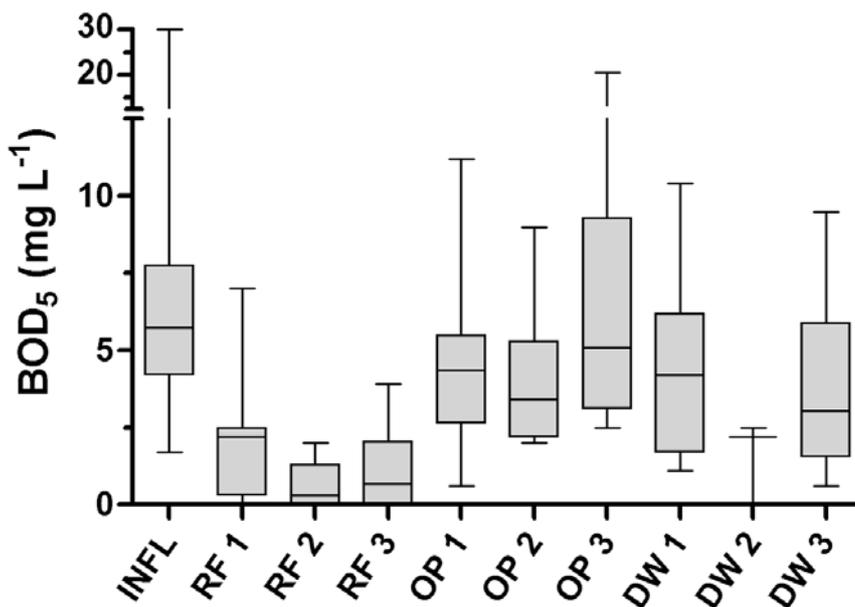


**Figure 3.13.** Specific conductivity box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed ponds 1, 2, 3 (DW-1, DW-2, DW-3).

Data from the monitoring of specific conductance is shown in Figures 3.12 and 3.13. Specific conductivity of the influent wastewater, and also that of all treatments, steadily increased from the start of the monitoring period in July of 2005 up until the end of the monitoring period in December of the same year. This increase coincided with the seasonal shift from winter in July to summer in December, and was a result of an increased evaporation rate during the increasingly warmer conditions (see Figure 3.6); something supported by the strong correlation between influent water temperature and conductivity (Spearman  $r_s = 0.888$ ;  $n = 33$ ;  $p < 0.0001$ ). This corresponded to an increase in wastewater salinity from  $0.8\text{g L}^{-1}$  in July, to  $1.2\text{g L}^{-1}$  in early December. Conductivity varied minimally within all treatments compared with pilot plant influent readings. Specific conductivity values ranged from  $1500\text{--}2400\mu\text{S cm}^{-1}$  (average of  $\approx 2000\mu\text{S cm}^{-1}$ ), with individual treatment train variation from influent readings of less than 1% from one pond to the next. This low-level variation was considered unlikely to be of any significant biological relevance in the current research context, and so conductivity data is not discussed further. Finally, for a concise statistical summary of the full listing of physicochemical parameter correlations, the reader is directed to the corresponding correlation matrices for the pilot plant influent as well as the three upgrade treatments (Appendix B).

### **3.3.5 Wastewater treatment performance: removal of particulate organics and oxygen demand**

Data from the monitoring of  $\text{BOD}_5$  within the three pilot treatment systems is shown in Figure 3.14.



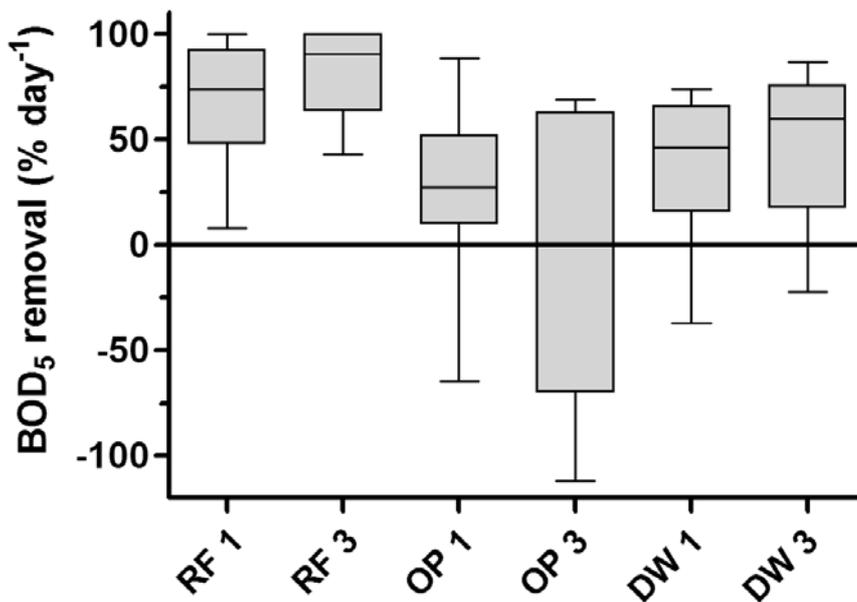
**Figure 3.14.** BOD<sub>5</sub> box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

As shown in Figure 3.14, pilot plant influent organic strength was both low and at times highly variable, with a median BOD<sub>5</sub> concentration of 5.75 mg L<sup>-1</sup> and a mean of 7.38 mg L<sup>-1</sup>. This sporadic and sometimes high-level variability in the Bolivar WSP effluent (i.e. pilot plant influent) is a common feature of such systems. Significant short-term fluctuations in WSP effluent quality for parameters such as BOD<sub>5</sub> and SS, can be largely apportioned to the relative ecological instability of WSP environments and also to their high sensitivity to changes in localised meteorological conditions; something exacerbated by their universally shallow depth and susceptibility to variable hydrodynamic conditions (Uhlmann, 1980). The result of these factors is a near-permanent ‘transient’ mode of operation, whereby a biological equilibrium ‘stable-state’ is neither reached nor maintained (Uhlmann, 1980). Although there was a single statistically-extreme (i.e. >3× the interquartile data range from the 75<sup>th</sup> percentile value) outlying BOD<sub>5</sub> value within the influent data set (30 mg L<sup>-1</sup>; see Figure 3.14), this particular data point was retained for the purposes of the following data analyses. Despite this event being associated with a higher than average daily SS value, because it did not directly coincide with a wind-induced outlying SS spike event (see Section 3.3.6) it was considered to reflect the normal variability in WSP effluent quality and so was

retained for data analyses. Given the highly skewed distribution for the influent BOD<sub>5</sub> data, the median value will be used for all subsequent treatment performance comparisons. This influent median concentration of 5.75 mg L<sup>-1</sup>—taking into account the mean HLR—translated to a median BOD<sub>5</sub> organic loading during the monitoring period of 4.2 g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>.

As described in Section 3.3.2, the daily influent loading for both Ponds 1 and 3 was taken as that of the primary pilot plant influent sampled from the header tank (see Figure 2.1). Pond 2 effluent data was not used for determining Pond 3 influent loading conditions due to the lack of analytical data for Pond 2 for some parameters (see Section 2.2.2.4). Furthermore, the high HLR and subsequently short HRT meant that pilot plant influent should have been relatively stable over the course of one complete three-pond hydraulic turnover (≈4.1 days); that is the influent wastewater quality was assumed to have been relatively stable over the course of the three to four day HRT for one entire three-pond treatment train.

Statistical analysis of the data from Figure 3.14 showed that there was no significant BOD<sub>5</sub> removal in the DW Pond series for any of the three ponds (1-way ANOVA;  $F_{(9,107)} = 8.845$ ;  $p > 0.05$ ). Similarly, no significant BOD<sub>5</sub> removal was achieved by the OP treatment train for any pond ( $p > 0.05$ ). In contrast, the RF train showed significantly reduced BOD<sub>5</sub> concentrations in all three filters compared with that of the influent ( $p < 0.001$ ). Moreover, by the end of each pilot treatment train, the BOD<sub>5</sub> of RF-3 effluent was significantly lower than that of both OP-3 ( $p < 0.001$ ) and DW-3 ( $p < 0.05$ ). It should be emphasized that whilst median values were invariably used to calculate parameter mass loading rates throughout this chapter, transformation of performance data often allowed for parametric statistical analyses, such that these were used in preference to non-parametric tests wherever possible. Relative percentage BOD<sub>5</sub> removal efficiencies for the three treatments are shown in Figure 3.15. Due to the low number of sample replicates for Pond 2 BOD<sub>5</sub> data (DW-2  $n = 3$ ; OP-2  $n = 7$ ; RF-2  $n = 6$ ; refer to Section 2.2.2.4), percentage performance data are not shown for the second pond of the respective treatments.



**Figure 3.15.** Box-plots showing daily percentage BOD<sub>5</sub> removal performance relative to pilot plant Influent concentration for Ponds 1 and 3 of each pilot treatment system ( $n \geq 12$  for all plots).

Long term mean percentage BOD<sub>5</sub> removals for Pond 1 data of Figure 3.15 were 36, 24 and 69%, for DW, OP and RF systems respectively and for Pond 3 data, 45, -10 and 84% for DW, OP and RF systems respectively. As described above, BOD<sub>5</sub> effluent concentrations were not significantly different from the pilot plant influent concentration for both the DW and OP series. When compared statistically to a theoretical zero average BOD<sub>5</sub> removal, however, mean percentage BOD<sub>5</sub> removals for DW Ponds 1 and 3 were found to be significantly ‘non-zero’, whereas they were only slightly greater than zero for OP-1 ( $p = 0.03$ ) and were effectively equivalent to zero removal for OP-3 (Table 3.4). This implied, on average, that greater-than-zero BOD<sub>5</sub> removals were achieved within the DW treatment series but not within the Open Ponds. Unlike the other two treatment trains, the RFs always yielded positive BOD<sub>5</sub> removals; with the DW and OP treatment series both experiencing net BOD<sub>5</sub> increases on at least two of the 12 or more sampling intervals. This was reflected in the corresponding coefficient of variations (CV’s) with respect to percentage BOD<sub>5</sub> removal efficiency, where there was shown to be considerably less variability in BOD<sub>5</sub> removal performance for the RFs than for the other two treatments (Table 3.4). The same analysis also showed the Duckweed Ponds to be more reliable than the parallel Open Ponds at removing loaded BOD<sub>5</sub>.



**Table 3.4.** Summary of BOD<sub>5</sub> performance data across all pilot plant treatments for Pond 1 and 3 data only.

BOD <sub>5</sub> performance parameter	Pilot treatment pond					
	DW-1	DW-3	OP-1	OP-3	RF-1	RF-3
Median influent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	5.8	5.8	5.8	5.8	5.8	5.8
Mean influent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	7.4	7.4	7.4	7.4	7.4	7.4
Median effluent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	4.2	3.1	4.4	5.1	2.2	0.7
Mean effluent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup> *	4.6	4.0	4.7	6.4	2.1	1.1
Median daily BOD <sub>5</sub> removal (% day <sup>-1</sup> )	46	60	27	0	74	90
Mean daily BOD <sub>5</sub> removal (% day <sup>-1</sup> ) <sup>‡</sup> *	36	45	24	-10	69	84
Long-term CV for BOD <sub>5</sub> removal (%)	100	80	163	702	38	23

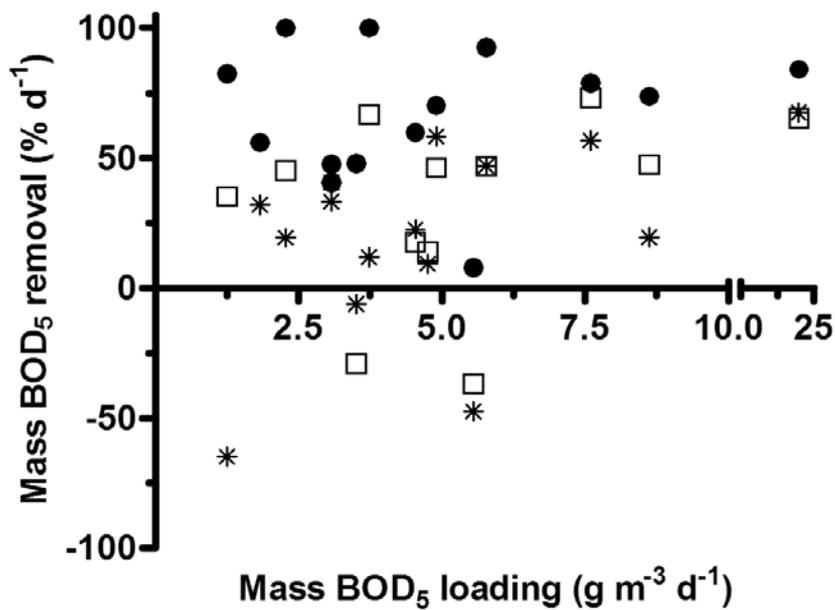
<sup>†</sup> Effluent BOD<sub>5</sub> concentration was tested relative to mean pilot plant influent BOD<sub>5</sub> concentration (1-way ANOVA)

<sup>‡</sup> Average BOD<sub>5</sub> removal % tested against a theoretical 'zero' daily mean (one sample *t*-test)

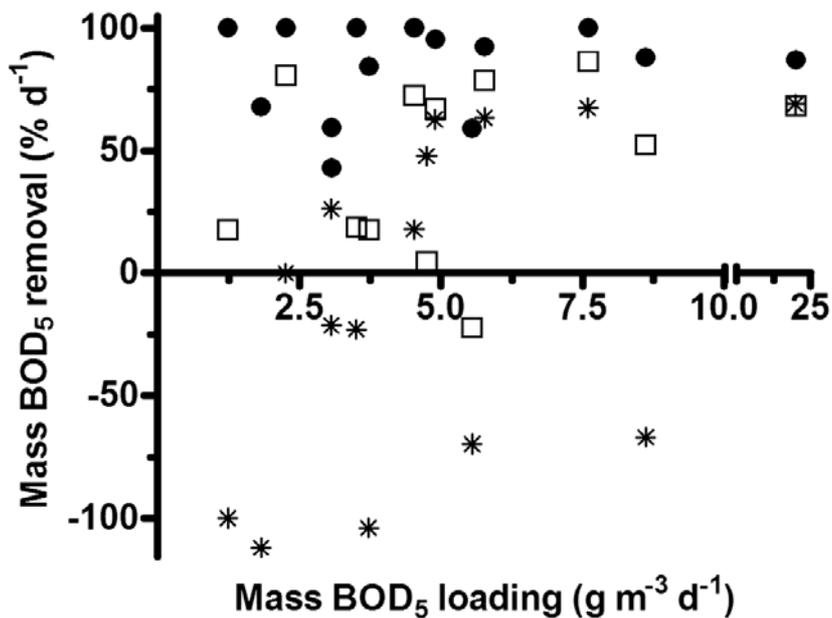
\* Shading intensity shows significance level: *p* > 0.05 (no shading); *p* < 0.05 (light); *p* < 0.01 (medium); *p* ≤ 0.001 (black)

The above analysis of performance *reliability* through comparing the corresponding BOD<sub>5</sub> removal CV's, highlighted the enhanced consistency in performance delivery of the RF system over both the DW and OP treatments. Not only did the RF treatment series deliver a better quality final effluent in terms of BOD<sub>5</sub> concentration, but it did so with enhanced consistency compared with both the DW and OP treatments. Interestingly, this trend for a high degree of consistency in performance delivery has been noted elsewhere. Hirsekorn (1974; pp. 7–8, in quoting Martin, 1970) noted that “*once the (rock) filters reached a state of equilibrium, the quality of the treated effluents would remain fairly uniform*” regardless of influent loading rate; something also observed by Swanson and Williamson (1980) during their rock filter investigations. This is in effect evidenced by the very stable effluent BOD<sub>5</sub> and also low CV of performance, and demonstrates the good buffering capacity that RFs have against a notoriously unpredictable WSP effluent water quality.

Whilst the above table and figures serve to reduce the long-term monitoring data into discrete statistical integers, they provide no real insight into the effects of BOD<sub>5</sub> loading rate on performance efficiency. In order to gain a more comprehensive understanding of the relationship between BOD<sub>5</sub> loading versus percentage removal efficiency, the condensed data from Figures 3.14 and 3.15 have been expanded and can be seen for Ponds 1 and 3 of the respective treatments in Figures 3.16 and 3.17 respectively.



**Figure 3.16.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. mass removal (as a percentage of daily loading rate) for Pond 1 data only. Individual data points represent performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●).

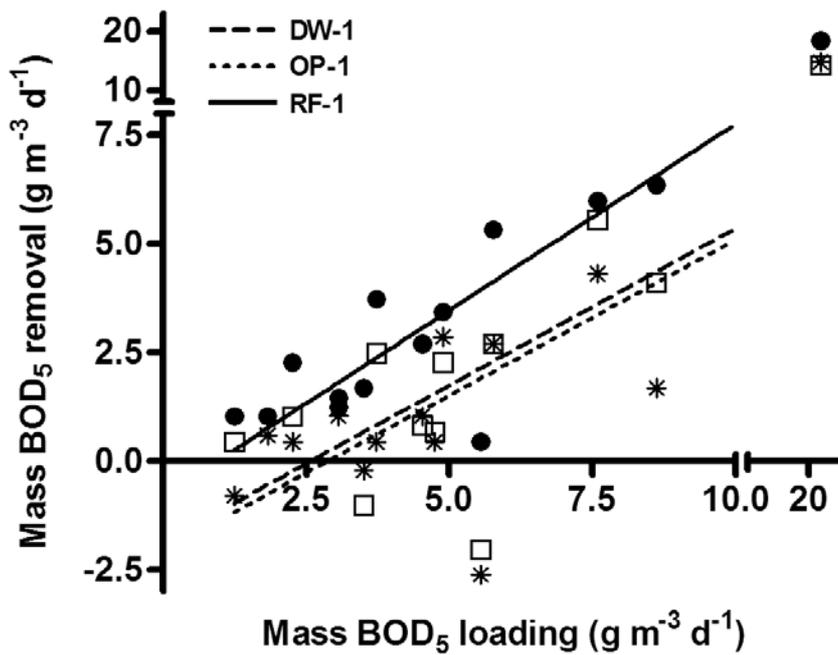


**Figure 3.17.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. mass removal (as a percentage of daily loading rate) for Pond 3 data only. Individual data points represent performance data for: Duckweed Pond 3 (□); Open Pond 3 (\*); and Rock Filter 3 (●).

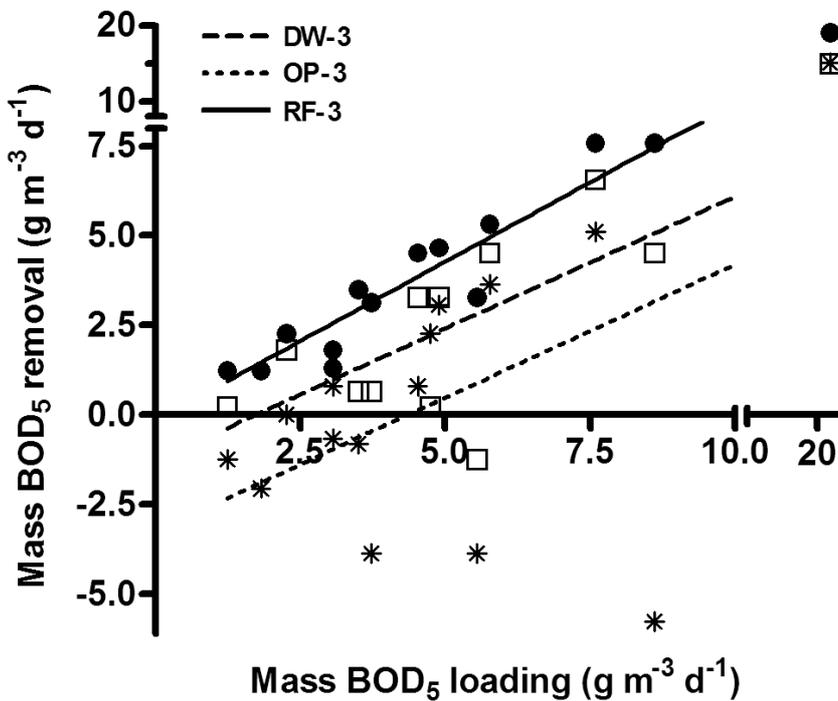
As evident in the above figures, there was a general trend for a greater percentage removal as well as reduced variation in BOD<sub>5</sub> removal performance at higher influent

mass loads. This trend was most apparent for the lower performing OP and DW treatment series, where percentage BOD<sub>5</sub> removals were commonly low and highly variable under low influent mass loads. This is a likely reflection of the first-order-type removal kinetics governing BOD<sub>5</sub> removal in such environments; whereby BOD<sub>5</sub> removal is largely load-dependent, especially at elevated mass loadings. The same removal patterns meant that treatment performance at low BOD<sub>5</sub> loads was commonly more variable at low influent loads—particularly for the less efficient DW and OP treatments. Because the influent wastewater was already so highly refined in this instance, and under the predominantly low BOD<sub>5</sub> loading conditions, the wastewater was effectively being moved through the Open Ponds in particular at an equilibrium ‘steady-state’ with little further treatment being achieved. This trend was actually commented on by Imhoff (1984), where it was noted that “low-loaded (maturation WSP) systems show smaller removal rates” because at low substrate concentrations there is inherently less scope for contaminant removal against normal background ‘steady-state’ levels. In other words, the potential for removal of a finite mass of loaded BOD<sub>5</sub> is effectively *substrate-limited* at reduced influent concentrations as a result of the ever-reducing concentration gradient between influent and steady-state BOD<sub>5</sub> levels.

An exception to the above discussed effect is found in the RF performance data, where 100% removals of loaded BOD<sub>5</sub> were frequently recorded (even at low mass loading rates). This suggests that the maximum capacity for BOD<sub>5</sub> removal is somewhat greater for the RFs than for the DW and OP treatments, and that the ‘steady-state’ concentration for RF effluent BOD<sub>5</sub> is significantly lower than in the DW or OP reactors. This is where it is also important to take into consideration *mass* removals in addition to those on a *percentage* basis. For example, on the 17<sup>th</sup> of December 2005, the RF train received a relatively low organic loading of 2.3g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup> and removed 100% of this loaded BOD<sub>5</sub> across all three RFs. On the other hand, the 6<sup>th</sup> of September 2005 saw the RF train loaded at a 10-fold higher rate of 22g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup> followed by some 15% lower removal of loaded BOD<sub>5</sub> (85% removal efficiency). On a mass basis, the 2.3g m<sup>-3</sup> d<sup>-1</sup> BOD<sub>5</sub> removal of the 17<sup>th</sup> December was 8-fold lower than the ≈19g m<sup>-3</sup> d<sup>-1</sup> BOD<sub>5</sub> removal on the 6<sup>th</sup> September, despite the higher *percentage* performance efficiency. Figures 3.18 and 3.19 below show the same data as for Figures 3.16 and 3.17 above, but this time on a mass loading versus mass removal basis.



**Figure 3.18.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of x- and y-axis breaks.



**Figure 3.19.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent performance data for: Duckweed Pond 3 (□); Open Pond 3 (\*); and Rock Filter 3 (●). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of x- and y-axis breaks.

When represented on a mass basis, there was again a noticeable relationship between mass loading and the mass of BOD<sub>5</sub> removed, indicating again the first-order-type load-dependent removal of BOD<sub>5</sub> across all treatment series. On a mass basis, this loading versus removal relationship was slightly more apparent for the higher performance RF and DW treatments compared to the less efficient OPs. By the last pond of each treatment series, there was a highly significant correlation between mass BOD<sub>5</sub> load and mass removal for RF-3 (Pearson  $r = 0.988$ ;  $n = 14$ ;  $p < 0.0001$ ), an equally significant correlation for the final DW-3 pond data ( $r = 0.913$ ;  $n = 12$ ;  $p < 0.0001$ ) and a less significant relationship for the OP-3 data of Figure 3.19 ( $r = 0.749$ ;  $n = 15$ ;  $p < 0.01$ ). This highlighted again the higher level of performance for the RF and DW systems over that of the OP treatment, whereby the quantity of BOD<sub>5</sub> removed by the RF and DW systems was more closely associated with the amount flowing into the systems. The reduced significance of this relationship for the OP treatment train again reflected the increased variability in BOD<sub>5</sub> removal performance for this system.

The fitted regression lines of Figures 3.18 and 3.19 provide some additional insights into the relationship of mass loading versus mass BOD<sub>5</sub> removal. Critical analysis of both the slopes and elevations of the fitted trendlines allows for more detailed between-treatment performance assessments than is afforded by the discrete correlation coefficient integer above. Looking at the linear regression data from the above Figures 3.18 and 3.19, there were again significant positive linear associations between the amount of loaded BOD<sub>5</sub> and the mass removed within each pilot treatment system. Regression coefficients were identical to the Pearson correlation coefficients above, with the slopes of all regression lines from both figures significantly greater than zero ( $p < 0.0001$ ). For the Pond 1 and Pond 3 data of Figures 3.18 and 3.19 respectively, there were no apparent differences between the slopes of the fitted regression lines (ANCOVA;  $F_{(2,35)} \leq 0.872$ ;  $p \geq 0.427$ ). With respect to the *elevations* of the regressed lines, however, there were significant differences between treatments for both the Pond 1 and Pond 3 data (ANCOVA;  $F_{(2,37)} \geq 7.04$ ;  $p \leq 0.003$ ). For the Pond 1 data of Figure 3.18, the elevation of the best-fit line was significantly greater for RF-1 than for both DW-1 and OP-1 ( $p < 0.01$ ), but elevations were equal for the DW and OP treatments ( $p = 0.72$ ). Similarly, for Pond 3 data (Figure 3.19) the elevation of the fitted line for RF-3 was again greater than both

the DW and OP treatments ( $p < 0.002$ ), but was again similar for both DW-1 and OP-1 ( $p = 0.09$ ).

Results from the above regression analyses effectively meant that whilst all three treatments displayed an equally linear pattern for BOD<sub>5</sub> loading versus removal, the RFs were able to remove a greater mass of loaded BOD<sub>5</sub> at any given mass loading rate than were the other two treatments; given that treatment performance in the above figures is effectively measured by the degree of *y*-axis *elevation* for each data point above (or indeed below) the point of zero removal (*x*-axis intersection). With respect to the work of others, there appears to be no published information regarding the treatment performance of duckweed ponds or rock filters on a ‘loading versus removal’ basis. Tanner *et al.* (2005) did report BOD<sub>5</sub> and total nitrogen ‘influent versus effluent’ concentration plots for their pilot-scale rock filter and duckweed pond systems; however, these provided no real insights into *mass removals* relative to loading rate for these water quality parameters. It is recommended that future investigations into the nature of treatment performance for both duckweed ponds and rock filters include information regarding the ‘loading versus removal’ capabilities of these systems under a range of influent mass loadings. This would serve to provide greater insights into the variability of performance efficiency under a given loading rate, particularly at low mass loads.

According to Mara (1974), Uhlmann (1979) and Kilani and Ogunrombi (1984), WSPs can for most practical purposes be seen as biochemical reactors in which BOD<sub>5</sub> removal can be adequately described by first-order kinetics. It is interesting to note that for the OP-1, the observed BOD<sub>5</sub> removal rates were close to what was predicted using published first-order removal rate constants. According to Uhlmann (1979; 1980), the first-order BOD<sub>5</sub> removal rate coefficient ( $K_1$ ) for WSPs operating at an average temperature of 20°C, an HRT  $\leq 2.5$  days, and an OLR  $\approx 4.2 \text{ g m}^{-3} \text{ d}^{-1}$ , is approximately  $0.24 \text{ d}^{-1}$ . Using this  $K_1$  value combined with the model of Uhlmann (1980) (Equation 3.1) for equal volume completely mixed reactors arranged in a series, the predicted BOD<sub>5</sub> concentrations in OP-1 effluent were very close to what was observed during the monitoring period (see Figure 3.20). The use of a completely mixed reactor equation was justified here by the results of hydraulic tracer studies presented in Section 3.3.1.

$$s = \frac{s_0}{\left(1 + \frac{K_1 \cdot t}{n}\right)^n} \quad \text{(Equation 3.1)}$$

where  $s_0$  = influent BOD<sub>5</sub> concentration (mg L<sup>-1</sup>)

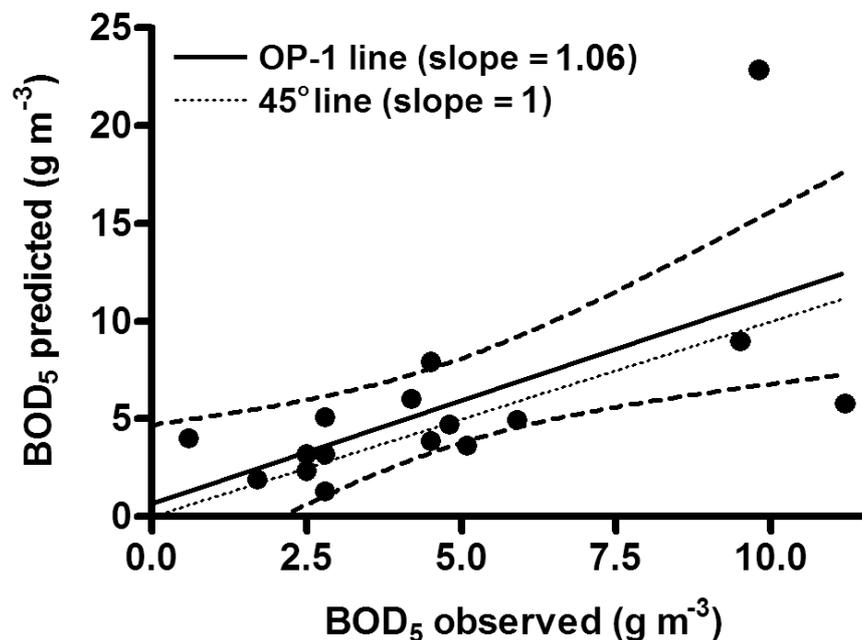
$s$  = effluent BOD<sub>5</sub> concentration (mg L<sup>-1</sup>)

$K_1$  = BOD<sub>5</sub> removal rate coefficient (day<sup>-1</sup>)

$t$  = reactor hydraulic residence time (days)

$n$  = number of succeeding ponds

Actually, the slope of the fitted regression line for OP-1 data in Figure 3.20 was identical (ANCOVA;  $F_{(1,28)} = 0.026$ ;  $p = 0.873$ ) to that of the theoretical perfect data fit (represented by the thin dashed line; slope = 1). Although the number of data points was not ideal ( $n = 16$ ), the high significance level of this comparison suggests that BOD<sub>5</sub> dynamics within Pond 1 of the OP treatment train was on average being adequately described by normal WSP first-order-type removal kinetics.



**Figure 3.20.** Scatter-plot of observed vs. predicted BOD<sub>5</sub> effluent concentrations for Open Pond 1 based on the model of Uhlmann (1979; 1980) for equal volume pond reactors arranged in a series. The solid line represents the fitted regression line for OP-1 observed and predicted data ( $\pm$  95% CI's; thick broken lines), and the thin broken line shows the theoretical perfect fit line for the data.

When considering the data from OP-3, however, this relationship broke down and no longer did observed and predicted values agree. In this case, the slope ( $m = 0.23$ ) of the fitted regression line for OP-3 data for 'observed versus predicted' BOD<sub>5</sub> values was extremely different (ANCOVA;  $F_{(1,26)} = 22.85$ ;  $p < 0.0001$ ) to that of the theoretical perfect fit line. This meant that the processes governing BOD<sub>5</sub> dynamics within OP-3 were certainly not following the classical first-order pattern. This breakdown in relationship for OP-3 is hardly surprising, given that there was a much less significant relationship between OP-3 mass loading and mass removal ( $r = 0.75$ ;  $p < 0.01$ ) compared with OP-1 ( $r = 0.91$ ;  $p < 0.0001$ ) and also no apparent removal of inflowing BOD<sub>5</sub> within OP-3 (see Figure 3.15 and Table 3.4).

The above analysis has shown that the small but insignificant 20% average removal of inflowing BOD<sub>5</sub> within OP-1 was indeed predictable according to classical first-order-type kinetic analysis; however, further down the line within OP-3, this was no longer the case. This posed the question then as to what was the source of this apparent separation of the fundamental treatment processes occurring within what were two presumably very similar ponds. If the majority of WSP effluent BOD<sub>5</sub> is known to be algal-based (see Section 1.2.5) and the processes governing solids removal in WSPs are also thought to be predominantly physical and hence single-phase exponential or first-order-type in nature (Sakata and Silveston, 1974; Stutz-McDonald and Williamson, 1979; Uhlmann, 1979; Reynolds *et al.*, 1990), then one would have expected the BOD<sub>5</sub> removals within the OP series to have continued in a linear fashion down-the-line. Instead of this, removal rates were observed to decrease even further in OP-3; as outgoing BOD<sub>5</sub> concentrations commonly exceeded that of OP-1 effluent and often even that of the influent. Uhlmann (1980) commented that WSP effluent BOD<sub>5</sub> often deviates from predicted values (based on calculated removal efficiencies) as a result of stochastic short-term variations in the *in situ* hydrodynamic conditions, and also due to the fact that the biological treatment processes within the pond environment are forced to function in a near-permanent transient state.

The curious 'worsening' of effluent quality with respect to BOD<sub>5</sub> concentration down the pond series within the OP treatment train was in this case thought to have been a result of a limited amount of algal re-growth and/or zooplankton proliferation within the



OP reactors as a result of above-ground quiescent impoundment. Incidentally, a similar conclusion was drawn Ouazzani (1995) following operation of pilot-scale ponds in Marrakesh, where it was suggested that poor particulate COD removals were a result of algal and zooplankton productivity exceeding the rates of normal solids sedimentation and microbial decomposition. These theories will be revisited within the coming sections and quantitatively assessed within Chapter 5.

One of the recognised limitations within the literature with respect to duckweed ponds, is their reduced capacity for organic matter removal—compared with that of conventional WSPs—as a result of the often low DO concentrations (Reed *et al.*, 1995). This can lead to a reduction in the level of achievable organic loading rates and also potential increases in the pond area required in order to achieve the same level of treatment (Caicedo *et al.*, 2002). The role of duckweed in the removal of organic materials and BOD<sub>5</sub> has been a subject of past controversy (Körner *et al.*, 2003). Although some species of *Lemnaceae* have been shown to be capable of direct uptake of simple organic compounds (see Chapter 1, Section 1.2.8.5.1), the direct role of duckweed in the attenuation of wastewater BOD<sub>5</sub> is thought to be minimal (Körner *et al.*, 1998). Indeed the findings of Al-Nozaily *et al.* (2000a) concluded that the role of duckweed in chemical oxygen demand (COD) removal within their duckweed pond system was marginal. The duckweed biomass is, however, thought to indirectly contribute to the overall removal process via the transfer of oxygen to underlying heterotrophic populations (Körner *et al.*, 1998) as well providing a limited amount of physical substrate for attached periphyton and other microbial growth (Rao, 1986; Körner and Vermaat, 1998; Hamersley *et al.*, 2003) which can then carry out treatment via the direct assimilative removal of small organic compounds during normal heterotrophic nutrition.

In instances where duckweed ponds are operated at a tertiary level (i.e. for final maturation pond effluent upgrading and algal solids removal), this reduced capacity for BOD<sub>5</sub> loading and subsequent removal may not pose such a significant problem due to the more refined nature of the wastewater in question. Operating a tertiary-level duckweed pond for algal removal could therefore feasibly be achieved using the same physical pond configuration as a conventional WSP (in terms of surface area and depth).

Furthermore, tertiary-level duckweed ponds operated under a relatively low organic loading, may—unlike secondary-level duckweed pond systems—be less prone to the development of pond anoxia as a result of their modest organic loading regime. This was in effect what was observed by Alaerts *et al.* (1996) following the four year operation of a full-scale duckweed pond system operated under a similarly low organic loading regime (4.8–6g BOD<sub>5</sub> m<sup>-2</sup> d<sup>-1</sup>), where aerobic conditions were reportedly maintained throughout the entire pond depth at all times. Given that this OLR is very similar to that of the current pilot Duckweed Ponds, and considering the DO data of Figure 3.10 above, it is reasonable to assume that aerobic conditions could also be maintained in a full-scale duckweed pond system at Bolivar.

Published BOD<sub>5</sub> performance data for duckweed pond systems varies largely with factors such as: reactor volume; hydraulic loading regime; and influent waste characteristics; although BOD<sub>5</sub> removal efficiencies are commonly in the range of 60–80% at organic loading rates in the order of 15–30g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup> (USEPA, 1988; Alaerts *et al.*, 1996; Karpiscak *et al.*, 1996; Bonomo *et al.*, 1997; van der Steen *et al.*, 2000; Baldizón *et al.*, 2002; Zimmo *et al.*, 2002; Ran *et al.*, 2004). Arguably the most crucial factor regarding the comparison of published performance data to that of the current work is the physical dimensions and corresponding volume of the pond reactors used elsewhere. There exists a large body of work on duckweed pond systems that has been carried out using reactor vessels of a significantly smaller and arguably non-representative size (Harvey and Fox, 1973; Sutton and Ornes, 1975; Oron *et al.*, 1984; Oron *et al.*, 1986; Oron *et al.*, 1987a; Oron *et al.*, 1987b; Oron *et al.*, 1988; Oron and Willers, 1989; Mandi, 1994; Oron, 1994; Körner *et al.*, 1998; Körner and Vermaat, 1998; van der Steen *et al.*, 1998; Vermaat and Hanif, 1998; Boniardi *et al.*, 1999; van der Steen *et al.*, 1999; Al-Nozaily *et al.*, 2000b; Al-Nozaily *et al.*, 2000a; Caicedo *et al.*, 2000; van der Steen *et al.*, 2000; Zimmo *et al.*, 2000; Awuah *et al.*, 2001; Al-Nozaily and Alaerts, 2002; Caicedo *et al.*, 2002; Öbek and Hasar, 2002; Awuah *et al.*, 2004). This significant volume of research has all involved the assessment of duckweed pond wastewater treatment efficiency using pond reactors of no larger than 170L and most commonly ≤40L. In addition to their small size, many of these pond systems have been operated in ‘batch mode’—a hydraulic regime arguably not representative of the typical flow-through nature of WSP hydraulics.

There are several important factors that come into play when dealing with such small-scale pond systems. Firstly the depth of so-called ‘mini-ponds’ is greatly reduced (commonly 20cm or less). This has obvious and significant implications with respect to the vertical sedimentation depth for suspended particulates and organics, and considering that the removal processes for such contaminants are almost exclusively physical, the subsequent effects of a greatly reduced hydraulic depth on the duckweed pond treatment efficiency are likely to be great (especially for physical water quality parameters such as particulate BOD<sub>5</sub>, algal biomass (chlorophyll *a*), SS and turbidity). The second factor is the likelihood of so-called ‘wall effects’ having a greater influence on measured treatment performance. It can be appreciated that with a decrease in pond reactor volume, comes an associated increase in the apparent surface area of physical pond surfaces (i.e. bottom and walls) relative to water volume. This increased surface-area-to-volume ratio in small-scale pond reactors has the potential to influence its overall treatment performance (e.g. Somiya and Fujii, 1984), particularly for treatment processes that are more heavily reliant upon the density of microbial biofilms (e.g. nutrient removal). The third factor is similar to the second and relates to the relative influence of the duckweed plant biomass on overall treatment performance in small reactors. As for factor two above, a reduction in experimental duckweed pond volume can have a significant influence on the plant ‘biomass-to-volume’ ratio within the system; something that can influence both the intensity of direct plant uptake and also the relative treatment activity of attached periphyton (Körner and Vermaat, 1998). This is especially important when pond reactors are not only small in volume but are very shallow, since this increases the ratio even further, and is also amplified by the operation of small-scale reactors in ‘batch’ mode, whereby the contact time between the duckweed biomass and the wastewater itself is significantly increased compared to that of a ‘flow-through’ pond.

The above size-related performance issues have been discussed in more detail elsewhere (Körner and Vermaat, 1998; Vermaat and Hanif, 1998). It has been made apparent by these authors that the overall result of using small-scale mini-ponds for investigations into wastewater treatment with duckweed is that there is significant potential for overestimating the true performance of such systems, particularly with respect to dissolved nutrient uptake. Whilst very small-scale batch reactors are useful for studies

more concerned with delineating the process chemistry and/or biology of duckweed ponds (e.g. Körner *et al.*, 1998; Körner and Vermaat, 1998; Vermaat and Hanif, 1998; Bonardi *et al.*, 1999; Caicedo *et al.*, 2000; Öbek and Hasar, 2002), extrapolating duckweed pond performance data obtained from experiments involving these small-scale batch reactors to the performance of much larger systems should only be done whilst considering the obvious applied limitations of such comparisons. Following this, performance comparisons will only be drawn between the current work and the work of others where similar scale or larger pond reactors have been used.

BOD<sub>5</sub> performance results for the DW system here were similar to those of other pilot-scale research of comparable or larger volumes. Zimmo *et al.* (2002) operated a very similar scale pilot duckweed pond system comprising four 3m<sup>3</sup> ponds in series instead of the three 2.8m<sup>3</sup> ponds used here. The authors reported an approximate 60% BOD<sub>5</sub> removal after the first duckweed pond and ≈85% removal after the *third* pond in the four pond series. These removals were comparable to those observed here; with median removals of 46 and 60% BOD<sub>5</sub> for DW Ponds 1 and 3 respectively. It should be noted that the pilot plant of Zimmo and co-authors above was loaded at a 4-fold higher OLR (≈20g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>) but had a 6-fold lower HLR (0.13m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) and was also operated under a weekly duckweed biomass harvesting regime. It is thought that the lower flow rate combined with the regular and permanent removal of accumulated plant biomass, could have contributed to the slightly higher BOD<sub>5</sub> removal performance of their pilot-scale duckweed pond system. Duckweed Pond performance reported in this thesis was also similar to that of Karpiscak *et al.* (1996) following six month monitoring of a large pilot-scale (700m<sup>3</sup>) duckweed pond. The authors recorded a mean BOD<sub>5</sub> removal efficiency of 53% at a slightly lower but similar OLR to that used here of 3.3g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>.

Performance results from the current Duckweed Ponds were again similar to those reported by Bonomo *et al.* (1997) from large-scale (400m<sup>3</sup>) pilot duckweed pond operation. The authors also observed a similar organic (COD) removal efficiency for their duckweed pond in the range of 55–75%. As was the case for Zimmo *et al.* above, the duckweed pond of Bonomo and co-workers was operated under a monthly biomass harvesting regime—possibly facilitating the slightly higher organic removal rates. The

pond was also loaded again at a much lower HLR ( $0.1\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ) than the current ponds, and was also operated under a slightly reduced OLR ( $\approx 3\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ ) despite the higher organic strength of their influent wastewater ( $42\text{mg BOD}_5\text{ L}^{-1}$ ) compared to that used here ( $5.8\text{mg BOD}_5\text{ L}^{-1}$ ). Ran *et al.* (2004) operated a similar scale pilot series of duckweed ponds to those here under an 8-fold higher BOD<sub>5</sub> loading regime ( $33\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ ), a 3-fold lower HLR ( $0.23\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ) and also under a weekly partial duckweed biomass harvesting regime. The authors reported good BOD<sub>5</sub> removal performance for their pilot duckweed ponds, with a two month average removal of around 70%—slightly higher than that achieved here. Baldizón *et al.* (2002) achieved 52% ( $\pm 25$ ) BOD<sub>5</sub> removal efficiency relative to influent loads in a larger-scale duckweed pond system with a significantly higher BOD<sub>5</sub> influent concentration ( $\approx 110\text{mg L}^{-1}$ ); although the authors give no indication of the HLR of the system and concluded themselves that the large degree of ‘noise’ in their performance data makes it difficult to draw definitive comparisons. Finally, Alaerts *et al.* (1996) have reported a BOD<sub>5</sub> removal efficiency of 95–99% in a full-scale duckweed pond system loaded at a comparable OLR ( $\approx 5.5\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ ) but under an increased retention time of 20 days and again with frequent duckweed harvesting (2–3 times per week). These discrepancies between the operating conditions of other systems and that reported here could have again contributed to the slight differences in BOD<sub>5</sub> treatment performance when compared to the current system.

The importance of routine harvesting of duckweed plant biomass for maximal pond performance has already been introduced (Section 1.2.8.5.1). Some authors have reported on the potential for a decline in the efficiency of BOD<sub>5</sub> removal in duckweed pond system over time due to the accumulation and steady degradation and remineralisation of accumulated plant biomass (Bonomo *et al.*, 1997). According to Szabó *et al.* (2000), the complete degradation and decomposition of sludge-accumulated duckweed plant biomass is likely to take place on timescales greater than the current 6 month experimental monitoring duration (i.e.  $>200$  days); with the half-life of organic matter degradation for decaying duckweed biomass in wastewater found to be in the order of 68 days. The data of Szabó *et al.* (2000) also suggested that the decaying duckweed biomass contributes significantly to effluent COD during the first 50 days of the long-term oxidative degradation process. Others have also shown that duckweed (*Lemna*) can leach somewhere in the order of 2.6% of the total daily fixed inorganic

carbon as DOC back into the water column (Baker and Farr, 1987); although this DOC was thought to be readily metabolised by attached periphyton and would therefore be unlikely to contribute significantly to aqueous BOD<sub>5</sub>. Considering the above factors, it may have been anticipated that the long-term performance of the DW Pond system would start to decline after approximately the first two months of continuous operation. Looking at the performance data, however, there was no evidence of a long-term decline in treatment performance within the current Duckweed Ponds during the course of the six month monitoring period. It is likely that the rate of duckweed biomass production was somewhat suppressed in the current pond system as a result of the absence of continuous plant harvesting and subsequently suppressed plant growth rates, such that this reduction in duckweed productivity could have somewhat dampened the effects of internal biomass/BOD<sub>5</sub> recycling within the pilot Duckweed Ponds.

In instances where a duckweed surface coverage is desired purely for the attenuation of algal populations, the continual harvesting of plant biomass from the WSPs could be considered a burden on the overall treatment plant operation. In these cases, if the duckweed surface cover is not periodically harvested, then there is the possibility that continual turnover of the standing duckweed biomass could lead to accelerated rates of sludge accumulation as well as potentially undesirable increases in effluent SS and BOD<sub>5</sub> from decaying plant matter. Szabó *et al.* (2000) found that the degree of sludge-accumulated duckweed biomass over the course of their 200 day degradation experiment was minimal, with only 3% of the total initial duckweed biomass organic carbon accumulating in the sediment and 83% solubilising into DOC. The results of Szabó and co-workers tie in well with the biochemical constitution of duckweed, given that duckweed is in the order of 93% water (w/w) and is recognised to possess very little structural tissue (i.e. recalcitrant organic carbon); meaning that they should be largely biodegradable.

As discussed above, a steady worsening of effluent water quality was not seen during the six month operation of Duckweed Ponds here. From this, it is thought that the use of a floating duckweed cover for algal solids removal would be unlikely to result in a significantly accelerated rate of sludge accumulation; taking into account the likely restriction of algal productivity and algal-derived solids accumulation. Although the 6

month monitoring duration here was probably too short to assess the true impact of this phenomenon, Rich (2003), following long-term operational experience in South Carolina, suggested that it was neither necessary to periodically harvest the duckweed biomass, nor did the decaying duckweed appear to result in a reduced effluent quality or significant benthic sludge accumulations. It is suggested that future investigations into duckweed pond treatment efficiency could incorporate assessments of both sludge accumulation and also sludge characterisation, in order to provide further insights into the above factors.

Interestingly, the duckweed degradation data of Szabó *et al.* (2000) raises an interesting point about the kinetics of duckweed biomass oxidation and its corresponding oxygen demand. The very slow rate of duckweed plant biomass degradation in domestic wastewater recorded by Szabó *et al.* (2000), highlights the apparent redundancy of the five-day 'BOD<sub>5</sub>' test for assessing the *true* degradative oxygen demand of any suspended plant biomass present within a typical duckweed pond effluent. Qualitative visual and microscopic observations made during daily analyses frequently recorded the presence of suspended decaying duckweed plant tissue. In line with the above discussion, however, it appears that these particulate plant organics were unlikely to have exerted any significant oxygen demand during routine BOD<sub>5</sub> analysis. This apparent understating of the *total oxygen demand* of suspended duckweed biomass in this instance was, however, thought to be representative of the general oxidative requirements of the wastewater; given that only a small fraction of the COD in WSP effluent is recognised as being biochemically labile under the conditions of the BOD<sub>5</sub> test (Davies-Colley *et al.*, 1995). Whilst no attempts were made to correct for this, it was considered necessary to raise the issue here, as the presence of duckweed plant tissue in collected samples has follow-on implications for later performance assessments. It would, therefore, be a final recommendation that all future performance assessments of duckweed ponds include COD as well as both soluble and total BOD<sub>5</sub> testing. Measurement of these parameters would allow for a more detailed assessment of total oxidative demand of both the suspended particulate and dissolved wastewater fractions.

With respect the BOD<sub>5</sub> treatment performance comparisons of the RFs, Swanson and Williamson (1980) recorded a lower mean BOD<sub>5</sub> removal efficiency of 55% (compared

with 84% mean removals here) during one year monitoring of their full-scale rock filter. Interestingly, this performance figure comes from a filter with a 5-fold higher average influent BOD<sub>5</sub> concentration (35g BOD<sub>5</sub> m<sup>-3</sup>) but an almost identical OLR (5.6g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>) due to the significantly lower hydraulic loading of their rock filter system ( $\approx 0.16$  compared with  $0.73 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ). Saidam *et al.* (1995) again reported a lower average BOD<sub>5</sub> removal efficiency in their large-scale (300m<sup>3</sup>) rock filters, achieving approximate 45% removal efficiencies for filters with a similar rock media size ( $\approx 12$ cm diameter) but significantly higher OLR (30g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>).

Mara *et al.* (2001) operated similar scale experimental rock filters to that of the current system at hydraulic loadings of 1.0 and  $2.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ , but reported a significant reduction in overall filter BOD<sub>5</sub> removal efficiency (32% drop) when operated at increased hydraulic loadings. This decline in filter performance efficiency was probably not surprising in the case of Mara and co-workers, given that at the highest HLR, the corresponding OLR was in the order of  $56 \text{ g BOD}_5 \text{ m}^{-3} \text{ d}^{-1}$ —some 13-fold greater than that applied to the current RFs—and was therefore likely to have been organically overloaded. von Sperling *et al.* (2007) have also reported substantial reductions in average BOD<sub>5</sub> removal efficiency from  $\approx 40\%$  down to just 3% in their similar sized pilot-scale rock filters when the hydraulic loadings were increased from 0.5 to  $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ . Once again, and as was the case for the rock filters of Mara and co-workers above, the rock filters of von Sperling *et al.* were probably organically overloaded at the highest HLR; given that at  $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  the OLR was again in the order of  $55 \text{ g BOD}_5 \text{ m}^{-3} \text{ d}^{-1}$ —although no information regarding filter DO concentration was provided. O'Brien and McKinney (1979, cited in Swanson and Williamson, 1980) also tested rock filters at HLRs in the range of  $0.5\text{--}3.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  and reported generally poor filter performance efficiency at higher volumetric loadings. The above performance comparisons suggest that the relatively high HLR applied to the rock filters reported in this thesis (i.e.  $0.73 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ) had no significant negative impact on filter performance with respect to BOD<sub>5</sub> removal capacity; with the entire three unit RF train achieving consistently high removal performance. Whilst this was the case for the current rock filters, the much lower organic strength of the influent was likely to have imparted favourable effects on overall filter performance, and so this must be taken into consideration when comparing the efficiency of these systems.



Although it was not directly investigated here, others have reported on the ability of rock filters to attenuate *soluble* organics from the infiltrating wastewater. According to the data of Hirsekorn (1974), there was negligible potential for soluble COD removal within rock filters (10–15%). Similarly, Swanson and Williamson (1980) reported no significant change in the concentration of soluble BOD<sub>5</sub> within their rock filter; citing the removal of particulate BOD<sub>5</sub> as the reason for the high observed total BOD<sub>5</sub> removal efficiencies. This provides further support to the idea that rock filters are indeed reactors for physical treatment and solids separation rather than ‘biological’ filters in the true sense (e.g. Swanson and Williamson, 1980; Rich, 1988). It should be noted that the frequent observation of ‘zero’ BOD<sub>5</sub> concentrations for rock filter effluent (as seen in the ‘100% removal’ data points of Figures 3.16 and 3.17) did not necessarily mean that the wastewater was devoid of soluble or dissolved organic materials. Periodic total organic carbon analyses of the effluent revealed that there were significant quantities (commonly 25–35mg L<sup>-1</sup>) of dissolved organic carbon (DOC) within final RF effluents. Recognising that only a small fraction of the total organic oxygen demand—and hence DOC—in WSP effluent is biochemically oxidised during the BOD<sub>5</sub> test (Davies-Colley *et al.*, 1995), this again serves to highlight the efficiency of the current RFs for *particulate* organic removal rather than DOC removal, and also highlights again the extraordinarily refined and recalcitrant nature of the final Bolivar WSP effluent.

Performance monitoring of the three pilot treatment systems has shown a general trend for a decline in the extent of BOD<sub>5</sub> removal within the pilot pond series from Pond 1 to Pond 3 of all treatments. Where significant removals occurred, typically the greatest removal of inflowing BOD<sub>5</sub> was realised following the first pond in each treatment train, after which the relative degree or ‘rate’ of BOD<sub>5</sub> removal diminished. This was a manifestation of the first-order-type processes governing BOD<sub>5</sub> removal in such environments, and resulted in both percentage and mass removals generally being highest under elevated influent BOD<sub>5</sub> loads. In finishing, it is concluded from the above performance analyses that the overall ranking of treatment performance with respect to BOD<sub>5</sub> removal potential places the RF system 1<sup>st</sup>, the DW treatment series 2<sup>nd</sup>, and the OP treatment 3<sup>rd</sup> in terms of BOD<sub>5</sub> removal rate along the pond series, absolute removal efficiency, and also performance reliability.

### **3.3.6 Wastewater treatment performance: suspended solids, turbidity and algal biomass removal**

Aqueous SS, turbidity, as well as the levels of suspended algal biomass, were periodically monitored during the course of the pilot plant performance assessments. Algal biomass dynamics were monitored during the course of this work by the proxy measure of chlorophyll *a*; since this parameter is widely recognised to be directly correlated with algal biomass density (Reynolds, 2006). Furthermore, because algal cells are known to rarely exist as discrete ‘planktic’ entities (Knoechel and Kalff, 1978), and due to the inseparable nature of algal cells and detritus/aggregate flocs within wastewater systems (Barley *et al.*, 2005), accurate quantitation of algal biomass densities via direct cell counting methods was considered to be both impractical and unachievable. Although algal community dynamics were periodically assessed on a species basis, the relative complexity and probable lack of quantitative precision associated with this sort of analysis meant that it was not performed in parallel to daily chlorophyll *a* measurements. As a result, the correspondingly low number of determinations for algal species dynamics meant that this data could not be discussed in the context of regular and more quantitative chlorophyll *a* treatment performance data here. Reference will instead be made to this population data set in a more ecological context within Chapter 5.

It should be noted first of all that for the purposes of results presentation and discussion here, the water quality parameters SS, turbidity, and chlorophyll *a* have been grouped together within the one section. This was firstly done for SS and turbidity due to their direct linear correlation within both the pilot plant influent ( $r = 0.990$ ;  $n = 24$ ;  $p < 0.0001$ ) and also the pooled pilot plant data from all 9 ponds of the three treatment trains ( $r_s = 0.878$ ;  $n = 204$ ;  $p < 0.0001$ ). Since the performance trends for turbidity data mirrored exactly those of SS, only the SS performance data will be referenced with respect to the performance of others treatment systems. This omission of turbidity data from the general discussion, therefore, served to avoid duplication of pilot upgrade performance assessments. Secondly, the chlorophyll *a* data was also grouped together with the SS and turbidity performance data for presentation and discussion purposes because of the highly significant interrelationship of these three water quality parameters in the influent data set; the extent of which can be seen in Table 3.5. The BOD<sub>5</sub>

performance data was discussed separately within the preceding section due to the weak nature or indeed absence of such correlations between that particular parameter and: SS ( $p = 0.045$ ); turbidity ( $p = 0.039$ ); and chlorophyll *a* ( $p = 0.905$ ) within the pilot plant influent. The cause of this apparent breakdown in relationship between what are commonly found to be interrelated water quality parameters will be discussed in more detail later.

**Table 3.5.** Pearson’s correlation matrix for pilot plant Influent water quality parameters: suspended solids (SS); turbidity; chlorophyll *a*; and BOD<sub>5</sub>.

<b>Pilot plant influent</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>Chlorophyll <i>a</i> (μg L<sup>-1</sup>)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Pearson <i>r</i> α Sig. level (2-tailed) <i>n</i>				
<b>Turbidity (NTU)</b>	Pearson <i>r</i> α Sig. level (2-tailed) <i>n</i>	.990(***) 0.000 24			
<b>Chlorophyll <i>a</i> (μg L<sup>-1</sup>)</b>	Pearson <i>r</i> α Sig. level (2-tailed) <i>n</i>	.657(**) 0.001 21	.774(***) 0.000 21		
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman <i>r<sub>s</sub></i> α Sig. level (2-tailed) <i>n</i>	0.524 0.045(*) 15	0.538 0.039(*) 15	0.035 0.905 14	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

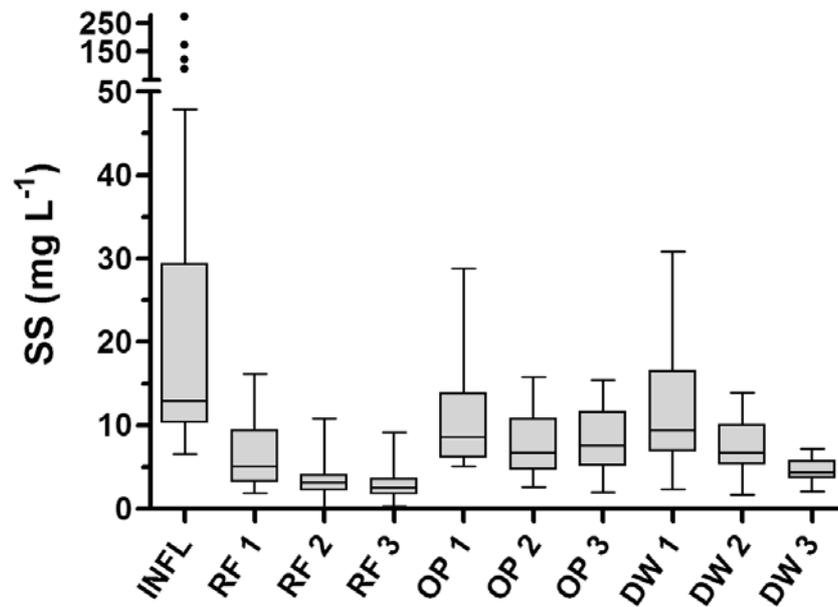
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

Prior to the presentation of the pilot plant performance data, it should be noted that during the six month monitoring period there were several instances of extreme influent SS and accompanying turbidity and chlorophyll *a* loading; with four recorded spikes in excess of 100mg SS L<sup>-1</sup> (55–96 NTU) and one of those as high as 270mg SS L<sup>-1</sup> (147 NTU). Likewise, these extreme SS spiking events corresponded to one spike of 93μg chlorophyll *a* L<sup>-1</sup> and three spikes in excess of 270μg chlorophyll *a* L<sup>-1</sup>. With the exception of the turbidity spike of 58 NTU and the chlorophyll *a* spike of 93μg L<sup>-1</sup>, all of these events were classified as statistically-significant outliers within the general data set (i.e. they were  $>1.5\times$  the IQR from the 75<sup>th</sup> percentile value), with several identified as statistically-extreme outliers ( $>3\times$  the IQR from the 75<sup>th</sup> percentile value)—providing statistical justification for their exclusion from the performance data analyses.

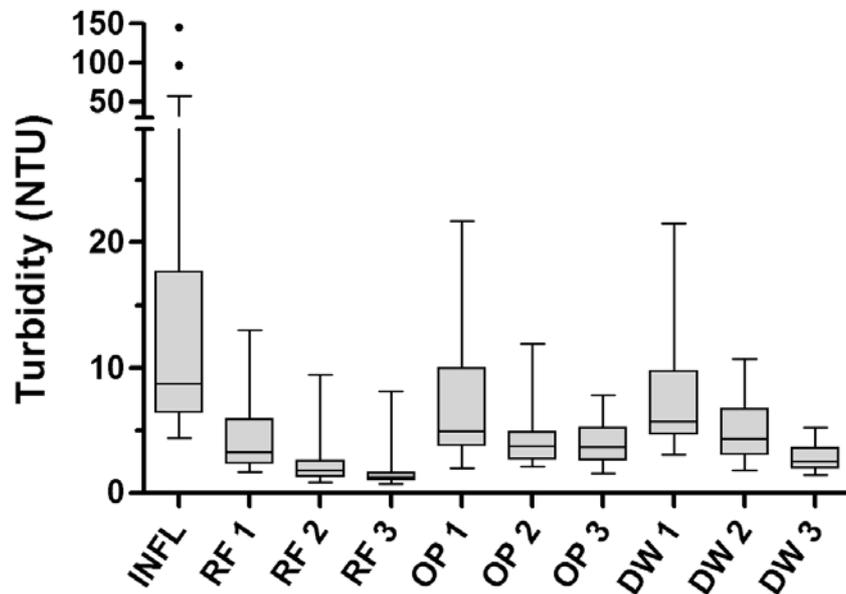
According to Uhlmann (1980), such high-magnitude short-term fluctuations in WSP effluent quality can be largely apportioned to the relative ecological instability of WSP

environments, as well as their heightened sensitivity to changes in localised meteorological conditions; something exacerbated by their universally shallow depth. With respect to the water quality spike events reported here, and more importantly than the above *statistical* basis for their exclusion, these spiking events were—as per Uhlmann (1980) above—all observed to be the direct result of extreme localised weather conditions (i.e. temporary wind-induced resuspension) and as such were not considered to be representative of the long-term average influent water quality. This observation was supported by a strong negative correlation between pilot plant influent (WSP effluent) SS and the relative proportion of volatile suspended solids (VSS) within each sample ( $r_s = -0.531$ ;  $n = 24$ ;  $p = 0.008$ ). In other words as the SS concentration increased, proportionally less of the total solids were volatile (i.e. they were more ‘fixed’ or inorganic in nature), suggesting that resuspension of recalcitrant materials was the likely cause of increased SS concentrations rather than algal biomass production. Furthermore, and in respect of the between-treatment performance of each upgrade system during these isolated spiking events, the order of treatment efficiency remained unchanged (i.e. the RF and DW Pond systems consistently out-performed the OP train).

Following on from the above factors, it was concluded that the omission of these extreme loading events served not to change the relative treatment efficiencies of the three-pond upgrades to each other, but rather provided a more accurate reflection of the relative performance of each system under ‘normal’ low-range influent loading conditions. This is reflected in Figures 3.21 and 3.22 (and in later Figure 3.29) whereby the influent SS, turbidity and chlorophyll *a* concentrations are all highly negatively skewed in terms of the overall data distribution.



**Figure 3.21.** Suspended solids box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). Filled circles (●) above the INFL data represent the four extreme spike outliers  $>3\times\text{IQR}$  from the 75<sup>th</sup> percentile value.

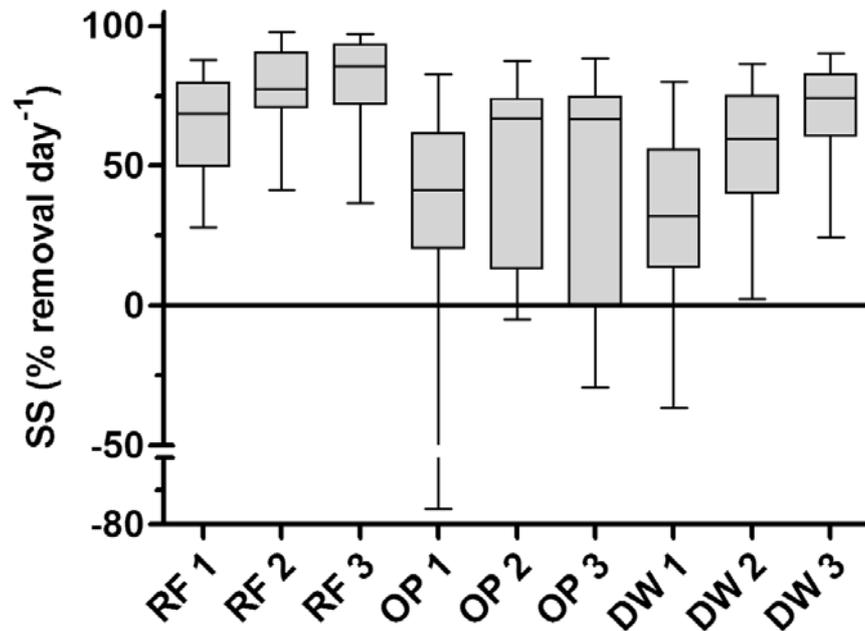


**Figure 3.22.** Turbidity box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). Filled circles (●) above the INFL data represent the three extreme spike outliers  $>3\times\text{IQR}$  from the 75<sup>th</sup> percentile value.

As shown in Figure 3.21, pilot plant influent SS levels were generally low but also highly variable in some instances, with a median SS concentration of  $13.0\text{mg L}^{-1}$  and a

mean of 20.3mg SS L<sup>-1</sup>. Similarly to SS, influent turbidity showed an identical pattern of being predominantly low but also displaying high-level variability at times (Figure 3.22), as reflected in the mean of 14.8 and median of 8.7 NTU. This random and high-level variability in SS and associated turbidity within the Bolivar WSP effluent (i.e. pilot plant influent) is a widely recognised and indeed common feature of such systems. The sources of such large-scale variability in effluent water quality have been discussed previously (Section 3.3.5). Given the highly skewed nature of the SS data, the median influent concentration of 13.0mg L<sup>-1</sup> translated to a median mass solids loading during the six month monitoring period of 9.5g SS m<sup>-3</sup> d<sup>-1</sup>.

Statistical analysis of the data from Figure 3.21 showed that influent SS was reduced significantly in the second and third ponds of the DW treatment series (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 107.1$ ;  $p \leq 0.01$ ) but not within DW Pond 1 ( $p > 0.05$ ). Qualitatively, the OP series displayed slightly higher and more varied effluent SS levels than the other two treatments. This increased variability was reflected in the slightly lower SS removal efficiencies within the OP treatment series, with no significant removal within OP-1 ( $p > 0.05$ ) and small but significant average removals in both OP-2 and OP-3 ( $p < 0.05$ ). For the RFs, highly significant reductions in SS concentration were recorded in all three-pond units relative to influent levels ( $p < 0.001$ ). These analyses suggested that final effluent concentrations of both the RF and DW treatment series were very much reduced compared to pilot plant influent levels ( $p < 0.001$ ) but were only slightly reduced along the OP series ( $p < 0.05$ ). With respect to the between-treatment performance comparisons, the SS concentration of the final RF-3 effluent was significantly lower than that of OP-3 ( $p < 0.001$ ), although it was similar to DW-3 ( $p > 0.05$ ). There was also no apparent difference in the final effluent quality of DW-3 and OP-3 with respect to SS concentration ( $p > 0.05$ ). Based solely on HLR, average SS removal efficiencies for the RF treatment here were some 15–20% more advanced than that predicted by the model of Swanson and Williamson (1980); suggesting a relatively high-level of treatment performance at the relatively low solids loading rates. The data of Figure 3.21 is represented below as percentage removal efficiencies for each pilot treatment pond (Figure 3.23).



**Figure 3.23.** Box-plots showing daily percentage suspended solids removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n \geq 20$  for all plots).

Long term median percentage daily SS removals for Pond 1 data across the three treatments were 32, 41 and 68% for DW, OP and RF systems respectively, and for Pond 3 data, 74, 67 and 86% for the respective DW, OP and RF systems. When compared statistically to a theoretical zero median SS removal, these average percentage SS removal efficiencies were all found to be significantly ‘non-zero’ across all treatment series (Table 3.6), indicating that all SS removals were on average greater than zero. As was the case for BOD<sub>5</sub> performance assessment in Section 3.3.5, the RF system was once again the only treatment to always yield a positive SS removal efficiency. The DW system was not far behind the performance of the RFs, however, with the DW Pond series realising just one single net increase in effluent SS (DW-1). The OP treatment series performed significantly worse in this regard, recording zero removals or net increases in effluent SS on at least three (OP-2) and up to six (OP-3) occasions. This trend is reflected in the corresponding CV’s for SS removal performance (Table 3.6), where OP performance is seen to be more variable than both the DW and RF treatments.

**Table 3.6.** Summary of suspended solids performance data for all pilot plant treatments for Ponds 1 and 3 only.

SS performance parameter	Pilot treatment pond					
	DW-1	DW-3	OP-1	OP-3	RF-1	RF-3
Median influent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	13.0	13.0	13.0	13.0	13.0	13.0
Mean influent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	20.3	20.3	20.3	20.3	20.3	20.3
Median effluent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup> *	9.5	4.4	8.6	7.6	5.1	2.5
Mean effluent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	11.9	4.7	10.8	8.4	2.8	6.4
Median daily SS removal (% day <sup>-1</sup> ) <sup>‡</sup> *	32	74	41	67	68	86
Mean daily SS removal (% day <sup>-1</sup> )	35	70	31	43	64	81
Long-term CV for SS removal (%)	85	25	134	96	27	19

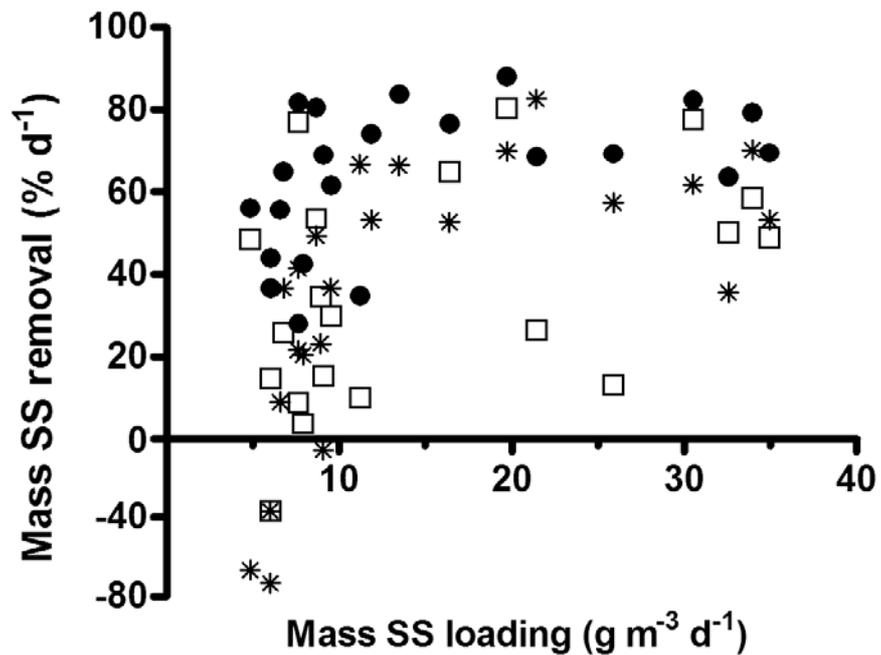
<sup>†</sup> Effluent SS concentration was tested relative to median pilot plant influent SS concentration (Kruskal–Wallis test)

<sup>‡</sup> Average SS removal % tested against a theoretical 'zero' daily median (Wilcoxon signed-rank test)

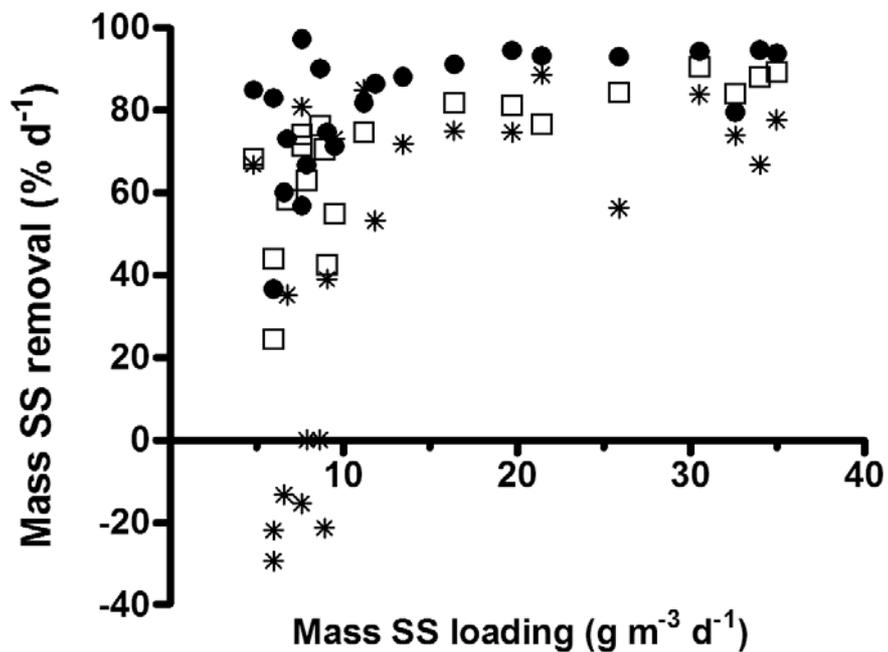
\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium);  $p \leq 0.001$  (black)

Comparison of individual treatment CV's for SS removal efficiency again provides insight into the overall performance reliability of each WSP upgrade system. As can be seen in both Figure 3.23 and Table 3.6 above, the enhanced consistency in performance delivery of the RF system over both the DW and OP treatments—as was the case for BOD<sub>5</sub> performance—was again apparent. Not only was the RF treatment capable of delivering a better quality final effluent in terms of its SS concentration, but it was able to do so with an enhanced consistency compared with the DW Ponds, and was considerably more reliable than the OP treatment train. This trend for both a greater consistency and superior reliability of treatment performance for the pilot RFs with respect to SS removal was similar to that for the BOD<sub>5</sub> data which was discussed above (Section 3.3.5). As was done for the BOD<sub>5</sub> performance data, the SS data from Figures 3.21 and 3.23 is again represented on a loading versus removal basis. This data is shown for all treatments in Figures 3.24–3.27 for Ponds 1 and 3 of the respective treatments.





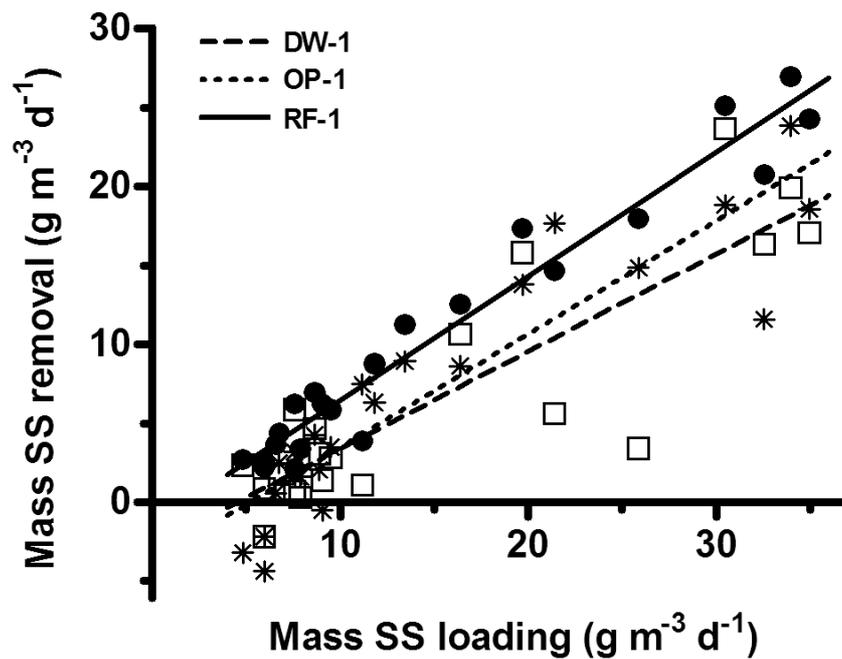
**Figure 3.24.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 1 data only (note the condensed y-axis scale for values below zero). Individual data points represent mean performance data for: Duckweed Pond 1 ( $\square$ ); Open Pond 1 ( $*$ ); and Rock Filter 1 ( $\bullet$ ). Individual data points show the mean of duplicate determinations.



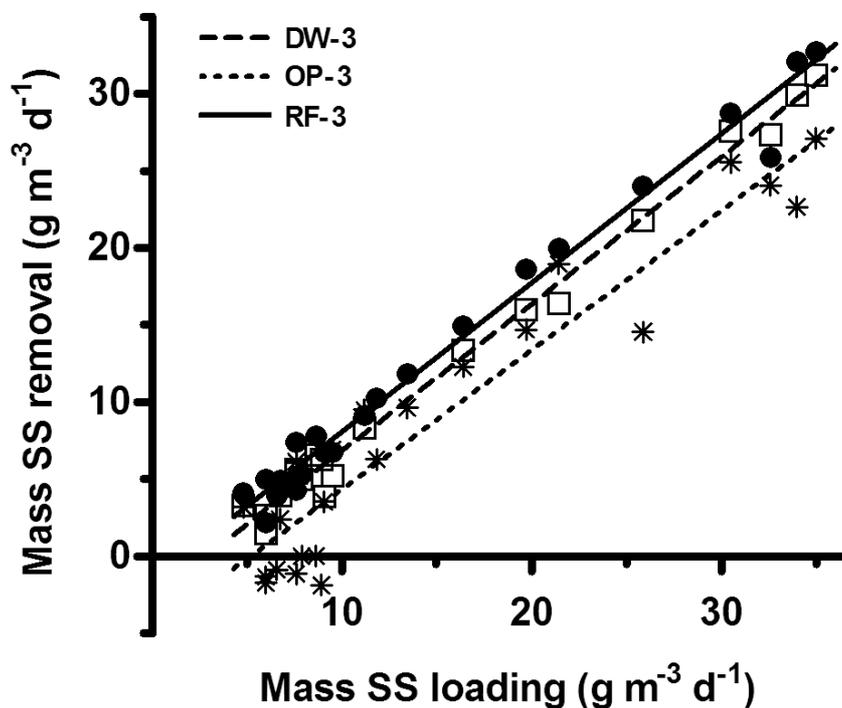
**Figure 3.25.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 3 data only. Individual data points represent mean performance data for: Duckweed Pond 3 ( $\square$ ); Open Pond 3 ( $*$ ); and Rock Filter 3 ( $\bullet$ ). Individual data points show the mean of duplicate determinations.

When considered on a mass loading versus percentage removal basis, there were similar trends for the SS performance data as were recorded for BOD<sub>5</sub> removal earlier. Qualitative visual analysis of Figures 3.24 and 3.25 showed a general trend across both Ponds 1 and 3 for an increase in percentage SS removal efficiency at higher influent solids loads, as well as a decrease in the variability of SS removal performance at higher mass loadings. This was again likely to be a reflection of the concentration-dependent ‘first-order-type’ processes governing solids removal in aqueous environments (Sakata and Silveston; 1974; Reynolds *et al.*, 1990)—an identical phenomenon to that previously discussed for BOD<sub>5</sub> removal above (Section 3.3.5).

As can be seen in Figures 3.24 and 3.25, under very low SS loading conditions (i.e.  $< 15\text{g m}^{-3}\text{ d}^{-1}$ ) the effluent quality of the DW and especially the OP treatment ponds appeared to be largely independent of influent SS. This was again likely to be an effect of the concentration gradient effect above, whereby low-level SS loading conditions promoted an inherently greater potential for variability in *percentage* removal performance, even to the point of yielding negative solids removals at very low influent SS loads. These net negative solids removals were also thought to have been a result of primary and/or secondary biomass production during pilot plant passage. In support of this theory, Ouazzani *et al.* (1995) also noted the negative influence of primary and secondary biomass production on the SS balance of conventional algal-based WSPs compared with macrophyte (water hyacinth) ponds. The authors attributed the poor SS and particulate COD removals in their large pilot-scale open WSPs to the fact that algal and zooplankton production rates often exceeded those of normal SS sedimentation and microbial degradation within their ponds.



**Figure 3.26.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data for: Duckweed Pond 1 ( $\square$ ); Open Pond 1 ( $*$ ); and Rock Filter 1 ( $\bullet$ ). Fitted lines represent best-fit lines from simple linear regression analyses. Individual data points show the mean of duplicate determinations.



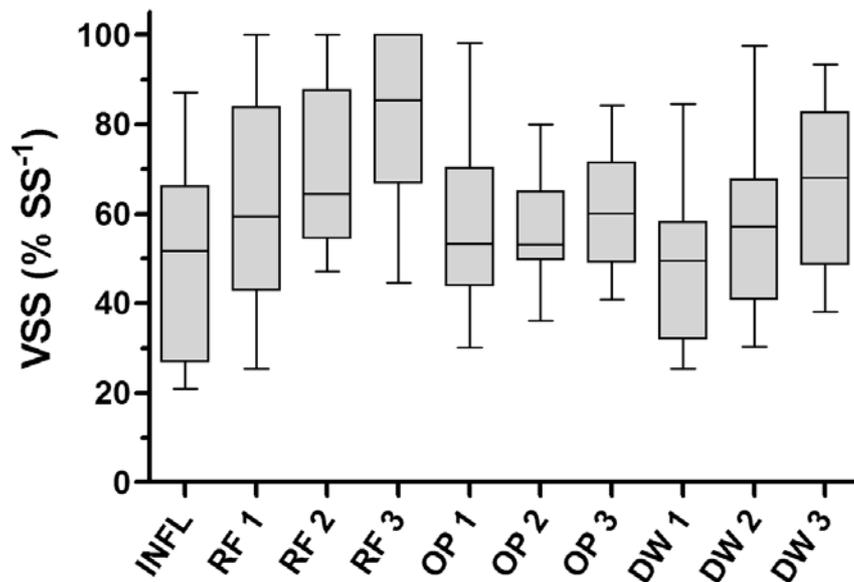
**Figure 3.27.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent mean performance data for: Duckweed Pond 3 ( $\square$ ); Open Pond 3 ( $*$ ); and Rock Filter 3 ( $\bullet$ ). Fitted lines represent best-fit lines from simple linear regression analyses. Individual data points show the mean of duplicate determinations.

When the SS data is represented purely on a mass basis (Figures 3.26 and 3.27), and as was previously the case for BOD<sub>5</sub> performance, there was again a noticeable direct relationship between mass loading and the mass of SS removed; particularly under elevated SS loads. When the data for all three ponds of each treatment were combined, this correlation between mass solids load and mass removal was highly significant across all treatments (Pearson  $r \geq 0.90$ ;  $n \geq 60$ ;  $p < 0.0001$ ). This reinforced the qualitative trends from Figures 3.26 and 3.27 above, and suggested that effluent SS was generally a direct reflection of influent concentration. As can also be seen in Figures 3.26 and 2.27, under conditions of low SS loading (i.e.  $\leq 15\text{g m}^{-3}\text{ d}^{-1}$ ) the OP treatment performed considerably worse in terms of overall solids removal potential than both the DW and RF treatments—a trend again reflected in the earlier Figures 3.24 and 3.25.

As was performed during BOD<sub>5</sub> data analyses in the previous section, simple linear regression analysis was performed and is shown for the data of Figures 3.26 and 3.27. Looking at the fitted regression data from these figures, there were again significant positive linear associations between the SS load and the mass removed within each pilot treatment system. Regression coefficients were identical to the Pearson correlation coefficients above (i.e.  $r \geq 0.90$ ), with the slopes of all fitted regression lines significantly greater than zero ( $p < 0.0001$ ). For the Pond 1 and Pond 3 data of Figures 3.26 and 3.27 respectively, there were no apparent differences between the slopes of the fitted regression lines (ANCOVA;  $F_{(2,59)} \leq 1.61$ ;  $p \geq 0.21$ ). With respect to the *elevations* of the regressed lines, however, this time there were significant differences between treatments for both the Pond 1 and Pond 3 data (ANCOVA;  $F_{(2,61)} \geq 9.90$ ;  $p \leq 0.0002$ ). For the Pond 1 data of Figure 3.26, the elevation of the best-fit line was significantly greater for RF-1 than for both DW-1 and OP-1 ( $p < 0.001$ ), but elevations were equal for the fitted lines of DW-1 and OP-1 ( $p = 0.59$ ). For Pond 3 data (Figure 3.27) the elevation of the fitted line for RF-3 was again greater than for DW-3 and OP-3 ( $p \leq 0.001$ ), but this time the elevation of the DW-3 regression line was significantly greater than that fitted to the OP-3 performance data ( $p < 0.001$ ).

Results from the above regression analyses suggested that whilst all three treatments displayed an ‘equally linear’ association between mass SS removal versus loading, the RFs were able to remove a greater mass of loaded SS at any given mass loading rate

compared with the other two treatments (remembering again that treatment performance in these figures is measured by the degree of *y*-axis elevation for each data point above or below the point of zero removal). Results also showed that by the end of the three-pond series, the DW treatment was more effective at removing SS under a given mass loading rate than was the OP treatment—a trend reflected in earlier performance analyses.



**Figure 3.28.** Relative volatile suspended solids fraction data (as a percent of total SS) for: pilot plant Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

Statistical analysis of the data from Figure 3.28 showed that influent VSS fractions were typically low, with a median volatile solids fraction of just 50%. Within both the DW and OP treatments, and despite appearing to increase slightly through the pond series, no significant change in influent VSS levels were recorded in any of the DW or OP reactors (1-way ANOVA;  $F_{(9,208)} = 6.84$ ;  $p > 0.05$ ). This lack of change in the relative proportions of ‘fixed’ and ‘volatile’ SS fractions within the OP series suggested that it was indeed performing adequately as an ‘open control’ pond treatment, in the sense that the *nature* of the SS within the inflowing WSP effluent was not changing significantly down the pond series as a result of temporary pilot plant impoundment. Unlike the DW and OP treatments, however, the relative VSS fraction in RF train effluent was increased significantly in both RF-2 ( $p < 0.01$ ) and RF-3 ( $p < 0.001$ ) relative to influent levels.

This increasingly ‘volatile’ nature of the SS within RF effluents suggested that the inorganic or fixed SS were accumulating more so within the confines of the RF than within the DW and OP treatments, and was also likely to have reflected some additional biomass production and sloughing within the RFs. This indicated that not only were the RFs removing more of the inflowing SS, but they were also producing a final effluent of a more labile or biodegradable nature.

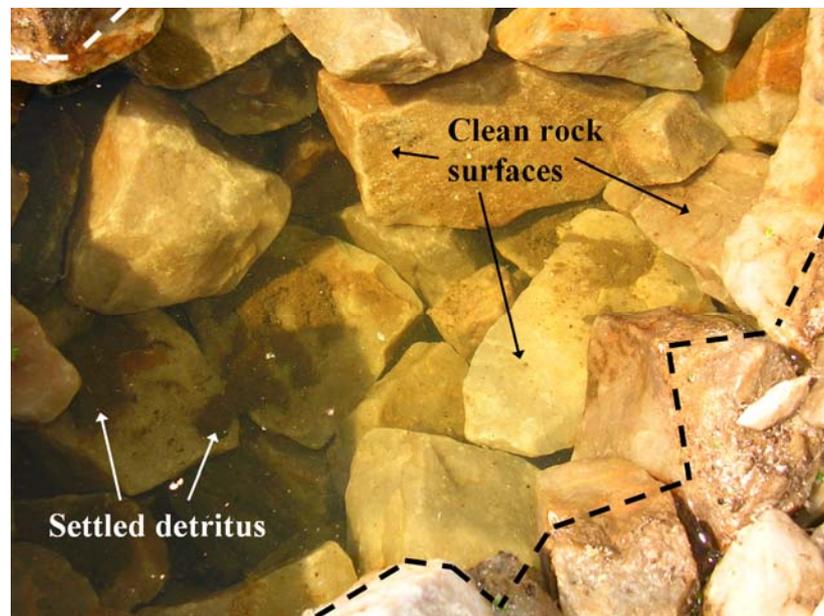
This trend for an approximate 30% increase in the fraction of VSS within RF effluent was unlike that reported by Hirsekorn (1974) and later Swanson and Williamson (1980), who both observed overall trends for a slight decrease ( $\approx 8\%$ ) in the fraction of VSS following RF passage; indicating the retention and degradation of a greater portion of the organic solids fraction within their RFs. The reasoning for an increasingly volatile effluent SS during RF treatment here was unclear; although it was thought to have been a reflection of the differing nature of the Bolivar WSP effluent compared to that of the previous authors, as well as a small amount of biomass production and subsequent sloughing within the higher flow velocity rock filters investigated here.

Interestingly, both of the above authors, as well as von Sperling *et al.* (2007) more recently, have reported on the presence of a biological “slime layer” on the internal surfaces within their rock filters, and both suggested that this biofilm could in some way be aiding overall filter performance (particularly with respect to the retention of settled materials). In a similar vein, Meiring and Oellermann (1995) suggested that algae passing through a shaded WSP environment would be expected to lose its vitality, thereby potentially making itself more susceptible to being adsorbed onto a biofilm such as that within a rock filter. Incidentally, this theory of Meiring and Oellermann was partly supported by the research findings of a later thesis Chapter (Chapter 9), in which the implications of dark-exposure on algal vitality and sinking velocity are rigorously discussed. Despite the suggestions of the above authors, Swanson and Williamson (1980)—following their observation of normal rock filter performance immediately after start-up—concluded that rock filters have no requirement for biological ‘pre-conditioning’, such that the *primary* mechanism behind effective filter performance is physical sedimentation. Following partial deconstruction of the current RF reactors, no biofilm was visually evident in any of the three filters (see Plate 3.1 and 3.2 for RFs 1

and 3 below respectively)—a likely consequence of their much lower organic loading regime. Based on this observation, it was thought that substrate biofilms were unlikely to have been contributing significantly to solids retention within the current RFs.

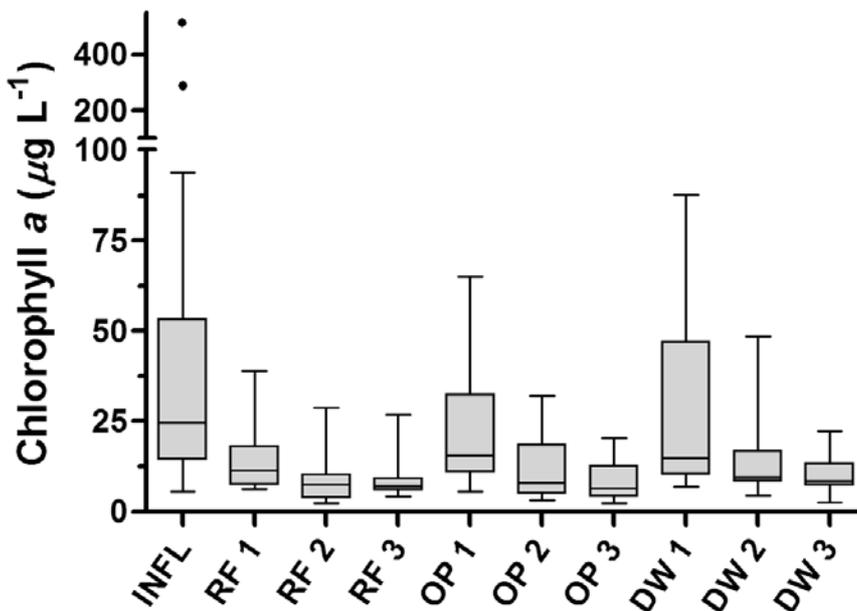


**Plate 3.1.** Detail of the relatively 'clean' biofilm-free internal rock media surfaces of RF-1, showing non-attached accumulations of flocculated materials. Broken lines indicate the water surface level.



**Plate 3.2.** Detail of the relatively 'clean' biofilm-free internal rock media surfaces of RF-3, showing non-attached accumulations of flocculated materials. Broken lines indicate the water surface level.

Similar to the above findings for SS, influent chlorophyll *a* data showed that there were generally low levels of suspended algal biomass, but again that levels were highly variable in some instances; with a very low median chlorophyll *a* concentration of  $24.6\mu\text{g L}^{-1}$  and a mean of  $36.8\mu\text{g L}^{-1}$ . The sources of this random and high-level variability in both SS and algal biomass density within the Bolivar WSP effluent (i.e. pilot plant influent) have already been discussed (see Section 3.3.5). Given the skewed nature of the influent data, this median concentration of  $24.6\mu\text{g L}^{-1}$  translated to a median mass loading during the monitoring period of  $18\text{mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$ . The chlorophyll *a* performance data is shown below in Figure 3.29.

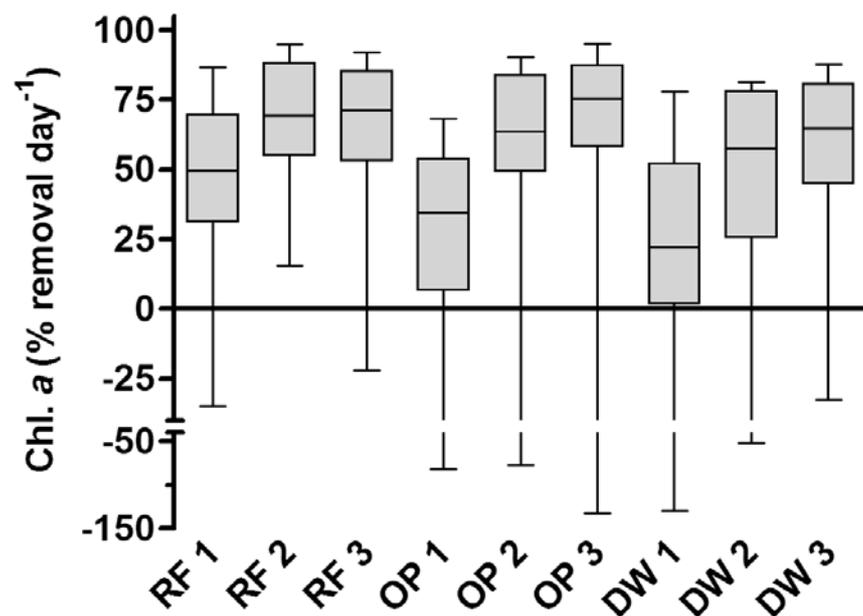


**Figure 3.29.** Chlorophyll *a* box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). Filled circles (●) above the INFL data represent the three extreme spike outliers  $>3\times\text{IQR}$  from the 75<sup>th</sup> percentile value.

Statistical analysis of the data from Figure 3.29 showed that influent chlorophyll *a* levels were reduced significantly down the pond series in DW-2 (1-way ANOVA;  $F_{(9,158)} = 8.51$ ;  $p < 0.05$ ) and DW-3 ( $p < 0.001$ ) but not DW Pond 1 ( $p > 0.05$ ). An identical trend was also seen for the OP treatment series, with no significant chlorophyll *a* removal in OP-1 ( $p > 0.05$ ) but significant removals in both OP-2 and OP-3 ( $p < 0.001$ ). Unlike the DW and OP treatments, the RF train displayed slightly more



advanced chlorophyll *a* removal potential; yielding significant removals in RF-1 ( $p < 0.05$ ) as well as Rock Filters 2 and 3 ( $p < 0.001$ ). With respect to the between-treatment performance comparisons, and despite the RF train qualitatively appearing to deliver greater performance, there were no significant differences in chlorophyll *a* levels for any of the three treatments down the pond series ( $p > 0.05$ ); implying that all three pilot treatment upgrades were producing an effluent with similar amounts of suspended algal biomass. This data is represented below as daily percentage removal efficiencies for each pilot treatment pond (Figure 3.30).



**Figure 3.30.** Box-plots showing percentage chlorophyll *a* removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n \geq 15$  for all plots).

Long-term median percentage chlorophyll *a* removals for Pond 1 data across the three treatments were 22, 35 and 50% for the DW, OP and RF systems respectively, and for Pond 3 data, 65, 75 and 71% for the respective DW, OP and RF treatments. When compared statistically to a theoretical zero median chlorophyll *a* removal, these average percentage chlorophyll *a* removal efficiencies were all found to be significantly ‘non-zero’ across all ponds of all treatment series except for the 22% removal of DW-1 (Table 3.7), suggesting once again that algal removals across all treatments were by the end of each the three-pond series greater than zero. As was the case for prior BOD<sub>5</sub> performance assessments, the RF system was once again the only treatment train not to

yield consistently negative removal efficiencies (i.e. RF-2), with just three daily negative chlorophyll *a* removals experienced for RFs 1–3, compared with a total of six for the DW and seven for the OP treatment train during the course of the 2005 monitoring period. This trend is reflected in the corresponding CV's for treatment performance with respect to chlorophyll *a* removal efficiencies (Table 3.7).

**Table 3.7.** Summary of chlorophyll *a* performance data across all pilot plant treatments for Ponds 1 and 3 only.

Chl. <i>a</i> performance parameter	Pilot treatment pond					
	DW-1	DW-3	OP-1	OP-3	RF-1	RF-3
Median influent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	24.6	24.6	24.6	24.6	24.6	24.6
Mean influent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	36.8	36.8	36.8	36.8	36.8	36.8
Median effluent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	15.0	8.4	15.6	6.4	11.4	7.2
Mean effluent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ ) <sup>†</sup>	26.0	10.2	23.1	8.4	15.0	8.9
Median daily Chl. <i>a</i> removal (% day <sup>-1</sup> ) <sup>‡</sup>	22	65	35	75	50	71
Mean daily Chl. <i>a</i> removal (% day <sup>-1</sup> )	15	56	26	56	47	61
Long-term CV for Chl. <i>a</i> removal (%)	357	64	145	104	65	56

<sup>†</sup> Effluent chlorophyll *a* concentration was tested relative to mean pilot plant influent concentration (1-way ANOVA)

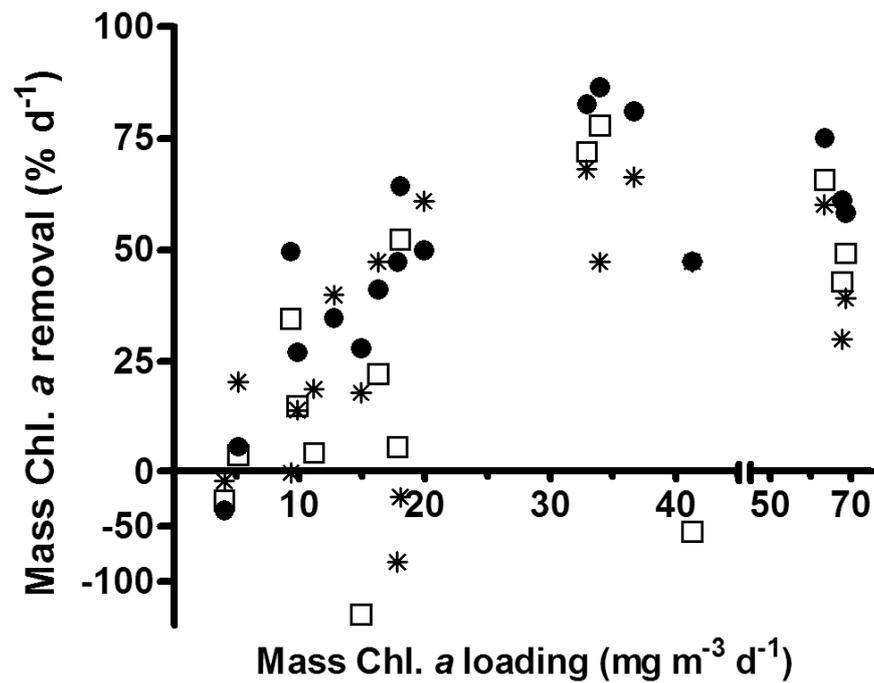
<sup>‡</sup> Average chlorophyll *a* removal % tested against a theoretical 'zero' daily median (Wilcoxon signed-rank test)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium);  $p \leq 0.001$  (black)

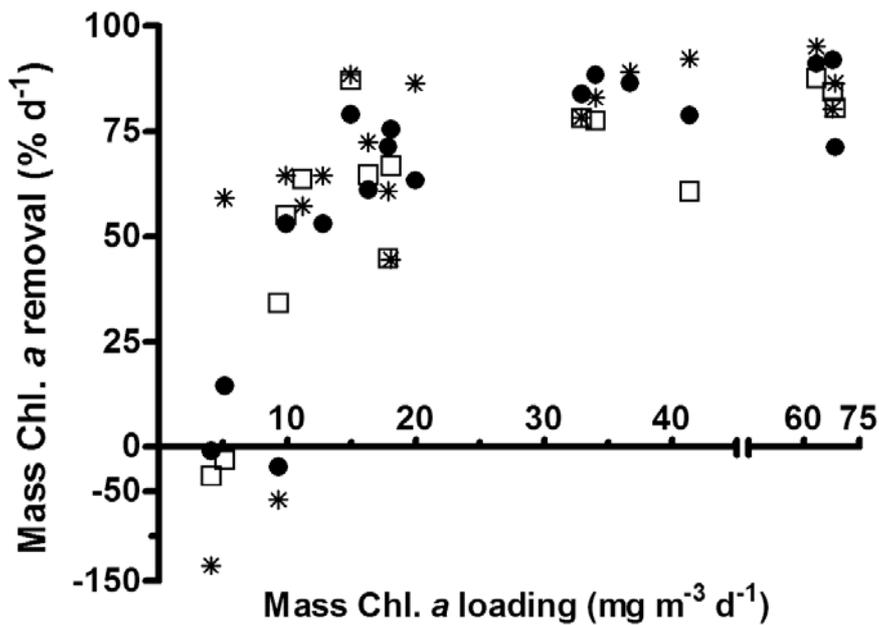
Comparison of the performance CV's for chlorophyll *a* removal (Table 3.7) together with the data of Figure 3.30, again showed an enhanced consistency in performance delivery for the RF system over both the DW and OP treatments—a trend noted previously for both BOD<sub>5</sub> and SS removal. Considering Pond 1 data only, RF-1 was the only pilot treatment series capable of consistently producing an effluent that contained significantly less chlorophyll *a* than its influent. Overall, however, and unlike the trends for BOD<sub>5</sub> and to a lesser extent SS removal efficiency above, the entire three-pond RF treatment series was in this case no more efficient at removing inflowing algal biomass than either the DW or OP pilot treatments; with all treatments producing an effluent containing significantly less chlorophyll *a* than that of the pilot plant influent at the  $p \leq 0.001$  level.

It is apparent for the chlorophyll *a* data, that although each treatment series was capable of removing a similar amount of loaded chlorophyll *a*, the RF treatment was able to remove suspended algal biomass at a greater rate down the three-pond series than both the DW and OP treatments (i.e. significant chlorophyll removals were realised in RF-1

but not DW-1 or OP-1). This trend for an increased ‘rate’ of removal down the pond series has been evident in all of the respective performance parameter plots so far (Figures 3.14, 3.21, 3.22, and 3.29) and suggests a greater capacity for rapid treatment within a rock filter compared with either a duckweed-covered or standard ‘open pond’. The above chlorophyll *a* data is represented again on a mass loading versus removal basis (Figures 3.31–3.34) for Ponds 1 and 3 of the respective treatments.

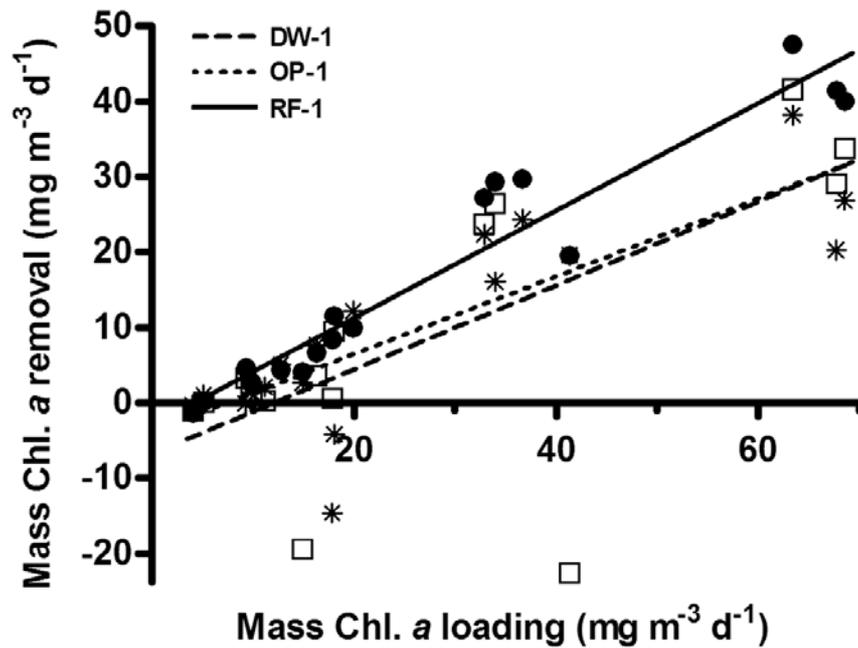


**Figure 3.31.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 1 data only (note the truncated *y*-axis scale for values below zero). Individual data points represent mean performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●). Individual data points show the mean of triplicate determinations.

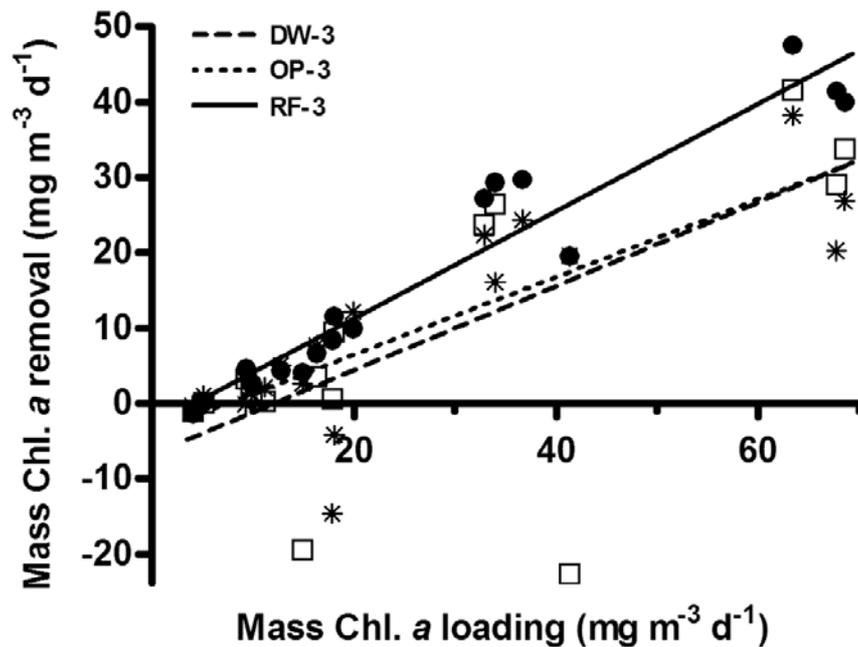


**Figure 3.32.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 3 data only (note the truncated *y*-axis scale for values below zero). Individual data points represent mean performance data for: Duckweed Pond 3 (□); Open Pond 3 (\*); and Rock Filter 3 (●). Individual data points show the mean of triplicate determinations.

Comparison of the performance on a mass loading versus percentage removal basis revealed similar trends for the chlorophyll *a* performance data to those reported for both BOD<sub>5</sub> and SS removal earlier. Visual analysis of Figures 3.31 and 3.32 reveals a general trend across both Ponds 1 and 3 for an increase in chlorophyll *a* removal efficiency at higher influent algal biomass loads. This again suggested that chlorophyll *a* removal was largely governed by concentration-dependent first-order-type removal processes, of which a detailed description has already been provided (Section 3.3.5). Another trend again evident in Figures 3.31 and 3.32 above was that under very low chlorophyll *a* loading (i.e. < 15mg m<sup>-3</sup> d<sup>-1</sup>), the removal performance and subsequent effluent quality of all treatments appeared to be largely independent of influent algal load. This again reflected the first-order removal processes, whereby very low-level chlorophyll *a* loading conditions promoted greater percentage variability in removal performance against normal background ‘steady-state’ effluent levels, to the point of sometimes yielding negative chlorophyll *a* removals at very low influent loads.



**Figure 3.33.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●). Fitted lines represent best-fit lines from simple linear regression analyses. Individual data points show the mean of triplicate determinations.



**Figure 3.34.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent mean performance data for: Duckweed Pond 3 (□); Open Pond 3 (\*); and Rock Filter 3 (●). Fitted lines represent best-fit lines from simple linear regression analyses. Individual data points show the mean of triplicate determinations.

When the chlorophyll *a* data is represented on a mass only basis (Figures 3.33 and 3.34), and as was the case for both BOD<sub>5</sub> and SS removal performance data above, there was again a noticeable direct positive association between mass loading and the mass of chlorophyll *a* removed, particularly under high influent loads. When the data for all three ponds of each treatment series were combined, this correlation between mass chlorophyll *a* load and mass removal was again highly significant for the DW Pond series ( $r = 0.843$ ;  $n = 45$ ;  $p < 0.0001$ ) the OPs ( $r = 0.890$ ;  $n = 54$ ;  $p < 0.0001$ ) and also the RF treatment train ( $r = 0.964$ ;  $n = 51$ ;  $p < 0.0001$ ).

Regression analyses of Figures 3.33 and 3.34 yielded equally high-level regression coefficients to the Pearson correlation coefficients above, with the slopes of all three fitted lines significantly greater than zero ( $p < 0.0001$ ). For the Pond 1 and Pond 3 data of Figures 3.33 and 3.34 respectively, there were no apparent differences between the slopes of the fitted regression lines (ANCOVA;  $F_{(2,44)} \leq 0.908$ ;  $p \geq 0.41$ ). With respect to the *elevations* of the regressed lines, however, there were significant differences between treatments for the Pond 1 data of Figure 3.33 (ANCOVA;  $F_{(2,61)} = 3.38$ ;  $p < 0.05$ ) but not for the Pond 3 data of Figure 3.34 ( $p = 0.58$ ). For the Pond 1 data, the elevation of the best-fit line was significantly greater for RF-1 than for both DW-1 and OP-1 ( $p \leq 0.033$ ), but elevations were equal for the fitted lines of the DW and OP treatments ( $p = 0.65$ ). For Pond 3 data (Figure 3.34) the elevations of the fitted regression lines this time were equal for all three treatments (ANCOVA;  $F_{(2,46)} = 0.552$ ;  $p = 0.58$ ); implying that all three treatments by the end of the three-pond series removed equivalent amounts of loaded chlorophyll *a* under the range of mass loading rates tested.

Results of the above regression analyses suggested that whilst all three treatments displayed an equally linear pattern for mass chlorophyll *a* removal versus loading, following the first pond of each three-pond series the RFs were able to remove a greater mass of the loaded algal biomass at any given mass loading rate than were the other two treatments (remembering again that treatment performance in Figures 3.33 and 3.34 is measured by the degree of *y*-axis *elevation* for each data point relative to the point of zero removal). Results also showed that by the end of the three-pond series, all three treatments were equally effective at removing chlorophyll *a* under a given mass loading rate; something supported by the data of Table 3.7. As mentioned previously (Section

3.3.5), published data regarding loading versus removal performance of duckweed ponds and rock filters is lacking, and so the trends reported here cannot be directly compared to those of other similar systems. It is again a recommendation that future work aims to present performance data on a 'loading versus removal' basis in order to provide additional insights into the nature of treatment efficiency for these upgrade systems.

The above results again suggest a trend for higher level treatment performance from the Rock Filters (at least as far as the first reactor in each series) compared with both the Duckweed and Open Ponds—a trend noted above for the performance parameters BOD<sub>5</sub> and SS. Tanner *et al.* (2005) noted a similar trend for their pilot-scale pond systems consisting of one open maturation pond and one planted wetland system with a duckweed surface cover. Both of their pilot pond systems experienced highly variable treatment performance and final effluent quality with respect to BOD<sub>5</sub>, SS, and chlorophyll *a*. Interestingly, once a rock filter was added to the end of both treatment series (occupying 25% of the total pond length), both the total performance and also the performance consistency of both systems rose dramatically (50% improvements in SS,  $\geq 50\%$  increase in BOD<sub>5</sub> removal, and  $\geq 80\%$  increase in chlorophyll *a* removals) to the point where the overall performance of the two treatments was virtually indistinguishable. This example serves to highlight the advanced treatment capacity and performance reliability of rock filters for upgrading final WSP effluent (with respect to BOD<sub>5</sub>, SS and chlorophyll *a*) over other systems such as macrophyte-based and conventional 'open' or algal-based ponds.

Whilst the heightened variability in chlorophyll *a* removal performance for the DW and OP systems was indeed a real outcome, these frequent and large-scale negative chlorophyll *a* removals experienced by both the DW and OP treatments were thought to have been influenced by both primary (algal for OPs and duckweed for DW Ponds) and also secondary (zooplankton) biomass production during pilot plant passage. As mentioned earlier during the discussion of BOD<sub>5</sub> performance data, and in spite of coarse filtration through a 2mm stainless steel mesh sieve (see Section 2.1), small suspended fragments of decaying duckweed plant material was regularly observed in the daily DW Pond samples. It was considered likely then that this plant tissue could have been contributing to the total measured chlorophyll *a* for DW Pond samples, and was

also likely to have been contributing to measured SS, as *algal* solids were partially replaced with decaying *duckweed* biomass. The extent of root development and root fragment shedding beneath a healthy duckweed plant mat can be seen below in Plate 3.3.



**Plate 3.3.** Photograph showing the highly developed root network of a low-density duckweed (*Lemna*) surface mat.

Following on from this suspicion, an analysis was later performed whereby triplicate samples of 15 medium-sized (1.5cm) duckweed roots were collected and acetone-extracted as per the standard chlorophyll *a* assay (Section 2.2.2.6). Results of this test showed that at a density of 15 whole root fragments  $L^{-1}$ , the amount of chlorophyll *a* coming from the duckweed plant tissue was in the order of 11–23% of the daily total. Furthermore, and as a result of steadily declining chlorophyll *a* levels from Pond 1 to Pond 3, the relative contribution of this duckweed biomass chlorophyll *a* to the total daily figure increased along the pond series from an average of 11.7% in DW-1, to 23.7% in DW-3. Considering the combined data from all three DW Ponds, the average contribution of suspended duckweed tissue to daily chlorophyll *a* measurements was thought to be in the order of 18% ( $\pm 6$ ). Whilst no attempt was made to try and correct for this *post hoc*, it should be noted that the algal removal capacity of the duckweed was likely to have been significantly underestimated as a result of the above factor, such that the true algal removal capability of the DW Ponds would be expected to be significantly greater than that quantified by chlorophyll *a* analysis here.



Although it was an initial aim of the current research to specifically investigate *algal* solids removal, following the performance data analysis it was apparent that there were significant difficulties involved with the analytical separation of chlorophyll *a* from a sample containing both suspended algal and duckweed biomass. Following this realisation, it was deemed unreasonable to attempt to separate the two chlorophyll *a* fractions based on that implied from published ratios of WSP chlorophyll *a* to SS or BOD<sub>5</sub>. Instead, it can only be recommended that all future investigations specifically concerned with assessing algal biomass removal within duckweed ponds should adopt a direct microscopic approach for the regular monitoring of algal biomass density in addition to the standard chlorophyll *a*, SS and BOD<sub>5</sub> analyses. This would enable the investigator to more accurately determine the relative proportions of suspended phytoplankton and macrophyte biomass, and would then allow for a more accurate assessment of both the algal removal efficiency as well as providing information on the relative contribution of senescent duckweed biomass back into the final pond effluent.

In addition to the likely contribution from duckweed tissue, the large-scale variability in percentage OP chlorophyll *a* removal efficiency could be related to the regular occurrence of zooplankton blooms within these ponds. The role of herbivorous zooplankton in the reduction of algal concentrations within WSPs is well documented (Kryutčhkova, 1968; Hussainy, 1979; Mitchell, 1980; Uhlmann, 1980; Hathaway and Stefan, 1995; Cauchie *et al.*, 1999; Tanner *et al.*, 2005). Sometimes very dense populations (up to 490 organisms L<sup>-1</sup>) of macrozooplankton were periodically recorded within the OP series in particular, and it was thought that the grazed algal biomass contained within their gastrointestinal tracts might have contributed to daily chlorophyll *a* measurements—particularly for non-selective substrate grazers (see below). This issue was raised by Hirsekorn (1974, p. 57) in that “*any crustaceans (zooplankton) present in the sample also contribute to the chlorophyll concentration, because algae consumed as a food source are present within the animal*” at the time of sample processing.

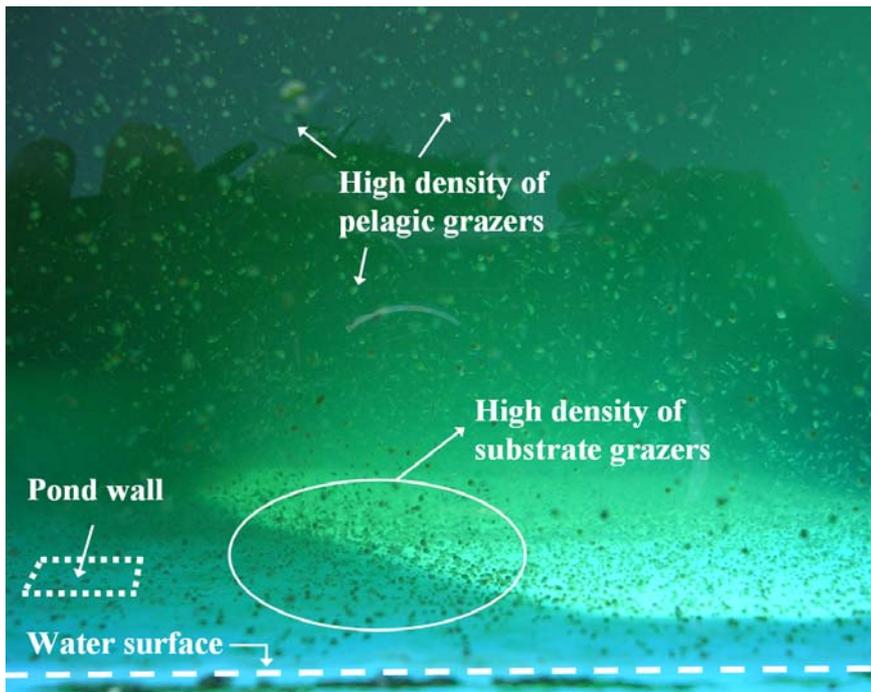
Following this, measurements were conducted in order to estimate the fraction of zooplankton-sequestered chlorophyll *a* in daily samples under zooplankton bloom conditions. Briefly, at a sampling interval where macrozooplankton density was in the order of 200 organisms L<sup>-1</sup>, both raw unfiltered and filtered (350µm) OP train samples

were processed for chlorophyll *a* analysis following the standard protocol (Section 2.2.2.6). Results of this analysis showed that individual macrozooplankton (*Daphnia* species) contained in the order of  $6.5 \times 10^{-3} \mu\text{g}$  chlorophyll *a* organism<sup>-1</sup>. Using OP-3 as an example, and under a mean OP-3 chlorophyll *a* concentration of  $\approx 9 \mu\text{g L}^{-1}$ , this translated to somewhere in the order of 10% of the average daily measured chlorophyll *a* being contained within the macrozooplankton biomass itself. Furthermore, and on a day-to-day basis, the contribution of *Daphnia* to the total chlorophyll *a* figure was anywhere in the range of 1–70% depending on the population density of the zooplankton bloom and also the influent chlorophyll *a* levels. Additionally, it is likely that this figure was a somewhat conservative estimate of the *actual* total zooplankton contribution, given that only the contribution from *Daphnia* species was taken into account and considering that *Daphnia* comprised on average only 60% of the total daily zooplankton biomass figure.

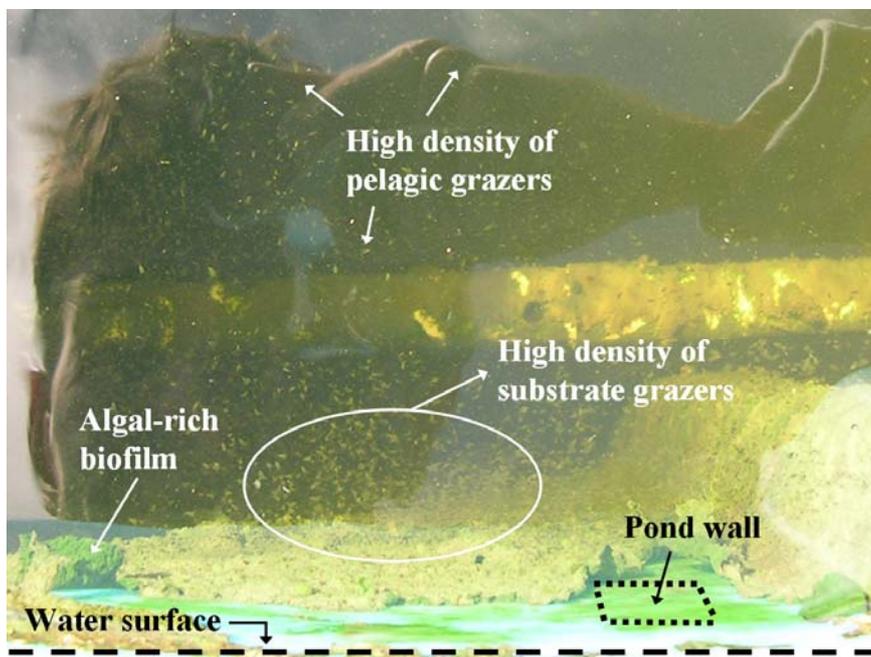
The above results suggest that a potentially concentrating effect is exerted on measured chlorophyll *a* levels by suspended zooplankton populations; something that is especially relevant for species capable of substrate grazing (e.g. *Daphnia* and ostracods; Horton *et al.*, 1979; Mitchell, 1980; Langis *et al.*, 1988). As also noted by the previous authors, substrate grazing was observed on a regular basis within the OP treatment train, whereby zooplankton populations were seen to be heavily—often preferentially—grazing on the internal surfaces of the HDPE reactors (shown in Plates 3.4 and 3.5). Furthermore, some macrozooplankton (e.g. copepods and *Daphnia*) are known to be ‘strong swimmers’ as well as having a tendency to swim against the direction of flow. This observation was made here for both copepods and *Daphnia* species, and incidentally, was also reported by Mitchell (1980) during experimental monitoring of a local WSP system in Gumeracha, South Australia. Mitchell (1980, p. 113) stated that “*observations made during a bloom of Daphnia carinata in pond 1 suggested that animals (actively) avoided the area immediately surrounding the outflow pipe.*” Even at moderate activity levels, *Daphnia* are capable of swimming at velocities in excess of  $20 \text{ m h}^{-1}$  (Dodson *et al.*, 1997). Similarly, copepods of similar size to the species encountered during the current work are reportedly capable of swimming at speeds in the order of  $30\text{--}90 \text{ m h}^{-1}$  (Enright, 1977). Under the highest HLRs tested here, the *in situ* fluid velocities were no greater than  $3 \text{ m d}^{-1}$  for any treatment pond, and so it is reasonable to suggest that these macrozooplankton were indeed capable of maintaining their position within the pilot

ponds despite the continuous hydraulic turnover. This meant that these organisms were almost certainly able to avoid being flushed out of the pilot ponds and may have been effectively accumulating in numbers within the pilot ponds.

Considering the above, it can be appreciated that if large numbers of these substrate grazers were withdrawn during daily pond sampling, the measured chlorophyll *a* from pilot pond samples would include both suspended and also non-suspended/attached (grazed) algal biomass fractions, whereas pilot plant influent chlorophyll *a* measurements represented the suspended 'planktonic' fraction only. This would have effectively resulted in an overstating of the true suspended chlorophyll *a* concentration within the pilot ponds, and a subsequent underestimation of planktonic algal removals within these systems. Whilst no attempt was made to apply a correction factor to the chlorophyll *a* performance data in order to account for the potentially confounding effects of zooplankton grazing, it is recommended that future work dealing with assessing algal dynamics in small-scale pond systems in the very least consider the likely influence of this phenomenon on the measured levels of chlorophyll *a*. Ideally, investigators could perform chlorophyll *a* analyses on filtered ( $\approx 300\text{--}400\mu\text{m}$ ) versus unfiltered influent and effluent samples in order to gauge the relative proportions of suspended and grazed chlorophyll fractions within their samples.

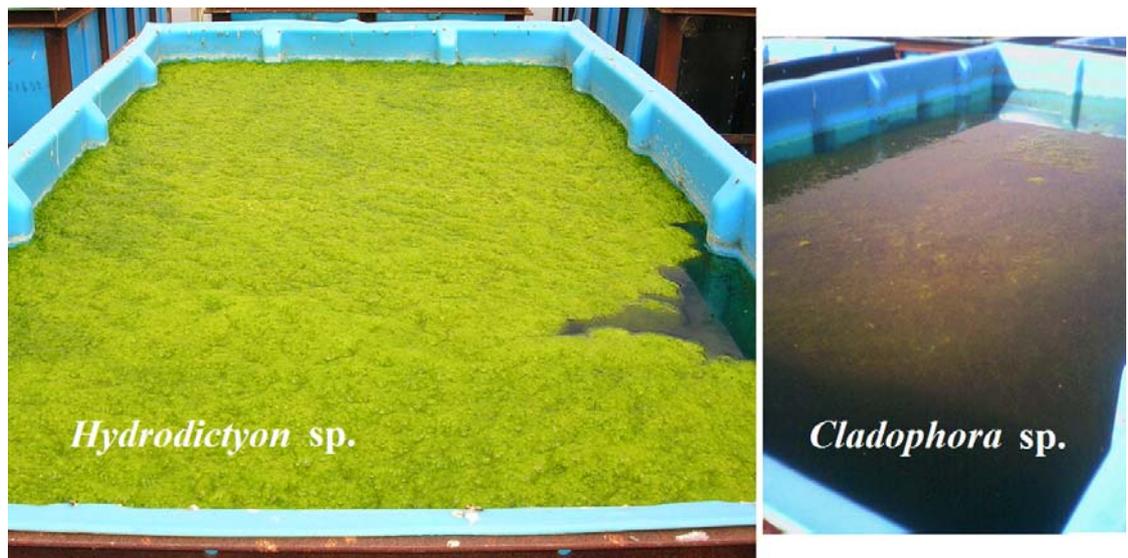


**Plate 3.4.** Aerial view looking down into one of the Open Ponds; showing the high densities of both pelagic (suspended) and substrate-grazing zooplankton populations (note the heavy grazing on pond wall biofilms).



**Plate 3.5.** Aerial view looking down into another of the Open Ponds; once again showing the high densities of both pelagic (suspended) and substrate-grazing zooplankton populations (note again the dense congregation of zooplankton close to the pond wall biofilm).

In addition to the likely effects of zooplankton on measured chlorophyll *a* concentration, the high-level variability in chlorophyll *a* levels within the OP series was thought to also be a consequence of periodic filamentous algal blooms which developed within these aboveground ponds; commonly under conditions of elevated temperature and irradiance (Plate 3.6). These filamentous green (chlorophyte) algae were identified during the course of the monitoring period as both *Cladophora* and *Hydrodictyon* species. Following their periodic appearance, these filamentous blooms were manually removed from the OP system because of an assumed potential for significant adverse impacts on pond hydraulics (e.g. short-circuiting) and incident irradiance. For example, Sand-Jensen (1989) reported that a 1.2mm thick cyanobacterial surface mat reduced the levels of incident irradiance by 93–99.9%; hence it was deemed necessary to quickly remove these algal blooms so as to reduce the potential for shading of underling suspended algal populations. Mitchell and Williams (1982) reported significant blooms of the filamentous green alga *Cladophora* in local WSPs at Gumeracha, South Australia, and although the authors found that such algae can play a definable role in WSP nutrient dynamics, the small scale of the current pilot ponds was thought to have significantly reduced the potential for natural wind-induced bloom dispersion, such that its immediate removal was necessary. It should also be noted that such algal blooms were never experienced in the DW or RF treatments.



**Plate 3.6.** Photograph of the periodic filamentous green (Chlorophyceae; *Cladophora* and *Hydrodictyon*) algal blooms experienced within the Open Pond series.

Throughout the above SS and chlorophyll *a* performance data analyses, as well as during the previous BOD<sub>5</sub> analyses, there has been a general trend for more advanced parameter removals within the *first* pond of each treatment series, followed by a less dramatic or more gradual reduction (where significant removals were recorded) within the following two ponds. This general trend can be seen in the respective Figures 3.14, 3.21, 3.22 and 3.29, and is most apparent within the higher performance RF treatment series in particular. This observation ties in with the reporting of Reed *et al.* (1988), who stated that the majority of wastewater SS and associated BOD<sub>5</sub> “will be removed in the primary cell of a pond system.” Reed and co-authors also commented that the removal of SS in a WSP system is achieved primarily through gravity sedimentation (i.e. physical means). Given the statistically significant correlation between influent SS and BOD<sub>5</sub> ( $r_s = 0.524$ ;  $p = 0.045$ ) as well as influent SS and chlorophyll *a* ( $r = 0.657$ ;  $p = 0.001$ ), this implies that predominantly physical mechanisms were likely to have been responsible for the attenuation of both BOD<sub>5</sub> and chlorophyll *a* (as well as SS and turbidity) within all three pilot plant treatments (although physically settled organic solids must ultimately undergo biological degradation; something supported by the reduced DO levels in both the RFs and DW Ponds). Furthermore, the largely inorganic nature of the pilot plant influent (average VSS fraction of 50%; Figure 3.28) implies that roughly half of all influent SS were effectively ‘biologically inert’ or colloidal in nature, such that the processes governing solids removal in general would be expected to be predominantly physical.

The qualitative observation of an apparent ‘mirroring’ of removal patterns down the pond series for BOD<sub>5</sub>, SS and chlorophyll *a* within the treatment ponds is also supported by the highly significant correlation between percentage SS and BOD<sub>5</sub> removal efficiency ( $r_s = 0.538$ ;  $n = 74$ ;  $p < 0.0001$ ), as well as the same high-level relationship between percentage SS and chlorophyll *a* removal ( $r_s = 0.470$ ;  $n = 132$ ;  $p < 0.0001$ ) when the data from all 9 experimental ponds is combined. This correlation of performance relationships for the above parameters implies that the most dominant or controlling removal mechanism governing BOD<sub>5</sub>, SS and chlorophyll *a* removal within all pilot treatments was indeed physical sedimentation. This finding is in agreement with the relevant literature, whereby effective treatment in both duckweed (Oron *et al.*, 1987b; Zirschky and Reed, 1988; Mara *et al.*, 1992; Smith and Moelyowati, 2001) and

rock filter systems (Hirse Korn, 1974; Stutz-McDonald and Williamson, 1979; Swanson and Williamson, 1980; Rich, 1988) is delivered through predominantly physical rather than biological processes; although once again all physically settled biomass must ultimately be biologically degraded. This finding also suggested that the potential contributions from other biologically-based treatment mechanisms toward the observed algal removals within the DW (e.g. antagonistic allelopathy) and RF (e.g. biofilm-entrapment) systems were insignificant. Having said this, the likely contribution from grazing interactions remained of potential significance, given that Hillman and Culley Jr. (1978) reported that a *Lemna* surface cover—when properly maintained—can provide favourable growth conditions for populations of *Daphnia* and other grazing metazoans. The likely importance of grazing on observed algal removals within all three pilot treatments will be discussed in Chapter 5.

It is appropriate here to highlight the work of Stutz-McDonald and Williamson (1979, p. 279), where it was said that temperature was likely to be “*an important, if not dominant, factor in influencing the settling rate of algae in rock filters*” due to temperature-related water density effects and their subsequent effects on water viscosity and particulate settling rate. Interestingly, Hirsekorn (1974) also observed an increase in rock filter performance under increased temperature, but offered the enhancement in biochemical reaction rates as an explanation for the increased performance rather than the physical reasoning of the previous authors. This implies that temperature could potentially have had a significant impact on the rate of particulate settling within the RFs (and presumably also within the quiescent DW Ponds) and could therefore be linked to both SS and chlorophyll *a* removal performance. Interestingly, a slight but non-significant negative correlation between temperature and SS removal efficiency was actually observed for the RF performance data here (Pearson  $r = -0.268$ ;  $n = 51$ ;  $p = 0.06$ ); something in apparent contradiction to the work of Stutz-McDonald and Williamson (1979). Similarly, no relationship was found between temperature and chlorophyll *a* removals (Spearman  $r_s = -0.016$ ;  $n = 51$ ;  $p = 0.92$ ). These results suggest that temperature alone may not be such a strong governing factor behind effective rock filter treatment, and is therefore unlikely to serve as a general predictor of rock filter performance efficiency.

In comparing the overall SS and chlorophyll *a* performance data of the current section to the work of others, it is first of all necessary that the same ‘exclusion criteria’ be applied to this body of research as was outlined for the BOD<sub>5</sub> treatment performance data comparisons above (Section 3.3.5). Briefly, SS and algal removal performance comparisons are only made between the current data and that derived from research conducted using pond reactors of a comparable or larger volume. This once again served to minimise the potential confounding effects resulting from performance comparisons made between studies involving pond reactors several orders of magnitude smaller in volume.

Average SS removal performance data for the DW treatment here was similar to that reported by Ran *et al.* (2004) during the operation of a similar pilot-scale duckweed pond system. The authors reported similar scale SS removals, with a two monthly average of 80% when operated under a comparable but slightly higher influent SS loading ( $\approx 16\text{g SS m}^{-3}\text{ d}^{-1}$ ) and longer HRT (8.6 days for two tanks in series). Similarly, Bonomo *et al.* (1997), following the operation of pilot-scale ( $430\text{m}^3$ ) duckweed pond system, reported similar magnitude SS removal efficiencies to those seen here, with 50–80% removals over their five month monitoring period; although these solids removals were achieved under a two-fold lower SS loading rate of  $\approx 4\text{g SS m}^{-3}\text{ d}^{-1}$ . Baldizón *et al.* (2002) achieved slightly lower solids performance results to those recorded here, reporting an average 52% ( $\pm 29$ ) SS removal efficiency from a large-scale duckweed pond system with an HRT in the order of 5 days and a significantly higher SS influent concentration ( $\approx 185\text{mg L}^{-1}$ ); although no information was provided regarding the hydraulic loading of the pond.

Zimmo *et al.* (2002) operated a very similar sized pilot duckweed pond system comprising four  $\times 3\text{m}^3$  ponds arranged in series (as opposed to the three  $2.8\text{m}^3$  ponds used here) and under a roughly two-fold higher SS mass loading rate of  $\approx 22\text{g SS m}^{-3}\text{ d}^{-1}$ . The authors reported an annual average SS reduction of 71%—almost identical to the six month average of 70% seen here. Interestingly, Zimmo and co-workers also observed that the vast majority of the total four pond duckweed train SS removal was realised after the first pond in series; something again mirrored by the current observations. Furthermore, their pilot plant also included the parallel operation of a standard algal-



based ‘open pond’ treatment train alongside the duckweed pond series, with this system yielding an average SS removal in the order of 37%—again very similar to the six month average OP train SS removal of 43% seen here.

With respect to the SS performance data of the RF treatment, results have again been comparable to those within the literature. von Sperling and de Andrada (2006), following the 8 month operation of an equivalent volume pilot-scale rock filter, reported a slightly lower magnitude SS removal efficiency of 73% (compared with 81% solids removal here). Although their rock filter was given a 30% lower HLR ( $0.5\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ), it received a 10-fold greater solids mass loading ( $\approx 90\text{g SS m}^{-3}\text{ d}^{-1}$ ) and so this was thought to have contributed to the reduced SS removal performance. Saidam *et al.* (1995) reported much lower average SS removal efficiencies for their large-scale ( $300\text{m}^3$ ), achieving approximate 45% solids removal efficiencies for filters with a similar rock media size distribution ( $\approx 12\text{cm}$  diameter) but a five-fold higher SS mass load ( $50\text{g SS m}^{-3}\text{ d}^{-1}$ ). Swanson and Williamson (1980) recorded a mean SS removal efficiency of 75% following 12 month monitoring of their full-scale rock filter. This was again a very similar magnitude percentage removal to that recorded for the current RF treatment train, and although the mean influent SS concentration of Swanson and Williamson (1980) was somewhat higher at  $50\text{mg L}^{-1}$ , the current rock filters were actually loaded at a slightly higher average SS mass loading rate ( $\approx 10$  compared with  $\approx 8\text{g m}^{-3}\text{ d}^{-1}$ ).

The above performance data comparison suggests that the approximate 5-fold higher HLR applied to the current rock filters (compared with that of Swanson and Williamson, 1980) had a negligible impact on filter performance with respect to its ability to remove infiltrating solids. This observation is contrary to the suggestions of Swanson and Williamson (1980), who proposed a linear decline in the SS removal efficiency of another pilot-scale rock filter from 90% down to 70% with an increasing HLR from 0.1 to  $0.5\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ . Interestingly, even the highest HLR of Swanson and Williamson (1980) was still some 30% lower than the  $0.73\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  HLR adopted here, and yet SS removals in the order of 70% were still able to be maintained. Similar to the suggestions of Swanson and Williamson (1980), the work of von Sperling *et al.* (2007) also reported a 30–35% decline in the  $\text{BOD}_5$  and SS removal efficiencies of their pilot-scale rock filters with an increase in HLR from 0.5 to  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ . In contrast, however, the work

of Mara *et al.* (2001) is in apparent agreement with the RF performance data reported here, whereby 63% SS removals were achieved at an HLR  $\approx 25\%$  higher than that used here ( $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ); although Mara and co-workers did observe a further 13% reduction in SS removal efficiency when the HLR was increased from 1.0 to  $2.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ . This shows that the apparent large-scale linear decline in SS removal efficiency with increasing HLR above the guideline value of  $0.3 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$  (Mara, 2003) is not necessarily a universal phenomenon that applies to all rock filters. Instead, the efficiency of SS removal is likely to be influenced by both the concentration and physical nature of the wastewater solids in question (i.e. organic or inorganic, dispersed or aggregated) as well as the rate of hydraulic loading and corresponding interstitial flow velocity within the void spaces of the rock bed; something that is a direct function of rock media size.

Following a literature survey, it was found that there exists only a very limited amount of performance data specifically regarding chlorophyll *a* removal in duckweed pond systems (see Section 1.2.8.5.3). Whilst many researchers have implied an advanced capacity for algal removal (via 'algal-associated' SS and BOD<sub>5</sub> removals) in duckweed pond systems (Hillman and Culley Jr., 1978; Ngo, 1987; Oron *et al.*, 1987b; Zirschky and Reed, 1988; Mara *et al.*, 1992; Mandi, 1994; Bonomo *et al.*, 1997), very few have attempted to assess this claim quantitatively through the reporting of either chlorophyll *a* levels (Özbay, 2002; Zimmo *et al.*, 2002), direct cell counts (Valderrama *et al.*, 2002), or by carrying out investigations into algal photo-physiology (Parr *et al.*, 2002) under a duckweed cover. Following this, one of the aims of this research (outlined in Section 1.2.8.5.3) was to investigate the capacity of a duckweed pond system to attenuate suspended algal populations using flow-through pilot-scale ponds operated without duckweed biomass harvesting.

Following the somewhat variable chlorophyll *a* performance data, it was identified that aqueous chlorophyll measurements were often skewed by the presence of chlorophyll-containing duckweed plant tissue, meaning that accurate assessments of *in situ* algal biomass dynamics were prevented. While direct cell counts and algal speciation data was periodically recorded during the course of this research, and despite this data revealing  $< 0.5\text{-log}_{10}$  order reductions in influent algal cell counts by DW Pond 3 (refer to Figure 5.3), as highlighted earlier, the complexity and relative subjectivity of this sort

of analysis meant that it was performed infrequently; such that sample sizes were considered too small to allow for meaningful statistical analysis. Consequently, no attempt was made to link the chlorophyll *a* performance data of the DW treatment Ponds to that of the limited literature base defined above.

Interestingly, Zimmo *et al.* (2002) reported similarly variable trends for chlorophyll *a* in their pilot-scale duckweed pond systems. The pilot pond system of Zimmo and co-workers was very similar in both size and configuration to the current pilot plant (see pp. 168 above) and consisted of parallel duckweed and algal-based treatment trains. The authors reported that under influent concentrations of 8–71 $\mu\text{g chlorophyll } a \text{ L}^{-1}$ , the duckweed pond train yielded a net increase in the levels of chlorophyll *a*, with effluent concentrations in the range of 42–157 $\mu\text{g L}^{-1}$ . Whilst chlorophyll *a* levels in their duckweed pond train were some 6–15 times lower than in the parallel ‘open pond’ system (270–2390 $\mu\text{g L}^{-1}$ ), it still suggested that negative chlorophyll *a* removals were frequently experienced within their duckweed ponds. Although the authors offered no discussion regarding their negative ‘algal’ removals, results from this chapter suggest that suspended fragments of plant biomass were almost certainly contributing to the undue elevation of chlorophyll *a* levels in their duckweed pond samples. It can only be reiterated that all future work concerned with assessing algal biomass removal within duckweed ponds should adopt a direct microscopic approach for the regular monitoring of algal biomass density in addition to the standard suite of chlorophyll *a*, SS and BOD<sub>5</sub> analyses. This approach represents the only means of accurately determining the relative proportions of suspended algal and DW biomass within the final effluent.

In passing, and to the author’s knowledge, there does not appear to be any previous work concerned with assessing the algal removal efficacy of a floating macrophyte cover compared with that of a synthetic pond cover. Whilst large-scale synthetic covers have been implemented for odour control and biogas production (DeGarie *et al.*, 2000), as well as algal control in potable water reservoirs (Hunter, 2002), there has so far been no comparisons between living and synthetic pond covers for algal control in WSPs. Given that Zirschky and Reed (1988, p. 1254) have said that “*mat formation is probably the most significant contribution that the duckweed plant makes to wastewater treatment*”, and taking into account the relatively high labour inputs required for continuous biomass

harvesting (Ward, 1987), there could be significant scope for artificial surface covers; especially in instances where it is required solely for algal control. Although it was the intention of research reported by Zirschky and Reed (1988) to compare the treatment efficacy of a duckweed cover with a synthetic pond liner, the follow-up monitoring was never performed. Given that unharvested dead and dying plant biomass has the potential to return significant amounts of BOD<sub>5</sub> and SS back to the WSP system, future research could look at assessing the two surface covers side-by-side so in order to determine corresponding ‘cost–benefits’ of each.

Unlike that for duckweed systems above, there is considerably more quantitative performance data available regarding chlorophyll *a* removals in rock filters. Hirsekorn (1974) achieved a lower average mean chlorophyll *a* reduction of 56% (compared with the 61% removals recorded here) in their ‘large rock’ media (2.5–3.8cm diameter) filters at a somewhat reduced HLR of 0.24m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>. Mara *et al.* (2001) recorded 28% higher mean chlorophyll *a* removal efficiency for their pilot-scale RFs under a comparably high hydraulic loading (1.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) but a 20-fold greater algal biomass loading (383mg chlorophyll *a* m<sup>-3</sup> d<sup>-1</sup>). Whilst the rock filters of Mara and co-workers did achieve 28% greater average chlorophyll *a* removals than the current filters at 27% greater hydraulic loadings, the vastly different mass loading rates of these filters must be taken into consideration when drawing performance comparisons between the two systems. The much higher influent chlorophyll *a* concentrations for the rock filters of Mara and co-workers almost certainly contributed to the higher average percent removals; given that their mean rock filter effluent chlorophyll *a* concentrations were more than double the median influent concentrations here. In other words, the concentration-dependent nature of algal removal processes afforded their filters more scope for chlorophyll *a* removal against the low-level steady-state background concentrations frequently seen during the current work. Since the previous authors offered no data regarding mass loading versus removal rock filter performance, it can only be assumed that this was the reason for the apparent performance differences between these two systems.

Swanson and Williamson (1980) observed very high mean chlorophyll *a* removal rates in their full-scale (8500m<sup>3</sup>) large rock media (≈10cm) filter, with mean influent levels of ≈310μg L<sup>-1</sup> and mean effluent algal biomass concentrations of ≈50μg L<sup>-1</sup> chlorophyll *a*.

The minimum chlorophyll *a* removal rate for their work was in excess of 50%, with average algal biomass removals in the order of 81% over the 12 month monitoring period—slightly more efficient than the 71% six month median RF train removal recorded here. Interestingly, their performance data comes from a rock filter with a 10-fold higher average influent algal biomass concentration ( $310\mu\text{g chlorophyll } a \text{ L}^{-1}$ ) but only a 2.5-fold higher chlorophyll *a* mass loading rate (49 compared with the  $18\text{mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$  reported here)—a consequence of the 5-fold lower HLR of their filter ( $\approx 0.16$  compared with  $0.73\text{m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ). This suggested again that the 5-fold higher HLR applied to the current rock filters had only a slight negative impact on filter performance with respect to its ability to attenuate algal solids.

Chlorophyll *a* removals of von Sperling *et al.* (2007) were slightly lower than those of the current RFs, with a median removal of 55% observed within their similar sized pilot-scale rock filters when loaded at a comparably high HLR of  $1.0\text{m}^3 \text{ m}^{-3} \text{ d}^{-1}$ . Tanner *et al.* (2005) reported an approximate 30% improvement in the chlorophyll *a* removal efficiency of their pilot-scale open maturation pond system when fitted with a rock filter on the back-end of the system. Such large-scale differences between the chlorophyll *a* removal performance of the OP and RF treatment trains were not seen here, although an approximate 20% greater average chlorophyll *a* removal efficiency was seen for the RF over the OP system when considering only the data from the first pond in each treatment series (Table 3.7). The reasons for a reduced gap in total three-pond train performance between the RFs and OPs here have already been discussed and relate to the concentration-dependent removal processes for chlorophyll *a* in the pilot pond systems.

As for SS above, there was again a general trend for reduced rock filter chlorophyll *a* removal performance with an increased HLR. Mara *et al.* (2001), following the operation of similar scale experimental RFs at HLRs of both  $1.0$  and  $2.0\text{m}^3 \text{ m}^{-3} \text{ d}^{-1}$ , reported a 32% reduction in chlorophyll *a* removal efficiency in when operated at an increased hydraulic loading. This drop in rock filter performance efficiency was perhaps not surprising in the case of Mara and co-workers, given that at the highest HLR, the corresponding chlorophyll *a* mass loading rate was in the order of  $650\text{mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$  (some 35-fold greater than that applied to the current RFs) and so the rock filters were probably overloaded at the higher flow rates. Given that the guideline HLR for

rock filters treating maturation pond effluent in the United Kingdom is  $0.3\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  (Mara, 2003), and as was the case for SS performance data comparisons above, this suggested that the relatively high HLR applied to the current RFs ( $0.73\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ) had a negligible impact on percentage treatment performance with respect to their ability to remove infiltrating algal biomass.

Performance monitoring of the three pilot treatment systems has shown a general trend for a decline in the extent of SS, turbidity and chlorophyll *a* removal within the pilot pond series from Pond 1 to Pond 3 of all treatments. Where significant removals were recorded, typically the greatest removal of loaded parameters was realised within the first pond of each three-pond treatment series, after which the relative degree or ‘rate’ of removal generally diminished as concentrations approached steady-state levels. This was said to be a manifestation of the concentration-dependent removal of SS and chlorophyll *a*, and resulted in both percentage and mass removals generally being highest under elevated influent mass loads. Following detailed SS and chlorophyll *a* analyses, it is concluded that the overall ranking of treatment performance potential places the RF system 1<sup>st</sup>, and both the DW and OP treatment series equal 2<sup>nd</sup> in terms of SS and chlorophyll *a* removal rate down the pond series, absolute removal efficiency, and also performance reliability. There was some evidence to suggest slightly enhanced and more reliable removals of SS for the DW Ponds over the OPs, and likewise some data that suggested greater chlorophyll *a* removal performance for the OPs over the DW series (e.g. Table 3.7 and Figure 3.25); however, the overall performance of these two treatments was largely indistinguishable. Finally, for a more concise statistical summary of the overall performance parameter correlations as discussed throughout this and the preceding section, in addition to some others not referenced during the discussion of results here, the reader is directed to the corresponding correlation matrices for the pilot plant influent as well as the three upgrade treatments (Appendix B).

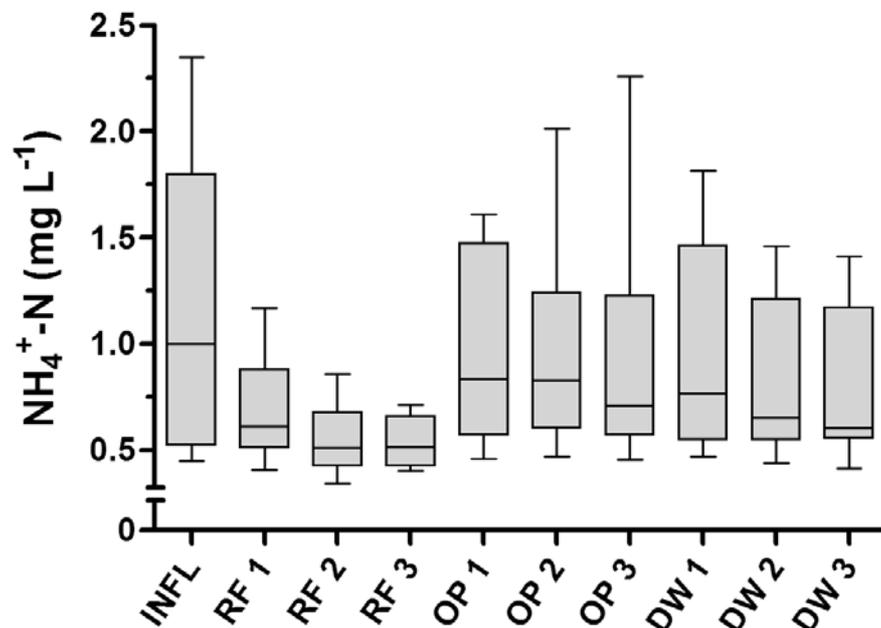
### **3.3.7 Wastewater treatment performance: nutrient removal**

Phytoplankton productivity is inherently governed by the availability of some 40 or so dissolved inorganic micronutrients. Amongst this consortium of nutritional requirements, N and P are generally regarded as the most essential ‘limiting’ nutrients (Reynolds, 2006). Typically, WSP systems are generally classified as being

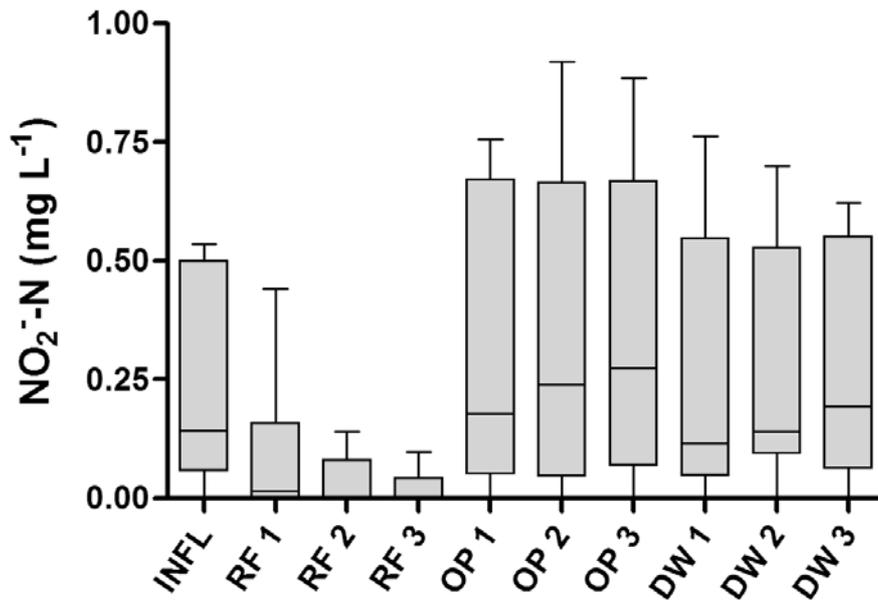
hypereutrophic in terms of their elevated nutrient resource status. With respect to the Bolivar system, however, and following the 2001 activated sludge treatment plant upgrade, the same trophic classification does not universally apply. So effective has the up-stream activated sludge plant installation been at sequestering nutrients, that the Bolivar WSPs are at times thought to actually be nitrogen-limited (Cromar *et al.*, 2005). Following the already low levels of dissolved nutrients within the influent wastewater, the levels of both  $\text{NH}_4^+\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  were periodically monitored during the course of the pilot plant performance assessments. Although the primary aim of the research was directly focused on algal biomass control through predominantly physical means, the monitoring of essential inorganic nutrient levels may provide for further insights into algal productivity control mechanisms arising from potential shifts in resource availability brought about by the respective pilot treatments.

### 3.3.7.1 Inorganic nitrogen dynamics

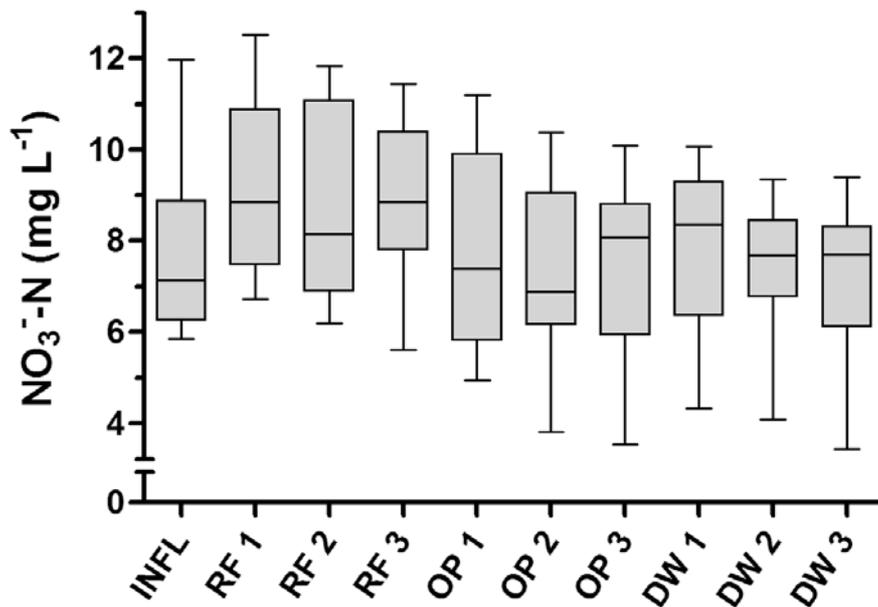
Data from six month performance monitoring of ammonia-, nitrite-, and nitrate-nitrogen are shown in Figures 3.35–3.37 respectively.



**Figure 3.35.** Ammonia-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median, and the ‘whiskers’ show the absolute data range.



**Figure 3.36.** Nitrite-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

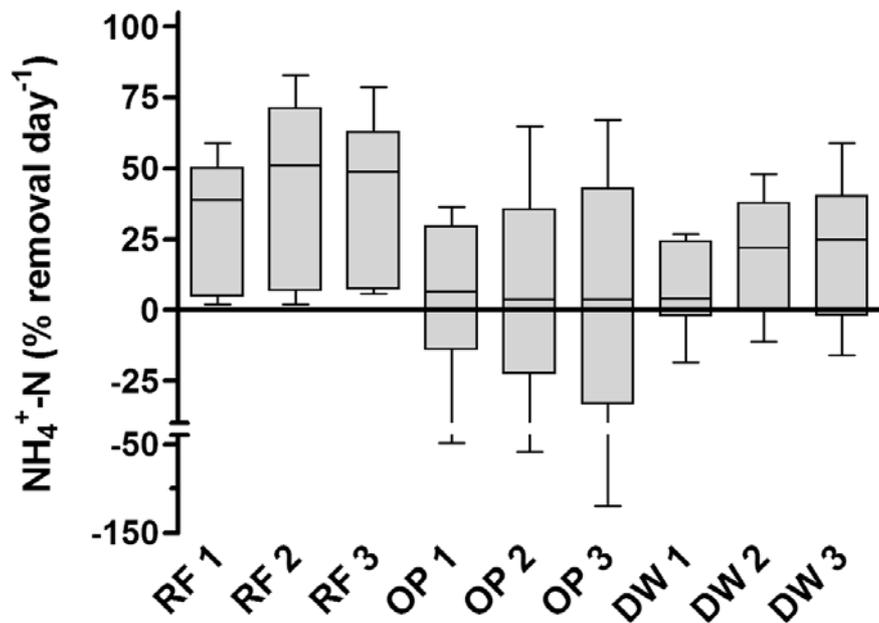


**Figure 3.37.** Nitrate-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

As evidenced in Figure 3.35, influent  $\text{NH}_4^+\text{-N}$  levels were generally very low, with a median value of 1.0 and a mean of  $1.2\text{mg L}^{-1}$ . This corresponded to a median mass influent loading of approximately  $0.73\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$ . Visual analysis of the data from Figure 3.35 showed that influent  $\text{NH}_4^+\text{-N}$  levels decreased by approximately 20% by the



last pond of the DW treatment series; although this apparent removal was not significant in any of the three DW Ponds (1-way ANOVA;  $F_{(9,100)} = 2.70$ ;  $p > 0.05$ ). The same was again true for the OP treatment train, with no significant change in any of the three OPs relative to influent  $\text{NH}_4^+\text{-N}$  loads ( $p > 0.05$ ). Unlike the other two treatments, the RF train did achieve significant removals of loaded  $\text{NH}_4^+\text{-N}$  in both RF-2 and RF-3 ( $p < 0.05$ ) but not for RF-1 ( $p > 0.05$ ); although there were no apparent differences between the final pond levels of  $\text{NH}_4^+\text{-N}$  in DW-3, OP-3 and RF-3 ( $p > 0.05$ ). Interestingly, this observation of significant  $\text{NH}_4^+\text{-N}$  removal in the current RFs is unlike the general trends reported in the literature. Most commonly, rock filters are thought to be incapable of  $\text{NH}_4^+\text{-N}$  removal, and in some instances actually release ammonia following the anaerobic digestion and remineralisation of settled organic materials.



**Figure 3.38.** Box-plots showing daily percentage ammonia removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n = 11$  for all plots).

Long term mean percentage  $\text{NH}_4^+\text{-N}$  removals for Pond 1 data of the three treatments were approximately 7, 5 and 30%, for DW, OP and RF systems respectively and for Pond 3 data, 23, -2 and 39% for the respective DW, OP and RF upgrade systems (Figure 3.38). As described above, there was no statistically-apparent decline in the influent  $\text{NH}_4^+\text{-N}$  concentration within individual ponds of the DW Pond series. When

compared statistically to a theoretical ‘zero’ average removal efficiency, the average 23%  $\text{NH}_4^+\text{-N}$  removal for DW Pond 3 was found to be significantly ‘non-zero’, whereas mean  $\text{NH}_4^+\text{-N}$  removals remained effectively equivalent to ‘zero removals’ for Ponds 1 and 3 of the OP treatment series (Table 3.8). As was the case for previously reported performance parameters, the RF system once again always yielded positive  $\text{NH}_4^+\text{-N}$  removals, whereas the DW and OP treatment series both experienced net  $\text{NH}_4^+\text{-N}$  gains on at least two (DW-2) and up to five (OP-2, OP-3) of the 11 sampling intervals (Figure 3.38). This is reflected in the corresponding CV’s for treatment performance with respect to percentage  $\text{NH}_4^+\text{-N}$  removal efficiency. Pond 1 CV’s for  $\text{NH}_4^+\text{-N}$  removal performance were 243, 532, and 77% for the DW, OP, and RF treatment systems respectively, and Pond 3 CV’s were 112, 2290, and 77% for the respective DW, OP, and RF treatment systems. This performance data is summarised in Table 3.8 below.

**Table 3.8.** Summary of ammonia removal performance across all pilot plant treatments for Pond 1 and 3 data only.

<b><math>\text{NH}_4^+\text{-N}</math> performance parameter</b>	<b><i>Pilot treatment pond</i></b>					
	<b>DW-1</b>	<b>DW-3</b>	<b>OP-1</b>	<b>OP-3</b>	<b>RF-1</b>	<b>RF-3</b>
Median influent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	1.00	1.00	1.00	1.00	1.00	1.00
Mean influent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	1.20	1.20	1.20	1.20	1.20	1.20
Median effluent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	0.77	0.60	0.84	0.71	0.61	0.50
Mean effluent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ ) <sup>†</sup>	1.03	0.78	1.00	1.00	0.69	0.53
Median daily $\text{NH}_4^+\text{-N}$ removal ( $\% \text{ day}^{-1}$ )	4.1	25	6.5	3.8	39	49
Mean daily $\text{NH}_4^+\text{-N}$ removal ( $\% \text{ day}^{-1}$ ) <sup>‡</sup>	6.7	23	4.8	-2.4	30	39
Long-term CV for $\text{NH}_4^+\text{-N}$ removal (%)	243	112	532	2290	77	77

<sup>†</sup> Effluent  $\text{NH}_4^+\text{-N}$  concentration was tested relative to mean pilot plant influent  $\text{NH}_4^+\text{-N}$  concentration (1-way ANOVA)

<sup>‡</sup> Average  $\text{NH}_4^+\text{-N}$  removal % tested against a theoretical ‘zero’ daily mean (one sample *t*-test)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium)

There are three general pathways for  $\text{NH}_4^+\text{-N}$  removal in WSP environments: volatilisation; biomass sequestration (microbial and algal); and nitrification–denitrification (Ferrara and Avcı, 1982; Middlebrooks *et al.*, 1982; Maynard *et al.*, 1999). Historically, the majority of inorganic nitrogen removal in WSPs was considered to be achieved via  $\text{NH}_4^+\text{-N}$  volatilisation, whereby an elevated pH results in a decrease in the degree of ammonia ionization and a subsequently increase in its volatility and loss to the atmosphere (Kreft *et al.*, 1958; Hemens and Mason, 1968). More recently, studies have shown biomass uptake and nitrification–denitrification to be more important

factors in WSP nitrogen removal than volatilisation (Ferrara and Avci, 1982; Baskaran and Farago, 2007; Camargo Valero and Mara, 2007a; 2007b).

With respect to DW Ponds, nitrogen removal is said to be achieved primarily via: ammonification; microbial nitrification–denitrification; plant biomass assimilation; and volatilisation (van der Steen *et al.*, 1998; Benjawan and Koottatep, 2007). In the absence of biomass harvesting, the small and largely variable  $\text{NH}_4^+$ -N removals achieved here for the DW treatment were thought not to have been from direct plant uptake. Similarly, the nitrite- ( $\text{NO}_2^-$ -N) and nitrate-nitrogen ( $\text{NO}_3^-$ -N) data from Figures 3.36 and 3.37 suggest that biological nitrification–denitrification was unlikely to have been responsible for removing any significant amount of  $\text{NH}_4^+$ -N. Furthermore, ammonia volatilisation was deemed unlikely to have been occurring within the pilot DW Ponds due to the relatively low aqueous pH (see Figure 3.11) and because of the thick (2–3cm) plant mat which blanketed the water surface having presumably prevented normal water–air gaseous exchange processes (van der Steen *et al.*, 2003). Although significant  $\text{NH}_4^+$ -N removals are widely reported in the relevant duckweed WSP literature, since there was no strong evidence of consistent  $\text{NH}_4^+$ -N removals in the current DW Ponds, no discussion of these performance results in the context of the others’ findings is offered.

Unlike duckweed ponds,  $\text{NH}_4^+$ -N removals are not commonly associated with rock filters. The observation of significant  $\text{NH}_4^+$ -N removal within the current RFs was unlike the more common trends for zero or negative removal efficiencies reported in the literature. With respect to the potential mechanisms of ammonia removal in rock filters, they are presumably the same as has already been discussed for WSPs above (i.e. volatilisation, biomass sequestration, and microbial nitrification–denitrification). As was the case for the DW Ponds above, volatilisation was considered unlikely to have played a significant role in RF  $\text{NH}_4^+$ -N removals. Once again, the pH data (Figure 3.11) recorded a steadily declining RF pH from an influent of 8.4 to 7.9 by the end of RF-3. Furthermore, volatilisation is thought not to be a significant loss factor for  $\text{NH}_4^+$ -N removal at pH values less than 8.0 (Vermaat and Hanif, 1998) due to the relatively low percentage ( $\approx 3\%$ ) of unionized free ammonia at  $\text{pH} < 8.0$  (20°C; Boyd, 1990). Additionally, because there was no apparent  $\text{NH}_4^+$ -N removal in the OP treatment series

under significantly higher average pH values (8.5–9.0), it again seems unlikely that the significant RF ammonia removals were achieved via high-pH-mediated volatilisation.

Whilst volatilisation was unlikely to have contributed to  $\text{NH}_4^+$ -N removal, there was evidence to suggest that microbial nitrification was occurring within the RFs. This included: a decreased pH; aerobic *in situ* conditions; relatively low  $\text{BOD}_5$ ; the sequential decline in both  $\text{NH}_4^+$ -N and  $\text{NO}_2^-$ -N; and a corresponding general increase in  $\text{NO}_3^-$ -N—all of which are associated with the occurrence of biological nitrification. Ammonia removal is known to correlate well with a decline in pH due to the consumption of alkalinity and production of  $\text{H}^+$  ions during the biological oxidation of ammonia (Lui, 1997; Heard *et al.*, 2002). Therefore, the slight reduction in pH through the RF treatment train (Figure 3.11) was likely to have been associated with microbial nitrification; although pH remained well above the levels at which it can impact negatively on nitrification ( $\text{pH} < 5.5$ ; Baskaran *et al.*, 1992). The DO data (Figures 3.9–3.10) also suggested that sufficient levels of oxygen were indeed present within the RFs to allow for  $\text{NH}_4^+$ -N removal via nitrification, given that Manthe *et al.* (1988) have stated that rock filter nitrification will generally not occur at DO levels below  $2.0 \text{ mg L}^{-1}$ . Furthermore, the stoichiometric oxygen demand for the biological oxidation of  $1 \text{ mg NH}_4^+$ -N is in the order of  $3.5 \text{ mg O}_2$ —a requirement again satisfied by the observed *in situ* rock filter DO levels.

All of the above factors strongly suggested that the RFs were indeed nitrifying, and were capable of removing  $\text{NH}_4^+$ -N even at very low mass ammonia loadings. In passing, and given the very low loading rates, the specific rates of  $\text{NH}_4^+$ -N removal within the RFs here were thought to be comparable to other reported removal rates for attached-biofilm processes in WSPs. At the median mass influent loading of  $0.73 \text{ g NH}_4^+$ -N  $\text{m}^{-3} \text{ d}^{-1}$ , average removal rates for the RFs were calculated to be in the order of  $2.7 \times 10^{-5} \text{ mg NH}_4^+$ -N  $\text{cm}^{-2} \text{ h}^{-1}$ ; approximately two orders of magnitude lower than the  $1\text{--}3 \times 10^{-3} \text{ mg NH}_4^+$ -N  $\text{cm}^{-2} \text{ h}^{-1}$  removals reported by Baskaran *et al.* (1992) for attached *in situ* algal–bacterial biofilms. Given that the influent  $\text{NH}_4^+$ -N concentrations here were some 100-fold lower here than for the above authors, and considering that microbial nitrification processes were likely to have been substrate-limited as a result, the RFs were thought to

have been relatively good at removing inflowing ammonia—especially under the reduced DO concentrations.

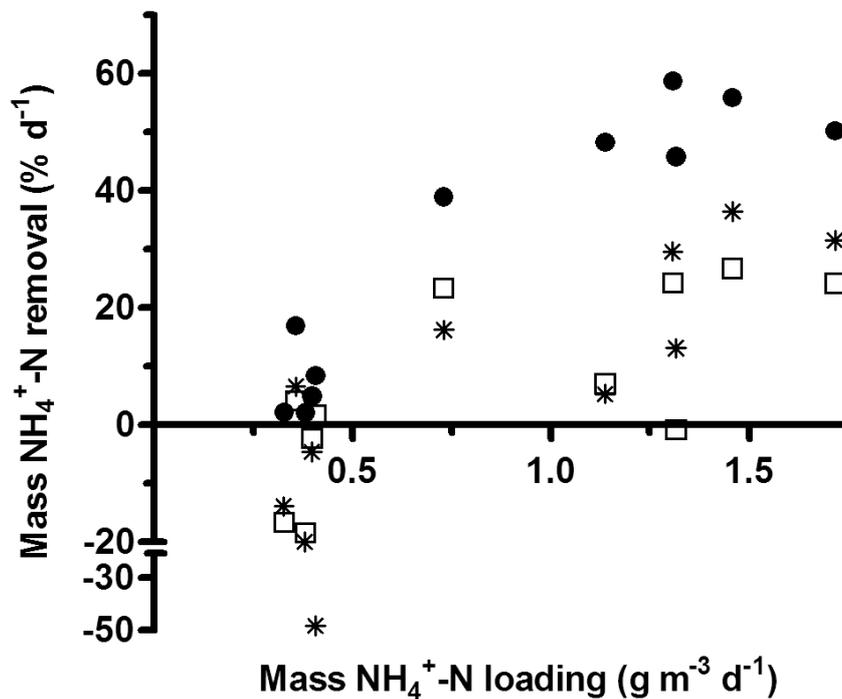
Following this evidence for biological nitrification occurring within the RFs, there was no data to suggest that the RFs were also denitrifying (i.e. no significant decrease in  $\text{NO}_3^-$ -N down the RF treatment series). It is possible that denitrification was not occurring within the RFs a consequence of the elevated DO concentrations not allowing for anaerobic microbial processes, and also the probable lack of a readily available carbon source to drive denitrification. It was also uncertain as to whether the daytime RF ammonia removals were able to be sustained *nocturnally*, given that 24 hour logging data showed the RFs to approach DO levels in the order of  $1\text{ mg L}^{-1}$  or less during the night (refer to Figure 4.7). In the absence of 24 hour  $\text{NH}_4^+$ -N data, the extent of nocturnal ammonia dynamics within the RFs remains unclear.

The highly variable  $\text{NH}_4^+$ -N removals observed within the OP treatment train were considered to have been due, in part, to variable rates of both primary and secondary biomass production. As presented in the previous Section (Plates 3.4–3.6) there were at times very high levels of both primary (algal) and secondary (zooplankton) biomass production within the OP system. Periodic filamentous algal blooms were thought to have been large enough such that during times of prolific growth, they could have been influencing the N dynamics within the relatively small volume pilot ponds. This theory is supported by the findings of Mitchell and Williams (1982), who reported that blooms of the same filamentous green alga (*Cladophora* species) can play a definable role in WSP nutrient dynamics.

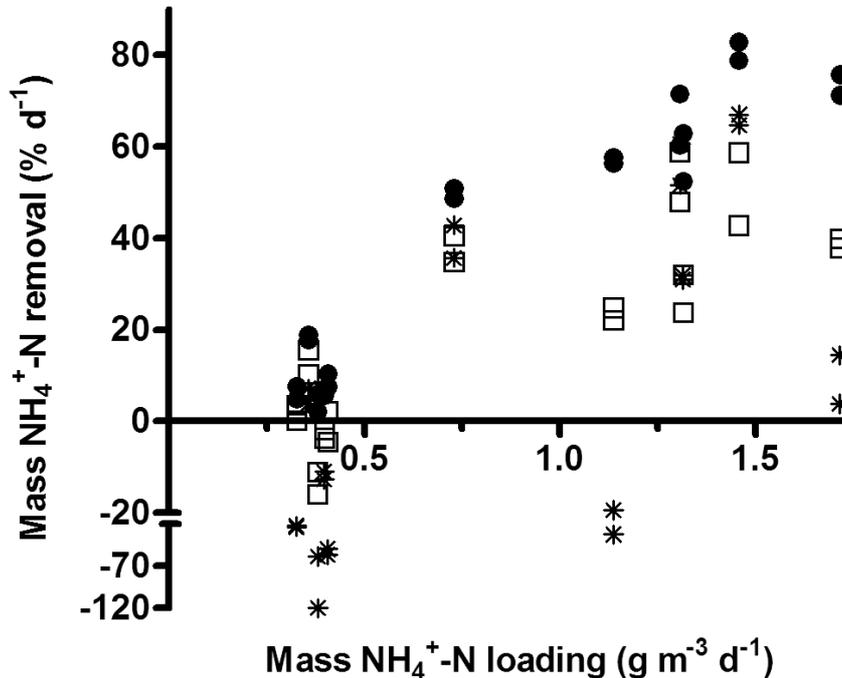
With respect to secondary (zooplankton) biomass production, Cauchie *et al.* (2000)—following the three year monitoring of a full-scale aerated WSP—noted that the lack of significant  $\text{NH}_4^+$ -N removal was likely to have been linked to the occurrence of dense populations of the large grazing zooplankton *Daphnia magna*. The authors indicated that these significant zooplankton blooms were thought to have counteracted the removal of  $\text{NH}_4^+$ -N through efficient feeding and remineralisation of a significant quantity of the organically-bound nitrogen assimilated by suspended algae and bacteria; something

highlighted previously by Ejsmont-Karabin (1983). The authors went on to say that as a result of this feeding activity, *Daphnia* can excrete significant quantities of  $\text{NH}_4^+\text{-N}$  which can then severely counteract any positive ammonia removals coming from algal or microbial biomass assimilation. Cauchie *et al.* (2000, citing Ejsmont-Karabin, 1984) quoted an average excretion rate for *Daphnia* of  $1.6\mu\text{g NH}_4^+\text{-N mg dry weight}^{-1} \text{ h}^{-1}$ , and combining this with the corresponding zooplankton biomass data for OP-3 for example (refer to Section 5.3.2; Figure 5.58), an average 7.6% of the corresponding daily influent  $\text{NH}_4^+\text{-N}$  flux could be attributed to recycling by *Daphnia* species alone; although on a day-to-day basis the figure was within the range of 1–20% of the daily influent  $\text{NH}_4^+\text{-N}$  value according to daily variations in *Daphnia* population density. Based on this information, it is highly likely that the conclusions of Cauchie and co-workers also applied here with respect to the generally poor and largely variable OP treatment ammonia removals. Furthermore, this daily average contribution of 7.6% is also thought to be a conservative estimate because it does not take into account the consortium of other zooplankton species commonly co-inhabiting the OPs; given that *Daphnia* on average comprised only 60% of the total daily zooplankton biomass value.

Whilst the average  $\text{NH}_4^+\text{-N}$  removal efficiencies of each of the three treatments were not as great as those seen for other performance parameters above (i.e.  $\text{BOD}_5$ , SS, chlorophyll *a*), they do suggest that the RFs were significantly more advanced in their ammonia removal capacity than either of the other pilot treatments. This general trend across the Pond 1 as well as Pond 2 and 3 data is again evident on a mass loading versus percentage removal performance basis within Figures 3.39 and 3.40 respectively. In this instance, and due to the relatively low number of daily samples for the  $\text{NH}_4^+\text{-N}$  data set ( $n = 11$ ), the data of Ponds 2 and 3 are combined in Figures 3.40 and 3.42 below.

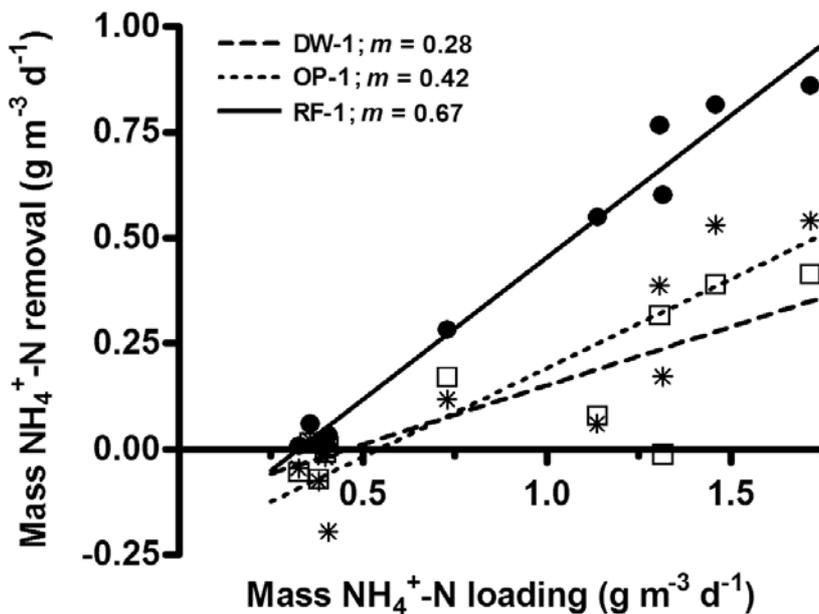


**Figure 3.39.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. percent mass removal (relative to daily loading rate) for Pond 1 data only. Individual data points represent mean performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●). Individual data points show the mean of triplicate determinations.



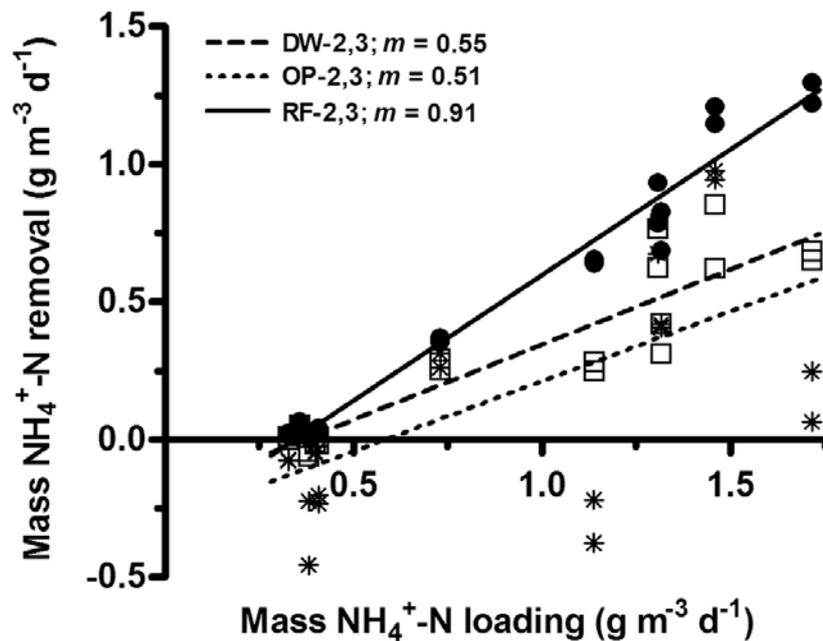
**Figure 3.40.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. percent mass removal (relative to daily loading rate) for the combined data of Ponds 2 and 3. Individual data points represent mean performance data for: Duckweed Ponds 2 and 3 (□); Open Ponds 2 and 3 (\*); and Rock Filters 2 and 3 (●). Individual data points show the mean of triplicate determinations.

In spite of the low sample sizes, the above Figures show again that the RF treatment series demonstrated a greater overall capacity for  $\text{NH}_4^+\text{-N}$  removal than did the DW or OP treatments. Interestingly, and like the respective figures for  $\text{BOD}_5$ , SS and chlorophyll *a* (Figures 3.16–3.17, 3.24–3.25 and 3.31–3.32), there were again visually apparent positive associations between mass loading rate and percentage removal performance in all three treatments for Pond 1 (Figure 3.39) and the combined Pond 2 and 3 data (Figure 3.40). Percentage  $\text{NH}_4^+\text{-N}$  removals were again observed to be generally higher under elevated ammonia mass loads—a consequence of the concentration-dependent removal kinetics as previously discussed.



**Figure 3.41.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data for: Duckweed Pond 1 (□); Open Pond 1 (\*); and Rock Filter 1 (●). Fitted lines represent best-fit lines from simple linear regression analyses, with regression slopes shown alongside the respective figure legends. Individual data points show the mean of triplicate determinations.





**Figure 3.42.** Scatter-plot showing NH<sub>4</sub><sup>+</sup>-N mass loading (pilot plant Influent) vs. total mass removal for the combined data of Ponds 2 and 3. Individual data points represent mean performance data for: Duckweed Ponds 2 and 3 (□); Open Ponds 2 and 3 (\*); and Rock Filters 2 and 3 (●). Fitted lines represent best-fit lines from simple linear regression analyses, with regression slopes shown alongside the respective figure legends. Individual data points show the mean of triplicate determinations.

When the NH<sub>4</sub><sup>+</sup>-N data was plotted on a mass basis only (Figures 3.41 and 3.42), there were again striking relationships between mass loading and the mass of NH<sub>4</sub><sup>+</sup>-N removed for each pilot treatment and across all ponds of each series; something noted for all of the previous water quality parameters. On a mass basis, this direct ‘loading versus removal’ relationship was slightly more apparent for the higher performance RF series compared with the other two treatments. Statistically, there was a very high-level association between mass load and mass NH<sub>4</sub><sup>+</sup>-N removal for the RF-1 data of Figure 3.41 (Pearson  $r = 0.989$ ;  $n = 11$ ;  $p < 0.0001$ ), a slightly less significant relationship for the OP-1 data ( $r = 0.895$ ;  $n = 11$ ;  $p < 0.001$ ) and a less significant relationship again for the DW-1 data of Figure 3.41 ( $r = 0.812$ ;  $n = 11$ ;  $p < 0.01$ ). Similar trends were seen for the combined Pond 2 and Pond 3 NH<sub>4</sub><sup>+</sup>-N data of Figure 3.42, with a highly significant relationship between mass load and mass removal for RFs 2 and 3 ( $r = 0.915$ ;  $n = 22$ ;  $p < 0.0001$ ) as well as for DW Ponds 2 and 3 ( $r = 0.822$ ;  $n = 22$ ;  $p < 0.001$ ), but this time a less significant relationship was apparent for the more variable OP-2 and 3 data ( $r = 0.607$ ;  $n = 22$ ;  $p < 0.01$ ).

Regression analyses of Figures 3.41 and 3.42 yielded identical high-level regression coefficients to the Pearson correlation coefficients above. Unlike trends for the previous water quality parameters, this time the slopes of the fitted regression lines for  $\text{NH}_4^+$ -N data were significantly different between the three treatments for Pond 1 data (ANCOVA;  $F_{(2,27)} = 11.48$ ;  $p < 0.001$ ) as well as for the combined Pond 2 and 3 data ( $F_{(2,60)} = 6.41$ ;  $p = 0.003$ ). The slopes of the regressed lines for the RFs of both Figures 3.41 and 3.42 were significantly greater than those of the DW and OP data ( $p < 0.01$ ), but were equal between the DW and OP treatments ( $p > 0.16$ ). This suggested that whilst the patterns between mass load and mass removal were indeed significantly linear for all treatments, there was an apparent breakdown in the interrelationship of  $\text{NH}_4^+$ -N removal processes between the three treatments. In this instance, the difference in slopes of the respective regression lines from Figures 3.41 and 3.42 reflected the differing capacity of each system for  $\text{NH}_4^+$ -N removal based on their variable *biological* nitrogen removal capabilities, because unlike  $\text{BOD}_5$ , SS and chlorophyll *a* above,  $\text{NH}_4^+$ -N removal is not so heavily reliant on *physical* processes. The significantly greater  $\text{NH}_4^+$ -N removal slope for the RFs over the other two treatments effectively meant that they were removing proportionally more  $\text{NH}_4^+$ -N at any given mass loading rate (given that a theoretical slope of 1.0 would represent 100% ammonia removal). This observation was further supported by the earlier performance data of Figure 3.38 and Table 3.8.

As highlighted earlier (Section 3.3.5), published data regarding the ‘loading versus removal’ performance of both duckweed ponds and rock filters is lacking, and so the above performance trends cannot be directly compared to those of other systems. Interestingly, it seems that the ability of the current RFs to remove inflowing  $\text{NH}_4^+$ -N was apparently limited at or below  $\approx 0.5 \text{ g NH}_4^+\text{-N m}^{-3} \text{ d}^{-1}$  (Figures 3.41 and 3.42). This indicated that under very low-level ammonia loadings, the biological capacity of the RFs to oxidise inflowing  $\text{NH}_4^+$ -N became effectively ‘rate-limited’ by the presumably shallow concentration gradient. It is anticipated then that with respect to the Bolivar WSPs, no significant  $\text{NH}_4^+$ -N removals would be expected at or below ammonia loading rates of  $\leq 0.5 \text{ g m}^{-3} \text{ d}^{-1}$ . At the same time, the current data also suggests that  $\text{NH}_4^+$ -N production would not be expected within a Bolivar rock filter; something that would be

highly beneficial in terms of satisfying discharge water quality guidelines and also reducing the chlorine demand during the disinfection of reclaimed Bolivar effluent.

As shown in the above figures, net ammonia gains were actually observed approximately 25% of the time in DW ponds and >40% of the time within the OP system, whereas no net increase in  $\text{NH}_4^+\text{-N}$  was ever observed in the RF effluent. This observation of significant  $\text{NH}_4^+\text{-N}$  removal in the current RFs is in contrast with other findings. High levels of  $\text{NH}_4^+\text{-N}$  in rock filter effluents are generally reported as being a significant performance problem associated with the technology, with effluent concentrations often exceeding that of the influent (Hirse Korn, 1974; Swanson and Williamson, 1980; Middlebrooks, 1988; Mara and Johnson, 2006). Mara *et al.* (2001) observed a 5% net increase in rock filter effluent  $\text{NH}_4^+\text{-N}$  concentrations when operated at a similar HLR of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  but a 25-fold greater ammonia mass loading rate of  $\approx 18\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$  compared with the current RFs. In the case of Mara and co-workers, it was suggested that this overall ammonia increase was possibly due to anaerobic degradation of accumulated  $\text{BOD}_5$ , SS and chlorophyll *a* within the RFs at the dissolved oxygen levels of  $0.2\text{mg L}^{-1}$ , and also due to the inhibition of other ammonia removal mechanisms such as volatilisation, assimilation and microbial nitrification. Similarly to Mara *et al.* (2001), pioneering work by Hirsekorn (1974) also realised net ammonia gains following rock filter treatment under similarly low ammonia mass loadings to the current RFs ( $\approx 1.6\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$ ). In this case, the negative  $\text{NH}_4^+\text{-N}$  removals seen by Hirsekorn (1974) were thought to have been a consequence of the very low effluent DO concentrations ( $< 0.5\text{mg L}^{-1}$ ) and much higher organic strength of their influent wastewater.

There have, however, been a limited number of reports for zero or indeed small-scale positive  $\text{NH}_4^+\text{-N}$  removals in rock filters. Saidam *et al.* (1995) showed no evidence of increased  $\text{NH}_4^+\text{-N}$  in their rock filter effluents (in spite of 3-fold reductions in DO concentration during filter passage). Strang and Wareham (2005) observed a small amount of  $\text{NH}_4^+\text{-N}$  removal in full-scale rock filters operated in New Zealand, recording some 5–8% removals at somewhat higher influent concentrations of  $8\text{--}20\text{mg L}^{-1}$ . Interestingly, no significant  $\text{NH}_4^+\text{-N}$  removal was recorded for their rock filters when

loaded at lower  $\text{NH}_4^+\text{-N}$  concentrations; with the authors suggesting that this was a result of the majority of  $\text{NH}_4^+\text{-N}$  having already been removed up-stream of the rock filters, thus providing too low an influent concentration gradient to see any real change following the relatively low-treatment-rate rock filter process—a similar conclusion to that made for this research. Finally, Johnson *et al.* (2007) observed a zero percentage  $\text{NH}_4^+\text{-N}$  removal in their smaller pilot-scale ( $1\text{m}^3$ ) rock filters when loaded at  $6\text{--}9\text{mg NH}_4^+\text{-N L}^{-1}$  ( $2.7\text{--}3.6\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$ ). In conclusion, whilst there have been a limited number of reports indicating  $\text{NH}_4^+\text{-N}$  removal during rock filtration, rock filters are generally not designed nor installed for achieving nitrogen removal, and as such, any degree of  $\text{NH}_4^+\text{-N}$  removal whatsoever would be considered an added benefit of what is primarily a physical solids removal process.

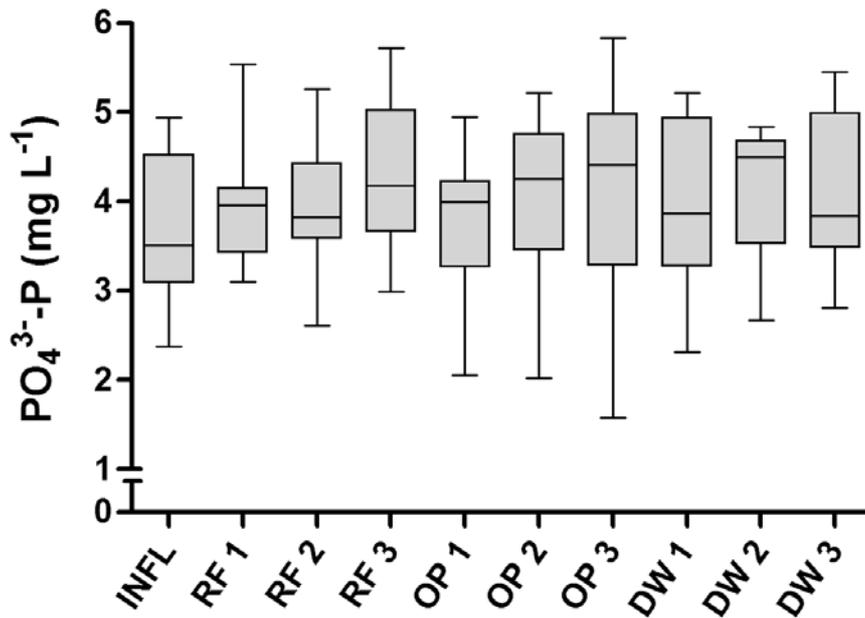
Duckweed pond systems generally achieve poor N removals without aeration (Reed *et al.*, 1995) or continuous plant biomass harvesting under optimal growth conditions (Körner *et al.*, 2003). With respect to the DW treatment reported here, the generally poor capacity for  $\text{NH}_4^+\text{-N}$  removal was thought to be a factor of both the high wastewater quality as well as the pilot plant operating conditions. Firstly, the absence of continuous plant biomass harvesting (unlike the conditions for most duckweed pond research) was thought to have significantly reduced the capacity of these DW Ponds for  $\text{NH}_4^+\text{-N}$  removal. Secondly, the relatively deep hydraulic depth (1m) of the current DW Ponds combined with the poorly developed and relatively small ( $\approx 1\text{--}4\text{cm}$ ) plant root stock, meant that nutrient uptake and mass-transfer potential would have been severely restricted as a result of the very low plant ‘biomass-to-volume’ ratio. This had the effect of significantly reducing the degree of physical contact between the duckweed plant mat and the aqueous nutrients—limiting their uptake potential (see Section 3.3.5 for the original discussion of this). Third, the relatively short HRT combined with a very low  $\text{NH}_4^+\text{-N}$  mass loading rate no doubt created a shallow concentration gradient within the pilot DW Ponds; something which gave the system a very low affinity for dissolved ammonia. One conclusion which can safely be drawn with respect to the Bolivar WSPs, is that undesirable duckweed mat die-off resulting from high and toxic levels of free ammonia would not be of concern should the system be implemented (based on the ammonia toxicity data of Caicedo *et al.*, 2000). Similarly, and although duckweed is

recognised to preferentially use  $\text{NH}_4^+\text{-N}$  as the preferred nitrogen source for growth (over  $\text{NO}_3^-\text{-N}$  for example; Porath and Pollock, 1982; Monselise and Kost, 1993), results from this work suggest that there should be no problems in maintaining a full duckweed surface cover even at such low Bolivar  $\text{NH}_4^+\text{-N}$  levels.

In conclusion, it should be re-stated that these pilot upgrade methodologies were investigated primarily to assess their capacities for algal solids removal, such that nutrient removal was not anticipated as an initial performance outcome. Given the already very low levels of dissolved nutrients in the Bolivar WSP effluent, it is assumed that any nutrient removals greater than zero efficiency would simply be considered as ‘fringe benefits’ of such pond upgrade systems. Having said this, results did show that the Rock Filters were significantly more capable of removing loaded  $\text{NH}_4^+\text{-N}$  than the other treatments, and also that the Duckweed Ponds were no worse at  $\text{NH}_4^+\text{-N}$  removal than the non-interventional Open Pond treatment; indicating that there was apparently no significant remineralisation of accumulated organic nitrogen (e.g. from decaying plant biomass) back into the system. Following the above analyses, the overall performance ranking for the three treatments with respect to their capacity for  $\text{NH}_4^+\text{-N}$  removal places the RFs 1<sup>st</sup>, the DW Ponds 2<sup>nd</sup>, and the OPs 3<sup>rd</sup> overall in terms of  $\text{NH}_4^+\text{-N}$  removal rate down the pond series, absolute treatment efficiency, and also performance reliability—a similar ranking order to that of above performance parameter assessments.

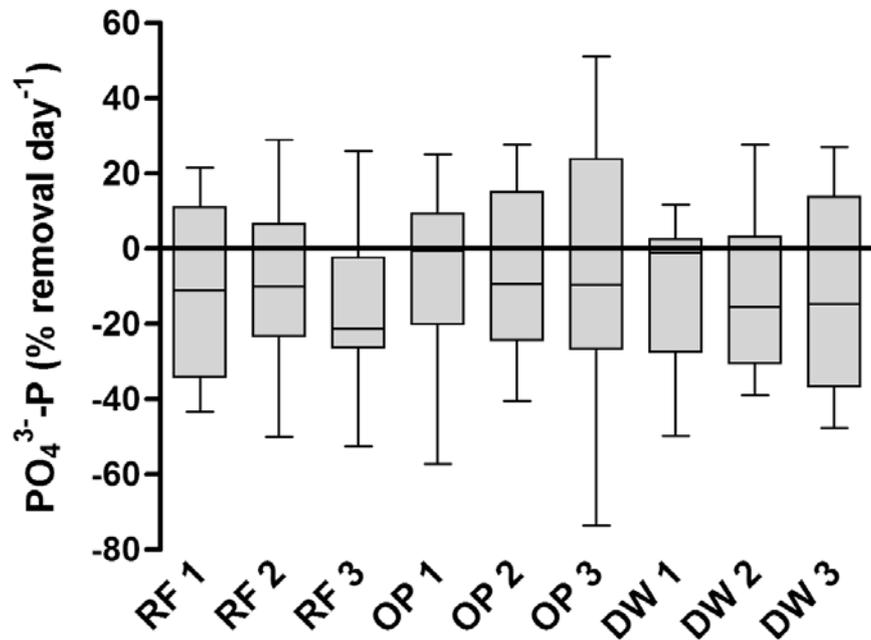
### **3.3.7.2 Soluble reactive orthophosphate removal**

Data from performance monitoring of soluble reactive orthophosphate ( $\text{PO}_4^{3-}\text{-P}$ ) levels is shown below in Figure 3.43.



**Figure 3.43.** Soluble reactive orthophosphate-phosphorous box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

As can be seen in Figure 3.43, influent  $\text{PO}_4^{3-}\text{-P}$  levels were generally in the order of 3–4  $\text{mg L}^{-1}$ , with a median influent concentration of 3.5  $\text{mg PO}_4^{3-}\text{-P L}^{-1}$  and a corresponding average mass influent loading of approximately 2.6  $\text{g PO}_4^{3-}\text{-P m}^{-3} \text{ d}^{-1}$ . Visual analysis of Figure 3.43 quickly shows that influent levels of  $\text{PO}_4^{3-}\text{-P}$  remained virtually unchanged through the pond series within the respective pilot treatments. There were no significant statistical differences between average influent  $\text{PO}_4^{3-}\text{-P}$  levels and those in any of the experimental treatment ponds (1-way ANOVA;  $F_{(9,100)} = 0.282$ ;  $p = 0.98$ ), such that there were no significant  $\text{PO}_4^{3-}\text{-P}$  removals and also no inter-treatment performance differences between any combination of the DW, OP and RF treatments.



**Figure 3.44.** Box-plots showing percentage orthophosphate-phosphorous removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n = 11$  for all plots).

On a percentage basis, all ponds across all treatments yielded frequent negative  $\text{PO}_4^{3-}\text{-P}$  removals (Figure 3.44). Long term mean percentage  $\text{PO}_4^{3-}\text{-P}$  removals for Pond 1 data of the three treatments were approximately  $-10$ ,  $-5$  and  $-9\%$  for DW, OP and RF systems respectively, and for Pond 3 data,  $-12$ ,  $-10$  and  $-17\%$  for the respective DW, OP and RF systems. Although long-term average trends for  $\text{PO}_4^{3-}\text{-P}$  removal were both negative and highly variable across all treatments, when compared to a theoretical zero mean removal rate, RF-3 was actually delivering a significant negative removal performance ( $t_{0.05(2)10} = 0.27$ ;  $p = 0.024$ ), whereas all other negative  $\text{PO}_4^{3-}\text{-P}$  removals remained effectively equivalent to ‘zero’ (Table 3.9) Whilst this was the case, when this slight negative removal efficiency was combined with the non-significant difference between RF-3 effluent and pilot plant influent  $\text{PO}_4^{3-}\text{-P}$  levels above, it was concluded that there was no real change in  $\text{PO}_4^{3-}\text{-P}$  concentration down-the-line within the RFs. Therefore, and since there were no performance trends of significant interest with respect to the  $\text{PO}_4^{3-}\text{-P}$  data, no further breakdown of treatment performance (i.e. mass-basis performance) is provided. A brief discussion of some factors relating to  $\text{PO}_4^{3-}\text{-P}$  dynamics in the DW, OP and RF systems is, however, offered below.

**Table 3.9.** Summary of orthophosphate-phosphorous performance data for all three treatments for Pond 1 and 3 only.

PO <sub>4</sub> <sup>3-</sup> -P performance parameter	Pilot treatment pond					
	DW-1	DW-3	OP-1	OP-3	RF-1	RF-3
Median influent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	3.5	3.5	3.5	3.5	3.5	3.5
Mean influent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	3.7	3.7	3.7	3.7	3.7	3.7
Median effluent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	3.9	3.8	4.0	4.4	4.0	4.2
Mean effluent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup>	4.1	4.1	3.9	4.0	4.0	4.3
Median daily PO <sub>4</sub> <sup>3-</sup> -P removal (% day <sup>-1</sup> )	-1.0	-15	-1.0	-9.5	-11	-21
Mean daily PO <sub>4</sub> <sup>3-</sup> -P removal (% day <sup>-1</sup> ) <sup>‡</sup>	-10	-12	-5.1	-9.7	-9.0	-17
Long-term CV for PO <sub>4</sub> <sup>3-</sup> -P removal (%)	188	209	430	394	238	124

<sup>†</sup> Effluent PO<sub>4</sub><sup>3-</sup>-P concentration was tested relative to mean pilot plant influent PO<sub>4</sub><sup>3-</sup>-P concentration (1-way ANOVA)

<sup>‡</sup> Average PO<sub>4</sub><sup>3-</sup>-P removal % tested against a theoretical 'zero' daily mean (one sample *t*-test)

\* Shading intensity shows significance level: *p* > 0.05 (no shading); *p* < 0.05 (light)

While phosphorous is generally a minor constituent of water, it is nevertheless an essential inorganic micronutrient for biological growth; so much so that its availability often governs the productivity of a given waterway (Boyd, 1990). Common mechanisms for phosphate removal in typical WSP environments include: biological assimilation; high-pH-mediated phosphate precipitation; and co-precipitation with carbonates (Surumpalli *et al.*, 1995; Mara *et al.*, 2001; Dodds, 2003). Co-precipitation of phosphates with calcium is known to be induced by high pH (Hemens and Mason, 1968), yet in spite of regularly recording daytime OP treatment pH values in excess of 9.0 (see Figure 3.11), significant PO<sub>4</sub><sup>3-</sup>-P removals were not observed.

Similarly, and in spite of a long-standing recognition for the potential of duckweed to assimilate wastewater-derived P (Harvey and Fox, 1973; Sutton and Ornes, 1975), there was no observed PO<sub>4</sub><sup>3-</sup>-P removal in the current DW Ponds. Non-removals for these DW Ponds was thought to have been a result of a number of factors, namely: a suppressed pH; the relatively deep pond depth and low HRT; and most notably because of the absence of continuous biomass harvesting. Indeed Zirschky and Reed (1988)—following a compressive review of duckweed in wastewater treatment—concluded that frequent and continuous biomass harvesting is the only way of guaranteeing permanent nutrient removals from a duckweed pond system. It should be re-stated that the operational regime of no biomass harvesting was chosen based on the assessment of the technology purely as an algal control mechanism. Since regular and ongoing duckweed



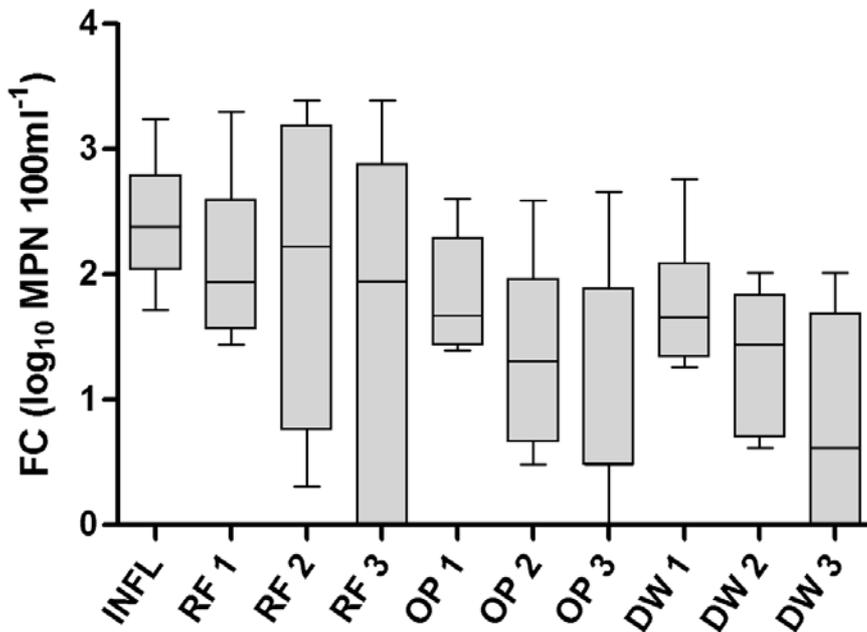
harvesting is the single biggest capital and operational demand associated with the technology, and since the economic viability of recovering these outlaid costs through the sale of harvested duckweed plant biomass (either for animal feed or for biogas production) has been brought into question (Ward, 1987), the efficiency of duckweed for upgrading the Bolivar WSP effluent was specifically investigated *without* harvesting.

With respect to the RF data, no significant overall decrease (biological assimilation) or increase (anaerobic remineralisation) in the levels of  $\text{PO}_4^{3-}\text{-P}$  was observed within the RF treatment train. This suggested that whilst phosphorous is not likely to be removed during RF treatment, at the same time there would be no real possibility of  $\text{PO}_4^{3-}\text{-P}$  levels significantly increasing as a result of rock filter anoxia in the current Bolivar setting. Hirsekorn (1974) also noted no significant change in phosphorous levels following rock filter treatment, instead the author proposed a possible 'equilibrium state' of rock filter operation, with an apparent balance between the rates of biological assimilation and organic  $\text{PO}_4^{3-}\text{-P}$  remineralisation within the rock filter.

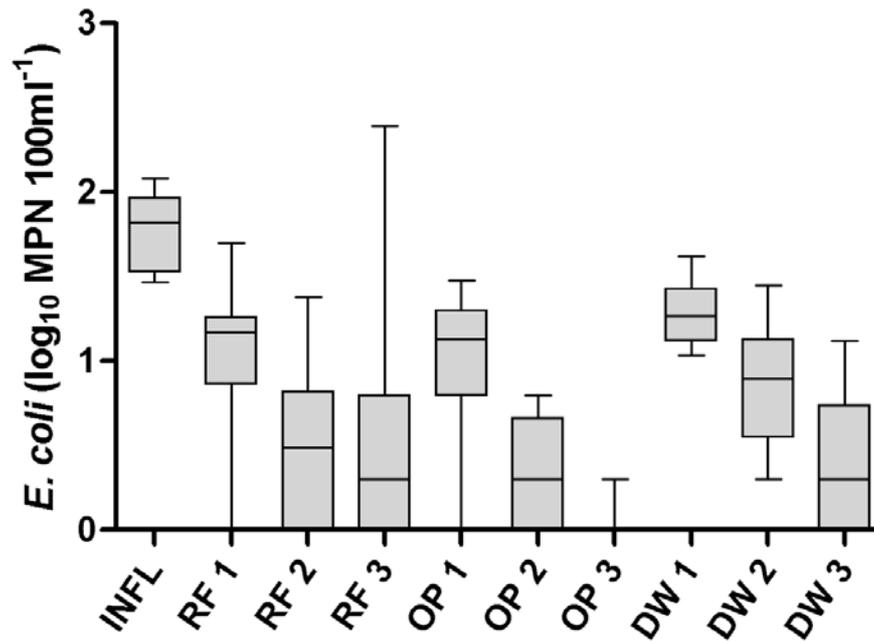
It should be re-emphasized that nutrient removal was not expected to be the primary treatment outcome of these WSP upgrade systems. Given the already low levels of dissolved nutrients within the Bolivar ponds, any significant non-zero nutrient removals would be considered as secondary to the primary goal of solids removal. Based on results presented here for both the  $\text{NH}_4^+\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  data, it would not be expected that passage through either a duckweed pond or rock filter upgrade would impart any significant adverse impacts upon resident algal populations from a nutritional standpoint. In both cases, the combined levels of  $\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  following DW or RF treatment would be expected to be high enough not to restrict the levels of algal productivity within the Bolivar WSPs. The overall performance ranking for the three treatments with respect to their capacity for  $\text{PO}_4^{3-}\text{-P}$  removal places all treatments on an equal footing in terms of:  $\text{PO}_4^{3-}\text{-P}$  removal rate down each pond series; absolute treatment efficiency; and also performance reliability; although the RF train did demonstrate a slightly 'more negative' treatment efficiency than the other two treatments.

### 3.3.8 Wastewater treatment performance: indicator organism removals

While there are numerous so-called ‘indicator organisms’ used as surrogate microbial pathogens in water quality monitoring, the monitoring of thermo-tolerant faecal coliforms (FC) and *E. coli* was adopted here following a general recognition—particularly for *E. coli*—of these organisms being the superior indicators of faecal pollution and wastewater treatment efficacy (Yanko, 2000). Data from performance monitoring of both FC and *E. coli* density is shown in Figures 3.45 and 3.46 respectively. As described earlier (Section 2.3), all average indicator organism densities are reported here as arithmetic mean values in accordance with the recommendations of Haas (1996).



**Figure 3.45.** Faecal coliform box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.



**Figure 3.46.** *E. coli* box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Duckweed Ponds 1, 2, 3 (DW-1, DW-2, DW-3).

As shown in Figure 3.45, pilot plant influent FC densities were consistently very low, with an average of approximately  $2.4\text{-log}_{10}$  ( $\pm 0.4$ ) and a maximum of  $3.2\text{-log}_{10}$  MPN  $100\text{ml}^{-1}$ . Overall Pond 1 average FC removals were similar, with a less than  $1\text{-log}_{10}$  removal observed across all three treatments. Individually, average removals were in the order of  $0.6\text{-log}_{10}$  units for DW-1,  $0.5\text{-log}_{10}$  for OP-1, and  $0.3\text{-log}_{10}$  for RF-1. With respect to Pond 3 data, mean FC removals down the pond series were in the order of  $1.5\text{-log}_{10}$  for DW-3,  $1.2\text{-log}_{10}$  for OP-3, and  $0.8\text{-log}_{10}$  for RF-3. Statistically, however, the only significant FC removals were seen for DW-3 (1-way ANOVA;  $F_{(9,91)} = 3.32$ ;  $p < 0.01$ ) and OP-3 ( $p < 0.05$ ), with no significant removal for RF-3 ( $p > 0.05$ ). There were also no significant differences between the FC densities of any pond for any of the three treatments ( $p > 0.05$ ).

With respect to *E. coli* performance data (Figure 3.46), influent counts were again very low, with an average of  $1.8\text{-log}_{10}$  ( $\pm 0.2$ ) and a maximum of  $2.1\text{-log}_{10}$  MPN  $100\text{ml}^{-1}$ . Pond 1 average  $\text{log}_{10}$  MPN *E. coli* removals were again similar for all treatment series, at  $0.7\text{-log}_{10}$  for DW-1,  $0.8\text{-log}_{10}$  for OP-1, and  $0.5\text{-log}_{10}$  for RF-1. In respect of Pond 3 data, mean *E. coli* removals relative to daily influent levels were in the order of  $1.3\text{-log}_{10}$

units for DW-3, 1.8- $\log_{10}$  for OP-3, and 1.4- $\log_{10}$  for RF-3. Statistically, the 0.5–0.7- $\log_{10}$  unit Pond 1 *E. coli* removals were significant for all three treatments (1-way ANOVA;  $F_{(9,91)} = 11.77$ ;  $p < 0.01$ ). Similarly, for the Pond 3 performance data, the  $>1$ - $\log_{10}$  *E. coli* removals were again highly significant for all three treatments ( $p \leq 0.01$ ). Indicator organism performance data is summarised in Table 3.10 below.

**Table 3.10.** Summary of indicator organism removals across all pilot plant treatments for Pond 1 and 3 data only.

Microbial performance parameter	Pilot treatment pond					
	DW-1	DW-3	OP-1	OP-3	RF-1	RF-3
Mean influent FC ( $\log_{10}$ MPN 100ml <sup>-1</sup> )	2.4	2.4	2.4	2.4	2.4	2.4
Mean effluent FC ( $\log_{10}$ MPN 100ml <sup>-1</sup> ) <sup>†*</sup>	1.8	0.9	1.9	1.2	2.1	1.6
FC removal ( $\log_{10}$ MPN 100ml <sup>-1</sup> d <sup>-1</sup> )	0.6	1.5	0.5	1.2	0.3	0.8
Mean influent <i>E. coli</i> ( $\log_{10}$ MPN 100ml <sup>-1</sup> )	1.8	1.8	1.8	1.8	1.8	1.8
Mean effluent <i>E. coli</i> ( $\log_{10}$ MPN 100ml <sup>-1</sup> ) <sup>†*</sup>	1.1	0.5	1.0	0.0	1.3	0.4
<i>E. coli</i> removal ( $\log_{10}$ MPN 100ml <sup>-1</sup> d <sup>-1</sup> )	0.7	1.3	0.8	1.8	0.5	1.4

<sup>†</sup> Effluent organismal density was tested relative to mean pilot plant influent organism density (1-way ANOVA)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium);  $p < 0.001$  (black)

As introduced in Chapter 1 (Section 1.2.1), there are numerous factors that have been associated with microbial disinfection in WSPs, including: microbiological attack (lytic bacteria and phage); predation (protozoan and metazoan); nutrient stress and algal competition; algal population structure; photosynthetically elevated pH; hydraulic retention time, wind action and the subsequent sedimentation rate; UV degradation; humic substances together with high dissolved oxygen leading to photo-oxidation; and also from an elevated temperature. Whilst the relative contributions of each of these factors toward overall pathogen die-off in WSPs remains under debate, it has been suggested that the sunlight-mediated factors (i.e. UV disinfection, photo-oxidation and photosynthetic pH and DO shifts) are the primary regulators of disinfection (Pearson *et al.*, 1987).

Performance data from the above Figures and Table shows that although the influent levels of both FC and *E. coli* were of a very low order of magnitude in the context of WSPs in general, significant  $\approx 1.5$ - $\log_{10}$  FC removals were able to be achieved for both the DW and OP treatment, as well as a similar magnitude  $\approx 1.5$ - $\log_{10}$  removal across all three treatments for *E. coli*. It was anticipated that the OP treatment would display an

advanced disinfection capacity over the other two treatments, given that there was considerably more scope for the primary light-mediated mechanisms of pathogen die-off in this exposed treatment system. In spite of the obvious absence of incident sunlight in both the DW and RF treatments, similar magnitude significant FC removals were seen for both the DW and OP treatment series, as well as having observed equivalent *E. coli* removals across all three treatments (Table 3.10). This implies that the so-called “dark removal” processes (i.e. biological attack and/or antagonistic interactions, protozoan and zooplankton grazing, biofilm attachment, flocculation and sedimentation; Ehrlich, 1966; Starkweather *et al.*, 1979; Moeller and Calkins, 1980; Pedrós-Alió and Brock, 1983; Seaman *et al.*, 1986; Boon and Shiel, 1990; Curtis *et al.*, 1992; Brücker *et al.*, 1998; Maynard *et al.*, 1999; Kim *et al.*, 2000; Davies-Colley *et al.*, 2003; Stevik *et al.*, 2004; Stott and Tanner, 2005; Tanaka *et al.*, 2005) were largely responsible for organism removals in both the DW and RF series, and also that the total disinfection efficiency of these dark processes was—at least under low microbial densities—equivalent to that of the light-mediated ones.

In comparing these results to the work of others, FC removals for the current DW Ponds were similar to those reported by Falabi *et al.* (2002) who observed a mean  $< 0.5\text{-log}_{10}$  FC reduction in their outdoor duckweed pond operated under a significantly lower HLR to those here ( $0.1\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ), a similar hydraulic depth (0.9m), but significantly greater influent FC density ( $10^6$  MPN  $100\text{ml}^{-1}$ ). Baldizón *et al.* (2002) also recorded similarly low FC removals of  $< 1\text{-log}_{10}$  units during the operation of a full-scale duckweed pond system under an HRT in the order of 5 days but again with a significantly higher influent FC density ( $10^6$  MPN  $100\text{ml}^{-1}$ ). van der steen *et al.* (2000) recorded in the order of  $2\text{-log}_{10}$  FC removals in their integrated duckweed pond system once again at significantly higher influent FC densities ( $10^5$  MPN  $100\text{ml}^{-1}$ ). Interestingly, van der steen and co-workers observed a slightly better ( $\approx 0.5\text{-log}_{10}$ ) FC removal efficiency in their parallel algal-based pond system—a result comparable to that recorded for the OP treatment here. Zimmo *et al.* (2002) operated parallel algal- and duckweed-based pond trains of a similar scale to those of the current research, and observed somewhat higher ( $\approx 4\text{-log}_{10}$  unit) FC removals for their open algal ponds compared with only  $2\text{-log}_{10}$  removals for the duckweed pond train at higher influent organism densities of  $10^4$  MPN  $\text{ml}^{-1}$ .

Karpiscak *et al.* (1996), following the six month monitoring of a large pilot-scale (700m<sup>3</sup>) duckweed pond system, recorded similarly low FC removal rates of  $\approx 0.5\text{-log}_{10}$  units. These low removals were observed in spite of being fed a significantly greater influent FC density (10<sup>6</sup> MPN 100ml<sup>-1</sup>) and a much lower HLR (0.12m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>). Interestingly, Karpiscak and co-workers did report greater removal efficiencies of 1- $\log_{10}$  units for the larger protozoan pathogens *Giardia* and *Cryptosporidium*; with the authors suggesting that pathogen removal in duckweed ponds is achieved primarily by physical sedimentation and hence removal efficiency is likely to be related to the size of the organism in question. Contrastingly to all of the above reports, Dewedar and Bahgat (1995) recorded no FC decay in a large wastewater reactor (15m<sup>3</sup>) over a period of five days when under a duckweed (*Lemna*) mat, in comparison with complete FC decay in their uncovered control. These results were unlike those observed during this research, with comparative removals seen for FC and *E. coli* in both the duckweed-covered and exposed 'OP' treatments.

With respect to rock filter performance comparisons, others have also reported commonly low-order removals for both FC and *E. coli* during rock filter treatment. Saidam *et al.* (1995) achieved equally low less-than 1- $\log_{10}$  reductions in total FC densities following rock filter passage for their large pilot-scale filters when operated at a two-fold lower HLR but a significantly greater influent FC density (10<sup>4</sup>–10<sup>5</sup> MPN 100ml<sup>-1</sup>). Mara *et al.* (2001) also achieved very low magnitude  $\approx 0.2\text{-log}_{10}$  FC removals in their pilot-scale rock filters under a comparable hydraulic loading (1.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) and similarly low-magnitude influent FC density (10<sup>3</sup> MPN 100ml<sup>-1</sup>). Tanner *et al.* (2005) again reported similarly low order *E. coli* removals (< 1- $\log_{10}$  units) in their pilot-scale rock filters when operated on the back end of both an open WSP and a planted wetland system. Finally, von Sperling and de Andrada (2006) reported a low *E. coli* removal efficiency of < 0.5- $\log_{10}$  units in their pilot-scale rock filters in spite of being fed influent wastewater of a significantly higher microbial density (10<sup>8</sup> MPN *E. coli* 100ml<sup>-1</sup>).

The data reported here, in addition to that of other similar studies, has indicated that effective pathogen removal would not be a predicted performance outcome of rock filter treatment. The relatively low-order FC and *E. coli* removal efficiencies in comparison with that achieved by a classical open WSP system suggest that the so-called *dark*

*removal* processes for pathogen removal in WSPs are significantly less efficient than light-mediated processes. Regardless of absolute treatment efficiency, all pilot upgrade treatments were producing a final effluent of sufficient quality to satisfy both the local and also World Health Organisation's minimum requirement for unrestricted irrigation of less than  $3\text{-log}_{10}$  MPN *E. coli*  $100\text{ml}^{-1}$  (WHO, 1989). Additionally, all pilot upgrade systems were also, on average, producing a 'Class A' final effluent in terms of microbiological quality (i.e. median  $< 1\text{-log}_{10}$  MPN *E. coli*  $100\text{ml}^{-1}$ ) suitable for unrestricted irrigation reuse according to local water quality guidelines (SAEPA, 1999).

As was the case for dissolved nutrients (Section 3.3.7), it must be remembered that the chosen WSP upgrade methodologies were not implemented to achieve pathogen removal nor improve the general microbiological quality of the final Bolivar effluent. Low efficiency indicator organism removals were somewhat anticipated for the DW and RF treatment systems, given that there was a fundamental exclusion of the primary ingredient for pathogen die-off—sunlight. Because of this, it was expected that there would be reduced scope for indicator organism removal, such that any additional removal performance above 'zero' reductions would be considered an added bonus of the overall Bolivar effluent upgrade process. Additionally, and following passage through the most expansive WSP network in the Southern Hemisphere, the microbiological quality of the final Bolivar effluent was already at such high levels that high magnitude organism removals ( $\geq 3\text{-log}_{10}$  units) were unattainable. Further to this, the heterotrophic microbial density in the pilot plant (final Bolivar effluent) was found to be in the order of  $7 \times 10^4$  CFU  $\text{ml}^{-1}$  (based on one-off heterotrophic plate counts); highlighting again the very low-level microbial activity and highly refined general nature of the Bolivar wastewater. Following assessment of indicator organism removal capacity, the final treatment performance ranking for the pilot upgrade methodologies places the OP and DW treatment series equal first, and the RFs 2<sup>nd</sup> in terms of absolute attenuation efficiency and also performance reliability; although the RF performance was not far behind that of the OP and DW treatments.

### **3.4 General research findings and chapter summary**

This chapter was concerned with investigating the relative treatment efficacy of a duckweed surface coverage and rock filtration in comparison to a non-interventional Open Pond ‘control’ treatment for the upgrading of maturation WSP effluent. Whilst there has been much prior research on both duckweed and rock filtration for upgrading WSPs, this work has presented the first known direct comparison of a RF and DW system for the upgrading of final maturation WSP effluent. Although Mara *et al.* (2001) used water lettuce (*Pistia* species) and rock filters in the same experimental study, both were fed with differing influent wastewaters, hence there was no way of making *direct* performance comparisons between the two upgrade methodologies in that instance. Similarly, work by Neder *et al.* (2002) assessed the algal removal efficacy of rock filtration and aquatic macrophytes side-by-side; however, the test macrophyte species was the exotic water hyacinth and there was also no attempt made to quantify algal biomass dynamics within their pilot systems (e.g. by monitoring chlorophyll *a* or algal cell density).

Early chapter results from hydraulic tracer analyses revealed each pilot pond treatment to be operating under a similarly well-mixed flow pattern, such that any subsequent differences in between-treatment performance efficiency were considered to have occurred independently of hydraulic operation. Significant reduction in DO levels in both the DW and RF treatment ponds compared with both the influent and also the OP treatment, with DO concentrations in the RFs being similar to levels in the DW Ponds by the end of pilot plant passage. Conversely, pH was significantly elevated within the OP treatment series compared with that in the DW and RF pond systems, but once again was effectively similar for both the DW and RF trains.

Pilot plant performance data presented during this chapter has shown an overwhelming trend for a generally enhanced treatment performance by rock filtration over a number of water quality parameters. Not only was the absolute treatment efficiency significantly greater for the RFs across a number of water quality parameters, but the reliability of treatment delivery was also significantly enhanced over both the DW and OP series—something evidenced in the corresponding performance CVs. In spite of reduced DO levels, the RF treatment series displayed a significantly more advanced BOD<sub>5</sub> removal



capacity than both the DW and OP treatments. Some factors relating to the high and often variable wastewater BOD<sub>5</sub> within the OP series were also discussed. Similar performance trends were observed also for both SS and chlorophyll *a* removals, with the RFs generally out-performing both the DW and OP treatments as well as displaying a higher 'rate' of removal down the pond series compared with both the DW and OP systems. Algal biomass removal was shown to be significantly more advanced down the pond series within the RFs over both the DW and OP systems; although the overall three-pond removals were similar (this will be discussed further in Chapter 10). These results suggest a greater capacity for algal solids removal during rock filtration compared with the other upgrade systems.

In the absence of plant harvesting, the duckweed surface mat attenuated in excess of 99% of incident light such that algal growth in the pilot ponds would have been greatly suppressed. In spite of this, sometimes highly variable chlorophyll *a* removals were recorded for the DW and OP treatment ponds. Variability in chlorophyll *a* measurements from confounding factors like duckweed plant tissue and high density zooplankton populations made absolute quantitation of free suspended algal biomass difficult in the DW and OP treatment series in particular. Methods for overcoming this problem were suggested, and could involve the analysis of both coarsely filtered and unfiltered wastewater samples as well as direct microscopic algal cell counts. Monitoring of pilot plant nutrient dynamics showed that the RFs were again significantly more capable of removing infiltrating ammonia than the DW or OP series; however, no significant phosphorous removals were evident in any of the three treatments. Indicator organism data revealed that this time the OP and DW systems were equally effective at microbial disinfection than was the RF treatment; however, Rock Filter performance was deemed to be not far behind that of the Open and Duckweed ponds.

Representation of parameter loading data for BOD<sub>5</sub>, SS, chlorophyll *a*, and NH<sub>4</sub><sup>+</sup>-N on a mass basis allowed for additional insights into the nature of treatment performance for each upgrade technology, and showed a general trend for increasing performance with an increased mass loading rate. This trend was seen across all of the above parameters and across all treatments (where significant parameter removals were recorded) and reflected the concentration-dependent removal kinetics for these parameters; resulting in

the greatest magnitude removals most commonly being realised within the first pond each three-pond series. The same analyses also revealed a greater separation of treatment efficiency at lower mass loadings, such that RF performance was consistently higher than the other two treatments at low loading rates. Conversely, and under conditions of high influent solids, algal or BOD<sub>5</sub> loading, there was a reduced separation in treatment efficiency, such that the relative performance differences between each of the three treatments were significantly reduced. These trends inferred that predominantly physical mechanisms were governing BOD<sub>5</sub>, SS and chlorophyll *a* removal within the pilot ponds; something backed up by the significant interrelationships observed for these three water quality parameters. Although treatment mechanisms were shown to be overwhelmingly physical, there was also evidence for biological treatment activity too; with complete microbial nitrification occurring in the RFs, as well as evidence of zooplankton grazing interactions within the OP series.

Performance rankings for each of the water quality parameters showed that the RFs were the most efficient of the three investigated WSP upgrade methodologies across all monitored parameters except faecal coliforms and possibly PO<sub>4</sub><sup>3-</sup>-P, where performance results were found to be similar or marginally less efficient. Furthermore, and in spite of no plant biomass harvesting at any stage, the DW Pond system delivered a statistically equivalent or better quality final effluent than the OP treatment series with respect to all monitored parameters; although DO levels in DW Ponds were significantly reduced compared with OPs. In spite of being more effective than the OP treatment, however, reliability of performance was more variable in the DW Ponds than within the RFs.

### ***3.5 Experimental improvements and suggestions for future research***

Throughout this chapter, there were a number of suggested improvements in experimental analyses or indeed suggestions for future scientific investigation. Below is a summary of these concepts in addition to several new ideas which could form the basis of future research into this area:

- There is scope for improvements with respect to chlorophyll *a* analyses of duckweed pond samples. This could come either in the form of direct algal counts, or could possibly involve the use of spectral chlorophyll absorption and fluorescence ‘signatures’ to identify and quantify chlorophyll *a* fractions from the various contributing groups (e.g. Yentsch and Yentsch, 1979; Stæhr and Cullen, 2003);
- Future work could also allow for separation of various chlorophyll *a* fractions (i.e. zooplankton-sequestered and free planktonic chlorophyll) from the total suspended pool. It is thought that this sort of analytical breakdown could be especially relevant for tertiary-level maturation WSPs;
- Future investigations into duckweed pond wastewater treatment could incorporate assessments of both the sludge accumulation rate and also sludge characterisations, in order to provide further insights into the likely effects of internal biomass recycling on overall treatment performance (particularly in the absence of sustained biomass harvesting);
- Future work could look at the efficacy of duckweed versus synthetic pond covers for WSP algal control. Synthetic systems could perhaps offer potential advantages over living macrophyte systems, especially for small-scale installations;
- Future pilot-scale investigations into WSP treatment process efficiency could include preliminary validation of *in situ* hydraulic flow conditions (rather than the *post hoc* verifications here) in order to optimise flow conditions and limit the degree of dead volume within experimental pond reactors;
- Future work with similarly refined wastewaters (as was the case here) could adopt the use of a 20 day ‘BOD<sub>20</sub>’ test rather than the standard BOD<sub>5</sub> assay. This would seek to more accurately assess the oxygen demand of the refined WSP effluent over a more extended timescale. Additionally, future work could also include monitoring of wastewater COD together with soluble BOD<sub>5</sub> in order to accurately characterise the oxidative requirements of the effluent;

- It is suggested that there is a need for a greater standardisation of experimental scale when it comes to reporting on WSP-related research. Future work could adopt a classification system based on specific the pond reactor scale (e.g. ‘micro-pond’ for reactors with a volume less than 10 litres, ‘mini-pond’ for 10–1000 litre reactors, ‘pilot-pond’ for ponds in the range of 1000–100,000 litres, and ‘full-scale’ for ponds with of a greater than 100m<sup>3</sup> capacity). This classification system would instantly allow for more standardised and insightful comparisons between reported results from a wide range of experimental pond systems of varying volume.

## **4 Relative performance of horizontal flow attached-growth media and rock filtration for the upgrading of WSP effluent**

### **4.1 Introduction**

As introduced in Chapter 1 (Section 1.2), WSPs represent an extremely robust, low-maintenance, cost-efficient wastewater treatment alternative. A major issue affecting WSP performance, however, is the unpredictable and often high concentrations of algal-based SS and accompanying BOD<sub>5</sub> in their effluent (see Section 1.2.5). Further upgrading of WSPs is therefore required if pond effluent is to be of a reliably high quality for either final waterway discharge or quaternary treatment processing prior to reuse applications (as is the case for the Bolivar wastewater treatment plant). This chapter describes the experimental performance data from the 2006 pilot plant monitoring *Period 2* (February–August 2006; see Table 2.1) under RF–OP–AGM treatment setup. For an in-depth review of these upgrade systems, the reader is directed to the relevant Sections in Chapter 1. The treatment efficacies of the three experimental interventions are detailed and discussed during this chapter, with special reference given to the discrete and relative treatment performances of each system, as well as the reliability or consistency of performance. As outlined in Chapter 1 (Section 1.4), the research presented here aimed to investigate, in parallel, the treatment efficacies of these selected pilot-scale WSP upgrades. Within these performance evaluations, and in line with the research aims, special reference is also made to the algal removal efficacy of each pilot WSP upgrade methodology.

Ellis (1983, p. 98) highlighted the fact that much of the prior work investigating rock filters for upgrading of WSPs “*has been the result of unrealistically extended retention periods.*” Whilst this statement was made some 25 years ago, the sentiments of Ellis still remain true to the present day, in that much of the data on rock filter performance has previously and continues to come from systems loaded at relatively low hydraulic rates. Whilst it was not a direct aim of this research to investigate the effect of HLR on rock filter performance, high volumetric loadings were inadvertently—by virtue of the highly refined nature of the tertiary-level maturation pond effluent—required in order to apply comparable organic loadings to the pilot-scale treatments (see Section 3.3.2 for the

initial reasoning). Therefore, results from this chapter serve to provide insights into both rock filter and AGM upgrade system performance at relatively high HLRs.

## **4.2 *Materials and methods***

For a detailed description of pilot plant construction, configuration, operation and monitoring protocols, please refer to Chapter 2.

### 4.3 Results and discussion

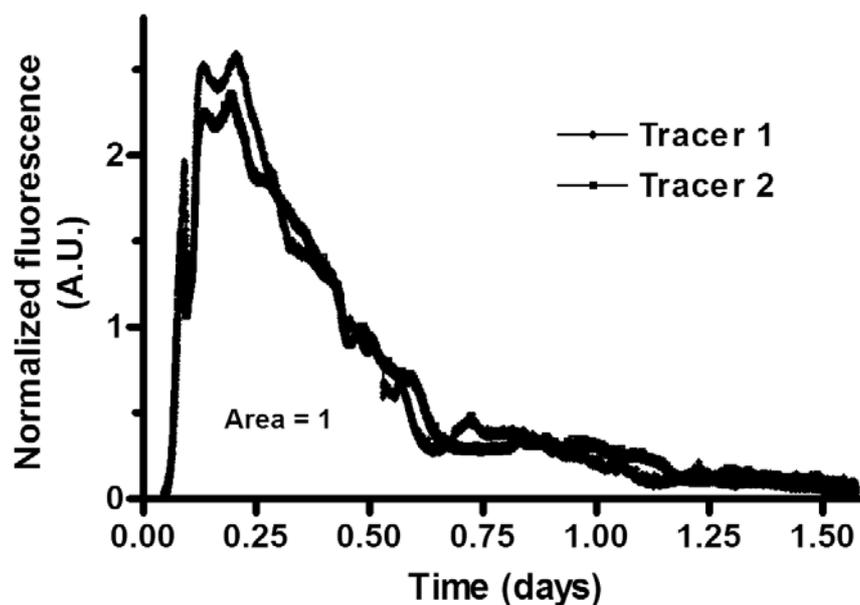
#### 4.3.1 Pilot plant flow hydraulics

As described in Section 2.1.2.1, the hydraulic operation of each pilot treatment pond was probed and characterised with the aid of the fluorescent dye rhodamine WT and an online fluorometer. It is reiterated that it was not the specific aim of this work to investigate the potential impact(s) of flow hydraulics on pond treatment performance. Rather, hydraulic flow patterns were characterised as part of the general description of the pilot plant itself, and also to aid in the identification of any particularly anomalous reactor flow patterns; something which might aid the later discussion of experimental results.

For a detailed description of pilot pond flow hydraulics for the RF and OP treatments, refer to Section 3.3.1. Otherwise, a detailed description of the flow patterns within the AGM reactors is provided below.

##### 4.3.1.1 Pilot pond flow hydraulics: attached-growth media reactors

Duplicate residence time distribution (RTD) curves from corresponding hydraulic tracer experiments for the pilot-scale AGM treatment reactors are given in Figure 4.1.



**Figure 4.1.** Duplicate single reactor normalised RTD curves for the Attached-Growth Media treatment; showing normalised rhodamine WT fluorescence (A.U.;  $y$ -axis) and time (days;  $x$ -axis).

As described previously (Section 3.3.1), tracer data from within-treatment duplicate tracer runs was averaged in order to yield one value for both the theoretical ( $\tau_{th}$ ) and actual mean residence time ( $\tau$ ), with tracer data also normalised to unity prior to RTD curve plotting in order to allow for direct comparison of the duplicate tracer experiments. Using Equation 2.1 in conjunction with the RTD curve data from Figure 4.1,  $\tau$  for single AGM reactors was calculated. Then, using this information together with Equation 2.2, the corresponding dead volume for AGM ponds was calculated (this was previously described for RF and OP reactors in Section 3.3.1).

As was the case for the DW, OP and RF treatment ponds of Chapter 3, duplicate single-pond RTD curves of for AGM reactors (Figure 4.1) again displayed a dispersed flow pattern similar to what would be expected from a relatively well-mixed tank reactor; with a maximum asymmetric fluorescence peak near to the  $y$ -axis followed by a slow and steady fluorescence decrease throughout an elongated tail (Levenspiel, 1999). Maximum tracer fluorescence was recorded in the AGM reactor effluent after only a fraction of the theoretical residence time, suggesting once more that the flow pattern within the AGM ponds was more mixed than plug—reflecting common *in situ* WSP hydraulics (Naméche and Vasel, 1996; Torres *et al.*, 1999). The asymmetric fluorescence peak, whilst being representative of a completely mixed tank reactor, was also indicative of a combination of short-circuiting and the existence of dead spaces within all pilot ponds (Bischoff and McCracken, 1966; Uhlmann, 1979; Levenspiel, 1999). The long tails for the same RTD curves represent the tracer dye quickly becoming well mixed and then slowly being diluted and washed out of the pond as the entire pond volume is gradually turned over. For a more detailed discussion of the hydraulic flow patterns of the pilot ponds in relation to *in situ* WSP hydraulics, the reader is referred to Section 3.3.1.

Duplicate tracer runs for AGM reactors recorded the rhodamine WT tracer arriving at the pond outlet within one and a half hours following inlet injection, with peak fluorescence recorded at an average time of 0.21 of  $\tau_{th}$ . Quantitative analysis of the duplicate tracer data from Figure 4.1 revealed relatively good hydraulic distribution within the AGM ponds, with  $\tau$  calculated from duplicate tracer runs of 19.93 and 20.94 hours compared to a  $\tau_{th}$  of 22.27 hours at 95.7% void space and an influent flow rate of



115L h<sup>-1</sup>. Calculated dead volume within each AGM reactors was in the order of 11 and 6% of pond void volume from the duplicate tracer studies respectively; or an average of 8.5%. This indicated the existence of dead volume and some degree of hydraulic short-circuiting within the AGM reactors, but overall flow conditions were considered to be more optimal than flow patterns within RF and OP reactors. A summary of hydraulic characterisations for all three treatments is provided below in Table 4.1.

**Table 4.1.** Hydraulic characterisation of individual pilot pond reactors for the three treatment systems: Rock Filter (RF); Open Pond (OP); and Attached-Growth Media (AGM). Individual parameter values represent the mean of duplicate tracer determinations.

Hydraulic parameter	<i>Experimental treatment</i>		
	RF	OP	AGM
Daily mean inflow rate (m <sup>3</sup> day <sup>-1</sup> )	2.76	2.76	2.76
Gross reactor volume (m <sup>3</sup> )	2.56	2.56	2.56
Void space volume (V <sub>p</sub> ; m <sup>3</sup> )	1.43	2.56	2.45
Void space volume (% total)	55.9	100	95.7
Theoretical residence time (τ <sub>th</sub> ; days)	0.52	0.93	0.89
Actual mean residence time (τ; days)	0.37 <sup>†</sup>	0.78 <sup>†</sup>	0.85
Dead volume (% V <sub>p</sub> )	27.9 <sup>†</sup>	13.63 <sup>†</sup>	8.50

<sup>†</sup> Based on previously described hydraulic characterizations of Chapter 3

As can be seen from the data of Table 4.1,  $\tau < \tau_{th}$  for all three pilot treatments; indicating the existence of dead volume within each of the pilot pond series. Results of tracer experiments showed that hydraulic flow conditions within the AGM ponds were apparently more ideal than those in both the RF and OP reactors (evidenced by the closer reflection of  $\tau_{th}$  in  $\tau$ ); although regions of dead volume still existed. For a more detailed discussion of factors relating to the existence of dead volume in these pilot ponds, the reader is redirected to Section 3.3.1. The same Section also contains a discussion of the potential effects of wind-induced mixing and thermal stratification on WSP flow hydraulics and also defines the likely impacts of these factors on the hydraulic conditions within these pilot reactors. Given the overwhelmingly similar hydraulic flow patterns within each of the pilot pond reactors, all subsequent performance differences between the three treatments reported within this chapter are considered to have occurred independently of hydraulic processes, and as such, the

hydraulic flow regime of each pilot system is not discussed in the context of later research findings.

### 4.3.2 Pilot plant loading conditions and influent wastewater characteristics

During the second operational *Period 2* (i.e. RF–OP–AGM configuration), average pilot plant influent flow rate was approximately  $110 \text{ L h}^{-1}$ , corresponding to an average HLR across all treatment ponds of  $1.03 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ . It should be noted that this *Period 2* HLR was some 45% greater than that applied to the experimental pilot plant during monitoring *Period 1* of the previous Chapter 3 (see Table 2.1 for definition of monitoring Periods 1 and 2). At this elevated HLR, corresponding theoretical mean HRTs for each single pond were 0.54 days per pond for the RF treatment, 0.97 days for each OP reactor, and 0.93 days for each AGM pond (based on RF and AGM void space volumes of 55.86% and 95.7% respectively). It should be noted that these theoretical residence times are actually longer than those given in Table 4.1 because the tracer experiments were performed *post hoc* under a slightly elevated flow rate ( $115 \text{ L h}^{-1}$ ). A summary of these and other operational parameters for the pilot-scale treatments is provided in Table 4.2.

**Table 4.2.** Summary of hydraulic and organic loading characteristics of the individual pilot-scale WSP upgrade treatment reactors during operational *Period 2*.

<b>Parameter</b>	<b>Treatment</b>		
	<b>RF</b>	<b>OP</b>	<b>AGM</b>
Hydraulic flow rate ( $\text{m}^3 \text{ d}^{-1}$ )	2.64	2.64	2.64
Theoretical fluid velocity ( $\text{m d}^{-1}$ )	4.02	2.24	2.33
Hydraulic loading rate ( $\text{m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ) <sup>a</sup>	1.03	1.03	1.03
Aerial surface loading rate ( $\text{m}^3 \text{ m}^{-2} \text{ d}^{-1}$ ) <sup>a</sup>	1.03	1.03	1.03
Theoretical mean HRT (d)	0.54	0.97	0.93
Actual mean HRT (d) <sup>†</sup>	0.39	0.82	0.89
Influent organic strength ( $\text{g BOD}_5 \text{ m}^{-3}$ ) <sup>‡</sup>	3.90	3.90	3.90
Organic loading rate ( $\text{g BOD}_5 \text{ m}^{-3} \text{ d}^{-1}$ ) <sup>‡,a</sup>	4.02	4.02	4.02

<sup>†</sup> Implied from the ratio of  $\tau:\tau_{th}$  measured during tracer experiments

<sup>‡</sup> Based on median influent  $\text{BOD}_5$  concentration during the operating period

<sup>a</sup> Based on gross reactor volume not void space volume

Hydraulic loading rates used here are significantly higher than those reported in the relevant literature for pilot-scale OP, RF and AGM systems. The reasoning behind the adoption of this higher volumetric loading regime was primarily based on the low organic strength of the pilot plant influent, and the corresponding need to obtain a comparable organic loading regime (see Section 3.3.2 for the initial discussion of this). The  $1.03\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  HLR adopted here was in the order of 2–5 times greater than those generally reported for rock filters and pilot-scale ‘open pond’ systems, and was some 3-fold higher than the recommended HLR for rock filters treating maturation WSP effluent in the United Kingdom (refer again to Section 3.3.2 for the initial review). The only exceptions to this are the rock filters of von Sperling *et al.* (2007) and Mara *et al.* (2001), where respective HLRs of 1.0 and  $1.0\text{--}2.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  were adopted.

In respect of reported HLRs for other AGM systems, Zhao and Wang (1996) operated similar pilot-scale attached-growth WSPs (AGWSPs) although at a significantly reduced HLR to that reported here ( $0.14\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ). McLean *et al.* (2000) operated large pilot-scale ( $9000\text{m}^3$ ) AGWSPs again at reduced HLRs in the range  $0.07\text{--}0.14\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ; however, since the specific surface area of McLean and co-workers’ attached-growth ponds was only  $2\text{m}^2\text{ m}^{-3}$ , this work is hesitantly included in the general ‘AGM’ literature. Lapolli *et al.* (2006) operated a pilot-scale AGWSP at an HLR of  $1.75\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ; however, their so-called ‘biomass attachment’ ponds again had a specific surface area of only  $2\text{m}^2\text{ m}^{-3}$ , such that they too are probably more accurately grouped together with the ‘hydraulic baffle’ research and are therefore tentatively considered part of the AGM literature base. The closest work from a volumetric loading perspective appears to be that of Shin and Polprasert (1987; 1988) who operated a similar pilot-scale AGWSP at HLRs in the range of  $0.16\text{--}0.32\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ .

A summary of the influent loading and water quality parameters during the six month monitoring *Period 2* is provided in Table 4.3 below. As described in Section 3.3.2, the pilot plant influent feed wastewater was classified as being of ‘weak’ organic strength according to Metcalf and Eddy (1991). Bearing in mind that the final Bolivar effluent is a highly refined tertiary-level maturation pond effluent, it is important to remember during the interpretation of performance data that the pilot plant influent is at the very

‘high-end’ of the typical WSP effluent water quality spectrum (see Section 3.3.2 for the initial discussion of this).

**Table 4.3.** Pilot plant loading conditions and pilot plant Influent water quality for the first pond reactor of each three-pond treatment series.

<b>Parameter<sup>†</sup></b>	<b>Loading range</b>	<b>Influent range</b>	<b>Median quality</b>
<b>BOD<sub>5</sub><sup>‡</sup></b>	0.3–18 g m <sup>-3</sup> d <sup>-1</sup>	0.3–17	3.9
<b>Chl. a</b>	6.9–96 mg m <sup>-3</sup> d <sup>-1</sup>	6.7–93 μg L <sup>-1</sup>	14.6 μg L <sup>-1</sup>
<b>SS<sup>‡</sup></b>	2.3–35 g m <sup>-3</sup> d <sup>-1</sup>	2.2–34	7.9
<b>Turbidity (NTU)</b>	1.9–24	1.9–24	3.7
<b>NH<sub>4</sub><sup>+</sup>-N<sup>‡</sup></b>	0.6–1.6 g m <sup>-3</sup> d <sup>-1</sup>	0.6–1.5	0.67
<b>PO<sub>4</sub><sup>3-</sup>-P<sup>‡</sup></b>	3.1–7.4 g m <sup>-3</sup> d <sup>-1</sup>	3.0–7.2	3.8
<b>FC<sup>a</sup></b>	1.2–2.7 m <sup>-3</sup> d <sup>-1</sup>	1.2–2.6	1.9
<b><i>E. coli</i><sup>a</sup></b>	1.1–2.6 m <sup>-3</sup> d <sup>-1</sup>	1.1–2.5	1.6

<sup>†</sup> BOD<sub>5</sub>, biochemical oxygen demand; Chl. a, chlorophyll a; SS, suspended solids;

NH<sub>4</sub><sup>+</sup>-N, ammonia nitrogen; PO<sub>4</sub><sup>3-</sup>-P soluble reactive orthophosphate; FC, faecal coliforms

<sup>‡</sup> Expressed as mg L<sup>-1</sup> unless otherwise indicated

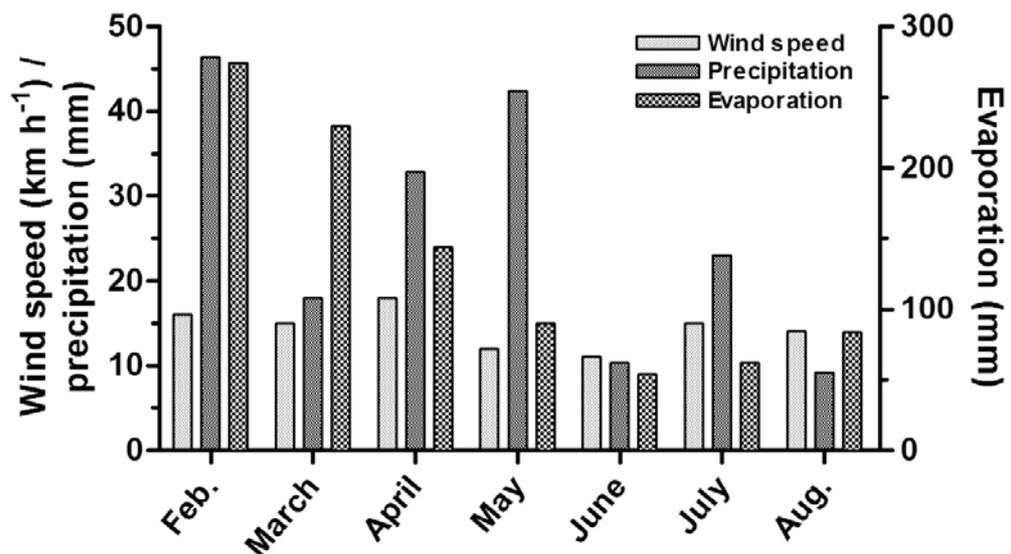
<sup>a</sup> Expressed as log<sub>10</sub> MPN 100mL<sup>-1</sup> unless otherwise indicated

In order to simplify the interpretation and discussion of pilot plant performance results, and as was done during Chapter 3, in-depth discussion of treatment performance for the three pilot upgrade systems (i.e. RF, OP and AGM) is only provided for Pond 1 and Pond 3 of each three-pond series. Additionally, and since the bulk of inter-study treatment performance comparisons have already been thoroughly discussed for the pilot-scale RF and OP systems in Chapter 3, no further detailed cross-references will be provided here. Instead, reference to the performance of these treatment systems will only be made regarding the work of others where comparable hydraulic loading regimes were applied and where similar or larger volumetric scale pond systems were used. For inter-study comparisons of AGM treatment performance, the same general exclusion criteria were applied to this body of research as was described during the previous Chapter (Section 3.3.5); in other words, performance comparisons between the current work and the results of others are primarily made where research was conducted using pond reactors of a comparable or larger volume. This again served to minimise the potential confounding effects resulting from performance comparisons made between studies involving pond reactors several orders of magnitude smaller in volume.

Regarding performance comparisons made between the RF and AGM systems, these were made with no consideration of the prior six month operational conditioning of the RFs compared with the newly established AGM ponds. Although the RF treatment train had already been operational for six months prior to commissioning of the AGM system, this was thought to have had no significant impact on the validity of performance cross-comparisons made between the two pilot treatments. Given that rock filters have been shown to require no biological ‘pre-conditioning’ for optimal physical SS and BOD<sub>5</sub> removal performance (Swanson and Williamson, 1980), the same was also assumed for the current AGM systems, such that AGM ponds should be operating at full *physical* treatment capacity immediately after start-up. Finally, for additional information regarding some other factors relating to pilot plant performance data analysis and interpretation, the reader is referred to Section 3.3.2.

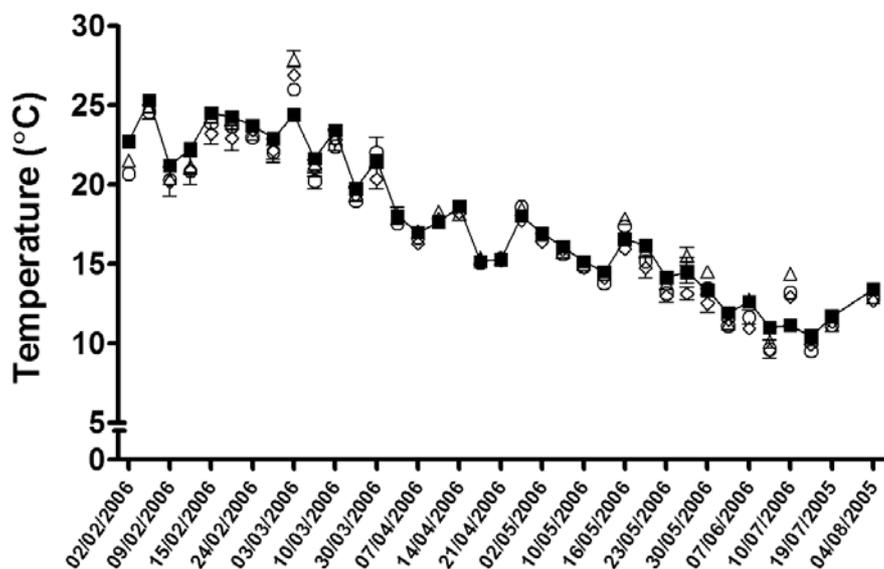
### 4.3.3 Environmental and physicochemical parameters

The pilot plant site received a daily average of 9.8 hours of sunlight in the summer season (December–February) and 5.3 hours during winter (June–August) at an average annual daily solar irradiance of 17MJ m<sup>-2</sup> (see Section 3.3.4 for data sourcing). A summary of the prevailing weather conditions experienced at the Bolivar WWTP during the 2006 monitoring *Period 2* is provided below.

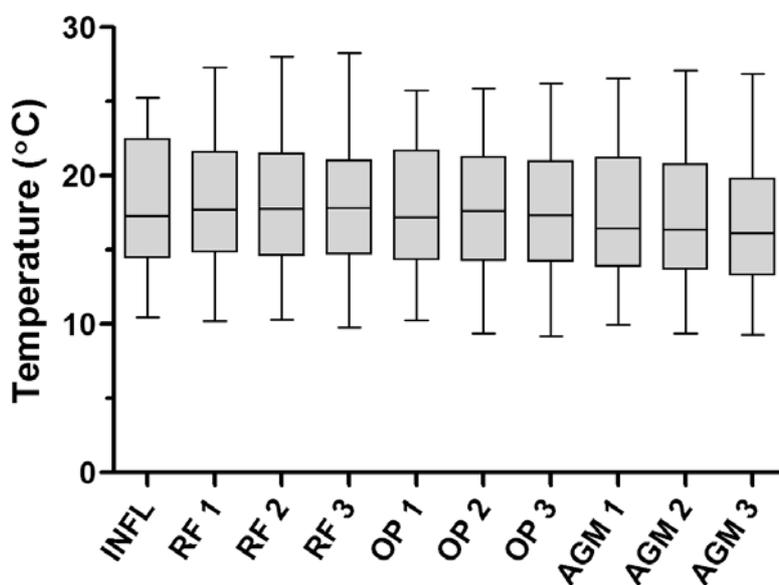


**Figure 4.2.** Selected mean monthly site weather conditions from February–August of 2006. Left y-axis shows average daily wind speed and monthly precipitation, and the right y-axis shows mean monthly evaporation (data courtesy of the Australian Government Bureau of Meteorology).

As outlined in Section 2.2.2.1, various physicochemical water quality parameters were monitored for the pilot plant influent and three experimental treatments during the 2006 operating *Period 2*. Results from these analyses are provided below.

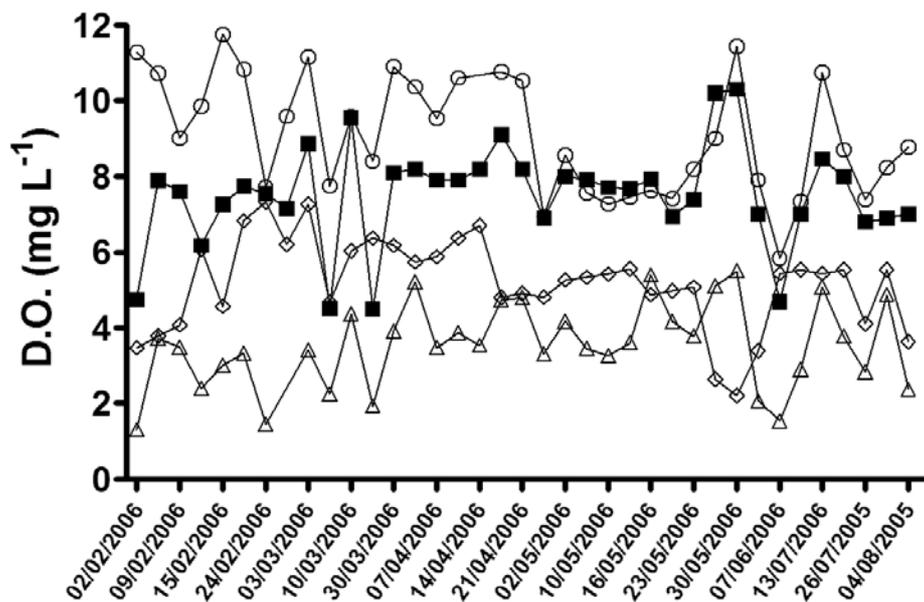


**Figure 4.3.** Water temperature data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Attached-Growth Media (◇) Reactors. For ease of interpretation, data points show only the mean temperature ( $\pm 1$  S.D.) averaged across each three-pond treatment series, with a line fitted only to the influent data set.

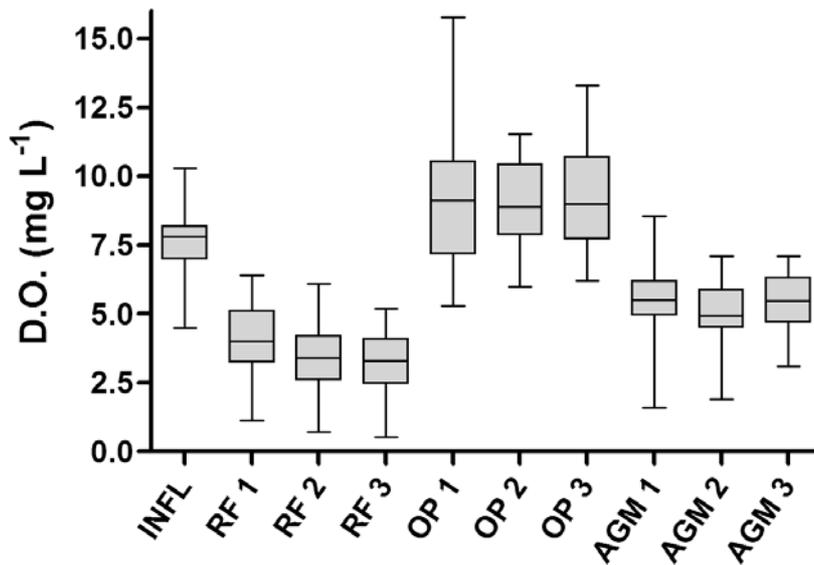


**Figure 4.4.** Water temperature box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The shaded ‘box’ represents the interquartile data range (IQR), the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

Data from water temperature monitoring is given in Figures 4.3 and 4.4. During the course of the six month monitoring period, the pilot plant water temperature decreased steadily from around 23.5°C in February of 2006, to approximately 12°C by late July–early August. Generally speaking, and as was the case during monitoring *Period 1* of Chapter 3, water temperature within the pilot pond systems varied minimally compared with that of the influent wastewater. Temperature was reduced on average by less than 1°C during pilot plant passage across all three treatments, and was not significantly different from that of the influent in any instance (1-way ANOVA;  $F_{(9,317)} = 0.301$ ;  $p = 0.97$ ). Generally speaking, these small and insignificant changes in water temperature along each pond series were thought to have been applied evenly across all three treatments, and were therefore thought to have had little or no influence on between-treatment performance outcomes.



**Figure 4.5.** Dissolved oxygen data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Attached-Growth Media (◇) Reactors. For ease of interpretation, data points show only the mean DO concentration averaged across each three-pond treatment series.



**Figure 4.6.** Dissolved oxygen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

Data from DO monitoring is given in the above Figures 4.5 and 4.6. During monitoring *Period 2*, as was the case for *Period 1* of Chapter 3, pilot plant influent DO levels again displayed significant temporal fluctuation according to localised meteorological conditions. Pilot plant treatment pond DO patterns mirrored that of the influent, generally: decreasing substantially for RFs; increasing slightly for OPs; and decreasing slightly for AGM Reactors. Average influent DO concentration was in the order of 7.8 ( $\pm 1.4$ ) mg L<sup>-1</sup>, with three-pond mean RF, OP and AGM concentrations of 3.1 ( $\pm 1.2$ ), 8.5 ( $\pm 1.8$ ), and 4.8 ( $\pm 1.3$ ) mg L<sup>-1</sup> respectively. Within treatments, DO levels remained relatively stable across all three-pond trains; with no significant changes in DO levels down the pond series from Pond 1 to Pond 3 in any treatment series ( $p > 0.05$ ).

In the RF treatment train, DO decreased significantly compared to influent concentrations in all three ponds (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 247.2$ ;  $p < 0.001$ ). In the OP series, the small approximate 1mg L<sup>-1</sup> DO increase was not significant in any of the three ponds ( $p > 0.05$ ), and for the AGM system, the average 40% reduction in the levels of inflowing DO was again highly significant across all three ponds ( $p < 0.001$ ). With respect to between-treatment DO levels, the OP series had significantly elevated oxygen concentrations than did the RF and AGM treatments across all three ponds in series ( $p \leq 0.01$ ). Between the RF and AGM series, DO levels were similar for ponds 1 and 2

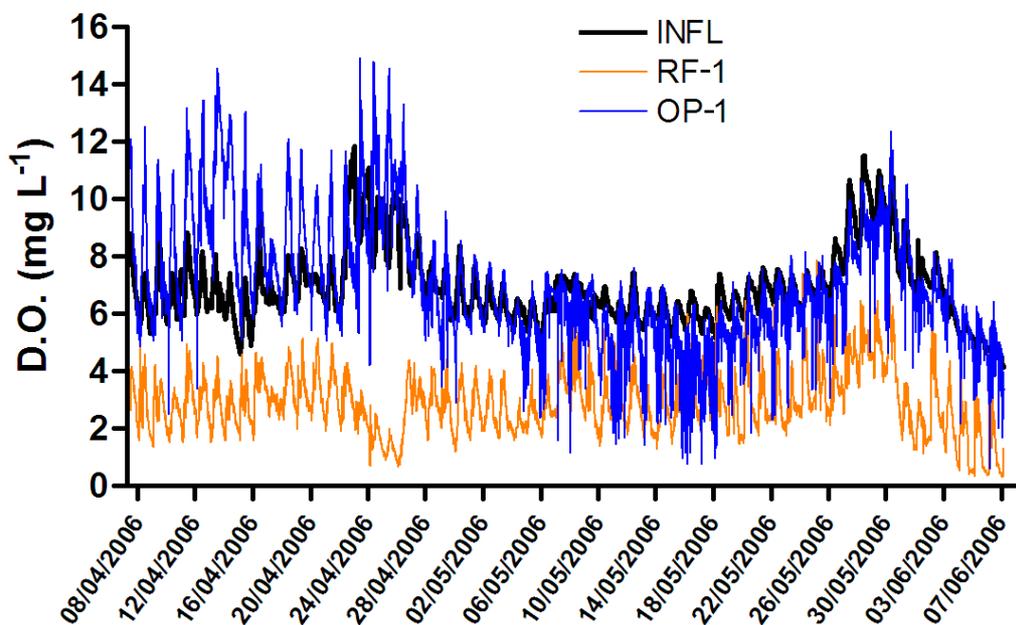


( $p > 0.05$ ) but significantly greater by the end of the line for AGM-3 compared with RF-3 ( $p < 0.01$ ). The significant decline in DO concentration within the rock filters is a well recognised operational disadvantage associated with the technology and has been discussed in detail elsewhere (see Section 3.3.4). The small but non-significant increase in DO down the pond series within the OP treatment train was most likely a reflection of a limited amount of photosynthetic re-oxygenation during the temporary impoundment of inflowing wastewater. This result was again considered to be of no great interest in the context of this research, and merely served to demonstrate an effective maintenance of *in situ* WSP effluent oxygen conditions within the *ex situ* pilot ponds.

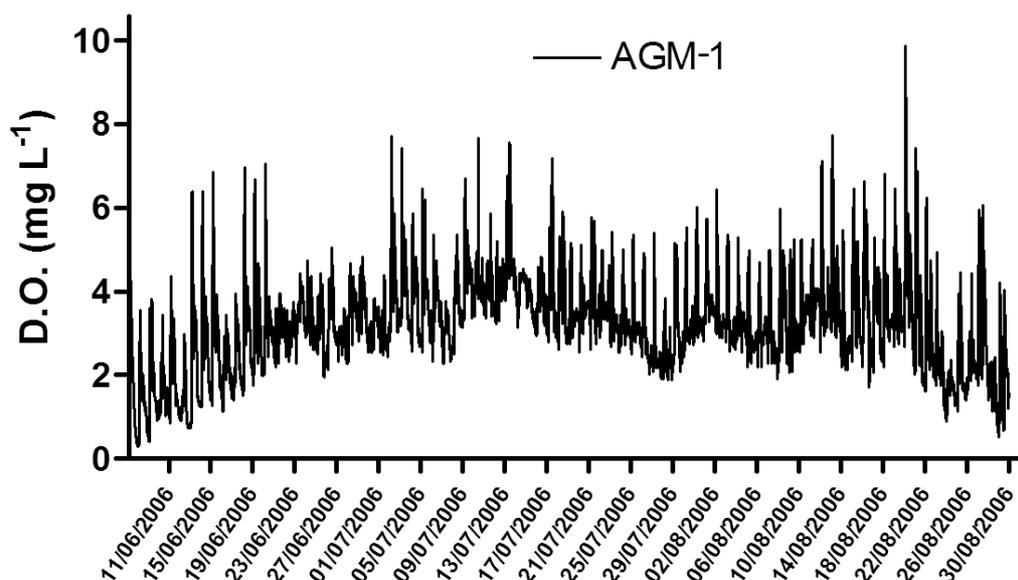
As for DO concentrations in the RFs, oxygen levels in AGM ponds were also significantly reduced down the pond series compared with influent levels. This trend has been reported elsewhere for other AGM systems (Shin and Polprasert, 1987; McLean *et al.*, 2000), whereby the elevated attached-biomass densities exert an increased oxygen demand on the system, leading to suppressed DO concentrations. Given the 4-fold greater surface area-to-volume ratio of the AGM ponds compared with the RFs, it may have been anticipated that DO levels would have been lower in the AGM ponds than in the RFs; however, DO concentration in AGM-3 was significantly higher than in RF-3. It was thought that this may have been a reflection of a greater accumulation of sediment oxygen demand during the previous six month *Period 1* operation for the RFs compared with the newly commissioned and comparatively sludge-free AGM reactors. It was also likely that the RF series was subjected to a greater respiratory oxygen demand as a result of the higher standing biomass density of resident invertebrate biota compared with the AGM system (refer to Section 5.3.2; Figure 5.58). Overall, the higher than anticipated DO concentrations observed within both the RF and AGM systems were most likely a consequence of the refined nature of the tertiary-level Bolivar WSP effluent. Given that this issue was discussed previously (see Section 3.3.4), no further explanation is provided here.

As referred to in Chapter 3 (Section 3.3.4), it was again likely that daily measured values for DO and pH in particular did not represent the *daily maxima* for each treatment pond series. It has been reported elsewhere that a daily peak in DO and accompanying pH concentration (according to photosynthetic processes) occurs somewhere during 1400–

1600 hours (Pearson *et al.*, 1987; Kayombo *et al.*, 2002). This classical ‘wax and wane’ daily cycle in DO and accompanying pH levels is a common phenomenon associated with WSP environments (Fritz *et al.*, 1979; Uhlmann, 1980) and relates directly to the levels of available sunlight and subsequent intensity of *in situ* photosynthetic processes. Given that DO concentration was measured daily at 1200 ( $\pm 2$ ) hours (Section 2.2.1), it was likely that the DO levels (as well as corresponding pH) reported here were somewhat lower than the daily maximum values. Critical evaluation of 24 hour online DO data from a number of time intervals during the 2006 monitoring *Period 2* supported this concept and revealed that the maximal DO (and likely accompanying pH) concentration generally occurred between 1600–1900 hours, with daily minimum DO levels recorded around 0700–0900 hours (see Figures 4.7 and 4.8 below). This suggested that the daily DO concentrations measured at 1200 ( $\pm 2$ ) hours represented approximately 70–80% of the daily maximum value. No attempts were made to correct for this 20–30% underestimation of maximum daily levels, rather the midday sampling interval appeared to coincide nicely with the approximate half-way-point between daily maximum and minimum oxygen concentration.



**Figure 4.7.** 24 hour online dissolved oxygen data from part of monitoring *Period 2* of 2006 for pilot plant: Influent (INFL); Rock Filter 1 (RF-1); and Open Pond 1 (OP-1).

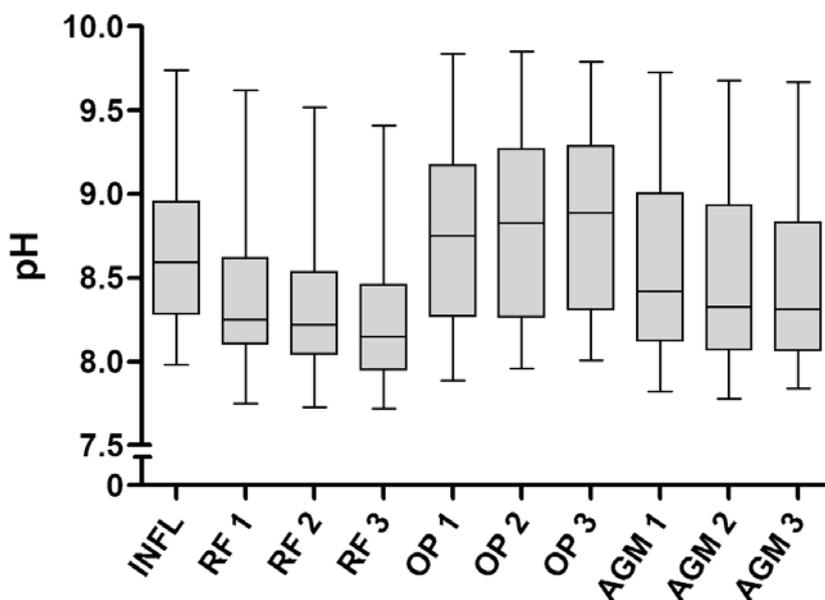


**Figure 4.8.** 24 hour online dissolved oxygen data from part of monitoring *Period 2* of 2006 for Attached-Growth Media Reactor 1 (AGM-1) only.

Data from Figures 4.7 and 4.8 shows that the levels of DO (and probably pH; although not monitored) in the influent, RF-1, OP-1, and AGM Pond 1, followed the typical diurnal pattern of oscillation. Twenty four hour DO concentrations in OP-1 were far more variable than for RF-1 and AGM-1, with recorded oxygen concentrations commonly fluctuating in the order of 6–8mg L<sup>-1</sup> over a single day period. Interestingly, the online data showed that the RFs (at least as far as RF-1) did not appear to be sinking into nighttime anoxia; suggesting that anaerobic NH<sub>4</sub><sup>+</sup>-N and sulphide production would not be common place in these RFs. This goes toward answering an earlier question posed in Section 3.3.7.1 as to whether there might be nocturnal anoxic NH<sub>4</sub><sup>+</sup>-N production, and, in the absence of complete RF anoxia being observed, results suggest that this would be unlikely to occur.

Further backing for the suggested absence of anoxic conditions within the rock filters comes from zooplankton population data presented in Chapter 5. The persistent observation of rotifers populations within the RF train suggests that aerobic conditions were effectively maintained within the rock filters (see Figures 5.26 and 5.30). Rotifers possess no respiratory organs and hence respire via their whole body surface. For this reason they are generally unable to persist in anaerobic environments, with only a few

very resistant species able to tolerate microaerobic or hypoxic habitats (Sládeček, 1983). Since rotifers are recognised to be one of the more sensitive macrobiotic indicators of the level of organic pollution and subsequent aerobic status (Gannon and Stemberger, 1978), their presence within the RFs was taken as further evidence of continuous aerobic operation. Based on the online DO data of Figures 4.7 and 4.8, it was apparent that influent DO concentration was the primary governing force behind the patterns of daily oxygen fluctuations within all pilot treatment ponds, with the treatments themselves simply defining the specific magnitude of diel DO variation.

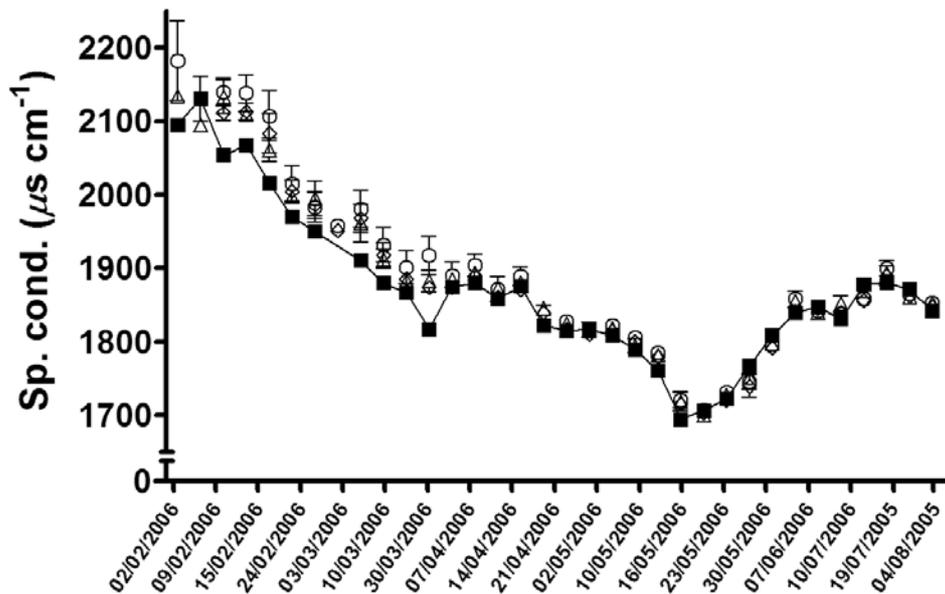


**Figure 4.9.** pH box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

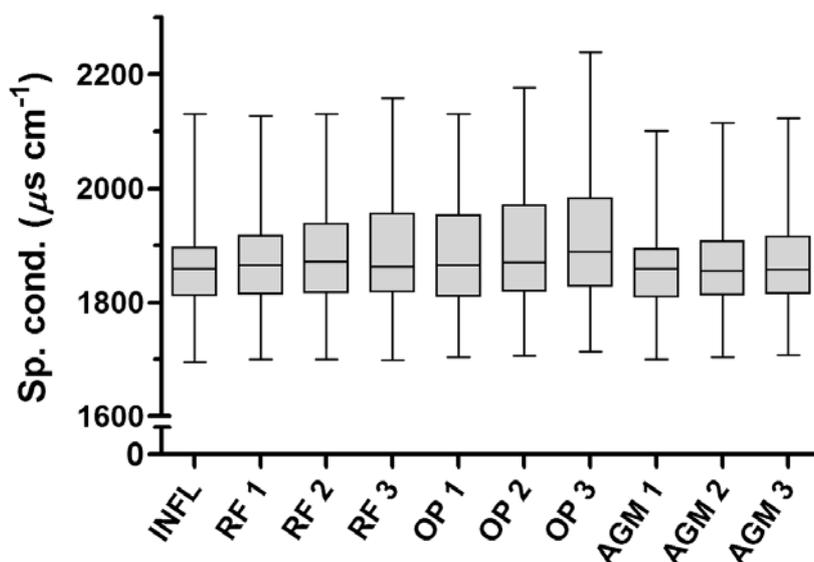
Data from *Period 2* monitoring of wastewater pH is shown above in Figure 4.9. Recorded pH values were always alkaline and were commonly >8.1 across all treatments (based on lower 95% CI of mean). Average influent pH was in the order of 8.6, with pH appearing to decrease slightly during both RF and AGM treatment ( $\approx 0.4$  units for the RFs and  $\approx 0.3$  units in AGM ponds) and increase down the pond series within the OPs by roughly the same magnitude. Statistically, the only significant change in influent pH was recorded for RF-3 (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 43.31$ ;  $p < 0.05$ ), with none of the other treatment ponds having a pH significantly different from influent levels ( $p > 0.05$ ). Regarding between treatment pH values, Open Pond pH was significantly greater across

all three ponds compared with pH in all three RFs ( $p \leq 0.01$ ), but was similar to parallel AGM ponds ( $p > 0.05$ ). Likewise, there was no significant difference between RF and AGM pH in any of the three pilot ponds ( $p > 0.05$ ).

Factors relating to the common observation of a decline in wastewater pH following RF treatment, as well as those surrounding the small increase in OP pH, have already been discussed (Section 3.3.4) and so the reader is referred there for additional information. Data on pH dynamics within attached-growth WSP systems is lacking. Zhao and Wang (1996) did report on relatively high (photosynthetically elevated) pH values of 7.8–9.3 within their pilot AGWSPs; however, their AGM was different to the current arrangement in that it comprised discrete lengths of AGM suspended vertically within the ponds and was therefore exposed to high levels of incident irradiance (as opposed to the darkened AGM Reactors here). McLean *et al.* (2000) reported pH values for their large pilot-scale AGWSPs to be in the range of 7–8.7; with a daily mean of 7.7—a similar range to those observed here. The reasons behind the small but insignificant drop in pH within the current AGM system were thought to be largely similar to those previously discussed for the RFs (Section 3.3.4).



**Figure 4.10.** Specific conductivity data for pilot plant: Influent (■); Rock Filters (△); Open Ponds (○); and Attached-Growth Media (◇) Reactors. For ease of interpretation, data points show only the mean conductivity ( $\pm 1$  S.D.) averaged across each three-pond treatment series, with a line fitted only to the influent data set.



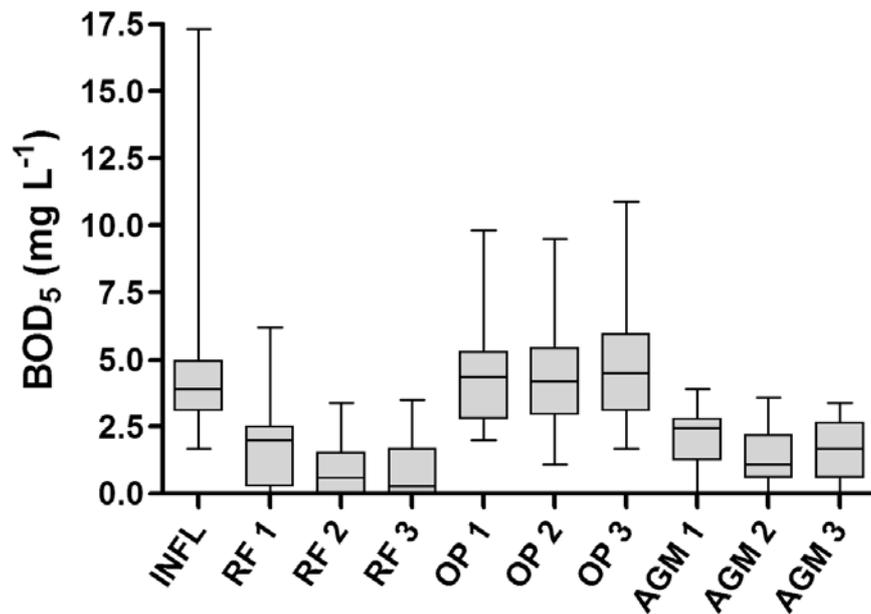
**Figure 4.11.** Specific conductance box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

Data from monitoring of specific conductance is shown in Figures 4.10 and 4.11. Specific conductivity of the influent wastewater, and also that of all treatments, steadily declined from the start of the monitoring period in February of 2006 up until the end of pilot plant monitoring in early August of the same year. Patterns of wastewater conductivity again closely mirrored the monthly rates of evaporation (see Figure 4.2) and were a simple reflection of the seasonal shift from summer in February to winter in August. This was again supported by the strong correlation between wastewater temperature and conductivity for the combined 9 pond pilot plant data ( $r_s = 0.640$ ;  $n = 326$ ;  $p < 0.0001$ ) but was considered to be of no great significance in the overall research context. Corresponding wastewater salinity was in the order of from  $1.1\text{g L}^{-1}$  in February, after which it decreased slightly to approximately  $0.9\text{g L}^{-1}$  in early August of 2006. Conductivity varied insignificantly across all treatments relative to initial pilot plant influent readings (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 4.085$ ;  $p = 0.91$ ) and also remained identical between the three treatments ( $p > 0.05$ ). Specific conductivity values ranged from  $1700\text{--}2250\mu\text{S cm}^{-1}$  (average of  $\approx 1900\mu\text{S cm}^{-1}$ ), with variation from influent readings less than 1% down each pond series. As discussed in Section 3.3.4, this low level variation was again considered unlikely to have had any significant biological relevance in the context of the current work, and so results for conductivity are not further discussed. For a concise statistical summary of the full listing of physicochemical

parameter correlations, the reader is directed to the corresponding correlation matrices for the pilot plant influent as well as the three upgrade treatments (Appendix C).

#### 4.3.4 Wastewater treatment performance: removal of particulate organics and oxygen demand

Data from *Period 2* performance monitoring of BOD<sub>5</sub> within the pilot plant influent and the three pilot treatment upgrade systems is shown in Figure 4.12.



**Figure 4.12.** BOD<sub>5</sub> box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

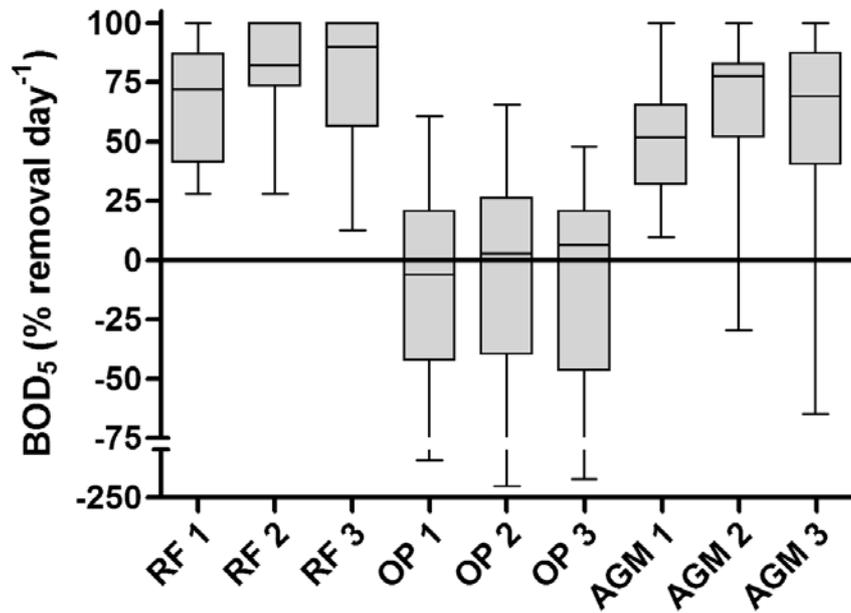
As shown in Figure 4.12, pilot plant influent organic strength was generally very low but spiked to much higher levels on occasion, with a median BOD<sub>5</sub> concentration of 3.7mg L<sup>-1</sup> and a mean of 4.5mg L<sup>-1</sup>. This sporadic and sometimes high-level variability in the Bolivar WSP effluent (i.e. pilot plant influent) is a common feature of WSP systems in general; with the reasons for such variability discussed elsewhere (Section 3.3.5). Given the highly skewed non-normal distribution of the influent BOD<sub>5</sub> data, the median value is again used for all subsequent loading rate calculations. This median influent

concentration of  $3.7\text{mg L}^{-1}$ , factoring in the mean HLR of  $1.03\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ , translated to an average organic loading rate during monitoring *Period 2* of  $3.8\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ .

Statistical analysis of the normalised data from Figure 4.12 showed that influent  $\text{BOD}_5$  was reduced significantly down the pond series in all three RFs (1-way ANOVA;  $F_{(9,205)} = 18.97$ ;  $p < 0.001$ ). The same was true for the AGM train, with significant removals of influent  $\text{BOD}_5$  in AGM-1 ( $p < 0.05$ ) as well as AGM Reactors 2 and 3 ( $p < 0.001$ ). Unlike the other two treatment pond series, however, no significant removal of loaded  $\text{BOD}_5$  was achieved in any of the OPs ( $p > 0.05$ )—a similar trend to that noted for OPs during Chapter 3. Qualitatively, the OP series appeared to have an even more variable  $\text{BOD}_5$  than the influent (shown by the larger interquartile data ranges); however, this increased variability was not statistically significant to that of the influent.

Regarding between-treatment performance comparisons, effluent  $\text{BOD}_5$  was significantly lower across all three RF and AGM Ponds relative to the corresponding levels in parallel OPs ( $p \leq 0.01$ ); however, between the RF and AGM treatment series, effluent  $\text{BOD}_5$  values were statistically similar for all respective ponds down the treatment train ( $p > 0.05$ ). Unlike Chapter 3,  $\text{BOD}_5$  data from Pond 2 of each treatment series has been included in this Chapter's performance analyses due to increased equipment availability and a greater number of analytical sample replicates ( $n \geq 21$  across all 9 ponds). Furthermore, and although the most extreme influent  $\text{BOD}_5$  data point of  $17.3\text{mg L}^{-1}$  was classified as an 'extreme outlier' within the influent data set (i.e.  $>3 \times$  IQR from the 75<sup>th</sup> percentile value), given that it did not coincide with an extreme outlying SS spike event (as was the case for the highest influent  $\text{BOD}_5$  value of Chapter 3), this particular influent data point was retained within the influent  $\text{BOD}_5$  data set because it was considered to reflect the normal variability in Bolivar WSP effluent quality.





**Figure 4.13.** Box-plots showing percentage BOD<sub>5</sub> removal performance relative to pilot plant Influent concentration for all three ponds of each pilot treatment system ( $n \geq 21$  for each plot).

Long term median percentage daily BOD<sub>5</sub> removals for Pond 1 data of the three treatments were 72, -6 and 52% for the RF, OP and AGM systems respectively, and for Pond 3 data, 90, 6 and 69% for the respective RF, OP and AGM treatments (Figure 4.13). When average BOD<sub>5</sub> removals were compared to theoretical zero median BOD<sub>5</sub> removals, RF and AGM removal performance was again highly significant ( $p < 0.001$ ) but remained equivalent to zero BOD<sub>5</sub> removals for all ponds of the OP series ( $p > 0.05$ ; Table 4.4). Unlike the other two treatment trains, the RF system always yielded positive BOD<sub>5</sub> removals, with the OP and AGM treatments both experiencing net BOD<sub>5</sub> increases at some stage (on two occasions each for AGM Reactors 2 and 3, and on average 8 times in each Pond for the OP series). This performance variability was reflected in the corresponding CV's for percentage BOD<sub>5</sub> removal efficiency (Table 4.4), where the OPs were shown to be considerably more variable in BOD<sub>5</sub> treatment performance than both the RF and AGM treatments.

**Table 4.4.** Summary of BOD<sub>5</sub> performance data across all three pilot plant treatments for Ponds 1 and 3 only.

BOD <sub>5</sub> performance parameter	Pilot treatment pond					
	RF-1	RF-3	OP-1	OP-3	AGM-1	AGM-3
Median influent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	3.7	3.7	3.7	3.7	3.7	3.7
Mean influent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	4.5	4.5	4.5	4.5	4.5	4.5
Median effluent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	2.0	0.3	4.0	4.5	2.4	1.7
Mean effluent BOD <sub>5</sub> (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup> *	1.6	1.0	5.0	4.7	2.1	1.6
Median daily BOD <sub>5</sub> removal (% day <sup>-1</sup> ) <sup>‡</sup> *	72	90	-5.8	6.3	52	69
Mean daily BOD <sub>5</sub> removal (% day <sup>-1</sup> ) <sup>‡</sup> *	65	78	-12	-18	51	57
Long-term CV for BOD <sub>5</sub> removal (%)	39	32	372	350	51	73

<sup>†</sup> Effluent BOD<sub>5</sub> concentration was tested relative to mean pilot plant influent BOD<sub>5</sub> concentration (1-way ANOVA)

<sup>‡</sup> Average BOD<sub>5</sub> removal % tested against a theoretical 'zero' daily median (Wilcoxon signed-rank test)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p \leq 0.001$  (black)

The above analysis of performance reliability, through comparing the corresponding BOD<sub>5</sub> removal CV's, highlighted the enhanced consistency in performance delivery of both the RFs and AGM upgrade systems over the OP treatment. Not only did the RF and AGM treatment series deliver a better quality final effluent in terms of BOD<sub>5</sub> concentration than the Open Ponds, but they achieved this with an enhanced degree of performance consistency as well. This trend has already been discussed in detail for the RFs in Chapter 3 (Section 3.3.5) and since it applies again to the RFs, as well as the AGM Reactors, it will not be re-described.

With respect to the treatment performance of other rock filters, only the work of others is referenced where similarly high HLRs were applied. Mara *et al.* (2001) operated similar scale experimental rock filters to the current system at very high HLRs of 1.0 and 2.0 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>. The authors reported a significant reduction in overall rock filter BOD<sub>5</sub> removal efficiency (from 46 down to 14%) when operated at the increased hydraulic loading of 2.0 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>. This decline in filter performance efficiency was arguably not surprising in the case of Mara and co-workers, given that at the highest HLR, the corresponding OLR was in the order of 56 g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>—some 15-fold greater than that applied to the current RFs—and was, therefore, probably organically overloaded. Similar to the rock filters of Mara *et al.* (2001) above, von Sperling *et al.* (2007) also reported substantial reductions in average BOD<sub>5</sub> from ≈40% down to just 3% in their similar sized pilot-scale rock filters when the hydraulic loadings were doubled from 0.5 to 1.0 m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>. Once again, however, the RFs of von Sperling *et al.*

were likely to have been organically overloaded at the highest HLR, given that at the hydraulic loading of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  the corresponding organic loading rate was in the order of  $55\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ —a rate similar to that of Mara *et al.* above. Despite the equivalent HLR of  $1.03\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ , the 40–95% greater mean BOD<sub>5</sub> removal efficiency of the current Rock Filters over those of the above authors was almost certainly a reflection of the much lower OLR of the filters here. This 15-fold lower organic loading afforded the current RFs >90% higher DO concentrations than those of Mara and co-workers, for example, with increased oxygen reserves presumably allowing for a more complete oxidation of loaded BOD<sub>5</sub> (a theory supported by the average 60% decline in DO concentration within the RFs; see Figure 4.6).

With respect to the absolute efficiency and performance reliability of other AGM systems, performance results are variable according to factors such as organic loading, volumetric scale, and specific surface area of the attached-growth media itself. Shin and Polprasert (1987) operated a similar pilot-scale AGWSP, with a specific surface area of  $1220\text{m}^2\text{ m}^{-3}$  and at organic loadings in the range of  $10\text{--}20\text{g COD m}^{-3}\text{ d}^{-1}$ , and reported only a slight enhancement in organic (COD) removal efficiency in their pilot-scale AGM ponds compared with open control ponds ( $\approx 5\%$ ). Given that common COD:BOD<sub>5</sub> ratios for WSP influent wastewater are in the order of 2.2:1 (Alaerts *et al.*, 1996), this suggests an equivalent OLR for the work of Shin and Polprasert in the order of  $4.6\text{--}9.2\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ —the same order of magnitude to the organic loadings applied to the current AGM Ponds. Like the performance of current AGM Ponds, however, Shin and Polprasert did report that their AGWSPs consistently produced an effluent of lower organic (COD) concentration than control ponds; suggesting a greater performance reliability of attached-growth ponds over open control ones. The authors went on to suggest that the increased microbial density within attached-growth systems provided for an increased resilience or ‘buffering capacity’ against hydraulic and organic shock-loadings—a notion later echoed by Peishi *et al.* (1993).

Peishi *et al.* (1993) reported 32% greater BOD<sub>5</sub> removals in an anaerobic AGWSP compared with a parallel anaerobic control pond when operated an OLR of  $\approx 42\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$  and 2% AGM packing density (AGM:water; v:v); although the authors offered no information regarding the specific surface area of their attached-growth pond

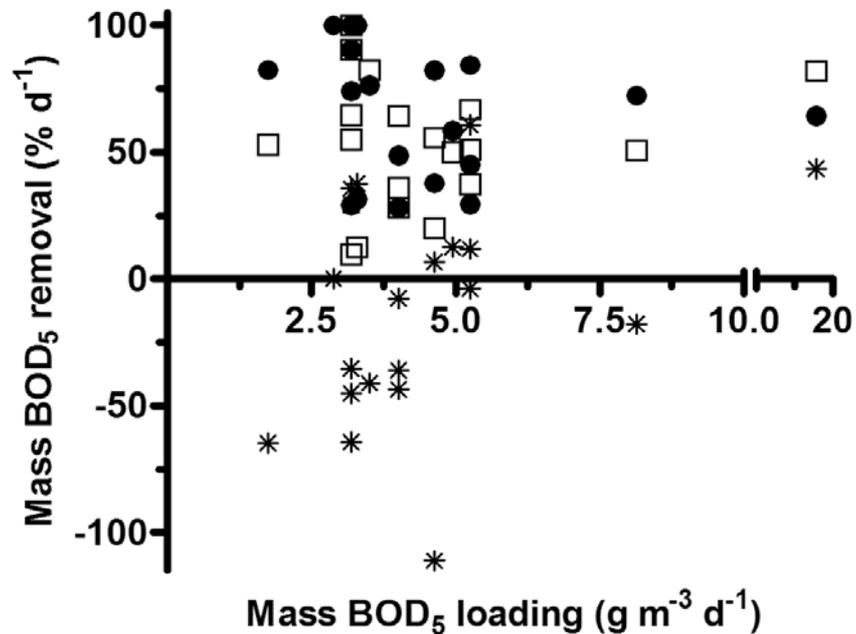
systems. Zhao and Wang (1996) observed some 15% improved BOD<sub>5</sub> removals in their pilot-scale AGM ponds compared to control ponds when operated at roughly twice the specific media surface area (270m<sup>2</sup> m<sup>-3</sup>) and an approximate 7-fold lower HLR (0.14m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>) to the current AGM system. Unlike the enhanced BOD<sub>5</sub> performance reliability of the current AGM ponds, levels of reported variability in BOD<sub>5</sub> removal performance appeared to be similar for both AGM and control ponds of Zhao and Wang. Furthermore, since the authors failed to state the organic concentration of their pilot AGWSP influent, no cross-comparisons can be made with respect to removal versus organic loading rate of these systems.

Rakkoed *et al.* (1999), following the operation of laboratory-scale (0.29m<sup>3</sup>) AGWSPs with 2-fold greater specific media surface area (300m<sup>2</sup> m<sup>-3</sup>) than the AGM used here, achieved very high BOD<sub>5</sub> removal efficiencies in the order of 96% when loaded at an organic loading of 36g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup> and a 40-fold lower HLR to that applied here (0.025m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>). In a second experiment, the authors doubled the HLR—increasing the OLR to 83g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>—and still achieved BOD<sub>5</sub> removal efficiencies in the order of 97%. Following practically identical performance results from parallel control ponds, however, the authors concluded that the organic treatment efficiency of both attached-growth and conventional WSPs were similar. In the case of Rakkoed *et al.* (1999), it was thought that the reactor volumes were too small and HRTs too long (20–40 days) to enable proper comparisons of treatment performance between conventional and AGM pond systems, such that any direct performance comparisons made between that system and the AGM reactors reported here are made tentatively. Following a final ‘shock-loading’ experiment with a greatly increased OLR (>200g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>) and significantly reduced hydraulic retention time (4 days), Rakkoed and co-workers were able to conclude that the AGM system did demonstrate a slightly better capacity to deal with organic and hydraulic shock loads; suggesting that the greater biomass densities facilitated higher rates of treatment under such conditions.

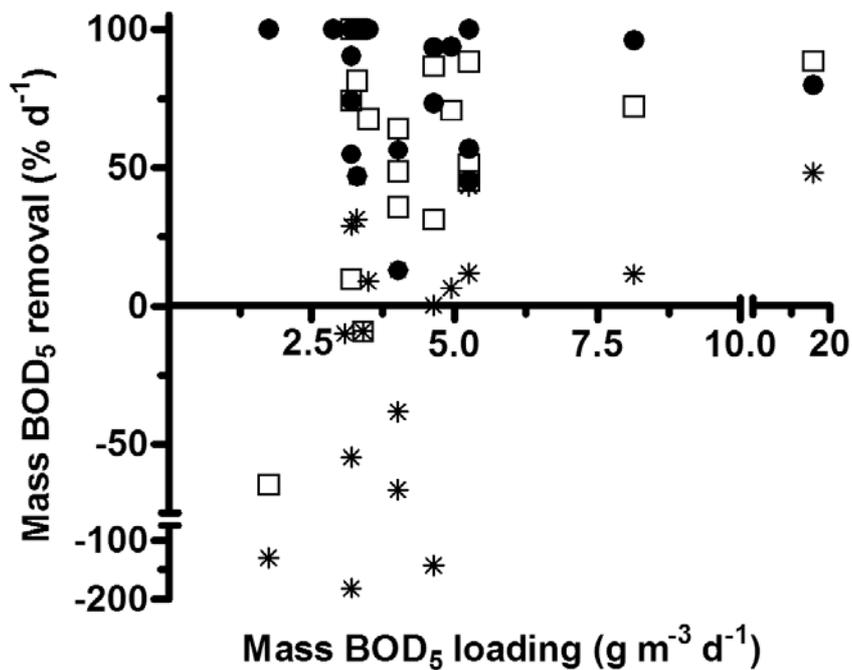
McLean (1999) achieved some 30% greater average organic (COD) removals in large pilot-scale (9000m<sup>3</sup>) AGWSPs compared with parallel control ponds when loaded at comparable organic loadings to those used in this research (7–15g COD or ≈3.2–7g BOD m<sup>-3</sup> d<sup>-1</sup>). Like observations made during the current work, the monitoring data of

McLean also indicated a greater reliability in the final effluent organic quality of AGM ponds compared with standard control ponds; however, the very low specific surface area ( $2\text{m}^2 \text{m}^{-3}$ ) of their attached-growth WSPs should be taken into consideration when comparing these results with those of the current system.

In order to gain a more comprehensive understanding as to the nature of  $\text{BOD}_5$  loading versus removal efficiency, the data from Figures 4.12 and 4.13 were plotted on a mass loading versus percentage removal basis, and are shown for Ponds 1 and 3 of each treatment in Figures 4.14 and 4.15 respectively.

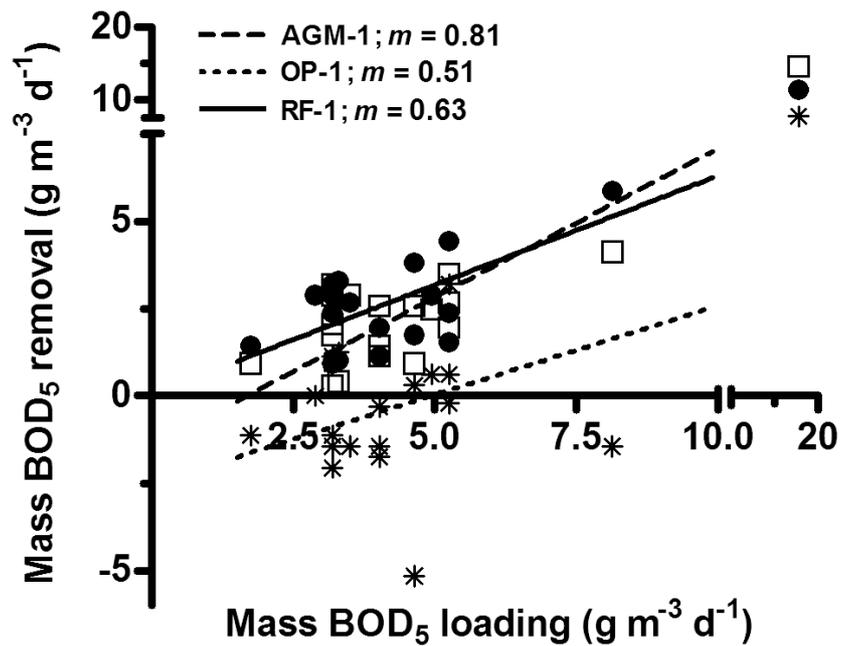


**Figure 4.14.** Scatter-plot showing  $\text{BOD}_5$  mass loading (pilot plant Influent) vs. mass removal (as a percentage of daily loading rate) for Pond 1 data only. Individual data points represent performance data from single determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□).

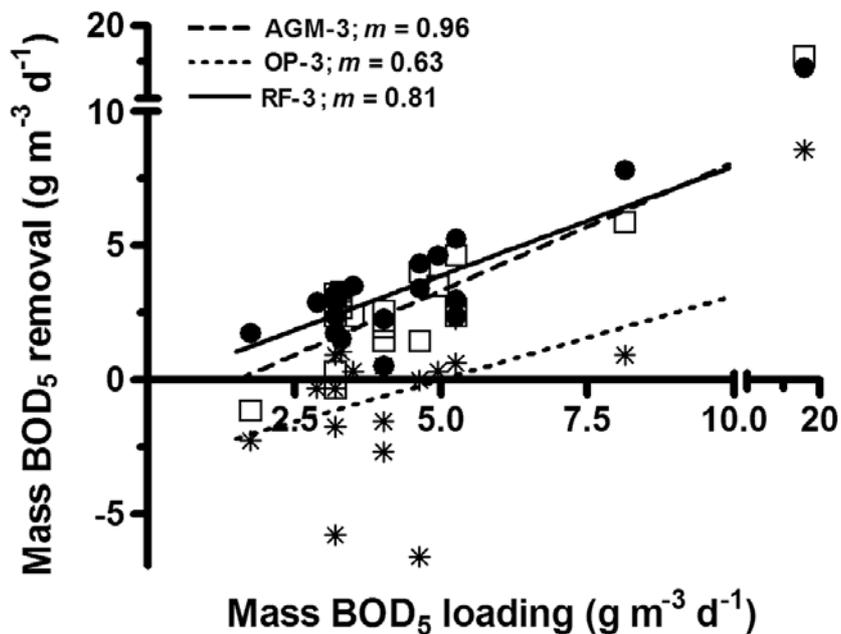


**Figure 4.15.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. mass removal (as a percentage of daily loading rate) for Pond 3 data only. Individual data points represent performance data from single determinations for: Rock Filter 3 (●); Open Pond 3 (\*); and Attached-Growth Media Reactor 3 (□).

As described in Section 3.3.2 and later in more detail within Section 3.3.5, the daily influent loading for both Ponds 1 and 3 is taken as that of the daily pilot plant influent sampled from the header tank (see Figure 2.1). As can be seen in the above Figures, the RFs and AGM Reactors were capable of delivering relatively high-level BOD<sub>5</sub> removal efficiency even at low influent loadings, whereas the BOD<sub>5</sub> removal performance of the OPs was far more variable and less efficient at the same loading rates—yielding negative removals on many occasions. Despite there only being two data points above 7g BOD<sub>5</sub> m<sup>-3</sup> d<sup>-1</sup>, there was again an apparent trend for higher-level treatment performance with increased organic loading (especially for the less efficient OPs). This was again a reflection of the first-order physical processes governing BOD<sub>5</sub> removal (described in Chapter 3). Figures 4.16 and 4.17 below show the same data as Figures 4.14 and 4.15 above, except the data is represented on a mass loading versus mass removal basis.



**Figure 4.16.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent performance data from single determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks. Individual treatment regression lines are shown with corresponding slope (*m*).



**Figure 4.17.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent performance data from single determinations for: Rock Filter 3 (●); Open Pond 3 (\*); and Attached-Growth Media Reactor 3 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks. Individual treatment regression lines are shown with corresponding slope (*m*).

When represented on a mass only basis, there was again a noticeable positive relationship between mass loading and the mass of BOD<sub>5</sub> removed. As seen in the above Figures 4.16 and 4.17, the relationship was evidently stronger for the higher performance RF and AGM treatments, with considerably greater variability for the OPs at low BOD<sub>5</sub> loadings. There was a highly significant positive relationship between mass load and mass removal for Pond 1 data of Figure 4.16 for the RF treatment (Pearson  $r = 0.912$ ;  $n = 22$ ;  $p < 0.0001$ ), an equally significant correlation for the AGM-1 data ( $r = 0.944$ ;  $n = 21$ ;  $p < 0.0001$ ) and a slightly less powerful relationship for OP-1 data ( $r = 0.688$ ;  $n = 21$ ;  $p < 0.001$ ). Similar trends between mass loading and BOD<sub>5</sub> mass removal were evident for the Pond 3 data of Figure 4.17, with a highly significant relationship for RF-3 ( $r = 0.931$ ;  $n = 22$ ;  $p < 0.0001$ ), an equally high-level correlation for the AGM-3 data ( $r = 0.943$ ;  $n = 21$ ;  $p < 0.0001$ ) and again a less powerful relationship for OP-3 data ( $r = 0.693$ ;  $n = 22$ ;  $p < 0.001$ ).

The fitted regression lines of Figures 4.16 and 4.17 provided additional insights into the relationship of mass loading versus mass BOD<sub>5</sub> removal. Critical analysis of both the slopes and elevations of the fitted trendlines provides for a more detailed between-treatment performance assessments than is afforded by the discrete correlation coefficient integer above. Looking at the linear regression data from Figures 4.16 and 4.17, there were again significant positive linear associations between the amount of loaded BOD<sub>5</sub> and the mass removed within each pilot treatment system. Regression coefficients were identical to the above Pearson correlation coefficients, with the slopes of all regression lines from both figures significantly greater than zero ( $p < 0.001$ ). For the Pond 1 data of Figure 4.16, there were small-scale significant differences between the overall slopes of the fitted lines (ANCOVA;  $F_{(2,55)} = 3.394$ ;  $p = 0.041$ ), with the AGM-1 trendline slope ( $m = 0.85$ ) significantly 'steeper' than both the OP-1 ( $m = 0.51$ ;  $p = 0.028$ ) and RF-1 data slopes ( $m = 0.63$ ;  $p = 0.031$ ). There were, however, no apparent differences between the slopes of the OP-1 and RF-1 data ( $p = 0.43$ ). For the Pond 3 data of Figure 4.17, this time there were no significant differences between the slopes of all three treatment regression lines (ANCOVA;  $F_{(2,56)} = 2.323$ ;  $p = 0.11$ ).

Even though the slope of AGM Pond 1 data from Figure 4.16 was greater than that of RF-1, this did not necessarily mean that the performance of AGM treatment Pond 1 was

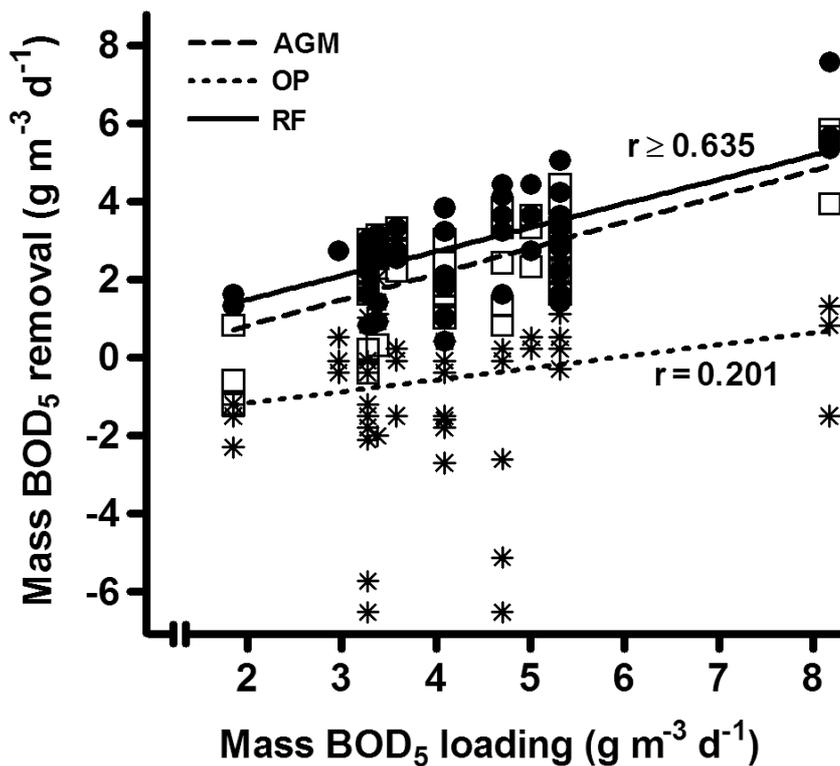


better than RF-1. As can be seen in Figure 4.16, the  $y$ -axis intercept of the RF-1 regression line was actually greater than that of AGM-1 data (inferring greater BOD<sub>5</sub> removals at lower loadings) and so a greater regression line slope does not mean greater BOD<sub>5</sub> removal performance unless the intercepts are equal. Given that the above regression difference between AGM-1 and RF-1 performance was only just outside the ' $p < 0.05$ ' cut-off ( $p = 0.041$ ), the small-scale Pond 1 slope difference was assumed to be of no real importance, especially in light of there being no significant differences between the slopes of the Pond 3 data regression lines (Figure 4.17;  $p = 0.11$ ).

Ignoring the small-scale differences between the slopes of the regressed lines of Figure 4.16, there were more importantly large-scale differences between the elevations of these fitted lines. Although the slopes were statistically equal, the elevations of the RF-1 regression line was much greater than for OP-1 data (ANCOVA;  $F_{(1,38)} = 44.88$ ;  $p < 0.0001$ ). Because the AGM-1 regression slope was significantly different to that of RF-1 and OP-1, it was not possible to test for differences between the elevations; although based on the above discussion, the performance of AGM-1 and RF-1 were considered equal and so AGM-1 performance can be ruled more advanced than OP-1. Similarly, and in spite of equivalent slopes for all treatment Pond 3 data, the elevations of both RF-3 and AGM-3 data were significantly greater than the parallel OP-3 data (ANCOVA;  $F_{(1,38)} > 30.92$ ;  $p < 0.0001$ ). This suggested that by the end of each three-pond series, whilst all treatments displayed an equivalent linear pattern for BOD<sub>5</sub> loading versus removal, the RFs and AGM Reactors were able to remove a greater mass of loaded BOD<sub>5</sub> at any given mass loading rate than were the parallel OPs (recalling that treatment performance in the above figures is essentially measured by the degree of  $y$ -axis elevation for each data point relative to the  $x$ -axis point of zero removal). As was the case during the Chapter 3 discussion of results, published data regarding the 'loading versus removal' performance of both rock filters and AGM ponds—for any parameter—is again lacking. Therefore, the above performance trends cannot be directly compared to those of other systems.

It was interesting to note that for the correlation analyses of Figures 4.16 and 4.17, one single high-level BOD<sub>5</sub> loading event was apparently responsible for maintaining the 'strong' relationship between mass loading and mass removal in the OP treatment data.

When this one-off statistically-extreme outlying loading event was removed from the overall analyses, the strength of the previous associations were greatly diminished to the point of statistical insignificance for the OP treatment Pond 1 data ( $r = 0.111$ ;  $n = 20$ ;  $p = 0.64$ ) and also for OP-3 ( $r = 0.288$ ;  $n = 20$ ;  $p = 0.21$ ) whilst the relationships remained unaffected in the RF and AGM treatments for both Pond 1 and 3 data ( $r \geq 0.685$ ;  $n \geq 20$ ;  $p \leq 0.0006$ ). This can be most easily seen in Figure 4.18, wherein there was no significant correlation for the combined three-pond OP series data in the absence of the outlier (Pearson  $r = 0.201$ ;  $n = 58$ ;  $p = 0.13$ ), but highly significant relationships for both the RF and AGM three-pond performance data remained ( $r \geq 0.635$ ;  $n \geq 57$ ;  $p < 0.0001$ ). This served to again highlight the significantly greater BOD<sub>5</sub> removal performance of both the RF and AGM treatments over the OPs, particularly at reduced influent mass loads.



**Figure 4.18.** Scatter-plot showing BOD<sub>5</sub> mass loading (pilot plant Influent) vs. total mass removal for the combined three-pond data of each treatment train (excluding the single extreme outlying loading event across all treatments). Individual data points represent performance data from single determinations for: Rock Filters (●); Open Ponds (\*); and Attached-Growth Media Reactors (□).

Presentation of BOD<sub>5</sub> performance data on a mass loading versus removal basis has highlighted the elevated performance of both the RF and AGM systems over that of the OP treatment. The quantity of BOD<sub>5</sub> removed by both the RF and AGM systems was more strongly associated with the amount flowing into the systems, as well as being removed more completely under any given mass BOD<sub>5</sub> load. The invariably reduced significance of associations between mass BOD<sub>5</sub> load and mass removal in the OP treatment train again reflected the increased variability in treatment performance for this particular pond system, and meant that BOD<sub>5</sub> effluent quality was far less predictable for the Open Ponds—especially under low loading rates.

Whilst it was not investigated, it should be noted that others have reported on an increase in AGWSP treatment performance efficiency with an increase in the ‘packing density’ of AGM into the system. Zhao and Wang (1996) found that pilot-scale AGWSP treatment performance increased with increasing AGM packing density from 0, 11 and 22% (AGM:water; v:v); with COD and BOD<sub>5</sub> removals increasing in efficiency under increased AGM packing densities. Peishi *et al.* (1993) observed a similar trend between AGM packing density and the corresponding COD and BOD<sub>5</sub> removal efficiencies in a small-scale model AGWSP, with pond performance consistently increasing up to the maximal AGM packing density of 40% (v:v). It is important to note, however, that with an increased AGM packing density (v:v) comes a corresponding *decrease* in hydraulic volume and accompanying HRT, such that performance efficiency of the AGWSP must therefore increase at a rate equal to (or indeed greater than) the rate of reduction in treatment efficiency owing to the reduced HRT.

The general upper-limit of AGM packing density within the literature appears to be in the order of 40%, with Shin and Polprasert (1987) finding an AGM packing density in the range of 5–10% to be most effective for organic (COD) removal and Zhao and Wang (1996) reporting an optimal figure of 22% for BOD<sub>5</sub>, COD and ammonia removal. Shin and Polprasert (1987) also discussed that whilst AGWSPs with 20 or 40% media packing density may have had a greater density of attached microbial biomass, the AGM itself blocked the majority of the pond environment from incident light penetration. This then restricted the degree of oxygenic algal photosynthesis—further limiting the already increased oxygen requirements of this bolstered microbial biomass and ultimately

stifling their capacity to oxidise organic materials. One can also appreciate that at very high standing biomass densities, the AGWSP would be increasingly vulnerable to the development of anaerobic conditions. It is suggested that the appropriate AGM packing density should be carefully pre-calibrated against the pond organic loading regime prior to installation, in order to ensure an appropriately balanced oxygen budget.

Performance monitoring of the three pilot treatment systems has shown a trend for greater absolute BOD<sub>5</sub> removals and also enhanced treatment consistency for the RF and AGM pond systems compared with the more conventional Open Ponds. Under the commonly low organic loadings, the RF and AGM series were invariably more efficient at attenuating inflowing BOD<sub>5</sub> than were the parallel Open Ponds, with the OP series frequently yielding negative BOD<sub>5</sub> removal efficiencies. Based on the above performance analyses, the overall ranking of treatment performance with respect to BOD<sub>5</sub> removal potential places the RF and AGM systems equal 1<sup>st</sup>, and the OP treatment 2<sup>nd</sup> overall in terms of BOD<sub>5</sub> removal rate, absolute treatment efficiency, and also performance reliability; although RF treatment efficiency was probably slightly more advanced than the AGM reactors.

#### **4.3.5 Wastewater treatment performance: suspended solids, turbidity and algal biomass removal**

As done for Chapter 3 (see Section 3.3.6), and for the purposes of results presentation and discussion here, the water quality parameters SS, turbidity and chlorophyll *a* have been grouped together within the one section. This was firstly done for SS and turbidity due to their high-level direct correlation within both the pilot plant influent ( $r_s = 0.842$ ;  $n = 36$ ;  $p < 0.0001$ ) and also the pooled *Period 2* pilot plant data from all 9 ponds of the three treatments ( $r_s = 0.840$ ;  $n = 312$ ;  $p < 0.0001$ ). Since the performance trends for turbidity data mirrored exactly those of SS, and as introduced in Section 3.3.6, only the SS performance data will be referenced during the coming discussion of results so as to avoid duplication of performance assessments. Secondly, the chlorophyll *a* data was also pooled together with SS and turbidity performance data in this instance because of the highly significant interrelationship of these three water quality parameters in the influent data set. The extent of these co-correlations can be seen in Table 4.5. Once again, the

BOD<sub>5</sub> performance data was discussed within the preceding section due to the uncharacteristic absence of such correlations between that particular parameter and: SS ( $p = 0.172$ ); turbidity ( $p = 0.926$ ); or chlorophyll *a* ( $p = 0.707$ ); within the pilot plant influent. Reasons for the absence of interrelationships between these commonly associated wastewater quality parameters have been discussed in Chapter 3 and were considered to have related to the highly refined nature of the Bolivar WSP effluent.

**Table 4.5.** Spearman's correlation matrix for pilot plant influent water quality parameters: suspended solids (SS); turbidity; chlorophyll *a*; and BOD<sub>5</sub>.

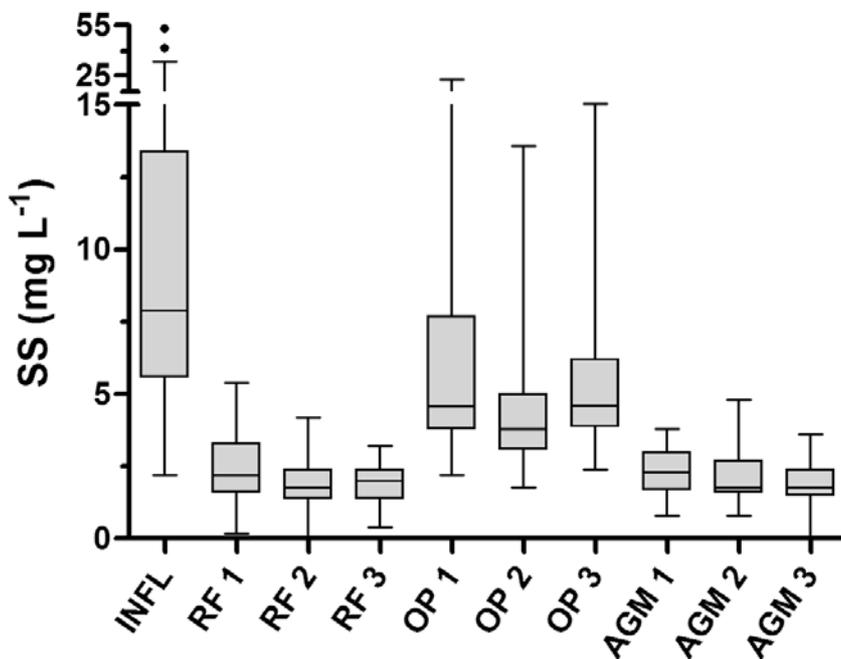
<b>Pilot plant influent</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>Chlorophyll <i>a</i> (μg L<sup>-1</sup>)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>				
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.842(***) 0.000 36			
<b>Chlorophyll <i>a</i> (μg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.760(***) 0.000 36	.741(***) 0.000 37		
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	0.302 0.172 22	-0.021 0.926 22	-0.085 0.707 22	

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

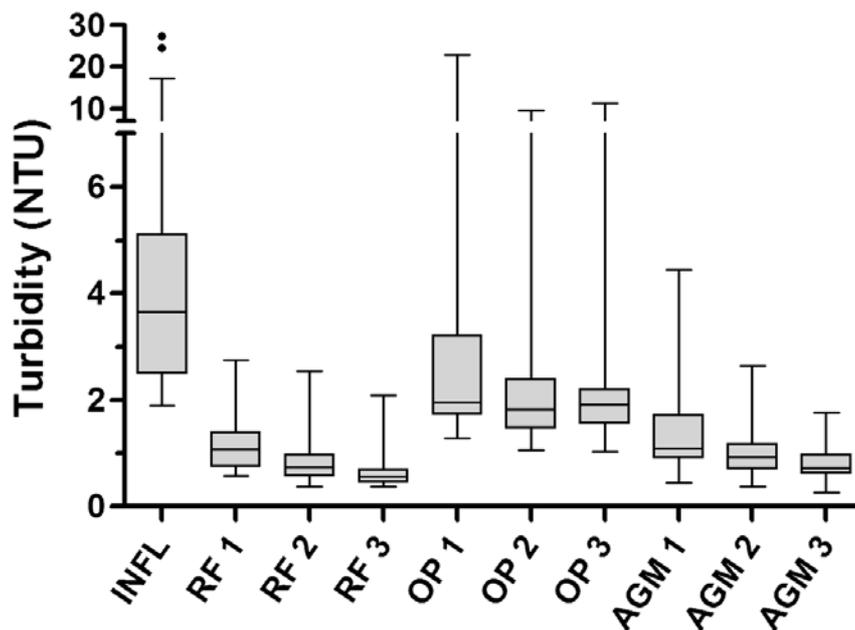
As was the case for the data analyses of Chapter 3, there were again instances of extreme outlying data points (i.e.  $>3 \times$  IQR from 75<sup>th</sup> percentile value) for both the SS and chlorophyll *a* influent data sets: two outliers for the SS data set of 43 and 54mg L<sup>-1</sup> (24 and 27 NTU); and one for chlorophyll *a* (111μg L<sup>-1</sup>). These outlying 'spike' events were again observed to be isolated incidents resulting from extreme wind-induced resuspension of benthic materials within the up-stream Bolivar WSP. This observation was again supported by the significant negative relationship between SS concentration and VSS fraction ( $r_s = -0.411$ ;  $n = 36$ ;  $p = 0.013$ ), indicating that SS spikes were largely fixed solids and not the result of algal blooms (see Section 3.3.6 for original discussion).

As for Chapter 3 performance analyses, these extreme outlying influent parameter values were again excluded from the current statistical analyses because it was thought that they did not accurately reflect the *normal* influent water quality state; an assumption further supported by the highly negatively skewed influent solids data (Figure 4.19). As

was also the case for previous Chapter 3 analyses, the order of treatment efficiency with respect to the between-treatment performance of each upgrade system during these outlying spike events remained unchanged, with the RF and AGM systems consistently out-performing the OPs during high solids and chlorophyll *a* spike loadings. Hence the omission of these extreme influent loading events did not alter the relative treatment efficiencies of the three-pond upgrades compared to each other, but instead served to provide a more accurate reflection of the performance of each system under normal low-level influent loading conditions.



**Figure 4.19.** Suspended solids box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range. Filled circles (●) above the INFL data represent the two extreme spike outliers  $>3\times$ IQR from the 75<sup>th</sup> percentile value.

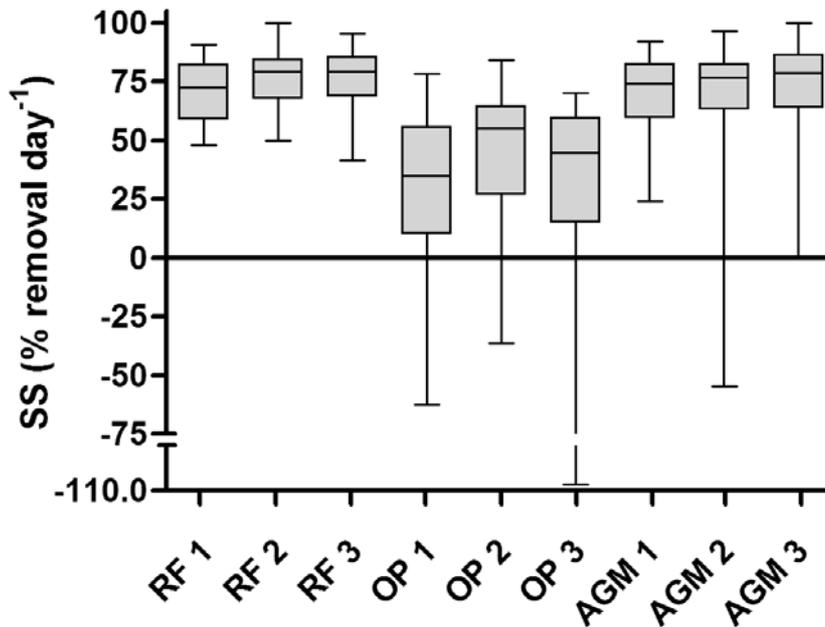


**Figure 4.20.** Turbidity box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). Filled circles (●) above the INFL data represent the two extreme spike outliers  $>3 \times \text{IQR}$  from the 75<sup>th</sup> percentile value.

As shown in Figure 4.19, pilot plant influent SS levels were generally very low but also highly variable on occasion, with a median SS concentration of  $7.9 \text{ mg L}^{-1}$  and a mean of  $10.0 \text{ mg SS L}^{-1}$ . This random and sometimes high-level variability in SS concentration within the Bolivar WSP effluent (i.e. pilot plant influent) is a common feature of such shallow pond systems; the sources of which have been discussed elsewhere (Section 3.3.5). Given the highly skewed nature of the influent SS data, the median influent concentration of  $7.9 \text{ mg L}^{-1}$  translated to a median mass solids loading during the six month monitoring period of  $8.14 \text{ g SS m}^{-3} \text{ d}^{-1}$ .

Statistical analysis of the data from Figure 4.19 showed that influent SS was reduced significantly down the pond series in all three RFs (1-way ANOVA;  $F_{(9,318)} = 31.52$ ;  $p < 0.001$ ) and similarly in all three AGM ponds ( $p < 0.001$ ), but only within Pond 2 and 3 of the OP series ( $p < 0.05$ ). The reasons behind the non-significant removal in OP-1 and less significant SS removals in Open Ponds 2 and 3 can be seen in Figure 4.19, whereby the OP series displayed both higher and more varied effluent SS levels than the other two treatments. Regarding between-treatment performance comparisons, the effluent SS concentration of all three RFs and all three AGM ponds was significantly

lower than that of the corresponding OPs ( $p < 0.001$ ). Between the RFs and AGM treatments, however, no such differences were apparent; with statistically identical effluent SS levels between the two treatment series across all pilot ponds ( $p > 0.99$ ). The data of Figure 4.19 is represented below as percentage removal efficiencies for each pilot treatment pond (Figure 4.21).



**Figure 4.21.** Box-plots showing percentage suspended solids removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n \geq 32$  for all plots).

Long term median percentage SS removals for Pond 1 data across the three treatments were 72, 35, and 74% for RF, OP and AGM treatments respectively, and for Pond 3 data, 79, 45 and 79% for the respective RF, OP and AGM treatments. When compared statistically to a theoretical zero median SS removal, these average percentage SS removal efficiencies were all found to be significantly ‘non-zero’ across all treatment series (Table 4.6), indicating that all SS removals were on average greater than zero. As was the case for BOD<sub>5</sub> performance assessment in Section 4.3.4, the RFs were once again the only treatment system to always yield positive SS removal efficiencies. The AGM system was not far behind the performance of the RFs, however, returning one single net increase in effluent SS for AGM-2. The OP treatment series performed significantly worse in this regard, recording zero removals or net increases in effluent SS on at least three (OPs 2 and 3) and up to five (OP-1) occasions. This trend for less



reliable treatment efficiency is again reflected in the corresponding CV's for treatment performance with respect to SS removal efficiencies (Table 4.6), where both RF and AGM treatments delivered significantly more consistent solids removal performance than the parallel OP series.

**Table 4.6.** Summary of suspended solids removal performance across all pilot plant treatments for Pond 1 and 3 data only.

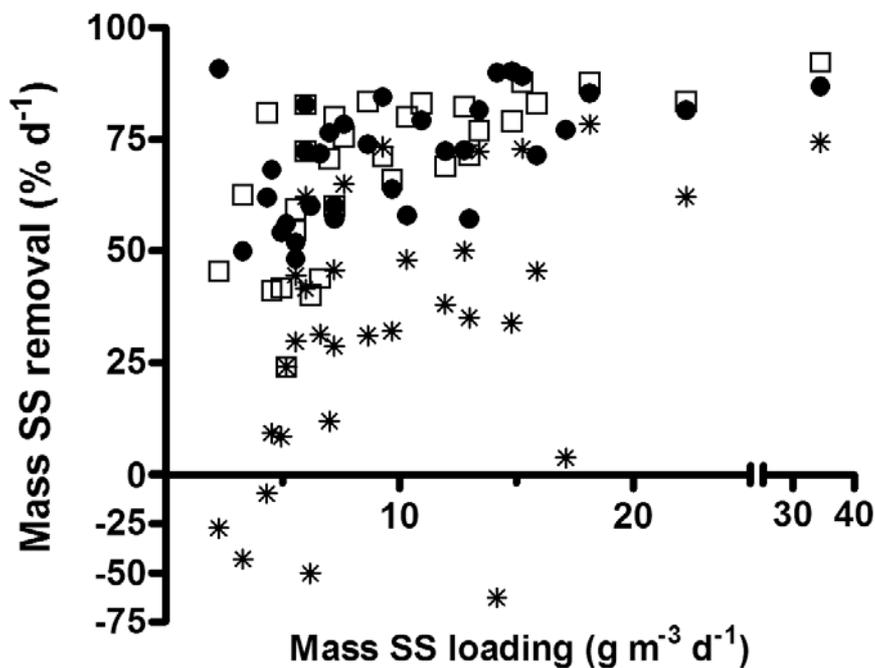
SS performance parameter	Pilot treatment pond					
	RF-1	RF-3	OP-1	OP-3	AGM-1	AGM-3
Median influent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	7.9	7.9	7.9	7.9	7.9	7.9
Mean influent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	10.0	10.0	10.0	10.0	10.0	10.0
Median effluent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup> *	2.2	2.0	5.0	4.6	2.3	1.8
Mean effluent SS (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	2.5	2.0	6.0	5.3	2.4	1.9
Median daily SS removal (% day <sup>-1</sup> ) <sup>‡</sup> *	72	79	35	45	74	79
Mean daily SS removal (% day <sup>-1</sup> )	71	76	30	34	69	73
Long-term CV for SS removal (%)	18	17	119	107	25	29

<sup>†</sup> Effluent SS concentration was tested relative to mean pilot plant influent SS concentration (1-way ANOVA)

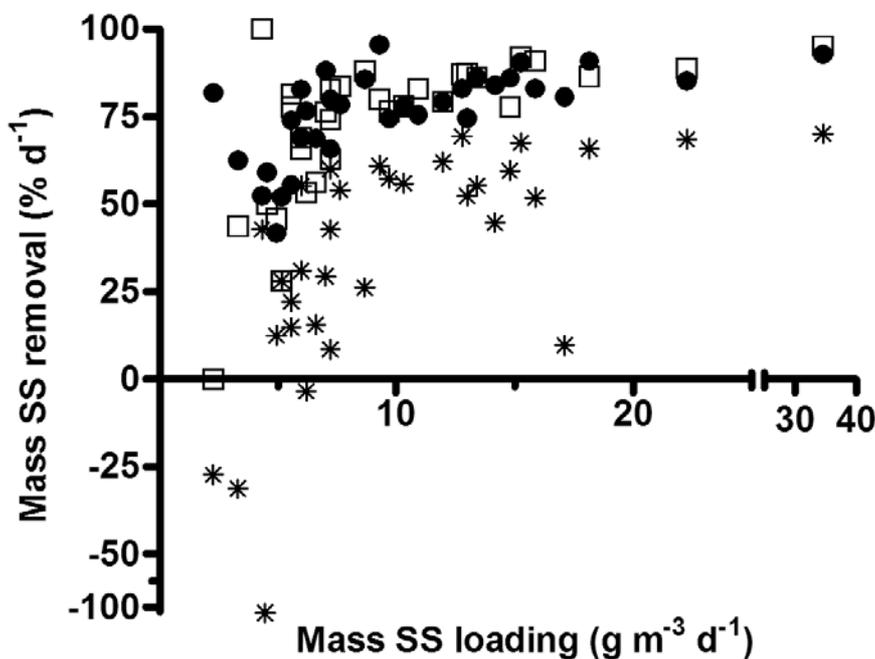
<sup>‡</sup> Average SS removal % tested against a theoretical 'zero' daily mean (one sample *t*-test)

\* Shading intensity shows significance level: *p* > 0.05 (no shading); *p* < 0.05 (light); *p* ≤ 0.001 (black)

Comparison of individual treatment CV's for SS removal efficiency again highlighted the enhanced consistency in treatment performance for the RF and AGM systems over the OPs. Not only did the RFs and AGM reactors deliver a better quality final effluent in terms of SS levels, but they achieved this with enhanced consistency compared with the OP treatment train. These trends for both greater absolute treatment efficiency, as well as enhanced performance reliability for the pilot RFs and AGM reactors were similar to the trends observed BOD<sub>5</sub> performance data of Section 4.3.4, and so will not be described in detail again. Suspended solids performance data from Figures 4.19 and 4.21 is re-presented below on a loading versus removal basis in order to provide a more detailed account of treatment performance relative to daily solids loading rate. This data is shown for all treatments in Figures 4.22–4.25 for Ponds 1 and 3 accordingly.



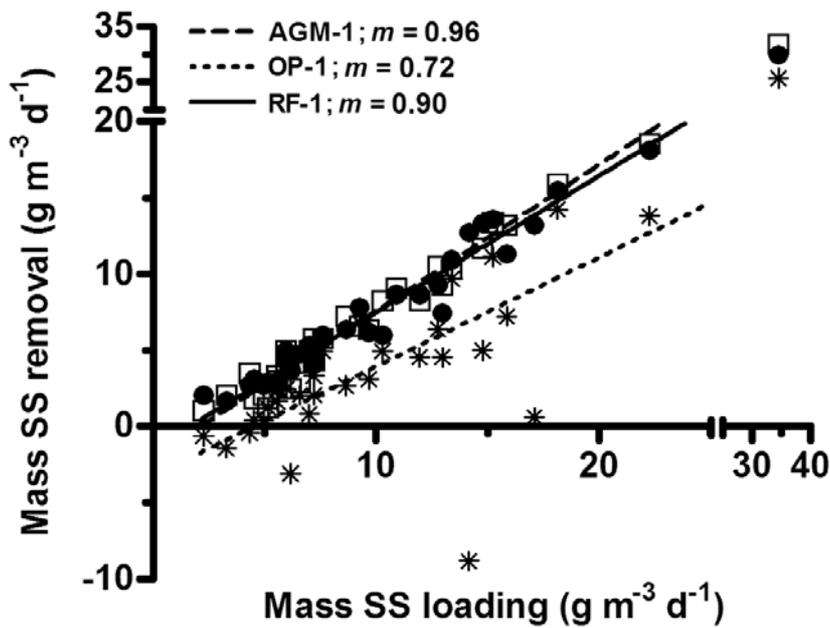
**Figure 4.22.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 1 data only (note the reduced y-axis scale for values below zero). Individual data points represent mean performance data from duplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□).



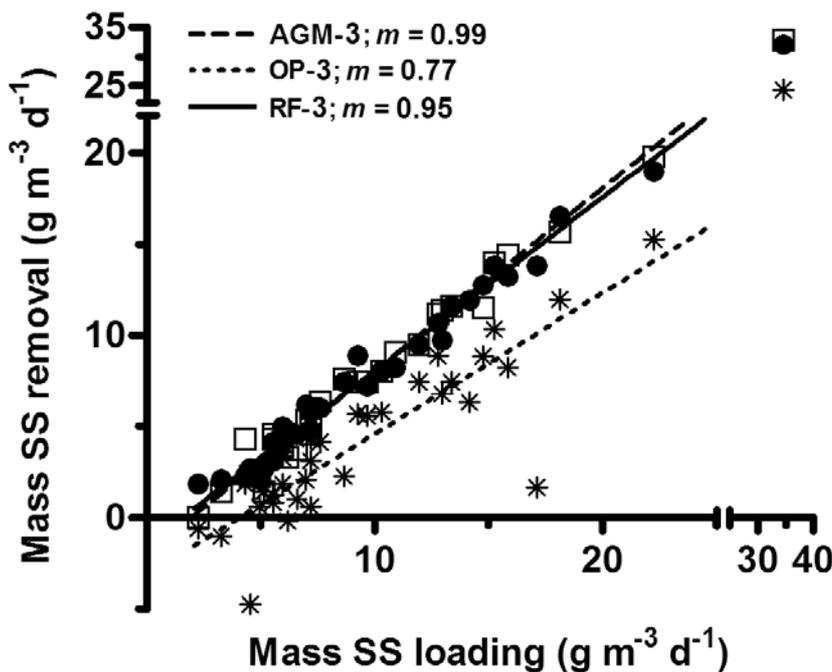
**Figure 4.23.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 3 data only (note the reduced y-axis scale for values below -50). Individual data points represent mean performance data from duplicate determinations for: Rock Filter 3 (●); Open Pond 3 (\*); and Attached-Growth Media Reactor 3 (□).

When considered on a mass loading versus percentage removal basis, there were again similar trends for the SS performance data as were recorded for BOD<sub>5</sub> performance data above. Visual analysis of Figures 4.22 and 4.23 above shows a general trend across both ponds 1 and 3 for an increase in percent SS removal efficiency at higher influent solids loads and *vice versa*. This meant again that under conditions of high influent SS loading, there was inherently more scope for greater solids removal, and so the larger the percentage magnitude removals were (again obviously up to the point of influent overloading). This noted trend was again a reflection of the first-order-type ‘concentration gradient’ effect, suggesting that SS removals within the pilot ponds were again governed by first-order-type processes as for BOD<sub>5</sub> above. Given that this issue was discussed at length in Chapter 3, no further explanation is provided here.

As can be seen in the Figures 4.22 and 4.23, SS removal efficiencies were especially variable for the lesser performing OP treatment at low influent loads. As was described in the previous Chapter (Section 3.3.6), these highly variable and sometimes negative SS removals were again thought to have been a result of primary and/or secondary biomass production during quiescent pilot plant passage. Periodically, and during favourable conditions, sometimes dense blooms (up to 490 individuals L<sup>-1</sup>) of large bodied metazoan zooplankton were observed within the OPs. During these times, the secondary biomass production rates would have exceeded those of normal SS sedimentation and microbial degradation within the ponds (see also Figures 5.35 and 5.39); an effect that would have been further amplified during periods of low influent SS concentrations. In addition to these factors, enhanced SS removals within the RF and AGM systems were somewhat anticipated. Given that the processes governing SS (including algal biomass) removal in wastewater environments are overwhelmingly physical in nature, and considering that particulate settlement is a direct function of water column depth (Swanson and Williamson, 1980; Reynolds, 1991), the RF and AGM treatments possessed an inherently greater capacity for solids retention than the OP reactors.



**Figure 4.24.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data from duplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and AGM Reactor 1 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks. Individual treatment regression lines are shown with corresponding slope (*m*).



**Figure 4.25.** Scatter-plot showing suspended solids mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent mean performance data from duplicate determinations for: Rock Filter 3 (●); Open Pond 3 (\*); and AGM Reactor 3 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks. Individual treatment regression lines are shown with corresponding slope (*m*).

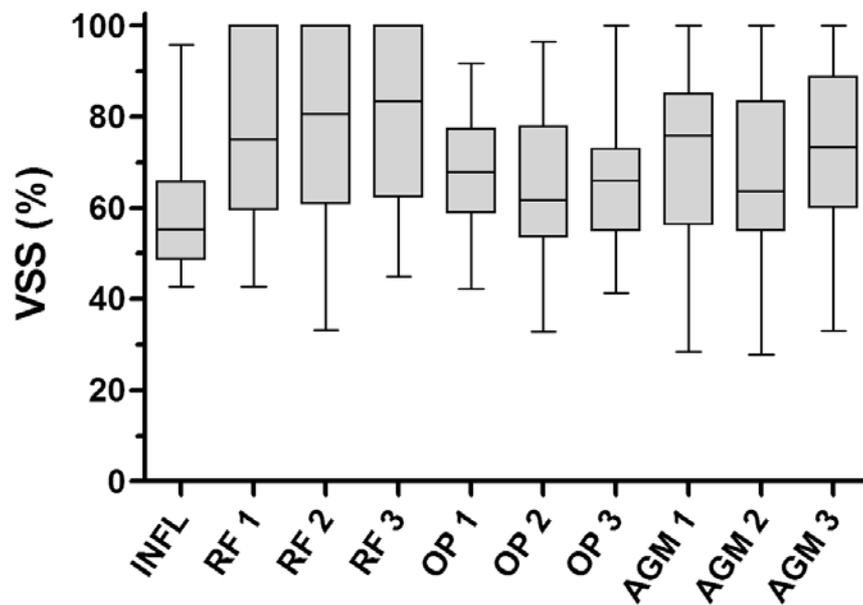
When the SS data is represented on a mass loading versus mass removal basis (Figures 4.24 and 4.25), and as was the case for BOD<sub>5</sub> performance data in the previous section, there was a noticeable direct positive relationship between mass loading and the mass of SS removed; the relationship being particularly strong under elevated solids loads. For the Pond 1 data of Figure 4.24, there were highly significant correlations between mass load and mass solids removals across all three treatments: for RF-1 (Pearson  $r = 0.986$ ;  $n = 33$ ;  $p < 0.0001$ ); AGM-1 ( $r = 0.991$ ;  $n = 32$ ;  $p < 0.0001$ ); and OP-1 ( $r = 0.779$ ;  $n = 33$ ;  $p < 0.0001$ ). Similarly, and for the Pond 3 data of Figure 4.25, there were again highly significant relationships for mass load versus mass SS removal in all treatments: RF-3 ( $r = 0.994$ ;  $n = 33$ ;  $p < 0.0001$ ); AGM-3 ( $r = 0.993$ ;  $n = 32$ ;  $p < 0.0001$ ); and OP-3 ( $r = 0.923$ ;  $n = 33$ ;  $p < 0.0001$ ). The existence of these significant correlations suggested that effluent SS was most commonly a direct reflection of influent concentration (i.e. concentration-dependent solids removal) and that mass removals were very predictable for all three treatments; although the strength of the relationship appeared qualitatively to be most robust for the RF and AGM treatments.

Looking at the fitted regression data from Figures 4.24 and 4.25 above, there were significant positive associations between the SS load and the mass of solids removed within each pilot treatment system. Regression coefficients were identical to the Pearson correlation coefficients above, with the slopes of all fitted regression lines significantly greater than zero ( $p < 0.0001$ ). For the Pond 1 and Pond 3 data of Figures 4.24 and 4.25 respectively, there were significant differences between the slopes of the fitted regression lines (ANCOVA;  $F_{(2,92)} \geq 3.91$ ;  $p \leq 0.023$ ); with the slopes of both the RF and AGM treatment regression lines significantly greater than the respective OP treatment data fits. Given that the  $y$ -axis intercepts of the RF and AGM trendlines were also both greater than the respective OP lines, this inferred a more advanced SS removal efficiency for the RF and AGM treatments over the OPs. There were, however, no differences between the slopes of the RF and AGM regression lines of either Figures 4.24 or 4.25 (ANCOVA;  $F_{(1,61)} \leq 3.03$ ;  $p \geq 0.089$ ), implying equivalently linear relationships between SS loading and removal for these two upgrade systems.

With respect to the elevations of the regressed lines from Figures 4.24 and 4.25, and since the regression slopes of OP-1 and OP-3 data were significantly different from the

corresponding RF and AGM data, it was not possible to test for differences between regression line elevations between the OP and other treatments. Regarding Pond 1 and Pond 3 RF and AGM data, however, there were no significant differences between the elevations of the fitted lines of Figures 4.24 and 4.25 (ANCOVA;  $F_{(1,62)} \leq 0.154$ ;  $p \geq 0.70$ ), suggesting equivalent mass SS removals for these two treatments at all loading rates. It should be pointed out here that the slope of 0.99 for the AGM-3 of Figure 4.25 does not imply near perfect solids removals across all mass loading rates. Because the regression line does not pass through the axial origin (i.e. 0,0) a slope of 1.0 in these plots does not infer complete SS removal. To illustrate this, when the regression line of AGM-3 is forced through the origin, the slope reduces from 0.99 to 0.86. Regardless of the precise magnitude of regression slopes, the greater the slope of the fitted lines for the above mass loading versus mass removal data, the greater the SS removal performance of that system is.

Results from the above regression analyses have shown the SS removal performance of both the RF and AGM treatments to be significantly more advanced than that of the parallel OP system. Generally speaking, the RF and AGM treatments were capable of removing a greater mass of SS under any given solids mass load than were the Open Ponds. This trend was reflected also in the earlier Figure 4.21, whereby SS removal efficiency in the OP series was shown to be both lower and more variable compared with the respective RF and AGM pond systems.



**Figure 4.26.** Relative volatile suspended solids fraction data (as a percent of total SS) for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

Data on the relative volatile fractions of total SS for the pilot plant influent and treatment ponds is shown in Figure 4.26. It should be stated that the sometimes high-level variability in VSS data from Figure 4.26 was considered to have been a reflection of the generally very low SS concentrations (commonly  $< 2\text{mg L}^{-1}$ ) and resultant increase in the degree of measurement error applied to such measurements when dealing with such small weights in the laboratory. A similar observation was noted by Martin (1970), with the author finding turbidity to be a more accurate measure of rock filter performance than SS at very low solids concentrations. Notwithstanding this, analysis of the data from Figure 4.26 showed that influent VSS fractions typically constituted about half of the total solids figure, with a median volatile solids fraction of 55%. No significant change in percentage VSS down the pond series within any of the OPs was recorded (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 42.5$ ;  $p > 0.05$ ); however, there were significant increases in VSS fraction within all three RFs ( $p \leq 0.01$ ) and also AGM Ponds 1 and 3 ( $p < 0.05$ ).

This lack of change in the relative proportions of ‘fixed’ and ‘volatile’ SS fractions within the OP series suggested—as for Chapter 3—that this system was indeed performing adequately as an ‘open control’ pond treatment in the sense that the *nature*

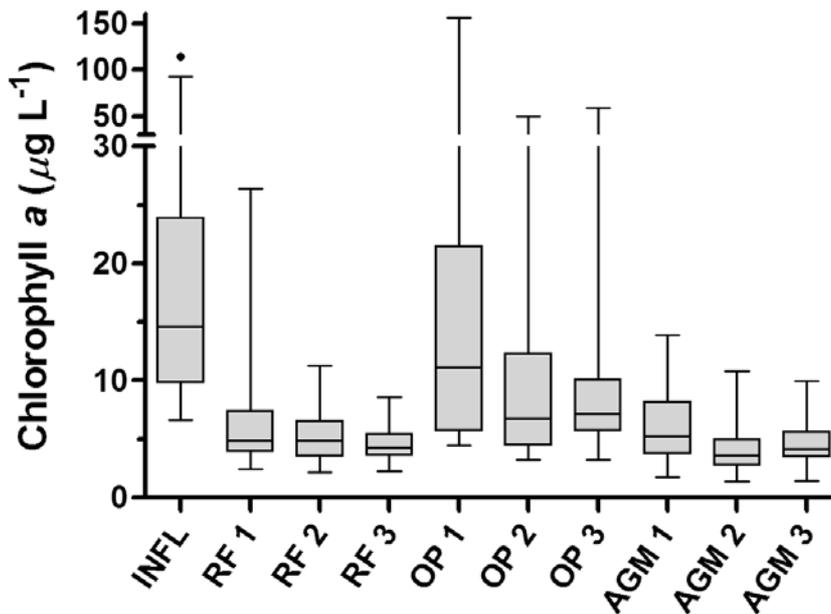
of the SS within the inflowing WSP effluent was not changing significantly down the pond series as a result of temporary pilot plant impoundment. The increasingly volatile nature of the SS within the RF and AGM effluent suggested that the inorganic or fixed solids were being removed more effectively within these systems than within the parallel OPs; allowing the RF and AGM trains to produce a final effluent both lower in total SS and also relatively more organic or 'biodegradable' in nature. In addition to this, it was likely that the increase in VSS within these two treatments was also a result of a small amount of biomass production and subsequent sloughing within these high specific surface area systems; especially so for the higher fluid velocity Rock Filters.

As for conventional WSPs, sludge accumulation in both RFs and AGM systems is a part of the overall treatment process resulting from accumulation of detached or 'sloughed' biomass and also sedimentation and entrapment of both fixed and volatile SS. As is the case for rock filters (e.g. Rich, 1988), anaerobic digestion of accumulated particulate organics is also thought to take place within AGM systems (Zhao and Wang, 1996). With reference to the work of others, Polprasert and Sookhanich (1995)—reporting on laboratory-scale ( $0.01\text{m}^3$ ) AGWSPs—observed that greater than 90% of total SS were volatile in nature, suggesting that the majority of the SS within their attached-growth ponds were comprised of organic biomass (presumably as a result of significant biomass sloughing). Although the fraction of VSS within the current AGM system did increase slightly down the pond series relative to influent levels (Figure 4.26), the failure to observe such high fractions of VSS was considered to have been a consequence of the vastly reduced organic strength of the Bolivar wastewater ( $< 5\text{mg BOD}_5 \text{L}^{-1}$ ) compared with the  $>1000\text{mg COD L}^{-1}$  wastewater of Polprasert and Sookhanich above; something that in turn prevented the development of such high-density attached-biomass. In this sense, the AGM ponds here were behaving less like a classical attached-growth pond system and more like the parallel RFs, in that they were predominantly acting as physical reactors for particulate settlement with very limited attached-growth biofilm development and an equally restricted biological treatment activity. Unlike a rock filter, however, the horizontal-flow AGM would offer the added advantages of an approximately 4-fold greater specific surface area and an approximate 100% increase in void space volume (Table 2.2), resulting in a reduction in dead volume and a subsequent increase in HRT and decrease in fluid velocity for more effective physical treatment.



Ignoring the respective 10- and 40-fold plus increases in bio-available substrate surface area within the respective RF and AGM systems compared to the OPs (Table 2.2), both the RF and AGM Reactors provide a significant increase in the number of horizontal ‘planes’ for physical sedimentation processes. Adding large numbers of sedimentation planes to the water column also has the effect of greatly reducing the discrete settlement depth for suspended particulates, and given that SS removal is known to be a direct function of settling depth (Reynolds, 1990), this alone could explain the higher solids removal performance of these two treatment systems over the OPs. In addition to this physical aspect, the effective ‘layering’ of substrate zones throughout the water column depth also serves to provide multiple sediment–water interfaces for acceleration of the recognised and important microbial and chemical exchange processes occurring within these zones. In this sense, having multiple ‘benthic sludge’ layers throughout the depth of the pond water column could offer potential benefits to overall pond treatment efficiency, given that sediments are recognised to contribute significantly to the overall wastewater treatment process through their role in N, P and heavy metal removal, as well as by supporting high-density micro- and macrobiotic populations (Naméche *et al.*, 1997). It is reasonable to conclude that these factors might have also contributed to the improved BOD<sub>5</sub> treatment performance for the RF and AGM systems described in Section 4.3.4.

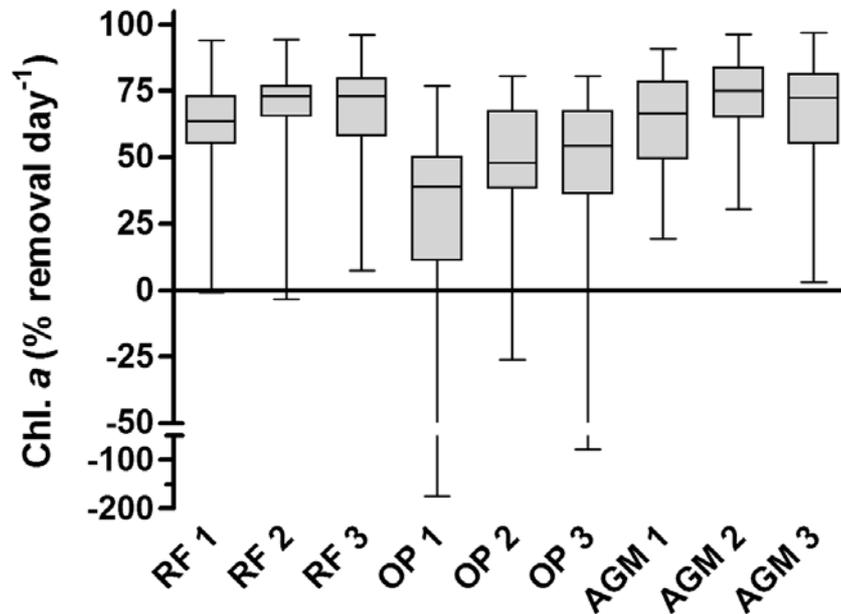
Similar to SS above, analysis of the chlorophyll *a* data showed that there were again generally low levels of suspended algal biomass within the pilot plant influent wastewater. Although chlorophyll *a* levels were commonly very low, there were instances of high-level variability, as shown by the large-scale difference between the median chlorophyll *a* concentration ( $14.6\mu\text{g L}^{-1}$ ) and corresponding mean ( $23.9\mu\text{g L}^{-1}$ ). The sources of such random and high-level variability in both SS and algal biomass density within the Bolivar WSP effluent (i.e. pilot plant influent) have already been discussed (see Section 3.3.5). Given the negatively skewed nature of the influent chlorophyll *a* data distribution, the median concentration of  $14.6\mu\text{g L}^{-1}$  was again used to calculate the mass loading rate; with an average loading during monitoring *Period 2* of  $15.0\text{mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$ . The pilot plant chlorophyll *a* data is shown below in Figure 4.27.



**Figure 4.27.** Chlorophyll *a* box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The filled circle (●) above the INFL data represents the single extreme outlying spike  $>3 \times \text{IQR}$  from the 75<sup>th</sup> percentile value.

Quantitative analysis of the data from Figure 4.27 showed that influent chlorophyll *a* levels were reduced significantly in all three RFs (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 135.6$ ;  $p < 0.001$ ) and all three AGM Reactors ( $p < 0.001$ ). For the OP treatment, however, there was no apparent reduction in chlorophyll *a* levels within OP-1 ( $p > 0.05$ ) but there were significant removals in both Open Ponds 2 ( $p < 0.01$ ) and 3 ( $p < 0.05$ ). Regarding between-treatment performance comparisons, the RFs and AGM ponds produced an effluent with significantly lower levels of chlorophyll *a* than the parallel OPs for all three respective ponds ( $p \leq 0.01$ ); however, chlorophyll *a* concentrations between the RF and AGM treatments were statistically identical for all parallel ponds down the treatment series ( $p > 0.05$ ). Considering the above chlorophyll *a* data, not only did the RFs and AGM ponds remove significantly more of the loaded algal biomass than the OPs, but they were also able to remove this inflowing algal biomass at a significantly enhanced rate down the pond series than were the OPs. This trend for an increased speed of removal down the pond series has been evident in all of the respective performance parameter plots so far (Figures 4.12, 4.19, 4.20 and 4.27) and suggests a greater capacity for rapid treatment within RFs and AGM systems compared with a standard OP Reactor.

The data of Figure 4.27 is again represented below as percentage removal efficiency for each pilot treatment pond (Figure 4.28).



**Figure 4.28.** Box-plots showing percentage chlorophyll *a* removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n \geq 34$  for all plots).

Long term median percentage daily chlorophyll *a* removals for Pond 1 data across the three treatments were 64, 39 and 67% for RF, OP and AGM treatments respectively, and for Pond 3 data, 73, 54 and 72% for the respective RF, OP and AGM treatments (Figure 4.28). When compared statistically to a theoretical zero median chlorophyll *a* removal, these average percentage chlorophyll *a* removal efficiencies were all found to be significantly non-zero across all ponds of all treatment series at the  $p \leq 0.05$  level (Table 4.7), suggesting that average long-term algal removals across all treatments were statistically greater than zero. Unlike prior performance analyses, this time the AGM pond system was the only treatment train not to yield negative parameter removals on at least one occasion; although the RFs were not far behind, with just two daily negative chlorophyll *a* removals of less than 3.5% each. Conversely to the AGM and RF series, the OP treatment frequently yielded net increases in chlorophyll *a* concentration down the pond series, with a treatment average of four negative removals per pond. This trend can be seen within the magnitude of the corresponding CV's for chlorophyll *a* removal

performance (Table 4.7), where both the RF and AGM treatment series delivered much more consistent chlorophyll *a* removals than did the parallel Open Ponds.

**Table 4.7.** Summary of chlorophyll *a* removal efficiencies across all three pilot plant treatments for Pond 1 and 3 data only.

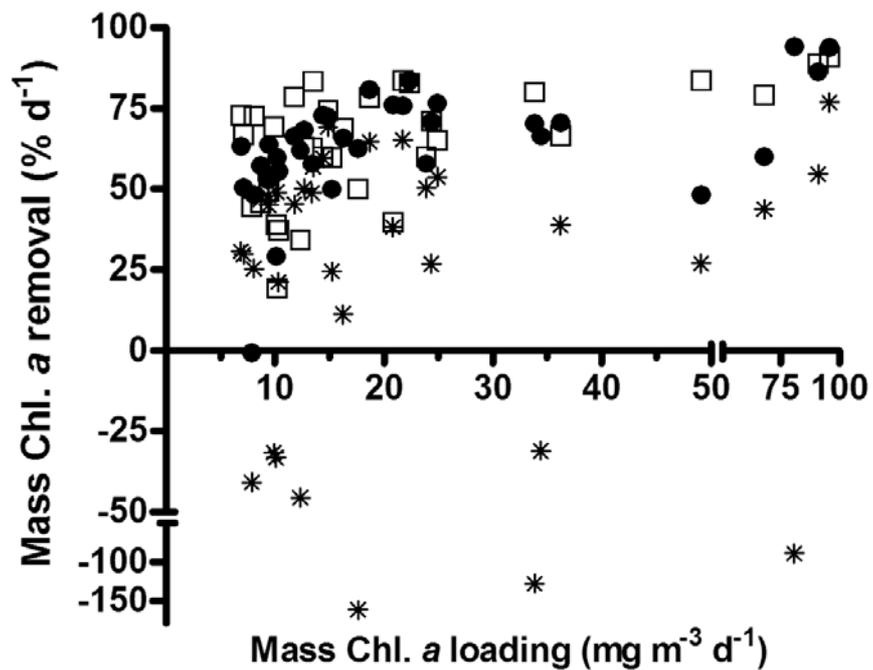
Chl. <i>a</i> performance parameter	Pilot treatment pond					
	RF-1	RF-3	OP-1	OP-3	AGM-1	AGM-3
Median influent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	14.6	14.6	14.6	14.6	14.6	14.6
Mean influent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	23.9	23.9	23.9	23.9	23.9	23.9
Median effluent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ ) <sup>†*</sup>	4.9	4.3	11.0	7.2	5.3	4.1
Mean effluent Chl. <i>a</i> ( $\mu\text{g L}^{-1}$ ; $\text{mg m}^{-3}$ )	6.8	4.8	20.0	11.0	6.0	4.6
Median daily Chl. <i>a</i> removal (% day <sup>-1</sup> ) <sup>‡*</sup>	64	73	39	54	67	72
Mean daily Chl. <i>a</i> removal (% day <sup>-1</sup> )	64	69	17	45	64	68
Long-term CV for Chl. <i>a</i> removal (%)	28	25	337	72	28	29

<sup>†</sup> Effluent chlorophyll *a* concentration was tested relative to median pilot plant influent concentration (Kruskal–Wallis test)

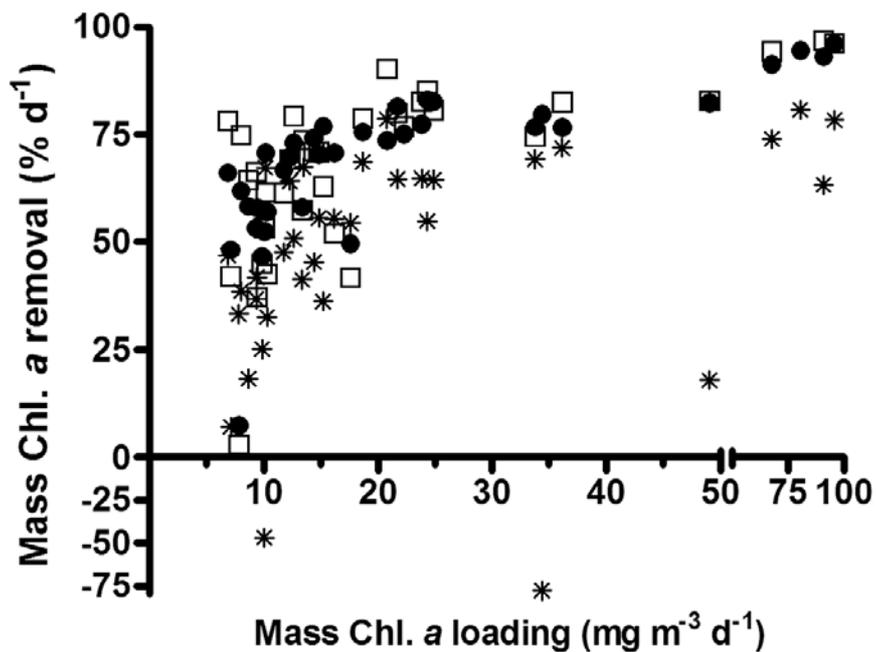
<sup>‡</sup> Average chlorophyll *a* removal % tested against a theoretical 'zero' daily median (Wilcoxon signed-rank test)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium);  $p \leq 0.001$  (black)

Looking at the performance CV's for chlorophyll *a* removal efficiency together with the data of Figure 4.28, gives an indication of the enhanced consistency in performance delivery for both the RF and AGM systems over the OP treatment—a trend noted for both BOD<sub>5</sub> and SS removals above. This trend for greater performance reliability has been discussed previously with respect to RFs and also AGM systems and so will not be reiterated here. As was done for the previous BOD<sub>5</sub> and SS data, chlorophyll *a* performance data from Figures 4.27 and 4.28 is represented on a loading versus removal basis below. This data is shown for all treatments for the data of Ponds 1 and 3 in Figures 4.29–4.32.

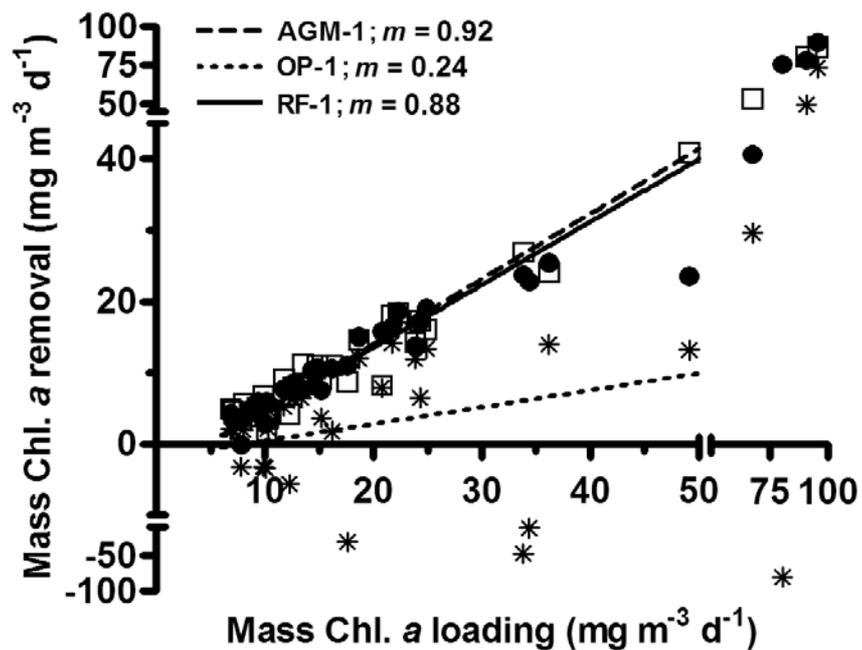


**Figure 4.29.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 1 data only. Individual data points represent mean performance data from triplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□).

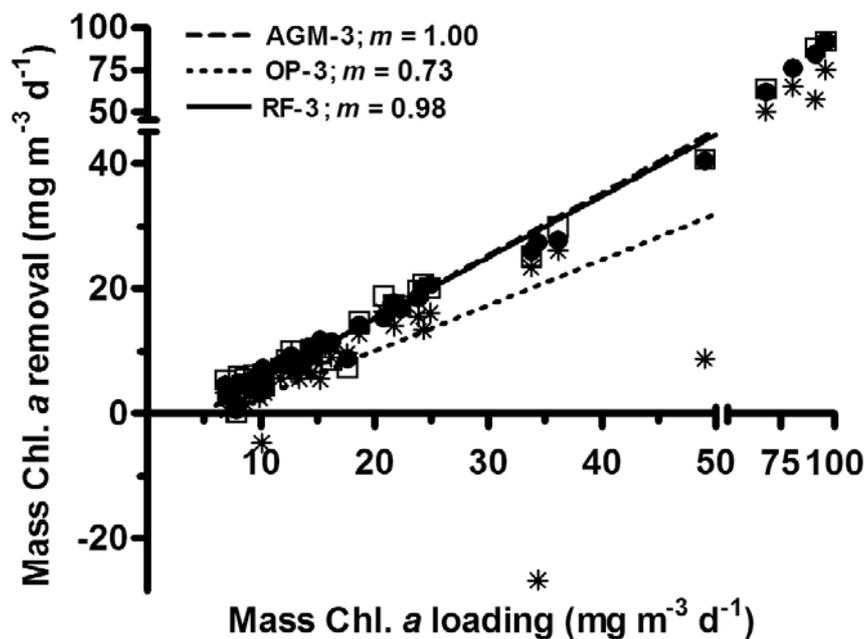


**Figure 4.30.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 3 data only (note the reduced y-axis scale for values below zero). Individual data points represent mean performance data from triplicate determinations for Rock Filter 3 (●); Open Pond 3 (\*); and Attached-Growth Media Reactor 3 (□).

When considered on a mass loading versus percentage removal basis, there were similar trends for the chlorophyll *a* performance data as were seen for both BOD<sub>5</sub> and SS removals above. The data of Figures 4.29 and 4.30 both displayed a general trend across both Ponds 1 and 3 for an increase in chlorophyll *a* removal efficiency at higher influent algal loads, suggesting again that chlorophyll *a* removal was predominantly governed by first-order-type processes. As can also be seen in the above Figures, and as was also the case for BOD<sub>5</sub> and SS earlier, under reduced chlorophyll *a* loadings, the removal performance of all treatments was more highly variable; especially for the OP series, where effluent algal concentrations were largely independent of influent algal loads at loading rates < 20mg m<sup>-3</sup> d<sup>-1</sup>. It was likely that for the OP treatment in particular, there were several confounding factors that adversely impacted on the measured algal removal capacity of the pond system. As described in the previous Chapter (Section 3.3.6), these related specifically to the periodic occurrence of both zooplankton and filamentous green algal blooms within the OP train, both of which served to elevate the levels of suspended chlorophyll *a* in daily effluent samples. Since this issue was discussed in great detail within Section 3.3.6, no further discussion is provided here. It should be emphasized, however, that these same problems did not affect the RFs or AGM ponds at any stage during the same monitoring period.



**Figure 4.31.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data from triplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks.



**Figure 4.32.** Scatter-plot showing chlorophyll *a* mass loading (pilot plant Influent) vs. total mass removal for Pond 3 data only. Individual data points represent mean performance data from triplicate determinations for: Rock Filter 3 (●); Open Pond 3 (\*); and Attached-Growth Media Reactor 3 (□). Linear regression lines were fitted to the entire data set, but for ease of presentation are shown only to the point of *x*- and *y*-axis breaks.

When the chlorophyll *a* data is represented solely on a mass basis (Figures 4.31 and 4.32), there was again a noticeable direct relationship between mass loading and the mass of chlorophyll *a* removed across all treatments; particularly for the RF and AGM treatments and especially under high influent loads. For the Pond 1 data of Figure 4.31, there were highly significant correlations between mass load and mass algal removals for RF-1 (Pearson  $r = 0.976$ ;  $n = 35$ ;  $p < 0.0001$ ) and AGM-1 ( $r = 0.992$ ;  $n = 35$ ;  $p < 0.0001$ ), but not for the more scattered data of OP-1 ( $r = 0.236$ ;  $n = 35$ ;  $p = 0.172$ ). For the Pond 3 data of Figure 4.32, there were again highly significant relationships for mass load versus mass SS removal in RF-3 ( $r = 0.988$ ;  $n = 35$ ;  $p < 0.0001$ ) and AGM-3 ( $r = 0.951$ ;  $n = 34$ ;  $p < 0.0001$ ) and this time also for OP-3 data ( $r = 0.818$ ;  $n = 35$ ;  $p < 0.0001$ ). The existence of these significant correlations suggested that effluent SS was predominantly a direct reflection of influent concentration for RFs and AGM Ponds 1 and all three treatments by the third pond in each series (i.e. concentration-dependent removal). This also suggested that chlorophyll *a* removals were largely predictable for RF-1 and AGM-1 as well as for all three treatments by Pond 3; although the strength of the relationship was evidently stronger for the RF and AGM treatments.

The relative strength of positive association between algal biomass load and the mass of algal solids removed for each of the pilot upgrade systems is highlighted by the fitted regression lines of Figures 4.31 and 4.32. Regression coefficients were identical to the Pearson correlation coefficients above, with the slopes of all fitted regression lines—except that of OP-1 ( $p = 172$ )—significantly greater than zero ( $p < 0.0001$ ). For the Pond 1 and Pond 3 data of Figures 4.31 and 4.32 respectively, there were significant differences between the slopes of the fitted regression lines (ANCOVA;  $F_{(2,98)} \geq 12.11$ ;  $p \leq 0.0001$ ). In both Pond 1 and Pond 3 data, the slopes of both the RF and AGM treatment regression lines were significantly greater than the respective OP treatment data fits ( $p < 0.001$ ), implying greater mass chlorophyll *a* removals for the RFs and AGM ponds at the observed range of loading rates. Between RF and AGM data of Figures 4.31 and 4.32, there were, however, no differences between the regression slopes of either treatment in Ponds 1 and 3 (ANCOVA;  $F_{(1,65)} \leq 1.12$ ;  $p \geq 0.294$ ), implying equally linear relationships between chlorophyll *a* loading and mass removal for these two pilot treatment systems.



With respect to the elevations of the regressed lines from Figures 4.31 and 4.32, since the regression slopes of OP-1 and OP-3 data were significantly reduced compared with the corresponding RF and AGM data, it was not possible to test for differences between regression line elevations between the OP and other treatments. With respect to the Pond 1 and Pond 3 RF and AGM data, however, there were no significant differences between the elevations of the fitted lines of the respective Figures 4.31 and 4.32 (ANCOVA;  $F_{(1,66)} \leq 0.400$ ;  $p \geq 0.53$ ), suggesting equivalent algal biomass removals for these two treatments at all encountered loading rates. It should again be pointed out that the slope of 1.00 for the AGM-3 of Figure 4.32 does not imply perfect chlorophyll *a* removals across all loading rates. Because the regression line did not intersect the origin, a slope of 1.0 does not confer complete removal. When the regression line of AGM-3 is forced through the origin, the slope reduces from 1.00 to 0.89. Once again regardless of the precise slope magnitude, the greater the slope of the fitted lines for the above mass loading versus mass removal data, the greater the performance efficiency of that system.

In comparing the SS and chlorophyll *a* performance data of the current Section to the work of others, and as described earlier (Section 4.3.4), RF performance results from this research will only be referenced to others' findings where similarly high HLRs were applied (i.e.  $\geq 1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ) to similar pilot-scale systems. With respect to the RF SS performance data, results were again comparable to those within the small body of relevant literature. The work of Mara *et al.* (2001) is in apparent agreement with the current RF performance data, whereby 63% SS removals were achieved (compared with the current 75% removals) at an HLR practically identical to that used here ( $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ) but under an 8-fold higher influent solids load ( $65 \text{ g SS m}^{-3} \text{ d}^{-1}$ ). Unlike SS, however, chlorophyll *a* removal performance was significantly greater for the rock filters of Mara and co-workers compared with the current RFs, with average 89% removals achieved at some 25-fold greater algal biomass loads ( $383 \text{ mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$ ). The RFs reported here also showed a greater solids removal performance than those of von Sperling *et al.* (2007). Following the operation of an equivalent volume pilot-scale RF, again at a practically identical HLR of  $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ , von Sperling and co-workers recorded lower median SS removals of 45% compared with the 75% removals achieved here. Despite the equivalent HLR, their RF received a 10-fold greater solids mass loading rate ( $\approx 90 \text{ g SS m}^{-3} \text{ d}^{-1}$ ) and so this was again thought to have

contributed to the reduced SS performance efficiency of their rock filter. Chlorophyll *a* removals were also lower for the rock filters of von Sperling *et al.* (2007), with average 56% removal efficiency at greatly elevated algal biomass loads ( $670\text{mg chlorophyll } a \text{ m}^{-3} \text{ d}^{-1}$ ) compared with the rock filters here ( $15\text{mg m}^{-3} \text{ d}^{-1}$ ).

Although SS removals were generally higher for the current RFs, the solids mass loads applied were much lower. It is not known what effect (if any) an increased average solids mass loading rate would have had on filter performance; however, data from Figures 4.22 and 4.23 suggests that Bolivar rock filters should be able to cope sufficiently well with higher influent SS loads. Additionally, there were likely to have been differences in the *nature* of the SS between the current system and those of the above authors; differences that might have had some bearing on solids removal efficiency. Although von Sperling *et al.* (2007) offered no information regarding the constitution of their influent solids, the work of Mara *et al.* (2001) suggests that their rock filter influent SS were significantly more organic in nature (71% compared with 55% VSS here). This could imply that there was inherently more scope for physical removal of the largely inorganic Bolivar SS than there was for the relatively more organic solids of Mara and co-workers. In fact on the basis of chlorophyll *a* concentrations, algal biomass accounted for roughly 60% of total SS for the rock filter influent of Mara *et al.* (as opposed to the  $\approx 18\%$  algal SS here), and taking into account the capacity of some algal species for buoyancy regulation, it is conceivable that this may have in some way contributed to their lower solids removals; although this concept remains purely speculative.

With respect to the SS and chlorophyll *a* performance of other AGM systems, performance data is overwhelmingly scarce. Shin and Polprasert (1987) reported that SS concentrations in the effluent of their pilot-scale AGM Reactors were consistently 10% lower than in parallel control ponds. The authors also reported an overall increase in effluent SS quality, as well as an increase in the *reliability* of AGM performance delivery over control ponds—a trend noted during this research. McLean (1999) and McLean *et al.* (2000) achieved a greater average SS removal efficiency in large pilot-scale AGWSPs compared with parallel control ponds, with  $\approx 45\%$  lower effluent SS concentrations compared with the control pond; very similar to the  $\approx 55\%$  lower effluent

SS seen here for the AGM ponds compared with the OPs. McLean's two year monitoring data also indicated a greater reliability in effluent SS levels for AGM ponds compared with parallel open ponds, with these control ponds recording close-to-zero or negative SS removal efficiencies on several occasions; something not observed for their AGM ponds. Rakkoed *et al.* (1999), following the operation of laboratory-scale ( $0.29\text{m}^3$ ) AGWSPs with 2-fold greater specific surface area ( $300\text{m}^2\ \text{m}^{-3}$ ), observed lower SS removals to those of the current AGM ponds (in the order of 45%) when loaded at an approximate 3-fold lower solids loading rate of  $3\text{g SS m}^{-3}\ \text{d}^{-1}$ . As discussed earlier, and given the very small volumetric scale of their attached-growth ponds, direct comparisons between the results of the current pilot-scale AGWSPs and those of Rakkoed and co-workers should be made with due caution.

Regarding chlorophyll *a* performance data, McLean (1999) and McLean *et al.* (2000) appear to have been the only authors to quantitatively report on algal removal efficiency in AGM ponds. The authors reported an overall approximate 16% reduction in algal SS (based on chlorophyll *a*) in large pilot-scale AGWSPs compared with an overall net 43% increase in chlorophyll *a* levels in their parallel control WSP; inferring a greater reliability for algal solids removal in their attached-growth ponds compared with open WSPs. Whilst average percentage chlorophyll *a* removals were significantly greater for the AGM ponds here ( $\approx 68\%$ ), algal biomass loads were slightly higher in the ponds of McLean and co-workers ( $\approx 20\text{mg chlorophyll } a\ \text{m}^{-3}\ \text{d}^{-1}$ ). Furthermore, and as introduced earlier (Section 4.3.2), the specific surface area of McLean's AGWSPs was some 75 times lower than the AGM Reactors reported here (at just  $2\text{m}^3\ \text{m}^{-3}$ ) and so it is highly likely that this contributed to their lower algal removals. Also, and unlike the current AGM Reactors, the attached-growth ponds of McLean *et al.* were exposed to incident sunlight—no doubt promoting algal growth within their system. Polprasert and Sookhanich (1995) did report on high concentrations of chlorophyll *a* in their AGWSP effluents ( $56\text{--}235\ \mu\text{g L}^{-1}$ ); however, in this case the high levels of suspended algae came from biomass sloughing and was seen as advantageous to the overall treatment of the toxic organic wastewater. Therefore, since the work of Polprasert and Sookhanich was not concerned with algal removal as such, results cannot feasibly be compared to those of the current AGM system. Aside from this limited volume of work, there appears to be

no additional research reporting on AGM systems and algal concentrations *per se*, especially so with respect to their potential for algal solids removal from WSP effluents.

As introduced in Chapter 1 (Section 1.2.8.7.3), particulate removal in AGM systems in general can be assisted by the growth of biofilms on the media surfaces that can then attract small infiltrating particles (either inorganic or organic in nature). In other types of biologically active filters with low water velocity, electrical interactions between the organic particles and the charged media surface can encourage particle attachment and removal (McDowell-Boyer *et al.*, 1986). It should be noted that the horizontal-flow AGM polypropylene substrate used here, is—based on the manufacturer’s specifications—negatively charged, and according to Huang *et al.* (1992), materials bearing a net charge (positive or negative) have been found to support the greatest microbial (*Pseudomonas* species) biofilm accumulation and hence the greatest biomass density. It is possible then that electrostatic surface charge interactions might have in some way contributed to the good treatment performance of the pilot AGM pond systems here, although no attempt was made to investigate such effects and so their potential influence on recorded performance remains purely hypothetical.

Whilst some authors have reported the persistence of well developed biofilms on their AGM, no attempts were made to probe the attached biofilm during the current work. Shin and Polprasert (1987) reported a linear increase in biofilm thickness in their pilot-scale AGWSPs up to a period of 30 days, after which time the biofilm reached a steady-state operational thickness of 60–80 $\mu\text{m}$ . Zhao and Wang (1996) reported similar findings, with AGM biomass thickness increasing up to 15–30 days following start-up, after which time it remained constant at a ‘steady-state’ biofilm thickness in the order of 30–100 $\mu\text{m}$ . Although no assessments of attached biofilm structure or density were made here, the AGM surface appeared (visually) to be relatively ‘clean’ and free from biological growth; although there was almost certainly a very thin biofilm invisible to the naked eye, given the omnipresence of biofilms in aquatic environments.

Following this, it was thought that instead of behaving like a classical AGM system, with high-density attached-biomass growth and high-rate microbial processes, the current horizontal-flow AGM was essentially functioning like an ‘artificial rock filter’.

In this sense, the AGM reactors provided for an increased solids removal capacity exclusively through the reduction in effective vertical settling distance for infiltrating suspended particulates; remembering that rate of SS removal is primarily a function of water depth as well as fluid velocity (Swanson and Williamson, 1980; Reynolds *et al.*, 1990). Compared with the OPs, for example, the particulate settlement depth was reduced by a factor of 50 within the AGM reactors, with only a slight  $\approx 4\%$  increase in fluid velocity as a result of the small reduction in void volume (Table 2.2). Compared with the RFs, however, the effective settlement depth within AGM ponds was reduced by a factor of only 2 (see Table 2.2), but this time there was a  $>40\%$  reduction in interstitial fluid velocity (see Table 4.2 for theoretical interstitial flow velocities).

Given that when compared to the RFs, both the discrete particulate settlement depth and also fluid velocity were reduced within the AGM reactors, it is unclear why the AGM system was not more advanced than the RFs in terms of its solids removal performance. It is perhaps possible that the solids loads were too low to enable true separation of the performance capabilities of the two upgrade systems. Indeed during the rare instances of high SS and algal biomass loads, the AGM treatment appeared (qualitatively) to be slightly more efficient at removing these parameters than the parallel Rock Filters (see Figures 4.22, 4.23, 4.29 and 4.30). It should be re-emphasized here that since this study was conceived specifically to look at methods for upgrading and managing the sometimes variable quality of the final Bolivar WSP effluent, there were essentially no means for 'active control' over the nature of pilot plant influent wastewater. This same passive reliance upon the natural variations in up-stream WSP performance and subsequent effluent quality regrettably allowed for no control over the magnitude or duration of the periodic increases in pilot plant parameter loadings. It can only be suggested that future performance assessments under increased mass loading rates would be required in order to determine the maximum treatment capabilities of the horizontal-flow AGM relative to rock filtration.

Performance monitoring of the three pilot treatment systems has shown a trend for greater absolute SS (also turbidity) and chlorophyll *a* removals as well as enhanced treatment consistency for the RF and AGM upgrade systems compared with the more conventional Open Ponds. Under the generally low solids and algal biomass loading

regime, both the RFs and AGM ponds were invariably more efficient at removing these parameters than were the parallel OPs, with the OP series frequently yielding negative removal efficiencies for both SS and chlorophyll *a*. Overall, the representation of pilot plant performance data on a loading versus removal basis has shown the RF and AGM treatments to be the strongest and most consistent performers with respect to both total solids and algal biomass removal potential. Throughout the above SS and chlorophyll *a* performance data analyses, as well as during the previous BOD<sub>5</sub> analyses, there has been a general trend for a more accelerated rate of parameter removal within the first pond of each treatment series, followed by a less dramatic or more gradual reduction (where appropriate) within the following two ponds; with this trend being most apparent for the higher performance RF and AGM treatment series. This observation was also noted in the previous Chapter (Section 3.3.5) and has been linked to the predominantly physical ‘first-order’ processes governing the removal of these parameters in wastewater environments.

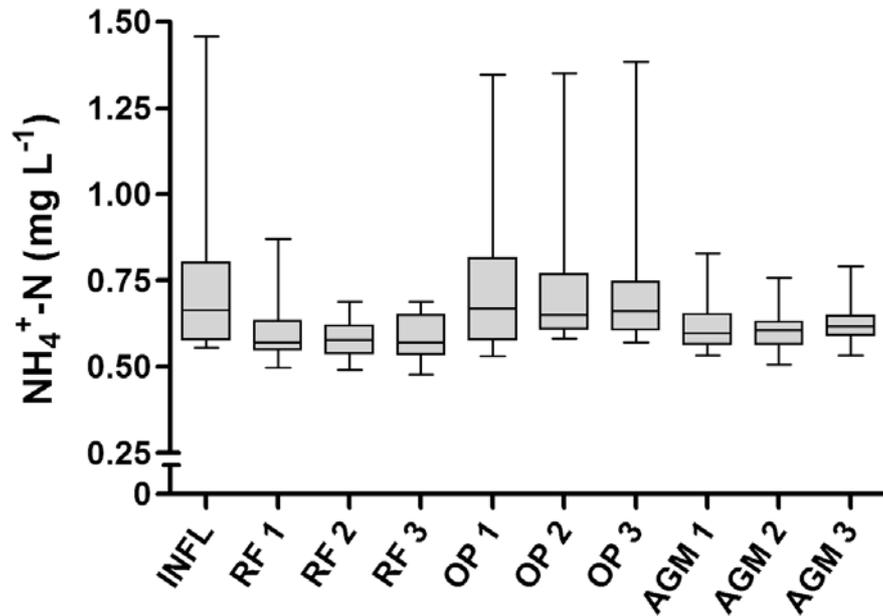
Following the above SS and chlorophyll *a* performance analyses, it is concluded that the overall ranking of treatment performance places both the AGM and RF treatments equal 1<sup>st</sup> and the OP treatment series 2<sup>nd</sup> in terms of parameter removal rate, absolute treatment efficiency, and performance reliability. Finally, for a more concise overview of the performance parameter correlations as discussed throughout this and the preceding section, in addition to some others not referenced during the discussion of results here, the reader is referred to the corresponding correlation matrices for the pilot plant influent as well as the three upgrade treatments (Appendix C).

#### **4.3.6 Wastewater treatment performance: nutrient removal**

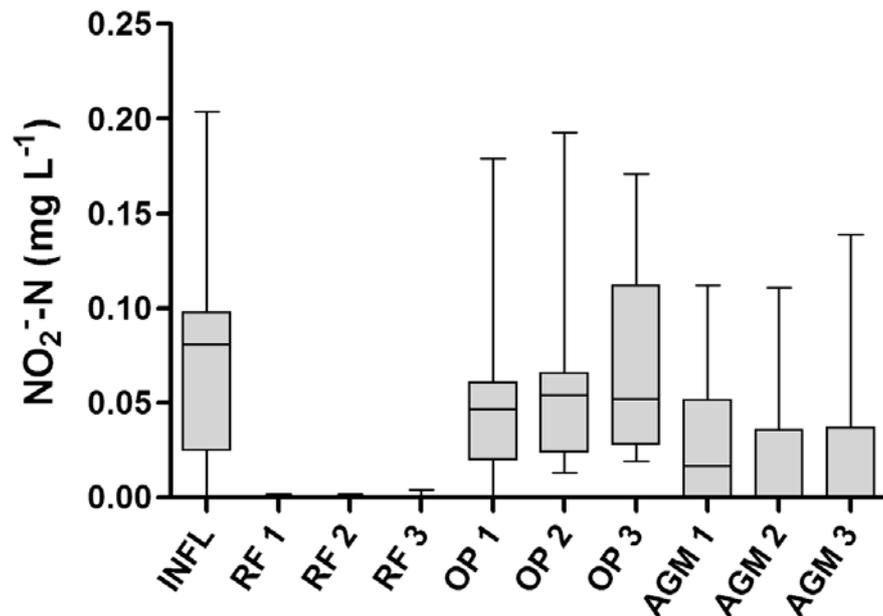
As described in the previous Chapter (Section 3.3.7), N and P are generally regarded as the most essential ‘limiting’ nutrients governing phytoplankton growth and productivity in aqueous environments. Following this, the levels of both NH<sub>4</sub><sup>+</sup>-N and PO<sub>4</sub><sup>3-</sup>-P were periodically monitored during the course of the pilot plant performance assessments as a means of identifying potential algal productivity control mechanisms arising from shifts in resource availability brought about by the respective pilot treatments.

#### 4.3.6.1 Inorganic nitrogen dynamics

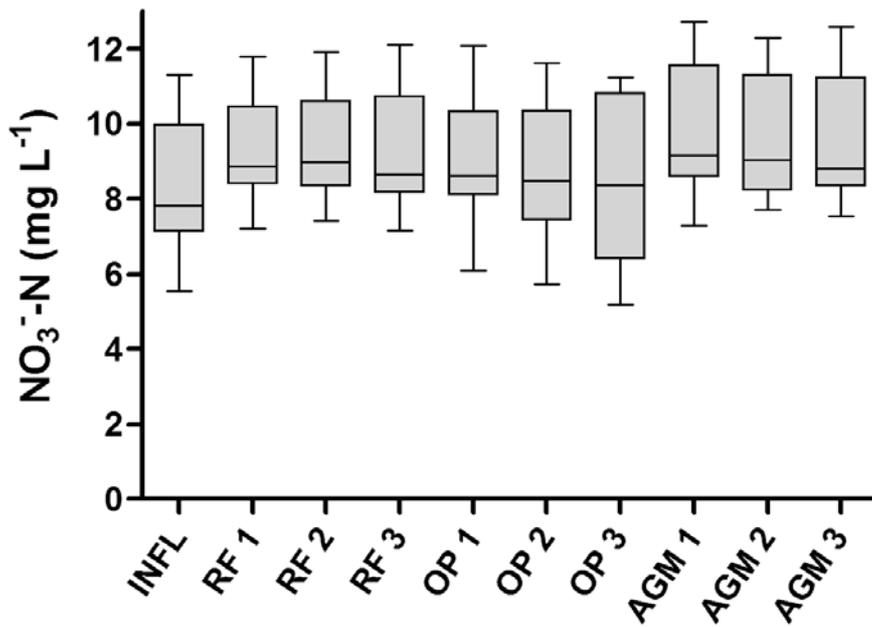
Data from six month performance monitoring of ammonia-, nitrite-, and nitrate-N are shown in Figures 4.33–4.35 respectively.



**Figure 4.33.** Ammonia-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).



**Figure 4.34.** Nitrite-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).



**Figure 4.35.** Nitrate-nitrogen box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

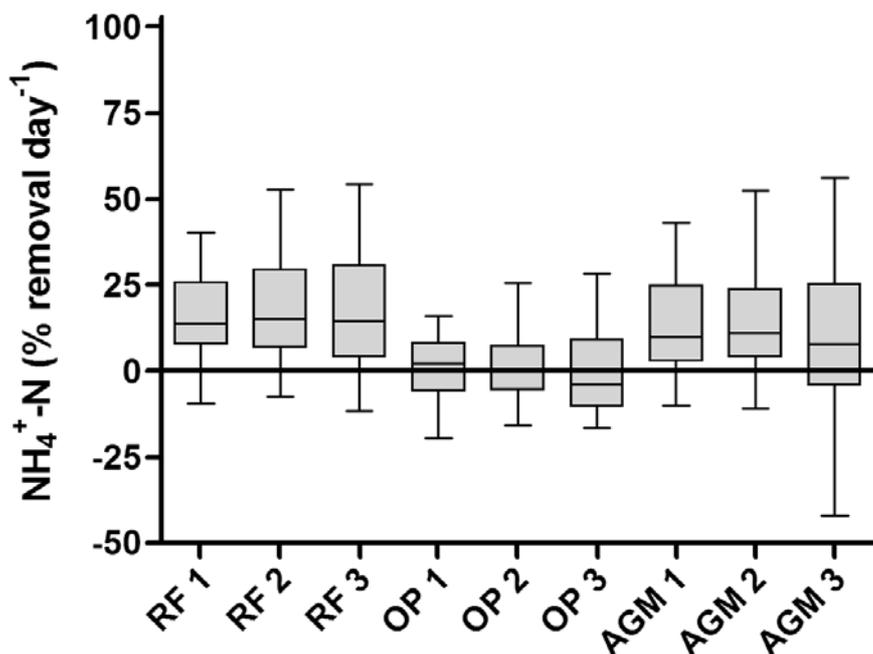
As shown in Figure 4.33, influent  $\text{NH}_4^+\text{-N}$  levels were generally very low, with a median value of 0.67 and a mean of  $0.75\text{mg L}^{-1}$ . This corresponded to a median mass influent loading of approximately  $0.69\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$  (just 5% lower than the  $0.73\text{g m}^{-3}\text{ d}^{-1}$   $\text{NH}_4^+\text{-N}$  loading rate applied during Chapter 3). Visual analysis of the data from Figure 4.33 showed that influent  $\text{NH}_4^+\text{-N}$  levels appeared to decrease down the pond series within both the RF and AGM series, but not the OPs; however, this qualitative observation was not supported statistically. Following data analysis of Figure 4.33, and although there were significant differences between influent and treatment median values within the overall test framework (Kruskal–Wallis test;  $\chi^2_{0.05,9} = 21.60$ ;  $p = 0.010$ ), the sample sizes were too low ( $n = 11$ ) to allow for powerful *post hoc* determinations of between-treatment performance. It was likely that  $\text{NH}_4^+\text{-N}$  concentrations within the RF and AGM series were actually reduced compared with influent levels; however, this qualitative observation could not be supported statistically.

The above theory was later verified when the data was grouped not according to individual Pond number, but by *treatment* within the ANOVA framework; thereby effectively tripling the sample size for each of the three treatment groups ( $n = 33$ ). When  $\text{NH}_4^+\text{-N}$  effluent data was grouped according to treatment (i.e. ignoring individual pond



performance in the analysis), there was a highly significant reduction in three-pond RF train  $\text{NH}_4^+\text{-N}$  concentration compared with influent levels (1-way ANOVA;  $F_{(3,128)} = 9.854$ ;  $p < 0.001$ ) as well as significantly reduced ammonia levels within the three-pond AGM treatment series ( $p = 0.016$ ), but still no significant difference between OP and influent  $\text{NH}_4^+\text{-N}$  levels ( $p = 0.99$ ). Furthermore, three-pond  $\text{NH}_4^+\text{-N}$  levels within both the RF train ( $p < 0.001$ ) as well as the AGM pond series ( $p = 0.016$ ) were significantly lower than ammonia concentration in the parallel Open Ponds. It is concluded then, that ammonia levels were indeed significantly lower following both RF and AGM upgrade treatment, but remained effectively unchanged down the OP series.

At the median mass influent loading of  $0.67\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$ , average removal rates for the RFs were practically identical to those reported in Chapter 3 (Section 3.3.7.1)—in the order of  $1.2 \times 10^{-5}\text{ mg NH}_4^+\text{-N cm}^{-2}\text{ h}^{-1}$ . For the AGM ponds, similar *mass* removal efficiencies under an approximate four-fold increased specific surface area meant that aerial removal rates were an order of magnitude lower than for the RFs—an average of  $2.3 \times 10^{-6}\text{ mg NH}_4^+\text{-N cm}^{-2}\text{ h}^{-1}$ . These removal rates were approximately two to three orders of magnitude lower than the  $1\text{--}3 \times 10^{-3}\text{ mg NH}_4^+\text{-N cm}^{-2}\text{ h}^{-1}$  removals reported by Baskaran *et al.* (1992) for attached *in situ* algal–bacterial biofilms. Considering that influent  $\text{NH}_4^+\text{-N}$  concentrations here were some 100-fold lower in the current systems, nitrification processes were almost certainly substrate-limited as a result of the shallow concentration gradients, such that high removal rates for both the RFs and AGM ponds were essentially unattainable. Despite the low N removals, it should be re-stated that these pilot upgrade methodologies were investigated primarily to assess their algal solids removal capabilities, such that high rates of nutrient removal were not anticipated as a performance outcome of these investigations. Furthermore, and as highlighted in Section 3.3.7.1, rock filters are generally considered to be incapable of  $\text{NH}_4^+\text{-N}$  removal, with most reports actually demonstrating ammonia production following the anaerobic digestion and remineralisation of settled organic materials. Following this, the small-scale  $\text{NH}_4^+\text{-N}$  removals achieved by the pilot RF and AGM upgrade systems was considered to be a beneficial secondary treatment outcome of effective solids removal.



**Figure 4.36.** Box-plots showing percentage ammonia removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n = 11$  for all plots).

Long term mean percentage  $\text{NH}_4^+\text{-N}$  removals for Pond 1 data of the three treatments were approximately 16, 1.4 and 13%, for RF, OP and AGM systems respectively, and for Pond 3 data, 17, 0.7 and 10% for the respective RF, OP and AGM upgrade systems. As described above, there were no statistically significant differences between the influent  $\text{NH}_4^+\text{-N}$  concentration and ammonia levels within individual ponds of all three treatments; although as emphasized, there were significant  $\text{NH}_4^+\text{-N}$  removals when grouped by treatment only. When compared statistically to a theoretical zero mean removal efficiency, average  $\text{NH}_4^+\text{-N}$  removals for both the RFs and AGM Reactors were significantly ‘non-zero’, whereas mean  $\text{NH}_4^+\text{-N}$  removals remained effectively equivalent to zero for Ponds 1 and 3 of the OP treatment (Table 4.8).

The occurrence of negative  $\text{NH}_4^+\text{-N}$  removal efficiencies for the RFs (and AGM ponds) during *Period 2* performance monitoring (Figure 4.36) was unlike the trends for no negative RF removals whatsoever during monitoring *Period 1* of the previous Chapter (see Figure 3.38). Analysis of performance data from Figure 4.36 revealed that the RF treatment recorded negative removals 15% of the time on average, with the AGM train not far behind at 21% of all ammonia ‘removals’ being negative. It was highly likely

that biological nitrification processes within both the RFs and AGM systems were frequently substrate-limited and that these very low influent  $\text{NH}_4^+\text{-N}$  concentrations then led to negative removal efficiencies. This is reflected in Figures 4.37 and 4.38, where it is apparent that percentage  $\text{NH}_4^+\text{-N}$  removal in the RFs was very poor at or below  $0.6\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$ . Given that median  $\text{NH}_4^+\text{-N}$  concentrations were some 35% lower during monitoring *Period 2* (this chapter) than during *Period 1* (Chapter 3), the concentration gradient for mass transfer processes *in situ* was almost certainly very weak, such that effective  $\text{NH}_4^+\text{-N}$  removals were effectively impossible some of the time. In spite of relatively infrequent and largely small-scale negative  $\text{NH}_4^+\text{-N}$  removals within the RF and AGM pond series, the OPs yielded negative removals much more frequently, with net  $\text{NH}_4^+\text{-N}$  gains realised 46% of the time. These trends are reflected in the corresponding CV's for treatment performance with respect to percentage  $\text{NH}_4^+\text{-N}$  removal efficiency. Pond 1 CV's for  $\text{NH}_4^+\text{-N}$  removal performance were 92, 696, and 117% for the RF, OP, and AGM treatment systems respectively, and Pond 3 CV's were 117, 2100, and 240 for the respective RF, OP, and AGM treatment systems. This performance data is summarised in Table 4.8.

**Table 4.8.** Summary of ammonia removal performance across all pilot plant treatments for Pond 1 and 3 data only.

<b><math>\text{NH}_4^+\text{-N}</math> performance parameter</b>	<b><i>Pilot treatment pond</i></b>					
	<b>RF-1</b>	<b>RF-3</b>	<b>OP-1</b>	<b>OP-3</b>	<b>AGM-1</b>	<b>AGM-3</b>
Median influent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	0.67	0.67	0.67	0.67	0.67	0.67
Mean influent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	0.75	0.75	0.75	0.75	0.75	0.75
Median effluent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ )	0.57	0.57	0.70	0.66	0.60	0.62
Mean effluent $\text{NH}_4^+\text{-N}$ ( $\text{mg L}^{-1}$ ; $\text{g m}^{-3}$ ) <sup>†</sup>	0.61	0.59	0.70	0.73	0.63	0.63
Median daily $\text{NH}_4^+\text{-N}$ removal ( $\%\text{ day}^{-1}$ )	14	15	2	-4	10	8
Mean daily $\text{NH}_4^+\text{-N}$ removal ( $\%\text{ day}^{-1}$ ) <sup>‡</sup>	16	17	1.4	0.7	13	10
Long-term CV for $\text{NH}_4^+\text{-N}$ removal (%)	92	117	696	2100	117	240

<sup>†</sup> Effluent  $\text{NH}_4^+\text{-N}$  concentration was tested relative to mean pilot plant influent  $\text{NH}_4^+\text{-N}$  concentration (1-way ANOVA)

<sup>‡</sup> Average  $\text{NH}_4^+\text{-N}$  removal % tested against a theoretical 'zero' daily mean (one sample *t*-test)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium)

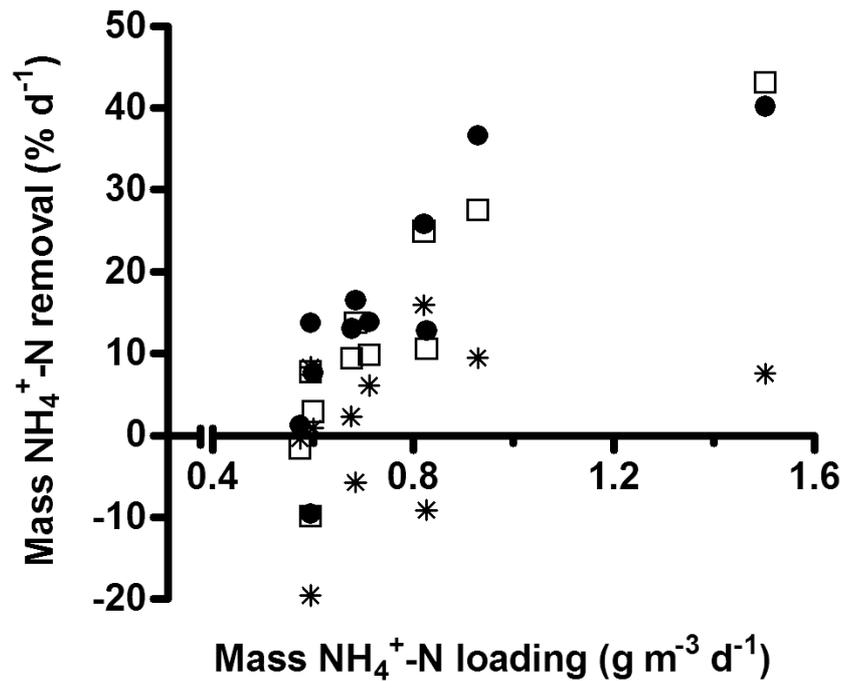
As described in Chapter 3, there are three general pathways recognised as being important for  $\text{NH}_4^+\text{-N}$  removal in WSP environments: volatilisation; biomass sequestration (microbial and algal); and nitrification–denitrification. As was also discussed in detail in Section 3.3.7.1, biological nitrification was considered to have

been the major pathway for  $\text{NH}_4^+$ -N removal within the pilot-scale RFs. The same conclusions were also drawn following analysis of this Chapter's performance data, with: the simultaneous disappearance of  $\text{NO}_2^-$ -N and slight (qualitative) increase in  $\text{NO}_3^-$ -N (Figures 4.34 and 4.35 respectively); the largely aerobic conditions (Figure 4.6); and a decline in wastewater pH down the pond series (Figure 4.9) all providing significant circumstantial evidence for microbial nitrification. Although RF dissolved oxygen levels were observed to fall below  $2\text{mg L}^{-1}$  on numerous occasions (Figure 4.7), they were also frequently above  $2.5\text{mg L}^{-1}$ ; the level stated by Baskaran *et al.* (1992) as being the lowest oxygen level at which effective nitrification can proceed.

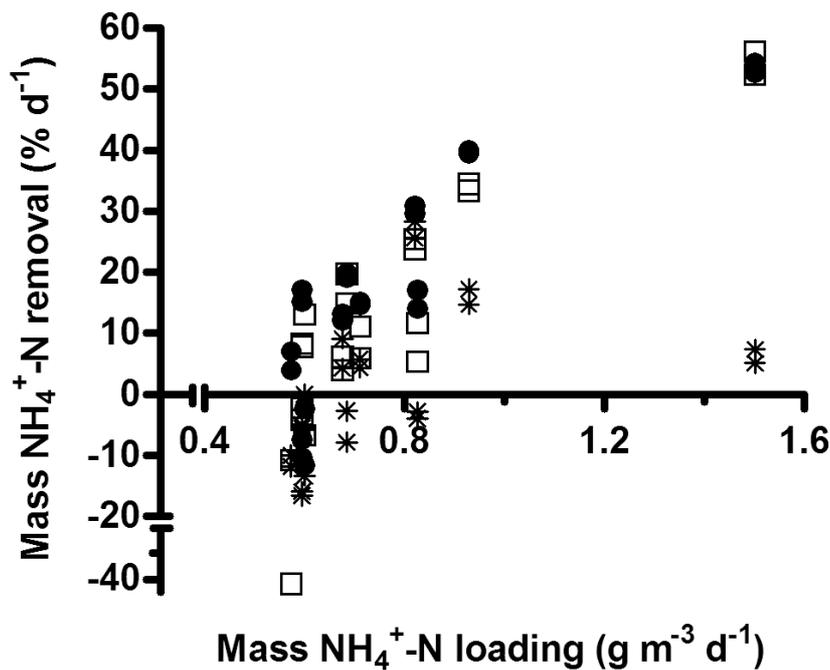
Given their physical similarities, it was perhaps unsurprising that similar trends were also apparent for the AGM treatment system, with qualitative evidence for reduced  $\text{NO}_2^-$ -N and elevated  $\text{NO}_3^-$ -N levels (Figures 4.34 and 4.35 respectively), sufficient quantities of DO (Figure 4.6) and a qualitative drop in pH (Figure 4.9) all pointing again to nitrification having been the primary factor behind the significant  $\text{NH}_4^+$ -N removals within the combined three-pond AGM performance data. Although others have suggested that the primary mechanism for  $\text{NH}_4^+$ -N removal in attached-growth WSP systems is via biomass uptake and sedimentation (Shin and Polprasert, 1987; 1988), this process was thought to have contributed relatively little to ammonia removals within the current AGM systems due to the much lower density of biomass development in the refined tertiary-level effluent (described in Section 4.3.5). The reasons behind the more highly variable and sometimes negative  $\text{NH}_4^+$ -N removals within the OP treatment have already discussed (Section 3.3.7.1) and were considered to have been largely due to variable rates of both primary (algal) and secondary (zooplankton) biomass production. These factors were thought to have resulted in increased and decreased  $\text{NH}_4^+$ -N removals respectively, and since they have been discussed elsewhere, no further elaboration of these processes is offered.

Whilst the mean percentage  $\text{NH}_4^+$ -N removal efficiencies for each of treatment were not as great as those seen for other performance parameters (i.e.  $\text{BOD}_5$ , SS, chlorophyll *a*), they do suggest that both the RFs and AGM ponds were significantly more advanced in their ammonia removal capacity than were the Open Ponds. This general trend across

the Pond 1 as well as Pond 2 and 3 data is again evident on a mass loading versus percentage removal performance basis within Figures 4.37 and 4.38 respectively. As was done for corresponding performance data of Chapter 3, and due to the relatively low number of daily samples for the  $\text{NH}_4^+\text{-N}$  data set ( $n = 11$ ), the data of Ponds 2 and 3 were again combined in the corresponding Figures 4.38 and 3.40 below.

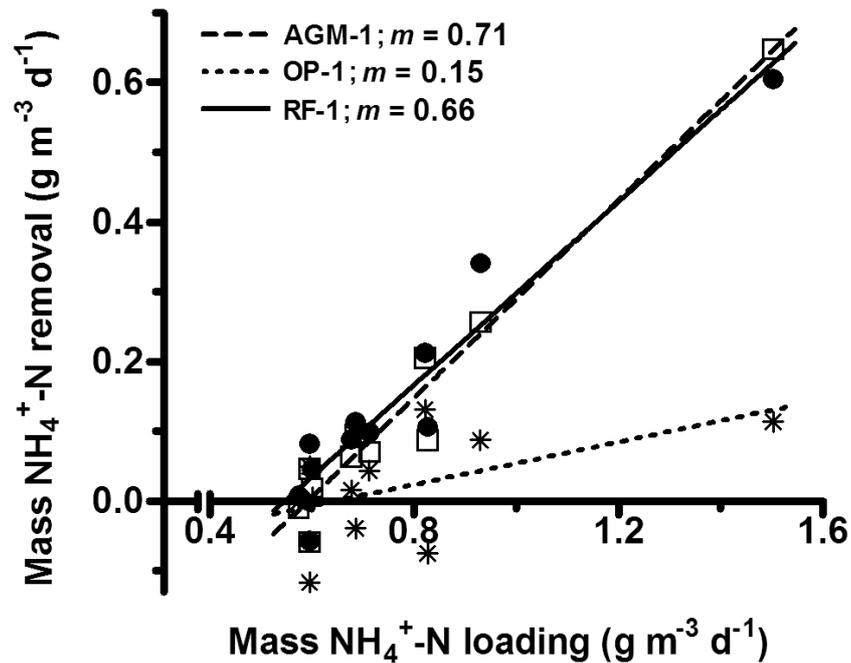


**Figure 4.37.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for Pond 1 data only. Individual data points represent mean performance data from triplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and Attached-Growth Media Reactor 1 (□).

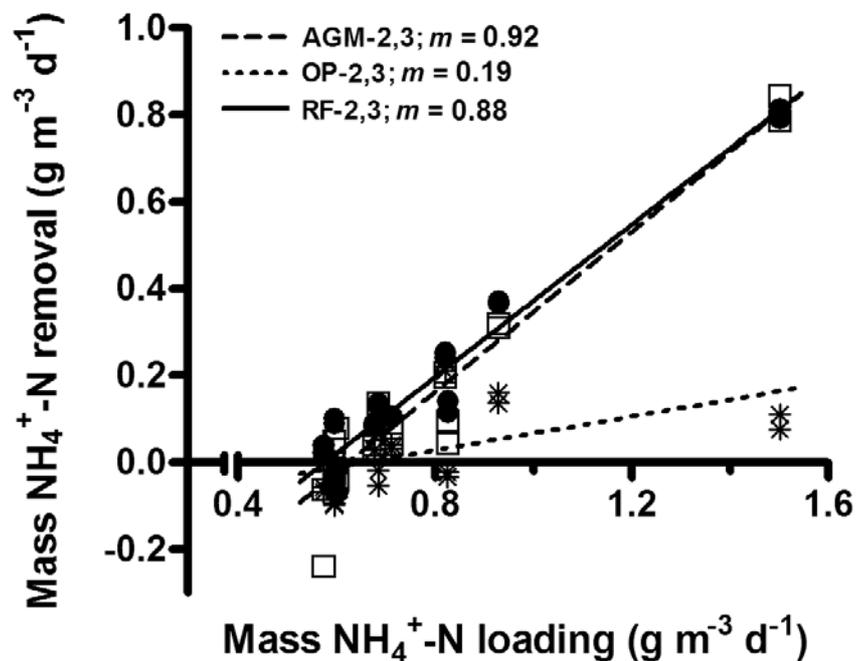


**Figure 4.38.** Scatter-plot showing NH<sub>4</sub><sup>+</sup>-N mass loading (pilot plant Influent) vs. percentage mass removal (relative to daily loading rate) for the combined data of Ponds 2 and 3. Individual data points represent mean performance data from triplicate determinations for: Rock Filters 2 and 3 (●); Open Ponds 2 and 3 (\*); and Attached-Growth Media Reactors 2 and 3 (□).

In spite of the low number of data points, the above Figures again demonstrate that the RF and AGM treatment series displayed greater overall capacity for NH<sub>4</sub><sup>+</sup>-N removal than the parallel OPs. As seen for the previous performance parameters (BOD<sub>5</sub>, SS and chlorophyll *a*), there were again apparent positive relationships between mass loading rate and percentage removal performance in all three treatments for Pond 1 (Figure 4.37) and the combined Pond 2 and 3 data (Figure 4.38); although these relationships were evidently stronger for the higher performance RF and AGM systems. Percentage NH<sub>4</sub><sup>+</sup>-N removals were again generally higher under elevated ammonia mass loads—a consequence of first-order removal processes already discussed.



**Figure 4.39.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. total mass removal for Pond 1 data only. Individual data points represent mean performance data from triplicate determinations for: Rock Filter 1 (●); Open Pond 1 (\*); and AGM Reactor 1 (□). Fitted lines represent best-fit lines from simple linear regression analyses, with regression slopes ( $m$ ) shown alongside the respective figure legends.



**Figure 4.40.** Scatter-plot showing  $\text{NH}_4^+\text{-N}$  mass loading (pilot plant Influent) vs. total mass removal for the combined data of Ponds 2 and 3. Individual data points represent mean performance data from triplicate determinations for: Rock Filters 2 and 3 (●); Open Ponds 2 and 3 (\*); and AGM Reactors 2 and 3 (□). Fitted lines represent best-fit

lines from simple linear regression analyses, with regression slopes ( $m$ ) shown alongside the respective figure legends.

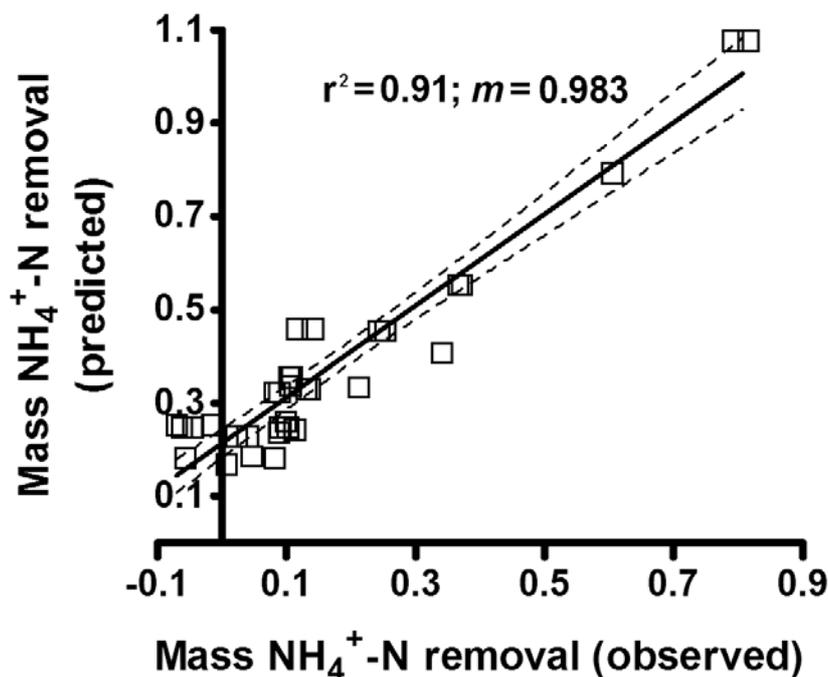
When the  $\text{NH}_4^+\text{-N}$  data was plotted on a mass basis only (Figures 4.39 and 4.40), there were again striking positive associations between mass loading and the mass of  $\text{NH}_4^+\text{-N}$  removed for each pilot treatment and across all ponds of each series—a trend noted for all of the previous water quality parameters. On a mass basis, this direct ‘loading versus removal’ relationship was significantly more apparent for the higher performance RF and AGM series compared with the OP treatment. Statistically, there was a very high-level association between mass load and mass  $\text{NH}_4^+\text{-N}$  removal for the RF-1 data of Figure 4.39 (Pearson  $r = 0.959$ ;  $n = 11$ ;  $p < 0.0001$ ), an equally significant relationship for the AGM-1 data ( $r = 0.980$ ;  $n = 11$ ;  $p < 0.0001$ ) and no significant relationship for the OP-1 data of Figure 4.39 ( $r = 0.532$ ;  $n = 11$ ;  $p = 0.092$ ). Similar trends were seen for the combined Pond 2 and Pond 3  $\text{NH}_4^+\text{-N}$  data of Figure 4.40, with a highly significant relationship between mass load and mass removal for RFs 2 and 3 ( $r = 0.972$ ;  $n = 22$ ;  $p < 0.0001$ ) as well as for AGM Ponds 2 and 3 ( $r = 0.960$ ;  $n = 22$ ;  $p < 0.0001$ ), but a much less significant correlation for the more highly variable OP-2 and 3 data ( $r = 0.524$ ;  $n = 22$ ;  $p < 0.05$ ).

Regression analyses of Figures 4.39 and 4.40 yielded identical regression coefficients to the Pearson correlation coefficients above. Critical analysis of the fitted regression lines for the  $\text{NH}_4^+\text{-N}$  data revealed significantly different regression slopes between the three treatments for Pond 1 data (ANCOVA;  $F_{(2,27)} = 21.69$ ;  $p < 0.0001$ ) as well as for the combined Pond 2 and 3 data ( $F_{(2,60)} = 46.05$ ;  $p < 0.0001$ ). Further to this, the slopes of the regressed lines for both the RF and AGM data of Figures 4.39 and 4.40 were significantly greater than those of the respective OP data ( $p < 0.0002$ ), but between themselves were equal ( $p \geq 0.52$ ). In this instance, the difference in slopes of the respective regression lines from Figures 4.39 and 4.40 reflected the differing capacity of the upgrade systems to remove  $\text{NH}_4^+\text{-N}$  based on variable biological nitrogen removal capabilities; remembering that unlike  $\text{BOD}_5$ , SS and chlorophyll  $a$  above,  $\text{NH}_4^+\text{-N}$  removal is not so heavily reliant on physical processes. The significantly greater mass  $\text{NH}_4^+\text{-N}$  removal slope for both the RFs and AGM upgrade series over the parallel OPs, effectively meant that they were removing proportionally more  $\text{NH}_4^+\text{-N}$  at all encountered mass loading rates. This observation was further supported by the earlier



data of Figure 4.36 and Table 4.8. As referenced on several occasions, published data regarding the ‘loading versus removal’ performance of both rock filters and AGM ponds (for any water quality parameter) is again lacking, therefore, the above performance trends cannot be directly compared to those of other systems.

In spite of the less effective RF  $\text{NH}_4^+$ -N removals reported during this chapter compared to those Chapter 3, the ‘patterns’ of  $\text{NH}_4^+$ -N removal were largely in line with those of the previous 2005 *Period 1* performance data. Using the RF performance data from previous regression analyses as the predictor variable (Section 3.3.7.1), there was a very good reflection of *Period 2* RF mass  $\text{NH}_4^+$ -N removals against what was expected based on *Period 1* performance data of Chapter 3 (Figure 4.41). The slope of the fitted regression ( $m = 0.98$ ) line was actually not significantly different from a theoretical perfect fit slope of 1.0 (ANCOVA;  $F_{(1,62)} = 0.094$ ;  $p = 0.760$ ), suggesting that there was an almost exact reflection of observed mass  $\text{NH}_4^+$ -N removals in those predicted based on the regression model from the previously acquired performance data. This suggested that whilst the RFs were now not achieving the same degree of ammonia removal performance as was reported in Chapter 3,  $\text{NH}_4^+$ -N mass removals were actually in line with what were being achieved during the previous data collection period; it was just that the very low influent concentrations precluded larger magnitude removals.



**Figure 4.41.** Entire Rock Filter train mass ammonia-nitrogen removals showing observed versus predicted performance (predicted values calculated based on Rock Filter regression performance analyses from Section 3.3.7.1). Fitted regression line (solid line) shown with 95% CI's (broken lines).

As previously discussed, significant  $\text{NH}_4^+$ -N removals are not commonly associated with rock filter performance. Instead, ammonia production is a commonly reported performance problem associated with the technology, whereby effluent  $\text{NH}_4^+$ -N concentrations often exceeding that of filter's influent (Hirse Korn, 1974; Swanson and Williamson, 1980; Middlebrooks, 1988; Mara and Johnson, 2006). Mara *et al.* (2001) observed 5% net increases in rock filter effluent  $\text{NH}_4^+$ -N concentrations when operated at an equivalent HLR ( $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ ) but a 25-fold greater ammonia mass loading rate of  $\approx 18\text{g NH}_4^+\text{-N m}^{-3}\text{ d}^{-1}$  compared with the current filters. It was again thought that in the case of Mara and co-workers, the rock filters were loaded at too high an OLR ( $24\text{g BOD}_5\text{ m}^{-3}\text{ d}^{-1}$ ) and subsequently contained insufficient oxygen levels ( $0.2\text{mg DO L}^{-1}$ ) to allow enable microbial nitrification. Whilst there have been a limited number of reports indicating no significant  $\text{NH}_4^+$ -N production or indeed ammonia removal during rock filtration (Saidam *et al.*, 1995; Strang and Wareham, 2005; Johnson *et al.*, 2007), rock filters are generally not designed nor installed for achieving nitrogen removal, such that any  $\text{NH}_4^+$ -N removal whatsoever would be considered an added benefit of what is overwhelmingly a physical solids removal process.

With respect to  $\text{NH}_4^+$ -N removal performance of other AGM systems, comparable work within the literature is limited. Shin and Polprasert (1987) operated a similar pilot-scale AGWSP, with an approximate 10-fold greater specific surface area ( $1220\text{m}^2 \text{m}^{-3}$ ) and at  $\text{NH}_4^+$ -N mass loadings in the comparably low range of  $0.5\text{--}2\text{g NH}_4^+\text{-N m}^{-3} \text{d}^{-1}$ . The authors reported ammonia removals in the range of 68–87%; some 65% greater than those achieved within the current AGM reactors. The authors also reported a trend for greater  $\text{NH}_4^+$ -N removals under reduced organic (COD) loads; however, no such trends between ammonia and  $\text{BOD}_5$  concentration were apparent for the AGM performance data reported here (Spearman  $r_s = 0.31$ ;  $n = 27$ ;  $p = 0.12$ ). It was considered likely that the higher  $\text{NH}_4^+$ -N removal efficiency of their pilot-scale AGM reactors was due to the elevated ammonia concentrations within their influent wastewater ( $9.5\text{mg NH}_4^+\text{-N L}^{-1}$ ) providing a greater concentration-gradient for substrate mass transfer thereby allowing for higher rate microbial processes.

Zhao and Wang (1996) observed  $\approx 40\%$   $\text{NH}_4^+$ -N removals in their pilot-scale AGM ponds compared to 25% removals in parallel control ponds when operated at roughly twice the specific media surface area ( $270\text{m}^2 \text{m}^{-3}$ ) and an approximate 7-fold lower HLR ( $0.14\text{m}^3 \text{m}^{-3} \text{d}^{-1}$ ) to the AGM system here. Since the authors failed to state the influent  $\text{NH}_4^+$ -N concentration of their pilot-scale AGM ponds, no comparisons can be made regarding removal performance versus ammonia loading rate of these systems. Zhao and Wang (1996) also noted a 10% improvement in  $\text{NH}_4^+$ -N removals with an increasing AGM packing density into the pond system (from 11 to 22% AGM v:v); however, since no such assessments of AGM packing density on treatment performance were made during the current work, it can only be recommended as a topic for future research.

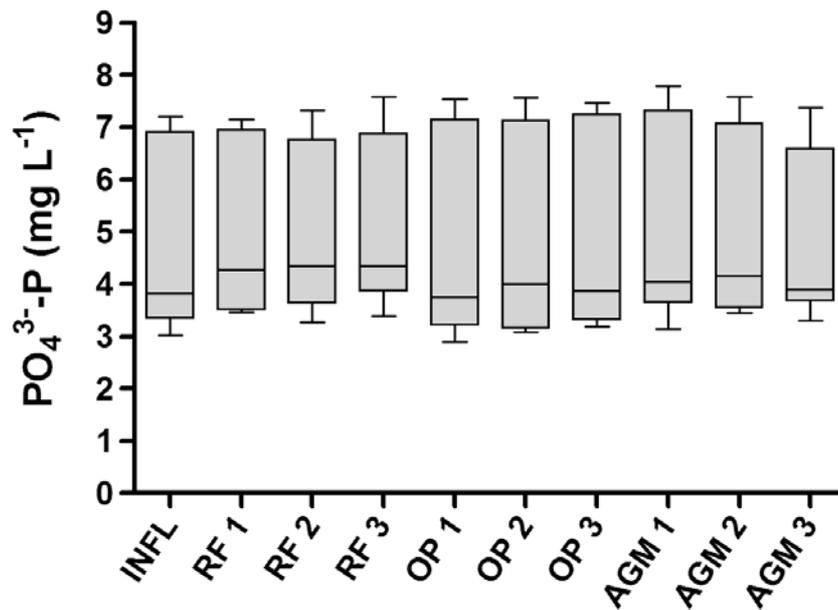
Rakkoed *et al.* (1999), following the operation of laboratory-scale ( $0.29\text{m}^3$ ) AGWSPs with 2-fold greater specific media surface area ( $300\text{m}^2 \text{m}^{-3}$ ), achieved higher level  $\text{NH}_4^+$ -N removal performance in the range of 44–99% when loaded at 25-fold higher ammonia mass loadings of  $16\text{g NH}_4^+\text{-N m}^{-3} \text{d}^{-1}$  but at a 40-fold lower HLR to that applied here ( $0.025\text{m}^3 \text{m}^{-3} \text{d}^{-1}$ ). The high order removals achieved by the AGM system of Rakkoed and co-workers were once again almost certainly a reflection of the 1000-fold higher influent ammonia concentrations ( $\approx 600\text{mg NH}_4^+\text{-N L}^{-1}$ ); presumably allowing for

significantly more effective substrate diffusion and higher rate biological treatment processes. Finally, McLean (1999) and McLean *et al.* (2000) reported enhanced *in situ* WSP nitrification and ammonia removals ( $\approx 50\%$  enhancement over parallel control WSPs) using large-scale ( $9000\text{m}^3$ ) low specific surface area ( $\approx 2\text{m}^2\text{ m}^{-3}$ ) attached-growth ponds incorporating vertically mounted polyethylene plate media. The higher  $\text{NH}_4^+\text{-N}$  removal performance of their system was likely to have been a result of simultaneous algal and bacterial uptake under the illuminated conditions, unlike the darkened AGM used here. Higher ammonia removals were again a likely reflection of the 50-fold higher influent concentrations ( $35\text{mg NH}_4^+\text{-N L}^{-1}$ ) allowing for higher-rate biological treatment as described above.

In conclusion, it should be re-stated that these pilot upgrade methodologies were investigated primarily to assess their capacities for algal solids removal, such that nutrient removal was not anticipated as an initial performance outcome. Given the already very low levels of dissolved nutrients in the Bolivar WSP effluent, it is assumed that any nutrient removals greater than zero would be considered ‘fringe benefits’ of primarily physical pond upgrade systems. Results did show, however, that both the RF and AGM systems were significantly more advanced in terms of  $\text{NH}_4^+\text{-N}$  removal potential than standard Open Ponds; suggesting that small amounts of additional ammonia removal could realistically be expected within these upgrade systems. Following the above analyses, the overall performance ranking for the three treatments with respect to their capacity for  $\text{NH}_4^+\text{-N}$  removal places the RFs and AGM ponds equal 1<sup>st</sup> and the OPs 2<sup>nd</sup> overall in terms of  $\text{NH}_4^+\text{-N}$  removal rate down the pond series, absolute treatment efficiency, and also performance reliability—a similar ranking order to that of all previous performance parameter assessments.

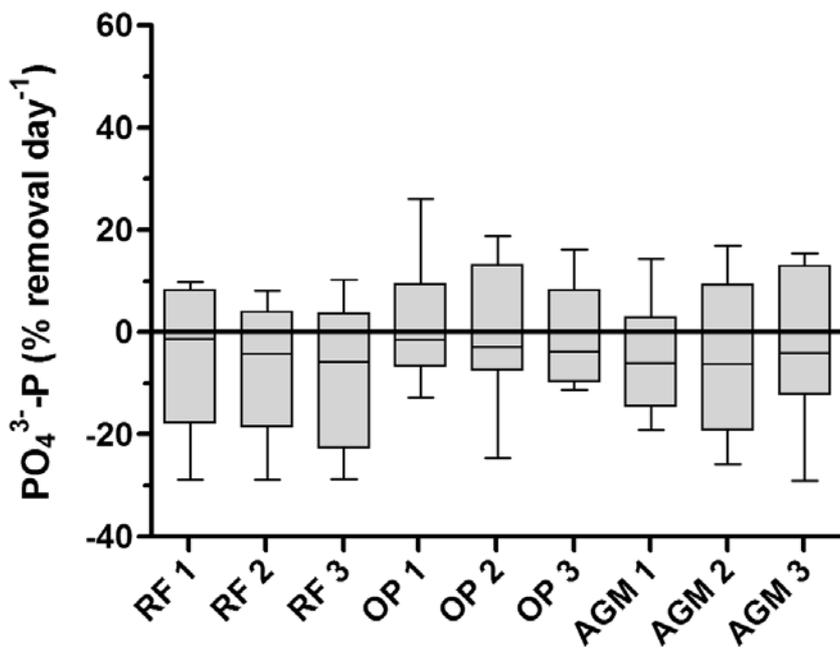
#### **4.3.6.2 Soluble reactive orthophosphate removal**

Data from performance monitoring of  $\text{PO}_4^{3-}\text{-P}$  levels is shown in Figure 4.42 below.



**Figure 4.42.** Soluble reactive orthophosphate-phosphorous box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.

As shown in Figure 4.42, influent  $\text{PO}_4^{3-}\text{-P}$  levels were generally in the order of 4–6  $\text{mg L}^{-1}$ , with a median influent concentration of 3.8 and a mean of 4.7  $\text{mg PO}_4^{3-}\text{-P L}^{-1}$ . Based on the median concentration, this corresponded to an average mass influent loading of approximately 3.9  $\text{g PO}_4^{3-}\text{-P m}^{-3} \text{ d}^{-1}$ . Following visual inspection of Figure 4.42, it is apparent that influent levels of  $\text{PO}_4^{3-}\text{-P}$  remained virtually unchanged following passage through each of the treatment pond series. Statistically, there were no significant differences between average influent  $\text{PO}_4^{3-}\text{-P}$  levels and those in any of the experimental treatment ponds (1-way ANOVA;  $F_{(9,90)} = 0.088$ ;  $p = 1.00$ ), such that there were no significant removals of influent  $\text{PO}_4^{3-}\text{-P}$  down any of the pond series and also no inter-treatment performance differences between any combination of the RF, OP and AGM treatments.



**Figure 4.43.** Box-plots showing percentage orthophosphate-phosphorous removal performance relative to pilot plant Influent concentration for all ponds and across all 3 pilot treatment systems ( $n = 10$  for all plots).

On a percentage basis, all ponds across all three treatments were seen to have yielded negative  $\text{PO}_4^{3-}\text{-P}$  removals on numerous occasions, with average percentage removal performance close to or below zero (Figure 4.43). Long term mean percentage  $\text{PO}_4^{3-}\text{-P}$  removals for Pond 1 data of the three treatments were approximately  $-5$ ,  $1$  and  $-4\%$  for RF, OP and AGM systems respectively, and for Pond 3 data,  $-9$ ,  $-2$  and  $-3\%$  for the respective RF, OP and AGM treatments. Although average removals were generally negative and quite often variable, when compared to a theoretical zero mean removal rate, no treatment pond was shown to be delivering significantly negative removal performance (Table 4.9). Since there were no performance trends of significant interest with respect to the  $\text{PO}_4^{3-}\text{-P}$  performance data of this chapter, no further breakdown of treatment performance (i.e. mass-basis performance) is provided.

**Table 4.9.** Summary of orthophosphate-phosphorous performance data for all three treatments for Pond 1 and 3 only.

<b>PO<sub>4</sub><sup>3-</sup>-P performance parameter</b>	<b><i>Pilot treatment pond</i></b>					
	<b>RF-1</b>	<b>RF-3</b>	<b>OP-1</b>	<b>OP-3</b>	<b>AGM-1</b>	<b>AGM-3</b>
Median influent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	3.8	3.8	3.8	3.8	3.8	3.8
Mean influent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	4.7	4.7	4.7	4.7	4.7	4.7
Median effluent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> )	4.3	4.0	3.8	3.9	4.0	3.9
Mean effluent PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> ; g m <sup>-3</sup> ) <sup>†</sup> *	4.9	5.0	4.7	4.8	5.0	4.8
Median daily PO <sub>4</sub> <sup>3-</sup> -P removal (% day <sup>-1</sup> )	-1.3	-6.0	-1.5	-3.7	-6.1	-4.1
Mean daily PO <sub>4</sub> <sup>3-</sup> -P removal (% day <sup>-1</sup> ) <sup>‡</sup> *	-4.5	-9.0	1.4	-1.5	-5.4	-3.0
Long-term CV for PO <sub>4</sub> <sup>3-</sup> -P removal (%)	294	155	797	632	197	467

<sup>†</sup> Effluent PO<sub>4</sub><sup>3-</sup>-P concentration was tested relative to mean pilot plant influent PO<sub>4</sub><sup>3-</sup>-P concentration (1-way ANOVA)

<sup>‡</sup> Average PO<sub>4</sub><sup>3-</sup>-P removal % tested against a theoretical 'zero' daily mean (one sample *t*-test)

\* Shading intensity shows significance level: *p* > 0.05 (no shading)

Common mechanisms behind phosphorous removal in typical open WSP environments have been described previously (Section 3.3.7.2). Similarly, and since RF PO<sub>4</sub><sup>3-</sup>-P performance results were similar to those of Chapter 3, no further discussion of results is offered here; instead, the reader is again directed to Section 3.3.7.2 for more information. With respect to the AGM performance data, there was no significant overall decrease (biomass assimilation) or increase (anaerobic release) in PO<sub>4</sub><sup>3-</sup>-P levels within the AGM pond system. This suggested—as for the RFs—that phosphorous is unlikely to be either removed or increase significantly a result of AGM treatment within the current Bolivar wastewater setting.

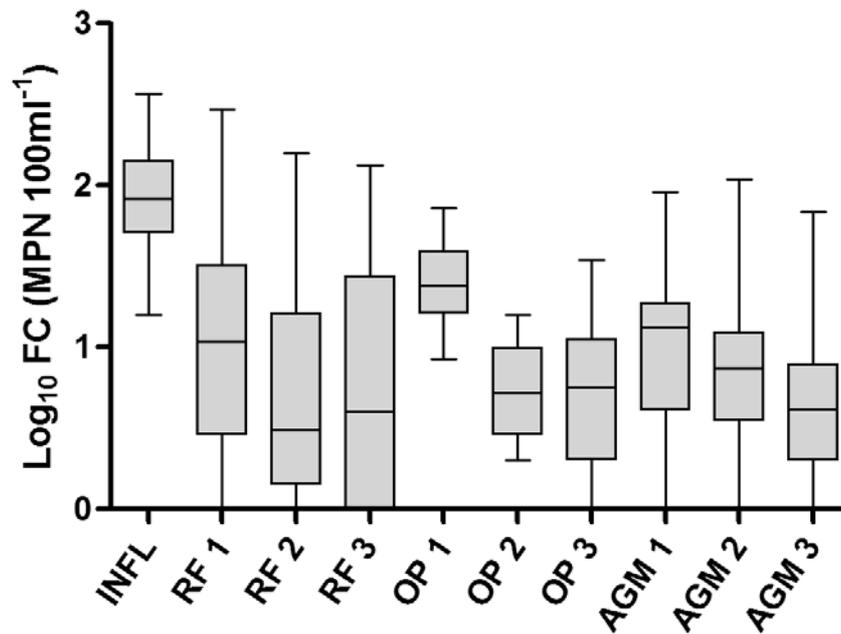
Phosphorous performance data for other attached-growth WSP systems is practically non-existent. Shin and Polprasert (1987) provide the only known results with respect to P removal using AGM for the upgrading of pond treatment performance. The authors reported total P removals to be some 20% better within pilot-scale AGWSP compared to a control pond without media, attributing the higher removals to a combination of biomass incorporation as well as some phosphate co-precipitation under the alkaline *in situ* conditions (although no pH data was provided). Since their work constitutes the only additional performance data detailing phosphorous dynamics in AGM pond systems, no further discussion of the current results in relation to the findings of others can be offered. It can only be suggested that this forms the topic for future research investigations into the area of AGM for wastewater treatment.

Based on results from both the previous  $\text{NH}_4^+\text{-N}$  and also the current  $\text{PO}_4^{3-}\text{-P}$  performance data, it would not be expected that passage through either a rock filter or attached-growth media pond upgrade would have any significant adverse impacts in terms of the nutritional requirements of suspended algal populations at Bolivar. In both cases, the combined levels of  $\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  following RF and AGM treatment would be expected to be high enough not to restrict algal viability and/or growth within the Bolivar WSPs. Similar to the conclusions of Chapter 3, the overall performance ranking for the three pilot treatments regarding their capacity for  $\text{PO}_4^{3-}\text{-P}$  removal, sees all treatments on an equal footing in terms of:  $\text{PO}_4^{3-}\text{-P}$  removal rate down the pond series; absolute removal efficiency; and also performance reliability.

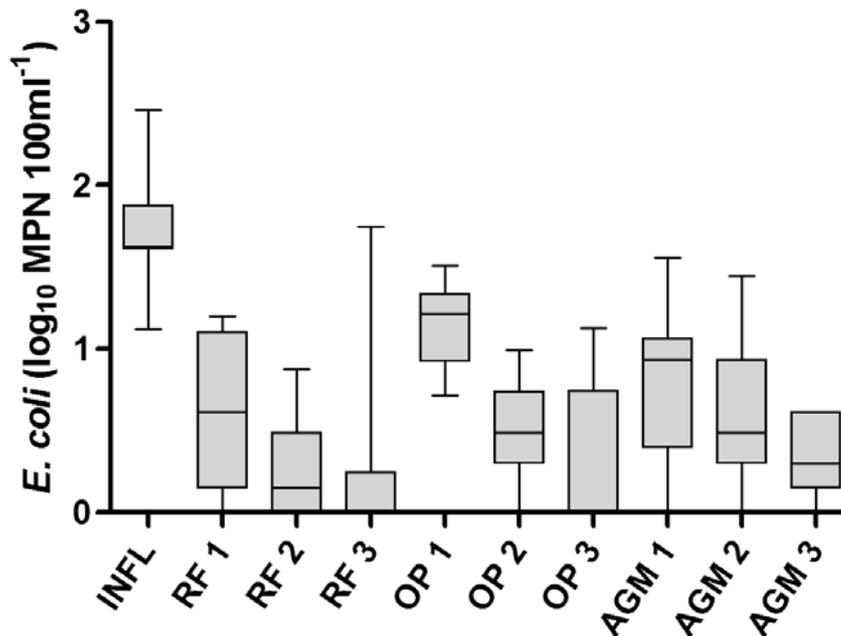
#### **4.3.7 Wastewater treatment performance: indicator organism removals**

Data from performance monitoring of both FC and *E. coli* density is shown in Figures 4.44 and 4.45 respectively. As described earlier (Section 2.3), all average indicator organism densities are reported here as arithmetic mean values in accordance with the recommendations of Haas (1996).





**Figure 4.44.** Faecal coliform box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3). The shaded ‘box’ represents the IQR, the horizontal bar shows the median value, and the ‘whiskers’ show the absolute data range.



**Figure 4.45.** *E. coli* box-plot data for pilot plant: Influent (INFL); Rock Filters 1, 2, 3 (RF-1, RF-2, RF-3); Open Ponds 1, 2, 3 (OP-1, OP-2, OP-3); and Attached-Growth Media Reactors 1, 2, 3 (AGM-1, AGM-2, AGM-3).

As shown in Figure 4.44, pilot plant influent FC densities were consistently very low, with a mean of approximately  $1.9\text{-log}_{10}$  ( $\pm 0.4$ ) and a maximum of  $2.6\text{-log}_{10}$  MPN  $100\text{ml}^{-1}$ . Overall Pond 1 average FC removals were significantly more advanced in the RF and AGM series than in the parallel OPs. Individually, average removals were in the order of  $0.8\text{-log}_{10}$  units for RF-1,  $0.5\text{-log}_{10}$  for OP-1, and  $0.9\text{-log}_{10}$  for AGM-1. Regarding Pond 3 data, mean FC removals down the pond series were of a similar order of magnitude for each pond series, with approximate  $1.2\text{-log}_{10}$  unit removals for all three treatments. Statistically significant FC removals were seen for both RF-1 and AGM-1 (1-way ANOVA;  $F_{(9,87)} = 5.37$ ;  $p < 0.05$ ) but not for OP-1 ( $p > 0.05$ ). For the Pond 3 data, the greater than  $1\text{-log}_{10}$  removals were highly significant for all three treatments ( $p < 0.001$ ); however, there were no apparent differences between FC densities in any pond for any of the three treatment series ( $p > 0.05$ ).

With respect to *E. coli* performance data (Figure 4.45), influent *E. coli* densities were again very low, with an average of approximately  $1.7\text{-log}_{10}$  ( $\pm 0.3$ ) and a maximum of  $2.5\text{-log}_{10}$  MPN  $100\text{ml}^{-1}$ . Pond 1 average *E. coli* removals were again significantly more advanced within the RF and AGM treatment series compared to the Open Ponds, with removals in the order of  $1.1\text{-log}_{10}$  units for RF-1,  $0.5\text{-log}_{10}$  for OP-1, and  $0.9\text{-log}_{10}$  for AGM-1. Regarding Pond 3 data, mean *E. coli* removals down the pond series were in the order of  $1.5\text{-log}_{10}$  for RF-3 and  $1.4\text{-log}_{10}$  for both OP-3 and AGM-3. Statistically significant *E. coli* removals were achieved in both RF-1 and AGM-1 (1-way ANOVA;  $F_{(9,87)} = 14.28$ ;  $p < 0.01$ ) but not OP-1 ( $p > 0.05$ ). For the Pond 3 data, the greater than  $1\text{-log}_{10}$  *E. coli* removals were again highly significant for all three treatments ( $p < 0.001$ ), with no significant differences between *E. coli* densities in any of the nine pilot ponds ( $p > 0.05$ ). Indicator organism performance data is summarised in Table 4.10 below.

**Table 4.10.** Summary of indicator organism removals across all pilot plant treatments for Pond 1 and 3 data only.

Microbial performance parameter	Pilot treatment pond					
	RF-1	RF-3	OP-1	OP-3	AGM-1	AGM-3
Mean influent FC ( $\log_{10}$ MPN 100ml <sup>-1</sup> )	1.9	1.9	1.9	1.9	1.9	1.9
Mean effluent FC ( $\log_{10}$ MPN 100ml <sup>-1</sup> ) <sup>†</sup> *	1.1	0.7	1.4	0.7	1.0	0.7
FC removal ( $\log_{10}$ MPN 100ml <sup>-1</sup> d <sup>-1</sup> )	0.8	1.2	0.5	1.2	0.9	1.2
Mean influent <i>E. coli</i> ( $\log_{10}$ MPN 100ml <sup>-1</sup> )	1.7	1.7	1.7	1.7	1.7	1.7
Mean effluent <i>E. coli</i> ( $\log_{10}$ MPN 100ml <sup>-1</sup> ) <sup>†</sup> *	0.6	0.2	1.2	0.3	0.8	0.3
<i>E. coli</i> removal ( $\log_{10}$ MPN 100ml <sup>-1</sup> d <sup>-1</sup> )	1.1	1.5	0.5	1.4	0.9	1.4

<sup>†</sup> Effluent organismal density was tested relative to mean pilot plant influent organism density (1-way ANOVA)

\* Shading intensity shows significance level:  $p > 0.05$  (no shading);  $p < 0.05$  (light);  $p < 0.01$  (medium);  $p < 0.001$  (black)

Performance data from the above Figures and Table shows that although the influent levels of both FC and *E. coli* were of a very low density in the general context of WSP environments, greater than 1- $\log_{10}$  FC removals were able to be achieved across all three treatments and similarly significant approximate 1.5- $\log_{10}$  *E. coli* removals were also realised in all pilot upgrade systems. Despite the higher potential for sunlight-mediated mechanisms of pathogen die-off in the exposed OPs, similar magnitude FC and *E. coli* removals were seen for all three treatment series (Table 4.10), suggesting that the dark-mediated processes (i.e. biological attack and/or antagonistic interactions, protozoan and zooplankton grazing, biofilm attachment, flocculation and sedimentation) were equally effective at surrogate pathogen removal as were the light ones. There was even evidence to suggest that the disinfection efficiency of the RF and AGM reactors was slightly more advanced than the parallel Open Ponds, whereby significant removals of both FC and *E. coli* were observed within Pond 1 of these two treatments but not in OP-1.

Factors governing microbial pathogen removal in WSP environments have been described elsewhere (Sections 1.2.1) as have the likely mechanisms behind indicator organism removal within the pilot RFs and OPs (Section 3.3.8). Since the RF indicator organism removal performance of the current system relative to others' findings has already been largely discussed (Section 3.3.8), only the comparably high-flow work of Mara *et al.* (2001) will be referenced here. Mara and co-workers achieved one order of magnitude lower FC removals ( $\approx 0.2$ - $\log_{10}$  unit) in their pilot-scale rock filters when operated under an almost identical hydraulic loading regime ( $1.0 \text{ m}^3 \text{ m}^{-3} \text{ d}^{-1}$ ) and similarly low-magnitude influent FC density ( $10^3$  MPN 100ml<sup>-1</sup>). The reasons for

achieving 1- $\log_{10}$  unit greater removals during the current research were thought to have related to the 6-fold lower organic loading of our RFs allowing for much higher DO levels within these filters ( $>3\text{mg L}^{-1}$ ) compared with those of Mara *et al.* ( $0.2\text{mg L}^{-1}$ ). These more oxygenated conditions may have fostered a greater density of resident grazers (protozoan and metazoan) which could have then facilitated the higher organism removals (Starkweather *et al.*, 1979; Seaman *et al.*, 1986)—a theory proposed also by Zhao and Wang (1996).

Given their strikingly similar structure and hydraulic flow regime, the processes involved in pathogen removal within attached-growth WSP systems are considered to be the same as the “dark processes” detailed above. As for the discussion of  $\text{NH}_4^+\text{-N}$  and  $\text{PO}_4^{3-}\text{-P}$  performance data above, data on indicator organism removals within the AGM literature is again scarce. Shin and Polprasert (1987) again provide the only known results with respect to indicator organism removal within attached-growth WSP systems. The authors reported no significant advancement in terms of FC removal in their AGWSPs compared with control ponds during both laboratory- and pilot-scale investigations. The authors theorised that the absence of significant performance differences between the two systems could have been due to a shading of the attached-growth pond environment reducing the capacity for UV sterilisation, but they also suggested that this probable reduction in disinfection efficiency may have been offset by an increased surface attachment rate of faecal microorganisms to the media surfaces.

As was the case for nutrient performance data above, it should again be emphasized that the chosen WSP upgrade methodologies were not implemented to achieve pathogen removal nor improve the general microbiological quality of the final Bolivar effluent. Given that the microbiological quality of the final Bolivar effluent was already at such highly polished levels ( $<2\text{-log}_{10}$  organisms  $100\text{ml}^{-1}$ ), large magnitude organism removals were unattainable. Despite the already high microbial quality of the influent wastewater, approximate 1- $\log_{10}$  unit removals were still able to be achieved by all pilot treatments, suggesting that small enhancements in the microbiological quality of the final Bolivar effluent could be expected within both a rock filter and attached-growth media WSP upgrade. Following assessment of indicator organism removal capacity, the final treatment performance ranking for the pilot upgrade methodologies places all three

treatments on equal ground in terms of absolute attenuation efficiency and also performance reliability.

#### **4.3.8 Serviceable life of a rock filter and attached-growth media WSP upgrade**

Whilst there have been reported cases of rock filters operating successfully for up to and in excess of 20 years (Middlebrooks, 1995; USEPA, 2002), rock filter design criteria as well as factors relating to their operational lifespan are generally poorly defined, and in some instances, highly variable and hotly contested (Swanson and Williamson, 1980; Rich, 1988; Middlebrooks, 1995; USEPA, 2002). Furthermore, inconsistencies in rock filter performance have indicated that too little is known about the mechanisms surrounding effective treatment (Middlebrooks, 1995); uncertainties that have in turn resulted in vastly differing opinions regarding *in situ* solids accumulation rates and subsequent serviceable life calculations (Swanson and Williamson, 1980; Reed, 1988).

The rates of refractory solids accumulation vary substantially within the literature based on uncertainties surrounding: the non-biodegradable fraction of WSP effluent VSS; the extent of rock filter anoxia and the subsequent degree of aerobic and anaerobic sludge digestion; and the effect of solids accumulation on interstitial fluid velocity and discrete particulate settlement. This has resulted in the calculated serviceable lifespan for rock filters having also varied considerably, based on wide-ranging differences in the magnitude of the above critical assumptions. For example, Swanson and Williamson (1980) calculated a serviceable lifespan of 55 years for their rock filter based on a 30mg L<sup>-1</sup> solids retention rate, whereas Rich (1988)—using the same solids accumulation rate—predicted that rock filter failure would occur in the order of just 7 years, or some 48 years earlier than the previous authors' calculation.

There seems to be considerable uncertainty regarding the rate of non-biodegradable 'fixed' solids accumulation within the void spaces of rock filters as well as the effect that DO concentration has on the rate of sludge digestion within these void spaces. Oxygen concentration in particular can have a profound effect on the relative biodegradability of accumulated volatile solids. For example, according to Reynolds

(2006), the refractory 'ash' content of algal biomass (Chlorophyta) is approximately 10% (w/w), suggesting a biodegradable organic fraction of 90%. This percentage is, however, known to increase with decreasing DO concentration. For example, according to Foree and McCarty (1970), the refractory percentage of algal biomass is in the order of 40% of the ash-free dry weight (or a total of 44% including 10% ash content as per Reynolds above) under anaerobic conditions. More recently, the work of Harvey and Macko (1997) suggested that approximately 25% of algal-based organic carbon is non-biodegradable under anoxic conditions.

Davies-Colley *et al.* (1995) have suggested that a typically high proportion (averaging about 82%) of VSS in WSP effluents are chemically oxidisable, whereas Rich (1988)—based on the data of Foree and McCarty (1970) above—suggested that the non-biodegradable fraction of volatile SS within the anaerobic confines of a rock filter would be more in the order of 50%. Given that the rock filters reported here were shown to have sustained significantly higher levels of dissolved oxygen than that in other rock filters (Hirse Korn, 1974; Swanson and Williamson, 1980; Saidam *et al.*, 1995; Mara *et al.*, 2001; Mara and Johnson, 2006), a compromise between the data of Davies-Colley and co-workers and Foree and McCarty above was adopted for the following serviceable life calculations (i.e. 70% of VSS was considered to be biodegradable; 30% refractory)—a figure also consistent with the work of Harvey and Macko (1997).

The early work of Swanson and Williamson (1980) focused specifically on investigating the mechanisms behind algal removal in rock filters. Their research demonstrated that sedimentation was the primary means of algal attenuation during rock filter passage, reporting that the efficiency of algal removal was a function of both hydraulic loading rate (fluid velocity) as well as the effective interstitial settling distance for particles in suspension to settle out within the confines of a rock filter. This work was in apparent agreement with the work of Reynolds *et al.* (1991), whereby the removal of particles in suspension was found to be a direct function of water depth, as well as a relating indirectly to fluid velocity. Something not discussed by Swanson and Williamson (1980) and Rich (1988), was that as a rock filter accumulates SS over time within the void spaces, the corresponding void space volume and filter HRT decrease proportionally. Because SS removal efficiency is known to be a function HRT—decreasing in

efficiency with a decreasing and HRT (Swanson and Williamson, 1980; Mara *et al.*, 2001; Johnson and Mara, 2002; Archer and Donaldson, 2003)—it could be expected that rock filters would become progressively ‘less efficient’ at solids retention during their prolonged operational lifespan. This suggests a ‘first-order-type’ decline in long-term treatment efficiency as opposed to the ‘zero-order’ relationship inferred by both Swanson and Williamson (1980) and Rich (1988).

Swanson and Williamson (1980) argued that progressive solids build-up would have the effect of merely reducing the particulate settling distance for infiltrating solids within the filter void spaces, suggesting an effective ‘trade-off’ between the two factors such that the progressive build-up of solids within the voids of the filter would not be expected to significantly affect its long-term removal efficiency. Results from *in situ* experiments conducted by Reynolds *et al.* (1990) on the settlement of algal-sized particles in an unobstructed moving fluid stream, suggested that absolute water depth and not flow velocity proved to be the primary variable determining the rate of sinking loss for particles in suspension. They concluded also, however, that flow velocity was still an important component in the rate of particle settlement, in the sense that it influences the horizontal (lateral) distance travelled by the particle during the time period required for complete settlement. So whilst the first part of Reynolds and co-worker’s findings are in support of Swanson and Williamson’s above hypothesis, the second aspect relating to fluid velocity suggests that as the void spaces fill with accumulated solids, there may be a proportionate increase in wastewater velocity within the void spaces of a rock filter over time.

Considering the above work of both Swanson and Williamson (1980) and also Reynolds *et al.* (1990), and depending on the specific flow-path-length of the filter, it is the opinion of this author that the ongoing accumulation of settled sludge would result in an ever-decreasing HRT and an accompanying gradual decline in the solids retention capacity of the rock filter, to the point where the interstitial flow velocity becomes such that sedimentation of infiltrating particulates is no longer possible and filter failure occurs. As a result of the ever-increasing fluid velocities within the filter, it is theorised that the solids removal efficiency would also decline at an identical rate, such that there would be an effective ‘off-set’ from the continuous solids build-up in the form a steady

reduction in solids retention efficiency. It is thought then, that this performance off-set would effectively result in the ‘slow death’ of the rock filter as opposed to the ‘continuously high treatment performance followed by rapid failure’ assumption adopted by both Swanson and Williamson (1980) and Rich (1988) in their serviceable life calculations.

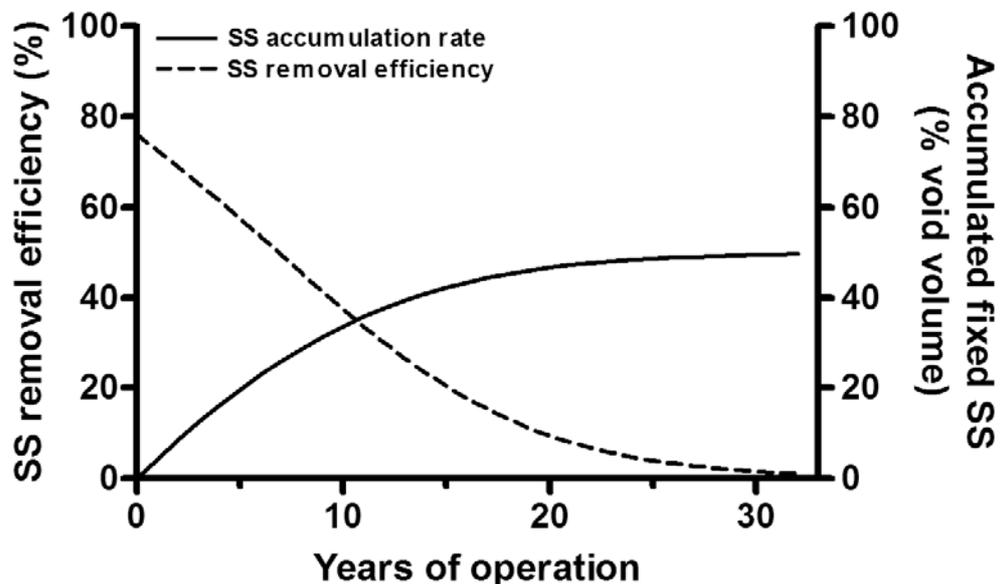
Using the combined performance data from both Chapter 3 and the current chapter, the yearly average SS (this time *including* the outlying spike events), the average VSS fraction, as well as the average percent SS removal efficiency were calculated. Based on: an average influent SS concentration of  $27.4\text{mg L}^{-1}$ ; an average VSS fraction of 52.5%; an average VSS biodegradability of 70%; and an average starting SS removal efficiency of 76%; the annual accumulated mass of non-biodegradable solids can be calculated. Whilst this information provides the mass of accumulated solids, it does not indicate the equivalent void volume occupied by this mass of settled material. Given that settled WSP sludge commonly contains a large volumetric water content (75–91%), not only the solid mass but also the *volume* occupied by this settled sludge must be taken into consideration. Based on the combined data of Rich (1988) and Naméche *et al.* (1997), an average sludge water content of 85% was adopted for the serviceable life calculations. In this sense, 1kg of accumulated SS does not simply occupy  $0.001\text{m}^3$  of void space volume, but 6.7 times this volume (given that the actual solid mass effectively represent only 15% of the total sludge volume). The total annual volume of accumulated non-biodegradable SS ( $SS_{\text{vol}}$ ) was calculated according to Equation 4.1.

$$(SS_{\text{vol}}) = SS_{\text{fv}} \times [100 / (1 - Sl_w)] \quad (\text{Equation 4.1})$$

where accumulated fixed + volatile SS mass ( $SS_{\text{fv}} = SS_{\text{f}} + SS_{\text{v}}$ )  
 accumulated fixed SS mass ( $SS_{\text{f}} = SS_{\text{d}} \times (SS_{\text{i}} \times [1 - VSS_{\text{f}}])$ )  
 accumulated VSS mass ( $SS_{\text{v}} = SS_{\text{d}} \times (SS_{\text{i}} \times VSS_{\text{f}}) \times (1 - VSS_{\text{bio}})$ )  
 annual diminished SS removal efficiency ( $SS_{\text{d}} \%$ ) =  $SS_{\text{e}} - (SS_{\text{e}} / [V_{\text{vd}} / V_{\text{vi}}]) + SS_{\text{e}}$   
 annual diminished void volume ( $V_{\text{vd}} = V_{\text{vi}} - SS_{\text{vol}}$ )  
 annual influent total SS mass loading ( $SS_{\text{i}} = (\text{HLR} \times SS_{\text{m}}) \times 365$ )  
 starting mean total SS removal efficiency ( $SS_{\text{e}} = 76\%$  for RF;  $71\%$  for AGM)  
 daily mean influent total SS concentration ( $SS_{\text{m}} = 27.4\text{g m}^{-3}$ )  
 initial void volume ( $V_{\text{vi}} = 55.86\%$  for RF;  $95.7\%$  for AGM)  
 HLR = 1.0  
 annual mean volatile SS fraction ( $VSS_{\text{f}} = 52.5\%$ )  
 mean VSS biodegradable fraction ( $VSS_{\text{b}} = 70\%$ )  
 mean sludge water content ( $Sl_w = 85\%$ )



Assuming a direct 1:1 ratio in terms of the reduction in SS removal performance with reducing void volume (based on the direct negative linear relationship between HLR and SS removal efficiency; Swanson and Williamson, 1980), and based on the data from both Chapter 3 and the current chapter, the longest possible serviceable life of a Bolivar rock filter operated at an hydraulic loading rate of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$  was calculated to be in the order of 30 years (Figure 4.46). Practically, however, the feasible serviceable lifespan would be more in the order of 15–20 years, after which the solids removal performance would have reduced by around 85% compared with ‘day zero’ operational efficiency.

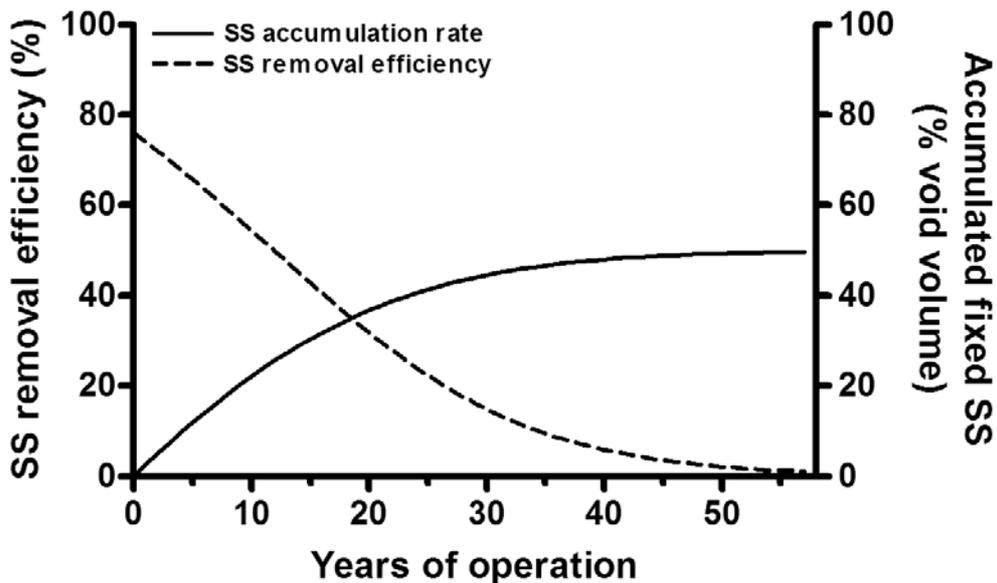


**Figure 4.46.** Theoretical serviceable life of a Bolivar-based rock filter (based on a void volume of 55.86%, HLR of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ , mean influent SS of  $27.4\text{g m}^{-3}$ , mean VSS of 52.5%, mean SS removal efficiency of 76% and a sludge water content of 85%).

As shown in Figure 4.46, further reductions in rock filter void space volume would not be realistically expected above and beyond approximately 35 years of operation. This ties in well with the suggestions of Rich (1988) in that complete plugging of all filter void spaces is considered impossible due to the constant head pressure from the upstream WSP preventing 100% filter clogging. It is interesting to note that based on the above calculations, the reduction in filter performance appears to plateau at the 50% void volume reduction mark. This is in apparent agreement with the earlier assumption

of Swanson and Williamson (1980) that a rock filter can operate effectively up until a point where the original filter void volume has been reduced by 50%.

Taking into account the combined three-pond average AGM solids removal performance of 71%, and using Equation 4.1 based on identical assumptions to those discussed above for a rock filter, the longest possible serviceable life of a horizontal-flow attached-growth media installation for the Bolivar WSP network would be in the order of 50 years—some 20 years more than that of an equivalent rock filter (Figure 4.47). This enhancement in serviceable life is a simple reflection of the 40% greater void volume of the AGM compared with the rock media, and affords the AGM systems an increased interstitial capacity for solids accumulation.



**Figure 4.47.** Theoretical serviceable life of a Bolivar-based AGM upgrade installation (based on a void volume of 95.7%, HLR of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ , mean influent SS of  $27.4\text{g m}^{-3}$ , mean VSS of 52.5%, mean SS removal efficiency of 71% and a sludge water content of 85%).

As shown in Figure 4.47, and as for the rock filter calculations above, complete plugging of the AGM is never achieved; however, complete failure of the AGM upgrade installation would nevertheless be expected after approximately 55 years of operation. Whilst the absolute serviceable life of an AGM installation would be around 50 years, the practical operational lifespan of such a system is suggested to be in the order of 30–35 years, after which time the capacity for solids retention would be reduced by roughly

85% of ‘day zero’ levels. It should be noted that these serviceable life estimates are generic calculations *per cubic metre* of rock filter and AGM upgrade installation, and actual serviceable lifespan would be expected to vary according to the *in situ* size and lateral flow-path-length of the specific full-scale installation.

Despite the pilot AGM ponds having a 4-fold greater specific surface area, roughly double the void volume, reduced interstitial fluid velocities, and arguably more ideal flow hydraulics than the parallel Rock Filters, the treatment performance of these two upgrade systems was shown to be virtually indistinguishable throughout the current chapter. Whilst the enhanced surface area and greater void volume for the AGM over the RFs did not result in significantly enhanced treatment performance, it would effectively translate to a longer serviceable life. The physical nature of the light-weight polypropylene attached-growth media would also mean that it would be a much ‘less-permanent’ installation, and—as suggested by Shin and Polprasert (1988)—could presumably be removed, washed and re-installed (especially for smaller-scale systems). This would also imply more straightforward decommissioning and cleaning protocols for AGM installations over rock filters, something that would reduce the period of downtime during decommissioning and cleaning and may also go toward off-setting the higher cost of the initial AGM installation. Having said this, the cost savings per m<sup>3</sup> for a rock filter are likely to be significant compared with the comparatively expensive *in situ* horizontal-flow attached-growth media, therefore, it is recommended that careful cost–benefit analyses should be conducted prior to installation of either upgrade system at the Bolivar treatment plant. These issues will be revisited in Chapter 10.

#### **4.4 General research findings and chapter summary**

The current chapter was concerned with investigating the relative treatment efficacy of rock filtration and attached-growth media in comparison to a non-interventional Open Pond ‘control’ treatment for the upgrading of maturation WSP effluent. Whilst there has been considerable prior research into rock filtration for upgrading WSPs, investigations into attached-growth ponds are comparatively lacking. Research from this chapter has also presented the first known performance comparison of a standard rock filter and an

operationally similar horizontal-flow AGM system for the upgrading of final maturation WSP effluent.

Results from hydraulic tracer analyses showed each pilot pond treatment to be operating under a similarly well-mixed flow pattern, such that any subsequent differences in between-treatment performance efficiency were considered to have occurred independently of reactor hydraulic flow regime. Performance data presented during this chapter has shown an overwhelming trend for enhanced treatment performance by both the RFs and AGM Reactors over the majority of monitored water quality parameters. In spite of significantly reduced oxygen concentrations, the absolute magnitude of treatment efficiency as well and the reliability of treatment performance were commonly significantly advanced within both the RF and AGM series across numerous parameters compared with the parallel OPs. Factors pertaining to the high and often variable wastewater quality within the OP series were also discussed.

BOD<sub>5</sub> removal performance was significantly more advanced within the RF and AGM pilot upgrade series, with the parallel Open Ponds yielding no significant removals of inflowing BOD<sub>5</sub> whatsoever. Similar performance trends were observed also for both SS and chlorophyll *a* removal, with the RF and AGM treatments consistently outperforming OP series and suggesting a greater capacity for total and algal solids removal during RF and AGM upgrade treatment. It is suggested that both rock filters and attached-growth media upgrade systems could provide Bolivar DAF/F plant operators with greater confidence in final WSP effluent quality. Monitoring of pilot plant nutrient dynamics showed that the RFs and AGM ponds were again significantly more capable of removing infiltrating ammonia than was the OP series; however, no significant changes in the levels of dissolved PO<sub>4</sub><sup>3-</sup>-P were evident in any of the three treatment series. Indicator organism data revealed all pilot systems to be equally effective at microbial disinfection.

Representation of parameter loading data for BOD<sub>5</sub>, SS, chlorophyll *a*, and NH<sub>4</sub><sup>+</sup>-N on a mass basis allowed for additional insights into the nature of treatment performance, and revealed a general trend for increasing performance with an increased mass loading rate. This trend was seen across all of the above parameters and across all treatments (where

significant parameter removals were recorded) and was a reflection of the first-order-type concentration-dependent removal kinetics for these water quality parameters. The same analyses also revealed a greater separation of treatment efficiency at lower mass loadings, such that RF and AGM treatment performance was consistently higher than the OP series at low loading rates. Conversely, and under conditions of high influent solids, algal or BOD<sub>5</sub> loading, there was a reduced separation in treatment efficiency, such that the relative performance differences between each of the three treatments were invariably reduced. These trends again inferred that predominantly physical mechanisms were governing BOD<sub>5</sub>, SS and chlorophyll *a* removal within the pilot ponds. Although treatment mechanisms were shown to be overwhelmingly physical, there was also evidence for biological treatment activity too; with microbial nitrification demonstrated to have been occurring in both the RF and AGM treatments as well as suggestions of grazing interactions within all three pilot series.

Final performance rankings for each of the water quality parameters showed the RFs and AGM ponds to be the most efficient WSP upgrade systems across all monitored parameters except for faecal coliforms and PO<sub>4</sub><sup>3-</sup>-P, where performance results were observed to be similar across all three treatments.

#### **4.5 Suggestions for future research**

Throughout this chapter, there were a number of suggested topics for future scientific investigation. Below is a summary of these concepts:

- More work is needed to investigate the treatment performance of the horizontal-flow AGM under increased mass and/or hydraulic loadings (BOD<sub>5</sub>, SS, algal biomass and NH<sub>4</sub><sup>+</sup>-N). Given that the majority of parameter removals were frequently achieved by the first pond of each three-pond series, it is likely that maximum loading rates would be significantly higher than those tested here;
- Future investigations could look into AGM packing density and its effect on attached-growth WSP treatment performance. Similarly, future work could involve the use of similar AGM but with different channel widths (e.g. 12mm channels as opposed to the 19mm channel size media used during the current

work) to assess any potential benefits arising from the increased specific media surface area (i.e.  $240\text{m}^2 \text{m}^{-1}$  compared with  $150\text{m}^2 \text{m}^{-3}$ );

- It is suggested that there is a need for additional work on phosphorous dynamics within attached-growth WSP systems.

## **6 Phytoplankton survival during prolonged darkness under conditions of ambient and reduced dissolved oxygen—literature review and introduction**

### **6.1 *Phytoplankton and photolithotrophy***

The vast majority of phytoplankton are obligate photoautotrophs; that is to say they gain all nutrition from inorganic sources (Neilson and Lewin, 1974; Prézelin *et al.*, 1991). Photoautotrophic phytoplankton use energy from sunlight to drive enzymatic cellular processes whereby inorganic carbon ( $\text{CO}_2$  and  $\text{HCO}_3^-$ ) from the aquatic environment is reduced and converted into organic carbon in the form of cellular biomass, whilst the inorganic reducing agent ( $\text{H}_2\text{O}$ ) is oxidised to oxygen (Neilson and Lewin, 1974). The useable spectra of light energy for photosynthesis is of wavelengths 400–700nm and is termed ‘photosynthetically active radiation’ (PAR) (Reynolds, 2006). The importance of light in the energetic life cycle of photoautotrophic phytoplankton is elementary.

Photosynthesis can proceed under a wide range of light climates, with variations coming from both the nature (spectral array) and intensity of the light source (Reynolds, 2006). Whilst there are no doubt definitive optimal photosynthetic light intensities (Photon Flux Densities; PFDs) for a given phytoplankton species (growing under defined environmental conditions), there are at the same time, super-saturating photo-inhibitory, sub-optimal to limiting irradiances, or indeed conditions of complete darkness, under which—if they persist for an extended period—cellular photosynthesis and growth may be reduced or cease altogether. Such conditions may lead to not only a reduction in cellular and hence population growth, but also potential adverse physiological effects for dark-exposed cells; adversities which may ultimately promote a reduction in cellular fitness or biological viability.

### **6.2 *Photophysiological acclimation by phytoplankton to changes in light climate: a survival strategy***

Phytoplankton are extraordinarily adept at survival—indeed proliferation—within a wide range of notoriously unstable aquatic environments. In order to survive, cells are forced to continually respond and adapt to localised environmental perturbations (e.g.

light intensity and quality, temperature and water chemistry) that occur not only on different time-scales but with differing frequencies (Prézelin *et al.*, 1991). In addition to this inherent environmental variability, phytoplankton are generally small, free-floating organisms and are thereby subjected to passive transport and distribution within their aqueous environment (Prézelin *et al.*, 1991). In order to be ecologically competitive, phytoplankton must therefore be able to withstand sub-optimal growth conditions at potentially any time and for an uncertain duration.

Free-floating phytoplankton within the pelagic environment rely exclusively on sunlight to drive their photosynthetic cellular processes. Since light is often a limiting factor for growth and productivity, especially in marine phytoplankton, it follows that the ability of algal cells to continually adapt to their ever-changing environment is an important if not central feature of phytoplankton physiological ecology (Richardson *et al.*, 1983; Falkowski, 1984; Neori *et al.*, 1984; Palmisano *et al.*, 1985). This overall cellular photophysiological adaptational response (first suggested by Steeman Nielsen and Jørgensen, 1968) to changes in light climate, is termed 'photoacclimation'. Photoacclimation occurs in direct response to changes in the PFD and spectral array of incident light and allows for an increased ability to utilise low-energy light, with adjustments cellular of pigments, photosynthetic processes and respiration in order to maintain vitality in sub-optimal conditions. For a more comprehensive introduction and description of the relevant literature, the reader is directed to a highly informative minireview by Falkowski and LaRoche (1991).

It should be emphasized that there is an important distinction between the terms 'photoacclimation' and 'photoadaptation' within the relevant literature. Generally photoacclimation refers to physiological *phenotypic* adjustments that arise in direct response to environmental perturbations, whereas photoadaptation encompasses changes in the *genotype* that arise either from mutations or from allelic distributional changes within the gene pool (Falkowski and LaRoche, 1991; MacIntyre *et al.*, 2002). The overall process of photoacclimation is complex, and, according to Falkowski and LaRoche (1991), involves many cellular modifications that can occur on a number of cellular levels. Morphologically, it can be accompanied by changes in: cell volume; the number and density of thylakoid membranes; the size of pyrenoids and other storage



bodies; and sometimes changes to the number of cellular plastids. Cytologically, photoacclimation involves changes in photosynthetic pigment (e.g. increased cellular chlorophyll and decreased maximum photosynthetic rates) and lipid content and composition. Physiologically, there are modifications to the minimum quantum requirement for oxygenic photosynthesis and in cellular growth rate. To some extent, all phytoplankton are capable of photoacclimation, with adaptational timescales shorter than or equivalent to a cell's generation.

Natural phytoplankton assemblages are surrounded by inherent environmental instability. Furthermore, they have little or no influence over important properties of their immediate growth environment and must constantly adapt by diverting synthesised energy from cellular metabolism to deal with environmental change (Prézelin *et al.*, 1991). As a direct consequence of this environmental variability, phytoplankton often exist in regions where solar radiation is too low to support normal autotrophic metabolism (e.g. below the photic depth, buried within sediments, or shaded at the base of thick periphyton communities; Tuchman *et al.*, 2006). Consequently, phytoplankton are adept at photoacclimating to changes in light climate: both spectral quality (Fisher *et al.*, 1996) and intensity (Flameling and Kromkamp, 1997).

Arguably the most prominent and indeed extreme example of the potential adversity resulting from a dynamic light climate is that endured by the polar phytoplankton. Algal species existing in these regions are subjected to lengthy periods of low-intensity light or darkness on a yearly basis as they become covered by thick ice sheets and snow, and as such, are well adapted to dark-survival during this 'over-wintering' period (Rodhe, 1955; Anita and Cheng, 1970; Palmisano and Sullivan, 1983; Palmisano *et al.*, 1985). Although prevalent in polar environments, dark-exposure is not only a feature of life at extreme latitudes. In the open ocean for instance, the vertical distribution of phytoplankton can extend well below the euphotic zone; indeed live phytoplankton are often recovered from well below the illuminated ocean layers (Jochem, 2000). Platt (1983), for example, recovered photosynthetically-competent phytoplankton (Bacillariophyceae, Coscinodiscophyceae, Dinophyceae) from the aphotic depths (1000m) of the deep-ocean. Similarly, a group of authors (Kiefer *et al.*, 1972; Tilzer *et al.*, 1977; Vincent, 1978) reported on the recovery of viable phytoplankton

(Chlorophyceae, Chrysophyceae, Coscinodiscophyceae) from the deep (up to 400m) aphotic zone of Lake Tahoe (California, Nevada). The presence of substantial phytoplankton biomass existing well below the photic zone in turn raises questions about both the time scales of cellular viability and also the physiological status of these autotrophic cells in dark conditions (Murphy and Cowles, 1997).

For phytoplankton existing in light-limited habitats, if sufficient light energy is unable to be sequestered during normal photosynthetic processes, then some form of adaptational photoacclimation response must be employed. Consequently, most unicellular phytoplankton has evolved to: (a) be metabolically diverse; (b) display opportunistic strategies; and (c) exhibit a high degree of physiological plasticity (Prézelin *et al.*, 1991). Lee and Rhee (1999) demonstrated the generic advantages of possessing such 'adaptational plasticity' through demonstrating that the cyanobacterium *Anabaena flos-aquae* was well able to adjust its nutritional requirements and subsequent growth rate in response to alterations in light climate (intensities ranging from limitation to photoinhibition). They echoed the above sentiments by concluding that this kind of adaptational responsiveness to a changing environment would ultimately assist in a species' competitiveness in the natural environment.

Any *in vivo* cellular deviations from 'the norm' as a result of implementing this physiological and adaptational plasticity, must therefore consume additional internal resources; resources which might otherwise be directed toward normal growth and reproduction. This can in turn severely restrict phytoplankton growth potential or even threaten immediate survival (Prézelin *et al.*, 1991). This has obvious follow-on implications with respect to competition and natural species succession (i.e. physiological ecology) of phytoplankton in the environment. The reader is directed at this point to several informative reviews on environmental variability and its implications for phytoplankton photosynthesis, ecology and natural species succession (Harris, 1978; Reynolds, 1984; Prézelin *et al.*, 1991).

### **6.3 *Phytoplankton and dark-survival***

Of the many ways in which their aquatic environment may vary (e.g. pH, temperature, salinity, DO), the reduction or complete absence of light could be considered to be of greatest consequence for resident phytoplankton. When algae are brought from conditions of saturating to sub-saturating irradiance, the cells undergo an ‘energy crisis’ whereby they need to harvest increasing amounts of light in order to maintain cellular growth rates (Falkowski and LaRoche, 1991). This so-called energy crisis is arguably far more severe for cells going from a light-saturated environment to one of complete darkness, especially when the dark period persists for an extended duration.

Because phytoplankton are subject to passive transport within the aquatic environment, they are often exposed to less than optimal growth conditions (such as darkness) for an uncertain and potentially extended duration. Survival strategies (such as photoacclimation) employed in response to these sub-optimal growth environments are invariably energetically expensive, as cells are forced to expend their energy reserves in order to deal with environmental stressors (Falkowski and LaRoche, 1991; Prézelin *et al.*, 1991). The capacity of phytoplankton to perform this photoacclimation response during and following a prolonged ‘dark stress’ event forms the basis for work presented in the current review Chapter as well as the later experimental Chapter 9.

There exists only a relatively limited body of prior research into the dark-survival of phytoplankton in general. Additionally, ‘dark-survival’ *per se* can constitute a period of dark-exposure in the order of a few days up to several months—even years—depending on the author’s interpretation and the practical application of the dark timescales relevant to their research interests. For general inclusion into this phytoplankton dark-survival review, the darkened period had to be greater than or equal to **three** days, with “prolonged darkness” referring to dark-survival over a period of **six** or more days. What little research does exist has focused mainly on marine phytoplankton species, and within those studies, the vast majority involves dark-survival assessment of species from: Bacillariophyta (diatoms); Ochrophyta (diatoms, yellow-green and golden algae); and Dinophyta (dinoflagellates).

It should also be noted that there exists a limited amount of published research into the dark-survival of Cyanobacteria (blue-green ‘algae’). The issue of including the *Cyanobacteria* in *phytoplankton* research remains a contentious one. Since Cyanobacteria still commonly fall under the general umbrella of ‘the phytoplankton’ (see Reynolds, 2006) they are not to be excluded from the current Chapter. Having said this, the fact that Cyanobacteria are fundamentally and taxonomically distinct from the majority of the phytoplankton (i.e. prokaryotic not eukaryotic) confers some obvious and fundamental differences in basic cellular physiology to that of the eukaryotic algae (Geider and Osborne, 1989; Sigee, 2005). Considering this, specific references to cyanobacterial dark-survival *per se* within the current Chapter are very limited. Where reference is made, however, it is only provided in either a methodological context (e.g. for cytometric staining comparison) or for general ‘illustrative’ purposes (i.e. not involving direct dark-survival comparisons with eukaryotic phytoplankton).

The ability to survive prolonged periods of darkness—commonly 6 or more days—has been demonstrated within the literature across a wide range of phytoplankton species, including, as above, some cyanobacterial species. The literature review of research relating to phytoplankton dark-survival included results from laboratory studies only, and excluded those reports based on more qualitative field ‘observations’. Dark-survival literature was also drawn specifically from research on ‘wild-type’ isolates only and excluded ‘mutants’. Furthermore, specific investigations into long-term resilience of morphologically-distinct ‘resting stages’ has been excluded, together with research focusing specifically on phytoplankton dark growth and/or heterotrophy *per se*; although there have been a limited number of more relevant research inclusions from the latter group. The literature base also includes a number of additional works pertaining generally to ‘phytoplankton dark-exposure’ as such, even if the research was not directly concerned with assessing dark-survival potential *per se*.

The compiled list of taxonomic phyla on which phytoplankton dark-survival research has been conducted is shown in Appendix F. Of this extensive listing, the majority of researchers have focused on marine phytoplankton species (most commonly Dinophyceae, Bacillariophyceae and Coscinodiscophyceae). Consequently, there has been a relatively limited volume of prior research dealing specifically with the green

algae (Chlorophyceae), as well as relatively limited research effort focusing on the dark-survival of freshwater species in general. The current research will focus on the dark-survival of two freshwater chlorophytes.

### 6.3.1 Dark-survival strategies

Within this body of literature, a number of cellular *strategies* are reportedly adopted by phytoplankton to deal with dark conditions. According to Dehning and Tilzer (1989), the dark-survival strategies of phytoplankton can be divided into three major categories: (1) reduced respiratory activity; (2) the formation of dormant ‘resting stages’; and (3) heterotrophic activity (including mixotrophy).

Phytoplankton dark-survival through reduced cellular respiration, or simply by pure persistence through the conservation or ‘winding-back’ of cellular metabolic processes, has been reported by several authors (Smayda and Mitchell-Innes, 1974; Anita, 1976; French and Hargraves, 1980; Dehning and Tilzer, 1989; Jochem, 1999). It has been postulated (Smayda and Mitchell-Innes, 1974; Anita, 1976) that species which lack the necessary cytological machinery for the development of dormant or ‘resting stages’, may invoke physiological-biochemical mechanisms for dark-survival; controlling energy expenditure from endogenous metabolism (or respiration) to the *bare minimum* required for long-term maintenance of cellular viability. This active reduction of ‘capital costs’ during low-light or darkness is viewed as an obvious mechanism by which phytoplankton may enhance their chances of survival under adverse light climates (Richardson *et al.*, 1983). These biochemically “quiescent” phases in phytoplankton have indeed been likened to those occurring during winter dormancy in higher plants (Anita, 1976). Dark-survival mechanisms can also involve the development of morphologically-distinct resting stages such as: ‘resting spore’ formation in diatoms (Anderson, 1975; Sicko-Goad *et al.*, 1989; McQuoid and Hobson, 1996); ‘cyst’ formation in dinoflagellates (Selvin *et al.*, 1988/1989; Chapman and Pfieste, 1995; Rengefors and Anderson, 1998; Lewis *et al.*, 1999); and ‘akinetes’ in Cyanobacteria (Rother and Fay, 1977; Jochem, 1999); and green algae (Coleman, 1975; McKnight *et al.*, 2000).

Similarly, the capacity for dark-assimilation of organic carbon, and even dark-growth, through alterations to modes of cellular nutrition—most commonly facultative heterotrophy—has been reported for: marine diatoms (Hellebust and Guillard, 1967; Hellebust, 1971; White, 1974; Deventer and Heckman, 1996; Tuchman *et al.*, 2006); green algal species of *Friedmannia* (Vincent and Goldman, 1980); *Scenedesmus* (Kulandaivelu and Senger, 1976a; 1976b; Abeliovich and Weisman, 1978; Jochem, 1999; Furusato *et al.*, 2004); *Micractinium* (Bouarab *et al.*, 2004); *Pediastrum* (Berman *et al.*, 1977); *Chlamydomonas* (Bennett and Hobbie, 1972; Laliberté and de la Noüe, 1993; Heifetz *et al.*, 2000); and *Chlorella* (Killam and Myers, 1956; Karlander and Krauss, 1966; Endo *et al.*, 1977; Ogawa and Aiba, 1981); as well as in red algal species of *Delesseria* (Lüning and Schmitz, 1988). Selvin *et al.* (1988/1999) even proposed a potential capacity for heterotrophy and/or phagotrophy as a strategy for dark-survival in some marine dinoflagellates; with phagotrophy reported elsewhere as being of nutritional significance amongst the Chrysophyceae (Bird and Kalff, 1987; Porter, 1988; Sanders *et al.*, 1990). These reports were echoed by the findings of Jochem (1999) who reported that bacterivory was likely to have augmented the dark-survival of the haptophyte *Chrysochromulina hirta*.

It should be noted, however, that the overwhelming consensus from this reporting on alternate trophic states (e.g. facultative heterotrophy or phagotrophy) in phytoplankton, is that they are invariably ‘lesser’ modes of nutrition (i.e. less productive and slower) in comparison to that of photolithotrophy, and, as such, are generally thought to be unimportant for phytoplankton in the natural environment; becoming relevant only under conditions of inorganic nutrient or light limitation (Killam and Myers, 1956; Sloan and Strickland, 1966; Wright and Hobbie, 1966; Bennett and Hobbie, 1972; Neilson and Lewin, 1974; Vincent and Goldman, 1980; Ogawa and Aiba, 1981; Gibson and Smith, 1982; Richardson and Fogg, 1982; Tsavalos and Day, 1994; Gervais, 1997) and even then, only under defined substrate conditions.

Reynolds (2006) discusses how osmotrophy (a bacterium-like ability to directly absorb selected dissolved organic compounds across the cell surface) is possible in some chlorophyte algae, including members of the Chlorococcales to which the genus *Chlorella* belongs. Regardless of specific cellular strategy, it is clear from the relevant

literature that different algal species utilise an array of mechanisms for dark-survival, and as a result, ultimately have largely differing capacities for withstanding a given period of dark-exposure. Indeed Anita and Cheng (1970), following assessment of the long-term dark-survival capacity of 31 species of marine phytoplankton from six taxonomic classes, observed no apparent correlation between the duration of dark-survivorship and phylogenetic classification. Instead the authors concluded that ecological origin had a far more dominant role in defining long-term resilience to dark conditions, with species isolated from cold water and benthic environments displaying a more advanced dark-survival capacity.

Given the relative ‘uncertainty’ surrounding phytoplankton dark-survival—in terms of both the dark-survival strategy and subsequent dark-survivorship—the current research included investigations into this area. Following preliminary investigations into WSP algal ecology, two model candidate phytoplankton species (*Chlorella vulgaris* (Chlorophyceae, Chlorococcales) and *Chlamydomas reinhardtii* (Chlorophyceae, Volvocales)) were chosen based on their recognised tolerance for high levels of organic pollution and hence natural prevalence within WSP environments (Oswald *et al.*, 1953; deNoyelles Jr., 1967; Palmer, 1969; Pearson *et al.*, 1987; Wrigley and Toerien, 1990). In addition, their unicellular nature and small (<10 $\mu$ m) physical size made them ideal candidates for experimental analysis via flow cytometry.

### **6.3.2 Phytoplankton cell death and dark-survival—implications for algal community ecology**

In spite of the immense global importance of phytoplankton primary production, many aspects of the ecology and physiology of these highly diverse organisms are poorly understood; one of which includes the process surrounding phytoplankton cell death (Berges and Falkowski, 1998; Lee and Rhee, 1999; Berman-Frank *et al.*, 2004). Recent research into the area has gone some way to defining cell death processes in phytoplankton (Lee and Rhee, 1997; Agustí *et al.*, 1998; Berges and Falkowski, 1998; Veldhuis and Kraay, 2000; Garbary and Clarke, 2001; Agustí and Carmen Sánchez, 2002; Segovia *et al.*, 2003; Berman-Frank *et al.*, 2004; Franklin and Berges, 2004; Segovia and Berges, 2005; Agustí *et al.*, 2006; Franklin *et al.*, 2006), with increasing

evidence for internally-mediated, autocatalysed, apoptotic-type ‘programmed cell death’ pathways being of significance for phytoplankton death in the natural environment (Kirchman, 1999; Veldhuis *et al.*, 2001; Franklin *et al.*, 2006).

Relatively recent advances in the field of cell biology have paved the way for new insights into the processes associated with cell death; insights which have, in turn, resulted in new concepts as well as the development of new methods that can be applied to quantify phytoplankton cell death (Agustí *et al.*, 2006). Early attempts aimed at quantifying phytoplankton cell death in freshwater ecosystems yielded varying conclusions about its importance as a ‘loss factor’ in natural systems, with cell death being identified as important in shaping some communities and of negligible influence in others (Jassby and Goldman, 1974; Knoechel and Kalff, 1978; Reynolds *et al.*, 1982). More recent research, however, has indicated that phytoplankton cell death could play a significant, if not defining role in phytoplankton community ecology and population dynamics (Agustí *et al.*, 2006; Franklin *et al.*, 2006). In line with these new concepts surrounding cell death, the loss of vitality (viability) is nowadays—next to grazing and sedimentation—considered to be the third most dominant loss factor responsible for reducing the size of phytoplankton populations in the field (Veldhuis and Kraay, 2000; Agustí and Carmen Sánchez, 2002).

Although much is known about the factors controlling phytoplankton growth and their physiology during cell division, there is relatively little understanding of the factors affecting cell death. According to Berges and Falkowski (1998), and more recently Franklin and Berges (2004) and Franklin *et al.* (2006), the physiological processes involved in natural phytoplankton mortality resulting from environmental stress (such as darkness for example) remain poorly understood. In light of the relatively concise body of research effort to date concerning natural phytoplankton cell death (i.e. excluding death from predation and sedimentation) and in line with the opinion of Furusato *et al.* (2004), the current level of understanding about what happens when phytoplankton are exposed to prolonged periods of darkness is considered insufficient. This is especially the case when phytoplankton is exposed to varying concentrations of dissolved oxygen during prolonged darkness.



The variable and species-specific rates of phytoplankton growth, as well as the rates of subsequent cell death under a given environment, have a significant influence on phytoplankton community ecology in nature. At the same time, the variable capacity of phytoplankton for dark-survival is also recognised to be a deterministic factor in the shaping of algal community composition under sub-optimal light climates, in that it can directly influence the outcomes of interspecies competition *during* as well as dictate the relative rates of cell death and subsequent species abundance and diversity *post*-darkness (Lee and Rhee, 1997). In other words, the relative amount of time a particular species is able to withstand dark conditions can ultimately determine both its immediate dark-survivorship and also its post-darkness vigour and competitive success (Jochem, 1999). This variable capacity for dark-survival may have direct practical implications in terms of how well different phytoplankton are able to survive during advanced in-pond upgrade treatment (e.g. within a rock filter or underneath a duckweed surface cover). Differential rates of dark-survival in this instance might also have additional downstream implications for the process efficiency of more intensive WSP effluent upgrade technologies (i.e. DAF/F) in terms of determining the algal species composition of the DAF/F plant influent and the relative ease with which this suspended algal biomass is ultimately able to be removed (see Section 1.3.1).

The seemingly advanced capacity for long-term survival under adverse environmental conditions such as darkness, whilst ecologically advantageous for the phytoplankton, is arguably a less desirable trait from the point of view of those involved in final WSP effluent polishing. The three advanced WSP upgrade methodologies that form the focus of the current research (duckweed coverage, rock filtration and attached-growth media addition) all involve, among other things, subjecting suspended algal populations to periods of low-light intensity or darkness for an undefined and potentially extended duration. In order to assist with the development and management of these advanced WSP upgrade technologies, it is important to try and understand the processes involved in algal cell death and their interrelationship with physiological dark-survival *in situ*.

### 6.3.3 Phytoplankton cell death dark-survival—implications for advanced WSP upgrade technologies

There has been considerable research effort into duckweed ponds, and less so for rock filters and attached-growth media, as advanced techniques for sequestering algal biomass from WSP effluents, with their effectiveness demonstrated by numerous authors (see Sections 1.2.8.4–1.2.8.7). The general consensus from the limited number of more detailed investigations into the ‘nature’ of this effective upgrade system performance (Stutz-McDonald and Williamson, 1979; Swanson and Williamson, 1980; Ellis, 1983; Rich, 1988; Reynolds *et al.*, 1990) have suggested that algal removal is achieved—first and foremost—as a result of physical processes (i.e. sedimentation, biofilm adsorption and entrapment) rather than from internal, cell-mediated, biological pathways *per se* (e.g. algal senescence and cell death). Similarly, additional work concerned with algal removal through alterations to the internal hydrodynamics of WSPs (e.g. Herdianto, 2003) has raised unanswered questions about how long algal cells may need to remain in dimly lit, hypoxic, benthic pond regions in order to be permanently (and terminally) removed from the system.

Several pertinent questions have arisen from these issues, and they served as the basis for the remaining research effort of Chapters 7 to 9.

- 1) What are the physiological consequences for viable cells both during and after this potentially extended dark period?
- 2) If there are adverse implications for cellular fitness and viability as a consequence of darkness, then what are the timescales of these processes (i.e. what period of dark-exposure is required in order to diminish algal cell viability or instigate cell death)?
- 3) Is the capacity for prolonged dark-survival in WSPs likely to be entirely species-dependent (even for two green algae with similar ecological distributions) in line with the trends reported within the wider literature?
- 4) Is the concentration of DO (‘high’ or ‘low’) likely to influence the long-term dark viability of WSP phytoplankton, given that conditions of reduced DO availability are likely to predominate within duckweed, rock filter and attached-growth media environments?

- 5) If a certain percentage of the population remains viable post-darkness, what are the re-growth kinetics for this surviving population (i.e. is there an extended lag period during population re-growth, and how quickly would cells be expected to ‘bounce back’ upon re-exposure to a more optimal light climate)?

As discussed above, dark survivorship has implications for aquatic phytoplankton both physiologically and also ecologically—ecologically as a direct result of their physiological ability to withstand dark-exposure. This has direct implications with respect to whether or not algal cells are able to physically endure the *in situ* conditions experienced during duckweed, rock filter or AGM upgrade treatment, and whether or not algal populations are then able to retain sufficient levels of viability to allow for their persistence in final pond effluents. This means that phytoplankton species with more advanced dark-survival capacities might be expected to be:

- a) more resistant to being physically removed by the advanced WSP upgrade process itself (by being more physically active during darkness and therefore being able to resist sedimentation or biofilm entrapment through mechanisms such as active buoyancy regulation or retained cellular motility);
- b) more resilient to biological removal through a more advanced dark-maintenance of metabolic vigour, allowing for enhanced resilience to biological attack from algal viruses or bacteria (e.g. Agustí *et al.*, 1998; Fuhrman, 1999; Brussaard *et al.*, 2001), or possibly even through an enhanced cellular resilience to predation (given that some phytoplankton (e.g. various marine Antarctic diatoms, and *Chlorella*) are able to traverse the digestive tract of zooplankton (copepods and *Artemia*) unscathed (e.g. Smayda, 1970) and so presumably a less physiologically robust or metabolically active phytoplankton would be less resilient to the digestion process);
- c) able to quickly re-grow in post-treatment effluent should cells be re-exposed to more favourable environmental conditions (e.g. type I and type II dark-survival strategists of Smayda and Mitchell-Innes, 1974 and Jochem, 1999).

Ecologically speaking, the above factors could have obvious implications in terms of regulating the phytoplankton community composition the effluent of an in-pond upgrade system. For example, whichever species display the greatest physiological

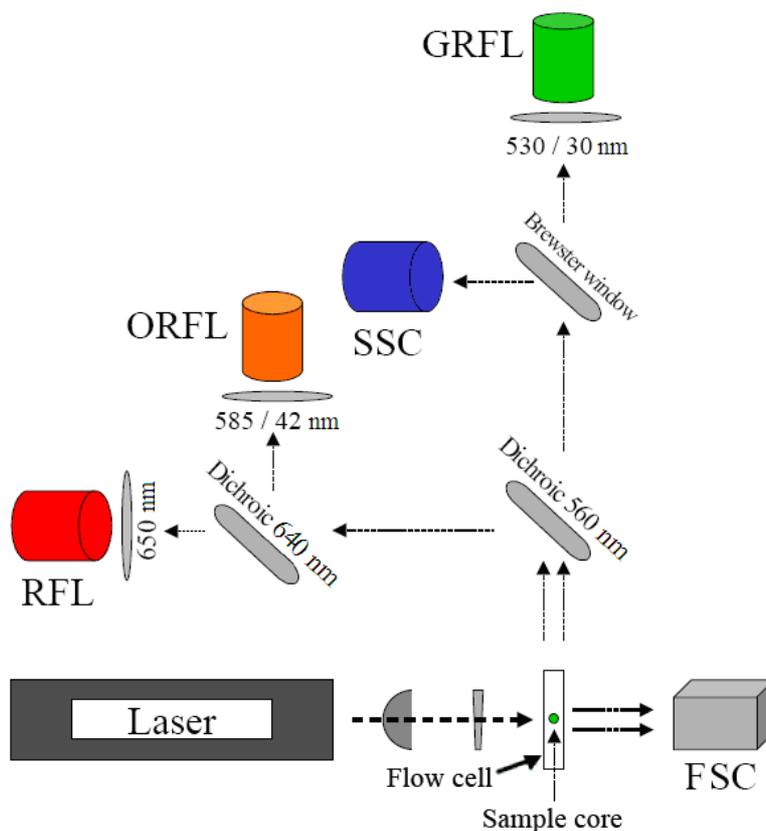
‘fitness’ during passage through a given advanced in-pond treatment process, will likely possess some competitive advantage over other species which may be rendered ‘less fit’ or indeed non-viable as a result of environmental conditions experienced during the treatment process. If this were indeed the case, then *in situ* treatment within a DW, RF, or AGM system could be viewed as a means of manipulating the physical environment in order to exert an ecologically selective (or perhaps universally destructive) negative pressure upon mixed phytoplankton assemblages within a given WSP effluent. Equally, conditions experienced during DW, RF, or AGM treatment may not be sufficiently adverse to impose a significant negative impact on algal cell viability, such that removal processes within the advanced treatment systems would be confirmed as being primarily physical.

Since there is currently no information available as to how such advanced in-pond treatment processes might be likely to affect algal viability, it was considered appropriate to further investigate the question in the laboratory. Following this, investigations into algal dark-survival under varying degrees of oxygen saturation were conceived. Given the universally heightened trophic (nutrient) state under which WSP systems operate, the current research was aimed at assessing the long-term dark viability of some ubiquitous WSP phytoplankton species under nutrient-replete conditions within the laboratory. The methodology selected to investigate these experimental aims was analytical flow cytometry.

#### **6.4 Analytical flow cytometry**

Flow cytometry (FCM) has been aptly described as a kind of “automated microscopy” with the added advantages of analytical automation, objectivity and speed (Veal *et al.*, 2000). FCM allows for rapid, real-time analysis of cells in suspension; permitting the instantaneous, simultaneous and quantitative measurement of multiple cellular properties from individual cells in rapid succession (Collier, 2000). The technique, depending on the individual instrument, has the ability to analyse thousands of cells per second by performing multiparameter (commonly a minimum of five) analyses on a wide range of cellular properties based on light-scatter signals and induced fluorescence.

FCM allows for measurement of both intrinsic and evoked optical signals from single cells within the moving fluid stream (Weaver, 2000). Modern flow cytometers incorporate a laser illumination source, two physical light scattering detectors (forward- and side-angle) and a minimum of three discrete photodiode fluorescence detectors collecting a range of fluorescence wavelengths. A detailed description of the mechanical and analytical processes involved in general FCM can be found in the work of Collier (2000) and Campbell (2001). For ease of reference, a schematic outline of the optical inner-workings of a standard flow cytometer is provided in Figure 6.1.



**Figure 6.1.** Laser excitation and detection optics layout for a standard bench-top (FACSCalibur, Becton Dickinson, USA) flow cytometer (modified from Campbell, 2001). Forward-angle light scatter, FSC; side-angle light scatter, SSC; red fluorescence, RFL; orange fluorescence, ORFL; green fluorescence, GRFL.

### 6.4.1 Flow cytometry in the biological sciences

FCM is a powerful bio-analytical tool that has for many years been an indispensable tool for research within the medical fields of hematology, immunology and oncology (Alberghina *et al.*, 2000). Although FCM was initially developed for applications in the

study of mammalian cell systems, the capabilities of the instrument for rapid enumeration and quantification of both structural and functional properties of individual cells at the 'single-cell' level has made it an ideal technology for applications in other fields. According to Troussellier *et al.* (1993), marine and environmental microbiologists were among the first to recognise the interdisciplinary potential of FCM, with the first reported instances of using flow cytometric analyses to probe phytoplankton in the aquatic environment arising in the late 1970's (Paau *et al.*, 1978) to early 1980's (Yentsch *et al.*, 1983). Since then, the potential of FCM as an analytical tool for aquatic microbiology has been well recognised, such that it is nowadays considered an invaluable tool for research into the areas of aquatic and environmental microbial ecology.

#### **6.4.2 Flow cytometry in phytoplankton research: viability assessment**

One of the foremost and indeed most prominent applications of FCM in the area of aquatic sciences has been in the fields of oceanography and phytoplankton ecology (Yentsch *et al.*, 1983; Premazzi *et al.*, 1989; Hofstraat *et al.*, 1994). More recently, FCM has been applied to the field of phycology in order to gain physiological insights at the 'single-cell' level; thereby providing the basis for integrating research at the 'micro-organismal level' with that concerning 'macro-scale' phytoplankton community ecology (Platt, 1989; Li, 1993; Jochem, 2000; Franklin *et al.*, 2001b). The tremendous ease and capacity for data collection from large numbers of individual cells is viewed as being one of the most important advantages of the flow cytometric method for microbiology (Winson and Davey, 2000). FCM has the additional advantage of requiring only very small sample volumes (commonly < 1ml, but dependent on cell density) in order to deliver large amounts of analytical data (Collier *et al.*, 2000; Veal *et al.*, 2000).

The inherent ability of FCM for the measurement of discrete cellular properties on a cell-by-cell basis, gives the investigator the means by which to quantify the distribution of a specific cellular property, or indeed properties, within an invariably heterogeneous microbial population; something in marked contrast to the majority of typical 'bulk' analyses (Winson and Davey, 2000). This notion has been raised previously by Platt

(1989) and later again in a review by Jochem (2000) who highlight the advantages of probing phytoplankton at the ‘single-cell’ level in order to elucidate and describe changes in ‘phytoplankton communities’ (given that any ecophysiological changes must inherently occur at the single-cell level prior to happening on a community scale).

Analytical FCM enables information on the biochemical and physiological characteristics of individual cells to be readily obtained in real-time under conditions close to the *in vivo* state (Veldhuis and Kraay, 2000). This attribute makes FCM especially useful for real-time assessments of the state of a cells’ physiology. To date, this *in vivo* probing of cellular physiology has been performed using an array of fluorescent probes specifically designed to target and measure a number of different cellular properties. Amongst the suite of physiological aspects investigated, phytoplankton cellular viability has been at the centre of much of this research. Given the previously discussed gap in knowledge surrounding processes associated with phytoplankton cell death (Section 6.3.2), FCM offers tremendous potential for probing the physiological viability of phytoplankton populations at the single-cell level.

One of the most basic questions a microbiologist may propose to ask of a microorganism is whether it is alive or dead (Vives-Rego *et al.*, 2000; Robinson *et al.*, 2003). Traditionally, microbiologists ascribed a state of cellular viability to cells so long as they remain culturable under classical techniques—the golden standard being growth on a solid agar medium. It follows then, that cellular viability in a microbiological context refers to the ability of an organism to grow and reproduce under appropriate growth conditions (Brussaard *et al.*, 2001). By definition, this precluded a vast number of organisms (i.e. viable but non-culturable) from analysis due to limitations imposed by the use of inappropriate growth media. The application of FCM as a microbiological tool has allowed for new insights into the area of cellular viability. Using the technique, it was no longer necessary to culture an organism in order to determine its viability (at least in terms of its metabolic status; Robinson *et al.*, 2003), instead, analyses could now be performed in real-time and ‘*in vivo*’.

By analogy with processes occurring in multicellular organisms, cell death can be necrotic in origin (swelling followed by immediate lysis, as in a response to injury) or

apoptotic (shrinkage and fragmentation, as in some forms of programmed cell death) (Berges and Falkowski, 1998). Cell death in phytoplankton is known to be accompanied by a series of discrete processes, some of which include: a loss of membrane integrity; swelling and/or vacuolisation; degradation of the photosynthetic pigments; and a cessation of metabolic function (Veldhuis *et al.*, 2001). The incorporation of specific fluorochromes and molecular probes (biological stains) into algal samples prior to FCM analysis, in addition to measurements of physical light scatter, allows for the direct assessment of cell viability through the monitoring of individual cellular parameters such as metabolic function, membrane integrity, and chlorophyll fluorescence.

#### **6.4.2.1 Physical light scattering**

Most commercial flow cytometers measure how much of the excitation light source is physically scattered by each cell at two points (Collier, 2000). The amount of light scattered at shallow or small angles relative to the angle of the excitation light beam is measured by a photodiode on the same plane as the excitation light source directly behind the sample interrogation point, and is termed low-angle or forward-angle light scatter (FSC). Conversely, light scattered at large angles relative to the direction of the excitation light source is termed side-angle light scatter (SSC).

##### **6.4.2.1.1 Forward-angle light scatter (FSC)**

FSC results from light diffracted at low or 'forward angles' ( $\approx 0-5^\circ$ ) around the cell, and as such, is solely dependent upon a cell's physical size and shape (Collier, 2000). FSC signal amplitude is commonly used as an index for relative cell size (Dorsey *et al.*, 1989) since it is known to correlate well with cell (Coulter) volume (Olson *et al.*, 1989). FSC signal amplitude is a more robust proxy for cell size determinations than SSC, due to its greater signal intensity, as well as being relatively unaffected by intracellular structure (Koch *et al.*, 1996).

Changes in cell size (and therefore in FSC signal) have been used to depict physiological state changes of some phytoplankton (Jochem, 2000), with an increase in cell volume following exposure to copper toxicity reported to correspond with an increase in FSC signal in the chlorophyte *Dunaliella tertiolecta* (Abalde *et al.*, 1995) and the diatom



*Phaeodactylum tricornutum* (Cid *et al.*, 1997). Since FSC signal height can be directly correlated with generic cellular size (Shapiro, 2003) as well as cell size for a wide array of phytoplankton groups (Chisholm, 1992), changes in cell size and volume were monitored during the course of the experiments by their proxy measure of corresponding FSC signal height.

#### **6.4.2.1.2 Side-angle light scatter (SSC)**

SSC results from light scattered (or diffracted) at wide or large angles ( $\approx 15\text{--}90^\circ$ ) relative to the excitation light source. As such, SSC signal is influenced by internal and external cellular structure and refractive index (Collier, 2000). Consequently, in FCM analyses, SSC signal amplitude (height) is used as a proxy for cell morphology and intracellular ‘structural density’ (i.e. granularity, vacuolisation and organelle content; Blum and Balber, 1996; Collier, 2000; Jochem, 2000). It is generally recognised that the amplitude of inherent physical light scatter signals (i.e. FSC and SSC) will diminish under sub-optimal environmental conditions such as nutrient limitation (Robinson *et al.*, 2003). Consequently, changes in both cellular size and intracellular structure were monitored during the course of this research by proxy measurement of their corresponding FSC and SSC signal amplitudes.

#### **6.4.2.2 Chlorophyll *a* autofluorescence**

In photosynthetic organisms, the main source of autofluorescence originates from photosynthetic pigments such as chlorophyll *a*, or more specifically, from a single pigment–protein light-harvesting complex within, called Photosystem-II (Vredenberg and Slooten, 1967; Krause and Weis, 1984). Photosystem-II (PS-II), which is predominantly responsible for chlorophyll *a* fluorescence, consists of a peripheral and a core light harvesting protein or ‘antenna’ complex. The former contains a species-dependent light-absorbing pigment, the latter an evolutionary conserved molecule; chlorophyll *a* (Beutler *et al.*, 2002). Under blue light excitation (peak  $\approx 420\text{nm}$ ), most of the energy transferred from the peripheral antenna to the core is utilised for photochemical reactions (i.e. photosynthesis) or lost as thermal energy, with a variable percentage re-emitted as red (peak  $\approx 685\text{nm}$ ) light (Krause and Weis, 1984; Gregor and Maršálek, 2004). Consequently, the overall *in vivo* chlorophyll fluorescence yield from

PS-II usually low (Campbell *et al.*, 1998). The magnitude of this re-emitted red autofluorescence signal serves as a tool for the *in vivo* determination of PS-II photosynthetic ‘activity’ (Vincent, 1981; Yentsch and Horan, 1989) as well as serving as a surrogate measure of aqueous chlorophyll *a* content (Beutler *et al.*, 2002). Chlorophyll fluorescence *in vivo* is, therefore, a direct reflection of the light-capturing ability and transfer efficiency of those PS-II accessory pigments which absorb light within the excitation spectrum (Vincent, 1981; Matorin *et al.*, 2004).

Active *in vivo* fluorescence measurements use an artificial light source (as opposed to natural solar radiation) to stimulate chlorophyll fluorescence (Kolber and Falkowski, 1993). This unique characteristic of *in vivo* chlorophyll *a* autofluorescence expressed in photoautotrophic phytoplankton can be detected by FCM as red (peak emission spectra 660–680nm) autofluorescence. Although the excitation wavelength of a standard blue argon-ion laser (488nm) is arguably not ideal for some investigations into chlorophyll *a* fluorescence (e.g. quantum efficiency of cellular photosystems), there is sufficient Stokes shift and quantum yield of chlorophyll *a* under 488nm excitation to provide sufficient autofluorescence signal for detection at wavelengths greater than 650nm using FCM.

*In vivo* chlorophyll autofluorescence—as measured by FCM—can serve as a surrogate measure of gross photosynthetic capacity and function (Furuya and Li, 1992; Kolber and Falkowski, 1993; Jochem, 2000; Veldhuis and Kraay, 2000) and indeed many researchers have found that the magnitude of *in vivo* fluorescence (per unit chlorophyll *a*) varies according to cellular photosynthetic capacity and physiological state of both the chloroplast and cell as a whole (Krause *et al.*, 1982; Krause and Weis, 1984; Alpine and Cloern, 1985). In compromised or dead cells, for example, cellular photosynthetic pigments will be degraded such that the *in vivo* chlorophyll *a* autofluorescence signal measured by FCM will diminish (Veldhuis and Kraay, 2000). This was reported by Brussaard *et al.* (1999), who observed a steady decrease in FCM-quantified cellular chlorophyll fluorescence intensity over time in viral-infected phytoplankton (Prasinophyceae and Prymnesiophyceae) cultures as a direct consequence of viral lysis and algal cell death. It is important to note, however, that chlorophyll *a* autofluorescence does not alone provide a quantitative assessment of the ‘quantum yield’ or ‘quantum

efficiency' of photosynthesis, and all chlorophyll *a* fluorescence measured and discussed in the remaining thesis chapters relates to what is most closely known as 'minimal' or 'steady-state' chlorophyll fluorescence ( $F_0$ ).

This autofluorescent signal detected by FCM is distinctly unique to algal analyses, and allows the user to instantaneously discriminate phytoplankton from other biological and non-biological particles in suspension (Yentsch and Yentsch, 1979). Depending on the specifics of individual cytometers, cellular chlorophyll fluorescence has been proven to be linearly correlated with cellular chlorophyll concentrations in laser cytometers (Li *et al.*, 1993; Jochem, 2000; Veldhuis and Kraay, 2000). Changes in cellular chlorophyll fluorescence have also been seen as indicators of a photoacclimation response to decreasing light intensities in marine environments (Falkowski and LaRoche, 1991). In addition to providing photosynthetic physiological insights, FCM-quantified chlorophyll *a* fluorescence has also been widely used as a surrogate measure of phytoplankton biomass and cell size (Chisholm, 1992; Dubelaar and Jonker, 2000) as well being used for the taxonomic identification of phytoplankton species in mixed environmental samples (Yentsch and Yentsch, 1979; Olson *et al.*, 1989; Hofstraat *et al.*, 1994). Veldhuis and Kraay (2000) do caution, however, that whilst this '*in vivo*' autofluorescence can serve as a surrogate for biomass estimation in studies concerning phytoplankton ecology, it is not recommended as a stand-alone measure due to inherent variations in fluorescence yield based on prior algal growth conditions and also individual machine specifications.

There is ongoing uncertainty within the literature surrounding the use of FCM for chlorophyll fluorimetry in phytoplankton research (Dubelaar and Jonker, 2000; Jochem, 2000). Most of this uncertainty is directed toward specific differences between inter-machine excitation energies, and also differences in fluorescence yields based on the cellular 'chlorophyll *a*: fluorescence' ratio—a manifestation of the package effect of Duysens (1956) (see Section 9.6.3.2 for further elaboration). In general, the efficacy of FCM for detailed investigations into photosynthetic efficiency is thought to be limited by the generally high photon flux densities of laser cytometers potentially causing 'exciton annihilation' or 'photo-bleaching' of cellular chlorophylls, resulting in reduced fluorescence yields (Dubelaar and Jonker, 2000). The high-energy laser excitation

source is also thought to restrict the analytical sensitivity of chlorophyll fluorimetry via FCM due to the necessary low excitation light intensity required for initial fluorescence probing during chlorophyll fluorescence analyses (Olson and Zettler, 1995; Olson *et al.*, 1996).

There also remains considerable uncertainty as to exactly what aspects of chlorophyll *a* PS-II fluorescence are quantified during FCM (i.e. minimum ( $F_0$ ) or maximum ( $F_M$ ) fluorescence yield of PS-II; Furuya and Li, 1992; Hofstraat *et al.*, 1994; Olson and Zettler, 1995; Collier, 2000), with the answer apparently a function of both the individual cytometer excitation intensity and sheath fluid velocity (Olson *et al.*, 1996). There has been some reported success with the use of specially modified “pump-and-probe” cytometers (Kolber and Falkowski, 1993; Olson and Zettler, 1995; Olson *et al.*, 1996) for improved control of sample excitation intensity, with these modifications providing for a more accurate determination of ‘photosynthetic efficiency’ (i.e. quantum yield of PS-II photochemistry).

Overall, FCM is not generally regarded as the ‘method of choice’ for precise investigations into photosynthesis and its quantum efficiency. Nevertheless, the added bonus of a cellular autofluorescence signal provides an extra dimension to standard cellular analyses via FCM, and hence results presented here will include those relating to chlorophyll *a* fluorescence. It should also be noted that specific instrumental cytometer validations are provided within the relevant results section (Section 9.7.3.1), thereby providing the necessary validation for the inclusion and discussion of chlorophyll *a* fluorescence measurements in the context of the current dark-survival research.

#### **6.4.2.3 Population cell density**

Another important indicator of an algal population’s ability to endure unfavourable conditions during and following an environmental stressor (such as darkness), is derived through monitoring of the population’s cell density. Changes in the number of cells in a given population during and following dark-exposure can provide an instantaneous measure of the algal population’s capacity to withstand and then recover from a period of dark-stress. Even before the conception of modern flow cytometers, researchers as far

back as the 1960's were using similarly automated particle counters (i.e. Coulter counters) for the rapid enumeration of natural phytoplankton assemblages (Mulligan and Kingsbury, 1968). Following that, the potential application of FCM as a technique for the enumeration of cells in suspension has been reported since the early 1980's (Stewart and Steinkamp, 1982), and since that time, it has gained widespread employ as a simple and effective means by which to count a wide range of particles (both biological and inert) suspended in an aqueous media.

Prior research has demonstrated the tremendous capacity of FCM for highly sensitive, accurate and reliable enumeration of cells in suspension. Ross *et al.* (1989) reported an excellent and reproducible correlation between flow cytometer (FACS IV) counts and actual cell density (based on Coulter counts) between  $10^2$  and  $10^6$  cells  $\text{ml}^{-1}$  using mammalian cells. It is also reported elsewhere that FCM is most accurate for cellular enumeration in the range of  $10^3$ – $10^6$  cells  $\text{ml}^{-1}$  (Tanaka *et al.*, 2000), with the general upper limit of accurate detection in the order of  $10^6$  cells  $\text{ml}^{-1}$  (Robinson *et al.*, 2003). Given that FCM is both an extremely rapid and sensitive cell counting technique, and one that is nowadays widely employed as a standard protocol for algal cell enumeration (Marie *et al.*, 2000; Robinson *et al.*, 2003; Marie *et al.*, 2005), population cell density was monitored during the current dark-survival work via FCM.

#### **6.4.2.4 Biological fluorochromes and flow cytometry**

The reaction of fluorogenic cellular 'probes' within cells was first described by Rotman and Papermaster (1966) and is now a universally exploited biological phenomenon especially suited to cellular studies involving flow cytometric analyses. Fluorogenic substrates (fluorochromes) are particularly useful for the assessment of cellular viability, and in many cases, additional information on cellular metabolic state, membrane properties and intracellular enzymatic activity, can be readily obtained (Sengbusch *et al.*, 1976).

Analytical FCM is now considered to be a mature technology, with a whole suite of biological fluorochromes (probes) now being applied during regular cytometric analyses. Viable cells can be easily identified directly using FCM and fluorescent probes

that target and identify properties of normal ‘healthy’ cells (Robinson *et al.*, 2003). Two of the more commonly invoked cellular properties used during viability assessment are: the integrity of the plasma membrane; and the presence of normal metabolic cellular processes. Stains such as diacetylfluorescein, more commonly referred to as Fluorescein Diacetate (FDA), and Propidium Iodide (PI) have gained widespread employ in studies concerning cellular viability (Jones and Senft, 1985; Berglund and Eversman, 1988; Selvin *et al.*, 1988/1989; Dorsey *et al.*, 1989; Ross *et al.*, 1989; Scorbati *et al.*, 1996). These two particular dyes, although both used in viability assessment, have differing fluorescence pathways based upon discrete physiological attributes. FDA on one hand works by its incorporation and expression in biologically active cells and is, therefore, generally seen predominantly as a marker for metabolic activity. PI on the other hand works on the basis of exclusion from structurally intact (i.e. viable) cells and, as such, is used as a marker for cell membrane integrity.

#### **6.4.2.4.1 Cellular metabolic activity**

The most commonly used probe for assessment of cellular metabolic processes is FDA. FDA has been widely used in studies involving bacteria (Diaper and Edwards, 1994; Porter *et al.*, 1995; Tanaka *et al.*, 2000; Morono *et al.*, 2004), yeast (Breeuwer *et al.*, 1995), Cyanobacteria (Brookes *et al.*, 2000a; Regel *et al.*, 2004), phytoplankton (Bentley-Mowat, 1982; Dorsey *et al.*, 1989; Gilbert *et al.*, 1992; Agustí *et al.*, 1998; Jochem, 1999; Lage *et al.*, 2001), protozoa (Iturriaga *et al.*, 2001), and a variety of mammalian cells (Sengbusch *et al.*, 1976; Jones and Senft, 1985; Prosperi *et al.*, 1986; Ross *et al.*, 1989). Since the initial reporting of the occurrence of enzymatic hydrolysis and subsequent fluorescence of esters of fluorescein (Guilbault and Kramer, 1966; Rotman and Papermaster, 1966), FDA found early employ in association with flow cytometric-type assays for the assessment of cellular processes (Sengbusch *et al.*, 1976). Since then, Bentley-Mowat (1982) reported the first use of FDA (in conjunction with fluorescence microscopy) for the assessment of phytoplankton viability in pollution studies, and soon after, Berglund and Eversman (1988) combined both FDA and FCM for the assessment of environmental pollutant stressors on the health status of algal cells.

Histologically speaking, FDA is a membrane-permeant 'vital' stain, which simply implies that it can readily cross the intact cytoplasmic membrane (Shapiro, 2003). Further to this, FDA is a colourless, hydrophobic, lipid soluble, non-polar, non-fluorescent, esterified molecule that diffuses freely across the cellular plasma membrane (Dorsey *et al.*, 1989). Once inside the cell, FDA is hydrolysed (cleaved) in the cytoplasm by non-specific enzymes called 'esterases' to produce the polar, hydrophilic, fluorescent by-product *fluorescein* which does not easily diffuse across the cell membrane and is subsequently retained and accumulated by cells with intact plasma membranes—allowing for easy detection (Prosperi *et al.*, 1986; Franklin *et al.*, 2001a). The non-specific cellular esterases responsible for FDA hydrolysis are also involved with phospholipid turnover in the cell membrane—a process shown to be correlated with cellular metabolic activity (Dorsey *et al.*, 1989; Breeuwer *et al.*, 1995; Franklin *et al.*, 2001b).

Once hydrolysed, the fluorescein anion is retained by intact cells (on timescales of minutes to hours), and with its high quantum yield, exhibits an intense green fluorescence when excited with blue light (Shapiro, 2003). Since the now charged hydrophilic fluorescein molecule exits healthy cells at a rate slower than the substrate FDA can enter, the end result is a net intracellular 'fluorescein fluorescence', whilst cells with compromised membranes are unable to accumulate fluorescein and so display little or no fluorescence (Prosperi *et al.*, 1986). Essentially, the more damaged a cell is, the lower its rate of enzymatic FDA hydrolysis, fluorescein accumulation and subsequent cellular fluorescence will be (Sengbusch *et al.*, 1976).

In general, assessments of metabolic activity in phytoplankton are based on parameters closely associated with cellular photosynthesis, such as ATP formation, radioactive ( $^{14}\text{C}$ ) carbon assimilation and oxygen evolution (Gilbert *et al.*, 1992). The central premise of the FDA assay is that the degree of cellular fluorescence is directly related to its physical and metabolic state (Gilbert *et al.*, 1992), such that 'healthy' cells will display greater fluorescein fluorescence when incubated with FDA than will 'less healthy' cells (Brookes *et al.*, 2000b). The extent of intracellular FDA hydrolysis and subsequent fluorescein accumulation can then be easily and quantitatively detected by FCM as

‘green’ cellular fluorescence, and so serves as a quantitative measure of both cell metabolic activity and membrane integrity.

When used in conjunction with FCM, the FDA assay is a very effective means of both probing the metabolic activity of discrete cells and also of assessing the population heterogeneity of phytoplankton metabolic status in the natural environment (Dubelaar and Jonker, 2000). FDA has been widely used for the measurement of cellular esterase activity in a variety of algal species (Bentley-Mowat, 1982; Selvin *et al.*, 1988/1989; Dorsey *et al.*, 1989; Gilbert *et al.*, 1992; Jochem, 1999; Brookes *et al.*, 2000b; Lage *et al.*, 2001; Agustí and Carmen Sánchez, 2002) where it provides an instantaneous measure of cell metabolic activity and function. The FDA assay has additional benefits with respect to phytoplankton analyses via FCM, since it does not interfere with physical (FSC/SSC) light scatter signals, it does not degrade the chlorophyll *a* autofluorescence signal (>660nm), nor does it impede normal cellular vitality, motility or carbon uptake in most instances (Selvin *et al.*, 1988/1989; Dorsey *et al.*, 1989; Lage *et al.*, 2001).

The early reporting by Rotman and Papermaster (1966) of the cellular FDA assay suggested that it was primarily a test of cellular membrane integrity, which was then likely to be closely correlated with cell viability. Since then, it was deduced that the magnitude of cellular fluorescence following FDA staining was linked more closely to the general *metabolic vigour* of (phytoplankton) cells (Bentley-Mowat, 1982). This realisation followed the direct observation by Bentley-Mowat (1982) of continued algal cell motility in toxic copper ( $10^{-4}$  M) exposed cells despite the absence of any cellular FDA-fluorescence. This meant that ‘non-fluorescence’ following FDA staining did not definitively indicate ‘non-viability’.

Prosperi *et al.* (1986) were among the first to report that the amount of fluorescein that accumulates intracellularly is dependent upon not only the hydrolysis rate of substrate FDA, but also on a cell’s membrane permeability properties (which influence both uptake of FDA and loss of fluorescein) and metabolic status (which will influence both initial hydrolysis and also energetic efflux rates). Cellular fluorescein fluorescence, therefore, reflects not only metabolic (esterase) activity, but also membrane integrity (i.e. cellular enzyme activity to cleave FDA and then membrane integrity to retain the



hydrolysed fluorescent product); both of which are widely recognised indicators of cell viability (Dorsey *et al.*, 1989). In this respect, there is a ‘duality’ of FDA staining outcomes, such that it can be difficult to discriminate the two components. Considering this, it is often desirable to incorporate another stain into the viability analysis in order to increase the diagnostic power of the FDA assay. This issue is discussed further in Section 6.4.2.4.3.

There are several factors surrounding the FDA assay that researchers need to be aware of prior to broad-spectrum application of the technique to a given analysis. Non-optimal pH values are recognised to adversely affect the FDA staining assay in particular, with a pH below 7 and above 8 potentially affecting cellular FDA hydrolysis and greatly reducing the fluorescein fluorescence yield (Franklin *et al.*, 2001a). This notable pH-sensitivity is primarily due to highly pH-dependent nature of fluorescein fluorescence (Brookes *et al.*, 2000b), but also because (intracellular) pH affects both the esterase activity and passive cellular efflux of hydrolysed fluorescein (Breeuwer *et al.*, 1995). In addition to pH-related effects, there is a recognised ‘species-specificity’ of the FDA viability assay when applied to phytoplankton research. An initial review of the relevant literature revealed a somewhat varied range of optimum FDA concentrations for use in FCM metabolic activity assays with phytoplankton. For example, 1.2 $\mu$ M FDA was used by Ross *et al.* (1989), 5 $\mu$ M final FDA concentration was recommended within the current protocols for cytometry manual (Robinson *et al.*, 2003), 8 $\mu$ M final FDA was used for the chlorophyte *Brachiomonas submarina* by Jochem (1999), 25 $\mu$ M final was used for *Chlorella* species (Franklin *et al.*, 2001a) and 40 $\mu$ M FDA by Brookes *et al.* (2000b) for the cyanobacterium *Microcystis aeruginosa*.

In addition to the above factors, it is essential that several ‘critical assumptions’ of optimal staining are satisfied during the course of cellular staining with FDA. A critical assumption of the FDA viability assay is that the factor limiting substrate FDA hydrolysis and fluorescein accumulation is *solely* cell esterase activity, and that: substrate (FDA) concentration; cell permeability to FDA; and subsequent FDA influx into the cells; remain constant and are not *rate-limiting* at any stage of the assay duration (Sengbusch *et al.*, 1976). In order to validate these conditions, it is essential to perform detailed preliminary assessments of FDA hydrolysis and fluorescein fluorescence

kinetics prior to final adoption of a given FDA staining protocol (Breeuwer *et al.*, 1995; Brookes *et al.*, 2000b; Franklin *et al.*, 2001a). In line with these preliminary methodological cautions, a thorough pre-optimisation of final FDA staining protocols to be used in the long-term dark-viability experiments was performed, along with detailed staining kinetics assessments for both candidate algal species across a range of FDA concentrations. Results of these preliminary FDA protocol optimisations are presented in Chapter 8. It should also be noted that whilst there have been other more recently applied derivatives of the original FDA molecule (e.g. carboxyfluorescein diacetate), Dorsey *et al.* (1989) concluded from their research on microalgal metabolic activity that FDA was just as effective as other more expensive molecular analogs. Following this, FDA has been used in conjunction with FCM as part of the current research investigations into phytoplankton viability during prolonged darkness.

#### **6.4.2.4.2 Cellular membrane integrity**

The integrity of a cells' cytoplasmic membrane is essential to normal cell function, and although some cells can survive transient small-scale breaches of their membranes, it is generally considered that cells with a demonstrable loss of membrane integrity are indeed dead (Shapiro, 2003). Permeability of the cytoplasmic membrane is consequently a commonly exploited marker for the identification of cells that are moribund or dead (Robinson *et al.*, 2003). It follows then, that the exclusion and subsequent non-expression of a dye by an intact cell membrane is regarded as the most straight forward, as well as one of the most high-ranking viability test one can perform and understand (Vives-Rego *et al.*, 2000; Robinson *et al.*, 2003).

Nucleotide-binding stains such as Propidium Iodide (PI) have been used to assess membrane integrity and hence cell viability in mammalian (Jones and Senft, 1985), bacterial (Scorbati *et al.*, 1996) and also algal (Franklin *et al.*, 2001a) cells. PI is a membrane-impermeant, positively charged, organic compound, that can only traverse the compromised cellular membranes of dead or dying cells, such that 'healthy' cells remain unstained (Franklin *et al.*, 2001a; Shapiro, 2003). Once inside the cell, PI forms a complex with double-stranded nucleic acids by intercalating between base pairs, after which it shifts its absorption spectrum and increases its fluorescence quantum efficiency

and resulting in a 20–30 times greater fluorescence emission yield than unbound dye molecules (Shapiro, 2003). Nucleic acid cell staining will, therefore, only occur in cells that are dead or have compromised membranes, with PI expression generally considered to be indicative of cell death (Robinson *et al.*, 2003; Shapiro, 2003).

As was the case for FDA above, there are again species-specific factors with respect to optimal PI staining conditions. To illustrate the potential magnitude of methodological variability, Fanklin *et al.* (2001a) reported an optimal PI concentration of  $7.5\mu\text{M}$  for FCM viability staining of the green alga *Chlorella*, whereas Cid *et al.* (1996) reported an optimal PI concentration of  $60\mu\text{M}$  for FCM viability assessment of the marine diatom (*Phaeodactylum*)—some 8-fold higher. It is also worth noting here, that Franklin *et al.* (2001a) actually tested a final concentration of  $60\mu\text{M}$  PI for *Chlorella* viability staining and the result was a gross over-staining of cells; with a significant quantity ( $\approx 40\%$ ) of ‘false-positives’ resulting from such a high PI concentration. In addition to this, Grégori *et al.* (2002) caution that one should be wary of the fact that optimum staining protocols (i.e. concentration and staining duration) may vary not only according to species, but also with population cell density in a given algal sample.

Following this, it is reasonable to assume that a given optimal staining regime may also vary according to other factors such as cellular growth status and general physiological health status. Indeed Gasol and del Giorgio (2000) discuss how staining properties can also display patterns of spatial heterogeneity within a given cell, whereby vital stains can often yield a gradient of staining intensity across individual cells—a staining artefact that can introduce undesirable analytical ambiguity in the separation of positively (live) and negatively (dead) stained cells and further complicate FCM resolution and subsequent live/dead discrimination. In addition to this variability, algal cells may be affected by fluid acceleration, electrical- and, most importantly, light-shock during FCM analyses (Dubelaar and Jonker, 2000)—the ultimate result of these being a potential for erroneous results. Following these cautions, and prior to the commencement of dark-survival experimentation and data collection, it was necessary to undergo thorough preliminary methodological validations and optimisations for both biological stains (PI and FDA) and for both algal species (*C. vulgaris* and *C. reinhardtii*) in order to guarantee optimal cell staining and also ensure that proper instrumental setup and data

acquisition parameters were in place. Results of these and other preliminary FCM protocol optimisations are presented in Chapter 8. Additionally, and as was the case for FDA above, despite the existence of more complex molecular analogues to PI (such as ethidium bromide, ethidium homodimer and TO-PRO-3), difficulties associated with definitive demonstration of dye-exclusion and confounding ‘false-positive’ staining have meant that PI-exclusion remains the method of choice for determinations of cell membrane integrity (Nebe-von-Caron *et al.*, 2000).

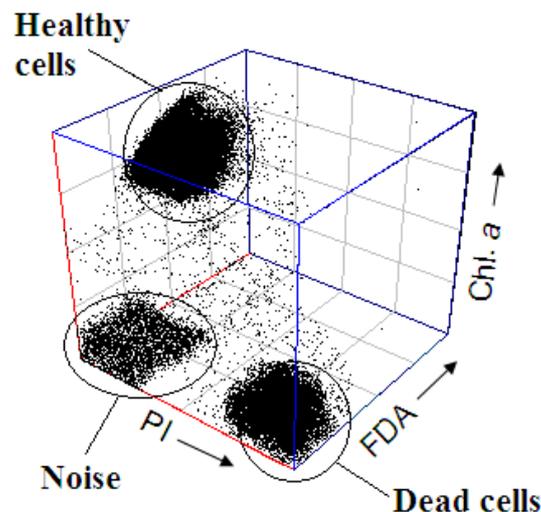
#### **6.4.2.4.3 Dual-staining for viability assessment**

Another important consideration when assessing cellular viability by way of membrane-impermeant dyes such as PI, is that in some instances (e.g. cells that are killed by ionizing radiation) a cell may retain membrane integrity for days after exposure despite being dead (Shapiro, 2003). It should be remembered during the course of contemplating a cell’s viability status, that an observed uptake of membrane-impermeant stains (such as PI) is a more reliable indicator of cellular non-viability than exclusion of the dye is of viability (Shapiro, 2003). In essence, it is important to remember that a ‘PI-positive’ cell is more likely to be *dead* than a ‘PI-negative’ cell is to be *viable*. This is a recognised limitation of using discrete viability probes, and fortunately, this problem can be largely overcome through the combination of two or more probes within a single viability assay.

Alberghina *et al.* (2000, p. 2) eloquently stated that “*The power of (flow) cytometry is best realised by multiparameter analysis, in which combinations of fluorescent reagents are used to simultaneously characterise two or more cellular properties*”. It can be appreciated that the simultaneous use of multiple fluorescent probes within a single sample during FCM analysis affords the investigator far greater analytical and discriminatory power. Whilst the information gained from the use of a single biological stain can be definitive in some instances, it can be ambiguous or even misleading in others. For example, if a cell stained only with FDA returns a very low fluorescein fluorescence reading during FCM analysis, the investigator may then be justified in assigning it an FDA-negative ‘non-viable’ status. However, the same cell when stained with PI only, may also return a ‘PI-negative’ reading and thus would be classified as ‘viable’ according to its positive membrane integrity status. The best means by which to

counter this sort of analytical artefact and provide improved physiological resolution to your viability diagnoses, is to perform dual PI–FDA staining of single samples.

Simultaneous staining of the one sample with more than one fluorochrome is an effective way of gaining multiple parameter information on target cells during a single analytical interval. Dual PI–FDA staining has been used successfully by others for viability assessment studies involving mammalian cells (Jones and Senft, 1985; Ross *et al.*, 1989), bacteria (Tanaka *et al.*, 2000) and phytoplankton (Franklin *et al.*, 2001a). Physiological characterisation of aquatic microorganisms can be performed rapidly and easily via dual staining with PI and FDA in combination with FCM (Tanaka *et al.*, 2000). PI and FDA make particularly good candidates for simultaneous staining in analytical FCM because they are both excited by blue 488nm single laser excitation source and because there is also good separation between their peak emission spectra (515–525nm for FDA and 615–625nm for PI). This allows for sufficient instrumental resolution between the two fluorescence channels, with no fluorescence quenching or emission spectra overlap occurring between these two fluorochromes. This improved discriminatory resolution of dual PI–FDA staining during viability assessments with FCM can be seen most easily in Figure 6.2 below based on a theoretical example of a 50/50 live/dead phytoplankton population.



**Figure 6.2.** Three-dimensional flow cytometric scatter plot from a theoretical mixed (50/50 live/dead) phytoplankton population showing: live ‘healthy’ cells (low PI–high FDA–high chlorophyll *a* fluorescence); dead cells (high PI–low FDA–low chlorophyll *a* fluorescence); and instrumental signal ‘noise’.

Considering the above example of FDA-negative/PI-negative cells, by using a dual PI–FDA staining protocol, the investigator is now equipped with the analytical resolution to categorise those cells more precisely as ‘viable but non-metabolically active’. It can be appreciated that this simple distinction may be of critical importance for viability studies, particularly on a community-ecology scale. Dual PI–FDA staining has therefore been adopted as part of this research for the assessment of phytoplankton viability following prolonged darkness.

#### **6.4.2.5 Long-term dark viability assessment—how viable is viable?**

Reynolds (2006, p. 296) has highlighted a personal—and arguably qualified—opinion on the need for diligence regarding the ascription of a ‘non-viable’ and especially ‘dead’ status to algal cells, stating that “*Unless an algal cell wall is entirely devoid of its contents... then it is not safe to assume that it is dead and incapable of physiological revival.*” Because the moment of cell death and the accompanying loss of biological viability are difficult to isolate, a multifactorial investigative approach that encompasses a range of processes known to be associated with cell death is the best way to distil the overall physiological process down to its individual components (Brussaard *et al.*, 2001). It is this rationale that has led to a combination of approaches (i.e. chlorophyll fluorimetry, cell membrane integrity, metabolic activity and cell counts) being implemented for this research into dark-survival and algal cell viability.

The capability of a cell population to re-grow following chronic exposure to some form of physiological stressor (in this case darkness) can be seen as the *ultimate* assessment of their ability to endure that particular stress event whilst retaining sufficient physiological vigour to allow for continued population expansion upon a return to more favourable growth conditions. Following this, a final experimental assessment of the algal population’s physiological revival or ‘re-growth’ potential post-darkness—as per the above recommendations of Reynolds (2006)—will serve as a fail-safe means of adding credence to the ‘viability conclusions’ drawn from the above cytometric analyses performed during the course of long-term dark-exposure.

## **6.5 Experimental questions and research aims**

As introduced (Section 1.2.3), algae are absolutely essential for effective WSP operation. In spite of this, however, the presence of large quantities of algal biomass in final WSP effluents represents one of the most significant performance problems associated with the technology (see Section 1.2.5), especially when final pond effluent is destined for quaternary-level treatment and/or reuse applications. As has also been highlighted (Sections 1.2.8.5.2, 1.2.8.6.1 and 1.2.8.7.1), the particular advanced in-pond WSP upgrade technologies chosen for investigation as part of this thesis (i.e. duckweed coverage, rock filtration and attached-growth media addition) all involve exposing the infiltrating effluent (and their suspended algal populations) to varying degrees of low light intensity or darkness in conjunction with significantly reduced concentrations of dissolved oxygen. The duration of this ‘shaded passage’ necessary to achieve algal cell death and subsequent settlement and removal from these systems is very poorly defined within the relevant literature. Zirschky and Reed (1988, p. 1254) suggested that most algal cells within a WSP environment will not settle until the cells are dead, and that “*the precise time required for algae death in a pond system is not well-defined*”. More recently, Bonomo *et al.* (1997)—referencing the work of Zirschky and Reed (1988)—postulated that effective algal removal in duckweed ponds was brought about by quiescent settlement and subsequent cell death in the darkened benthic regions. These theories regarding algal survival and cell death in such environments remains largely uninvestigated to this day, and so this served as the applied rationale for the following dark-survival research.

The work of Zirschky and Reed (1988), and later of Bonomo *et al.* (1997), spawned several questions relating to the innate biological capacities of different algal species to withstand such conditions whilst traversing these advanced in-pond upgrade processes. Not only was their survival *during* this darkened passage of interest, but also the likelihood of cells being able to re-grow *post-darkness* (should they be re-suspended and/or and re-exposed to light conditions) was a question of significant practical relevance. Following on from these initial questions, time-course analyses of dark-survival capacity for two model algal species (*Chlorella vulgaris* and *Chlamydomonas reinhardtii*) were performed over a period of approximately two months. These two algal species were initially selected due to their recognised high-level tolerance for

organic pollution and their classification within the relevant literature as being both universally ubiquitous and numerically dominant algae within WSP environments in general (e.g. deNoyelles Jr., 1967; Palmer, 1969; Shillinglaw and Pieterse, 1977; Hussainy, 1979; Véber *et al.*, 1982; Dor *et al.*, 1987; Pearson *et al.*, 1987; Rivera *et al.*, 1988; Harris, 1989; Banat *et al.*, 1990; Wrigley and Toerien, 1990; Bartosh and Banks, 2007). Further to this, algae of the genera *Chlorella* and *Chlamydomonas* have been identified by others as so-called “problem” organisms in terms of their relative ease of removal during DAF/F treatment (see Section 1.3.1). Given the universally nutrient-rich hypertrophic state under which WSP systems operate, the current research was aimed at assessing the long-term dark viability of ubiquitous WSP phytoplankton species under nutrient-replete, controlled conditions. Owing to their small size and unicellular nature, the above candidate species were also favourable candidates for examination via analytical FCM.

### **6.5.1 Research aims:**

1. To assess the long-term dark-survival capacities of two ubiquitous WSP algal species through a multi-faceted experimental approach (i.e. membrane integrity and metabolic activity viability assays, chlorophyll *a* analyses (quantity and fluorimetry), population cell density, cell size and intracellular density).
2. To characterise and define the optimal experimental FCM protocols to be used during long-term dark-viability assessments of the two model species (*C. vulgaris* and *C. reinhardtii*).
3. To investigate the kinetics of dark-survival (i.e. cellular photoacclimation response) in these two algal species during simulated and prolonged dark-exposure.
4. To investigate whether dissolved oxygen concentration has a significant and/or influential role in the long-term dark-survivorship of the chosen algal species.
5. To assess the potential for population re-growth of these two phytoplankton species following extended exposures to dark conditions.
6. To apply results from laboratory-based dark-survival experiments to their wider-ranging implications with reference to the selected in-pond WSP upgrade technologies.



## 7 Phytoplankton survival during prolonged darkness under conditions of ambient and reduced dissolved oxygen—materials and methods

### 7.1 Algal stock culture maintenance and experimental cultures

Non-axenic starter cultures for two species of green (Chlorophyceae) freshwater phytoplankton: *Chlorella vulgaris* (order Chlorococcales; strain no. CS-42; Beyerinck); and *Chlamydomonas reinhardtii* (order Volvocales; strain no. CS-51; Dangeard); were obtained from the CSIRO Microalgae Research Centre Culture Collection (Marine Research Division, Hobart, Australia). Upon receipt of cultures they were immediately sub-cultured into 60ml aliquots of sterile (15mins at 121°C), 0.2µm filtered (cellulose nitrate membrane, Whatman®), buffered, modified Woods Hole MBL culture medium (see Appendix G for chemical composition) at pH 7.2. Duplicate 60ml algal stock cultures were continuously maintained in 100ml borosilicate conical flasks in an illuminated orbital incubator (InnOva™ 4340, New Brunswick Scientific) under a continuous 24 hour PFD of 60µmol photons m<sup>-2</sup> s<sup>-1</sup> PAR (400–700nm) provided by a combination of ‘cool white’ and ‘gro-lux’ fluorescent lighting at 20 (± 1°C) and with constant shaking at 55 RPM.

Standard growth curves for both algal species were enumerated by flow cytometry, with specific culture growth rates ( $r_n$ ) calculated according to Equation 7.1 (Reynolds, 2006).

$$r_n = \ln(N_t/N_0) / (t) \quad (\text{Equation 7.1})$$

where  $N_0$  is population density at  $t = 0$

$N_t$  is population density at time  $t$

$t$  = time in days

Stock solutions of the antibiotics Cefotaxime (Sigma® Chemical Company; sodium salt; #C7039) and Carbenicillin (Sigma®; di-sodium salt; #C3416) were made up at 50mg ml<sup>-1</sup> in 0.2µm-sterilised Milli-Q® water and stored at –20°C until required. Algal cultures were maintained in an axenic state by continuous antibiotic addition (50µg ml<sup>-1</sup>

Cefotaxime; 100 $\mu\text{g ml}^{-1}$  Carbenicillin final concentration) during all sub-culturing according to the methods of Brussaard *et al.* (1999) and Yu *et al.* (2001). Once established, continuous algal cultures were sub-cultured every 8 days by transferring 0.02% (v:v) of the existing culture into fresh sterile 0.2 $\mu\text{m}$ -filtered MBL growth media. All algal culture manipulations were performed in a Class 2 laminar-flow cabinet (NuAire, Plymouth, Minnesota, USA) wherein standard aseptic techniques were employed. Algal stock cultures were periodically assessed for the absence of microbial (bacteria and moulds) contaminants by spread plating (100 $\mu\text{l}$ ) serially-diluted ( $10^{-1}$ – $10^{-4}$  in MBL media) algal culture onto R2A agar (Oxoid) plates followed by 24 hour dark incubation at 31°C and final examination.

## **7.2 Dark-survival experimental design, sampling protocols and analyses**

### **7.2.1 Experimental design rationale**

As introduced in Chapter 1 (Sections 1.2.8.5.2, 1.2.8.6.1 and 1.2.8.7.1) and as was also observed during the course of the ecurrent research investigations (see Sections 3.3.4 and 4.3.3), some advanced in-pond WSP upgrades (such as a rock filters, AGM or duckweed pond systems) will invariably reduce the concentration of dissolved oxygen in the infiltrating wastewater. For this reason, it was decided to not only investigate algal survivorship in darkness at ‘ambient’ DO concentrations, but also to incorporate a ‘low D.O.’ treatment in order to elucidate potential physiological effects resulting from chronically reduced DO levels as well as their subsequent effects upon overall phytoplankton dark-survivorship. Following on from these initial aims, the necessary experimental designs and protocols were conceived in a ‘4-by-3’ format, yielding four treatments each with triplicate replication. These four experimental treatments were conceived in order to investigate the initial research aims, and were as follows:

1. ‘Light / aerobic’ – comprising continuous algal culture illumination under ambient ( $\approx 8\text{mg L}^{-1}$ ) levels of dissolved oxygen;
2. ‘Light / low D.O.’ – comprising continuous algal culture illumination under reduced ( $\approx 2\text{mg L}^{-1}$ ) levels of dissolved oxygen;

3. 'Dark / aerobic' – comprising continuous dark-incubation of algal cultures under ambient ( $\approx 8\text{mg L}^{-1}$ ) levels of dissolved oxygen;
4. 'Dark / low D.O.' – comprising continuous dark-incubation of algal cultures under reduced ( $\approx 2\text{mg L}^{-1}$ ) levels of dissolved oxygen.

Initial algal cell densities of  $1 \times 10^6$  cells  $\text{ml}^{-1}$  were chosen for all dark-survival experiments and across all treatments in accordance with the findings of Ross *et al.* (1989) who reported that FCM was most accurate for cellular enumeration between  $10^2$  and  $10^6$  cells  $\text{ml}^{-1}$ ; although the current FACScan flow cytometer was found to be most accurate in the range of  $10^3$ – $10^6$  cells  $\text{ml}^{-1}$ . This ensured that both no dilution of dark-incubated samples would be necessary prior to FCM analysis (based on the assumption that culture density would not *increase* during dark-incubation) and also that cell densities would be maximised so as to allow for speedy acquisition of the necessary population sample size (in the order of  $10^4$  cell events). Day 8, late exponential- to early stationary-phase cultures were used for all dark-survival experimentation due to the desire for a high starting (day zero) culture density in order to achieve the most sensitive and time-efficient FCM analyses (Robinson *et al.*, 2003; Marie *et al.*, 2005). This standard initial cell density also aimed to satisfy the recommendations of Grégori *et al.* (2001) who cautioned that optimal cytometric staining protocols may vary undesirably with changes in cell density, and so cell densities were maintained at numbers as close as practicable to  $10^6$   $\text{ml}^{-1}$  during all FCM staining manipulations.

The current dark-survival experiments were designed around a non-destructive sampling protocol due to the desire for ongoing assessment of the dark-survival kinetics of the same algal population. Additionally, space limitations within the illuminated incubator (Plate 7.1) prevented the adoption of a destructive sampling protocol due to the large number of replicate flasks which would have been required. There were also important considerations surrounding the physical size of individual treatment flasks. It was thought to be desirable for the volume of algal culture in each treatment flask to be as large as possible in order to minimise the unwanted effects of a diminishing culture volume during the extended and non-destructive sampling program. Following this, 500ml Borosilicate flasks were used for all experimental incubations.



**Plate 7.1.** Photograph showing the internals of the illuminated orbital incubator and the randomised arrangement of the experimental treatment flasks for both algal species in all four treatments.

For the ‘dark’ treatments, 500ml flasks were double-wrapped in aluminium foil and then covered again with durable masking tape for additional protection against any unwanted damage to the opaque covering during the two month experimental duration. For ‘low D.O.’ treatments, filtered medical-grade  $N_2$  gas was used for sparging of the culture medium in order to strip away the majority of the dissolved oxygen. Individual treatment flasks were bubbled with  $N_2$  in the laminar-flow cabinet for precisely 2 minutes each. This pre-treatment was effective at removing approximately 80% of the oxygen from the culture media, leaving a starting oxygen concentration of  $\approx 2\text{mg L}^{-1}$  for ‘low D.O.’ treatments.

Sterile cotton wool bungs were inserted in the necks of ‘aerobic’ treatment flasks, whilst rubber stoppers were inserted and secured tightly with electrical tape into the necks of ‘low DO’ treatment flasks. Prior to fitting, the tapered wall of the rubber stoppers was coated with a thin film of high pressure vacuum grease in order to create a hermetic seal and prevent re-oxygenation during the long-term experiment (Plate 7.2). Prior to commencement of the experiment, this airtight sealing technique was assessed with respect to its ability to prevent re-aeration of the culture medium, with results of these tests showing no evidence of oxygen exchange over a period of two weeks between the internal culture medium and the outside atmosphere (data not shown). Finally, an

aluminium foil cap (represented by broken line in Plate 7.2) was molded over and around the top of all ‘dark’ treatment flasks in order to further ensure conditions of absolute darkness for the duration of the experiment.



**Plate 7.2.** Double aluminium foil wrapped and sealed flask used for ‘dark / low D.O.’ treatments (broken line represents the positioning of the opaque aluminium foil cap).

Prior to the commencement of an experiment, all flasks were pre-rinsed with 0.2 $\mu$ m-filtered distilled water and sterilised via autoclaving (121°C for 15 min). Liquid culture medium used for all dark-survival experimentation was 0.2 $\mu$ m-filtered, sterile, buffered Woods Hole MBL media as detailed in Section 7.1. All laboratory manipulations concerning experimental start-up were performed aseptically in a Class 2 laminar-flow cabinet (see Section 7.1).

Following cultivation of the necessary algal culture stocks (Section 7.1), and the determination of their respective cell densities (see below; Section 7.2.3.2.1), quantities of stock cultures were added to experimental flasks (already containing MBL media) so that the starting ‘day zero’ cell densities were as close as possible to 1.0 $\times$ 10<sup>6</sup> cells ml<sup>-1</sup> for both species. This resulted in a starting ‘day zero’ volume of  $\approx$ 425ml per flask. Following ‘day zero’ sampling, all 24 treatment flasks were placed in the illuminated orbital incubator where they were maintained under the standard culture conditions described earlier (Section 7.1). Experimental algal culture inocula to be used for dark-survival assessments were not gradually ‘shade-adapted’ as others have done (Griffis and Chapman, 1988), with the goal being to avoid any ‘dark-conditioning’ of cells prior

to the long-term dark-exposure experiment. To prevent experimental cultures from sedimentation and accumulation at the flask base during the two month study (as a result of the vortex ‘eddy’ action from continuous orbital mixing), all flasks were hand-mixed every second day (except at two intervals) for the duration of the 64 day experiment. This served, as much as possible, to maintain a homogeneous cell suspension in all treatments, whilst minimising the potential for benthic cellular agglomeration which could lead to self-shading in light treatments and unwanted cell clumping; the latter of which can be problematic for FCM analyses. Following all hand-mixing and sampling events, flasks were replaced randomly into the incubator (Plate 7.1) in order to minimise potential confounding influences resulting from physical positioning within the incubator itself.

As previously mentioned, and given the universally hypertrophic state under which WSP systems operate, this research involved the long-term dark viability assessment of two ubiquitous WSP phytoplankton species under nutrient-replete conditions within the laboratory. Since algal stock cultures were re-inoculated at ‘day zero’ into fresh, nutrient-replete MBL growth medium, it was assumed that there were sufficient nutrients to not pose any limitation upon survival (or indeed growth), and, as such, dissolved nutrients were not monitored at any time during the course of these experiments.

### **7.2.2 Experimental sampling protocols—65 and 7 day dark-survival experiments**

Sampling of all treatment flasks during the two month (65 day) experiment was carried out in a non-destructive manner, with repeated sampling of each experimental flask intended for days 0, 7, 14, 24, 34, 44, 54 and 64. ‘Day zero’ sampling was performed by sub-sampling the experimental culture inoculum following commencement of each experimental run. Following the ‘day 7’ sampling interval, and due to the large number of treatment flasks and the time-consuming nature of the subsequent analyses, it was no longer feasible to sample both light and dark treatments on the same day. Consequently, sampling regimes for ‘light’ and ‘dark’ treatments from day 7 onwards were staggered over two consecutive days. This meant that ‘light’ samples (for both species) were taken

on day 14 whilst 'dark' treatments were sampled on the following 'day 15' (i.e. *light* flasks were sampled on days 7, 14, 24, 34, 44, 54 and 64, whereas *dark* flasks were sampled on days 0, 7, 15, 25, 35, 45, 55 and 65). Whilst this situation was not ideal, the small time difference between 'light' and 'dark' treatments was considered to be negligible in the scheme of the 8 week experimental duration, such that both light and dark treatment results from the two month experiment are reported in Chapter 9 as though they were both taken on the latter day of the consecutive two day sampling intervals (i.e. days 0, 7, 15, 25, 35, 45, 55 and 65).

Following the initial 65 day dartheek-survival experiment, a secondary follow-up experiment was conducted in order to distill the kinetics of dark acclimation during the course of the first week of dark-exposure. Consequently, a 7 day dark-survival experiment was performed, in which all four treatments were identical to that of the 65 day experiment, but during which only the alga *C. vulgaris* was experimentally assessed (see Section 9.4 for more information). Sampling of all treatment flasks during the secondary 7 day dark-survival experiment was carried out in a non-destructive manner (as above), with repeated sampling of each experimental flask conducted on days 0, 2, 4 and 7. In addition to following the above sampling procedures, one extra step was added to the daily sampling protocol. This extra step involved supplemental dissolved inorganic carbon (DIC) addition ( $\text{NaHCO}_3$ ) in sufficient quantities to yield a final concentration similar to that of the original MBL media (Appendix G), and was performed following the perceived DIC limitation of some treatments during the two month dark-survival experiment (refer to Section 9.10 for further elaboration). Since the volume of stock  $\text{NaHCO}_3$  added at each sampling interval was in the order of  $400\mu\text{l}$  (to a total volume in the order of 400ml), this was considered to have imposed no significant dilution effects on daily algal population cell density.

Prior to and after every sampling interval, all treatment flasks were weighed to within two decimal places and individual flask weights recorded. Any reduction in flask weight between sampling intervals was calculated and taken as a measure of evaporative loss during the course of the experiment. If necessary, a correction factor could later be applied in order to counter any potential concentration effects from undue volumetric losses, particularly with respect to culture cell density in un-sealed 'aerobic' treatment

flasks. Results showed, however, that no evaporation occurred at any point in the sealed ‘low D.O.’ treatment flasks and that average 10-day evaporative losses were always less than 0.25% v:v in un-sealed ‘aerobic’ treatment flasks. Following this, the application of a volumetric correction factor to the final data was deemed redundant and was, therefore, not performed.

All sampling manipulations were performed in a Class 2 laminar-flow cabinet, with ‘dark’ treatment sampling conducted in conditions as close as practicable to total darkness and with great care taken to prevent exposure of dark cultures to any light whatsoever—following the recommendations of Hellebust and Terborgh (1967). Umebayashi (1972) and Smayda and Mitchell-Innes (1974) have also highlight the need for strict control of dark sampling conditions during dark-survival experimentation, claiming that even the slightest re-exposure to very-low (sub-compensation) light intensities during darkness can be enough to significantly and beneficially alter phytoplankton dark survivorship. Very weak, diffuse background lighting during sampling intervals was provided non-directly by standard white fluorescent lighting, with PFD during sampling quantified (Skye Instruments quantum sensor) as being below the instrumental limit of detection (i.e.  $\leq 0.000\mu\text{mol photons PAR m}^{-2} \text{ s}^{-1}$ ).

### **7.2.2.1 Summary of sampling protocol**

A concise overview of the daily sampling protocol adopted during the long-term dark-survival experiment is provided below:

1. Flasks were carefully removed from the incubator, weighed to two decimal places and weights recorded;
2. Flasks were thoroughly hand-mixed ( $\approx 20$  rotations in each direction) and then transferred one at a time to the laminar-flow cabinet for sampling;
3. ‘Aerobic’ treatment flasks were sampled under a normal atmosphere, whereas ‘low D.O.’ flasks were sampled under low pressure (15 PSI)  $\text{N}_2$  sparging to avoid re-oxygenation of the flask headspace during sampling. Sterile 5ml pipette tips (Eppendorf) attached to silicone hosing were used to direct the flow of  $\text{N}_2$  gas into the flask whilst sampling took place;



4. Composite samples (22ml total volume consisting of  $2 \times 8.5\text{ml}$  and  $1 \times 5\text{ml}$  aliquots) were withdrawn aseptically into labeled, sterile 10ml tubes. Samples withdrawn from dark treatment flasks were stored in an aluminium-foil-wrapped test tube rack that was then placed in a cardboard box in order to minimise potential re-exposure to light post-sampling. Sample tubes from ‘low D.O.’ treatment flasks also had the tube headspace sparged with  $\text{N}_2$  gas and were also parafilm-sealed to reduce re-aeration potential prior to final sample processing and analysis;
5. Once all sampling was completed, ‘aerobic’ treatment flasks had their sterile cotton bungs re-inserted, whilst ‘low D.O.’ treatment flasks had the rubber stoppers re-inserted and securely re-sealed (with electrical tape around the entire length of the flask) and all aluminium foil caps finally replaced;
6. Prior to their return to the incubator, all treatment flasks were re-weighed (as per Step 1 above) to allow for evaporative losses during long-term incubation to be calculated.

Every second day following hand-mixing, and following every sampling interval, qualitative visual observations were made on the physical appearance (e.g. dispersion, aggregation, bleaching, flask adhesion) of the algal cultures. It was envisaged that these observations could then be later applied to aid in the interpretation of subsequent FCM data, or perhaps go toward explaining any unforeseen anomalies encountered during later data analyses.

#### **7.2.2.2 Assessment of re-growth potential—experimental design and analysis**

Following completion of the two month dark-survival experiment, an experiment aimed at assessing the illuminated algal re-growth potential post-darkness was conducted. Following the final two month sampling interval for both ‘light’ and ‘dark’ treatments (i.e. days 64 and 65), 0.6ml of the aged treatment cultures were sub-cultured into fresh, sterile MBL medium containing antibiotics according to the routine protocols outlined in Section 7.1. Re-growth cultures were again incubated according to the same standard

protocols, with sampling intervals on days 3, 7 and 10 to allow for population cell density assessments and determination of the experimental culture's re-growth potential.

### 7.2.3 Dark-survival experimental analyses

Following their collection, all samples from each sampling interval were analysed for a number of various parameters. Analysis of experimental cultures included monitoring of: pH; DO; and chlorophyll *a* concentration. Additional 'cytometric' sample analyses (performed via FCM) included assessment of: population cell density; cell size (FSC-height); intracellular density (SSC-height); chlorophyll *a* fluorescence; PI fluorescence (membrane integrity); and FDA fluorescence (metabolic activity).

#### 7.2.3.1 Gross culture analyses

Aqueous pH (WTW pH-320, Nova Analytics Inc.) and dissolved oxygen (WTW Oxi-330) were measured *in vitro* once the samples had been withdrawn from treatment flasks (see 'Step 4' above) in order to eliminate the risk of microbial or cross-contamination. Algal culture chlorophyll *a* was quantified by acetone extraction according to the standard trichromatic method 10200 H (APHA, 1992). Briefly, samples (6–8ml) were filtered onto GF/C (1.2 $\mu$ m nominal pore size; Whatman<sup>®</sup>, UK) and then extracted in ice-cold 90% acetone (5ml) in aluminium-foil-wrapped 10ml centrifuge tube at 4°C for 24–48 h. Following extraction, samples were centrifuged at 3000g (Sigma 6K15) at 4°C and then the absorbance (ABS) read at 630, 647 and 664nm against a 90% acetone blank (Shimadzu UV–1700). Chlorophyll *a* concentration was then calculated according to the Equation 7.2 as follows:

$$\text{Chl. } a = [(11.85 \times \text{ABS-664nm}) - (1.54 \times \text{ABS-647nm}) - (0.08 \times \text{ABS-630nm})] \times (V_A / V_F) \quad (\text{Equation 7.2})$$

where Chl. *a* = chlorophyll *a* concentration ( $\mu$ g L<sup>-1</sup>)

ABS-664nm = absorbance read at 664nm (and so on)

V<sub>A</sub> = volume of 90% acetone used during sample extraction (ml)

V<sub>F</sub> = volume of experimental sample filtered during the analysis (L)

Total- (TC), dissolved inorganic- (DIC), total organic- (TOC) and dissolved organic carbon (DOC) were quantified using a Shimadzu TOC–5000A Total Organic Carbon analyser according to methods outlined in the operating manual. DOC fractionation of TOC was determined by filtering samples through 0.45 $\mu$ m syringe filters (Acrodisc<sup>®</sup>, PALL Corporation) and analysed as for TOC. Instrument sensitivity analysis was performed in order to determine the limit of detection for DIC across a range of anhydrous sodium carbonate and sodium bicarbonate standards (according to the manufacturer's instructions). The instrument was found to be reliably accurate in the range of 0–5mg DIC L<sup>-1</sup> ( $r^2 = 1.000$ ;  $n = 4$ ;  $CV \leq 5\%$ ); however, analytical resolution was diminished at lower DIC concentrations 0–1mg L<sup>-1</sup>, with the reliable limit of detection identified to be somewhere in the order of 200–300 $\mu$ g DIC L<sup>-1</sup>.

### **7.2.3.2 Optimisation of sample treatment, staining protocols, and cytometric analyses**

Although there already exists numerous and detailed protocols for phytoplankton analyses with FCM, the recognised species-specificity (particularly of cell staining practices) of these sorts of cytological analyses made it essential to optimise the staining protocols in particular before conducting the final experimental analyses (see Section 6.4.2.4.1–6.4.2.4.2 for more information). In line with the recommendations of Grégori *et al.* (2002) outlined in Section 6.4.2.4.2, and for the purposes of methodological ease and continuity, a single staining protocol for each fluorochrome was initially optimised both algal species at a cell density of  $\approx 1 \times 10^6$  ml<sup>-1</sup>. This cell density was then adopted during all subsequent experimental staining assays.

#### **7.2.3.2.1 Enumeration of population cell density**

Phytoplankton cell density enumerations were done according to the method of Marie *et al.* (2005), of which a brief description is provided here. Approximately 1ml of 0.2 $\mu$ m-filtered Milli-Q<sup>®</sup> water was transferred into a FACScan sample tube. The sample tube plus water was then accurately weighed to four decimal places and the starting weight recorded. The tube was then loaded onto the cytometer sample injection platform and a chronometer simultaneously started as the cytometer was engaged. The cytometer was then allowed to run for a minimum of 10 minutes (commonly 20–30 minutes) after

which time the sample tube was removed from the cytometer simultaneously as the chronometer was stopped (commonly on an exact minutely interval for convenience). The sample tube was then re-weighed and the final weight recorded. Cytometer flow rate (R) in  $\mu\text{l min}^{-1}$  could then be calculated according to Equation 7.3. Since 0.2 $\mu\text{m}$ -filtered Milli-Q<sup>®</sup> water was used for daily FCM flow rate calibrations, no density correction factor was required—as outlined by Marie *et al.* (2005)—because Milli-Q<sup>®</sup> water was assumed to have a density of 1.000. Cytometer flow rate for the cell enumeration protocol was also recalculated daily in order to minimise potential error resulting from small day-to-day variations in instrument pressure and subsequent sheath fluid velocity.

$$R = (Wt_i - Wt_f) / (t \times d) \quad (\text{Equation 7.3})$$

where  $Wt_i$  = initial tube weight (mg)

$Wt_f$  = final tube weight (mg)

t = time (minutes)

d = density of the solution (Milli-Q<sup>®</sup> = 1.000)

Cell density enumeration for experimental algal culture samples was then performed by mounting  $\approx 500\mu\text{l}$  of *raw* algal sample (i.e. no added beads, stains etc.) onto the cytometer, allowing the flow to stabilise for about 15 seconds and then commencing data acquisition for a precise time (i.e. 2 minutes for cultures with  $\approx 10^6$  cells  $\text{ml}^{-1}$  and 3–5 minutes for  $10^5$ – $10^4$  cells  $\text{ml}^{-1}$ ). Cell density was then determined according to: the daily cytometer flow rate (R); the number of cell events recorded; and the sample acquisition run time. Following the methodological recommendations of Marie *et al.* (2005), all FCM data was collected at an event rate of no more than 700 events per second (commonly <600) to avoid cytometer coincidence (a phenomenon where two cells are so close together in the sample stream such that they are recorded as a single event); something common at event rates between 1000–1400 cells  $\text{s}^{-1}$  (Gasol and del Giorgio, 2000). Additionally, Veldhuis and Kraay (2000) reported that actual cell numbers may be underestimated by as much as 70% at event rates above 1500 cells  $\text{s}^{-1}$ , with potential adverse effects on scatter and fluorescence signals also likely at such high

event rates. Following this, and where necessary, cultures were serially diluted so that the acquisition event rate for all samples satisfied the above criteria.

#### **7.2.3.2.2 PI, FDA and cytometry bead stock solutions**

A stock solution of propidium iodide (Molecular Probes<sup>®</sup>; lot no. 45B5–18) was made up in Milli-Q<sup>®</sup> water to a final concentration of  $1\text{mg ml}^{-1}$  ( $1.5 \times 10^{-3}$  M). PI stock was then  $0.2\mu\text{m}$  filter-sterilised (Acrodisc<sup>®</sup>) and stored as  $500\mu\text{l}$  aliquots in low-adsorption  $1.5\text{ml}$  screw-cap tubes (AXYGEN Scientific Inc.) at  $4^\circ\text{C}$  in the dark until required.

A stock solution of fluorescein diacetate (Sigma<sup>®</sup>; F-7378; lot no. 062K5318) was made up in reagent-grade dimethylsulfoxide (DMSO) to a final concentration of  $10\text{mg ml}^{-1}$  ( $0.024$  M) and then stored as  $250\mu\text{l}$  aliquots in low-adsorption  $1.5\text{ml}$  screw-cap tubes at  $4^\circ\text{C}$  in the dark. To assist in dissolving the pure FDA, the stock solution was heated slightly ( $45\text{--}50^\circ\text{C}$ ) and vortex-mixed prior to decanting and aliquot storage. FDA stocks were always assessed visually for the presence of any signs of yellow-green fluorescein fluorescence following both the initial stock formulation and subsequent thawing. FDA stocks displaying any apparent yellow-green coloration were discarded immediately.

A laboratory stock solution of internal standard  $2.5\mu\text{m}$  PeakFlow<sup>™</sup> green fluorescent reference beads (Molecular Probes<sup>®</sup>; lot no. 69C1–1) was made from the manufacturer's stock solution. The laboratory stock was made up to a final bead density of  $\approx 1 \times 10^6$  beads  $\text{ml}^{-1}$  in  $0.05\%$  Tween–20 and  $2\text{mM}$  sodium azide as per the manufacturer's stock. This working laboratory bead stock solution was again stored at  $4^\circ\text{C}$  in the dark until required.

#### **7.2.3.2.3 PI and FDA staining optimisations**

Dual PI–FDA staining protocols were pre-optimised with respect to: stain concentrations; staining incubation time; and were also validated with respect to their ability to discriminate 'live' from 'dead' cells. In order to test the 'live/dead' discriminatory resolution, pre-prepared mixtures of live and dead cells were tested using the dual PI–FDA flow cytometry protocol. Eight day old (approaching stationary-phase) cells were used for all staining optimisation protocols in order to maximise consistency

between optimal staining protocols and later experimental staining conditions (see Section 7.2.1 for further clarification). Day 8 algal cultures for *C. vulgaris* and *C. reinhardtii* were re-suspended in 0.2 $\mu$ m-filtered buffered MBL culture medium to final cell densities of  $\approx 1.0 \times 10^6$  cells ml<sup>-1</sup> prior to all staining optimisations. Heat-killed algal cells were used as positive and negative controls during PI–FDA staining optimisation protocols. After preliminary trials, the final procedure used for heat-attenuation was similar to that of Franklin *et al.* (2001a). Briefly, a tightly capped 50ml centrifuge tube containing  $\approx 15$ ml of algal culture was incubated in a pre-heated ( $\approx 120^\circ\text{C}$ ) electric heating block for an initial 10 minute equilibration period, during which time the algal culture reached a temperature of  $\approx 97^\circ\text{C}$ . This was then followed by a final 10 minute heat-attenuation period and final culture cooling in a room-temperature ( $\approx 20^\circ\text{C}$ ) water bath.

Due to the widely recognised pH-sensitivity of particularly the FDA staining procedure (see Section 6.4.2.4.1), algal culture pH was monitored prior to all experimental staining manipulations and FCM analyses to ensure optimal compliance during cytological staining. During preliminary PI–FDA staining optimisations, algal culture pH was also maintained at a pH 7.0–8.0 (most commonly around 7.60). Where aqueous pH exceeded 8.0, experimental samples were diluted accordingly using 0.2 $\mu$ m-filtered MBL media (pH 7.2). General FCM protocols for optimal PI and FDA staining were adapted from a combination of the existing protocols of: Ross *et al.* (1989); Dorsey *et al.* (1989); Williams *et al.* (1998); Jochem (1999); Brookes *et al.* (2000); Franklin *et al.* (2001a); and Robinson (2003). Based on a review of previously published methods (see also Section 6.4.2.4.1), four FDA concentrations were initially chosen for testing during preliminary staining optimisations: 2.4 $\mu$ M; 12 $\mu$ M; 24 $\mu$ M; and 36 $\mu$ M. Optimal staining incubation time would also be determined based on critical analysis of the FDA hydrolysis and fluorescence–time kinetics data obtained during testing of the above substrate concentrations (refer to Section 8.2.2.1).

Similar to that for FDA above, three PI concentrations were also pre-selected (based on those reported in the literature above) for initial assessment during staining optimisations: 1.5 $\mu$ M; 3 $\mu$ M; and 7.5 $\mu$ M; along with differing staining incubation durations: 1; 5; 10 and 20 minutes. Pre-prepared mixtures of live and dead (heat-killed;

see above) algal cells for both species were prepared at live:dead cell ratios of: 100:0; 75:25; 50:50; 25:75 and 0:100. These mixed cell preparations were then later analysed according to the pre-optimised staining protocols in order to assess general FCM methodological accuracy and ‘live vs. dead’ discriminatory resolution. Cytometer instrument gain settings were adjusted so that the bulk (>99.9%) of ‘raw’ un-stained cells appeared in the first decade of the log-scale fluorescence as per the methodological recommendations of Brookes *et al.* (2000).

During all staining optimisation protocols, biological stains were always added directly to the algal sample tube at the desired final concentration. Stained cells were never washed prior to FCM analysis and were kept at room temperature with light-exposure minimised by an aluminium foil covering as per the methods of Dorsey *et al.* (1989). Dark storage prior to FCM analysis in this case served to ensure both consistent sample pre-treatment and also to effectively ‘dark-adapt’ algal cells prior to chlorophyll *a* fluorescence measurements (Falkowski and Owens, 1980; Matorin *et al.*, 2004). Unstained ‘raw’ cells were always analysed in parallel with stained cells to enable calibration of any background fluorescence for PI-FDA protocols. Despite this, unstained cells never recorded any undue fluorescence signals, such that it was never necessary to correct for erroneous background scatter or fluorescence for any measured fluorescence channel at any stage during the dark-survival experiments. Following initial staining optimisation procedures, cytometer instrument settings for each fluorescence channel were saved to disk and could then be re-loaded for all subsequent analyses as required. This ensured that daily measurements for a given fluorescence parameter were as consistent as possible throughout the course of the long-term dark-incubation experiment.

Some cells and their analytical procedures require that they be fixed prior to FCM analyses. All FCM protocols and corresponding experimental manipulations reported herein were always performed using unfixed or ‘raw’ cells. During experimental manipulations and FCM analyses, algal samples were always stored at room temperature ( $\approx 20\text{--}22^\circ\text{C}$ ) and, where practicable, away from direct light in accordance with *Current Protocols* (Robinson *et al.*, 2003). Results from these staining pre-optimisations and preliminary methodological validations are provided in Chapter 8.

#### **7.2.3.2.4 The optimal PI–FDA staining protocol**

Following initial thawing, the FDA stock solution (0.024M; Section 7.2.3.2.2) was stored at room temperature in the dark prior to formulation of the daily working stocks. Daily FDA working solutions were prepared according to the methods of Dorsey *et al.* (1989) and Jochem (1999). Briefly, the FDA stock solution was thawed completely and kept in the dark at room temperature. 200 $\mu$ l of the 0.024M FDA stock solution was then transferred into a sterile 10ml tube containing 9800 $\mu$ l of ice-cold Milli-Q<sup>®</sup> water by direct-injection from the pipette, followed immediately by rapid mixing of the working solution in order to minimise FDA precipitation. This daily working solution was then stored on ice in the dark to minimise potential substrate FDA degradation. Even though the FDA daily working solution may have appeared slightly opaque, it remained in suspension and did not precipitate out. This yielded a daily FDA working solution of 0.48mM, which was then stored on ice until needed. Pipetting 50 $\mu$ l of this daily FDA working solution into 950 $\mu$ l of sample yielded a final FDA concentration of 24 $\mu$ M. Daily FDA working solutions were always made in duplicate so that they could be discarded in case of apparent contamination, precipitation or degradation during daily staining manipulations. FDA working solutions were also kept for a maximum of 4 hours in accordance with the methods of Dorsey *et al.* (1989) and Jochem (1999).

In accordance with initial staining pre-optimisations and methodological evaluations (see Section 7.2.3.2.3), final PI–FDA staining of experimental samples was then performed according to the following protocol. 50 $\mu$ l of the FDA daily working solution (0.48mM) was firstly transferred into a FACScan cytometer sample tube containing 939 $\mu$ l of algal sample, followed by vortex mixing and incubation at room temperature for 5 minutes. 1 $\mu$ l of PI stock solution ( $1.5 \times 10^{-3}$  M; see Section 7.2.3.2.2) was then added to the sample tube, followed again by mixing and incubation for a further 5 minutes. This protocol resulted in no apparent ‘false-positive’ PI staining of viable cells during procedural optimisations. This optimised staining protocol yielded final concentrations of 1.5 $\mu$ M for PI and 24 $\mu$ M for FDA. As per the method of Dorsey *et al.* (1989) algal cells were not washed following staining, but were kept in the sample tube containing PI and FDA until analysed on the cytometer.



Just prior to final FCM analysis, 10 $\mu$ l of freshly sonicated (10 minutes) stock green fluorescent bead suspension (see Section 7.2.3.2.2) was added to give a final bead density of  $\approx 1 \times 10^4$  beads ml<sup>-1</sup>. The addition of standard reference beads served as both an internal fluorescence and physical light scatter standard and also allowed for instantaneous instrumental control during FCM analyses. All samples were stored at room temperature in the dark prior to FCM analyses.

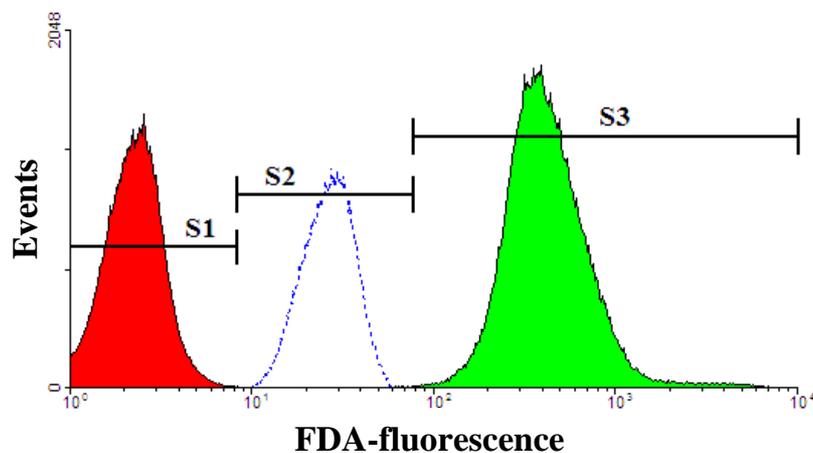
### **7.2.3.3 Flow cytometric data acquisition, analysis and presentation**

All flow cytometric analyses and sample data acquisition were performed using a FACScan<sup>™</sup> (Becton Dickinson, USA) cytometer equipped with a single argon-ion (488nm, 15mW) laser excitation source. The cytometer was calibrated on a weekly basis using Becton Dickinson CaliBRITE<sup>™</sup> beads (cat. no. 349502) and associated software. Physical light scatter signals (FSC and SSC) were collected along with three-colour fluorescence over a range of different wavelengths (see Section 6.4.2 for more information), with cytometer signal data presented as arbitrary units of signal amplitude ‘height’ (FSC and SSC) or fluorescence (PI, FDA and chlorophyll *a*). Samples were ‘gated’ during data acquisition using plots of FSC vs. SSC-height and FSC-height vs. chlorophyll *a* fluorescence.

To allow for sufficient analytical resolution, and in line with the recommendations of Marie *et al.* (2005), no less than 10<sup>4</sup> gated events (commonly 2–4 $\times 10^4$ ) were recorded for each individual sample. Individual population sample sizes of this order were also reported elsewhere as being adequate in cytometric phytoplankton analyses (Balfourt *et al.*, 1992; Cid *et al.*, 1996). All flow cytometric data was collected in logarithmic mode, with subsequent processing and analyses performed using CELLQuest<sup>™</sup> version 3.2.1 analytical software (Becton Dickinson, USA). Following the recommendations of Shapiro (2003), all raw data reporting and statistical analyses were performed based on distribution-independent population geometric means or medians and not arithmetic means (due to the invariably ‘non-normal’ or skewed population frequency distributions for any given parameter). Graphical depictions of raw cytometer data files (i.e. two- and three-dimensional scatter plots and histograms only) were compiled using the freeware

programme WinMDI (Joseph Trotter; version 2.8; available at <http://facs.scripps.edu/software.html>).

Due to the complex and multifaceted nature of flow cytometric data, the data was—where appropriate—manipulated and presented in a number of various formats. This allowed the same data set to be represented in a number of different ways in order to show important and discrete aspects of the same cell population. As an example of this, these data manipulations involved such things as like expressing long-term cellular FDA fluorescence as either: the population average; the population average normalised to cell volume; the population average as a relative percentage of time zero values; or the population average of cells lying within three discrete fluorescence ‘activity states’. This final method of data presentation was derived from Franklin *et al.* (2001a; 2001b) and involves the use of a population ‘marker’ system (Figure 7.1). This technique effectively segregates the entire cell population into discrete *regions* based on their relative ‘fluorescence activities’ and was adopted here in order to monitor long-term population shifts in relative per-cell FDA fluorescence.



**Figure 7.1.** Graphical depiction of the three-marker system used for differentiating the fluorescence activity states of the total algal cell population during prolonged dark-exposure. The cellular FDA-fluorescence histogram of a hypothetical mixed population depicts: the normal or ‘healthy’ fluorescence state (S3); a cell population with a reduced fluorescence state (S2); and an FDA-negative ‘non-viable’ cell population (S1). The y-axis shows the number of counted cell events and x-axis shows  $\log_{10}$  cellular FDA-fluorescence activity (A.U.) (Figure adapted from Franklin *et al.*, 2001a).

This method of data analysis utilises three discrete markers within a single population histogram, thereby splitting the population into separate regions of differing fluorescence states or activities. The three population markers (S1, S2 and S3), by dividing the entire population distribution into discrete regions of fluorescence intensity, allows the investigator to monitor long-term changes in cellular properties on a population scale. Methodologically, all baseline ‘control’ S3 markers for FDA fluorescence for both algal species were defined at ‘day zero’ of the dark-survival experiment (using day 8 algal cultures; see Section 7.2.1). Following this, any subsequent population deviations (i.e. decrease in FDA-fluorescence) from these initial S3 fluorescence boundaries signified a shift from the ‘normal’ fluorescence state. Day zero S3 markers were set so that generally >98% of cells fell within these S3 ‘control’ fluorescence states. Similarly, the percentage of positive-control heat-killed cells falling into their respective S1 ‘non-viable’ regions during methodological optimisations generally exceeded 99% of the total gated population.

This marker system gives the investigator far greater analytical resolution with respect to the distribution of cell population activity rather than dealing with a single population average FDA metabolic activity value. This benefit was highlighted somewhat earlier by Sengbusch *et al.* (1976) where it was discussed that not only can the FDA assay give ‘bulk’ assessments of mean cellular activity or fitness, but the frequency distributions from FCM outputs give detailed insights into the population structure in terms of the homogeneity (or otherwise) of overall population metabolic activity. In other words, the *shape* of the frequency distribution curve (i.e. Gaussian or skewed) gives the operator instantaneous insight into the overall population fitness; providing additional context to viability outcomes implied from the FDA assay.

Standard green fluorescent beads were run during every flow cytometric session and in most experimental samples (excluding raw cell preparations for cell enumerations and chlorophyll *a* fluorescence) as internal standards. It is relatively common to report FCM data with reference to, or standardised against, measured fluorescence values of these standard fluorescent beads. In the current analyses, however, and following the extremely low day-to-day deviations in measured bead fluorescence and light scatter (see Section 8.2.2.3), it was decided that bead-standardisation of cellular fluorescence

and scatter data was not necessary—an analytical tact also taken by Vaultot *et al.* (1986). Since this bead-standardisation step changed neither the phytoplankton data (relative to each other) nor the outcome of subsequent statistical analyses, all data were left as standard numerical values so as to avoid further and unnecessary complication of already complicated multi-parametric datasets. Furthermore, the regular cytometer calibration ensured that maximal control and instrument precision was inherently associated with all cytometric analyses. Other authors (e.g. Dorsey *et al.*, 1989) have also reported using standard fluorescein beads in FDA cellular fluorescence analyses with FCM as a way of universally standardising per-cell fluorescence (metabolic activity) in units of ‘fluorescein molecular equivalents’ (this is done primarily to enable inter-laboratory comparisons of cellular FDA fluorescence data obtained from different cytometers). Since another type of green-fluorescent bead was used during the current analyses (i.e. non-fluorescein), and because only one cytometer was used for all experimental analyses, this fluorescence standardisation step was not performed.

#### **7.2.3.4 Chlorophyll fluorimetry**

Chlorophyll fluorimetry was assessed under 488nm (15mW Argon-ion) excitation illumination using the FACScan cytometer, and was done following a 20 minute dark-acclimation period. Cellular *in vivo* chlorophyll *a* fluorescence signals were recorded in log mode and expressed in arbitrary units (A.U.). Chlorophyll *a in vivo* ‘variable fluorescence’ ( $F_0$ ) was also measured using a FLUOstar™ Galaxy (BMG Lab Technologies) microplate spectrofluorometer following a similar method to that of Gregor and Marsalek (2004).  $F_0$  is as a measure of the *in vivo* photosynthetic (light absorption) capacity of the photosynthetic pigments within algal cells, and it is recognised that  $F_0$  correlates well with the chlorophyll concentration of natural waters measured *in vitro* (Beutler *et al.*, 2002; Matorin *et al.*, 2004). Briefly, algal samples (300 $\mu$ l) were loaded into opaque black (BMG Lab Technologies) polypropylene 96-well microplates and dark-adapted for 15–20 minutes to allow for dissipation of non-photochemical quenching and to ensure that cellular photosystem activity was restored to baseline levels prior to fluorescence probing (Olson *et al.*, 1996). Non-saturating broad-band excitation light at 390 nm ( $\pm$  50nm) was administered and then emitted chlorophyll *a* fluorescence ( $F_0$ ) measured at 660 nm ( $\pm$  10nm) in conjunction with the

following instrumental parameters: gain, 100; number of excitation flashes, 4; read time delay, 0.5s; integration time, 40 $\mu$ s; top read mode; shake duration, 30s (prior to commencing initial plate reading only). All fluorescence readings were corrected against 0.2 $\mu$ m filter-sterilised MBL media blanks (Appendix G).

### **7.2.3.5 Data treatment, statistical analyses and interpretations**

Prior to all statistical analyses, normality of data distributions were assessed by way of Kolmogorov–Smirnov and Shapiro–Wilk normality tests, with supplemental Q–Q normal probability plot analysis in some instances. Homogeneity of data variances were also checked using Bartlett’s or Levene’s tests (depending on the statistical software used). Where raw data satisfied the underlying assumptions of normality and homoscedasticity, parametric statistical analyses (ANOVA, Pearson correlations and *t*-tests) were always performed on the raw un-transformed data. Where these assumptions were not satisfied, data transformations ( $\log_{10}$ ) were performed in an attempt to normalise the data, followed again by normality testing and parametric statistical analysis where appropriate. Where parametric statistical assumptions were not be satisfied (i.e. in instances where data could not be log-normalised or where *n* was too small) Kruskal–Wallis, Spearman rank correlations and Mann–Whitney *U*-tests were employed to test for differences in treatment outcomes.

Differences between the slopes of fitted regression lines were assessed by way of Analysis of Covariance (ANCOVA) using GraphPad PRISM v. 4.03 (GraphPad software, San Diego, CA, USA) and according to the methods of Zar (1996). Differences between treatment outcomes during and following the two month dark-survival experiment were distilled by way of either general linear (type I) repeated-measures (RM) 1-way ANOVA (where data was normally-distributed and  $n \geq 7$ ) with Tukey’s honestly significant difference multiple comparison *post hoc* testing, or by non-parametric Kruskal–Wallis testing (where data was non-normal or  $n < 7$ ) with Dunn’s multiple comparison *post hoc* testing. For ANOVA testing, the variance ratio (*F*) and associated degrees of freedom between (A) and within groups (B) are provided along with the corresponding sample size (*n*) and corresponding *p* value. Kruskal–Wallis test results are provided with the corresponding Chi-square approximated test statistic ( $\chi^2$ ),

associated  $\alpha$  significance level ( $_{0.05}$ ), between-groups degrees of freedom ( $_A$ ) and corresponding  $p$  value. Data correlations were elucidated by way of either parametric Pearson ( $r$ ) correlation or non-parametric Spearman rank ( $r_s$ ) correlation (according to the assumptions of data normality as above) using GraphPad PRISM v. 4.03 and SPSS v. 14.0.0 (SPSS Inc., Chicago, IL, USA). All significance testing was performed at or below  $\alpha \leq 0.05$  level. Any remaining statistical testing or data transformations were performed using GraphPad PRISM v. 4.03. Data tables were formulated using Microsoft® Office Excel 2003. All graphical data, unless otherwise stated, was compiled using GraphPad PRISM v. 4.03.

## **8 Results and discussion of flow cytometric methodological optimisations—the importance of critically assessing optimal staining protocols**

This short Chapter presents the results of methodological optimisations performed prior to the final flow cytometric assessment of long-term phytoplankton dark-survival capacity. Given that both *C. vulgaris* and *C. reinhardtii* are morphologically quite similar (i.e. size and shape) and share a similar taxonomic lineage (Chlorophyceae), it was relatively unsurprising that both species behaved very similarly during these initial FCM methodological validations. Considering this, and for ease of presentation, only the data derived from *C. vulgaris* is presented and discussed in detail below.

### **8.1 Phytoplankton enumeration**

During the course of this research, cells counts using FCM were found to be extremely rapid, highly sensitive and reproducible. A preliminary assessment of FCM for the enumeration of cell density for *C. vulgaris* and *C. reinhardtii* was performed as part of the initial methodological validations. The FACScan cytometer was checked for counting linearity and instrument sensitivity by analysing serial dilutions (where appropriate) of stock algal cultures ( $10^3$ – $10^6$  cells ml<sup>-1</sup>). Subsequent regression analysis of counting calibration data revealed cell counts to be highly accurate for both *C. vulgaris* ( $n = 4$ ;  $r^2 = 0.9999$ ) and *C. reinhardtii* ( $n = 4$ ;  $r^2 = 0.9998$ ). These results were similar to the findings of Ross *et al.* (1989), as described in Section 6.4.2.3, who also reported very accurate and reproducible (slope = 0.94,  $r^2 = 0.99$ , triplicate CV's < 6%) cell counts between  $10^2$  and  $10^6$  cells ml<sup>-1</sup> using mammalian cells.

### **8.2 Flow cytometric discrimination of live vs. dead phytoplankton—optimising the dual PI–FDA assay**

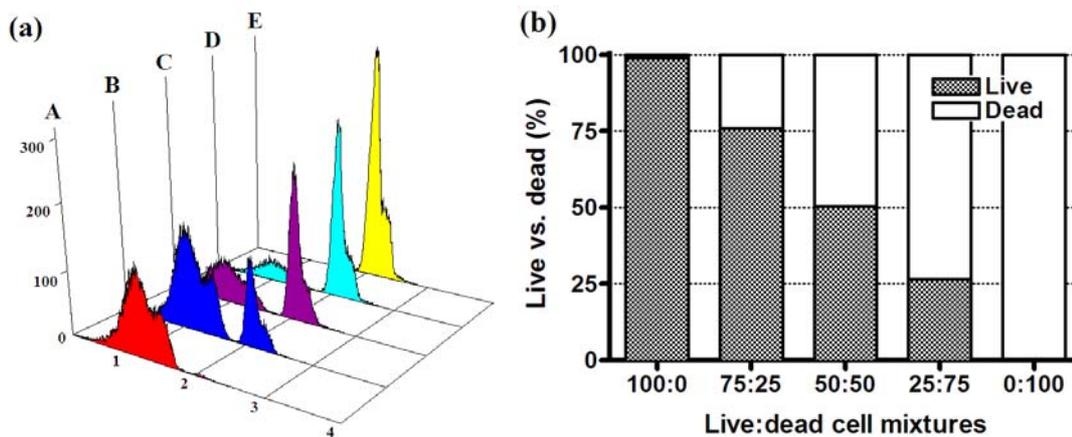
As highlighted earlier (Section 6.4.2.4.1), the species-specificity (amongst other issues) associated with cellular viability assays, such as that of the PI–FDA, made it necessary to undergo pre-optimisations of all cytological staining and analytical FCM protocols. Since both algal species behaved very similarly (if not identically) during this research, only the data derived from cytometric optimisations involving *C. vulgaris* are provided.

### 8.2.1 Flow cytometric assessment of cell membrane integrity—optimising the PI assay

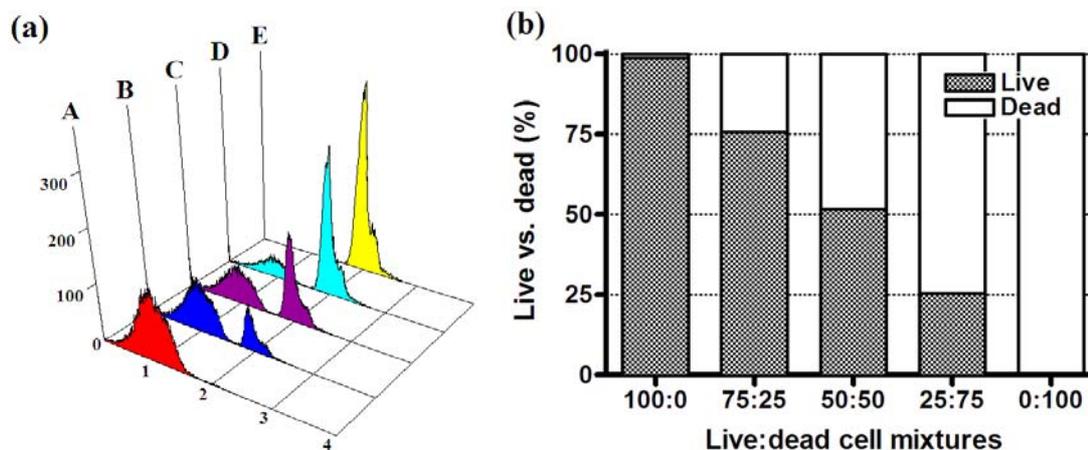
Following on from the variable reporting of optimal PI staining conditions (see Section 6.4.2.4.2), several variations of both final stain concentration and also staining incubation time for the PI viability assay were assessed. Briefly, stain concentrations of 1.5, 3, and 7.5 $\mu$ M, along with staining incubation durations of 1, 5, 10, and 20 minutes were pre-selected for protocol optimisation. Various mixtures of live and dead (heat-killed; see Section 7.2.3.2.3) cells were also used in order to optimise the discriminatory resolution of the PI assay. Following these preliminary optimisations, a final staining duration in the order of 5–10 minutes was selected as optimal (in terms of fluorescence yield and relative ‘live vs. dead’ discriminatory resolution). This optimal PI staining duration was also similar to that of Williams *et al.* (1998) and Franklin *et al.* (2001a). Although not a precise time integer, a staining duration within the range of 5–10 minutes was both satisfactory and far more practicable than a precise staining duration. Furthermore, it is worth noting that the PI staining procedure was also very stable over time, with very little false-positive staining of ‘live’ (negative control) cells even after 20–25 minutes of PI incubation; something contrary to the reporting of others (e.g. Franklin *et al.*, 2001a).

Assessment of PI concentration found that optimal staining (fluorescence yield and ‘live vs. dead’ resolution) was achieved at 1.5 $\mu$ M for both species. Concentrations of 3 and 7.5 $\mu$ M PI resulted in significant and unwanted over-staining (possibly due to leaky membranes as discussed by Franklin *et al.*, 2001a) and probable fluorescence quenching—resulting in reduced PI fluorescence yields. Cytometer instrument settings were adjusted so that dead cells emitted peak PI fluorescence within the first decade of the log-fluorescence scale. This meant that any cells in subsequent analyses recording PI fluorescence signals greater than this pre-determined  $\approx$ 1-log ‘cut-off’ could be classified as PI-positive or ‘non-viable’. 1.5 $\mu$ M PI resulted in both good separation of ‘live’ and ‘dead’ cell populations during FCM data acquisition, as shown in Figures 8.1 and 8.2 for *C. vulgaris* and *C. reinhardtii* respectively.





**Figure 8.1.** (a) Overlaid frequency distribution plots depicting live: dead *C. vulgaris* FCM discrimination in various PI-stained ( $1.5\mu\text{M}$ ) mixtures of live: dead (heat-killed) cultures: (A) 100% live; (B) 75% live; (C) 50% live; (D) 25% live; (E) 0% live (primary  $x$ -axes show  $\log_{10}$  PI fluorescence yield (A.U.),  $y$ -axes show number of cells counted); (b) stacked histogram depicting the accurate discriminatory capacity for the various mixtures of live: dead PI-stained ( $1.5\mu\text{M}$ ) *C. vulgaris* ( $n \geq 20,000$  cells for each live: dead mixture).



**Figure 8.2.** (a) Overlaid histogram plot depicting live: dead *C. reinhardtii* discrimination via FCM and PI ( $1.5\mu\text{M}$ ) staining of mixed ratio live: dead (heat-killed) cultures: (A) 100% live; (B) 75% live; (C) 50% live; (D) 25% live; (E) 0% live (primary  $x$ -axis shows  $\log_{10}$  PI fluorescence yield (A.U.),  $y$ -axis shows number of cells counted); (b) stacked histogram depicting the accurate discriminatory capacity for the various mixtures of live: dead PI-stained ( $1.5\mu\text{M}$ ) *C. reinhardtii* ( $n \geq 20,000$  cells for each live: dead mixture).

Subsequent regression analyses of the data from Figure 8.1 for *C. vulgaris* and Figure 8.2 for *C. reinhardtii* revealed very good reflections of the pre-prepared live:dead mixtures in the respective FCM-quantified ratios for both species (both  $r^2 > 0.999$ ; slope = 0.99;  $n = 5$ ). Two-tailed one-sample  $t$ -tests revealed that regression intercepts

were not significantly different from the data origin for either *C. vulgaris* ( $t_{(0.05(2),2)} = 2.736$ ;  $p = 0.112$ ) or *C. reinhardtii* ( $t_{(0.05(2),2)} = 1.734$ ;  $p = 0.225$ ), meaning that the fractions of ‘known’ and ‘FCM-quantified’ live and dead cells were statistically equal. From this, the PI staining method was considered sufficiently accurate in terms of its ability to reliably discriminate viable from non-viable (heat-killed positive control) cells *in vitro*.

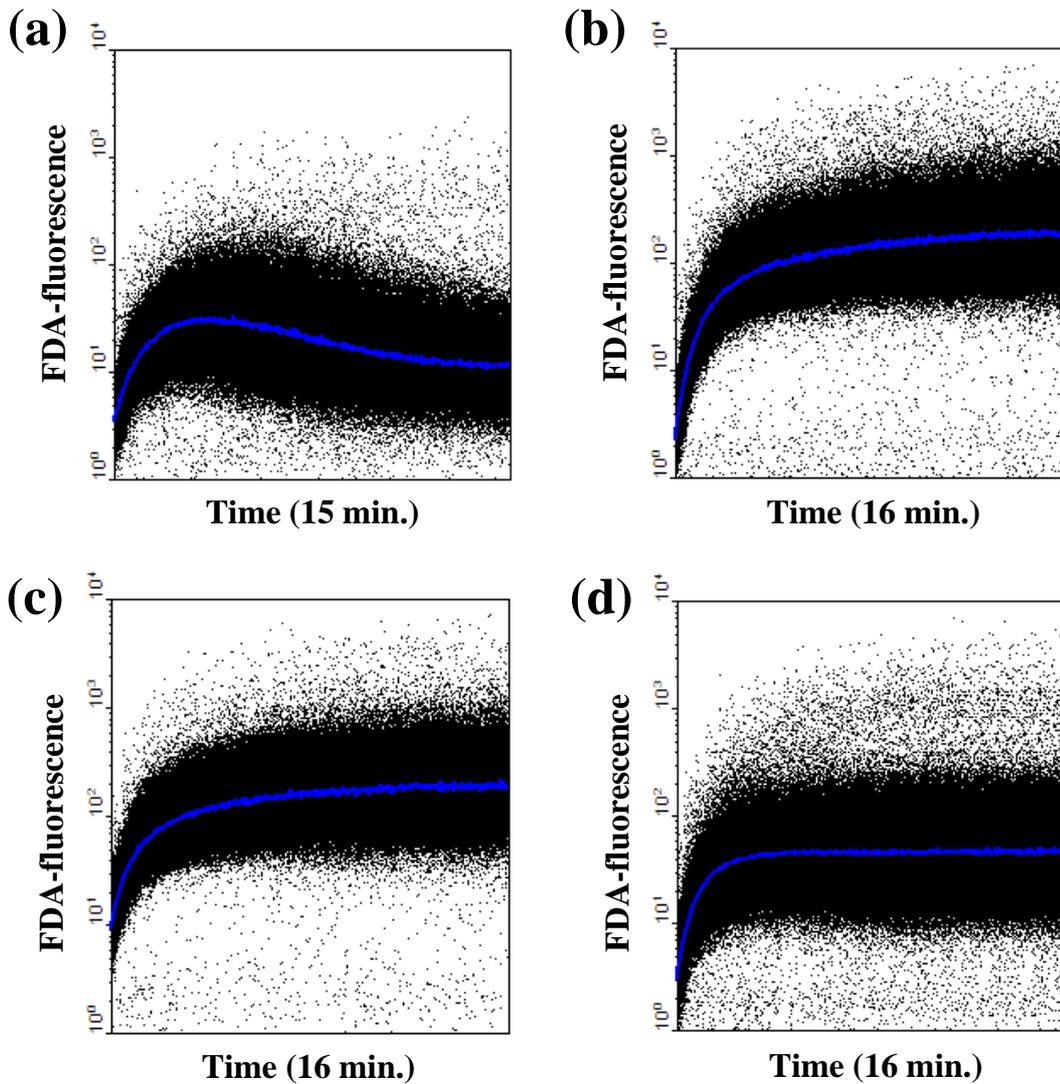
## **8.2.2 Flow cytometric determination of phytoplankton metabolic activity—optimising the FDA assay**

The accumulation of fluorescein fluorescence in intact cells following staining with FDA is dependent on: the amount of substrate that penetrates the cell; the amount of enzyme (esterase) activity that hydrolyses the substrate FDA; and the rate of fluorescein efflux (Prosperi *et al.*, 1986). As outlined in Section 6.4.2.4.1, a critical assumption of this viability assay is that the factor limiting FDA hydrolysis and fluorescein accumulation is *solely* cell esterase activity, and that: substrate (FDA) concentration; cell permeability to FDA; and subsequent FDA influx into the cells; remain constant and are not rate-limiting at any stage of the assay duration (Sengbusch *et al.*, 1976). Following this, several different FDA concentrations were pre-selected for preliminary optimisation as part of the FDA viability assay. Following initial assessment of these pre-selected FDA concentrations, it was necessary to evaluate the above ‘critical assumptions’ in order to verify that they were indeed satisfied (or otherwise), so as to accurately identify the most optimal final FDA concentration for later experimental use. This critical evaluation was achieved via a detailed assessment of staining kinetics for the four chosen FDA concentrations. Since both *C. vulgaris* and *C. reinhardtii* behaved very similarly with respect to FDA staining optimisations, only the protocols optimised for *C. vulgaris* are discussed in detail below.

### **8.2.2.1 Determination of optimal FDA concentration and assessment of substrate FDA hydrolysis kinetics**

Based on the variable reporting of optimal FDA staining concentration in the relevant literature (see Section 6.4.2.4.1), a number of different FDA staining concentrations were pre-selected for initial testing. Final concentrations of 2.4, 12, 24 and 36 $\mu$ M FDA

were assessed with respect to their uptake and hydrolysis kinetics and fluorescence emission properties for both algal species, with results from these initial assessments shown in Figure 8.3 below.

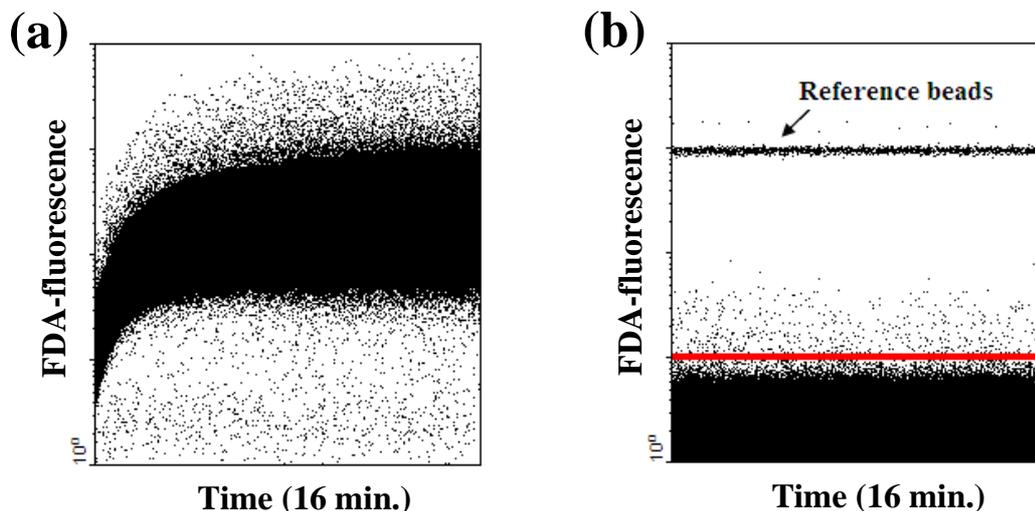


**Figure 8.3.** FDA uptake and hydrolysis kinetics dot-plots (with plotted mean fluorescence kinetics line) for FCM staining protocol optimisation of *C. vulgaris*: (a) 2.4 $\mu\text{M}$ ; (b) 12 $\mu\text{M}$ ; (c) 24 $\mu\text{M}$  and (d) 36 $\mu\text{M}$ .

Following these initial trials, it was found that maximal FDA (fluorescein)-fluorescence yield was obtained for both algal species at a final FDA concentration of 24 $\mu\text{M}$  (Figure 8.3c). Although 12 $\mu\text{M}$  FDA returned similar results to 24 $\mu\text{M}$ , it was decided that the higher FDA concentration was most appropriate in order to ensure that optimal substrate

concentration was maintained for the duration of the FCM sample analysis and to prevent any possible FDA substrate-limitation.

Live:dead staining of *C. vulgaris* with 24 $\mu$ M FDA revealed good ( $\approx 2\text{-log}_{10}$  units) analytical separation between live (positive control) and heat-killed (negative control) algal cells; allowing the ‘live’ and ‘dead’ fluorescence limits to be clearly defined. This can be seen most easily in Figure 8.4 below. Cytometer instrument settings were adjusted so that dead cells displayed peak FDA fluorescence at or below the first decade ‘cut-off’ on the log-fluorescence scale. This meant that any cells in subsequent analyses recording an FDA-fluorescence higher than this pre-determined lower non-viable ‘cut-off’ fluorescence value (i.e.  $10^1$ ) could be classified as FDA-positive ‘viable’.

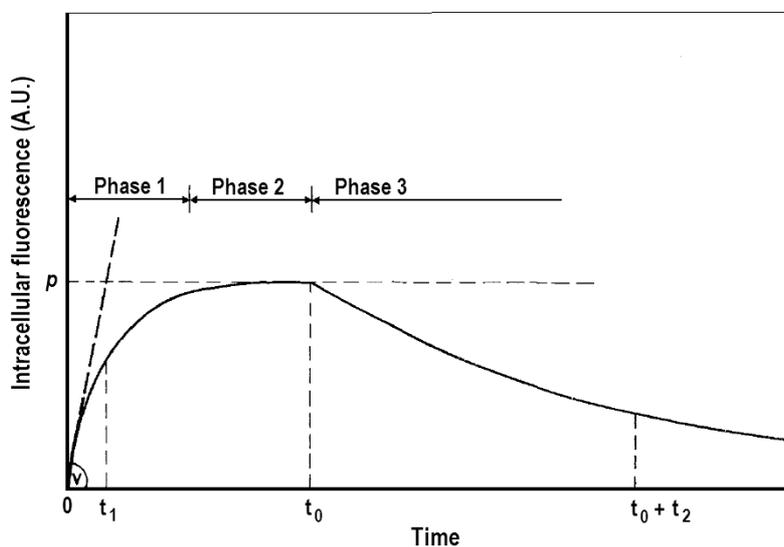


**Figure 8.4.** (a) Live *C. vulgaris* FDA (24 $\mu$ M) hydrolysis kinetics and fluorescence yield, y-axis shows relative FDA (fluorescein) fluorescence yield ( $n = 8 \times 10^4$  cells). (b) Heat-killed negative control *C. vulgaris* FDA (24 $\mu$ M) hydrolysis kinetics and fluorescence yield, y-axis shows relative FDA fluorescence yield ( $n = 3 \times 10^5$  cells). NB. >99.9% of algal cells in Figure 8.4(b) are below the solid  $10^1$  fluorescence cut-off line.

Visual analysis of the hydrolysis and fluorescence kinetics plots from Figure 8.3 showed that substrate FDA diffused quickly into algal cells where it was rapidly hydrolysed to the fluorescent product fluorescein. This observation was supported by the work of Breeuwer *et al.* (1995), whereby FDA is generally assumed to diffuse freely into intact healthy cells. Fluorescence kinetics plots also revealed that an optimal FDA-fluorescence yield was observed after approximately 8–10 minutes staining duration, and that this fluorescence yield was stable up to a total of approximately 16 minutes

staining incubation (and most likely in excess of 20 minutes). This approximate 8–10 minute peak timing for maximum FDA-fluorescence yield is similar to the 8 minute optimum interval of Dorsey *et al.* (1989) for metabolic studies involving FDA viability assessment of marine phytoplankton. In addition, staining with 24 $\mu$ M FDA resulted in higher geometric mean FDA-fluorescence yields for both *C. vulgaris* and *C. reinhardtii* than was seen for all other FDA concentrations trialed.

In order to perform a more detailed and critical analysis of the FDA uptake and hydrolysis kinetics plots from Figure 8.3 above, a detailed knowledge of the kinetics of FDA staining was sourced from Sengbusch *et al.* (1976) (shown below in Figure 8.5).



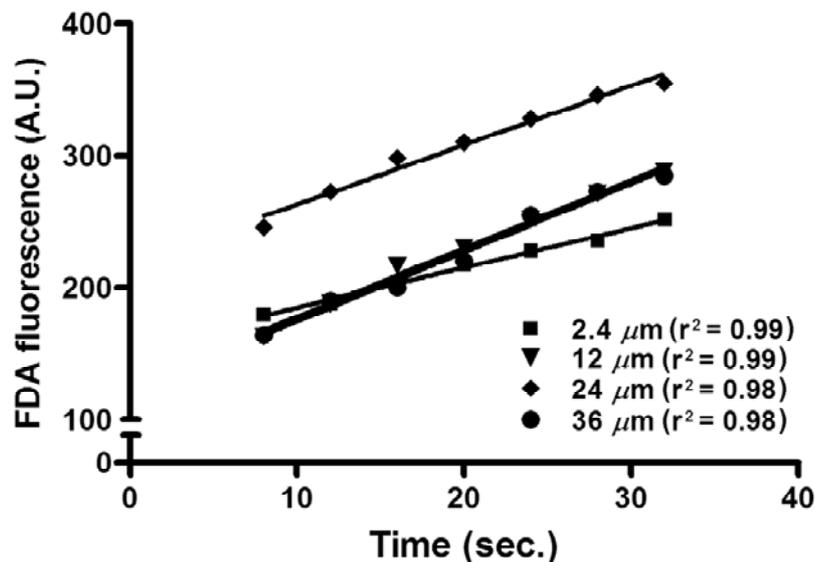
**Figure 8.5.** Parameters describing the time dependence of intracellular FDA hydrolysis:  $v$  = initial velocity,  $t_1$ ,  $t_2$  = relaxation times,  $\rho$  = plateau level, or the time at which the extracellular substrate concentration gradient is made zero (modified from Sengbusch *et al.*, 1976).

The typical kinetics curve of intracellular FDA hydrolysis shown in Figure 8.5 can be described according to three discrete Phases:

1. the initial Phase 1; which is characterised by a linear first-order increase in the intracellular fluorescein concentration with a mean initial rate ( $v$ ) and an exponential approach to an equilibrium concentration with a time constant  $t_1$ ;
2. the equilibrium Phase 2; in which the intracellular fluorescein concentration maintains a constant level ( $\rho$ ); and

- the ‘efflux’ Phase 3; at time =  $t_0$  the substrate FDA concentration is exhausted such that an exponential decrease in the intracellular concentration of fluorescein is seen.

Critical assessment of the initial (linear) part of the ‘Phase 1’ kinetics lines for each individual FDA hydrolysis curve at all of the tested concentrations (2.4, 12, 24 and  $36\mu\text{M}$ ; Figure 8.3) was conducted (only data for *C. vulgaris* is shown). It was deduced from these analyses that the initial part of Phase 1 FDA hydrolysis kinetics curve was indeed linear (and hence non-substrate-limited) at all tested concentrations (Figure 8.6).



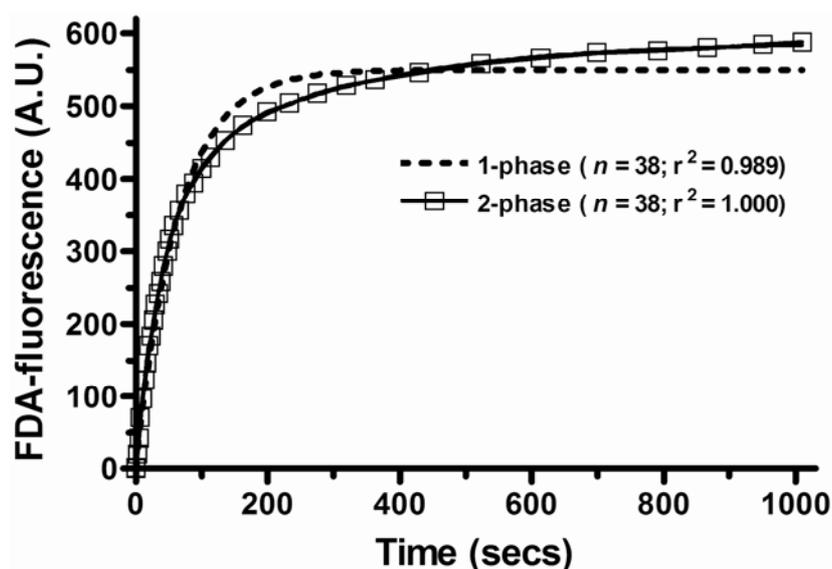
**Figure 8.6.** Graphical plot of the initial linear portion of the Phase 1 FDA hydrolysis curve for *C. vulgaris* at: 2.4; 12; 24; and  $36\mu\text{M}$ ; with associated linear regression ( $r^2$ ) coefficients given in parentheses.

Also assessed was the difference between the slopes of the initial first-order portion of the Phase 1 kinetics curves from Figure 8.6. Analysis of covariance (ANCOVA) was used to test for differences, and following this it was found that the slope of the linear portion of the Phase 1 curve at  $2.4\mu\text{M}$  FDA was significantly lower than the remaining higher concentrations (ANCOVA;  $F_{(3,20)} = 19.04$ ;  $p < 0.0001$ )—indicating substrate-limitation at low FDA concentrations. For the remaining three FDA concentrations (12, 24 and  $36\mu\text{M}$ ), analyses showed that the slopes of the linear portion of the Phase 1 hydrolysis curves (Figure 8.6) were not significantly different (ANCOVA;  $F_{(2,15)} = 2.32$ ;  $p = 0.133$ ). This ensured that initial Phase 1 FDA hydrolysis was indeed linear (and non-

limited) at 12, 24 and 36 $\mu$ M, and also that there were no significant differences between the slopes of this initial linear phase of the overall hydrolysis curve. Because there were no differences between these slopes, it can be assumed that FDA uptake and hydrolysis for *C. vulgaris* (*C. reinhardtii* was assumed to have behaved similarly) is saturated at  $\geq 12\mu$ M. This agrees well with the reported maximum water solubility for FDA of approximately 10 $\mu$ M (Breeuwer *et al.*, 1995), suggesting that concentrations above approximately 12 $\mu$ M FDA, whilst they will not necessarily yield any increased cellular fluorescence, may guard against substrate-limited hydrolysis as was observed at lower (2.4 $\mu$ M) FDA concentrations.

Based on the above, it was concluded that both cellular uptake and subsequent hydrolysis of FDA was neither rate- nor substrate-limited at the optimal concentration of 24 $\mu$ M. Interestingly, the optimal FDA staining protocol adopted here was similar to that of Franklin *et al.* (2001b) who reported an optimum FDA concentration of 25 $\mu$ M for 10 minutes staining duration for algal (*Chlorella*) viability assessment following copper toxicity. It was also similar to that of Brookes *et al.* (2000) who adopted an FDA staining protocol for cyanobacterial (*Microcystis*) viability assessment of 40 $\mu$ M for 7–10 minutes staining duration.

The hydrolysis of FDA has been shown—under optimum substrate concentrations and usually only for a finite incubation period—to follow first-order reaction kinetics in yeast (Breeuwer *et al.*, 1995), mammalian cells (Sengbusch *et al.*, 1976) and some phytoplankton (Gilbert *et al.*, 1992; Franklin *et al.*, 2001a). Optimal (24 $\mu$ M) FDA uptake and hydrolysis kinetics presented here could most accurately be explained by a two-phase exponential association (Figure 8.7), suggesting first-order-type exponential FDA hydrolysis kinetics over the  $\approx 16$  minute staining incubation timescale.



**Figure 8.7.** FDA ( $24\mu\text{M}$ ) hydrolysis and fluorescein-fluorescence kinetics for *C. vulgaris* (fitted curves are derived from single- and two-phase exponential associations).

Other researchers have highlighted problems associated with a rapid (5 minute) loss of algal cell FDA-fluorescence following initial staining incubation (Jochem, 1999); presumably due to fluorescein efflux and/or substrate limitation. As can be clearly seen in Figure 8.7, this trend was not observed at the optimum  $24\mu\text{M}$  final FDA concentration; instead, an extended Phase 2 fluorescence ‘plateau’ was observed, with no apparent active cellular efflux of hydrolysed fluorescein evident over the 16 minute timescale. Following this observation, later experimental samples were always analysed within a maximum of 20 minutes following initial staining in order to further guard against potential long-term product efflux and cellular fluorescence losses.

The above observation also ties in with the reporting of Breeuwer *et al.* (1995), who concluded that the rate of active fluorescein efflux in yeast (*Saccharomyces cerevisiae*) cells was significantly lower than the rate of passive uptake and enzymatic hydrolysis. This suggests that both the overall *rate* and also the gross *accumulation* of the hydrolysed fluorescent product are not significantly influenced by active cellular efflux of fluorescein (at non-limiting substrate concentrations). Interestingly, this trend for prolonged ( $\approx 20$  minute) and stable fluorescein fluorescence emission during staining incubation—as observed during this research—is unlike that reported elsewhere (Sengbusch *et al.*, 1976; Prospero *et al.*, 1986). Jochem (1999) also reported cellular



leakage or efflux of cleaved fluorescein in the green alga *Brachiomonas submarina*, with a subsequent and large-scale loss of cellular FDA-fluorescence yield being observed for that alga after just 4 minutes of staining incubation.

According to the *Current Protocols in Cytometry* (Robinson *et al.*, 2003), the FDA assay works well in some instances, but the rate at which the generated fluorescein diffuses out of cells varies greatly. The importance of critical assessment of FDA staining kinetics was highlighted most eloquently, however, by Breeuwer *et al.* (1995). Breeuwer and co-workers cautioned that if one had a limiting concentration of FDA during the staining procedure, then a situation may occur where there is a rate-limited FDA uptake due to low concentration gradient for passive diffusion of FDA into cells; given that FDA transport into cells most likely occurs via passive diffusion. Additionally, and recognising that there is an active efflux (pumping) of the hydrolysed fluorescein product out of algal cells (Sengbusch *et al.*, 1976; Prosperi *et al.*, 1986; Bunthof *et al.*, 1999), there may also be a more significant cellular loss of fluorescein due to the lower initial *rate* and *mass* of intracellular fluorescein accumulation resulting from the initially substrate-limited uptake. This is in essence reflected in Figure 8.3(a), where it appears that cellular fluorescein fluorescence is either substrate (FDA)-limited after approximately 5 minutes staining duration, or, that the rate of fluorescein efflux (loss) from the cells becomes greater than the rate of FDA hydrolysis. More than likely it is a combination of both of these factors (i.e. a truncated plateau Phase 2 has been reached early due to the reduced 'substrate-limited' FDA hydrolysis rate, after which time the Phase 3 efflux rate is greater than the hydrolysis rate of the now low concentrations of residual FDA); the ultimate result being insufficient accumulation and/or retention of fluorescein as well as subsequent and undesirable loss of cellular fluorescence when cells are incubated at the lowest ( $2.4\mu\text{M}$ ) FDA concentration.

A similarly undesirable and apparent 'over-staining' of *C. vulgaris* cells at an FDA concentration of  $36\mu\text{M}$  was also observed (Figure 8.3d). Breeuwer *et al.* (1995, p. 1615) reported a decrease in the rate of FDA hydrolysis at high FDA concentrations due to a possible "*precipitation of FDA, resulting in lower concentrations of free (substrate) FDA*". This 'substrate precipitation' could possibly account for the sub-optimal cell staining and fluorescence yields at the highest ( $36\mu\text{M}$ ) FDA concentrations (Figure

8.3d). Another possible artefact of this apparent over-staining is a phenomenon known as ‘fluorescence quenching’. This ‘quenching’ effect is a process which decreases the intensity of the fluorescence emission of a fluorophore and is known to occur when intracellular concentrations of fluorescein are too elevated (Waggoner, 1990; Breeuwer *et al.*, 1995).

Another possible explanation for the low yield fluorescein fluorescence at the high ( $36\mu\text{M}$ ) FDA concentration could be that relating to ‘product inhibition’ (discussed by Sengbusch *et al.*, 1976). It is possible that some form of ‘product inhibition’ (i.e. negative feedback) resulting from high concentrations of rapidly accumulated fluorescein product, may have also resulted in a reduced cellular FDA-fluorescence at elevated substrate concentrations. As described by Sengbusch *et al.* (1976), a higher substrate FDA concentration leads to an accelerated initial rate of intracellular fluorescein accumulation. This heightened accumulation of fluorescent product then slows down the hydrolysis rate of new substrate FDA by “product-inhibition” of intracellular enzyme (esterase) activity. As a result, the equilibrium phase (Phase 2 of Figure 8.5) is reached earlier than would normally be expected if there were no ‘negative feedback’ product-inhibition and, as such, the rate of fluorescein efflux (Phase 3) is now—in proportion to the rate of accumulation (Phase 1)—far greater than that in product-uninhibited cells. The overall result of this product-inhibition is a lower intensity ‘equilibrium phase’ cellular fluorescence, and also a likely accelerated onset of the Phase 3 net loss of intracellular fluorescence; trends that were both apparent in Figure 8.3(d).

Regardless of the mechanisms involved (be they substrate-limitation, fluorescence quenching or substrate precipitation) it seems that the ultimate result of ‘too-high’ and ‘too-low’ a concentration of FDA was a reduction in the recorded cellular fluorescence signal (as seen in both Figures 8.3a and 8.3d). It should be noted, however, that the most optimal FDA concentration of  $24\mu\text{M}$  reported here is most likely dependent on the incubation temperature, sample pH, as well as the particular algal species used during the viability assay. The author strongly recommends at this point, that individual staining optimisations should always be performed prior to the adoption and

implementation of staining protocols for FCM analyses, even if the protocols being adopted are from identical species.

### **8.2.2.2 Effect of culture growth phase, population cell density, and pH on staining protocol optimisation**

Prosperi *et al* (1986) reported that cellular metabolic state not only influences initial FDA hydrolysis, but also the energy-dependent efflux of hydrolysed fluorescein. Considering this, it is of considerable importance that during initial staining optimisation procedures one tries as much as possible to mimic the cell's physiological status as close to what it may actually be '*in vivo*' during the subsequent experimentation. It can be appreciated that it is less practical to optimise staining conditions on exponentially growing cells when the final experiment involves the staining and analysis of static, ageing cultures. This point was echoed by the earlier findings of Bentley-Mowat (1982, p. 204), who stated that the strength of cellular fluorescence following FDA staining was linked to "the metabolic vigour of the cells". Bentley-Mowat observed that rapidly dividing algal cells displayed an intense fluorescence, whereas static cultures showed a feeble reaction with a marked reduction in fluorescein fluorescence yields. The fact that the research presented here utilised early stationary-phase cells for the initial PI-FDA staining optimisations (and not exponentially growing 'hyperactive' cultures) means that there should be a reduced likelihood of observing vastly different FDA uptake, hydrolysis, and fluorescence kinetics in the final prolonged dark-survival experiments. In other words, the use of 8 day old cultures for pre-determination of the optimal FDA staining incubation time (i.e. 8–10 min) should be more relevant to the staining kinetics of the longer-term statically ageing cultures encountered during later experimentation than if 'day 3' exponential-phase cells were used during these initial staining optimisations.

It has been emphasized elsewhere that optimum staining protocols can vary not only according to species, but also with changes in the population cell density of a given algal sample (Grégori *et al.*, 2002). Franklin *et al.* (2001a) looked at the effects of algal (*Chlorella*) culture cell density on FDA uptake and hydrolysis kinetics and found that the amount of fluorescein accumulated per cell increased with decreasing cell density

( $10^2$ – $10^5$  cells  $\text{ml}^{-1}$ ). This potential staining artefact was offset during the current analyses by using relatively high, non-limiting FDA concentrations ( $24\mu\text{M}$ ) as well as standard cell densities ( $\approx 1 \times 10^6$  cells  $\text{ml}^{-1}$ ) for all staining optimisations and during all experimental analyses. The possible effects of relatively small variations in population cell density on the overall efficacy of the FDA viability assay (and the implied phytoplankton metabolic activities) are, therefore, not considered during the Chapter 9 presentation of long-term dark-survival experiments.

Brookes *et al.* (2000), regarding the FDA assay, stressed that the pH needs to be in the range of 5–8 and also highlighted the ‘ageing culture’ effect on elevating pH; with the authors reporting on a significant loss of fluorescein fluorescence at  $\text{pH} > 9$  for a cyanobacterial (*Microcystis*) FDA assay. Others have quoted that optimal intracellular pH in yeast was 7.0 (Breeuwer *et al.*, 1995) and that maximal fluorescein fluorescence was observed at  $\text{pH}$  7.8–8.0 for the phytoplankton *Selenastrum capricornutum* (Franklin *et al.*, 2001a). Early work by Guilbault and Kramer (1966) reported that fluorescein exhibits a maximal fluorescence yield at  $\text{pH}$  8.0 and that the maximal rate of enzymatic hydrolysis occurs at  $\text{pH}$  7.0. Having stated this, however, they went on to report that the greatest sensitivity and reproducibility in kinetics measurements was achieved at  $\text{pH}$  8.0 in buffered media. Guilbault and Kramer (1966) also reported that “spontaneous hydrolysis” of esterified FDA occurred at elevated temperature (30–40°C) and  $\text{pH}$  (8.5). Consequently, all staining manipulations carried out as part of this dark-survival research were performed at room temperature ( $\approx 20$ – $24^\circ\text{C}$ ) and at a  $\text{pH}$  no greater than 8.0 (most commonly 7.60; see Section 7.2.3.2.3).

### **8.2.2.3 Instrument drift, internal standards and data transformation**

The inclusion of internal light scatter and fluorescence standards (in the form of standard cytometry beads) allows for identification, quantitation, and then correction of any instrument drift between non-concurrent analyses, and also enables standardisation of cellular fluorescence within a given data set (Marie *et al.*, 2005).  $2.5\mu\text{m}$  PeakFlow™ green fluorescent beads were added to all samples as required for internal fluorescence calibration and standardisation (see Section 7.2.3.2.2). Given that the FACScan cytometer was calibrated on a weekly basis, and considering that the same instrument

settings were used for *all* FCM analysis, it follows that only a very small daily shifts in standard bead fluorescence yield were recorded over both the 7 and 65 day dark-survival experiments. Daily measured parameter CVs for SSC-, FSC-height and FL1 (green fluorescence) from the standard beads were always between 0.59 and 3.8% for the 65 day experiments and between 0.44 and 1.2% for the final 7 day dark-survival experiment. Long-term instrument ‘drift’ never exceeded 10% for a given set of samples, with the long-term daily mean percentage drift (relative to 65 day mean for each parameter) well below 1% ( $\pm 4.4\%$ ). Further statistical analysis of the standard bead data revealed no significant long-term variations among either the measured bead SSC- or FSC-height or FL1 fluorescence (1-way RM-ANOVA; SSC,  $F_{(3,7)} = 0.770$ ;  $p = 0.5234$ ; FSC,  $F_{(3,7)} = 0.875$ ;  $p = 0.470$ ; FL1,  $F_{(3,7)} = 2.12$ ;  $p = 0.141$ ).

Furthermore, and even though there were likely to have been relatively small day-to-day variations in cytometer fluorescence and/or light scatter measurements, it is reasonable to assume that these small instrument drifts would have been applied ‘across the board’ for all samples measured on any given day, such that any small daily instrumental drift would, by default, be applied equally to all data from that particular sampling interval. This is evidenced by the fact that daily pairing (matching) of sample data (within the RM-ANOVA framework) was significantly effective in controlling for any small day-to-day variations in the 65 day light scatter parameters (1-way RM-ANOVA matching; SSC,  $F_{(3,7)} = 67.31$ ;  $p < 0.0001$ ; FSC,  $F_{(3,7)} = 15.33$ ;  $p < 0.0001$ ). This ‘internal standardisation’ of instrument drift is inherently applied by default to all samples measured on any given day, such that the relative differences *between* experimental treatments measured on that day should have remained unaffected. This high degree of instrumental accuracy and analytical reproducibility made it redundant to normalise any of the algal FCM data against the corresponding daily bead light scatter and fluorescence values, since doing so would have resulted in no meaningful change to the acquired data.

Finally, and although Dorsey *et al.* (1989) performed regular (2 hourly intervals) ‘bead calibrations’ of instrument fluorescence signals during experimental runs in order to check for instrument drift, such measures were not carried out here because daily analytical duration during FCM analyses rarely exceeded two hours. Instead, the above *post hoc* validations of the levels of variability within multiparameter measurements of

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standard fluorescent beads (analysed during the course of all sampling intervals) were performed. In this sense, and after all of the data was acquired and collated, significant daily drifts in instrument measurements (for physical light scatter and fluorescence properties) could then be identified, and, where required, the necessary correction factors applied to daily experimental data. As discussed above, however, such correction factors were never required.

## **9 Phytoplankton survival during prolonged darkness under conditions of ambient and reduced dissolved oxygen—results and discussion**

In a similar context to the definition of Anita (1976), the term “dark-survival”, as used extensively throughout this Chapter, refers explicitly to the retention of algal cell viability without growth (i.e. without significant increase in cellular mass or numbers) during prolonged exposure to darkness. Implicit in this definition is the denial of any recognised possibility of growth, either autotrophically with light or heterotrophically through assimilation of a suitable organic carbon source, since both of these ‘substrates’ were effectively absent during these experiments. A more thorough discussion of issues surrounding this ‘assumption of absence’ of both light and organic carbon substrates for phytoplankton growth will be provided within the coming Sections. Also, and as introduced in Chapter 6 (Section 6.3), experimental results will be discussed with reference to relevant published results from algal dark-survival investigations conducted over a period of no less than 3 days and will generally exclude cyanobacterial studies except where due relevance is identified. For clarity and ease of interpretation, and as was the case during the previous experimental Chapters 3, 4 and 5, results will be discussed in context as they appear, followed later by a more general discussion of some relevant factors in the context of the work at large. This will then be followed by a final presentation of the overall research findings at the Chapter’s end.

### **9.1 Validation of experimental design**

It is well known that prior light climate can influence dark respiration (Hellebust and Terborgh, 1967; Yallop, 1982), chlorophyll *a* concentration (Veldhuis and Kraay, 2000), dark-growth, as well as dark-survival (Karlander and Krauss, 1966b) in phytoplankton. Bearing this in mind, experimental algal culture inocula used for dark-survival assessments were not gradually ‘shade-adapted’ as others have done (Griffis and Chapman, 1988), with the goal being to avoid any ‘dark-conditioning’ of cells prior to the long-term dark-exposure experiment (see also Section 7.2.1). Further justification for sudden dark-exposure is also found in the work of Palmisano and Sullivan (1983), wherein long-term (five month) dark-survival of marine polar diatoms was found to be generally enhanced by the preconditioning of cells through a simulated ‘summer–winter’

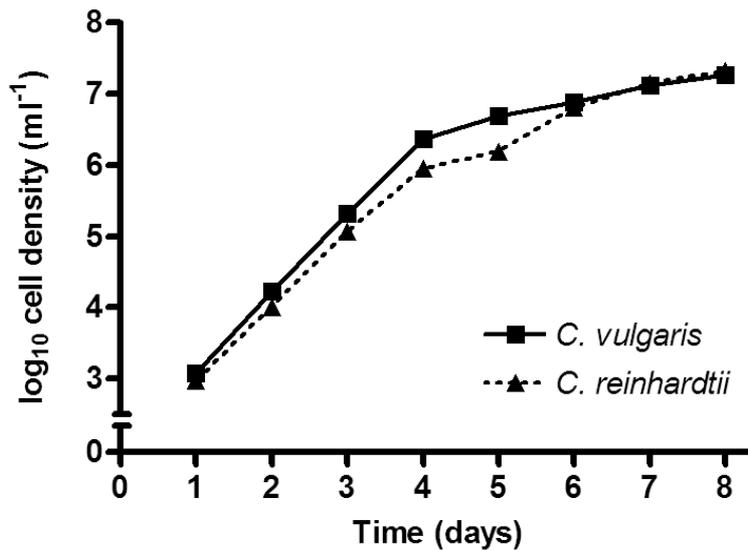
transitional gradient (i.e. decreasing light and temperature). In addition to this, sudden darkening of cultures was deliberately performed here in order to replicate the instantaneous dark transition that phytoplankton would be expected to be subjected to whilst traversing an advanced in-pond upgrade such as an *in situ* rock filter or dense duckweed cover (ignoring the fact that algal cells would already be exposed to an *in situ* sinusoidal light:dark photoperiod within the WSP environment).

During the present dark-survival experiments, and in line with the assumption of Griffis and Chapman (1988) as part of their long-term (10 week) phytoplankton (diatom, dinoflagellate, coccolithophore) viability experiments, dissolved inorganic nutrients were not considered to be a limiting factor for long-term dark-survival. Since algal stock cultures were re-inoculated at ‘Day zero’ into fresh, nutrient-replete MBL growth medium (see Section 7.2.1), it was assumed that there were sufficient nutrients to not impose any limitation upon the survival (indeed growth) potential of the experimental phytoplankton cultures. Further validation for the use of nutrient-replete experimental cultures is found within Ferris and Christian (1991), who stated that nutrient-replete cells grown under controlled culture conditions tend to be more ‘physiologically endowed’ than those in natural populations, and therefore are presumably in the best possible physiological state to be able to respond to environmental changes (such as sudden and prolonged darkness in this instance). Following this, it was assumed that the measured dark-survival potentials would most likely reflect the maximal rates of survival possible for both *C. vulgaris* and *C. reinhardtii* under the manipulated environmental conditions.

## **9.2 Algal stock culture maintenance, standard growth curves and growth rates**

Algal stocks were maintained as previously described (Section 7.1). A typical 8 day culture growth curve for each species is shown here in Figure 9.1.





**Figure 9.1.** Standard 8 day algal growth curves for *C. vulgaris* and *C. reinhardtii* (individual data points are the result of duplicate determinations).

Standard growth curves for both algal species revealed that following routine sub-culturing, algal stock cultures approached stationary growth phase after approximately 8 days. Average daily culture growth rates ( $r_n$ ; Equation 7.1) for *C. vulgaris* and *C. reinhardtii* over the 8 days were 1.09 and 1.13 respectively; equating to mean 8 day culture doubling times of 1.44 and 1.10 days for *C. vulgaris* and *C. reinhardtii* respectively. Day zero cell densities cannot be shown in Figure 9.1 as they were below the accurate detection limit of the FCM counting method.

### 9.3 Effect of culture growth phase on dark-survival

Although not investigated, it was considered unlikely that culture growth phase at the time of initial dark-incubation would have had a significant impact on overall long-term phytoplankton dark-survivorship. For the current research, Day 8, late exponential- to early stationary-phase cultures of *C. vulgaris* and *C. reinhardtii* (Day 8  $r_n$  values of 0.33 ( $\pm 0.05$ ) and 0.40 ( $\pm 0.03$ )  $d^{-1}$  respectively) were used for dark-survival experiments. Although the use of exponential-phase cultures is commonplace in phytoplankton research in general and could arguably have been more ideal for this research, the need for high ( $10^6$  cells  $ml^{-1}$ ) starting cell densities (see Section 7.2.1) precluded their use here. Also, due to the large total volume of experimental cultures required for a single experimental run (i.e. 5 L per species), it was necessary to have a highly concentrated

culture stock in order to achieve the desired ‘Day zero’ cell densities of  $10^6$  ml<sup>-1</sup>; something not achievable using exponential-phase inocula.

Interestingly, Montaini *et al.* (1995) proposed that cells in the exponential-phase of culture growth have weaker (thinner) cell membranes than do stationary-phase cells. Montaini *et al.* (1995) also suggested that their observation of increased tolerance to freezing in *Tetraselmis suecica* (Prasinophyceae)—something observed originally by Fenwick and Day (1992)—was potentially attributable to the use of more ‘robust’ late exponential or stationary-phase cells. In the context of the current research, the use of non-exponentially-growing early stationary-phase cells could arguably be more representative of WSP phytoplankton populations, given the ‘continuous-culture’ type situation *in situ* combined with an almost certainly less optimal pond growth environment. At the same time, the fact that non-exponentially-growing cells were used for the current dark-survival experiments could imply that the algal cells were in a ‘less-than-optimal’ physiological state at the start of the dark period; thereby potentially underestimating their *true* dark-survival potential. Indeed, Popels and Hutchins (2002) reported that dark-incubated exponential cultures of *Aureococcus anophagefferens* (Pelagophyceae) recovered more quickly upon re-illumination than did stationary-phase cells; however, the influence of growth phase was deemed by Popels and Hutchins to have had only a relatively minor effect on algal dark-survival and recovery post-darkness, with even late stationary-phase cultures capable of re-growth post-darkness—albeit somewhat more slowly.

Following this, it is suggested that the use of non-exponential-phase algal cultures for the dark-survival experiments reported here would have—at best—had a negligible impact on the long-term dark survival outcomes reported herein. Furthermore, the fact that early stationary-phase cultures were used for these experiments could imply that the dark-survival (and post-dark re-growth) capabilities of *C. vulgaris* and *C. reinhardtii* measured here would be likely to represent a ‘worst case scenario’ in terms of what may indeed be possible for algal cells from a more optimal growth phase. It should also be noted that although the population cell density used for this research was quite high, it was by no means impossibly high in the context of the Bolivar WSP environment. For example, peak Bolivar WSP effluent chlorophyll *a* concentrations during the course of

the 2005 monitoring data presented in Chapter 3 were between 2–4 times greater than the ‘Day zero’ chlorophyll *a* levels here, and peak *Chlorella* cell densities recorded in the Bolivar pond effluent during May of 2005 were  $4 \times 10^5$  cells ml<sup>-1</sup> (data not shown). Based on this, the adoption of high initial population cell densities was considered not to be unrepresentative of *in situ* WSP conditions, such that any effects stemming from this high starting cell density were deemed to have had no significant bearing on the measured dark-survival potential of both *C. vulgaris* and *C. reinhardtii*.

#### **9.4 Phytoplankton dark-survival kinetics: 65 versus 7 day investigations**

The dark-survival capabilities of both *C. vulgaris* and *C. reinhardtii* under the current experimental conditions were first assessed with respect to their experimental reproducibility within the laboratory. Long-term dark-survival results presented in this Chapter come from the second of duplicate two month experimental runs that produced very similar, if not identical results. Due to space limitations, and at the risk of further complicating an already complex data set, dark-survival results from the initial two month experiment are not presented. Given that results from the duplicate 65 day dark-survival experiments for both *C. vulgaris* and *C. reinhardtii* revealed a very similar capacity for dark-survivorship for both species, a further and more detailed ‘7 day’ dark-survival investigation was performed (using *C. vulgaris* only) in order to further distill the kinetics of cellular dark-acclimation between ‘Day zero’ and the first sampling day (i.e. Day 7) of the two month experiment. As described previously (Section 7.2.2), this 7 day dark incubation experiment was run for a period of one week, with sampling intervals on days 0, 2, 4, and 7.

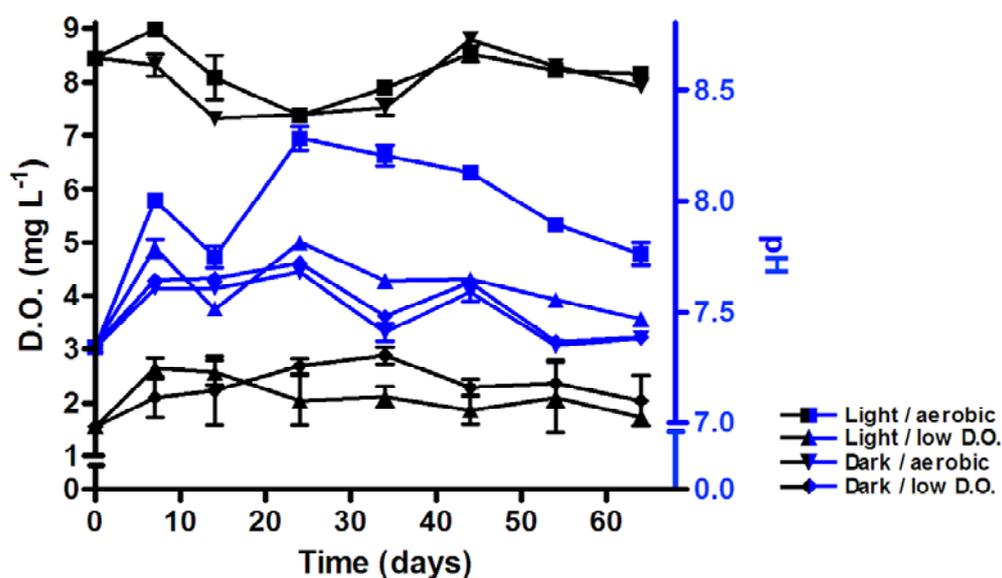
Filling in the gaps between ‘Day zero’ and ‘Day 7’ from the initial 65 day experiment, whilst scientifically desirable, also had practical relevance with respect to advanced in-pond upgrade systems. This 7 day timescale would be of particular practical significance with respect to the approximate hydraulic retention time of a rock filter or duckweed pond system *in situ*, and so it is suggested that results from this more detailed short-term investigation may provide for a more detailed understanding of the kinetics of algal dark-acclimation and survival within these particular environments. Results from both

the long-term two month dark-survival experiment and the secondary 7 day experiment are presented sequentially within their relevant results Sections, with necessary cross-references made between the two experimental runs where appropriate.

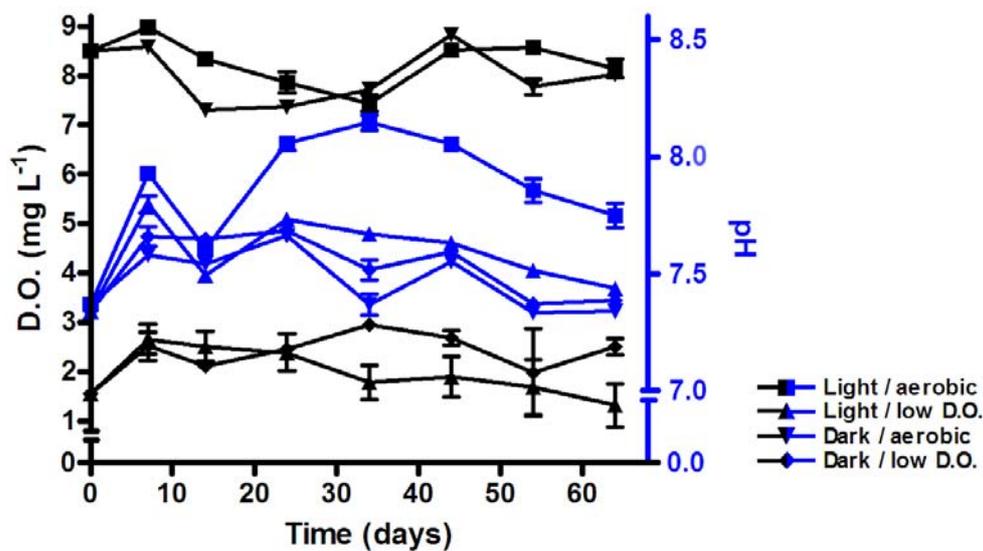
## 9.5 Prolonged darkness and water quality: dissolved oxygen; pH; and dissolved inorganic carbon

### 9.5.1 Results from the two month dark-survival experiment

Aqueous DO and pH were monitored during the course of the 65 day dark-incubation experiments and are shown here in Figure 9.2 for *C. vulgaris* and 9.3 for *C. reinhardtii*.



**Figure 9.2.** Aqueous dissolved oxygen and pH for *C. vulgaris* over the 65 day experiment for all 4 treatments: *x*-axis represents the experimental duration (days); left *y*-axis (in black) shows dissolved oxygen concentration (mg L<sup>-1</sup>); and the right *y*-axis (in blue) depicts aqueous pH. Data points shown mean values  $\pm$  1 S.D from triplicate treatment cultures.



**Figure 9.3.** Aqueous dissolved oxygen and pH for *C. reinhardtii* over the 65 day experiment for all 4 treatments: x-axis represents the experimental duration (days); left y-axis (in black) shows dissolved oxygen concentration (mg L<sup>-1</sup>); and the right y-axis (in blue) depicts aqueous pH. Data points shown mean values  $\pm$  1 S.D from triplicate treatment cultures.

Dissolved oxygen levels in both ‘aerobic’ and ‘low D.O.’ treatments remained relatively stable over the 65 day experimental duration for *C. vulgaris*, with no significant differences between the 65 day light and dark ‘aerobic’ and light and dark ‘low D.O.’ treatment concentrations (1-way RM-ANOVA;  $F_{(3,7)} = 439.3$ ;  $p > 0.05$ ). Similarly, for *C. reinhardtii* there were also no significant differences between the 65 day DO concentration in light and dark ‘aerobic’ and light and dark ‘low D.O.’ treatments (1-way RM-ANOVA;  $F_{(3,7)} = 416.1$ ;  $p > 0.05$ ). This essentially meant that regardless experimental light climate, both the ‘light’ and ‘dark’ treatments were effectively subjected to the same DO concentration according to their specific DO regime. Results showed that there was also no significant change in the ‘Day 65’ DO concentration compared with the ‘Day zero’ concentration in ‘low D.O.’ treatments for both *C. vulgaris* and *C. reinhardtii* (Kruskal–Wallis test;  $\chi^2_{(0.05,4)} = 10.23$ ;  $p > 0.05$ ); suggesting that no significant re-oxygenation (photosynthetic or atmospheric) of the ‘low D.O.’ treatment flasks took place over the two month experimental duration.

This lack of photosynthetic re-oxygenation in the ‘light / low D.O.’ treatment was a curious observation, and could to some extent be potentially accounted for by the fact that O<sub>2</sub> production rate (per unit biomass) of algal (*Chlorella*) cultures has been reported

to be lowest during stationary growth phase (Bartosh *et al.*, 2002); such that the rate of photosynthetic re-oxygenation would be expected to be at its lowest in the atmospherically-isolated and statically ageing 'light / low D.O.' treatment cultures. It is also possible (although perhaps unlikely) that the oxygen balance within the sealed 'light / low D.O.' treatment flasks was approximately even (i.e. the quantity of evolved oxygen was matched by the algal culture's respiratory oxygen demand, resulting in neither a long-term O<sub>2</sub>-deficit nor surplus). The observed lack of photosynthetic re-aeration was considered more likely to have been a manifestation of the apparent DIC-limitation within the hermetically-sealed 'light / low D.O.' treatment flasks—a consequence of the initial autoclave heat-sterilisation step de-gassing or stripping dissolved CO<sub>2</sub> (and probably also the added HCO<sub>3</sub><sup>-</sup>) from the growth medium (e.g. Jaworski *et al.*, 1981) and then atmospheric isolation preventing normal CO<sub>2</sub> solubilisation from taking place. This would have presumably resulted in a further repression of algal culture photosynthesis and an even greater reduction in its capacity for O<sub>2</sub> evolution. This issue of chronic DIC-limitation in the context of the current research findings will be discussed in more detail later (Section 9.10).

The relatively stable DO concentration in the 'dark / low D.O.' treatments appears also to be a curious observation at first. One might have expected that DO reserves would be gradually exhausted by respiring cells during the two month dark period. There are a number of potential factors that come into play with respect to this maintenance of aerobic conditions within 'dark / low D.O.' treatment flasks, one of which pertains to a generally reduced cellular metabolic rate in darkness, and the other, a dark-mediated interference with normal aerobic respiration. Both of these factors will be discussed more in depth within Sections 9.8 and 9.10.

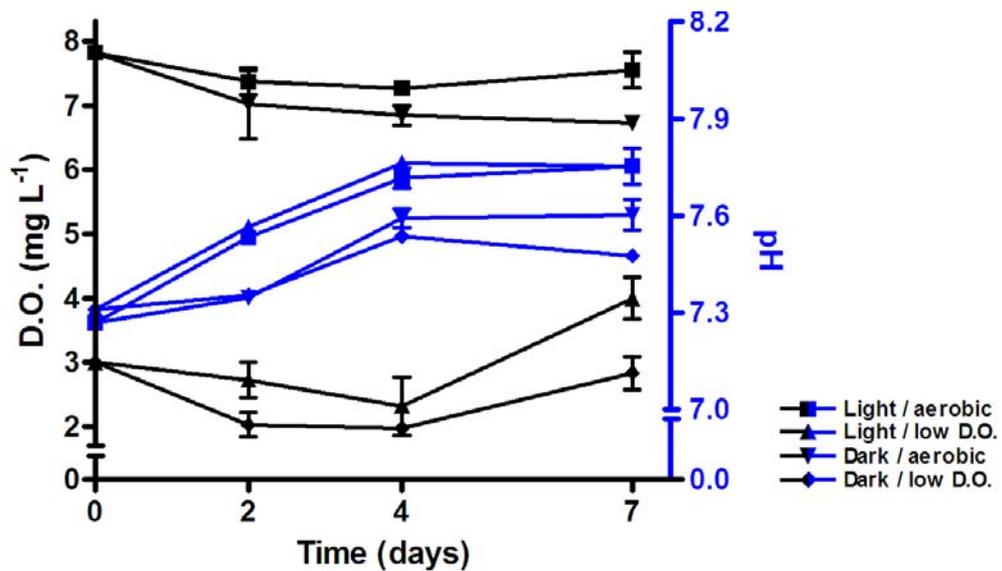
With respect to aqueous pH, and as can be seen in Figures 9.2 and 9.3 above, there were no differences in 65 day culture pH in any treatment for both *C. vulgaris* (1-way RM-ANOVA;  $F_{(3,7)} = 19.85$ ,  $p > 0.05$ ) and *C. reinhardtii* (1-way RM-ANOVA;  $F_{(3,7)} = 15.99$ ,  $p > 0.05$ ) except where the 'light / aerobic' treatment was involved. This observed elevated pH in 'light / aerobic' treatments is a well known feature of statically ageing algal cultures, and although the effects of a slightly elevated pH are assumed to be negligible in the general context of the research presented here, it did have potential

implications for the efficacy of the FDA assay (see Section 6.4.2.4.1). The serial dilution (in pH 7.2 MBL media) of all 'light / aerobic' samples prior to FDA staining analyses, however, meant that potential pH effects were assumed to have been of negligible importance in the context of the current results. Although others have reported relatively large (2 unit) decreases in algal (*Chlorella*) culture pH during 3 day dark-exposure (Karlander and Krauss, 1966a), aqueous pH was stably maintained in the current work through the use of heavily buffered culture medium (see Appendix G).

With respect to DIC, and although relatively high levels of inorganic carbon ( $\text{NaHCO}_3$ ) were added during initial preparation of the MBL culture medium ( $\approx 12\text{mgL}^{-1}$ ), later analysis of the experimental algal cultures showed that they were most likely DIC-limited, or more appropriately, DIC was present at or below the  $0.2\text{--}0.3\text{mgL}^{-1}$  instrumental detection limit (see Section 7.2.3.1). This 'stripping' of added DIC was assumed to be a result of the high pressure autoclave heat-sterilisation process and was unfortunately not discovered until after the commencement of the final long-term 65 day dark-survival experiments. Nevertheless, the absence of significant quantities of DIC did yield some interesting results (particularly for the atmospherically-isolated 'light / low D.O.' treatment) and so, where relevant, issues regarding these results will be discussed in more detail within later Sections.

### **9.5.2 Results from the 7 day dark-survival experiment**

Aqueous DO and pH were again monitored during the course of the 7 day dark-survival experiments, with results shown below in Figure 9.4 for *C. vulgaris*.



**Figure 9.4.** Aqueous dissolved oxygen and pH for *C. vulgaris* over the course of the 7 day experiment for all 4 treatments ( $x$ -axis represents the experimental duration (days); left  $y$ -axis (in black) shows DO concentration ( $\text{mg L}^{-1}$ ); and the right  $y$ -axis (in blue) depicts aqueous pH). Data points shown mean values  $\pm 1$  S.D from triplicate treatment cultures.

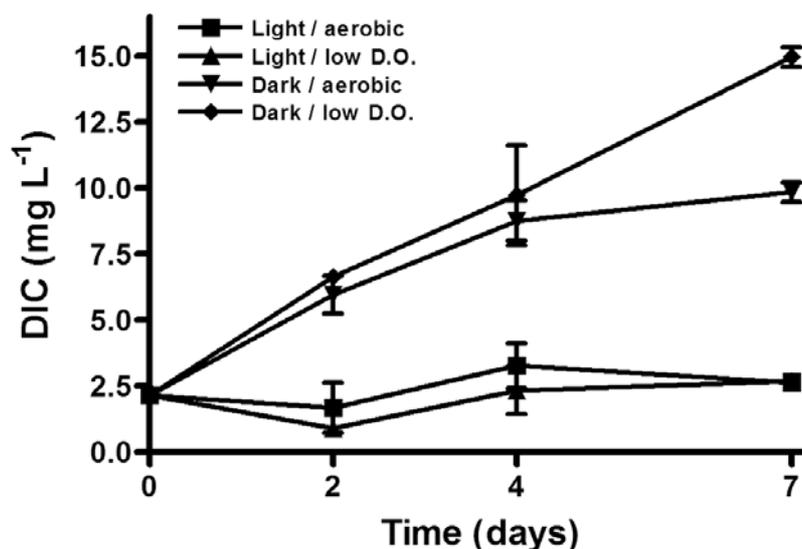
Dissolved oxygen levels in both ‘aerobic’ and ‘low D.O.’ treatments again remained relatively stable over the truncated 7 day experimental duration for *C. vulgaris*, with no significant differences between the overall 7 day light and dark ‘aerobic’ and light and dark ‘low D.O.’ treatment dissolved oxygen concentrations (1-way RM-ANOVA;  $F_{(3,3)} = 167.8$ ;  $p > 0.05$ ). There was also no significant change in the ‘Day 7’ DO concentration compared with the ‘Day zero’ concentration in ‘low D.O.’ treatments for *C. vulgaris* (Kruskal–Wallis test;  $\chi^2_{(0.05,5)} = 15.75$ ;  $p > 0.05$ ); suggesting once again that no significant re-oxygenation of the specifically reduced and hermetically-sealed DO treatments took place over the one week experimental duration. Although not significant, it does appear that some degree of photosynthetic re-oxygenation within the ‘light / low D.O.’ treatment has occurred by Day 7—a trend supported by the accompanying increase in pH. Unlike the two month experimental cultures, the addition of sufficient quantities of DIC in the 7 day incubations (see below) may have allowed for this increased photosynthetic activity in the ‘low D.O.’ treatment flasks.

In respect of aqueous pH, the data of Figure 9.4 suggests that there were no significant differences among the 7 day culture pH in any of the treatments for *C. vulgaris* (1-way



RM-ANOVA;  $F_{(3,3)} = 7.091$ ,  $p > 0.05$ ) except where both ‘light’ treatments were involved. In the current context, this small increase in pH for both ‘light’ treatments was assumed to be related to normal photosynthetic processes and was considered to be of negligible importance in the context of this dark-survival research. There was also no significant change in the ‘Day 7’ culture pH compared with the ‘Day zero’ concentration in ‘dark’ treatments for *C. vulgaris* (Kruskal–Wallis test;  $\chi^2_{0.05,5} = 16.08$ ;  $p > 0.05$ ); however, there was a significant increase in aqueous pH for both ‘light’ treatments ( $p < 0.05$ ), again most likely due to normal photosynthetic activity.

Following the supplemental addition of inorganic carbon ( $\text{NaHCO}_3$ ) at every sampling interval (see Section 7.2.2), DIC was monitored and was found to be generally in the order of  $\geq 2 \text{mg L}^{-1}$  for all treatments over the 7 day experimental duration (Figure 9.5). As can be seen for the ‘dark’ treatments, DIC appeared to accumulate over the course of the 7 day period following its repeated addition at every sampling interval and presumably also from non-photosynthetic consumption during continuous dark-exposure. At these concentrations, DIC was assumed to be non-limiting for ‘light’ treatments based on the very low levels of inorganic carbon reportedly required for saturation of photosynthesis in *Chlorella* (0.1%  $\text{CO}_2$ ; Myers, 1944) and some marine phytoplankton (2.0–2.2  $\text{mmol DIC L}^{-1}$ ; Raven, 1991), as well as the recognised high affinity for DIC possessed by phytoplankton in general (Goldman and Graham, 1981; Raven, 1991).



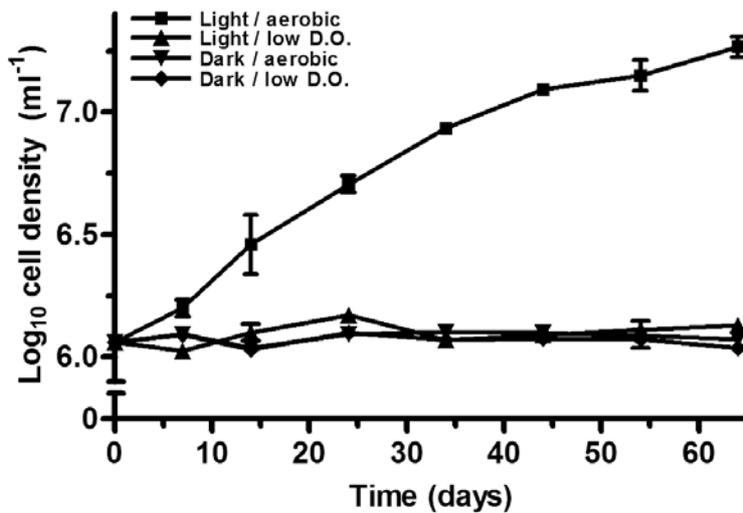
**Figure 9.5.** Aqueous total dissolved inorganic carbon (DIC) levels for *C. vulgaris* over the course of the 7 day experiment for all 4 treatments (x-axis represents individual sampling intervals (days); and the y-axis shows DIC concentration (mg L<sup>-1</sup>). Data points shown mean values  $\pm$  1 S.D from triplicate treatment cultures.

## 9.6 Prolonged darkness: implications for population cell density; cell size; and intracellular density

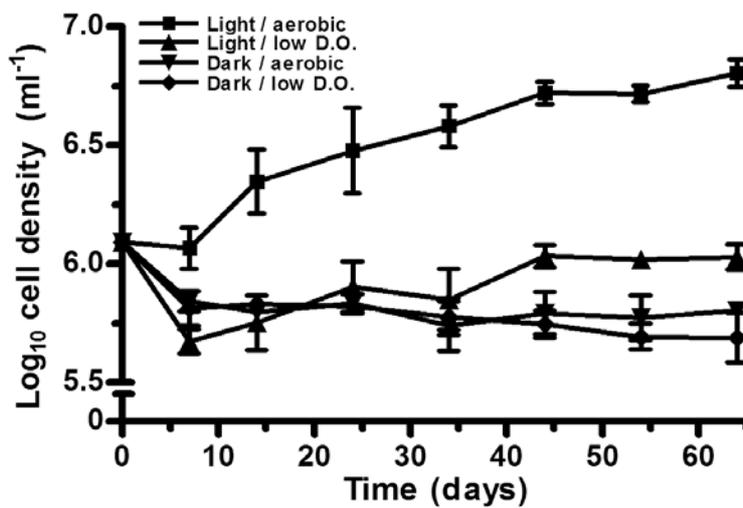
The ability of an algal population to maintain a constant population density during prolonged darkness provides one of the most basic insights into their ability as a population to withstand the particular dark stress event. Other physical population attributes, such as cell size and intracellular density (granularity), serve also to monitor morphological changes during prolonged darkness and can in turn be related to physiological alterations and linked to potential dark-survival strategies. Long-term phytoplankton dark-survivorship was assessed during the course of these experiments through the monitoring of: population cell density; cell size (FSC signal amplitude); and intracellular density or ‘granularity’ (SSC signal amplitude).

### 9.6.1 Darkness and population cell density: results from the 65 day experiment

Periodic assessments were made of population cell density for both *C. vulgaris* and *C. reinhardtii* during the current long-term dark-survival experiments. Results of these respective analyses are shown in Figures 9.6 and 9.7.



**Figure 9.6.** Two month *C. vulgaris* population cell density for all treatments (data points show the mean of three replicate cultures  $\pm$  1 S.D.)



**Figure 9.7.** Two month *C. reinhardtii* population cell density for all treatments (data points show the mean of three replicate cultures  $\pm$  1 S.D.)

Analysis of the data from Figures 9.6 and 9.7 above showed that the 'light / aerobic' treatments had significantly higher overall two month population cell densities for both *C. vulgaris* (1-way RM-ANOVA;  $F_{(3,6)} = 25.76$ ;  $p < 0.001$ ) and *C. reinhardtii* (1-way RM-ANOVA;  $F_{(3,6)} = 42.46$ ;  $p < 0.001$ ) than the other three treatments. The same analyses showed also that there were no apparent differences ( $p > 0.05$ ) between the long-term population cell densities for all remaining treatments (i.e. 'light / low D.O.', 'dark / aerobic', and 'dark / low D.O.')—a trend most easily observed through the overlapping treatment lines in Figures 9.6 and 9.7 above. Further analysis of the same

data suggested that 65 days of continuous dark-exposure, regardless of DO concentration, resulted in no discernable population cell growth or decline in *C. vulgaris* (1-way ANOVA;  $F_{(4,10)} = 2037$ ;  $p > 0.05$ ) and a small but significant long-term population decline for dark-exposed *C. reinhardtii* (1-way ANOVA;  $F_{(4,10)} = 155.7$ ;  $p < 0.01$ ) relative to ‘Day zero’ cell densities. Within the two ‘dark’ treatments themselves, however, there was no significant difference between the Day 65 values in either algal species ( $p > 0.05$ ), implying that DO concentration ( $\approx 8.1$  or  $2.2 \text{ mg L}^{-1}$ ) had no real influence on the ability of algal cultures to maintain their initial population cell density during prolonged dark-exposure.

Selvin *et al.* (1988/89) reported a wide array of dark-survival capacities (in terms of the ability to maintain pre-dark population cell density) in three dinoflagellates (*Protogonyaulax*, *Gymnodinium* and, *Prorocentrum* species) during 5 days of dark-exposure. Whilst some phytoplankton maintained (*Protogonyaulax*) or gradually reduced (*Gymnodinium*) their population cell densities during dark conditions, *Prorocentrum* species actually yielded some dark growth over the 5 days of dark-exposure; although this population growth increase was small ( $< 0.2$ -log<sub>10</sub> units). Griffis and Chapman (1988) also reported wide ranging dark-survival capabilities for a number of phytoplankton genera. Species of *Gonyaulax*, *Ensiculifera* and *Scrippsiella* (Dinophyceae) failed to survive two weeks of darkness, whereas *Coccolithus* species (Prymnesiophyceae) survived up to 38 weeks of continuous darkness. Gervais (1997) showed also that even phytoplankton species within the same genus (*Cryptomonas*) can display markedly differing dark-survival capacities (as measured by changes in population cell density over  $\approx 10$  days of darkness). This trend was not seen for the current phytoplankton species, however, with similarly effective maintenance of pre-dark population densities in both species despite there being a higher-level taxonomic divergence for *C. vulgaris* and *C. reinhardtii*.

Anita and Cheng (1970) observed no dark growth in any of the 31 species of marine phytoplankton tested during their prolonged (2–24 week) and pioneering dark-survival research. Instead, the authors discovered an innate and tenacious capacity for dark tolerance in many phytoplankton species. More recent work by Ferroni *et al.* (2007) demonstrated that the coldwater marine chlorophyte *Koliella antarctica* was capable of

maintaining pre-dark population cell densities over a similar 60 day dark period to that tested here. Overall, it was found that no significant population growth occurred during long-term dark-exposure of either *C. vulgaris* or *C. reinhardtii* under the current experimental conditions. Small-scale declines in cell culture density were, however, observed for long-term dark-exposed populations of *C. reinhardtii*; although there were some additional issues relating to this finding and these will be discussed below.

#### **9.6.1.1 Possible sources of error in FCM-quantified population cell density—sticky cells**

A potential source of error during the quantitation of population density using FCM arises from issues relating to cellular aggregation or ‘clumping’. Due to the way the FCM software ‘gates’ around a particular cell size distribution, there is potential for underestimation (not overestimation) of the true cell culture density. Cellular aggregation depends on the relative ‘stickiness’ of cells—a factor which varies according to both species and physiological state (Fisher *et al.*, 1996). Balfourt *et al.* (1992) reported that their *Chlorella* cultures appeared to clump or aggregate under illuminated culture, with percentage population clumping increasing during the light period and reversing during the dark ‘night’ period (possibly as a result of photosynthetically-elevated pH or due to bacterial proliferation). Kroon *et al.* (1992) hypothesised that clumping could be a physiological response to high light intensity exposure during the sinusoidal photoperiod (as a result of cellular polymer excretion and aggregation in an effort to ‘self-shade’ and protect themselves from photodamage during excessive PFDs). For the current analyses, this issue of underestimating population cell densities due to cell clumping was only an issue in *light-exposed* treatments and only for *C. reinhardtii* (see Section 9.7.1 and Figure 9.2.5 for further elaboration) and was of no real consequence to the ‘dark’ treatment data reported above for either species.

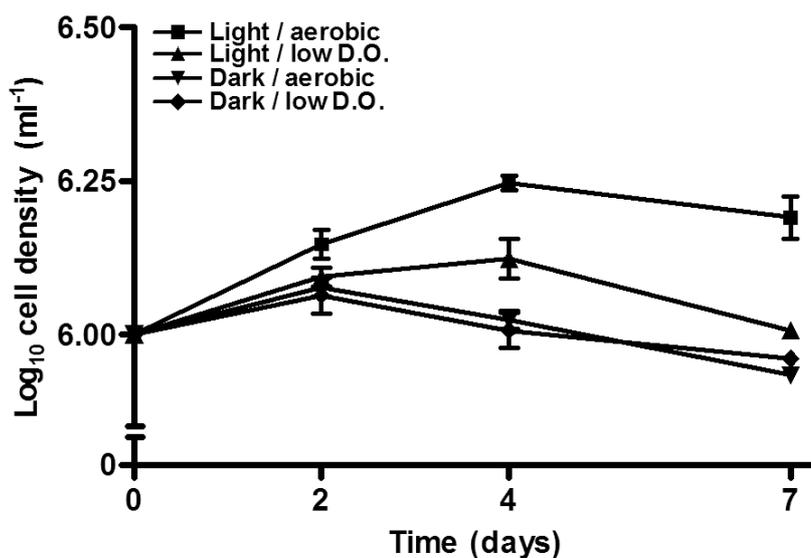
Another issue that was of more relevance to the ‘dark’ treatment data (again particularly for *C. reinhardtii*) is the issue of cellular adhesion. Qualitative visual observations of the light treatment flasks for *C. reinhardtii* (again not *C. vulgaris*) showed that there appeared to be some biofilm-adhesion to the flask base, and to a lesser degree the lower walls of the flask, during the long-term 65 day experiment. This would have resulted in

an effective loss of planktonic algal cells from the culture medium and a subsequent underestimation of actual population cell density. This trend can in effect be visualised in Figure 9.7, whereby *C. reinhardtii* cell culture density experienced a sudden  $\approx 0.5$ - $\log_{10}$  decrease from Day zero to Day 7 for the 'light / low D.O.' and 'dark' treatments, after which it remained relatively constant. This possible adhesion loss can also be visualised in Figure 9.7 for the 'light / aerobic' treatment, whereby there was unexpectedly no apparent culture proliferation from Day zero to Day 7 (possibly due to supposed biofilm attachment losses); however, from Day 7 onwards (presumably once all suitable adhesion sites were saturated) the population cell density steadily increased.

This adhesion of algal cells to hard substrates has been noted for green (Chlorophyceae) algal cells by Sekar (2004), with substrate-adhesion more pronounced at high population densities and under high ( $>7$ ) culture pH (muchlike that of ageing 'light / aerobic' cultures reported here). Other researchers have reportedly coated the inner surfaces of the experimental flasks with a 'non-stick' (silicone in isopropanol) surface in order to reduce algal cell losses from adhesion to the flask walls (Gervais, 1997); although this was not done for the research reported here. In conclusion, it is therefore likely that population cell density was slightly underestimated for *C. reinhardtii* across all treatments. This has some follow-on implications for cellular chlorophyll *a* calculations, and hence the issue is again referred to in Section 9.7. Finally, it should be noted that the consequences of this 'adhesion effect' were considered to have been relatively consistent across all four treatments for *C. reinhardtii*, such that relative differences in cell density *between* treatments should have remained relatively unaffected.

### **9.6.2 Darkness and population cell density: results from the 7 day experiment**

As was done for the long-term 65 day dark-survival experiment, periodic assessments of the population cell density for *C. vulgaris* were also made during the subsequent 7 day dark-survival investigation. Results are shown in Figure 9.8.



**Figure 9.8.** Seven day *C. vulgaris* cell density following incubation under experimentally-manipulated light and dissolved oxygen conditions (data points show mean of three replicate cultures  $\pm$  1 S.D.)

Statistical analysis of the data from Figure 9.8 showed that the 'light / aerobic' treatment had a significantly higher overall 7 day population cell density for *C. vulgaris* than both of the 'dark' treatments (1-way RM-ANOVA;  $F_{(3,2)} = 10.27$ ;  $p < 0.05$ ) but was not significantly different to that of the 'light / low D.O.' treatment ( $p > 0.05$ ). At the same time, however, this analysis also showed that there were no apparent differences between the overall 7 day population cell densities for the 'light / low D.O.', 'dark / aerobic' and 'dark / low D.O.' treatments—a trend again most easily observed through the close proximity of the plotted lines for these 3 treatments in Figures 9.8. Statistical analysis of the 'Day 7' versus 'Day zero' data only, showed that the 'light / aerobic' treatment had increased (1-way ANOVA;  $F_{(4,10)} = 111.3$ ;  $p < 0.001$ ), the 'light / low D.O.' treatment had remained unchanged ( $p > 0.05$ ), the 'dark / aerobic' treatment had decreased ( $p < 0.01$ ) and the 'dark / low D.O.' treatment had not changed its population cell density over the 7 day experimental duration ( $p > 0.05$ ). Looking qualitatively at Figure 9.8 it is difficult to give any real weight to these statistical outcomes, given that the statistically significant decrease in 7 day cell culture density for the 'dark / aerobic' treatment represented a numerical decrease from 6.00 to 5.93- $\log_{10}$  cells  $\text{ml}^{-1}$ . The real significance of this is discussed again below.

Microbiologically speaking, the recorded 7 day population density reduction for the above 'dark / aerobic' treatment, although significant at the  $p < 0.01$  level, constituted a population cell density reduction of  $< 0.1\text{-log}_{10}$  units and was almost certainly a simple manifestation of daily operator/instrument error. The ever-present low-level sampling and measurement error was thought to have been exacerbated in this instance by the lack of any real change in population cell density, such that it became a case of compounding 'multiplicity of error' during every experimental manipulation stage (i.e. from treatment culture sampling to final FCM analyses) that eventually became large enough to be declared significant by the statistical analyses (most likely due to the presence of zero variability in the triplicate 'Day zero' counts that were performed on a single culture inoculum). From this, it is concluded that there was no *real* change in population cell density during the 7 day experiment in any treatment except for the 'light / aerobic', which underwent a slight and somewhat anticipated culture growth.

Others have reported a 'carryover' of cellular growth when algal (*Chlorella*) cultures are transferred abruptly from light to dark conditions, such that cells appear to 'continue growing' in darkness for up to 5 days at an ever-decreasing rate, after which time no further growth is observed (Karlander and Krauss, 1966a). A similar carryover of light growth during the initial stages of prolonged dark-exposure was qualitatively apparent in the data of Ferroni *et al.* (2007) during 60 day dark-exposure of the marine chlorophyte *Koliella Antarctica*. This observation was not recorded during the current work; rather, cellular growth appeared to have been completely arrested upon transfer to dark conditions, with no significant growth in darkness observed for *C. vulgaris* either within the first two or up to a total of 65 days. This failure to observe 'carryover' growth following culture transfer to darkness was a likely manifestation of having used near stationary-phase cultures, as opposed to the exponentially-growing cultures used by both Karlander and Krauss (1966a) and Ferroni *et al.* (2007).

### **9.6.3 Prolonged darkness and phytoplankton cell size, volume and intracellular density**

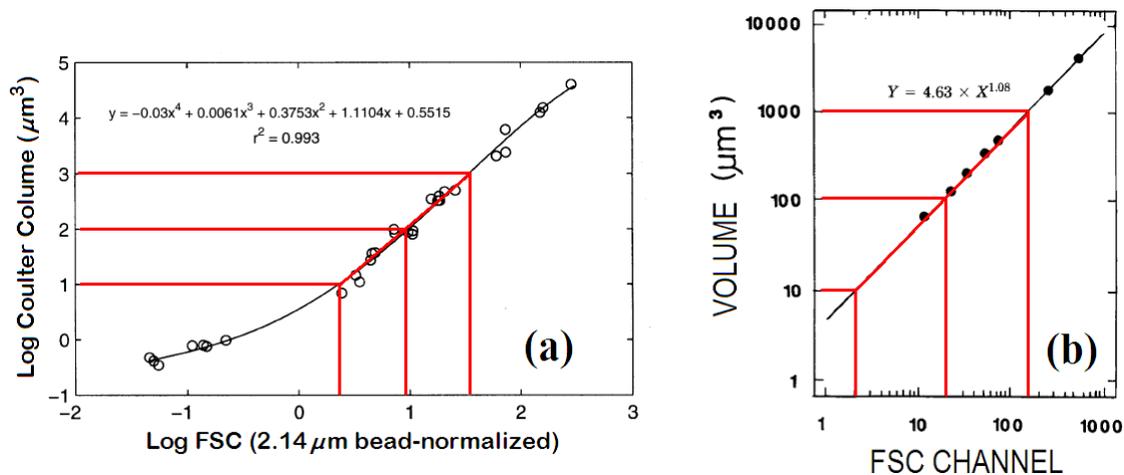
As introduced earlier (Section 6.4.2.1.1), forward-angle light scatter (FSC) results from light diffracted around the cell and so is solely dependent upon the cell's physical size



and shape (Collier, 2000). Since FSC signal amplitude can be directly and accurately correlated with cell size (Chisholm, 1992; Shapiro, 2003), changes in cell size and volume were monitored during the course of the experiments by their proxy measure of corresponding FSC signal height.

### 9.6.3.1 FSC-height versus cell volume

Prior work has demonstrated that FSC-height and cell size (i.e. volume for coccoid cells) can be empirically correlated. Empirical equations of Koch (1996) and Shalapyonok *et al.* (2001) reveal a non-linear polynomial-type function of cell volume to FSC signal height. Published data of Demers *et al.* (1989) and Shalapyonok *et al.* (2001) also showed, however, that FSC-height—as measured by FCM—is highly linear within the size range of algal cells involved in the current research (i.e. 10–1000 $\mu\text{m}^3$ ). This relationship can be seen in Figure 9.9 below.



**Figure 9.9.** Graphical representation of published data showing the analytical linearity between FSC signal amplitude and cell volume: (a) modified from Shalapyonok *et al.* (2001); and (b) modified from Demers *et al.* (1989). Axial markings indicate the approximated relevant regions of the fitted curves (10–1000 $\mu\text{m}^3$ ) pertaining to the cell size ranges used in this research.

Based on the existence of this linear relationship, and recognising that the specific empirical ‘equation of the line’ is entirely machine-specific (Gasol and del Giorgio, 2000; Bouvier *et al.*, 2001), a calibration curve between standard analytical bead size (diameter) and FSC-height was constructed using the current FACScan cytometer. The standard beads used had precise diameters of 2.5, 3 and 6 $\mu\text{m}$ , with corresponding

volumes of 8.18, 14.14 and 113.1 $\mu\text{m}^3$  respectively. The subsequent standard calibration curve, regardless of the low  $n$ , displayed a highly linear trend (regression  $r^2 = 0.997$ ;  $n = 3$ ;  $y = 0.0986x + 0.491$ ) when bead diameter ( $\mu\text{m}$ ;  $y$ -axis) was plotted against FSC signal amplitude (A.U.;  $x$ -axis). Finally, the mean percentage deviation between actual and calculated volumes (using the fitted equation) was 7.3% for the three beads tested, so this was taken as accurate evidence that the relationship between algal cell volume and FSC-height was sufficiently linear for the current machine (at least within the FSC signal heights recorded during the dark-survival analyses) and was also precise enough to allow FSC signal height to be used as an accurate reflection of actual algal cell volumes.

It is important to note at this point that there are recognised limitations and potential sources of error when using FSC-height to calculate cell volume. Physical light scatter signals can vary according to physical cell morphology (shape), cell growth phase and also its refractive index (Cunningham and Buonacorsi, 1992; Bouvier *et al.*, 2001; Shapiro, 2003). Gasol and del Giorgio (2000) summarise the above limitations by stating that the empirical relationship between FSC-height and cell volume is likely to be highly species specific, thereby potentially prohibiting direct comparisons of cell volume changes between *C. vulgaris* and *C. reinhardtii* for the current research. Fortunately, the work of both Cunningham and Buonacorsi (1992) and Shalapyonok *et al.* (2001) showed that this FSC versus cell volume relationship was in fact highly linear across a range of measured phytoplankton sizes ( $\approx 2$ – $45\mu\text{m}$  diameter) and spanning several taxonomic phyla—effectively negating these prior ‘interspecies’ concerns.

The above concern was further alleviated by the fact that the FSC:SSC ratios were not significantly different (Mann–Whitney  $U$ -test;  $U_{0.05(2)3,3} = 0.000$ ;  $p = 0.100$ ) for both algal species in healthy culture populations (FSC:SSC of 0.276 ( $\pm 0.0027$ ) and 0.270 ( $\pm 0.0026$ ) for day-8 *C. vulgaris* and *C. reinhardtii* cultures respectively). Similarly, and regarding the two month dark-survival data, when SSC-height was plotted against FSC-height for both species grouped according to ‘light’ and ‘dark’ treatments, the slopes of the regression lines were again similar between the two algal species (i.e. the ratio of FSC- to SSC-height was found to be consistent between algal species even in ageing cultures). For ‘light’ treatment data, the slopes between *C. vulgaris* and *C. reinhardtii*

data were not significantly different (ANCOVA;  $F_{(1,92)} = 2.802$ ;  $p = 0.098$ ) and for the ‘dark’ treatment data, there was again no difference between the slopes of the *C. vulgaris* and *C. reinhardtii* FSC- versus SSC-height data (ANCOVA;  $F_{(1,92)} = 0.173$ ;  $p = 0.680$ ). What this all effectively means is that there was no apparent difference between the way the current flow cytometer measures physical light scatter properties between the two algal species in question and so there should be no reason why the abovementioned standard equation for converting FSC-height to cell volume cannot be applied to both algae. This also implies that any and all subsequent inter- and intra-species comparisons between changes in cell volume are indeed valid and can be made without reservation. This apparent similarity in measured FSC:SSC ratios between the two algal species was perhaps unsurprising, given their taxonomic and morphological similarities.

The final calculation of cell biovolume from FSC signal heights during the current work was further simplified by the fact that both *Chlorella* and *Chlamydomonas* species are most adequately described by a standard spherical geometric model in terms of their biovolume calculation (Hillebrand *et al.*, 1999), meaning that cell volume ( $V$ ) could be easily calculated from the FSC-derived cell diameter ( $d$ ) using the standard Equation 9.1.

$$V = \frac{\pi}{6} \cdot d^3 \quad \text{(Equation 9.1)}$$

### **9.6.3.2 The importance of cell volume for interpreting FCM data**

Whilst FCM provides the experimental investigator with tremendous analytical power and highly detailed multiparameter data sets, without proper data ‘crunching’ (i.e. processing, manipulation, transformation) and unless one can present the data in a logical format, it often represents a complex and demanding task to arrive at meaningful conclusions. One such necessary data transformation was highlighted initially—although somewhat inadvertently—by Winokur (1949) and has been more recently emphasized by Alpine and Cloern (1985), Dorsey *et al.* (1989) and again by Raven and Kübler (2002) and relates to the need for normalisation of cytometric cellular fluorescence signals to cell size (i.e. volume). Although not commonly performed in the literature, it

can be misleading if cellular fluorescence signals are not ‘cell-volume-normalised’, especially for chlorophyll *a* absorption and fluorescence signals which are known to vary non-linearly according to cell size (Alpine and Cloern, 1985; Raven and Kübler, 2002) as a result of size-based differences in photon capturing efficiencies (Duysens, 1956; Kirk, 1975a; Kirk, 1975b)—a phenomenon known as the intracellular self-shading or the “package effect” (see Section 9.7.3 for more information). Raven and Kübler (2002) also highlight the fact that an increase in cell radius can decrease the cellular capacity for solute influx and efflux (on a volume basis) as a result of a thicker diffusion boundary layer and a reduced membrane lipid surface area (relative to volume) for catalysing these solute fluxes. This has potential implications for the FDA cell viability assay in particular because it involves quantitative measurement of substrate uptake and hydrolysis through the measurement of cellular fluorescence properties.

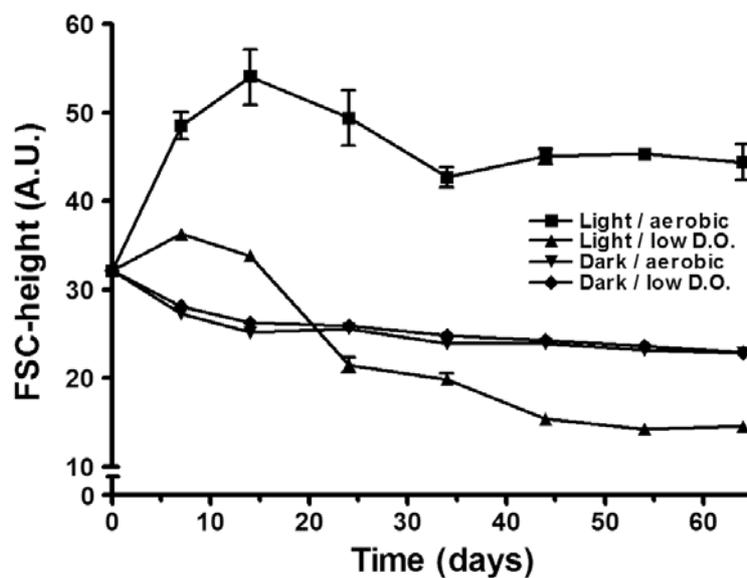
It should be emphasized that there is potential for erroneous conclusions when representing long-term cell fluorescence data derived from physiological parameters (e.g. PI, FDA, chlorophyll *a*) as standard ‘population mean’ values without taking into account the effects that changes in cell size may have had on the measured fluorescence signal over time. This becomes especially important in instances where different experimental treatments result in non-uniform changes to cell volume over time (as was observed during the current work; see Section 9.6.3.3). It is not appropriate, however, to simply divide the mean parameter fluorescence (e.g. chlorophyll *a* or FDA) for a cell population by its mean FSC signal amplitude, because the raw FSC signal value is actually a surrogate for ‘equivalent cell diameter’ and not ‘volume’ *per se*. Due to the fact that cell volume varies over a far greater magnitude than does cell size measured by spherical diameter (Dorsey *et al.*, 1989), cell *volume* rather than cell diameter is more important in the context of these fluorescence signals, because cellular fluorescence signals normalised to diameter may not reflect the true magnitude of physiological changes that may be obtained by normalising the same fluorescence signal to the corresponding cell volume.

Far from being well understood and widely agreed upon, and following much research effort into the area, the protocols for scaling cellular metabolic processes with the size of algal cells still remain unclear. Despite many attempts to develop quantitative

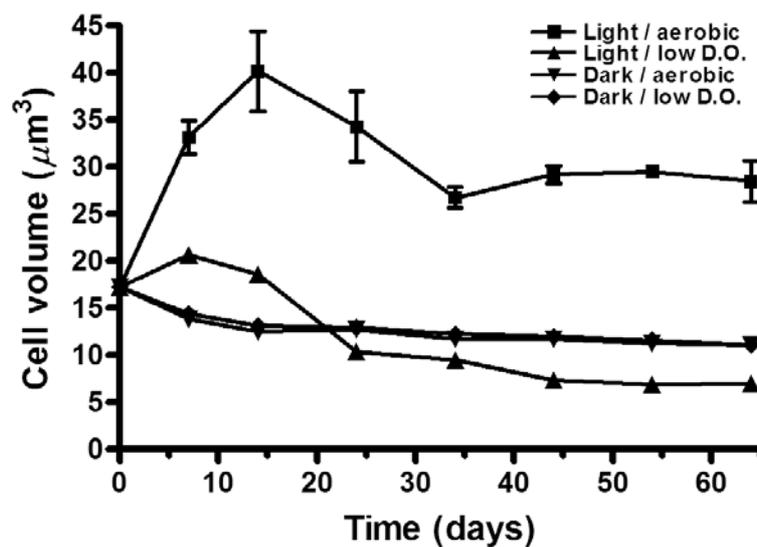
mechanistic models of these size-dependent variations in algal metabolic rates, there is still considerable uncertainty regarding the general nature of the relationship between metabolic rate and cell size (Raven and Kübler, 2002). In light of the fact that this is a complex and as yet unresolved issue, average FCM-quantified fluorescence signals (PI, FDA and chlorophyll *a*) for a given sample were normalised to the corresponding mean cell volume in order to—as best as possible—account for the abovementioned size-related factors. It should be noted also, that both ‘standard’ (raw fluorescence signals) and ‘cell-volume-normalised’ fluorescence values will be given in the following sections in order to highlight any differences (or otherwise) between results derived from the two data formats.

### **9.6.3.3 Prolonged darkness and phytoplankton cell size and intracellular density: results from the 65 day experiment**

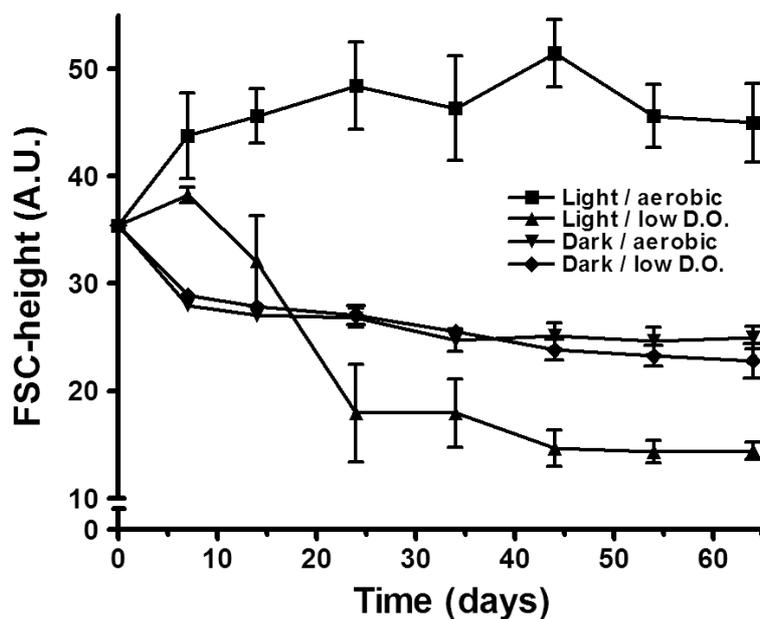
Since FSC and SSC signal height (amplitude) serve as surrogate measures of cell size and intracellular density or ‘granularity’ respectively, FSC and SSC signals were periodically monitored during the course of the dark-incubations in order to assess changes in both size and intracellular density during prolonged darkness for both *C. vulgaris* and *C. reinhardtii*. Results from these analyses presented in Figures 9.10–9.13 below.



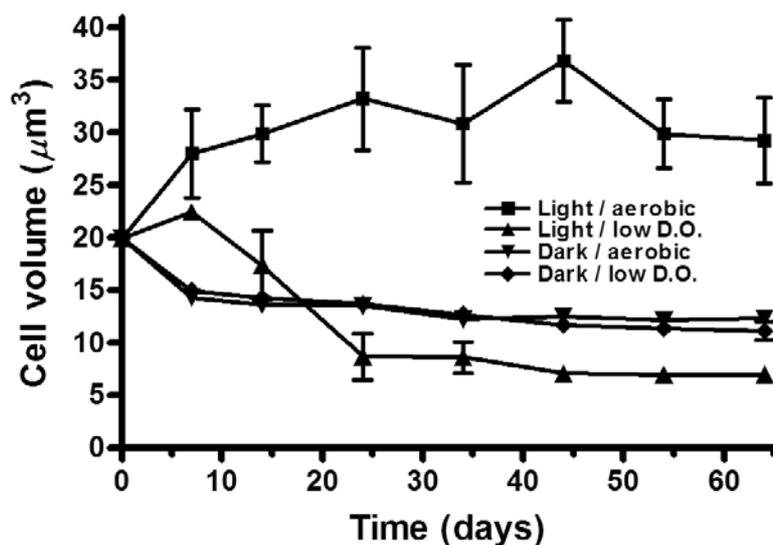
**Figure 9.10.** 65 day *C. vulgaris* population mean FSC signal amplitude for all four experimental treatments (data points represent the mean of triplicate algal cultures  $\pm$  1 S.D).



**Figure 9.11.** 65 day *C. vulgaris* population average cell volume for all four treatments. Cell volumes calculated according to the predefined equations of Section 9.6.3.1 (data points represent the mean of three replicate cultures  $\pm$  1 S.D).



**Figure 9.12.** 65 day *C. reinhardtii* population mean FSC signal amplitude for all four experimental treatments (data points represent the mean of triplicate algal cultures  $\pm$  1 S.D).



**Figure 9.13.** 65 day *C. reinhardtii* population average cell volume for all four experimental treatments. Cell volumes calculated according to the pre-stated equations of Section 9.6.3.1 (data points represent the mean of three replicate cultures  $\pm$  1 S.D).

As can be seen from Figures 9.10 and 9.11 for *C. vulgaris* and Figures 9.12 and 9.13 for *C. reinhardtii*, converting the respective FSC signal amplitudes to their equivalent cell volumes merely changes the data units and changes neither the overall treatment trends nor the relative long-term differences between the four treatments. Consequently,

changes in cell size will only be discussed with reference to equivalent *cell volume* and not the corresponding raw FSC signals.

Two month experimental data showed that statically ageing 'light / aerobic' treatments for both *C. vulgaris* and *C. reinhardtii* contained algal populations with both larger (1-way RM-ANOVA;  $F_{(3,7)} \approx 26$ ;  $p \leq 0.001$ ) and more varied cell size distributions than all other treatments. This observation simply reflected the normal physiological relationship between cell size and growth-phase (Zettler *et al.*, 1996) and was of no great interest given that during the normal growth cycle of *C. reinhardtii*, for example, parent cells commonly sub-divide into eight daughter cells prior to cell division and can grow to many times their original size during this time (Bisova *et al.*, 2005). This also probably goes toward explaining the larger variation (S.D.) in cytometer FSC signals and corresponding cell volume in light treatments compared to non-growing dark-incubated cultures. Similarly, the trend for statically ageing cultures of *C. vulgaris* to increase their cell size over time has also been reported since very early on (Pearsall and Loose, 1937) and so will not be discussed further here.

Algal cells of both species in the 'light / low D.O.' treatment decreased markedly in Day-zero-relative size over the first month and eventually stabilised over the following 35 days to be significantly smaller on average at 'Day 65' than at 'Day zero' for both *C. vulgaris* (1-way ANOVA;  $F_{(4,10)} = 217.5$ ;  $p < 0.001$ ) and *C. reinhardtii* (1-way ANOVA;  $F_{(4,10)} = 65.24$ ;  $p < 0.001$ ). Although raw data FSC signals and corresponding cell volumes for both *C. vulgaris* and *C. reinhardtii* appeared to increase slightly from days 0–7 in 'light / low D.O.' treatments, this was most likely a simple manifestation of the inoculum 'carryover growth' effect described above (Section 9.6.2), especially considering that cell volumes decreased relatively quickly thereafter. Other authors have reported that dying cells are often observed to give reduced FSC signals in FCM analyses (Brussaard *et al.*, 1999; Shapiro, 2003). As is discussed in later Sections, this significant long-term cellular shrinkage ( $\approx 60\%$  reduction of 'Day zero' volume as at 'Day 65') for 'light / low D.O.' treatment algal cells was likely to have been directly linked to the correspondingly high rates of cell death in these treatment cultures, with cell death later suggested to have resulted from necrotic pathways.



During the course of prolonged dark-exposure, Day-zero-relative algal cell volume decreased steadily and significantly (1-way ANOVA;  $F_{(14,30)} = 192.0$ ;  $p < 0.01$ ) by  $\approx 35\%$  for *C. vulgaris* at Day 65, compared with an average volume reduction of  $\approx 41\%$  (1-way ANOVA;  $F_{(14,30)} = 47.16$ ;  $p < 0.01$ ) for *C. reinhardtii* after the same time. This observed reduction in cellular volume agrees well with the fact that a decrease in cell volume is generally observed for most algal groups as a low-light acclimation response (Ferris and Christian, 1991) and also with the fact that many authors have reported cellular shrinkage during dark-exposure. Briefly, Franklin and Berges (2004) reported notable cell shrinkage for dark-exposed dinoflagellates (*Amphidinium carterae*) over the course of 20 days of darkness. Wolfe *et al.* (2002) reported on the Haptophyte alga *Emiliania huxleyi* decreasing its cell volume (by 50–70%) within 24 hours of being transferred to darkness, with cell volume increasing quickly upon re-exposure to light conditions. Conversely, Finkle *et al.* (1950)—reporting on one of the first algal dark-exposure studies—observed that prolonged (up to 10 week) dark-exposure of *C. vulgaris* (Emerson strain) resulted in ‘granular’ cells that were much larger than normal size. This trend was not observed here for the Beyerinck strain of *C. vulgaris*; instead dark-exposed cells appeared to steadily shrink in size. Intraspecies physiological differences (e.g. in cellular growth characteristics) have been reported elsewhere for different strains of *C. vulgaris* (Karlander and Krauss, 1966a) and so it is hypothetically possible that this sort of physiological variability in the dark-acclimation response does exist even within the same algal species.

Falkowski and Owens (1980) reported a similarly notable decrease (to that reported here) in cell volume (15–40%) with decreasing irradiance (down to  $2\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for the marine phytoplankton *Skeletonema costatum* and *Dunaliella tertiolecta* during short-term shade-adaptation experiments (a physiological outcome that was said to be coupled to the simultaneous reduction in cell division and population growth). This suggestion of Falkowski and Owens is a particularly noteworthy point with respect to results of the current long-term dark-incubations, in that it is difficult to ascertain whether the cells actually *decreased* in cell volume during prolonged darkness, or, if they simply, on average, didn’t change at all one way or the other. It can be appreciated that at experimental ‘Day zero’, perhaps there was still a significant number of very large cells in active ‘growth phase’ (i.e. Day 8 culture inoculum; see Figure 9.1) such

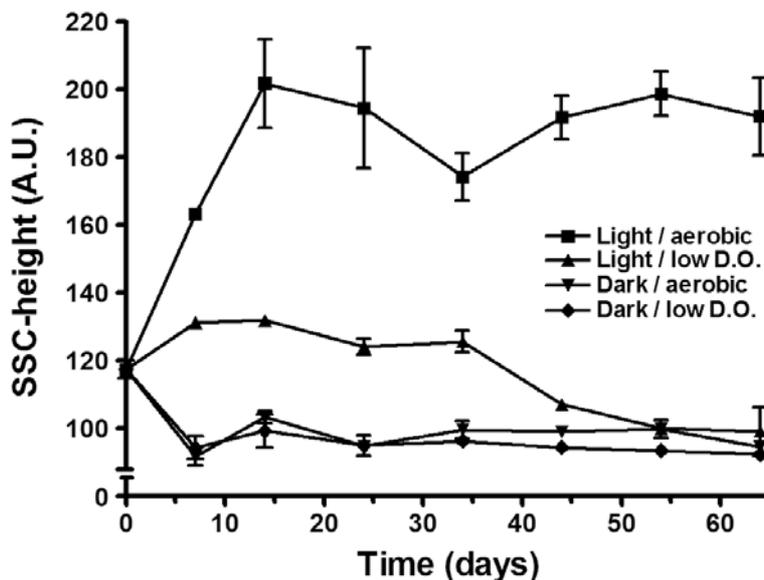
that the *overall* population average cell size was somewhat elevated (from ‘carry-over’ cells that were still growing and dividing during early stationary-phase at culture ‘Day 8’). Then, at the time that those remaining cells had finished growing and dividing (i.e. by Day 7 of the dark period), the total mean population cell size had decreased somewhat after which it appeared to be relatively constant for the remainder of the two month dark experimental duration.

This trend is a seemingly logical conclusion and can be (qualitatively) evidenced in the corresponding Figures for 65 day cell volume (9.11 and 9.13) for both algal species. Quite clearly, the biggest rate of decline in mean population cell size was realised between days zero and 7, after which time the rate of reduction in cell volume was seen to slow considerably. Irrespective of this hypothesis, a steady decrease in algal cell volume was indeed observed for both ‘dark’ treatments in both algal species from Day 7 onwards, with Day 65 cell volume still significantly smaller than Day 7 for both ‘dark’ treatments and both species ( $p < 0.001$ ) but with no difference between the 65 day ‘dark’ treatment cell volumes for either species ( $p > 0.05$ ).

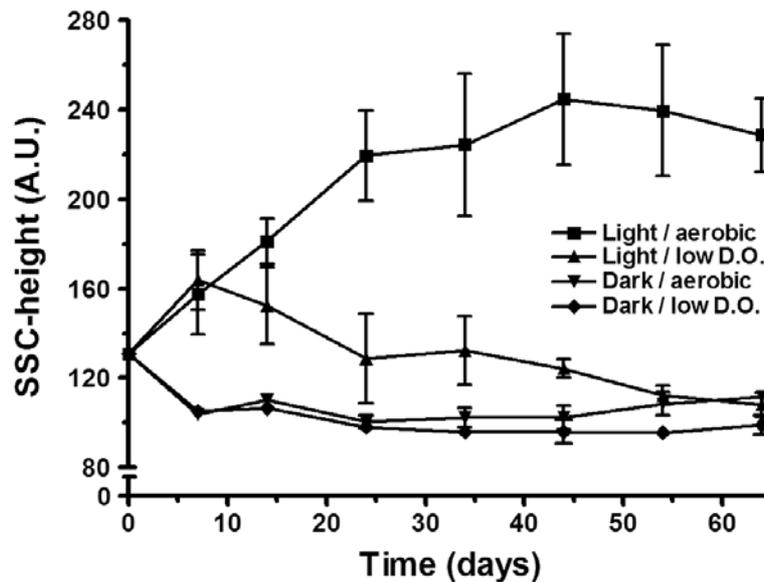
Montechiaro *et al.* (2006) reported a 50% reduction in cell volume for the cyanobacterium *Phormidium autumnale* after 21 days of darkness. This significant reduction in cell volume was deemed to be a result of continued ‘homeostatic intracellular resource consumption’ over the three week dark period. This is in direct contrast to the apparent adaptational response of *C. vulgaris* and *C. reinhardtii* in the current dark-survival experiments, and suggests a more conservative resource consumption strategy in these green algae compared with that of *P. autumnale*. Indeed Montechiaro *et al.* (2006) proposed two different cellular adaptational strategies in response to changes in light climate. ‘Strategy 1’ is said to be an “acclimation response”, whereby cells change or modulate their metabolic status or cellular composition. ‘Strategy 2’ was termed a “homeostatic response”, whereby phytoplankton cells maintain a balanced cell composition, despite the dramatic changes in their physicochemical (light) environment, and actively conserve and maintain cellular structures and functions. Incidentally, this survival strategy concept was actually proposed somewhat earlier by Smayda and Mitchell-Innes (1974) following dark-survival assessments of marine planktonic diatoms. Smayda and Mitchell-Innes,

following the discovery of markedly different dark-survival capacities in seemingly related species, suggested that there exists distinct ‘survival strategies’ for vegetative phytoplankton cells during prolonged dark-exposure—termed ‘Type I’ and ‘Type II’ survival strategies. These dark-survival strategies will be referred to again in the context of later Sections.

Species of *Chlamydomonas* are recognised as having a capacity for resting spore (thick-walled resistant ‘zygospores’ from sexual reproduction) formation (Coleman, 1975), with this non-vegetative stage potentially capable of increased resilience to prolonged darkness. Although no detailed microscopic examination of experimental cultures was performed at any stage during this research, based on the observation of no apparent dark proliferation of the presumably vegetative culture inocula and also taking into consideration the lack of change in the population FSC:SSC ratio, it was assumed that original vegetative cell population remained in this state for the entire experimental duration. The formation of so-called ‘resting stages’ was, therefore, not considered to have influenced the long-term dark-survival capacity of *C. reinhardtii* during the current research.



**Figure 9.14.** 65 day *C. vulgaris* population mean SSC signal amplitude for all four experimental treatments (data points represent the mean of triplicate algal cultures  $\pm$  1 S.D).



**Figure 9.15.** 65 day *C. reinhardtii* population mean SSC signal amplitude for all four experimental treatments (data points represent the mean of three replicate algal cultures  $\pm 1$  S.D).

Long-term results from monitored SSC signal amplitudes for both *C. vulgaris* and *C. reinhardtii* are shown in Figures 9.14 and 9.15. Light-exposed statically ageing 'light / aerobic' treatment algal cultures recorded significant long-term increases in SSC signal height relative to all other treatments for both *C. vulgaris* (1-way RM-ANOVA;  $F_{(3,7)} = 35.65$ ;  $p < 0.001$ ) and *C. reinhardtii* (1-way RM-ANOVA;  $F_{(3,7)} = 23.35$ ;  $p < 0.001$ ), inferring an increased intracellular density over the 65 day experimental duration. Winokur (1949) reported a similar trend in ageing cultures of *C. vulgaris* (statically aged in the light for 32 days), whereby cells gradually became more intracellularly dense (i.e. individual cell mass increased relative to cell volume) over the 32 day experimental duration. Blum and Balber (1996) have also reported that some organisms in stationary growth phase can exhibit SSC signals up to 3–4-fold lower than actively growing exponential-phase cultures. This observation of heightened SSC signals in ageing 'light / aerobic' treatment cultures, like that of cell size above, was again perceived to be of limited interest with respect to the current dark-survival research objectives and so will not be discussed any further.

Algal cells in the 'light / low D.O.' treatment (as observed for FSC signal data above) displayed similar long-term trends in SSC signal amplitudes for both species. SSC

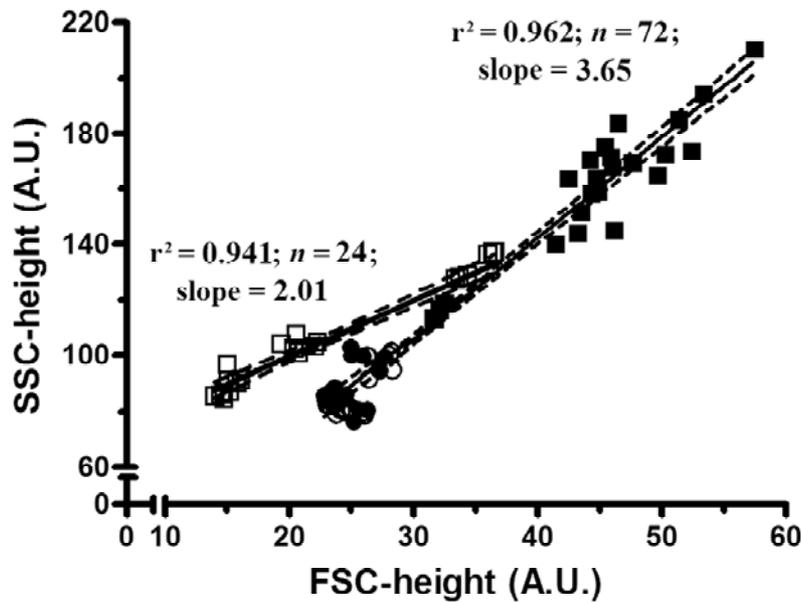
signals for both *C. vulgaris* and *C. reinhardtii* increased slightly at first (again a likely artefact of carryover growth as per Section 9.6.2 above) and then decreased gradually over the following 7 weeks of dark-incubation. This, combined with the parallel trend of steadily decreasing FSC signal heights, suggested that these cells were likely to have undergone a 'necrotic-type' cell death. Generally speaking, large-scale declines in FSC and SSC signal amplitudes during flow cytometric analyses are hallmark signs of cellular necrosis (i.e. degenerative cell death involving membrane damage and subsequent leakage of cellular constituents). Additional support for this notion of cell death in 'light / low D.O.' treatments will be provided in later Sections 9.7 and 9.8, with results from 'light / low D.O.' treatment discussed in greater detail in Section 9.10.

One trend commonly reported in the literature for dark-exposed *Chlorella* is a tendency for cells to lose cellular chlorophyll (Finkle *et al.*, 1950; Killam and Myers, 1956) and become granular (Finkle *et al.*, 1950). Dehning and Tilzer (1989) demonstrated that long-term (3 month) dark-exposure in *Scenedesmus acuminatus* (Chlorophyceae) led to both a reduction in cellular biomass (dry weight) and an approximate doubling in cellular biovolume due to the catabolism of cellular reserves and enhanced cytoplasmic granularisation and cellular vacuolisation. Although dark-exposed algae in the current experiments appeared to shrink instead of swelling (unlike *S. acuminatus* above), they did exhibit reduced SSC signal amplitudes over time; thereby apparently becoming slightly 'less dense' or 'more granular' during prolonged dark-exposure. Once again, however, when looking qualitatively at Figures 9.14 and 9.15 above, it is apparent that the bulk of the 65 day drop in recorded 'dark' treatment SSC signal heights was again realised between days zero and 7, after which there is no further decrease from Day 7 to 65 for *C. vulgaris* (unpaired *t*-tests;  $p \geq 0.198$ ) nor for *C. reinhardtii* (unpaired *t*-tests;  $p = 0.080$ ). It appears then that there were similar Day zero 'carryover' issues to those already discussed for long-term cell volume measurements above. Following this, it can be concluded that there was no real long-term change in intracellular structural density (inferred by corresponding SSC signal amplitudes) during the 65 days of darkness for either algal species and under either 'aerobic' or 'low D.O.' conditions.

In light of these relative uncertainties surrounding the interpretation of light scatter signals, it was conceived that perhaps by *combining* the two physical light scatter signals

(FSC and SSC) one may feasibly be able to assess the long-term stability of algal ‘cellular makeup’ (i.e. size-to-density ratio), thereby controlling for the abovementioned inoculum ‘carryover effect’ and also effectively normalising against any inter-species differences at the same time. It was thought that this numerical ‘FSC:SSC ratio’ might serve as a proxy for the general ‘nature’ of a given cell population by correlating average cell size to average intracellular density, with any long-term shift from the Day zero ‘normal’ range indicating some alteration to general physical or morphological composition and perhaps also corresponding cellular fitness. According to Blum and Balber (1996, p. 217), the relative “*change in FSC/SSC ratio is a particularly sensitive index of cellular reorganizations*” in protozoa. This suggested that the abovementioned FSC:SSC ratio concept was a viable one and was likely to have useful application to phytoplankton analysis also. Following this, the corresponding FSC:SSC signal ratios were calculated for all treatments during the two month dark-survival experiment.

For the current data, the ratio of FSC:SSC appears to be relatively well conserved and remained stable in both algal species over for the entirety of the two month experimental duration (1-way RM-ANOVA; *C. vulgaris*;  $F_{(3,21)} = 13.73$ ;  $p > 0.05$  and *C. reinhardtii*;  $F_{(3,21)} = 19.19$ ;  $p > 0.05$ ) but only as long as the cells retained their viability (as determined by PI membrane integrity). In other words, there was no significant difference between the FSC:SSC ratios of any treatment *except* when compared with the ‘light / low D.O.’ treatment, in which case there were then significant differences between the ‘light / low D.O.’ and all other treatments for both algal species ( $p \leq 0.01$ ). This similarity between FSC:SSC ratios for healthy ‘light’ and ‘dark’ treatment groups suggested that despite algal cells having reduced their physical size during the long-term dark-incubation, they apparently did not significantly alter their overall cellular constitution (i.e. ‘size-to-density’ ratio). This trend can be easily visualised when FSC and SSC signals are plotted (Figure 9.16), with deviation from the ‘healthy’ trendline (as plotted from viable ‘dark’ and ‘light / aerobic’ treatments) seen only for moribund or dead cells of the ‘light / low D.O.’ treatment.



**Figure 9.16.** *C. vulgaris* 65 day FSC vs. SSC signal amplitudes from: 'light / aerobic' (■); 'light / low D.O.' (□); 'dark / aerobic' (●); and 'dark / low D.O.' (○) treatments. Both regression slopes were significantly non-zero ( $p < 0.0001$ ), with fitted regression lines shown  $\pm$  95% CI's (broken lines).

Statistically, there were no significant differences between the slopes of the linear regression lines for FSC versus SSC in 'healthy' treatment cultures (i.e. excluding 'light / low D.O.' treatment cells only) for both algal species (ANCOVA;  $F_{(5,132)} = 0.863$ ;  $p = 0.51$ ). This can be again evidenced in more detail in Table 9.1 below, whereby significant differences only exist between FSC versus SSC regression profiles when 'light / low D.O.' treatment cells are included in the analyses; suggesting that changes in the interrelationship between physical light scatter properties could possibly be used as an indicator for changes in physical cell structure and linked to cellular viability status.

**Table 9.1.** FSC vs. SSC signal regression slope comparisons for both algal species during the two-month dark-survival experiment. Significant differences between treatments were identified via ANCOVA, with level of significance indicated by shading intensity: no shading signifies no difference ( $p > 0.05$ ); light shading indicates a difference at  $p < 0.05$ ; intermediate shading is significantly different at  $p < 0.01$ ; and black shading is different at  $p < 0.001$ .

<b><i>C. vulgaris</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	0.0001			
Dark / aerobic	0.956	0.0001		
Dark / low D.O.	0.265	0.0014	0.302	

<b><i>C. reinhardtii</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	0.0065			
Dark / aerobic	0.594	0.0179		
Dark / low D.O.	0.520	0.0125	0.919	

In this sense, so long as the dark-exposed algae adhered to healthy the ‘line-of-best-fit’ in Figure 9.16, they could be subjectively classified as having retained their structural ‘normality’ and are likely to have remained viable—a theory that was proven in later viability analyses. It is, therefore, possible that it might be even more insightful to compare changes in the ratio of physical light scatter signals (i.e. FSC and SSC) rather than comparing the changes in each parameter separately. Changes in this FSC:SSC ratio could then be used to identify larger scale changes in one parameter compared to the other, thereby accounting (and possibly correcting for) the interconnectivity and cross-over between forward- and side-angle light scatter signals (see Section 9.6.3.4). It is also possible that this ratio might be more highly conserved between different instruments, thus allowing for more direct comparisons between physical light scatter properties of a given algal species measured in one location and that of the same species analysed in a different laboratory. To the author’s knowledge, this concept of using physical light scatter signal ratios as indicators of morphological or structural changes and cellular viability is novel to phytoplankton research.



#### **9.6.3.4 The interrelationship between FSC and SSC signals in FCM analysis**

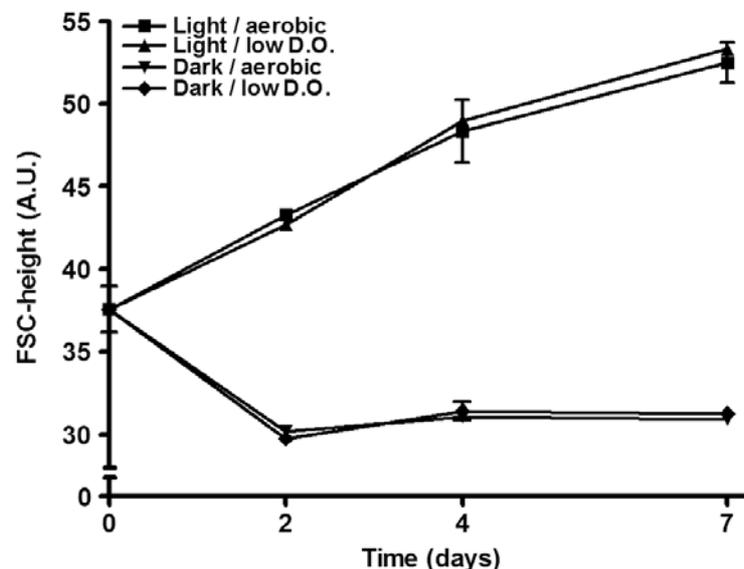
There is a recognised interconnection between physical light scatter signals (FSC and SSC) measured during FCM. This linkage between FSC and SSC has been discussed by Jochem (2000), whereby for smaller picoplanktonic algae, SSC is actually used in preference to FSC as measures of cell size. Shapiro *et al.* (2000) commented also on how FSC signal, while being largely size-dependent, also depends strongly on the surface roughness and/or internal granularity of the cells in question. This implies a probable interconnection between side- and forward-angle light scatter signals in FCM analyses and, as such, it is suggested that investigators should perhaps look at the results from FSC and SSC data in unison so that this ‘dichotomy’ in physical light scatter signals can be more insightfully interpreted in the context of the dataset at hand. This concept served as the necessary rationale for looking at the light scatter signal ratio as a means of incorporating this inherent signal overlap into the final analysis.

To illustrate this FSC–SSC interconnection, results from the current research showed that a decrease in FSC signal height (i.e. relative cell size) was always accompanied by a corresponding decrease in SSC signal amplitude and vice versa. Such was the extent of the correlation, that a highly significant statistical relationship was found between the FSC and SSC signals of healthy cells in both the two month (*C. vulgaris*; Pearson  $r = 0.981$ ;  $n = 72$ ;  $p < 0.0001$ ; and *C. reinhardtii*; Pearson  $r = 0.962$ ;  $n = 72$ ;  $p < 0.0001$ ) and the latter 7 day (*C. vulgaris* Pearson  $r = 0.973$ ;  $n = 48$ ;  $p < 0.0001$ ) dark-survival experiments. This significant interrelationship between cellular physical light scatter properties reflects the ‘duality’ of these two physical light scatter signals during flow cytometric analysis. This finding complements the previous observations by Shapiro *et al.* (2000), whereby FSC signal is susceptible to be influenced by morphological characteristics generally associated with defining the corresponding SSC signal (i.e. cell surface texture and/or internal granularity). A few questions arise from these findings, such as: “Which parameter is distorting or influencing the other, or is it mutually interactive?”; and “How then could one best interpret data from physical light scatter signals?” It is thought that perhaps by using the ratio of one parameter to the other (e.g. FSC:SSC) one could possibly accounting for apparent the co-variation in one signal as a direct result of the other.

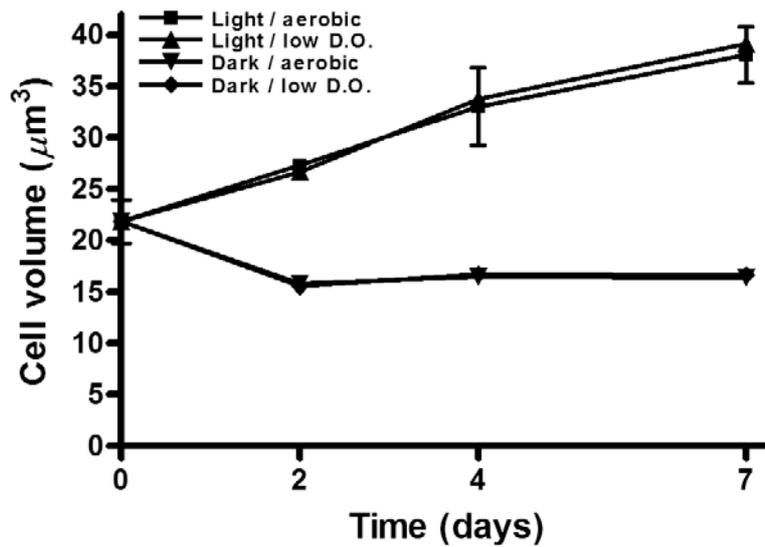
Shapiro *et al.* (2000) also suggested that, unlike cytometric *fluorescence* signals, quantitative cytometric measurements of physical light scatter (i.e. FSC and SSC) are far from being ‘standardised’; most likely due to the inherent capacity for signal distortion resulting from the natural heterogeneity of cellular ultrastructure. This emphasizes again the need for vigilant interpretations of FSC and SSC data from flow cytometric analyses, and investigators should exercise due caution when attempting to directly apply quantitative light scatter data from external research to their own, especially when trying to draw quantitative conclusions or parallels between the two.

### 9.6.3.5 Prolonged darkness and phytoplankton cell size and intracellular density: results from the 7 day experiment

Follow-up one week dark-survival experiments were conducted in order to further distill the time kinetics of dark-acclimation between days zero and 7 of the 65 day experiment. Once again, FSC and SSC signals were monitored during the course of 7 day dark-incubation to identify short-term changes in both size and intracellular density following dark-exposure of *C. vulgaris*, with results shown below.



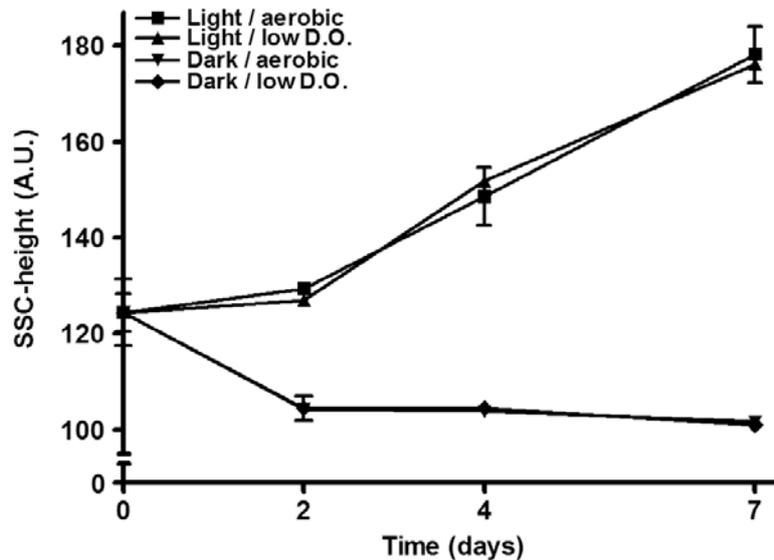
**Figure 9.17.** 7 day *C. vulgaris* FSC signal amplitude for all 4 experimental treatments (data points show the mean of 3 replicate cultures  $\pm$  1 S.D).



**Figure 9.18.** 7 day *C. vulgaris* cell volume ( $\mu\text{m}^3$ ) for all 4 treatments (data points show the population average of triplicate cultures  $\pm$  1 S.D).

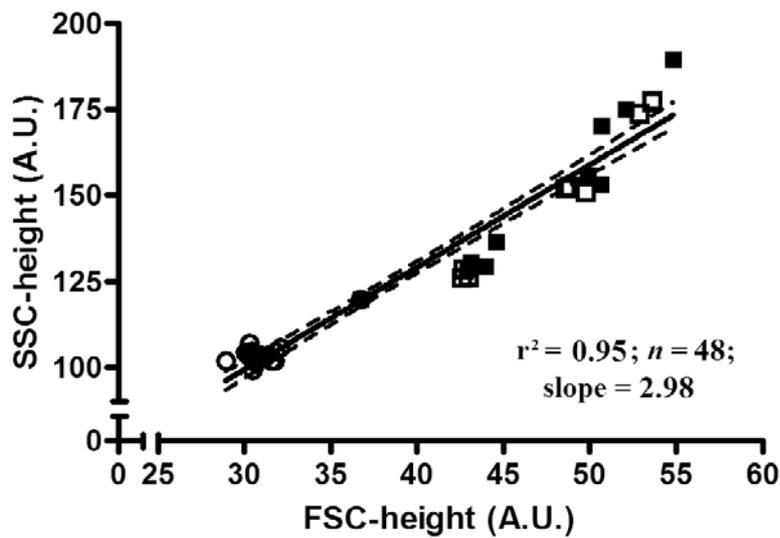
In line with the above results from the 65 day experiment, average cell volumes for both ‘light’ treatments increased significantly during 7 days of continuous culture illumination (1-way ANOVA;  $F_{(6,14)} = 30.79$ ;  $p < 0.001$ ). Unlike the long-term results of the 65 day experiment, however, the previously ailing 'light / low D.O.' treatment cultures now had a similar cell size distribution to that of the 'light / aerobic' treatment at Day 7 ( $p > 0.05$ ). According to Figure 9.18, the average cell volume for both ‘dark’ treatments decreased rapidly and significantly by some 25% (1-way ANOVA;  $F_{(6,14)} = 17.42$ ;  $p < 0.001$ ) following the initial two days of dark-exposure. The data shown in both Figures 9.17 and 9.18, however, provide further backing to the Day zero culture inoculum ‘carryover’ idea proposed earlier in Section 9.6.3.3. As can be seen in the above two Figures, it appears that again the overwhelming and significant majority of the reduction in cell FSC signal amplitude (and corresponding cell volume) for ‘dark’ treatments is realised between ‘Day zero’ and the first sampling interval (in this case Day 2), after which time average cell volume remained unchanged for both dark treatments ( $p > 0.05$ ). Initial cell volume reduction between Day zero and Day 7 for the 65 day experiment was  $\approx 20\%$ ; a figure very similar to the approximate 25% volume reduction recorded here for the follow-up 7 day dark-survival experiment. From this, it is concluded that no significant reduction in mean cell volume was observed as a result of 7 day dark-exposure, and also that DO concentration ( $8.1\text{mg L}^{-1}$  ‘ambient’ or  $2.2\text{mg L}^{-1}$  ‘low’) had no significant effect on cell volume during dark conditions.

Practically identical results were seen for SSC signal amplitudes (and presumably the corresponding intracellular densities) during the 7 day experiment, with results shown in Figure 9.19 below.



**Figure 9.19.** 7 day *C. vulgaris* SSC signal amplitude for all 4 treatments (data points show the mean of 3 replicate cultures  $\pm$  1 S.D).

Once again, both of the ‘light’ treatment SSC signal heights significantly increased over the 7 day experimental duration (1-way ANOVA;  $F_{(6,14)} = 40.11$ ;  $p < 0.001$ ); most likely a result of normal energetic cellular growth in the light conditions. Similarly, both ‘dark’ treatments (regardless of DO concentration) resulted in significant reductions ( $\approx 20\%$ ) in SSC signal amplitudes over the first two days of darkness (1-way ANOVA;  $F_{(6,14)} = 22.73$ ;  $p < 0.001$ ) and no significant change thereafter ( $p > 0.05$ ). Assuming that this two-day drop in FCM-quantified SSC signals was a manifestation of inoculum carryover (as discussed previously), it is concluded that the intracellular structure of *C. vulgaris* was not significantly influenced by 7 days of dark-exposure.



**Figure 9.20.** *C. vulgaris* 7 day FSC vs. SSC signal amplitudes from: 'light / aerobic' (■); 'light / low D.O.' (□); 'dark / aerobic' (●); and 'dark / low D.O.' (○) treatments. Fitted regression line (solid) shown with 95% CI's (broken lines). Pooled regression slope was significantly non-zero ( $p < 0.0001$ ).

Statistical analysis of the data from Figure 9.20 revealed that that there was no significant difference between the slopes (ANCOVA;  $F_{(3,40)} = 1.236$ ;  $p = 0.309$ ), elevations ( $F_{(3,43)} = 2.46$ ;  $p = 0.076$ ) or intercepts of the individual fitted regression lines for FSC versus SSC signals of all four treatments; hence the individual regression lines have been pooled into a single fitted line. This serves merely in this instance to illustrate again that the dark-incubated cells (lower left quadrant of Figure 9.20), although they are spatially separated from the 'light' treatments on the above plot, still adhere to the generic cellular 'size-to-density ratio' one may expect to find in any normal population. From this, it can be concluded that the FSC:SSC ratios of all experimental treatments were effectively the same, such that cellular size-to-density ratio remained relatively constant regardless of experimental treatment conditions during the final 7 day dark-survival experiment.

### **9.6.3.6 The effects of darkness on cell volume and intracellular density—ecological implications for phytoplankton sinking velocity**

Most of the chemical constituents which make up the protoplasm of living algal cells are heavier than water (Walsby and Reynolds, 1980). The sinking velocity of a suspended planktonic cell, therefore, depends upon the difference in density between the cell and its

surrounding aqueous medium. Phytoplankton intracellular density (and corresponding settling velocity) is largely dictated by its chemical composition. The relative proportions of more dense (protein and carbohydrate) and less dense (lipid) molecular constituents, as well as the quantities of heavier ( $\text{SO}_4^{2-}$ ) and lighter ( $\text{NH}_4^+$ ) ionic accumulations within the vacuole, will ultimately define a cell's sinking velocity (Smayda, 1970; Smayda, 1974; Fisher *et al.*, 1996).

Prezlin *et al* (1991), referencing the earlier work of Walsby and Reynolds (1980) and Sze (1986), discussed how many non-motile phytoplankton can physiologically adjust their relative (intracellular) density to enable themselves to regulate their vertical position in the water column (i.e. 'float' or 'sink'). An ability to do this would confer obvious advantageous for the cells in question when it comes to things like chemotaxis (e.g. following a nutrient gradient) or phototaxis (e.g. avoiding inhibiting or limiting light intensities). Although direct 'gravimetric' determinations of cell density were not made, SSC signals derived from FCM (as a surrogate measure of intracellular structural density) could perhaps be used to make inferences about changes to phytoplankton settling velocities following prolonged darkness. Considering this information then, results presented here suggest that active cytological adjustment of intracellular (structural) density as a result of dark-exposure was not a significant photoacclimation response for either *C. vulgaris* or *C. reinhardtii*. Based on this apparent lack of intracellular modification, no decrease in settling velocity would be expected to result from long-term dark-exposure in these algal species.

According to Stokes' law, the sinking velocity of inert particles in quiescent suspension increases quadratically with size. For phytoplankton, sinking velocity (for cells more dense than the surrounding water) is known to increase in direct proportion to cellular radius (i.e. 10-fold increase in cell radius results in 10-fold increase in sinking velocity; Raven and Kübler, 2002). According to these empirical laws, a small decrease in cell size (radius) confers an obvious advantage in terms of its corresponding decrease in sinking velocity and reduced loss rate from the pelagic zone to benthic sediments (Reynolds, 1984). Phytoplankton cell volume, and corresponding sinking velocity, therefore has wide-ranging ecological implications in terms of sinking losses and phytoplankton periodicity and species succession *in situ*. For a more in depth discussion

of this complex subject, the reader is directed to relevant and comprehensive reviews by Smayda (1970) and Reynolds (1984).

This 'size versus sinking velocity' effect is also potentially magnified by exposing phytoplankton cells to non-optimal growth conditions (Smayda, 1970), such that alterations in cell size (and hence sinking velocity) during non-optimal *dark* conditions could be expected to be of even greater consequence for resident phytoplankton (in terms of whether they will ultimately 'float' or 'sink'). During the course of prolonged dark-exposure here, Day-zero-relative algal cell volume decreased steadily by  $\approx 35\%$  for *C. vulgaris* at Day 65 compared with an average of  $\approx 41\%$  for *C. reinhardtii* after the same time. Taking into account the inoculum carryover effect of Section 9.6.3.3, cell volume still decreased significantly from Day 7 to 65 by  $\approx 20\%$  for 'dark' treatment *C. vulgaris* and *C. reinhardtii*. Given the direct linear relationship between cell size (radius) and sinking velocity (Raven and Kübler, 2002), this cellular shrinkage could have obvious implications for the cells' sinking velocities. Using this relationship of Raven and Kübler (2002), and from Day 7 onwards, the sinking velocities of both *C. vulgaris* and *C. reinhardtii* could be expected to have decreased by some 7–8% following two months of continuous darkness. Whether the observed long-term reduction in cell size (from Day 7 to 65) comes from an *active* physiological 'dark-acclimation' response mechanism, or simply through *passive* cellular resource consumption and subsequent biovolume reduction, there is a clearly advantageous ecological incentive for dark-exposed cells to reduce their sinking rate (by whatever means) in terms of limiting their rate of loss to sediments.

Smayda (1970) discusses how cellular aggregation during senescence (e.g. like that seen in the diatom *Nitzschia closterium*) is a recognised factor contributing to an increased settling velocity in some senescent phytoplankton. This is of particular practical relevance, given that Fisher *et al.* (1996) deemed the sinking rates of cell aggregates to be of greater ecological importance than the sinking rates of discrete single-cells. Similar to Smayda (1970), Titman and Kilham (1976) also reported that cellular clumping during culture had a significant effect on increasing the sinking rate of diatom (*Cyclotella*) species. If there was indeed some culture aggregation or 'clumping' in *C. reinhardtii* here, then it appears that dark-incubation served to protect these cells from

any adverse effects resulting from ‘biofloculation’ and an increased settling velocity. As shown in the FCM cytograms of Figure 9.24 (Section 9.7.1), ‘dark’ treated *C. reinhardtii* cultures retained more closely their uniform Day zero cell size distributions (FSC- versus SSC-amplitude), whereas ‘light / aerobic’ treatment cultures contained a considerably greater proportion of ‘dead’ cells and generally had a less uniform and more fragmented population distribution. Dark-exposure would, therefore, not be expected to result in a significantly increased sinking velocity for either *C. vulgaris* or *C. reinhardtii* as a result of enhanced rates of cellular aggregation. For a further discussion of these and some additional issues as they relate to the applied context of the current research, the reader is referred to Section 9.15.

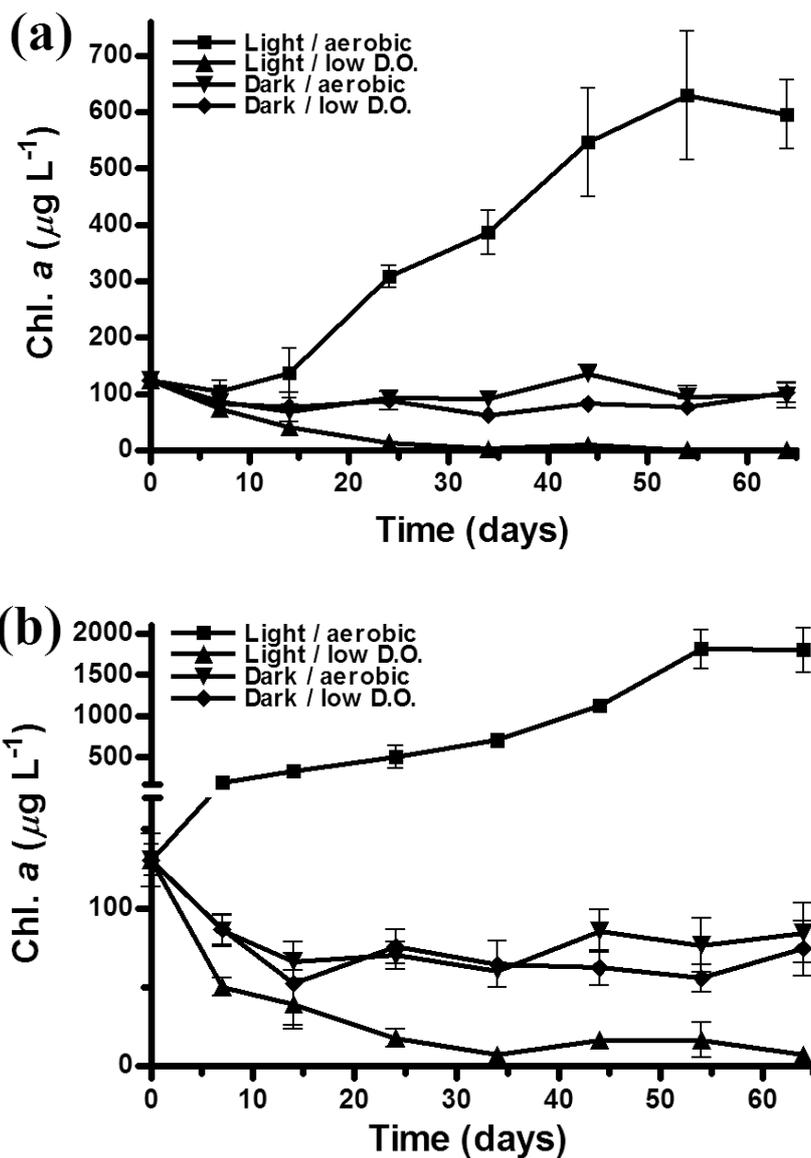
## **9.7 Darkness and phytoplankton photosynthesis— photosynthetic pigments and chlorophyll a fluorescence activity**

### **9.7.1 Dark-survival and cellular chlorophyll a: results from the 65 day experiment**

The quantity of photosynthetic cellular pigment (e.g. chlorophyll *a*) possessed by a given algal cell is inextricably linked to the cell’s photophysiological requirement for inorganic carbon and the corresponding availability of necessary light energy required for its fixation. Although there has been much prior research effort attempting to define the absolute chlorophyll *a* contents of freshwater phytoplankton, these quantities are known to be so variable that they have little value when applied by themselves (Reynolds, 2006). Changes in cellular chlorophyll content can occur in direct response to changes in light climate, thereby enabling phytoplankton to compensate for changes (increase or decrease) in incident light intensity by optimising the ability of their photosystem ‘machinery’ to harvest the available light (Falkowski and Owens, 1980). This form of photosynthetic acclimation is both complex in strategy and varied in nature of response (especially between species) and so at this point, the reader is again directed to several external reviews for a more in depth understanding (Harris, 1978; Richardson *et al.*, 1983; Falkowski and LaRoche, 1991; Prézelin *et al.*, 1991).



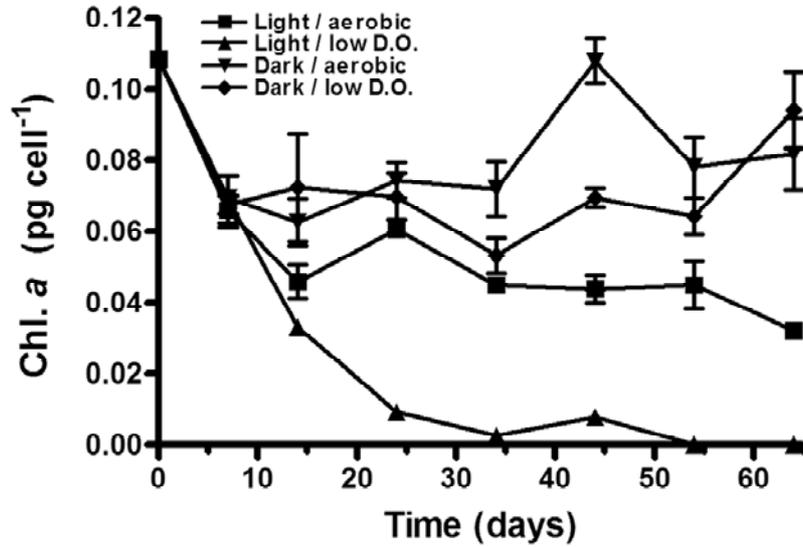
Cellular chlorophyll *a* was quantitatively monitored during the course of the two month dark-survival experiment in order to identify potential acclimation-type alterations in cellular pigments during the course of, and following, prolonged dark-exposure in *C. vulgaris* and *C. reinhardtii*. Results of these analyses are presented and discussed below.



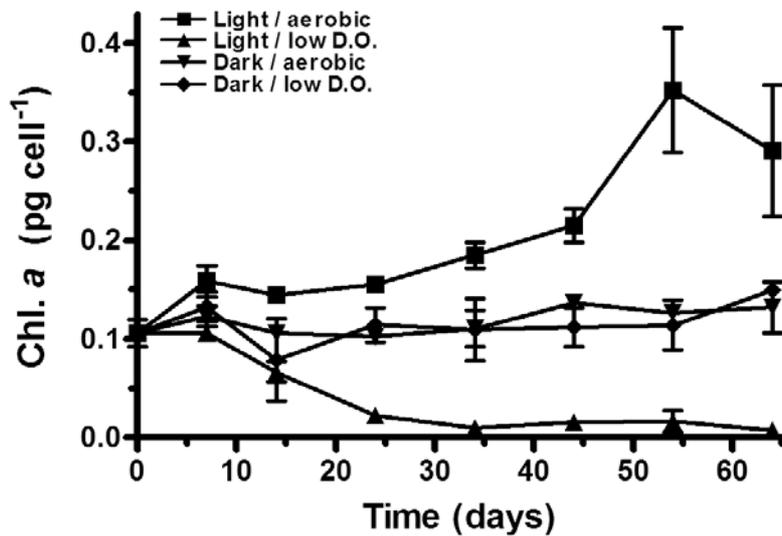
**Figure 9.21.** Two month aqueous chlorophyll *a* concentration for *C. vulgaris* (a) and *C. reinhardtii* (b) across all experimental treatments (data points show the mean of triplicate culture determinations  $\pm$  1 S.D.)

Total aqueous chlorophyll *a* measurements from Figure 9.21 paint a somewhat misleading picture of the *actual* cellular chlorophyll *a* dynamics during the two month

experiment. In order to determine the changes in pigment concentration on a cellular level, the above data was normalised according to the corresponding daily population cell densities, with results of this data transformation shown in Figures 9.22 and 9.23 below.



**Figure 9.22.** Two month chlorophyll *a* per-cell dynamics for *C. vulgaris* across the four experimental treatments (data points show the mean of triplicate algal cultures  $\pm$  1 S.D.).



**Figure 9.23.** Two month chlorophyll *a* per-cell dynamics for *C. reinhardtii* across all four experimental treatments (data points show the mean of triplicate cultures  $\pm$  1 S.D.).

Long-term data from Figure 9.22 showed that prolonged darkness did not result in a significant change to the levels of cellular chlorophyll *a* for *C. vulgaris*, with both ‘dark’

treatments showing 'Day 65' cellular chlorophyll levels similar to that at 'Day zero' (1-way ANOVA;  $F_{(4, 10)} = 46.34$ ;  $p > 0.05$ ). The same could not be said for the two 'light' treatments, however, with both displaying significantly reduced per-cell chlorophyll *a* levels following two months of continuous illumination ( $p < 0.001$ ); although the 'light / low D.O.' treatment cells had significantly less cellular chlorophyll than those of the 'light / aerobic' treatment ( $p < 0.05$ ).

Loss of cellular pigment is a recognised feature of algal senescence in ageing illuminated cultures (Franklin *et al.*, 2004) and warrants no further discussion in the context of this dark-survival work. Long-term 'dark' treatment, regardless of DO concentration, yielded similar results for *C. vulgaris* (1-way RM-ANOVA;  $F_{(3,6)} = 17.37$ ;  $p > 0.05$ ), with the observed 'spike' at Day 44 a likely manifestation of the later discovered variability in the efficacy of chlorophyll pigment extraction in acetone for *C. vulgaris* (this was probably also responsible for the apparent 'see-sawing' of chlorophyll *a* results over time in Figure 9.22). Falkowski and Owens (1980) did discuss, however, that chlorophyll metabolism is "highly dynamic" in some species, implying that changes in cellular pigment content can occur within relatively short time scales. Whilst absolute cell chlorophyll *a* concentrations were likely to have been slightly underestimated in the current work (through incomplete pigment extraction in 90% acetone), the *relative* long-term changes and also the differences between treatments were assumed to have been exposed to a constant level of error and should therefore have remained unaffected. It was later determined that a more complete chlorophyll *a* extraction was achieved without maceration using 100% dimethyl sulfoxide (similar to the findings of Speziale *et al.*, 1984) and so at this point it would be recommended that this solvent be used for any future pigment extractions involving either *C. vulgaris* or *C. reinhardtii*.

In similar fashion to that of *C. vulgaris*, data from Figure 9.23 showed that prolonged darkness did not result in any significant change to the levels of cellular chlorophyll *a* for cultures of *C. reinhardtii*, with both 'dark' treatments again showing Day 65 cellular chlorophyll levels similar to that at Day zero (1-way ANOVA;  $F_{(4, 10)} = 11.05$ ;  $p > 0.05$ ). Long-term 'dark' treatment, regardless of DO concentration, again yielded similar

results with respect to cellular chlorophyll *a* levels over the course of the 65 day experiment for *C. reinhardtii* (1-way RM-ANOVA;  $F_{(3,6)} = 18.02$ ;  $p > 0.05$ ).

Published results from others' assessments of cellular chlorophyll *a* content following prolonged darkness are far from unified. Yentsch and Reichert (1963) reported an overall increase in cellular chlorophyll *a* for dark-exposed *Dunaliella tertiolecta* (Prasinophyceae) following 5 days of darkness; although the trend was somewhat erratic (likely due to measurement and/or operator error). Hellebust and Terborgh (1967), again following dark-exposure in *D. tertiolecta*, found that although photosynthetic rates and activities diminished by in excess of 90% of initial values, cellular chlorophyll content and population cell density remained unaffected by 7 days of darkness. Deventer and Heckman (1996) observed a similar trend, reporting significantly greater conservation of cellular chlorophyll *a* in long-term (43 day) dark-exposed *Scenedesmus quadricauda* (Chlorophyceae) compared with statically ageing cultures kept for the same duration in the light, with the authors reporting no significant long-term change in cellular chlorophyll *a* concentration during the prolonged dark event. Bunt and Lee (1972), reporting on the long-term (3-month) dark-survival of a variety of marine Antarctic sea-ice phytoplankton, observed no major change in pigment (chlorophyll *a*) concentrations relative to cellular carbon for two diatoms and one unidentified Chlamydomonad. Wolfe *et al.* (2002) reported also that the per-cell chlorophyll *a* content of *Emiliana huxleyi* when transferred into darkness showed no real trends over time (i.e. it was relatively stable during the dark period). The findings of these studies were all very similar to those of the current research, and they serve to highlight the apparent 'dark conservation' of cellular photosynthetic pigments in some phytoplankton species.

Steeman Nielsen and Jørgensen (1968) were the first to report the so-called "photophysiological adaptational" response (now known as 'photoacclimation') in phytoplankton, discovering that both *Chlorella* and *Chlamydomonas* species displayed photophysiological acclimation responses to changes in light intensity. The authors reported significant increases in cellular chlorophyll *a* concentration with decreasing light intensity (down to compensation point) for *Chlorella* species; although this trend was not seen here (probably due to the absence of light altogether). Steeman Nielsen and Jørgensen (1968) also discussed, however, that this was not a universal acclimation

response phenomenon, with the diatom *Cyclotella meneghiniana* (Coscinodiscophyceae) maintaining constant levels of cellular chlorophyll when grown at both ‘high’ and ‘low’ light intensities (*C. meneghiniana* instead photoacclimates to differing light intensities by adjusting the maximal rate of its photosynthetic processes).

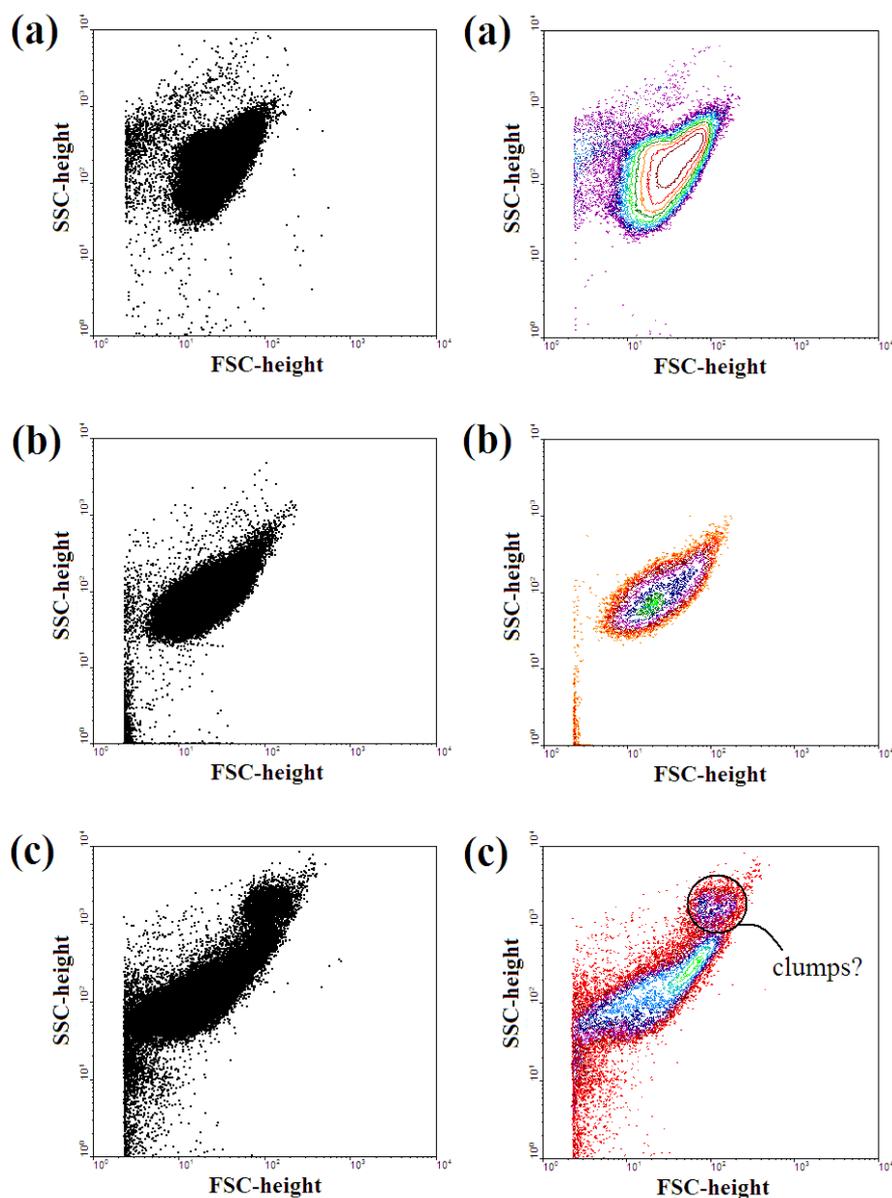
Results of Karlander and Krauss (1966b) provided further weight to the non-universal nature of photoacclimation responses, by reporting that the chlorophyll content of dark-incubated *C. vulgaris* (Emerson strain) actually decreased over a period of 7 days of darkness—a result in direct contradiction to that of Steeman Nielsen and Jørgensen (1968) above. This trend for interspecific differences in photoacclimation responses was again emphasized by Falkowski and Owens (1980) for two marine phytoplankton (*Skeletonema costatum* and *D. tertiolecta*), with the fundamental differences between the two species’ strategies of photoacclimation cited as potentially relating to the separate ecological niches occupied by the two species. The fact that *C. vulgaris* and *C. reinhardtii* reacted similarly to darkness in almost every parameter measured during the current research suggests that these species may inhabit similar environmental niches and, therefore, might be expected to behave similarly to a given dark-stress event *in situ*.

Unlike the results of Figure 9.22 for *C. vulgaris*, however, *C. reinhardtii* cells in the statically ageing ‘light / aerobic’ treatment of Figure 9.23 significantly increased their levels of cellular chlorophyll following continuous illumination for 65 days ( $p < 0.05$ ), with the ‘light / low D.O.’ treatment cells again losing a significant percentage ( $\approx 95\%$ ;  $p < 0.01$ ) of their original cellular chlorophyll after the two month experimental duration. The increasing levels of cellular chlorophyll *a* in statically ageing illuminated cultures of *C. reinhardtii*, whilst secondary in importance to ‘dark’ treatment results in the context of this research, was nonetheless a curious observation, given that one might expect these cultures to lose (as was seen for *C. vulgaris*) rather than accumulate their photosynthetic pigments. As discussed briefly above, Deventer and Heckman (1996) noted that cultures of *Scenedesmus quadricauda* maintained under a constant 12:12h light:dark regime for 43 days had significantly lower levels of chlorophyll *a* (and significantly increased levels of the chlorophyll *a* breakdown product chlorophyllide *a*) than cultures maintained in continuous darkness for the same duration. The authors cited an increased severity of nutrient starvation effects in the light compared with in darkness

as a possible explanation for this effective ‘dark-protection’ of chlorophyll *a* in *S. quadricauda*. This idea of ‘dark-protection’ will be referred to again in the context of later discussion (Section 9.10).

Regarding the above results, this apparent increase in ‘light / aerobic’ treatment *C. reinhardtii* cellular chlorophyll *a* (Figure 9.23) was not considered to be a real phenomenon; rather, it was thought that there were several sources of error involved in the quantitation of photosynthetic pigment. As can be seen in Figure 9.23, both the *quantity* and also the *variability* of chlorophyll *a* appear to increase with increasing *C. reinhardtii* culture age. Evidence for this large-scale variability is found in the fact that as the ‘light / aerobic’ treatment cultures statically aged, they became increasingly more heterogeneous (compared to the more evenly dispersed homogeneous ‘Day zero’ and long-term ‘dark’ treatment cultures) and were, therefore, more difficult to analyse and ‘gate’ during FCM protocols. The extent of this culture heterogeneity can be seen in Figure 9.24.

Qualitative analysis of daily FCM cell population scatterplots showed that cellular ‘clumping’ was insignificant in all treatments for *C. vulgaris* over the 65 day study, but also that it could have been of significance for *C. reinhardtii* over the course of the two month experiment. This made it particularly difficult to perform accurate *C. reinhardtii* population cell density enumerations for ‘light / aerobic’ treatment samples and was, therefore, likely to have resulted in an underestimation of actual cell density due to FCM gating process treating any multi-cell clusters or ‘clumps’ as single-cells (see also Section 9.6.1.1). This concept can be evidenced further in the population cell density data of Section 9.6.1 (Figures 9.6 and 9.7), whereby the Day 65 population cell density for *C. reinhardtii* is some 65% lower than that of *C. vulgaris* after the same time (despite the two species having shown similar culture growth characteristics previously; Figure 9.1).



**Figure 9.24.** Cytograms from FCM analyses showing *C. reinhardtii* cultures at: day zero (a); Day 64 of 'dark / aerobic' treatment (b); and at Day 64 of 'light / aerobic' treatment (c). Figures on the left hand side show 2-D FCM scatter plots, and figures on the right show 2-D contour plots depicting relative cell population proportions from high (central) to low (outer) cell numbers ( $x$  and  $y$  axes show  $\log_{10}$  forward and side-scatter respectively).

This likely underestimation of population cell density for 'light / aerobic' treatments of *C. reinhardtii* would have resulted in an overstatement of average per-cell chlorophyll  $a$  content, especially when the culture chlorophyll  $a$  levels (Figure 9.21b) were observed to have increased some 10-fold during the 2 month experiment from an average of  $130$  to  $1800\mu\text{g L}^{-1}$ . In conclusion, it is the opinion of the author that the per-cell chlorophyll

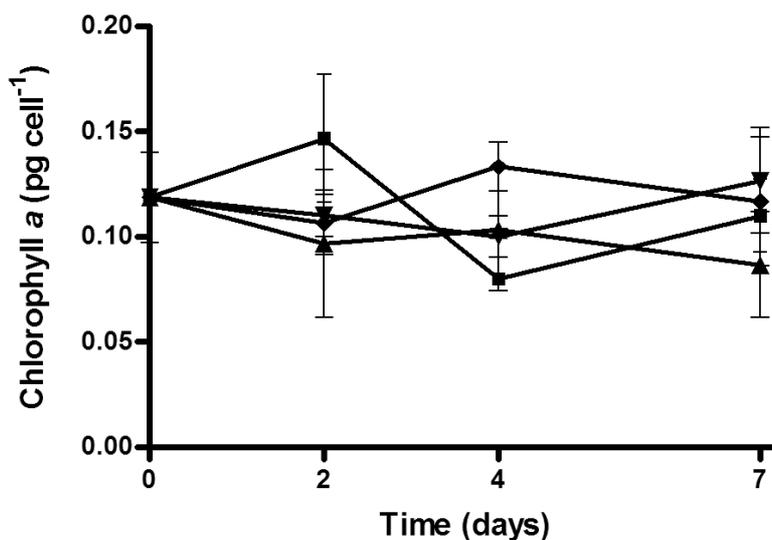
*a* levels for *C. reinhardtii* only, and only in the 'light / aerobic' treatment, were somewhat overestimated (particularly towards the latter half of the two month experiment). It should be reiterated, however, that there was no such problem for the 'dark' treatments, because those cultures appeared to have remained much more evenly dispersed and homogeneous in terms of their respective FSC versus SSC signal distributions (see Figure 9.24). This clumping artefact was, regrettably, uncontrollable and is a recognised problem associated with the use of FCM where cells are present as non-discrete entities. The author would recommend that the use of surfactants (such as sodium dodecyl sulphate or Tween<sup>®</sup>20) be trialed in future analyses with aged *C. reinhardtii* cultures as a possible means of effective cellular dispersion prior to counting via FCM.

Overall, results from chlorophyll *a* analyses showed that both algal species were equally competent at prolonged dark-maintenance of photosynthetic pigments under both ambient and 'low D.O.' conditions. This long-term dark-maintenance of cellular constituents (chlorophyll *a* in this instance) ties in with the observed long-term stability of FSC:SSC ratios for 'dark' treatment cells in both algal species (Section 9.6.3.4), suggesting again the capacity for the effective preservation of a 'healthy' cellular constitution during prolonged darkness. It was apparent that there were some issues surrounding the variable extraction efficacies of photosynthetic pigments in the acetone solvent used; however, this source of error was considered to have been applied relatively uniformly for both species and across all treatments. Cells of both algal species in the 'light / low D.O.' treatment had, after 65 days, all lost significant quantities of cellular chlorophyll *a* compared to both 'Day zero' levels and in comparison with all other treatments. It is thought that phytoplankton in this treatment were exposed to conditions of inorganic carbon limitation or starvation and that exposure to these conditions in some way resulted in elevated rates of algal cell death; as implied from the large observed losses of essential photosynthetic pigments. Further clarification of this hypothesis will be provided by the results of PI-FDA viability assessments in Section 9.8. Based on the chlorophyll *a* data, *C. reinhardtii* did appear—at least qualitatively—to be slightly more capable of surviving these conditions of DIC starvation in the light; however, both species were obviously highly susceptible to such conditions and both displayed significant ill-effects as a result.



### 9.7.2 Dark-survival and cellular chlorophyll *a*: results from the 7 day experiment

Follow-up one week dark-survival experiments were performed in order to further distill the time kinetics of dark-acclimation between Days zero and 7 of the above-discussed 65 day experiment. Once again, the levels of cellular chlorophyll *a* were monitored as part of the suite of analyses performed during the one week dark-incubation experiment. Results of the 7 day pigment analysis for *C. vulgaris* are shown in Figure 9.25.



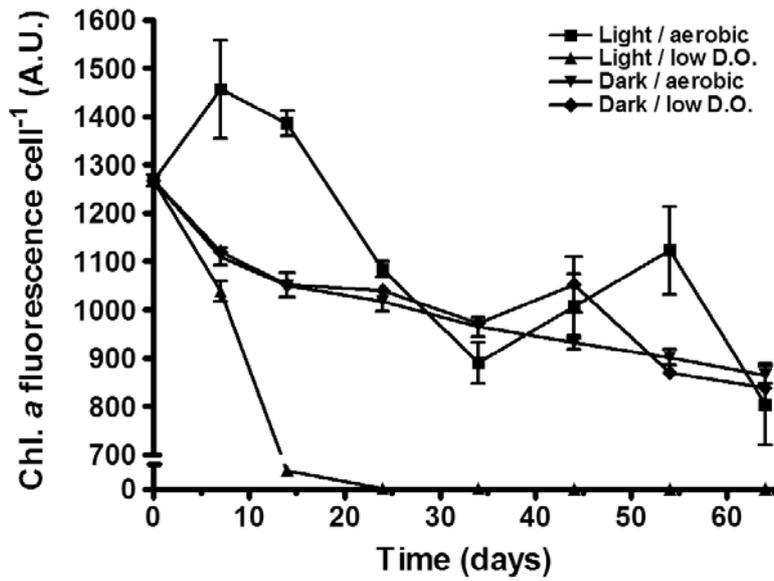
**Figure 9.25.** 7 day *C. vulgaris* chlorophyll *a* per-cell for all experimental treatments (data points show mean of three replicate cultures  $\pm$  1 S.D.).

Results from the truncated one week timescale of Figure 9.25 showed no significant change in ‘per-cell’ chlorophyll *a* levels in any treatment following the 7 day period (1-way ANOVA;  $F_{(4,10)} = 1.175$ ;  $p = 0.379$ ) and also no significant difference between the overall 7 day trends among any of the four treatments (1-way RM-ANOVA;  $F_{(3,3)} = 0.651$ ;  $p = 0.602$ ). When comparing the above 7 day results with the corresponding Figure 9.22 from the 65 day experiment, there is a distinct difference in apparent cellular chlorophyll *a* concentrations after the same 7 day experimental duration (despite having similar starting chlorophyll levels). This can only be explained by the unfortunate differential extraction efficacies of chlorophyll *a* in 90% acetone (something noted qualitatively during the course of these experiments and as discussed in Section 9.7.1 above). In spite of this, data from the follow-up 7 day dark-survival experiment does provide further backing to the conclusions of the long-term 65 day

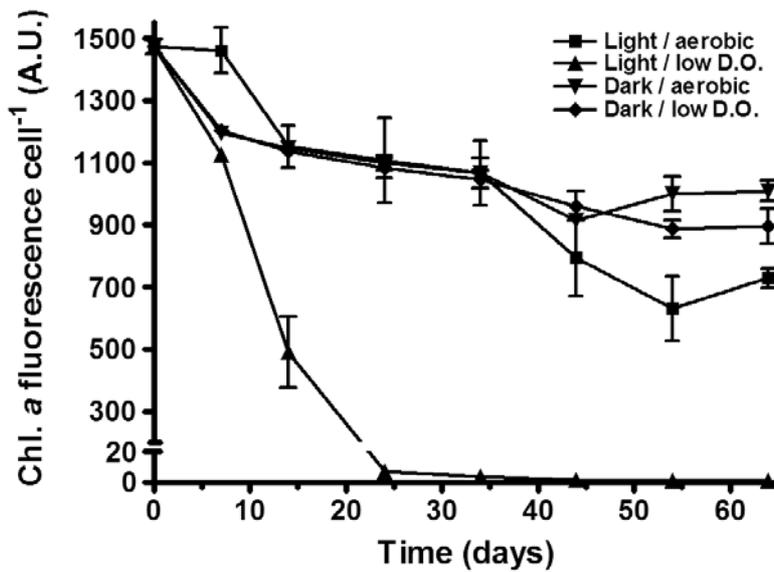
experiment, in that dark-exposure did not result in any significant change in the levels of cellular chlorophyll *a* relative to those at ‘Day zero’. From this, it is again concluded that *C. vulgaris* was sufficiently adept at enduring conditions of continuous darkness (from 7 up to 65 days), with this alga being able to successfully maintain its cellular chlorophyll at pre-dark levels for an extended period of dark-exposure.

### **9.7.3 Dark-survival and *in vivo* chlorophyll *a* fluorescence: results from the 65 day experiment**

As introduced in Section 6.4.2.2, the use of FCM for investigating chlorophyll *a* fluorescence in phytoplankton has received mixed reviews. Overall, there is good agreement within the relevant literature that FCM is generally accurate as a surrogate measure for phytoplankton biomass and, consequently, also generally correlates well with cellular chlorophyll *a* content. As was cautioned in Section 6.4.2.2, however, FCM is generally not accepted ‘across the board’ as a reliable indicator of cellular photosynthetic activity (i.e. through *in vivo* measurements of chlorophyll *a* fluorescence activity) due to the machine-specific nature of the magnitude of the chlorophyll *a* fluorescence signal in relation to the actual quantity of cellular chlorophyll; something known as the ‘package effect’ of Duysens (1956) and later of Kirk (1975a; 1975b). There were a number of reasons why these concerns were thought to be of lesser importance with respect to the valid use of FCM-quantified chlorophyll *a* fluorescence signals in this research. For a complete discussion of these issues, the reader is directed to the coming short Section 9.7.3.1. For now, however, results of the FCM-quantified chlorophyll fluorescence data from the current dark-survival investigations for *C. vulgaris* and *C. reinhardtii* will be presented and discussed.



**Figure 9.26.** Two month *C. vulgaris* chlorophyll *a* fluorescence (FCM-quantified) per-cell (note the broken *y*-axis scale). Data points show the mean of three replicate cultures ( $\pm 1$  S.D.).



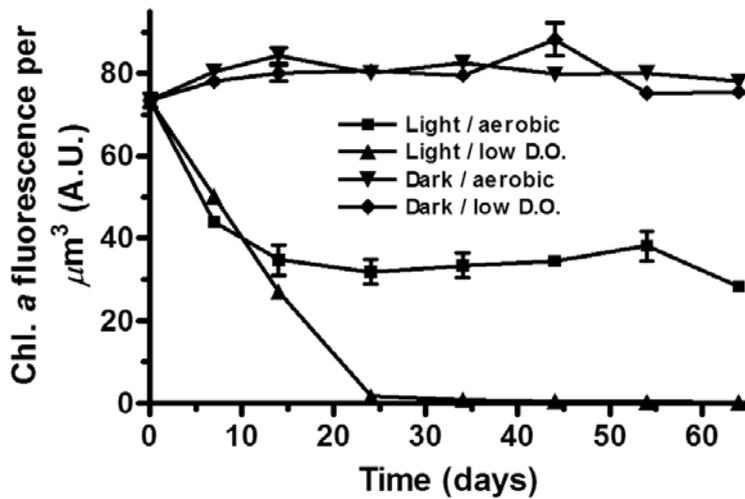
**Figure 9.27.** Two month *C. reinhardtii* chlorophyll *a* fluorescence (FCM-quantified) per-cell (note the broken *y*-axis scale). Data points show the mean of three replicate cultures ( $\pm 1$  S.D.).

Critical analysis of the data from Figures 9.26 and 9.27 showed that cell populations in both of the ‘dark’ treatments and also the statically ageing ‘light / aerobic’ treatment, all displayed similar 65 day chlorophyll *a* fluorescence activities for both *C. vulgaris* (1-way RM-ANOVA;  $F_{(3,7)} = 21.91$ ;  $p > 0.05$ ) and *C. reinhardtii* (1-way RM-ANOVA;

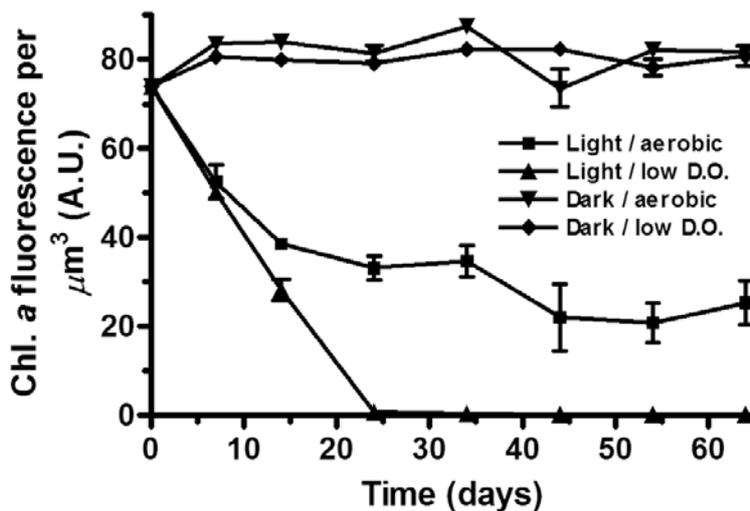
$F_{(3,7)} = 20.05$ ;  $p > 0.05$ ). Cells in all other treatments also displayed significantly higher 65 day mean cellular chlorophyll *a* fluorescence than did cells in the 'light / low D.O.' treatment for both species ( $p < 0.001$ ).

Following on from some cautions raised earlier (see Section 9.6.3.2), it was deemed prudent to normalise the chlorophyll *a* fluorescence data from Figures 9.26 and 9.27 against the corresponding mean daily cell volumes. This was in line with the earlier reporting of: Winokur (1949); Duysens (1956); of Kirk (1975a; 1975b); Alpine and Cloern (1985); Dorsey *et al.* (1989); and more recently Raven and Kübler (2002); whereby photosynthetic capacity (chlorophyll *a* quantum absorption and fluorescence efficacy) is known to vary as a function of cell volume, such that smaller cells are inherently more efficient at capturing photons than larger cells due to a decreasing chlorophyll *a*-specific absorption coefficient with increasing cell radius. This phenomenon—a manifestation of the 'self-shading' or 'package effect' as above—means that individual chlorophyll *a* molecules (assuming a constant chlorophyll *a* concentration and uniform spherical geometry) have a lower probability of absorbing incoming photons in larger unicellular phytoplankton than they do in smaller cells (Kirk, 1994; Raven and Kübler, 2002). The follow-on implications of this realisation with respect to this research are that there is an apparent necessity for expressing chlorophyll *a* (concentration and fluorescence) relative to cell size (i.e. per unit cell volume). This was emphasized by Raven and Kübler (2002) in their discussion of the need to express cellular fluorescence parameters relative to cell size, or more aptly, per unit 'cell volume'. Sosik *et al.* (1989) also showed that the 'package effect' influenced the FCM chlorophyll *a* fluorescence signals from marine diatom and dinoflagellate species; thereby reaffirming the desirability of normalising cellular chlorophyll *a* fluorescence against cell volume.

Cell-volume-normalised results from the subsequent data transformations for *C. vulgaris* and *C. reinhardtii* are provided in Figures 9.28 and 9.29 respectively.



**Figure 9.28.** Two month *C. vulgaris* chlorophyll *a* fluorescence (FCM-quantified) per unit cellular volume ( $\mu\text{m}^3$ ). Data points show the mean of triplicate cultures ( $\pm 1$  S.D.).

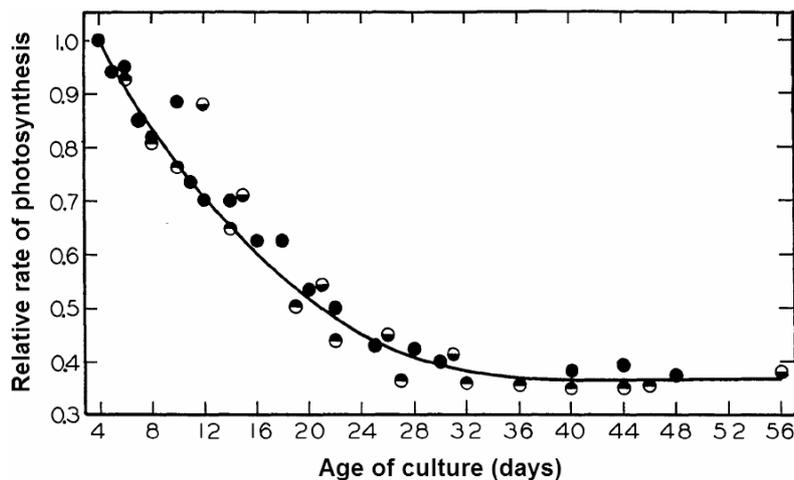


**Figure 9.29.** Two month *C. reinhardtii* chlorophyll *a* fluorescence (FCM-quantified) per unit cellular volume ( $\mu\text{m}^3$ ). Data points show the mean of triplicate cultures ( $\pm 1$  S.D.).

An initial and striking feature of the above two Figures compared with the corresponding ‘raw’ (non-volume-normalised) data of Figures 9.26 and 9.27, is the dramatic difference in the overall chlorophyll *a* fluorescence trends. Where the long-term fluorescence per data from the two ‘dark’ and the ‘light / aerobic’ treatments of *C. vulgaris* and *C. reinhardtii* were previously indistinguishable, there are now large-scale differences between the 65 day chlorophyll *a* fluorescence activities of both algal species. Statistical analysis of the overall 65 day *C. vulgaris* chlorophyll *a* fluorescence data (cell-volume-normalised) for all 4 treatments (Figure 9.28) showed that both ‘dark’

treatments displayed significantly higher levels of long-term cellular chlorophyll *a* fluorescence than all other treatments (1-way RM-ANOVA;  $F_{(3,6)} = 85.13$ ;  $p < 0.001$ ) and that both 'dark' treatments had similar long-term per-cell chlorophyll *a* fluorescence activities regardless of DO concentration ( $p > 0.05$ ). Algal cells in the 'light / aerobic' treatment also displayed significantly greater two month chlorophyll *a* fluorescence activities than algae in the 'light / low D.O.' treatment ( $p < 0.01$ ), indicating again (in line with the per-cell chlorophyll *a* pigment data of Section 9.7.1 above) that algal cells in the 'light / low D.O.' treatment were suffering significant long-term ill-effects. Analysis of the 'Day 65' cell-volume-normalised data only, showed that after two months of experimental incubation, the chlorophyll *a* fluorescence activity of the 'dark' treatments was significantly greater than that of all other treatments (1-way ANOVA;  $F_{(3,8)} = 4967$ ;  $p < 0.001$ ) and also that *C. vulgaris* in both 'dark' treatments had similar per-cell chlorophyll *a* fluorescence regardless of aqueous oxygen concentration.

It is worth mentioning briefly, that the observed trend for a steady reduction in chlorophyll *a* fluorescence during static culture ageing in 'light / aerobic' *C. vulgaris* cultures (Figure 9.28), displays a similar pattern (in terms of both magnitude and kinetics) to the gradual loss of relative photosynthetic rate noted by Pratt (1943) during illuminated ageing of *C. vulgaris* over a similar timescale (Figure 9.30).



**Figure 9.30.** Relative rate of photosynthesis in continuously illuminated ageing cultures of *C. vulgaris* over various culture ages (modified from Pratt, 1943).

Similarly, analysis of the two-month *C. reinhardtii* chlorophyll *a* fluorescence data (cell-volume-normalised) for the 4 experimental treatments (Figure 9.29) showed again that both ‘dark’ treatments showed significantly higher long-term cellular chlorophyll *a* fluorescence than all other treatments (1-way RM-ANOVA;  $F_{(3,6)} = 85.13$ ;  $p < 0.001$ ) and that both ‘dark’ treatments (regardless of DO concentration) had similar long-term per-cell chlorophyll *a* fluorescence activities ( $p > 0.05$ ). Algal cells in the 'light / aerobic' treatment (as seen for *C. vulgaris* above) displayed significantly greater two month chlorophyll *a* fluorescence than algae in the 'light / low D.O.' treatment ( $p < 0.01$ ). Analysis of the ‘Day 65’ cell-volume-normalised data for *C. reinhardtii* yielded identical statistical trends to those reported above for *C. vulgaris*. Following two months of experimental incubation, the Day 65 chlorophyll *a* fluorescence activity of the ‘dark’ treatments was significantly greater than that of all other treatments (1-way ANOVA;  $F_{(3,8)} = 637.8$ ;  $p < 0.001$ ), and again, *C. reinhardtii* in both ‘dark’ treatments displayed similar per-cell chlorophyll *a* fluorescence regardless of DO concentration. These treatment differences between the chlorophyll *a* fluorescence activities of both *C. vulgaris* and *C. reinhardtii* following the current two month dark-survival experiment can easily visualised in Table 9.2 below.

**Table 9.2.** Statistical significance tables for two month chlorophyll *a* cellular fluorescence (normalised to cell volume;  $\mu\text{m}^{-3}$ ) of *C. vulgaris* and *C. reinhardtii* for all treatments (1-way RM-ANOVA with Tukey’s multiple comparisons). Shading shows level of significant difference between treatment means: no shading signifies no difference ( $p > 0.05$ ); medium shading shows significance at  $p < 0.01$ ; and black shading indicates a significant difference at  $p < 0.001$ .

<b><i>C. vulgaris</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$F_{(3,6)} = 85.13$			
Dark / aerobic	$F_{(3,6)} = 85.13$	$F_{(3,6)} = 85.13$		
Dark / low D.O.	$F_{(3,6)} = 85.13$	$F_{(3,6)} = 85.13$	ns	

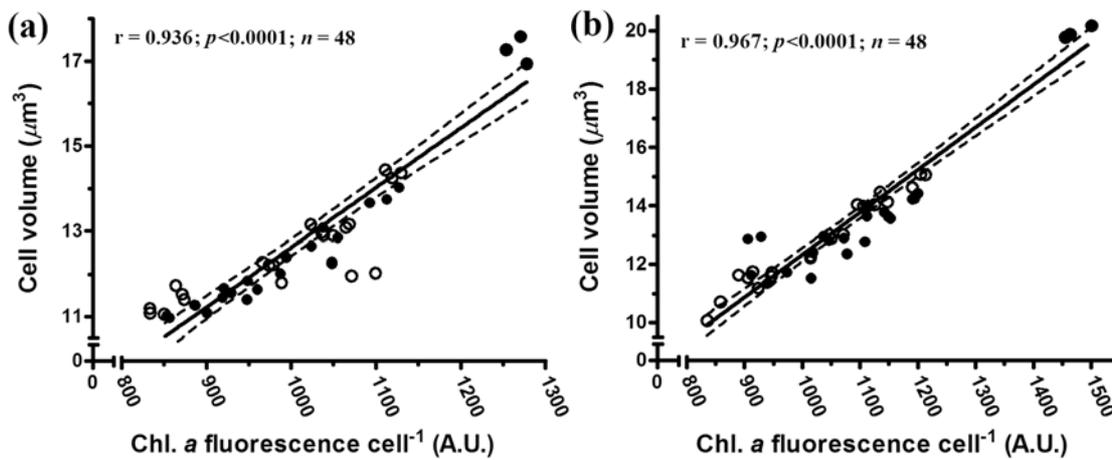
  

<b><i>C. reinhardtii</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$F_{(3,6)} = 91.07$			
Dark / aerobic	$F_{(3,6)} = 91.07$	$F_{(3,6)} = 91.07$		
Dark / low D.O.	$F_{(3,6)} = 91.07$	$F_{(3,6)} = 91.07$	ns	

Many researchers have found that the magnitude of *in vivo* chlorophyll fluorescence (per unit cell volume or normalised to cellular chlorophyll *a*) varies according to cellular photosynthetic capacity and physiological state of both the chloroplast and cell as a whole (Krause *et al.*, 1982; Krause and Weis, 1984; Alpine and Cloern, 1985; Neale *et al.*, 1989). Popels and Hutchins (2002) reported that the photosynthetic capabilities of the alga *Aureococcus anophagefferens* (Pelagophyceae) resumed quickly following 30 days of dark-exposure, suggesting that the photosynthetic apparatus remained intact during the prolonged dark period—a trend also reported by other authors (Tilzer *et al.*, 1977; Platt *et al.*, 1983, Dehning and Tilzer, 1989). This trend was also quantitatively observed for the two algal species reported here, with both *C. vulgaris* and *C. reinhardtii* retaining pre-dark photosystem activities (as determined by cell-volume-relative *in vivo* chlorophyll *a* fluorescence activities) even after 65 days of continuous darkness.

It is clear from comparisons of the above ‘raw’ and ‘cell-volume-normalised’ chlorophyll *a* fluorescence data (Figures 9.26 to 9.29) that including the effects of a diminishing cell volume in the final analysis yields a markedly different overall picture about the capacities of the two algal species in terms of their prolonged dark-survival. Although dark-exposed phytoplankton cells had a somewhat diminished ( $\approx 30\%$ ) long-term gross chlorophyll *a* fluorescence activity compared with pre-dark levels, it was—on a per-cell basis—no different to that seen in statically ageing cultures under continuous illumination for the same period. In fact, when the necessary effects of cell size dynamics were factored into the long-term data, dark-exposed algal cells of both *C. vulgaris* and *C. reinhardtii* were significantly ‘better off’ (in terms of being able to conserve their ‘Day zero’ chlorophyll fluorescence activities) than their light-incubated counterparts; something supported by their rapid population re-growth following re-illumination of the ‘dark’ treatment cultures (see Section 9.9.1). These results also tie in well with the previously discussed concept of ‘dark conservation’ of cellular chlorophyll *a* (see Section 9.7.1). The extent to which changes in cell volume influenced chlorophyll *a* fluorescence signals, was also evidenced by the good correlations between cell size and recorded chlorophyll *a* fluorescence values for ‘dark’ treatments during FCM analyses (Figure 9.31).





**Figure 9.31.** 65 day cell volume vs. chlorophyll *a* fluorescence for *C. vulgaris* (a) and *C. reinhardtii* (b) for 'dark / aerobic' (●); and 'dark / low D.O.' (○) treatments (showing Pearson's correlation coefficient (*r*) and fitted regression lines  $\pm$  95% CI's).

Figure 9.31 serves to highlight again the previously discussed necessity for normalising cellular fluorescence signals to cell volume. The fact that cell size and chlorophyll *a* fluorescence activity were directly correlated during FCM analysis, implies that the recorded fluorescence signal varied as a direct consequence of cell size—a trend reported on by numerous other authors (Demers *et al.*, 1989; Zettler *et al.*, 1996; Olson *et al.*, 2000). This observation was perhaps unsurprising, given that a direct linear relationship between cell size (volume) and cellular chlorophyll *a* content ( $\text{pg cell}^{-1}$ ) has been reported elsewhere (Reynolds, 2006). Although chlorophyll *a* fluorescence signal (and photon absorption efficiency) varies non-linearly with cell volume (Raven and Kübler, 2002), for the purposes of this work, a linear relationship was assumed in light of the relatively small algal cell volumes across all treatments ( $\leq 40\mu\text{m}^3$ ) and relatively small magnitude ( $\approx 35\%$ ) cell volume changes observed during the current work. Moreover, the fact that light absorption efficiency (and presumably the resulting fluorescence signal) increases non-linearly with decreasing cell volume (Raven and Kübler, 2002)—such that smaller cells are inherently more efficient at capturing and presumably re-emitting photons (Kirk, 1975; Alpine and Cloern, 1985)—should mean that normalising cellular fluorescence signals to cell volume in shrinking cells (as was observed for 'dark' treatments here) is more likely to yield *conservative* cell-volume-relative fluorescence estimates rather than overstating them.

In the context of the current results, whilst prolonged darkness has indeed resulted in reduced cellular chlorophyll *a* fluorescence activities (and implied photosynthetic activity), it also appears that this reduction in chlorophyll fluorescence activity has not been ‘disproportionately high’ relative to the corresponding reduction in cell volume recorded over the same period. These ‘fluorescence versus cell volume’ issues are both complex in nature and difficult to standardise, with their interpretation representing a significant challenge for future research. This issue will again be discussed in Section 9.8.

### **9.7.3.1 Validation of flow cytometric chlorophyll *a* fluorescence measurements**

With respect to the current research, and as introduced in Section 9.7.3, there were some initial concerns surrounding the proper use of FCM-quantified chlorophyll *a* fluorescence data in the context of assessing phytoplankton photophysiological viability following dark-exposure. These concerns were indeed assessed and were subsequently found to be of lesser importance for a number of reasons:

1. Due to the relatively low excitation energy (15mW) (Neale *et al.*, 1989; Olson and Zettler, 1995) of the laser illumination source and intermediary sheath fluid stream flow rate ( $\approx 30\mu\text{l min}^{-1}$ ), phytoplankton cells should have been exposed to a relatively low-intensity excitation source for a relatively short duration. This means that the current FACScan cytometer was likely to have been measuring an intermediary of chlorophyll *a* fluorescence somewhere between  $F_0$  and  $F_m$  (i.e. neither minimum or maximum PS-II yield; see Section 6.4.2.2 for more information) such that the measured algal *in vivo* chlorophyll *a* fluorescence signal should be neither light-limited (low resolution) nor over-saturated (low sensitivity).
2. Because the FCM-quantified chlorophyll *a* fluorescence signal (cell-volume-normalised) correlated well with cellular chlorophyll *a* content (cell-volume-normalised) for both *C. vulgaris* (Spearman  $r_s = 0.760$ ;  $n = 96$ ;  $p < 0.0001$ ) and *C. reinhardtii* (Spearman  $r_s = 0.673$ ;  $n = 96$ ;  $p < 0.0001$ ) during the course of the two month dark-survival experiment. Additionally, because *in vivo* chlorophyll *a* signals were normalised to cellular chlorophyll *a* content, it was thought that this

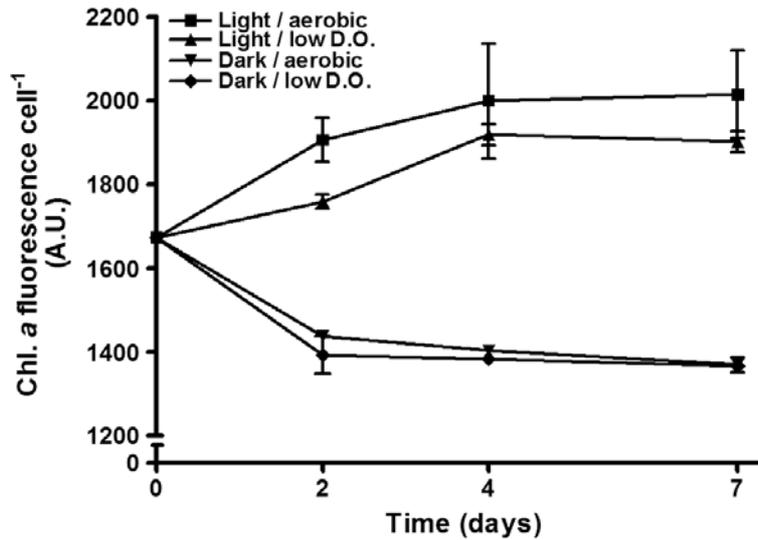
served to minimise the inter- and intraspecies problems associated with quantitative comparisons of FCM-derived chlorophyll *a* fluorescence signals (Sosik *et al.*, 1989), whilst complementing the recognised validity of using “*in vitro:in vivo*” chlorophyll determinations for the assessment of phytoplankton physiology (Kruskopf and Flynn, 2006).

3. Because cellular *in vivo* ( $F_0$ ) chlorophyll *a* fluorescence (as measured on a low excitation energy bench-top fluorometer; Section 7.2.3.4) correlated well with the high-excitation intensity FCM-quantified chlorophyll *a* fluorescence for both *C. vulgaris* (Pearson  $r = 0.706$ ;  $n = 96$ ;  $p < 0.0001$ ) and *C. reinhardtii* (Pearson  $r = 0.656$ ;  $n = 96$ ;  $p < 0.0001$ ).
4. Because the phytoplankton species used in this research are both small and coccoid. This meant that the potential for misleading fluorescence measurements (resulting from the ‘package effect’) is minimised.
5. Due to the good correlations between per-cell FDA fluorescence (metabolic activity; Section 9.8.1) and *in vivo* chlorophyll *a* fluorescence (photosynthetic activity) measurements for both *C. vulgaris* (Spearman  $r_s = 0.540$ ;  $n = 96$ ;  $p < 0.0001$ ) and *C. reinhardtii* (Spearman  $r_s = 0.508$ ;  $n = 96$ ;  $p < 0.0001$ ) during the course of the dark-survival experiment; suggesting a quantifiable link between general cell metabolic and photosynthetic ‘activities’.
6. Because heat-attenuation (see Section 7.2.3.2.3) also led to significant reductions in FCM-quantified chlorophyll *a* fluorescence signals (approximately 30%), this suggests that the chlorophyll *a* fluorescence signal measured by the current flow cytometer is indeed related to *in vivo* photosynthetic activity.

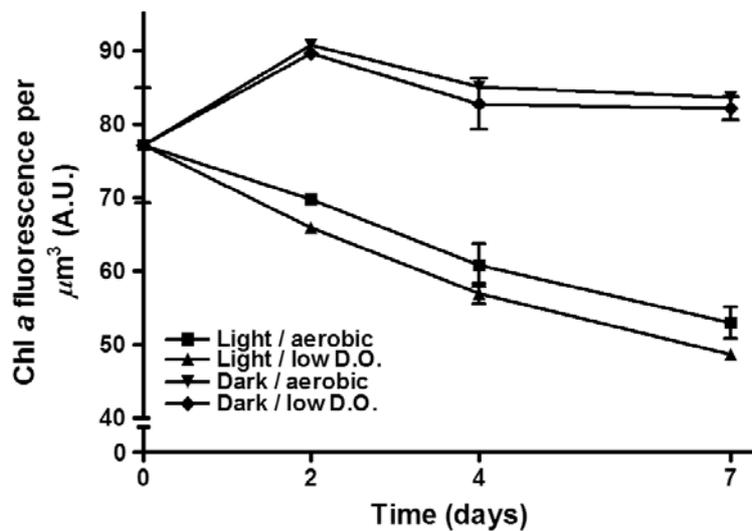
Following on from these suggested validations of the cytometric chlorophyll *a* fluorescence signals, it was assumed that the *in vivo* chlorophyll *a* fluorescence signals measured by the FACScan cytometer during the current research were sufficiently representative of cellular chlorophyll content and also were likely to adequately reflect *in vivo* cellular photosynthetic capacities.

### 9.7.4 Dark-survival and phytoplankton *in vivo* chlorophyll *a* fluorescence: results from the 7 day experiment

As for the two month dark-survival experiment above, *in vivo* FCM-quantified chlorophyll *a* fluorescence results from the follow-up 7 day dark-survival experiment for *C. vulgaris* are presented below in both the raw (Figure 9.32) and cell-volume-normalised (Figure 9.33) data formats.



**Figure 9.32.** 7 day *C. vulgaris* cellular chlorophyll *a* fluorescence (note the truncated y-axis). Data points show the mean of three replicate cultures ( $\pm 1$  S.D.).



**Figure 9.33.** One week *C. vulgaris* chlorophyll *a* fluorescence (FCM-quantified) normalised per unit cell volume ( $\mu\text{m}^3$ ). Data points show the mean of triplicate cultures ( $\pm 1$  S.D.).

As was the case for the 65 day dark-survival results (Section 9.7.3), normalising the cellular chlorophyll *a* fluorescence activity data to cell volume ( $\mu\text{m}^{-3}$ ) vastly transformed the individual ‘light’ and ‘dark’ treatment outcomes. Comparisons between the above 7 day data of Figure 9.33 and that of the 65 day experiment for *C. vulgaris* (Figure 9.28) revealed virtually identical trends over the course of the first week experimental duration. After 7 days of darkness, the cellular chlorophyll *a* fluorescence (per  $\mu\text{m}^3$ ) for both ‘dark’ treatments was similar to that at Day zero (1-way ANOVA;  $F_{(12,26)} = 87.86$ ;  $p > 0.05$ ), whereas after the same time of continuous illumination, both ‘light’ treatments had cells with significantly reduced cellular chlorophyll *a* fluorescence activities ( $p < 0.001$ ) compared with ‘Day zero’ values. As was observed for the 65 day results, there was again no overall difference here between the ‘aerobic’ and ‘low D.O.’ dark treatments ( $p > 0.05$ ); suggesting no apparent difference in algal dark-survivorship at ambient or low oxygen concentrations. It should also be mentioned that the previously observed strong correlation between 65 day *in vivo* cellular chlorophyll *a* fluorescence and cell volume for both *C. vulgaris* and *C. reinhardtii* was again recorded here during the final 7 day dark-survival experiment across all treatments (Pearson  $r = 0.946$ ;  $p < 0.0001$ ;  $n = 48$ ). Whilst this trend will not be further discussed, it does serve to highlight the reproducibility of the observed correlation between the two parameters during the current FCM analyses.

In summary, results from cellular chlorophyll *a* (both mass and fluorescence activity) analyses during the current experimentation have shown that both *C. vulgaris* and *C. reinhardtii* both have similar and well-developed dark-survival capacities. Both species appeared to competently maintain their normal pre-dark levels of cellular chlorophyll *a* during both shorter-term (7 day) and extended (65 day) dark-exposure. The existence of ambient ( $\approx 8.1 \text{mgL}^{-1}$ ) or actively reduced ( $2.2 \text{mg L}^{-1}$ ) dissolved oxygen levels appeared to have little or no influence on the overall dark-survival capabilities of the two algal species (based on their chlorophyll *a* fluorescence activities). Chlorophyll fluorescence data for both algal species showed that although cells had ‘shrunk’ slightly during long-term dark-exposure, the inferred ratio of chlorophyll *a* fluorescence activity to cell size remained relatively constant, despite the recorded individual reductions in both parameters. Following this, it is concluded that prolonged dark-exposure (up to 65 days) at either ‘ambient’ or ‘low’ DO concentration is likely to have a negligible impact

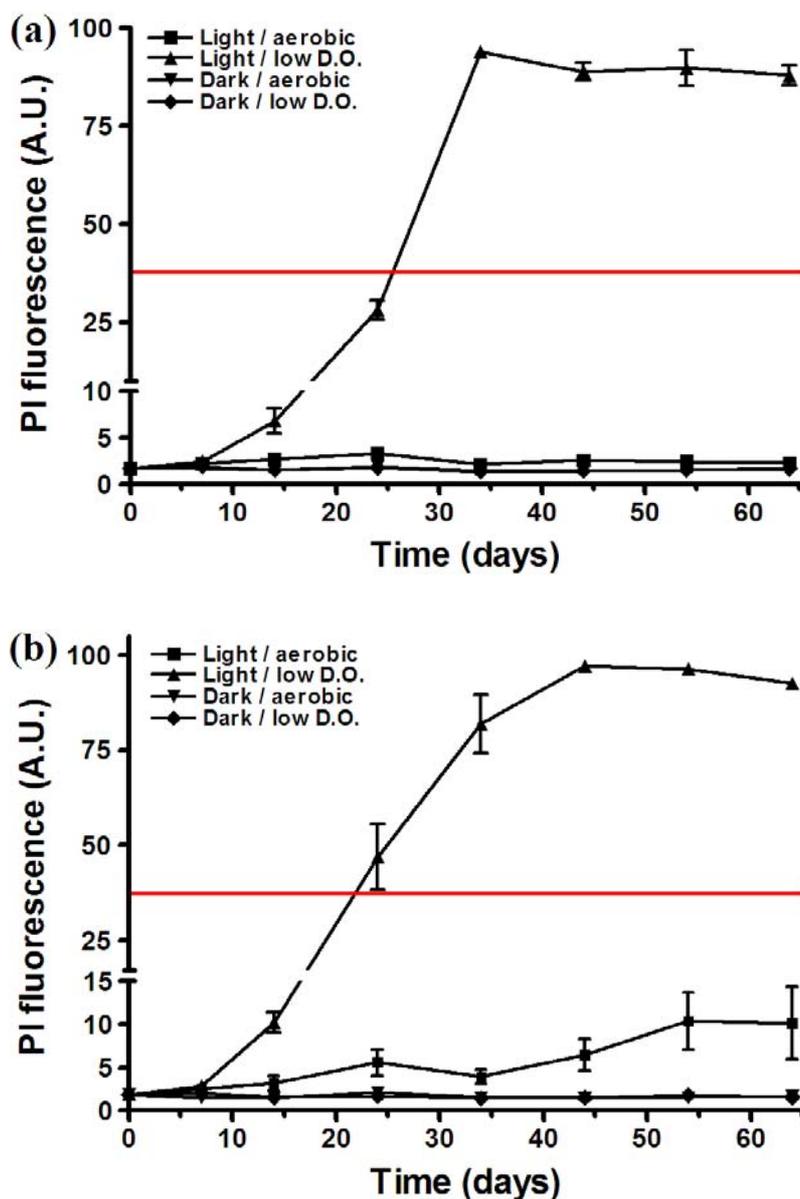
on the ability of both *C. vulgaris* and *C. reinhardtii* to maintain their cellular chlorophyll *a* fluorescence activities.

## **9.8 Prolonged darkness: phytoplankton metabolic activity and membrane integrity**

Long-term dark-viability assessments of *C. vulgaris* and *C. reinhardtii* involved analysis of cellular metabolic activity and membrane integrity (through proxy measurements of FDA and PI fluorescence respectively) and the quantitation of any subsequent changes to these parameters resulting from prolonged dark-exposure. Results of these analyses are provided below.

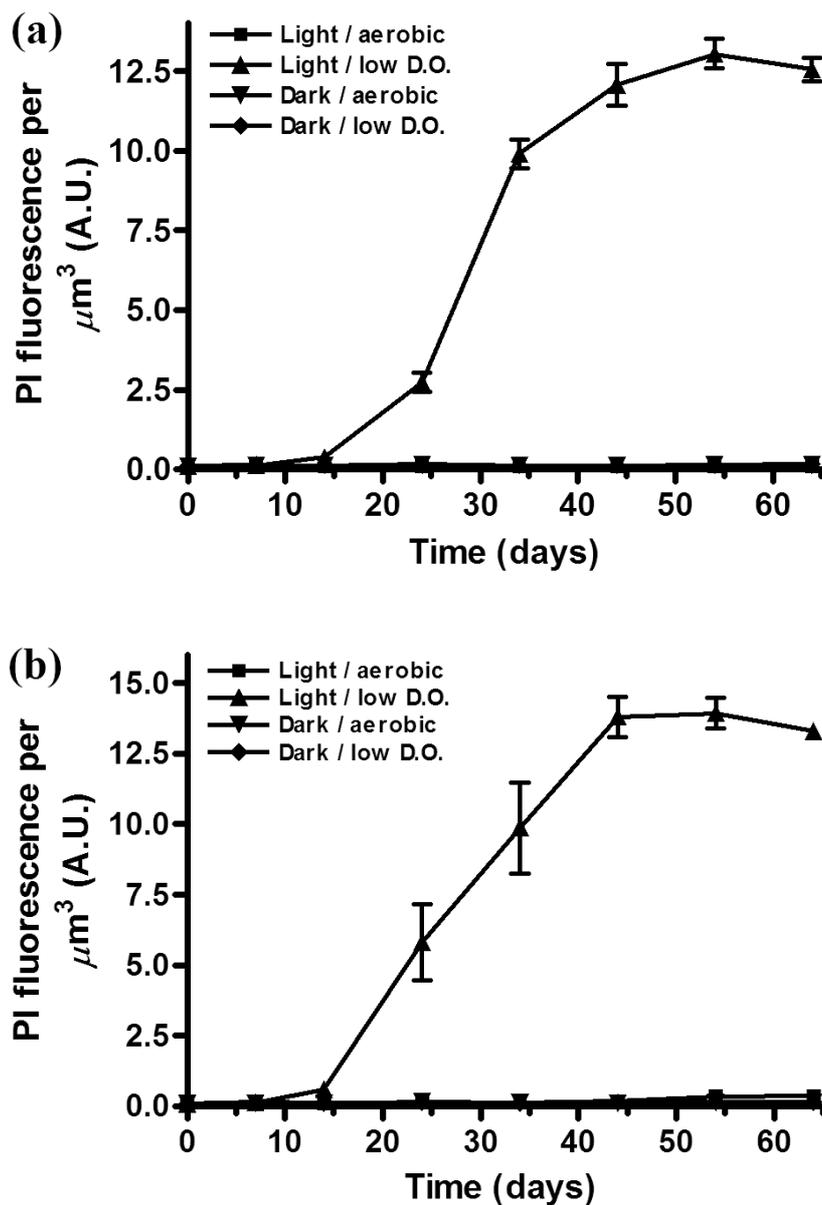
### **9.8.1 Dual PI–FDA viability assessment: results from the 65 day dark-survival experiment**

PI staining results from the long-term cell membrane integrity assessments performed throughout the 65 day dark-survival experiment are provided in Figure 9.34 below. Analysis of Figure 9.34 revealed that algal populations in all experimental treatments *except* for those of the 'light / low D.O.' treatment remained (on average) PI-negative 'viable' throughout the entire 65 day duration. 'Dark' treatment cultures appeared to be the most greatly conserved in terms of their ability to maintain original 'Day zero' population average membrane integrity (based on PI fluorescence). The slight increase in long-term population average PI fluorescence for the 'light / aerobic' treatments was considered to be of no real interest in the dark-survival research context, and most likely a simple reflection of there being an increase in the relative number of dead cells in the statically ageing illuminated cultures as a result of ongoing culture growth and decline.



**Figure 9.34.** Two month *C. vulgaris* (a) and *C. reinhardtii* (b) mean PI fluorescence for all four treatments. The horizontal  $y$ -axis line (red) indicates the pre-determined lower ‘cut-off’ limit of the PI-positive ‘non-viable’ regional marker (i.e. cells below the line are PI-negative ‘viable’, and cells above the line are PI-positive ‘non-viable’). Both  $y$ -axes represent cellular PI fluorescence (A.U.) and  $x$ -axes show elapsed time (days). Data points show the mean of 3 replicate cultures ( $\pm 1$  S.D).

As for the chlorophyll *a* fluorescence data of the previous Section, mean population PI fluorescence signals were again normalised to cell volume in order to account for any potential ‘cell-volume-relative’ effects during the course of the two month experiment. Results of this data transformation are presented in Figure 9.35.



**Figure 9.35.** Two month *C. vulgaris* (a) and *C. reinhardtii* (b) PI cellular fluorescence (normalised to cell volume;  $\mu\text{m}^3$ ) for all treatments. Both  $y$ -axes represent cellular PI fluorescence ( $\mu\text{m}^{-3}$ ) and  $x$ -axes show elapsed time (days). Data points show the mean of 3 replicate cultures ( $\pm 1$  S.D.).

Unlike the previously discussed chlorophyll *a* data (and also the forthcoming FDA fluorescence results), there was no real change to the overall outcomes from the FCM data in either algal species when cellular PI fluorescence was normalised to cell volume (i.e. there was no significant difference between mean cellular PI fluorescence in any treatment comparisons *except* where 'light / low D.O.' treatment data was included). This result can be most easily visualised in Table 9.3.



**Table 9.3.** Statistical significance tables for two month PI cellular fluorescence (normalised to cell volume;  $\mu\text{m}^3$ ) of *C. vulgaris* and *C. reinhardtii* for all treatments (1-way RM-ANOVA with Tukey's multiple comparisons). Shading shows level of significant difference between treatment means: no shading signifies no difference ( $p > 0.05$ ); medium shading shows significance at  $p < 0.01$ ; and black shading indicates a significant difference at  $p < 0.001$ .

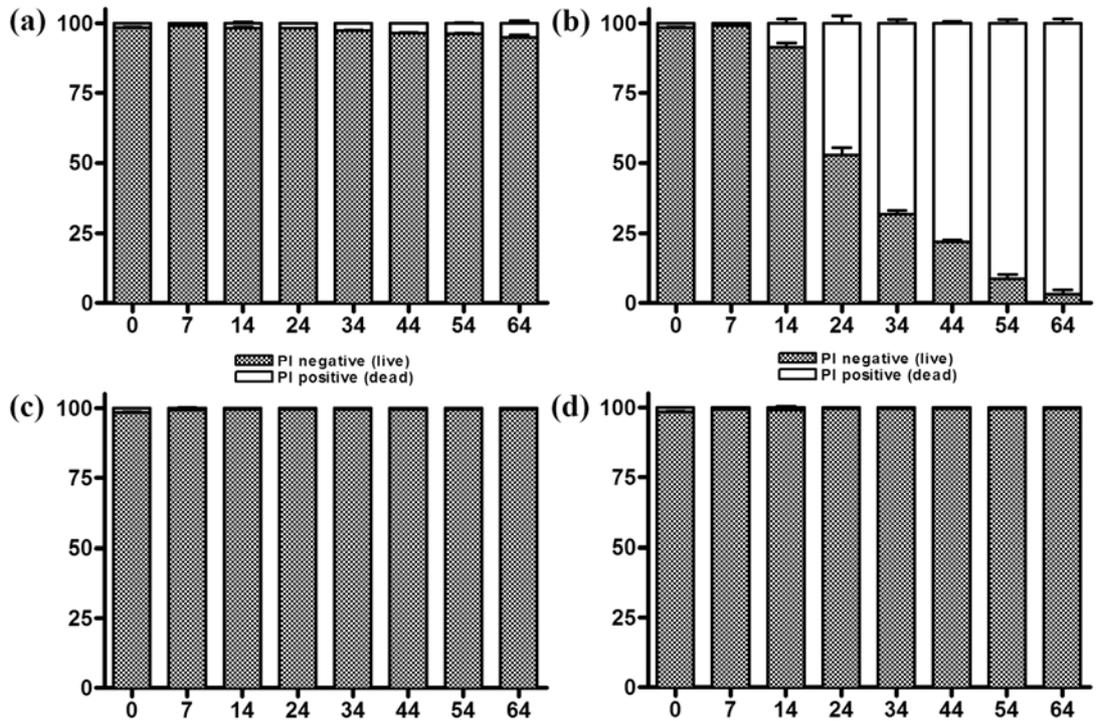
<b><i>C. vulgaris</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$F_{(3,6)} = 10.18$			
Dark / aerobic	ns	$F_{(3,6)} = 10.18$		
Dark / low D.O.	ns	$F_{(3,6)} = 10.18$	ns	

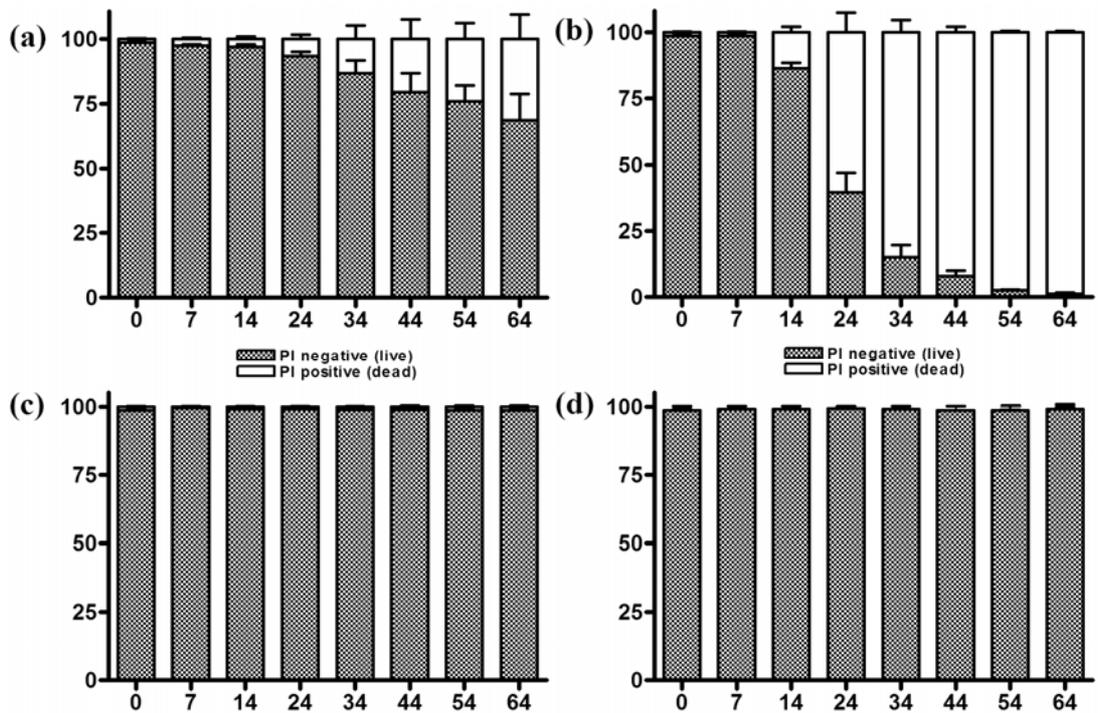
<b><i>C. reinhardtii</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$F_{(3,6)} = 12.42$			
Dark / aerobic	ns	$F_{(3,6)} = 12.42$		
Dark / low D.O.	ns	$F_{(3,6)} = 12.42$	ns	

Comparing only the 'Day 65' mean population PI fluorescence values, there again was a significantly elevated per-cell PI fluorescence for algal cells in the 'light / low D.O.' treatment only for both *C. vulgaris* (1-way ANOVA;  $F_{(3,8)} = 3179$ ;  $p < 0.001$  and *C. reinhardtii* (1-way ANOVA;  $F_{(3,8)} = 2034$ ;  $p < 0.001$ ) but no difference between any other treatment combination at 'Day 65' ( $p > 0.05$ ). Essentially, this meant that the only treatment with a significant percentage of the total cell population being PI-positive (i.e. dead) at 'Day 65' was that of the 'light / low D.O.' treatment combination. Despite there being a considerable portion ( $\approx 30\%$ ) of 'dead' cells in the *C. reinhardtii* 'light / aerobic' treatment at 'Day 65' (Figure 9.34b), this was ruled insignificant in terms of the overall population average cellular PI fluorescence value.

The PI viability data from Figure 9.35a for *C. vulgaris* and 9.35b for *C. reinhardtii* is represented in terms of the relative percentage of 'live' and 'dead' cells in Figures 9.36 and 9.37 for *C. vulgaris* and *C. reinhardtii* respectively.



**Figure 9.36.** Two month *C. vulgaris* PI assay viability over the four treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (x-axis represents time (days) and vertical bars show daily average live vs. dead cell populations (%) from 3 replicates  $\pm 1$  S.D.)



**Figure 9.37.** Two month *C. reinhardtii* PI assay viability over the four treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (x-axis represents time (days) and vertical bars show daily average live vs. dead cell populations (%) from 3 replicates  $\pm 1$  S.D.)

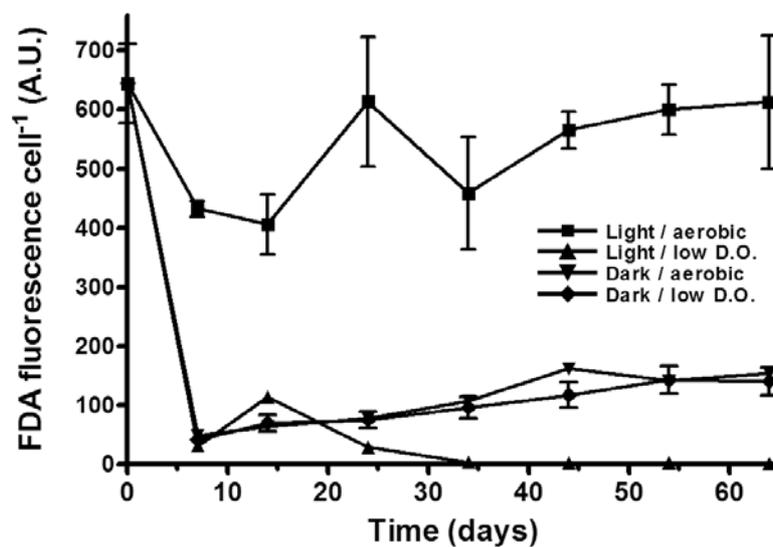
As can be seen in Figure 9.36, *C. vulgaris* cultures in all treatments except for the 'light / low D.O.' conditions effectively retained their cell membrane integrities over the full course of the 65 day experiment. This apparently high proportion of cell death in 'light / low D.O.' treatment cultures was alluded to earlier (Section 9.6.3.3) through the observation of parallel reductions in FSC and SSC signal amplitudes and their corresponding reductions in cell volume and intracellular density. It was suggested, as a result of these observed reductions in FSC and SSC signals, that cells in 'light / low D.O.' treatments might have undergone significant rates of cell death, and that the nature of this cell death was most likely necrotic (based on the observed simultaneous reductions in both light scatter parameters) (Vitale *et al.*, 1997). This idea of cell death via necrosis (as opposed to apoptosis) for algal cells in 'light / low D.O.' treatments was supported by the significant losses in cell population plasma membrane integrity (as shown by the correspondingly high population PI fluorescence values). Whilst cell death resulting from apoptotic pathways cannot be ruled out in this instance, all available evidence suggests that it was most likely necrotic in origin.

Very similar results to those for *C. vulgaris* above were recorded for treatment cultures of *C. reinhardtii*, except this time the statically ageing 'light / aerobic' treatment cells also appeared to suffer greater ill-effects as a result of the continuously illuminated culture 'stagnation'. The significant amount of PI positive 'dead' cells in the statically ageing 'light / aerobic' treatment cultures for *C. reinhardtii* (Figure 9.37) was again considered to be of no great interest in the context of dark-survival *per se*, and, as mentioned previously, was most likely a simple reflection of the normal energetic cellular cycle in ageing algal cultures (i.e. sequential population growth and death). Furthermore, and on a population scale, this fraction of dead cells was actually not large enough to bring the mean *population* PI fluorescence anywhere near the PI positive cut-off limit for the overall population to be effectively declared 'non-viable' (see Figure 9.34b). Following this, it is concluded that prolonged dark-exposure, under either ambient or reduced DO conditions, had no significant negative effects on algal cell membrane integrity over the course of the two month dark-survival experiment.

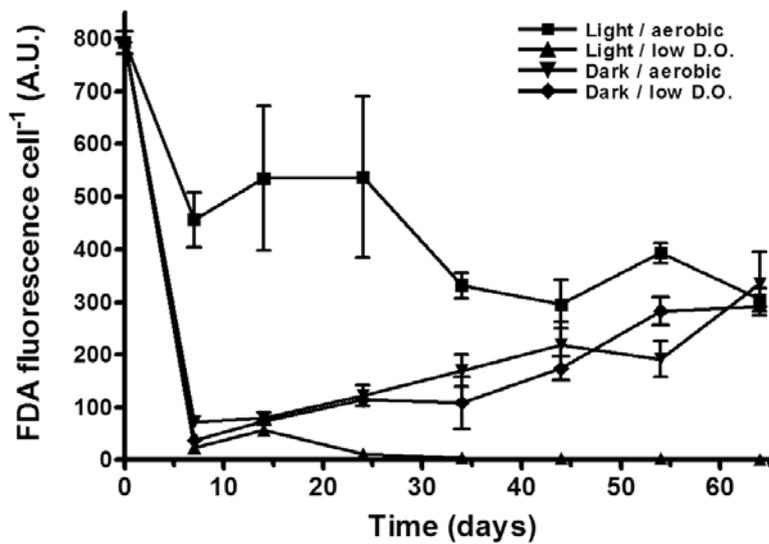
Generally speaking, there has been very little previous work concerning the assessment of algal cell membrane integrity as a consequence of prolonged darkness. Franklin and

Berges (2004) assessed algal (Dinophyceae) cell membrane integrity following 8 days dark-exposure using the relatively new SYTOX<sup>®</sup> Green (Molecular Probes) membrane integrity probe and reported significantly higher rates of chlorosis and cell lysis in *Amphidinium carterae* than was recorded for either of the algal species during the current research. Segovia *et al.* (2003) did report on the cell lysis of *Dunaliella tertiolecta* following 4 days of dark-exposure; however, this was assessed histologically via transmission electron microscopy. Although the PI assay has been employed for phytoplankton viability assessment in other scenarios (e.g. following copper toxicity (Franklin *et al.*, 2001a) or for monitoring algal cell death in environmental samples (Agustí *et al.*, 2002)), to the author's knowledge there has been no previous application of so-called 'vital' staining techniques for the assessment of cellular membrane integrity following prolonged dark-exposure in phytoplankton.

FDA staining results from the long-term cell metabolic activity assessments performed throughout the course of the 65 day dark-survival experiment are given for *C. vulgaris* (Figure 9.38) and *C. reinhardtii* (Figure 9.39) below.

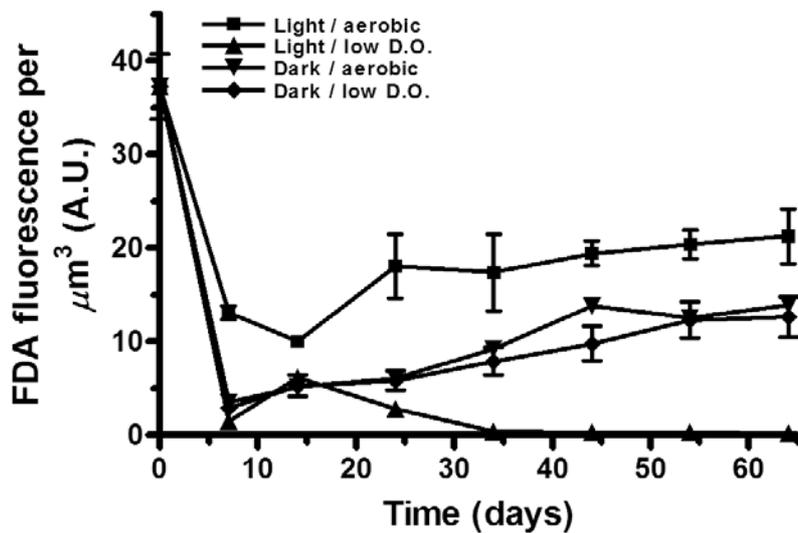


**Figure 9.38.** Two month *C. vulgaris* FDA fluorescence per-cell. Data points show the mean of three replicate cultures ( $\pm 1$  S.E.M.).

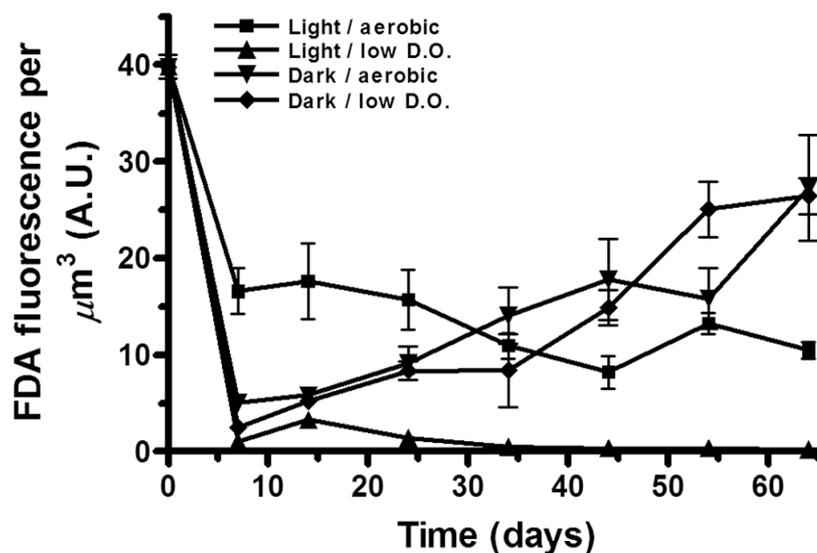


**Figure 9.39.** Two month *C. reinhardtii* FDA fluorescence per-cell. Data points show the mean of three replicate cultures ( $\pm 1$  S.E.M.).

Prior to critical analysis of the cellular FDA metabolic activity data, daily population average FDA fluorescence values were again normalised to the mean population cell volume for each treatment. Cell-volume-normalised cellular FDA fluorescence data are provided in Figures 9.40 and 9.41 for *C. vulgaris* and *C. reinhardtii* respectively.



**Figure 9.40.** Two month *C. vulgaris* cellular FDA fluorescence (per unit cell volume;  $\mu\text{m}^{-3}$ ). Data points show mean of three replicate cultures ( $\pm 1$  S.E.M.).



**Figure 9.41.** Two month *C. reinhardtii* cellular FDA fluorescence (per unit cell volume;  $\mu\text{m}^{-3}$ ). Data points show mean of three replicate cultures ( $\pm 1$  S.E.M.).

Visual inspection of the data in Figures 9.38 to 9.41 again shows the difference in outcome one obtains from taking into account the effects of changes in cell volume during the course of the dark-survival experiments. Statistical analysis of the overall 65 day cell-volume-normalised *C. vulgaris* data (Figure 9.40) for all 4 treatments showed that 'light / aerobic' treatment cells had significantly elevated FDA metabolic activity than all other treatments (1-way RM-ANOVA;  $F_{(3,6)} = 29.55$ ;  $p < 0.001$ ). The same analysis showed also that algae in both 'dark' treatments had significantly greater long-term FDA metabolic activities than cells in the 'light / low D.O.' treatment ( $p < 0.01$ ), but revealed no overall difference between the activities of both 'dark' treatments themselves ( $p > 0.05$ ). Relative to 'Day zero', *C. vulgaris* cellular FDA metabolic activities for both 'dark' treatments were significantly reduced at all time intervals (1-way ANOVA;  $F_{(14,30)} = 34.92$ ;  $p < 0.01$ ). Analysis of the 'Day 65' data only from Figure 9.40, showed that Day 65 'light / aerobic' FDA metabolic activity was, on average, not significantly different from that of both 'dark' treatments (1-way ANOVA;  $F_{(3,8)} = 22.38$ ;  $p > 0.05$ ) but was significantly greater than that of 'light / low D.O.' treatment ( $p < 0.001$ ). Both 'dark' treatments also showed significantly higher cellular FDA fluorescence than algal cells in the 'light / low D.O.' treatment ( $p < 0.01$ ). Figures 9.40 and 9.41 also showed large-scale declines in cellular FDA fluorescence within the first 7 days. It was considered that this could again have been partly due to the inoculum carryover effect (see Section 9.6.3.3) whereby cells were more actively growing in the

late exponential to early stationary-phase ‘Day 8’ culture stock than they were following 7 days of experimental incubation.

Critical analysis of the two-month *C. reinhardtii* FDA fluorescence data (Figure 9.41) revealed no significant difference in overall per-cell FDA metabolic activity in 'light / aerobic' treatment cells than in either of the ‘dark’ treatments, nor between the two ‘dark’ treatments themselves (1-way RM-ANOVA;  $F_{(3,6)} = 7.369$ ;  $p > 0.05$ ). There was, however, a significantly reduced long-term cellular FDA activity in 'light / low D.O.' cells compared with that of all other treatments ( $p < 0.05$ ). Relative to ‘Day zero’, average *C. reinhardtii* cellular FDA metabolic activities for both ‘dark’ treatments were again significantly reduced at all sampling intervals (1-way ANOVA;  $F_{(14,30)} = 16.43$ ;  $p < 0.05$ ). This data from the above-discussed statistical analyses of the long-term FDA metabolic activities for both *C. vulgaris* and *C. reinhardtii* is summarised in Table 9.4.

**Table 9.4.** Statistical significance tables for two month FDA cellular fluorescence (normalised to cell volume;  $\mu\text{m}^3$ ) of *C. vulgaris* and *C. reinhardtii* for all treatments (1-way RM-ANOVA with Tukey’s multiple comparisons). Shading shows the level of significant difference between treatment mean FDA fluorescence: no shading signifies no difference ( $p > 0.05$ ); light shading represents a significant difference at  $p < 0.05$ ; medium shading shows significance at  $p < 0.01$ ; and black shading indicates a significant difference at  $p < 0.001$ .

<b><i>C. vulgaris</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$F_{(3,6)} = 29.55$			
Dark / aerobic	$F_{(3,6)} = 29.55$	$F_{(3,6)} = 29.55$		
Dark / low D.O.	$F_{(3,6)} = 29.55$	$F_{(3,6)} = 29.55$	ns	

<b><i>C. reinhardtii</i></b>	Light / aerobic	Light / low D.O.	Dark / aerobic	Dark / low D.O.
Light / aerobic				
Light / low D.O.	$\chi^2_{(0.05,4)} = 15.01$			
Dark / aerobic	ns	$\chi^2_{(0.05,4)} = 15.01$		
Dark / low D.O.	ns	$\chi^2_{(0.05,4)} = 15.01$	ns	

Analysis of the ‘Day 65’ data only from Figure 9.41 revealed a significantly higher population average cellular FDA fluorescence activity in both ‘dark’ treatments than for both the statically ageing 'light / aerobic' (1-way ANOVA;  $F_{(3,8)} = 20.25$ ;  $p < 0.05$ ) and

also the ailing 'light / low D.O.' treatment ( $p < 0.001$ ). It was thought that the gradual long-term decline in mean cellular FDA metabolic activity for 'light / aerobic' treatment *C. reinhardtii* was due to both the ever-increasing mean cell volume (Figure 9.13) and also because of the rising percentage of dead cells within that treatment population (see Figure 9.34b) bringing down the overall average FDA fluorescence activity status. On the other hand, the slight increase in per-cell FDA fluorescence over the course of the 65 day experiment for 'dark' treatments in both algal species (see Figures 9.40 and 9.41) was likely to have been a result of the gradually declining cell volume during prolonged darkness (see Figures 9.11 and 9.13). This could again be a manifestation of the previously described 'package effect' (Section 9.7.3), whereby smaller cells are inherently more efficient at absorbing photons such that it ultimately leads to a higher relative fluorescence yield on a per-unit-volume basis. As outlined in Section 9.6.3.2, this cell-volume-relative variation in substrate FDA uptake and fluorescence yield is known to vary as a direct consequence of both changes to the efficiency substrate influx and efflux resulting from variations in the relative thickness of cellular diffusion boundary layers, and also from changes in relative membrane lipid surface area and membrane transporters governing solute flux—all of which increase in efficiency with decreasing cell volume (Raven and Kübler, 2002).

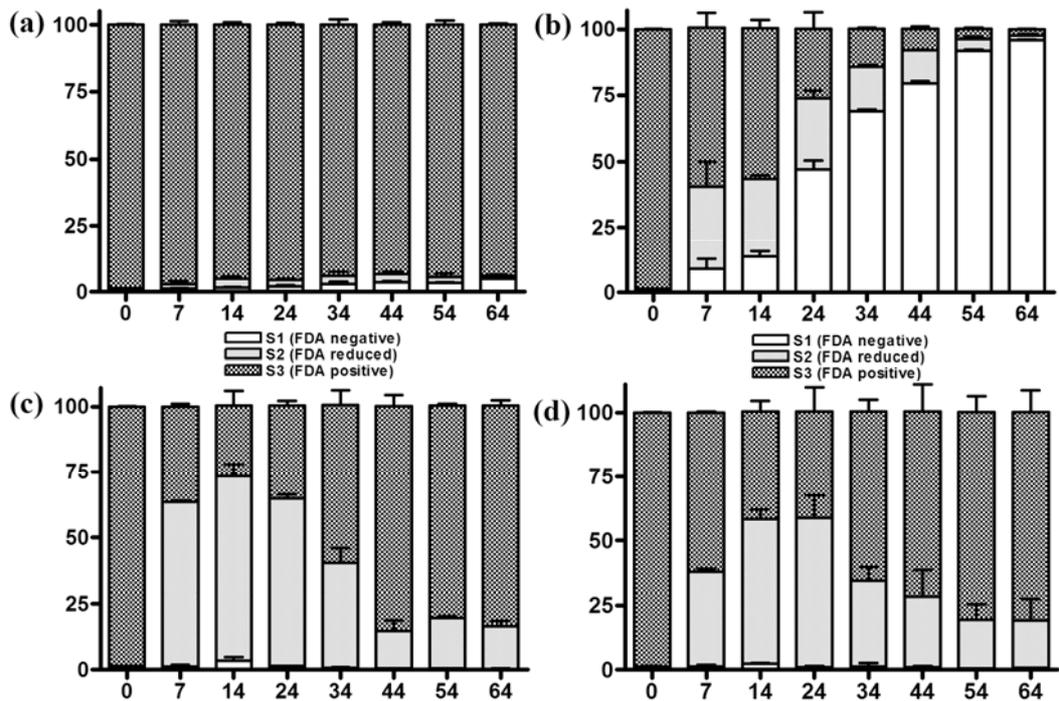
Although cellular FDA fluorescence signals were normalised to cell volume, the *linear* nature of the volumetric correction factor applied to cellular FDA fluorescence values in the current work (refer to Section 9.7.3) appeared to over-correct for volumetric changes in relative cellular fluorescence (as seen in Figures 9.40 and 9.41). This potential overestimation resulting from the adoption of a linear volumetric correction factor could be explained by the fact that the specific 'volume:fluorescence' relationship is more likely to be *non-linear* (although still undefined) according to Raven and Kübler (2002). The end result of having applied this linear-type correction factor was a per-cell FDA fluorescence that appeared to escalate disproportionately with a reducing cell volume, giving the impression of an 'ever-increasing' metabolic activity over time in the dark. Whilst it is indeed possible that this could represent some form of highly efficient photoacclimation response during prolonged darkness, it was considered more likely to have been an artefact of the way that cellular fluorescence is quantified during FCM analysis, and in the absence of a more suitable (i.e. non-linear) volumetric correction



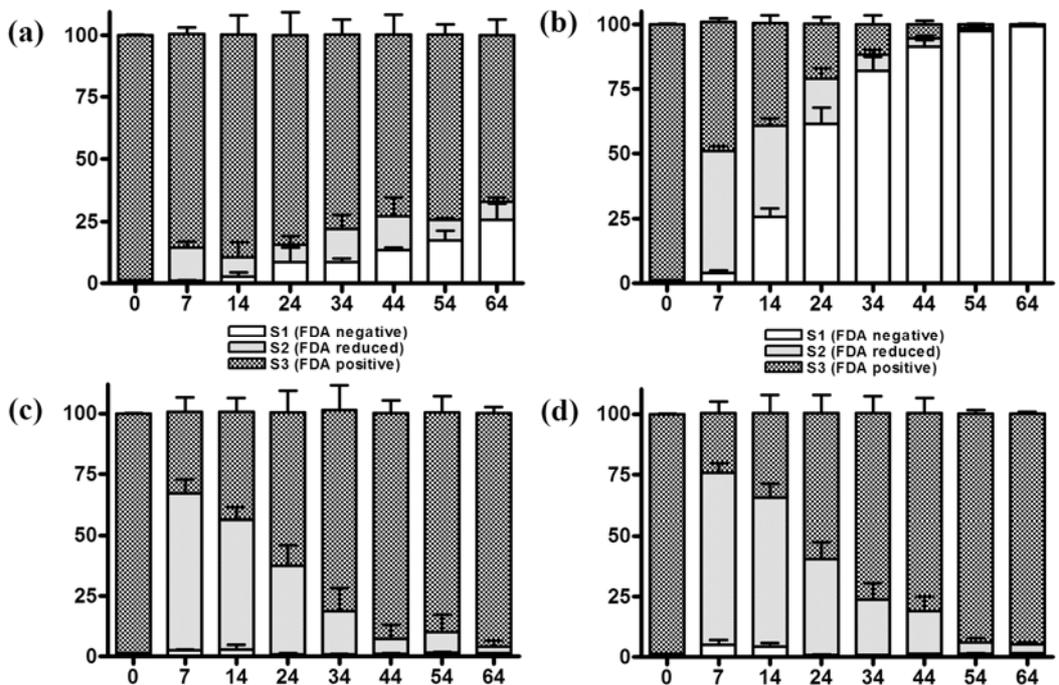
factor, no further data transformations were attempted. It should be noted also, that since a similarly increasing fluorescence trend was not seen in 'light / aerobic' treatments for either algal species, potential methodological factors (e.g. variability in daily staining efficacy) were deemed unlikely to have been influencing the observed results.

The daily FDA metabolic activities for both *C. vulgaris* and *C. reinhardtii* during the course of the 65 day dark-survival experiment were also classified according to their relative fluorescence 'activity states' (see Section 7.2.3.3 for initial description). Daily population FDA fluorescence histograms were segregated into the three fluorescence activity states: 'S3' normal or healthy fluorescence state; 'S2' reduced FDA fluorescence state; and an FDA-negative or non-viable 'S1' fluorescence activity state. Instead of reducing the entire algal cell population for each treatment down to a single population average fluorescence value (as was the case for the above analyses), the daily population fluorescence distribution was expanded into effective 'sub-populations' based on their relative metabolic activities. Results of this data manipulation are presented for *C. vulgaris* and *C. reinhardtii* in Figures 9.42 and 9.43 respectively.

Both the PI 'live versus dead' (Figures 9.36 and 9.37) and also the S1, S2, S3 cellular 'FDA activity state' data (Figures 9.42 and 9.43) show phytoplankton dark-survival trends from the un-manipulated (i.e. non-cell-volume-normalised) per-cell fluorescence data. This data treatment was not performed *post hoc* on these particular data sets due to the fact the original PI 'live:dead' and FDA 'S1,S2,S3' activity states were defined using the non-cell-volume-normalised raw data and so it was necessary to adhere to these original fluorescence guidelines. Notwithstanding this, it should also be stated that performing this data transformation was shown to be of no apparent consequence with respect to the PI viability outcomes (as discussed previously) and was of no real consequence for the dark-survival outcomes based on the FDA metabolic activity data. If anything, the above non-cell-volume-normalised raw S1, S2, S3 FDA metabolic activity state data provides a 'worst case' outlook in terms of the 65 day survival of 'dark' treatments compared with that of the 'light / aerobic' treatment (given the proportionately greater increase in 'dark' treatment cellular FDA fluorescence when normalised to cell volume; Figures 9.38–9.41) such that there are no grounds for further validating the use of raw data in Figures 9.42 and 9.43 above.



**Figure 9.42.** Two month *C. vulgaris* FDA metabolic activity states (S1 – non-viable; S2 – compromised; S3 – viable) over the four treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (x-axes represent elapsed time (days) and vertical bars (y-axes) show daily average percentage of the total population in each FDA fluorescence activity state  $\pm$  1 S.D. from 3 replicates cultures)).

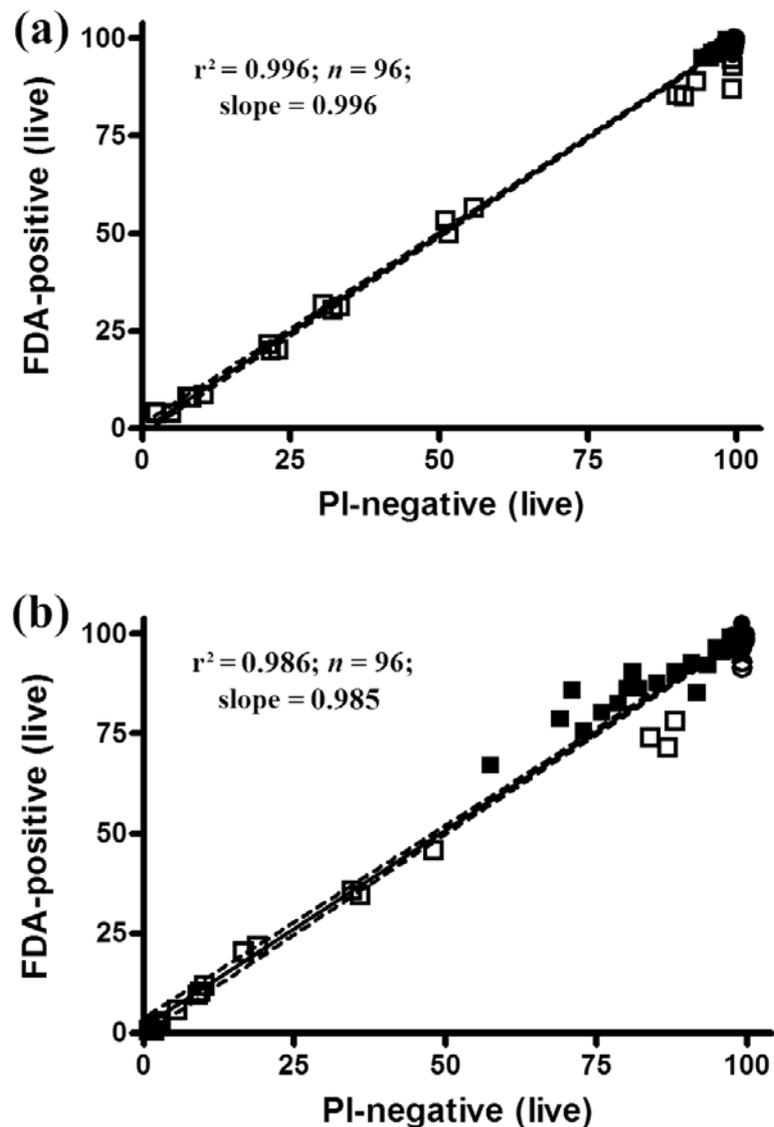


**Figure 9.43.** Two month *C. reinhardtii* FDA metabolic activity states (S1 – non-viable; S2 – compromised; S3 – viable) over the four treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (x-axes represent elapsed time (days) and vertical bars (y-axes) show daily average percentage of the total population in each FDA fluorescence activity state  $\pm$  1 S.D. from 3 replicates cultures)).

Analysis of the 'dark' treatment data for both *C. vulgaris* (Figure 9.42c, d) and *C. reinhardtii* (Figure 9.43c, d) showed that during the course of the 65 day experimental duration, there wasn't a significant portion of the total treatment cell population in the S1 non-viable FDA activity state. Although statistically there was a significantly increased percentage of S1 non-viable cells at Day 14 for both 'dark' treatment *C. vulgaris* cultures (1-way ANOVA;  $F_{(14,30)} = 6.200$ ;  $p \leq 0.05$ ) and similarly for 'dark / low D.O.' treatment *C. reinhardtii* cultures on Days 7 and 14 ( $F_{(14,30)} = 3.102$ ;  $p \leq 0.05$ ), this trend did not continue for the remainder of the experimental duration. Indeed after two complete months of dark-exposure, and regardless of DO concentration, both algal species had less than 1.5% of their entire cell populations classified as FDA-negative 'non-viable'. The same could not be said for algal cells in the 'light / aerobic' treatment, which again suffered high levels of cellular death to end up at 'Day 65' with more than 97.5% of cells in the S1 'dead' state for *C. vulgaris* and >99% S1 dead for *C. reinhardtii*. It should be noted, however, that the apparent steady decline in the relative percentage of 'dark' treatment cells in the S2 reduced metabolic activity state from Day 7 onwards was again likely to have been a manifestation of the cell-volume-related effects of FDA staining and fluorescence yield discussed above, and corresponded well with the apparent increase in overall per-cell FDA fluorescence seen in Figures 9.38–9.41. It is concluded, therefore, that prolonged darkness resulted in a significant reduction in cellular metabolic activity in both *C. vulgaris* and *C. reinhardtii*; although not to the extent that cells were classified as FDA-negative 'non-viable'.

#### **9.8.1.1 Agreement between the discrete viability assessments of the PI and FDA assays**

In order to quantify the agreement (or otherwise) between the two biological stains during the dual cellular viability assessments, pooled regression analyses were performed on PI and FDA data from the 65 day FCM analyses across all four treatments for both algal species. Results showed that there was indeed very good agreement between the two stains in terms of their discrete abilities for accurate viability assessment of both algal species throughout the experimental duration (Figure 9.44).



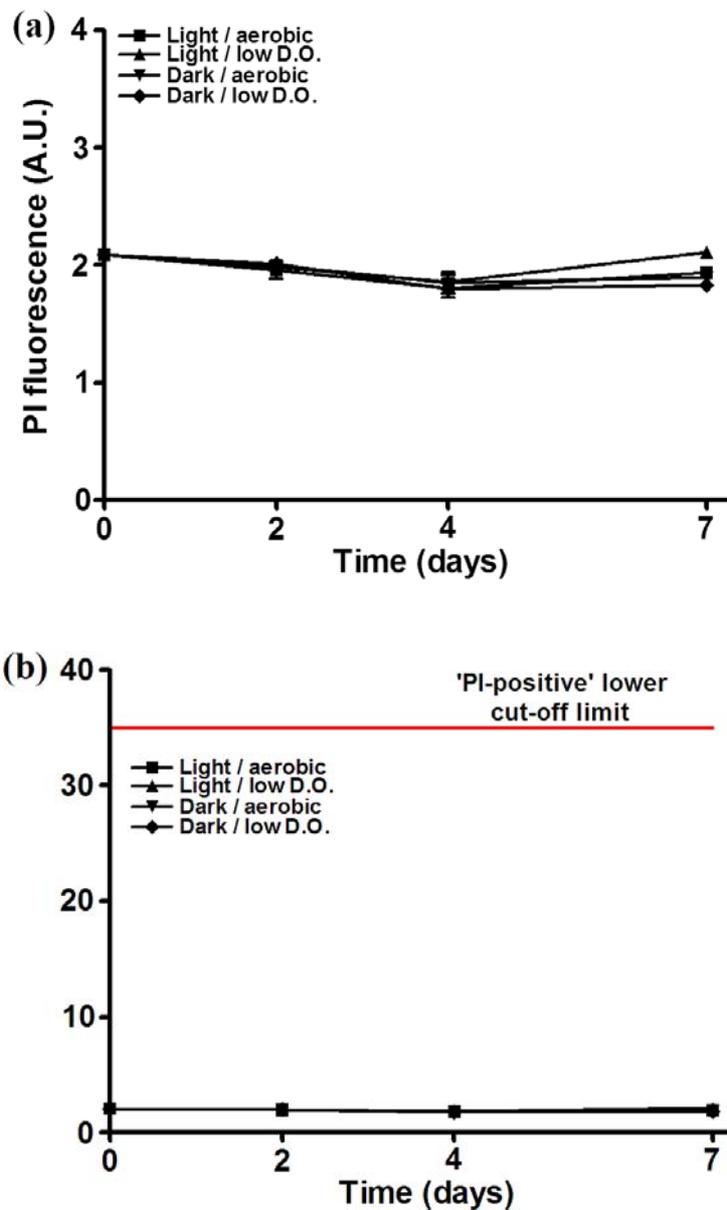
**Figure 9.44.** Sensitivity analyses of dual PI–FDA staining for viability (live vs. dead) discrimination of: (a) *C. vulgaris* and; (b) *C. reinhardtii* for all four experimental treatments: 'light / aerobic' (■); 'light / low D.O.' (□); 'dark / aerobic' (●); and 'dark / low D.O.' (○). Both *y*-axes reflect the percentage of FDA-positive cells in a given sample, whilst *x*-axes show the percentage of PI-negative cells from the same sample. Individual linear regression coefficients shown (with slope) for the fitted regression lines (broken lines represent 95% CI's for the fitted line).

This sensitivity analysis of dual PI–FDA staining results from the 65 day dark-survival experiment confirmed the high level of agreement between the two viability assays; as evidenced by the fact that neither of the regression slopes from Figure 9.44 were significantly different from a theoretical perfect fit slope of 1.000 (1-way ANOVA;  $F_{(2,6)} = 2.954$ ;  $p = 0.128$ ). Although the 'live: dead' discriminatory power of the dual PI–FDA assay had already been firmly established during prior methodological

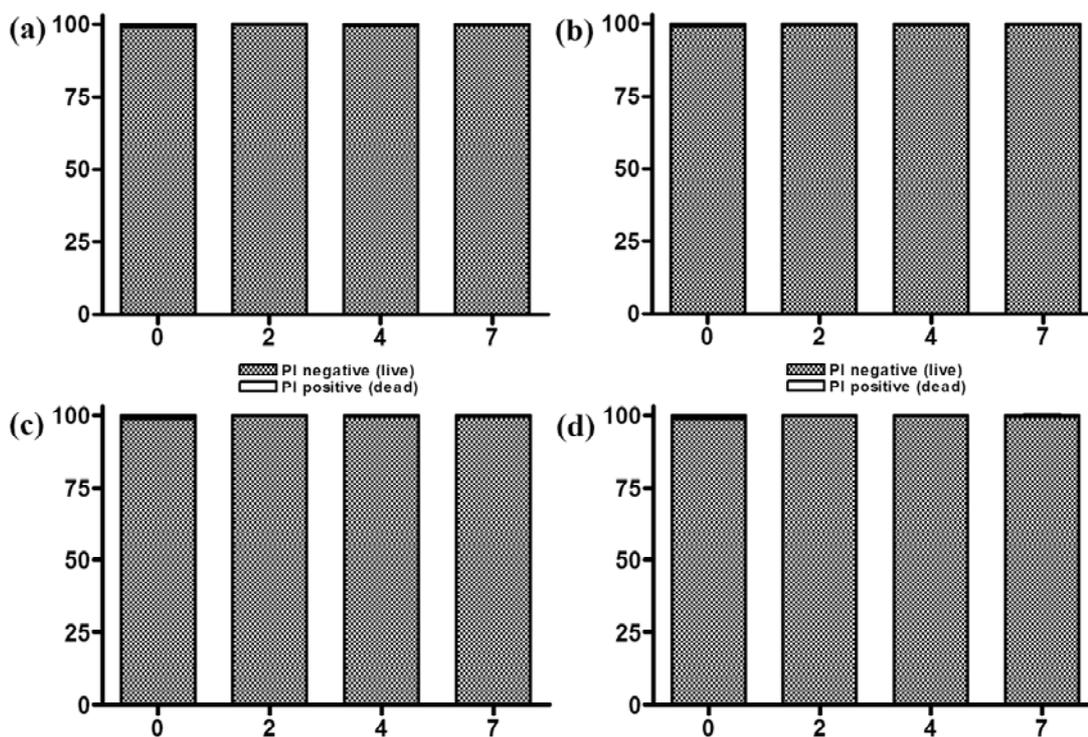
optimisations (see Section 8.2.1), this demonstrated that the PI–FDA staining method used here was also sufficiently robust with the use of both ‘ideal’ and ‘non-ideal’ target cells. In other words, the PI–FDA assay was not only accurate when using ‘fresh’ 8 day old cultures and heat-killed positive controls, but was also sensitive with respect to viability resolution when applied to statically aged cultures subjected to a range of environmental conditions.

### **9.8.2 Dual PI–FDA viability assessment: results from the 7 day dark-survival experiment**

Results from PI cell membrane integrity assessments performed for *C. vulgaris* during the course of the one week follow-up dark-survival experiment are provided in Figure 9.45. Similar to the 65 day experimental data, normalising the 7 day PI cellular fluorescence per unit cell volume ( $\mu\text{m}^{-3}$ ) was again performed; however, results of this transformation are not shown for reasons discussed below. Although the cell volume did decrease slightly for both ‘dark’ treatments over the 7 day period ( $\approx 25\%$  reduction; Figure 9.18), since the initial per-cell PI fluorescence was so low, the reduction in cell volume was insignificant in real terms with respect to magnitude of cellular PI fluorescence and so did not change the overall ‘PI negative’ viable status of all treatments (see Figure 9.46 below). As was observed during the first 7 days of the previously discussed two month experiment, the follow-up 7 day dark-survival experiment again revealed that all treatments sufficiently retained their full ‘Day zero’ viability status as at ‘Day 7’, such that no further elaboration of the capacity of *C. vulgaris* for successful dark-survival after such a dark-exposure duration is required.

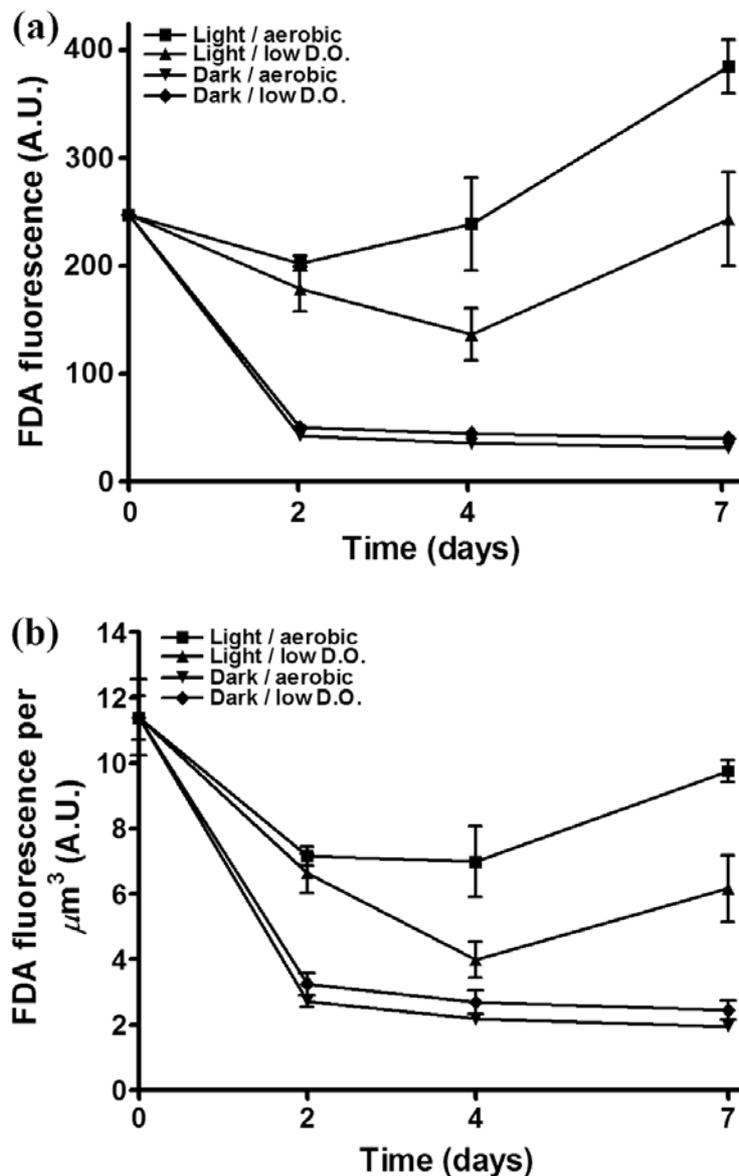


**Figure 9.45.** (a) Seven day *C. vulgaris* cellular PI fluorescence for all treatments (note the reduced  $y$ -axis scale). (b) Same data as for (a) but with an expanded  $y$ -axis scale. The horizontal line indicates the pre-determined lower 'cut-off' limit of the PI-positive 'non-viable' regional marker (i.e. cells below the line are PI-negative 'viable', and cells above the line are PI-positive 'non-viable'). Both  $y$ -axes reflect the relative PI cellular fluorescence (A.U.), whilst  $x$ -axes show the elapsed time at each sampling interval. Data points show the mean of 3 replicate cultures ( $\pm 1$  S.D.).



**Figure 9.46.** 7 day *C. vulgaris* PI-assay viability for the four experimental treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (*x*-axis represents time (days) and vertical bars show daily average live vs. dead cell populations (%) from 3 replicate cultures  $\pm$  1 S.D.)

Results from FDA cell metabolic activity assessments for *C. vulgaris* during the course of the follow-up 7 day dark-survival experiment are also provided below. Since the cellular enzymes (esterases) responsible for FDA hydrolysis during the FDA metabolic assay are turned over intracellularly on timescales in the order of several hours (Yentsch *et al.*, 1989), it was deemed to be an appropriate technique for detecting relative changes in cellular metabolic activity over a timescale of several days during this follow-up 7 day dark-survival experiment.



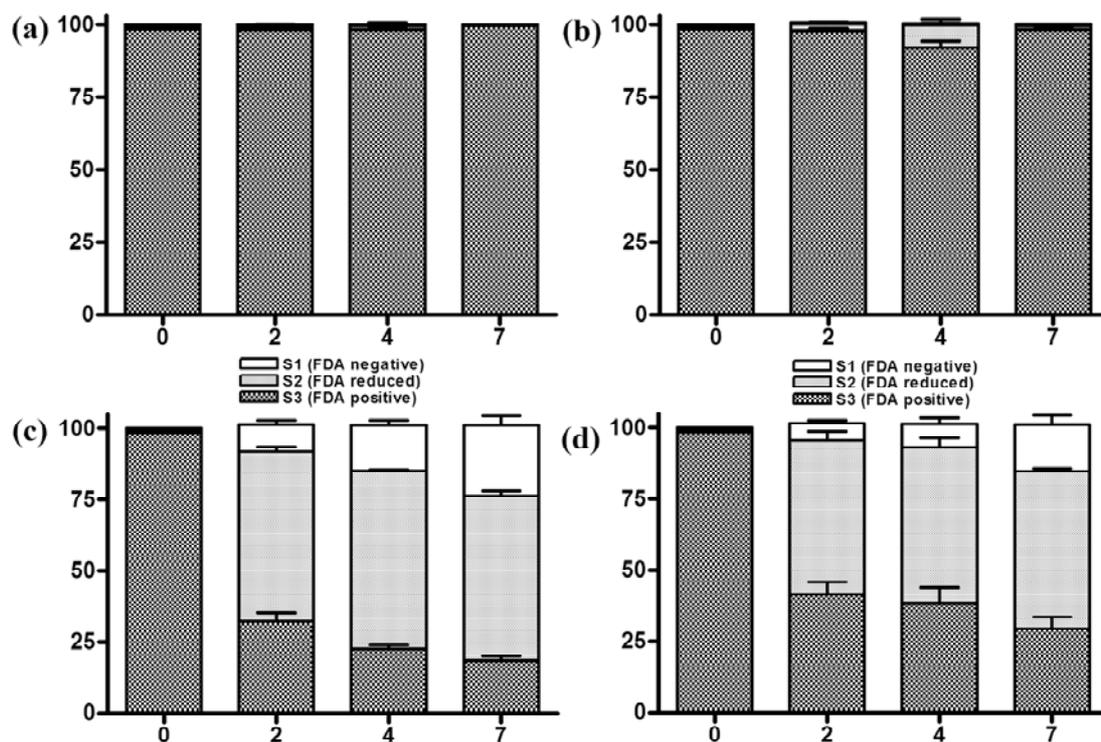
**Figure 9.47.** (a) Seven day *C. vulgaris* FDA fluorescence per-cell, and (b) FDA fluorescence per-cell normalised to cell volume ( $\mu\text{m}^{-3}$ ). The  $y$ -axes show respective cellular FDA fluorescence, whilst  $x$ -axes show the elapsed time at each sampling interval. Data points show mean of triplicate cultures ( $\pm 1$  S.E.M.).

Normalisation of the 7 day FDA cellular fluorescence per unit cell volume ( $\mu\text{m}^{-3}$ ) was again performed, with results still placing the ‘dark’ treatments well below that of the ‘light’ treatments over the truncated 7 day experimental duration. Seven day raw cellular FDA fluorescence (Figure 9.47a) was significantly greater for both ‘light’ treatments than both ‘dark’ treatments (1-way RM-ANOVA;  $F_{(3,2)} = 15.42$ ;  $p < 0.01$ ), with the same ANOVA showing no apparent differences within ‘light’ or ‘dark’ treatments themselves regardless of DO concentration ( $p > 0.05$ ). Identical results were seen for the



cell-volume-normalised data (Figure 9.47b), meaning that although algal cell volume did decrease by  $\approx 25\%$  over the 7 days for both 'dark' treatments, it was not sufficient to account for the observed reduction in FDA metabolic activity (given that the cell membranes were in tact according to PI viability staining). As evident in Figures 9.47(a) and (b), normalising cellular FDA fluorescence to cell volume did bring the FDA metabolic activity status of the 'light' and 'dark' treatments closer together; although not enough for them to be considered statistically similar.

Cellular FDA metabolic activity results from the 7 day dark-survival experiment were also divided into their respective regional S1, S2 and S3 fluorescence 'activity states' as was done for the 65 day experimental data above. Results of this data manipulation are shown in Figure 9.48 below. As shown in Figure 9.48, there were significantly more cells with reduced or indeed no FDA metabolic activity when exposed to continuous darkness over a period of 7 days than for cells in the light. As for Figure 9.47 above, there was again no real difference between the overall population FDA activities of either 'dark' treatment (Figures 9.48c and 9.48d), implying that the observed reduction in FDA cellular activity during darkness was occurring independently of DO concentration (i.e.  $\approx 8.1$  or  $2.2 \text{ mg L}^{-1}$ ). This general trend of there being no distinction between the dark-survivorship of phytoplankton in 'dark / aerobic' and 'dark / low D.O.' treatments has been observed virtually across the entire suite of cellular analyses during the two month dark-survival experiment, and so it can be concluded with relative confidence that a reduced DO concentration during both prolonged and short-term darkness has no significant bearing on cellular survival for either of these phytoplankton species. Results from the follow-up 7 day dark-survival experiment showed that even for algal (*C. vulgaris*) cells with no prior history of exposure to dark conditions, cell-volume-normalised FDA metabolic activity for both 'dark' treatments was reduced (from that at 'Day zero') by roughly 80% (or approximately twice the magnitude reduction seen for 'light' treatments at the same interval) after the first two days of darkness. Whilst it was likely that this observation was again to some extent a reflection of the previously discussed Day zero inoculum carryover effect (see Section 9.8.1), it is hypothesised that this could also serve to highlight the innate photoacclimational responsiveness of these phytoplankton species to stark changes in light climate.



**Figure 9.48.** 7 day *C. vulgaris* FDA metabolic activity states (S1 – non-viable; S2 – compromised; S3 – viable) over the four treatments: (a) Light / aerobic; (b) Light / low D.O.; (c) Dark / aerobic; (d) Dark / low D.O. (*x*-axis represents time (days) and vertical bars show daily average percentage of the total population in each FDA fluorescence activity state  $\pm$  1 S.D. from 3 replicates).

It should be noted that there was an apparent disagreement between the viability rulings of the PI and FDA assays for the 7 day experiment. As can be seen in Figures 9.46(c) and (d), in excess of 99% of algal cells across all treatments effectively retained their membrane integrity during the 7 day dark-survival experiment, and yet according to the FDA viability assay (Figure 9.48), up to 20–25% of algal populations were classified as FDA-negative ‘non-viable’. This is an important observation, and whilst >99% of cells were obviously still viable (based on having retained their cell membrane integrity), a certain percentage of the population did display ‘sub-viable’ levels of FDA metabolic activity. This, therefore, seems an appropriate time to reiterate the importance of performing simultaneous viability assays, as experimental conclusions based solely on either the PI or FDA assays during the current research would have yielded erroneous conclusions regarding the true nature of algal cell physiology during prolonged darkness. This issue will be referred to again in Section 9.8.2.2.

The large extent of variability reported by others regarding chlorophyll *a* (mass and fluorescence activity) dynamics during phytoplankton dark-survival (see Section 9.7) is again found for reports of algal metabolic activity during prolonged dark-exposure. Hellebust and Terborgh (1967) reported that exponentially-growing, continuously illuminated cultures of *D. tertiolecta* when transferred to complete darkness at 18°C temporarily increased their metabolic (photosynthetic) enzyme activity during the first 24 hours of darkness, after which there was a rapid decrease in photosynthetic capacity and enzyme activity to approximately 25% that of pre-dark conditions following 3 days of continuous darkness (a similar order of magnitude decrease to that recorded here after roughly same dark period). Selvin *et al.* (1988/1989) and Dorsey *et al.* (1989) were among the first to report on cellular FDA metabolic activity in phytoplankton following exposure to prolonged darkness. Selvin *et al.* (1988/89) reported a wide array of cellular FDA metabolic activities in three dinoflagellates (*Protogonyaulax*, *Gymnodinium* and *Prorocentrum* species) during 5 days of dark-exposure. Whilst some species (*Protogonyaulax*) completely lost all signs of cellular FDA metabolic activity following just a few days of darkness, others (*Gymnodinium* and *Prorocentrum*) retained >95% of their cell population's pre-dark FDA fluorescence and were, therefore, considered to have remained viable following the period of darkness.

Dorsey *et al.* (1989) noted that during prolonged (20 day) dark-exposure, some phytoplankton (Prasinophyceae) significantly reduce their FDA metabolic activity, and although they may have appeared compromised or FDA-negative 'non-viable', re-exposure to light conditions saw a full return to FDA-positive 'viable' fluorescence status, such that cells were deemed merely to have been 'quiescent' or inactive during this dark period. This proposed metabolic 'quiescence' in phytoplankton during unfavourable dark conditions seems quite logical on a fundamental level, given that higher plants, such as deciduous macrophytes, do essentially the same thing during terrestrial low-light, low-productivity winter periods. These biochemically 'quiescent' phases in phytoplankton have indeed been likened to those occurring during dormancy or hibernation of higher plants and animals (Anita, 1976). In a similar vein, other researchers have also drawn parallels between the low temperature survival mechanisms of unicellular phytoplankton (*Chlorella*) and higher plants (e.g. Hatano *et al.*, 1976; Bartosh and Banks, 2007).

Jochem (1999) recorded similarly variable algal dark-survival capacities based on cellular FDA metabolic activities during approximately 2 weeks of dark-exposure. Following the observed interspecific metabolic variability during prolonged darkness, Jochem (1999)—following in the footsteps of earlier work by Smayda and Mitchell-Innes (1974)—concluded that different algal species have different dark-survival ‘strategies’ according to their inherent ability to alter metabolic cellular processes under adverse dark conditions. Phytoplankton species assigned to ‘Type I’ survival strategy (*Brachiomonas submarina*, *Pavlova lutheri*, *Chrysochromulina hirta*) appeared to recognise the problem of darkness and associated phototrophic energy limitation, and were seen to react by reducing their metabolic activities within a few days; thereby enabling these species to sustain their population abundance following  $\approx$ 2 weeks in the dark. Species displaying the second ‘Type II’ dark-survival response on the other hand (*Prymnesium parvum*, *Bacteriastrum* species) seemed to lack any adjustment in metabolic activity upon dark-exposure and were thought to have essentially ‘grown themselves to death’. As reported by Jochem (1999), carrying on “as usual” in the case of *P. parvum* resulted in constant FDA activity readings but an inevitable decrease in cellular abundance and a greatly reduced re-growth potential following eventual culture re-illumination.

Like that observed by Hellebust and Terborgh (1967) above, Franklin and Berges (2004) also observed an initial increase in time-zero-relative FDA fluorescence during the first 10 days of dark-exposure for the dinoflagellate *A. carterae*, after which time (3 weeks darkness) the FDA metabolic activity returned to levels comparable to light-incubated controls. These examples, therefore, serve to highlight the inherent variability in dark-survival responses between different phytoplankton taxa and also should be used to reiterate the importance of methodological precision and control during such cytological measurements, especially where relatively small shifts in measured parameters can significantly alter the inferred conclusions.

Possible mechanisms for phytoplankton survival under dark conditions include algal heterotrophy and reduced cellular metabolism—the latter involving all catabolic processes being slowed (Tilzer *et al.*, 1977). Because dark respiration rates are generally lower than light-enhanced rates (Graham *et al.*, 1995), algal cells that are subjected to

prolonged dark conditions would be expected to have reduced energetic requirements and so would also be likely to undertake a slower respiratory consumption of cellular 'photosynthate' storage products (typically carbohydrates according to Geider and Osborne (1989)). Montaini *et al.* (1995) proposed that the observed long-term (5 month) dark-survival of the Prasinophyte *Tetraselmis suecica* was attributable to the alga possessing large amounts of intracellular reserve material which could be efficiently catabolised in order to satisfy the necessary energy requirements for dark-maintenance of cellular integrity and motility. This was somewhat contradicted by the suggestions of Jochem (1999), who proposed that the majority of small phytoplankton (<10 $\mu$ m diameter) are not likely to possess significant quantities of cellular reserves for prolonged dark-survival and so must either switch to alternate modes of nutrition or reduce their metabolic activity to a necessary minimum. Since alternate trophic states were all but ruled out in the context of the current research (Section 9.12), it can only be suggested that both *C. vulgaris* and *C. reinhardtii* either possessed significant quantities of cellular reserves to sustain dark-viability, or, were sufficiently capable of reducing cellular metabolic processes to such an extent that complete population survival was achieved during and following two months of continuous darkness. Although cellular storage products were not monitored during the course of the current experiments, it is proposed that the advanced dark-survival capabilities of these two phytoplankton resulted from a combination of the above two factors; although the relative contributions of each to overall dark-survivorship remain unknown.

It should also be noted that the observed dark reduction in cellular metabolism (and implied reduction in rates of oxidative respiration) is supported by the lack of oxygen-debt accumulation in particularly the sealed 'dark / low D.O.' treatment flasks for both species (see Figures 9.2 and 9.3). If the algal cultures in this hermetically-sealed treatment displayed 'normal' rates of cellular respiration then it could be expected that the DO reserves would be quickly depleted and ultimately would have resulted in the development of anoxic conditions. The fact that there was no apparent exhaustion of the available DO does imply a significantly reduced rate of metabolic cellular respiration during darkness—a concept that has been confirmed elsewhere (French, 1934; Ganf, 1974; Geider and Osborne, 1989; Reynolds, 2006).

Algal respiration rates are known to generally decrease with decreasing PFD as part of a general photoacclimation response to changing light climate (Falkowski and Owens, 1980; Ferris and Christian, 1991). The rate of phytoplankton dark respiration has been reported to decrease exponentially (5 to 10-fold) with increasing time in the dark; stabilising at a constant rate after approximately 8 to 16 hours of darkness (Geider and Osborne, 1989; Markager *et al.*, 1992). Assuming that a low and stable respiratory rate was established during the current experiments, and using the data of French (1934), the respiratory O<sub>2</sub> demand of *Chlorella* following ≈30 hours of darkness would be expected to be in the order of 0.02 mol O<sub>2</sub> mol<sup>-1</sup> C d<sup>-1</sup> (i.e. 1:50 O<sub>2</sub> to C molar ratio). Stoichiometrically then, and using a cell carbon to chlorophyll *a* mass ratio of 50:1 (Reynolds, 2006), there was a chlorophyll-derived average of ≈4.00mg C L<sup>-1</sup> (or ≈3.33×10<sup>-4</sup> M) in the ‘dark’ treatments for both *C. vulgaris* and *C. reinhardtii* over the entire 65 day experimental duration. Based on these calculations, the implied respiratory O<sub>2</sub> demand of the ‘dark’ treatment algal cultures would be expected to have been in the order of 0.080mg O<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>—an oxygen consumption rate identical to the lower-end dark-respiration spectrum reported by Reynolds (2006). This equates to something in the order of 5.15mg O<sub>2</sub> required per litre of algal culture of over the course of the entire 65 day dark-survival experiment, or approximately twice that which was available according to the 65 day average DO concentration of ≈2.2mg L<sup>-1</sup>.

Since atmospheric re-aeration was previously ruled out for ‘low D.O.’ treatment flasks (Section 7.2.1), and since there could have been no photosynthetic re-oxygenation during the strict dark conditions, it can only be assumed that the algal culture respiration rate must have been somewhat lower than the values suggested above; perhaps diminishing proportionately in response to the diminished O<sub>2</sub> availability in these treatment flasks. It is possible then, that at the already reduced ‘low D.O.’ oxygen concentration, the algae were subjected to significantly reduced oxygen tensions and may therefore have had an inherently reduced capacity for oxygen uptake due to the low concentration gradient; again suggesting a ‘positive-feedback’ type scenario. Additionally, since the treatment flask dissolved oxygen measurements were actually performed *ex situ* (i.e. outside of the treatment flasks themselves; Section 7.2.3.1), it is possible that there may have been some degree of sample re-aeration during both the pipetting transfer stage and also from the measurement (probe immersion) process itself;

both of which might have lead to a potential overestimation of the actual DO concentration within ‘low D.O.’ treatment flasks. Regardless of the precise stoichiometry, however, it was evident that overall cellular metabolic processes were somewhat subdued during prolonged darkness, and as already discussed above, this was thought to have contributed greatly to the observed long-term dark-survival capabilities of both *C. vulgaris* and *C. reinhardtii* here.

### **9.8.2.1 The effects of a changing cell volume on FDA-quantified physiological activity**

The need to be conscious of changes to cell volume over time, and how this might then influence one’s physiological observations, was first raised (although somewhat inadvertently) by Winokur (1949). Winokur (1949), following photosynthetic investigations involving ageing illuminated cultures of *C. vulgaris*, observed that part of the observed reduction in culture photosynthetic capacity ( $\text{mm}^3 \text{O}_2 \text{ evolved h}^{-1}$ ) over a 30 day period was attributable to a parallel reduction in average cell volume over the same period. Although Winokur (1949) did concede that the overall loss of photosynthetic capacity from ageing *C. vulgaris* cultures as a whole was more than could be accounted for by the observed reduction in cell volume, it does highlight the need for experimental diligence in this area. Attempting to correct for changing cell volumes over time during the course of the current research yielded mixed results. Unlike the dark-induced reduction in chlorophyll *a* fluorescence activity (which was considered to have been largely accounted for by diminishing cell volume; Section 9.7.3), the reduction in FDA metabolic activity was significantly more than could be simply attributed to ‘cellular shrinkage’ during the 65 day experiment. From this, it could be tentatively concluded that both *C. vulgaris* and *C. reinhardtii*, when subjected to prolonged dark conditions, appear to reduce their general levels of maintenance metabolism whilst more actively maintaining their levels of general photosynthetic activity; possibly to allow for rapid re-growth following re-exposure to suitable light conditions (a hypothesis supported by the rapid re-growth rates observed in Section 9.9.1).

As highlighted previously (Section 9.6.3.2), these issues surrounding the correct units of expression for fluorescence measurements derived from and relating to aspects of cellular physiological activity, are far from being universal and clear-cut. As demonstrated within the above Sections, expressing fluorescence activity (e.g. chlorophyll *a* or FDA) ‘per-cell’ or ‘per unit cell volume’ can yield markedly different experimental outcomes. Since these issues are very much still under debate (Raven and Kübler, 2002), no attempt will be made to consolidate them here. In the very least, however, this does serve to highlight the complex nature of cellular fluorescence data derived from flow cytometric analyses and the arguably even more complex and challenging associated task of ‘crunching’ and formatting the raw data into some logical, interpretable and meaningful format from which one can then attempt to draw the correct conclusions.

### **9.8.2.2 The importance of multiple markers for viability resolution**

As introduced (Section 6.4.2.4.3), the powerful duality of the PI–FDA staining assay provides the analytical resolution necessary for accurate ‘live versus dead’ discrimination during FCM analyses. Using the 7 day experimental data as an example, if one were to look exclusively at the PI viability data from Figures 9.45 and 9.46, it could only be concluded that algal cells in all treatments were “equally viable”. Similarly, if only the FDA metabolic activity data from Figures 9.47 and 9.48 was used for viability assessment, then one would be justified in concluding that both ‘dark’ treatments contained significantly “less viable” algal populations than did the ‘light’ treatments—a significant portion of which would have incorrectly been considered FDA-negative ‘non-viable’. In this sense, it is not surprising that there is a recognised ‘higher ranking’ of membrane integrity measurements in the overall assessment of cellular physiological status (Vives-Rego *et al.*, 2000).

Whilst the detection of metabolic activity is less stringent than other measures of viability such as culturability or membrane integrity, it does suggest the *absence* of cell death (Vives-Rego *et al.*, 2000). So whilst metabolic activity does not directly guarantee the capacity for reproductive growth, it does provide useful insight into a cells’



physiological status, especially when combined with other measures of viability. In cases of injury, dormancy or extreme starvation, metabolic functions might also be occurring at or below the method detection limit (Vives-Rego *et al.*, 2000) and this is where the true value of multiple viability markers lies. In the context of the current research, the conspicuous lack of PI uptake in long-term dark-exposed algal cells suggests that the observed decrease in FDA fluorescence was due to a metabolic inhibition of intracellular esterases and *not* a consequence of changes in membrane integrity (where hydrolysed fluorescein may have leached from compromised cell membranes). An interesting point of discussion here is that if we were to observe a significant decrease in fluorescence signal from a single viability marker (e.g. cellular FDA fluorescence or chlorophyll *a* activity) during prolonged dark-exposure, how do we then decipher whether we have observed either an active photoacclimation-type response to the environmental dark stressor, or, if we have instead recorded an early indicator of impending cellular death? This is again where it becomes highly desirable—if not essential—to incorporate multiple measures of cellular viability so that the most accurate determination of actual cell survivorship can be realised.

Since disintegration of the cell membrane structure is indicative of late-stage cell mortality (Veldhuis and Kraay, 2000) it should be taken as the ultimate *final word* on the status of cellular viability. In this sense, membrane integrity provides a quantitative measure of cell death (i.e. alive or dead) whereas other measures of viability, such as metabolic activity, serve more as qualitative measures of ‘general vitality’. It is recommended, therefore, that all future work in this area adopt a combination of methods from both the ‘qualitative’ and ‘quantitative’ sides of general viability assessment in order to achieve the most comprehensive evaluation of phytoplankton cellular viability as a whole.

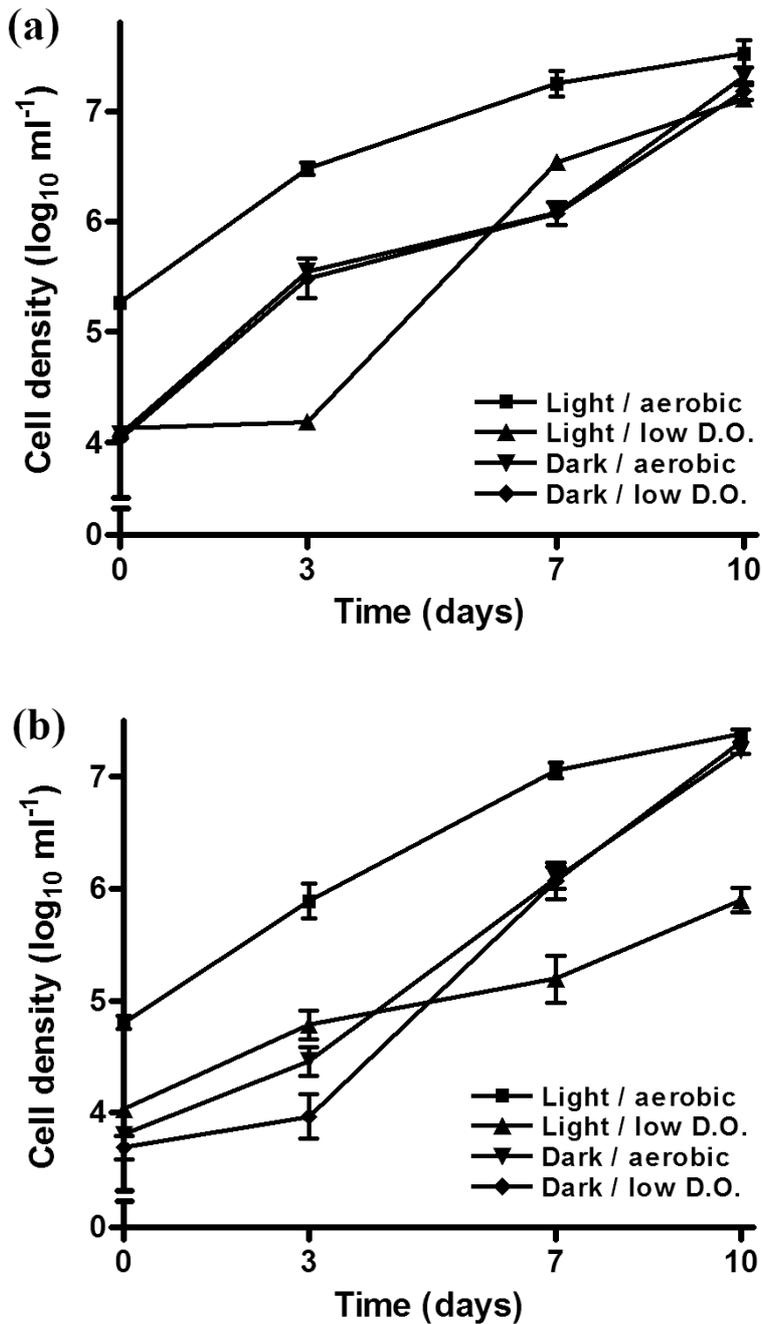
### **9.9 *Phytoplankton re-growth potential following prolonged darkness***

As introduced in Chapter 6 (Section 6.4.2.5), the capacity of cells to re-grow following chronic exposure to some form of environmental and/or physiological stressor (in this case darkness) serves as the ultimate assessment of their ability to endure that particular

stress event, whilst retaining sufficient physiological vigour to allow for continued population expansion upon a return to more favourable growth conditions. So far, the assessments of phytoplankton capacity to withstand prolonged dark-exposure have focused on ‘single-cell’ level cytological investigations. Whilst these cytometric investigations have provided tremendous physiological insights in to the vitality of the experimental algal populations on a cell-by-cell basis, they offer no definitive verdict with respect to the overall capacity (and rate) to which the population as a whole can re-grow following re-exposure to a more favourable light climate. It can be appreciated that from an ecological standpoint, one could perform a suite of FCM analyses on a cell population and find that 100.000% of algal cells display no discernable signs of physiological viability and conclude based on this that the population is not likely to be able to recover. At the same time, if this conclusion is never validated in a culture situation, then these previous determinations will invariably carry less weight (given the often high orders of magnitude cell densities in phytoplankton populations and the theoretical requirement for only 1 viable cell amongst 999,999 dead ones in order to promote full-scale population recovery). Hence, for final qualification of the results from ‘single-cell’ level flow cytometric viability determinations, re-growth experiments of long-term dark-exposed phytoplankton cultures were conducted and the results presented below.

### **9.9.1 Re-growth potential of *C. vulgaris* and *C. reinhardtii* following 65 day dark-exposure**

Freshly sub-cultured aliquots of long-term ‘Day 65’ dark-exposed cultures (see Section 7.2.2.2) for both *C. vulgaris* and *C. reinhardtii* displayed seemingly unrestricted capacities for population re-growth post-darkness, attaining stationary-phase culture densities ( $>1 \times 10^7$  cells ml<sup>-1</sup>) after 10 days of re-illumination at 20°C (Figure 9.49).



**Figure 9.49.** 10 day algal re-growth curves for (a) *C. vulgaris* and (b) *C. reinhardtii* (data points show mean of three replicate cultures  $\pm$  1 S.D.)

Following just three days of re-illumination, both *C. vulgaris* and *C. reinhardtii* appeared to have become fully ‘re-activated’; displaying chlorophyll *a* fluorescence activities equal to that of healthy ‘Day zero’ pre-dark cultures (data not shown). Critical analysis of the data from Figure 9.49a showed that triplicate *C. vulgaris* ‘dark’ treatment re-growth cultures, during the first 3 days of re-illumination, had statistically identical

(unpaired  $t$ -test;  $p = 0.87$ ) average maximum culture growth rates ( $r_n$ ) of  $1.13 (\pm 0.04)$  and  $1.11 (\pm 0.15) \text{ d}^{-1}$  for the 'dark / aerobic' and 'dark / low D.O.' treatments respectively. Whilst these re-growth rates were only approximately half those of the optimal exponential culture growth rates for *C. vulgaris* ( $r_n = 2.50$ ; Figure 9.1), three-day 'dark' treatment re-growth  $r_n$  values were actually slightly higher (although not significantly;  $p > 0.05$ ) than that of the 'light / aerobic' treatment at  $0.93 (\pm 0.07) \text{ d}^{-1}$ . As can clearly be seen in Figure 9.49, algal cells from the previously ailing 'light / low D.O.' treatment re-grew at a significantly reduced rate ( $r_n = 0.44 \pm 0.06$ ) compared with all of the other treatments (1-way ANOVA;  $F_{(3,8)} = 39.01$ ;  $p < 0.001$ ).

Similar overall trends were seen for *C. reinhardtii* during 10 day re-growth experiments. 'Dark' treatment *C. reinhardtii* re-growth cultures after 7 days of re-illumination had maximum  $r_n$  values of  $0.96 (\pm 0.01)$  and  $1.21 (\pm 0.03) \text{ d}^{-1}$  for the 'dark / aerobic' and 'dark / low D.O.' treatments respectively. Once again, and although these re-growth rates were around half of the maximum exponential culture growth rates achieved for *C. reinhardtii* previously ( $r_n = 2.42$ ; Figure 9.1), dark treatment re-growth  $r_n$  values were again higher (although only significantly for the 'dark / low D.O.' treatment; 1-way ANOVA;  $F_{(3,8)} = 36.23$ ;  $p < 0.01$ ) than that of the 'light / aerobic' treatment at  $0.83 (\pm 0.08) \text{ d}^{-1}$ —a likely reflection of the lower initial starting population density for 'dark' treatments (see Figure 9.49b).

'Light / low D.O.' treatment *C. reinhardtii* also re-grew at a significantly reduced rate ( $r_n = 0.54 \pm 0.14$ ) to that of all other treatments ( $p \leq 0.01$ ). For the 'light / low D.O.' treatment cultures, whilst there was considerable population cell death during the two months of continuous illumination and DIC starvation, quite obviously the small number ( $\approx 2\%$  or  $2 \times 10^4$  of an initial  $1 \times 10^6$  cells for both species) of surviving cells were sufficiently viable to allow for a relatively rapid and full-scale population recovery following 10 days of re-illumination for *C. vulgaris* and a partial recovery for *C. reinhardtii*; although the shape of the re-growth curve for 'light / low D.O.' *C. reinhardtii* would suggest that a full recovery (i.e.  $10^7$  cells  $\text{ml}^{-1}$ ) would be achieved sometime shortly after the 10 day monitoring period. This observation was similar to findings of Berges and Falkowski (1998), where it was shown that cultures of *D. tertiolecta* underwent catastrophic cell death following 6 days of dark-exposure (after

which time overall culture 'vital signs' approached zero), yet the culture population as a whole was still able to fully re-grow—albeit with a somewhat lengthy lag-phase—even after more than two weeks of total darkness. The same was also true for the extensive cell death observed for 'light / low D.O.' treatment cultures during the current research, whereby the death of the vast majority ( $\approx 98\%$ ) of the population merely paved the way for the more resilient 2% of the cell population to re-grow once conditions were again favourable.

Vaulot *et al.* (1986) reported a 25% slower or retarded re-growth cycle in the alga *Hymenomonas carterae* when dark-exposed for 3–4 days and then re-exposed to light conditions. Similarly, Popels and Hutchins (2002), after investigating the dark-survival capacity of *Aureococcus anophagefferens* (Pelagophyceae), reported that the alga could indeed re-grow after a period of 30 days darkness; however, the length of the lag time during population recovery was shown to increase with increasing duration of dark-exposure. It is possible that the same result may have been observed for *C. vulgaris* and *C. reinhardtii* here (i.e. between 'Day zero' and Days 1 or 2), unfortunately, and due to the first re-growth sampling interval being on 'Day 3' following re-exposure to light conditions, more detailed resolution of the re-growth time kinetics following initial culture re-illumination cannot be provided. Regardless of this, results from the re-growth experiments serve to demonstrate that prolonged dark-exposure (regardless of 'high' or 'low' dissolved oxygen concentration) did not restrict the capacity of either *C. vulgaris* or *C. reinhardtii* to re-grow upon re-exposure to light conditions. Re-growth results showed that following approximately two months of dark-incubation, both algal species were essentially no worse off than their constantly illuminated, statically ageing counterparts.

The rapid re-growth potentials observed for both 'dark' treatments and for both algal species in the current research tied in well with the observed long-term maintenance of cellular pigments and chlorophyll *a* fluorescence activity during the 65 day dark period (see Sections 9.7.1 and 9.7.3). As hypothesised earlier (Section 9.8.2.1), it appeared that algal cells may have indeed been maintaining their cellular photosystem activity so as to allow for rapid population expansion upon re-exposure to light conditions. Popels *et al.* (2007) actually suggested that phytoplankton could possibly metabolise the

photosynthetic enzyme RUBISCO as another catabolic protein source during prolonged dark-exposure. The high-level maintenance of *in vivo* chlorophyll *a* fluorescence observed for both species during and up to 65 days of continuous darkness would suggest that these phytoplankton were not going down this pathway of ‘photosynthetic catabolism’ during the dark period. Instead, cells appeared to actively maintain photosynthetic infrastructure during two months of darkness and were then observed to be capable of rapid re-growth following culture re-illumination. This observation is again supported by the knowledge that phytoplankton are known to preferentially catabolise carbohydrates (before proteins and lipids) in order to satisfy their immediate energy requirements during dark respiration (Handa, 1969; Geider and Osborne, 1989).

Despite this long-term metabolic ‘dark maintenance’ coming at an obvious energetic cost, it appears that a dark period in the order of 60 days was not sufficient to adversely impact on overall phytoplankton survivorship (as measured on both a ‘single-cell’ and also a ‘population’ scale). The findings here are similar to those reported earlier by Handa (1969) following 10 day dark-exposure of continuously illuminated cultures of the marine diatom *Skeletonema costatum*. Handa (1969), despite observing a rapid and significant consumption of cellular resources during darkness (sum total of total lipid, carbohydrate and protein metabolism during the 10 day dark period corresponded to a total loss of  $\approx 43\%$  of cellular organic carbon), showed that recalcitrant cell wall carbohydrates and photosynthetic pigments were conserved and that cultures were able to photosynthesise and quickly re-grow upon re-exposure to light conditions. This can also be linked to the reporting of Richardson and Fogg (1982), whereby some algal species have been observed to resume maximal growth rates either immediately, or very soon after, transfer from a two week period of very low light intensity ( $2\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) or darkness, back to a more favourable light climate ( $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). While this does not necessarily indicate that these organisms retain the ability to photosynthesise *maximally* whilst exposed to low-level irradiance, it does suggest that some phytoplankton can maintain biochemical machinery in excess of their immediate demands under very low light and even dark conditions, and/or that the recovery time of this biochemical machinery upon return to more a optimal light climate can be fast (Richardson *et al.*, 1983).

As is the case for individual algal dark-survival capacity, the capability for rapid post-dark re-growth is by no means a universal trait possessed across the board by all phytoplankton species. Smayda and Mitchell-Innes (1974) discussed how the rate of culture re-growth has been observed to display variability according to algal species and also the specific duration of the dark period. Finke *et al.* (1950) reported a relatively long lag period during culture re-growth for 10 week dark-exposed *C. vulgaris*; something not observed for the same species here. Richardson and Fogg (1982) also found some species (Dinophyceae) to exhibit a considerable lag period prior to resuming maximal growth rates upon transfer from low ( $2\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) to high ( $60\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) light intensities. Similarly, Wolfe *et al.* (2002) reported an almost immediate return to exponential growth in the *Emiliana huxleyi* following re-illumination after 5 days of dark-exposure, whereas they observed very poor post-dark recovery in the dinoflagellate *Alexandrium fundyense* after the same period of darkness after re-exposure to light conditions. Finally, Yentsch and Reichert (1963) saw no re-growth whatsoever in five-day dark-exposed *D. tertiolecta*—a similar observation to that reported later by Berges and Falkowski (1998) and Segovia *et al.* (2003) for the same species when exposed to dark conditions over a similar time-scale. Jochem (1999) linked the post-dark re-growth capacities of different phytoplankton species to their overall physiological dark-survival strategy (i.e. ‘Type I’ and ‘Type II’ strategists; see Section 9.8.2), with both *C. vulgaris* and *C. reinhardtii* apparently displaying the more advanced ‘Type I’ response throughout the research presented in this Chapter.

Bartosh and Banks (2007) demonstrated a similarly tenacious dark-survival capacity for *C. vulgaris* to that reported in this Chapter, with culture re-growth possible even after 21 weeks of darkness; although this work was carried out at a much lower temperature ( $4^{\circ}\text{C}$ ) than the current investigations ( $20^{\circ}\text{C}$ ). Whilst the reduced temperature in the work of Bartosh and Banks was likely to have augmented the long-term dark-survival capability of *C. vulgaris* (e.g. Anita 1976; Smayda and Mitchell-Innes, 1974; Dehning and Tilzer, 1989; Lewis *et al.*, 1999; Bartosh *et al.*, 2002; Popels and Hutchins, 2002), the same species was still highly resilient to prolonged ( $\approx 9$  week) darkness at the much higher temperature adopted during the current research. Interestingly, Bartosh and Banks (2007) also observed that *Scenedesmus subspicatus* was significantly less resilient to prolonged darkness than *C. vulgaris*, with the alga failing to re-grow after  $\approx 14$  weeks of

dark-exposure despite belonging to the same taxonomic Order as *Chlorella* (Chlorococcales). Bartosh and Banks (2007) concluded that since algae of the genus *Chlorella* are not known to possess any structural adaptations nor to develop morphologically-distinct resting stages, long-term dark-survival relies exclusively on the brute “hardiness” of vegetative cells. It is, therefore, likely that both *C. vulgaris* and *C. reinhardtii* would be very resilient to prolonged *in situ* dark passage within a WSP advanced upgrade scenario (such as a dense duckweed cover or within a rock filter) and would be expected to be both physiologically viable and also capable of re-growth if re-exposed to light conditions, even following a greatly extended two month dark period.

As is always the case when using laboratory-based studies for predicting environmental outcomes, there are several potential sources of error that may have influenced the observed dark-survival and subsequent re-growth results of the current research. As highlighted earlier (Section 7.2.2), and in spite of adopting the utmost preventative measures in order to eliminate any light exposure (particularly during sampling), the fact that experimental sampling during the dark-survival study was performed with the unaided eye meant it was highly likely that long-term dark-exposed phytoplankton were periodically exposed to very low intensity light (i.e. below the quantum sensor’s limit of detection;  $\text{PAR} < 0.000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ); light that could have in some way prolonged or augmented the long-term dark-survival capacities of the two investigated algal species. Although this periodic low-light exposure would have been well below the photosynthetic compensation point for both species, other researchers have cautioned—indeed confirmed—that such light regimes can have a supportive effect on long-term algal dark-survival (Hellebust and Terborgh, 1967; Umebayashi, 1972; Smayda and Mitchell-Innes, 1974). The adoption of low-intensity back-lighting regime at sampling intervals during this research was considered to have been as dark as practicable, and was arguably less intense than the sampling illumination source used by Ferroni *et al.* (2007) during their long-term phytoplankton dark-survival assessments (i.e. dim green ‘safe-light’; 15W green Philips globe;  $\text{PAR} < 0.1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ).

Although the precise implications of this occasional low-light-exposure were unquantifiable, periodic exposure to very low PFD during sampling could have potentially both augmented overall algal dark-survival and also allowed for an



accelerated rate of re-growth following re-illumination. When considering this in an applied sense, however, the possibility of periodic low-level re-illumination during the prolonged dark period may not be a misrepresentation of *in situ* conditions. For example, under even the thickest (2–3cm) of duckweed surface foliage mats ( $\approx 8.3\text{kg m}^{-2}$  fresh weight), the degree of incident irradiance attenuation at a depth range of 0.1m below the surface mat did not exceed 99.5% ( $\pm 0.04$ ) under an incident light intensity of  $600\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (see Section 3.3.3). This means that algal cells traversing a duckweed-covered WSP will in the very least be expected to be exposed to periodic low-level irradiation; conditions that might be sufficient to facilitate their dark-survivorship *in situ*. Nonetheless, it must be cautioned that this is perhaps a fatal flaw of all non-destructive sampling regimes adopted during dark-survival experimentation, and so it would be the author's firm recommendation that *destructive* sampling protocols be adopted during any future work involved with dark-survival assessment of phototrophic organisms.

Another potentially important factor when extrapolating the results of laboratory re-growth experiments to what may be expected *in situ*, is the fact that these re-growth assessments were performed under arguably more 'optimal' conditions (i.e. continuous illumination, nutrient-replete and axenic) than those which might be reasonably expected to exist within a WSP environment. This could mean that the shape or slope of the algal re-growth curves may not be expected to be so elevated *in situ* as they were here under controlled laboratory conditions. Additionally, the single-species nature of the laboratory monocultures inherently prevented any competition factors from exerting an effect upon algal dark-survival and/or re-growth performance. Any interspecies differences in dark-survival capabilities, and the associated differential rate of dark-induced algal cell death, will almost certainly affect the outcome of interspecies competition and post-dark recovery *in situ*; although little is known about these processes in phytoplankton (Lee and Rhee, 1999).

Considering the above, it is likely that the relatively advanced capacity for post-dark re-growth observed during the current research represents a 'best case' scenario for both *C. vulgaris* and *C. reinhardtii* in terms of the anticipated rapidity of population re-growth and recovery following a prolonged dark-exposure event *in situ*. In the same sense, it could also be argued that the research findings reported here represent a 'worse

case' scenario from the viewpoint of WSP operators, in terms of these results reflecting both the best possible algal dark-survival capabilities and also the fastest re-growth rates one might reasonably expect to see following algal passage through an advanced WSP effluent upgrade process.

### **9.10 Dark-survival, dissolved oxygen concentration and dark respiration—discussion of the 'low D.O' treatment results**

Following a rigorous literature search, and to the best of the authors' knowledge, there is a very limited body of research reporting on the effects of dissolved oxygen concentration on phytoplankton dark-survival. There have, however, been a limited number of reported cases of phytoplankton viability *per se* under dark–anoxic conditions (Wiedeman and Bold, 1965; Moss, 1977; Platt *et al.*, 1983; Anderson *et al.*, 1987; Pearson *et al.*, 1987; Richardson and Castenholz, 1987; Vermaat and Sand-Jensen, 1987; Blanco, 1990; Detmer *et al.*, 1993; Sun *et al.*, 1993; Harvey *et al.*, 1995; Gervais, 1997; Casamayor *et al.*, 2001; Ruangdej and Fukami, 2004; Wilk-Woźniak and Żurek, 2006), of which a brief discussion will be provided below.

Platt *et al.* (1983), Detmer *et al.* (1993), Casamayor *et al.* (2001) and Wilk-Woźniak and Żurek (2006), whilst they are included in the above list, all involve mainly bulk observations from uncontrolled field surveys. The work of both Richardson and Castenholz (1987) and also Harvey *et al.* (1995) is also included tentatively, since these works involve dark–anoxic assessment of Cyanobacteria (*Oscillatoria* and *Synneococcus*) rather than true phytoplankton species (see Section 6.3 for an initial discussion of Cyanobacteria in algal research). Harvey *et al.* (1995), despite also assessing the dark–anoxic survival of a diatom (*Thalassiosira*), defined the point of phytoplankton cell death as the point at which CO<sub>2</sub> uptake reached 4% of pre-dark levels and considered this point to have been reached after just five days of darkness; such that it is highly likely that these cells actually remained viable even after their so-called 'death'. Furthermore, Harvey *et al.* (1995) made no attempts to define cellular viability following Day five 'cell death' (e.g. via microscopic, photosynthetic, or re-growth assays) and so their results are not considered here. Anderson *et al.* (1987) and Blanco (1990) are also included in the above list tentatively, since these works both involve

investigations into dinoflagellate *resting cyst* germination rather than *vegetative cell* dark-survival. The work of Gervais (1997) is again included with reservation, as it involved *aerobic* dark-survival assessment of phytoplankton (*Cryptomonas*) that were simply isolated from the oxic–anoxic chemocline of a freshwater lake. Vermaat and Sand-Jensen (1987) are again cautiously included in the above list, since this work involved dark–anoxic assessments of macroalgae (*Ulva lactuca*) not phytoplankton *per se*. Finally, the work of Sun *et al.* (1993) and Ruangdej and Fukami (2004) are also careful inclusions, since ‘algal’ survival under dark–(within sediment) anoxic conditions was only assessed indirectly through the monitoring of proxy chlorophyll *a* concentration.

Following this, the work of Wiedeman and Bold (1965), Moss (1977), and also Pearson *et al.* (1987) remain as the only real exceptions from the above literature base. Even then, the anaerobic or “air excluded” Petri dish treatments employed by Wiedeman and Bold (1965) are considered to have been of a somewhat dubious oxygen status (as highlighted also by Pearson *et al.*, 1987c), with the authors also failing to define exactly how they quantified cellular “growth” following the dark-exposure period. Similarly, the work of Pearson *et al.* (1987) involved heterotrophic growth assessments of phytoplankton following just 4 days of darkness; experimental conditions that place their work on the very brink of exclusion here based on the initial literature ‘inclusion criteria’ outlined in Section 6.3. In light of this very limited body of work, the potential influence of DO concentration on phytoplankton survival during prolonged darkness was investigated. It must be reiterated that strict anoxia was not a condition assessed during the current research; instead the potential effects of a reduced or ‘hypoxic’ DO environment on long-term dark-survival was investigated (see Section 6.3.3 for the initial experimental rationale).

Results from the current long-term dark-survival investigations have demonstrated that DO concentration had no discernable impact on phytoplankton dark-survivorship in either *C. vulgaris* or *C. reinhardtii*. Moreover, this conspicuous absence of DO concentration influence on dark-survival was thoroughly demonstrated across the entire range of monitored cellular viability parameters. In the very few instances where there were statistically significant differences between 'dark / aerobic' and 'dark / low D.O.'

treatments (in terms of observed physiological vitality), DO concentration had no *real* bearing on overall phytoplankton dark-survivorship or the subsequent ability for post-dark re-growth. Following this, it is concluded that oxygenic status (within the tested conditions of  $\approx 2.2$  and  $8.1 \text{ mg L}^{-1}$ ) of the aqueous environment did not influence long-term dark-survival for these two phytoplankton species.

This manifest lack of any real DO ‘concentration effect’ on phytoplankton dark-survival in the research presented here does not mean that it hasn’t been cited elsewhere. Moss (1977), for example, observed a significant effect of oxygen concentration on the long-term dark-survival of benthic diatoms. The author reported a good capacity (>3 week) for phytoplankton survival when under ‘dark / aerobic’ conditions; however, when dark-exposed under anaerobic conditions, algal photosynthetic capacity reduced quickly to the point where no detectable photosynthesis was occurring after approximately 6 days, with dark–anaerobic cultures also showing no discernable revival potential following 5 days of re-illumination. Moss (1977) also saw a similar result under ‘light / anaerobic’ conditions, with the rate of photosynthesis declining with diminishing DO concentration and again stopping altogether upon anaerobiosis. Following his observations, Moss (1977) concluded that the absence of light alone was less important for phytoplankton survival than the combined effects of darkness and anoxia.

In opposition to the above suggestion of Moss (1977), Richardson and Castenholz (1987) actually observed a *protective* effect of environmental anaerobiosis on the prolonged (10 day) dark-survival of the cyanobacterium *Oscillatoria terebriformis*. The authors found that when ‘dark / aerobic’ conditions were present, *O. terebriformis* viability was lost within 3 days (regardless of the presence of an exogenous carbon source), yet under ‘dark / anaerobic’ conditions (with 30mM supplemented fructose), *Oscillatoria* viability was sustained for a period of 10 days. Additionally, Detmer *et al.* (1993) reported an unexpected increase of aphotic-zone phototrophic pico- and nanoplankton under simultaneous ‘dark / anoxic’ conditions in the central Baltic Sea below a depth of 120m. They observed that cell abundances of phototrophic nanoflagellates were some 6–8 times higher in ‘anoxic–aphotic’ layers than in the overlying ‘oxic–aphotic’ layer. Furthermore, these phototrophic organisms from ‘dark / anoxic’ depths were also capable of photosynthetic productivity when re-oxygenated

and returned to light conditions, with the authors stating that it remained unclear how these originally “oxyphototrophic” organisms were maintaining survival—even growth—under such seemingly sub-optimal conditions.

In order to generate sufficient energy during prolonged darkness, algal cells (obligate autotrophs only) must rely solely on respiratory metabolism of stored material (Montaini *et al.*, 1995). This oxidative process quite obviously consumes oxygen and has the potential to then, in turn, affect the survival of dark-exposed cells if oxygen becomes a limiting resource. Regarding the current results, it is considered that the absence of any significant effect of DO concentration on prolonged algal dark-survival abilities was possibly due to there not being a large enough difference between the ‘aerobic’ and ‘low D.O.’ treatment oxygen concentrations (i.e. approximately 8.1 versus 2.2 mg L<sup>-1</sup>; or ≈90 versus 25% DO saturation at 20°C). Perhaps if the ‘low D.O.’ treatments had DO concentrations of 0.5–1 mg L<sup>-1</sup> (or indeed completely anaerobic), then any differences—adverse or protective—between the ‘aerobic’ and ‘low D.O.’ treatment dark-survival would have been exacerbated, such that they would have then become more readily distinguishable during viability analyses. Whilst this may indeed have been the case, the ‘low D.O.’ treatment oxygen concentration was specifically engineered to reflect the *in situ* DO concentration within an advanced WSP upgrade systems (see Sections 3.3.4 and 4.3.3) and so the observed dark-survival results are, therefore, still seen as a valid reflection of this specific applied scenario.

Interestingly, and as referred to previously (Section 9.8.2), there was no apparent build-up of an ‘oxygen debt’ in any of the ‘low D.O.’ treatments; most notably in the ‘dark / low D.O.’ treatments, where there was assumed to have been no photosynthetic re-oxygenation potential. The absence of oxygen debt accumulation here was unlike the trend observed by Moss (1977), where dark-incubated, mixed, non-axenic phytoplankton (Bacillariophyceae) samples in sealed flasks (similar to that of ‘low D.O.’ treatments here) quickly deoxygenated to the point of anoxia after approximately 8 days; resulting in complete cessation of photosynthesis once all oxygen had been depleted. The lack of such observations during the current work was indeed curious, and in addition to the previously offered explanations of Section 9.8.2, can perhaps be partly explained by the lack of microbial oxygen demand (unlike the natural samples used by

Moss, 1977). Heterotrophic microbes are required to oxidise a variety of organic substrates such as those coming from leaky or dead algal cells, which, in their absence, will not normally combine with molecular oxygen (Varma and DiGiano, 1968). Due to the axenic status of the current dark-survival experiments, there could have been no microbial oxidation of the dissolved and particulate organic carbon released from algal cells over the two month period. This is again of particular relevance for the 'dark / low D.O.' treatment, where DO concentrations were maintained at consistently low levels ( $\approx 2.2 \text{ mg L}^{-1}$ ) for extended periods in the hermetically-sealed vessels without any apparent exhaustion of the already limited oxygen reserves. Since there was no microbial  $\text{O}_2$  demand, it can only be concluded that the low DO concentrations must have been sufficient to sustain cellular maintenance metabolic processes—albeit at likely reduced rates. Also contributing to the lack of oxygen debt in 'light / low D.O.' treatments, could be the likelihood of there having been reduced rates of cellular respiration during the prolonged period of illumination. For example, Pratt (1943) reported that the rates of respiration in ageing illuminated cultures of *C. vulgaris* decreased by  $>50\%$  after a period of 3 weeks of continuous illumination; a trend supported by the results of FDA metabolic analyses reported here (Figure 9.40).

The absence of an observed build up of oxygen debt in the 'dark / low D.O.' treatment algal cultures could possibly also be explained by the reporting of Karlander and Krauss (1966b). The authors reported on a possible interference of the normal respiratory system for *C. vulgaris* in the absence of light, with an inhibited terminal oxidase system (cytochrome oxidase) postulated as the likely locus of the so-called “dark-block”. They suggested that the synthesis of a particular cytochrome oxidase enzyme necessary for oxidative cellular respiration may be light-mediated, such that dark-exposed cells undergo a gradual reduction in cytochrome oxidase levels, ultimately to a critical level whereby cellular metabolism is adjusted towards anaerobic pathways. It should also be noted that if this was indeed the case, then this hypothesised ‘aerobic respiratory inhibition’ would have probably been exacerbated by the reduced  $\text{O}_2$  pressure within ‘low D.O.’ treatments—a phenomenon also observed by Karlander and Krauss (1966b). This hypothesis for a reduced respiratory activity in darkness is supported by the findings of Graham *et al.* (1995), which showed that the rates of cellular respiration in darkness were significantly reduced compared with those under high-intensity light.

Furthermore, respiration is also considered to play less of a role in the general energetics of algae, with dark respiration rates often equating to only 10% of light-saturated photosynthesis (Parsons *et al.*, 1977; Geider and Osborne, 1989). This reduced importance of cellular respiration in phytoplankton compared with vascular plants, for example, is largely due to the relative differences in the levels of non-photosynthetic biomass of vascular plants (high levels) compared with the low levels in unicellular phytoplankton (Geider and Osborne, 1989).

Briggs and Whittingham (1952) observed that *Chlorella* cultures treated with cylinder nitrogen (0.5% O<sub>2</sub>) had a dark respiration rate one third that of cells exposed to 21% O<sub>2</sub>; however, this effect was quickly annulled when cultures were re-illuminated. Considering this, it could be possible that the 'dark / low D.O.' treatment cultures during the current research may have again exerted a reduced respiratory O<sub>2</sub> demand as a direct result of the lowered DO concentration; something that may have potentially contributed toward the absence of complete anoxia within these treatment flasks over two months of continuous darkness. Overall, some combination of the above discussed factors could perhaps go toward explaining the lack of apparent O<sub>2</sub> consumption and the subsequent development of anoxic conditions in hermetically-sealed 'dark / low D.O.' treatment flasks for *C. vulgaris*. While the same could also be true for 'dark / low D.O.' treatments of *C. reinhardtii*, it was not as thoroughly researched within the literature and so is not discussed.

If the abovementioned factors contribute towards explaining the lack of oxygen debt in the 'dark / low D.O.' treatment cultures, then what can be said for the 'light / low D.O.' treatment? Whilst the hermetically-sealed 'dark' cultures obviously had no means by which to re-oxygenate the 'low D.O.' aqueous medium (i.e. neither photosynthetic nor gas exchange), presumably the 'light' cultures would have been able to do so through normal photosynthetic pathways; however, this was not the case. This curious observation has already been highlighted in Section 9.5.1 and will be discussed here in more detail. The conspicuous lack of photosynthetic re-oxygenation in the 'light / low D.O.' treatment cultures (see Figures 9.2 and 9.3) could to some extent be accounted for by the fact that O<sub>2</sub> production rate (per unit biomass) of algal (*Chlorella*) cultures has been reported to be at its lowest during stationary growth phase (Bartosh *et al.*, 2002),

such that the rate of photosynthetic re-oxygenation would be expected to be significantly reduced in the statically ageing 'light / low D.O.' treatment cultures. This notion was also supported by the work of Pratt (1943), who reported a 50% decrease in relative photosynthetic rate and associated O<sub>2</sub> production in statically ageing illuminated cultures of *C. vulgaris* over a period of 20 days. Sargent (1940) recorded an even larger decrease in the photosynthetic rate of illuminated ageing *Chlorella* cultures, citing a >80% decline in relative culture photosynthesis over a period of just 15 days. Further validation of this notion comes from Kulandaivelu and Senger (1976a, p. 157), who stated that “cultures of *Chlorella*... when allowed to grow over a prolonged period without the addition of fresh (nutrient) medium demonstrate a rapid loss of the photosynthetic capacity after 2–4 days.”

In addition to this likely reduction in photosynthetic productivity, another probable area of influence, which was not considered by Moss (1977) above, is the possibility that DIC resources were steadily depleted within the atmospherically-isolated 'light / low D.O.' flasks. It should be reiterated at this point that all 'low D.O.' treatment cultures were thought to have likely suffered chronic DIC starvation due to the initial autoclave heat-sterilisation step de-gassing or stripping dissolved CO<sub>2</sub> (and presumably also HCO<sub>3</sub><sup>-</sup>) from the MBL growth medium, and hermetic sealing then preventing normal atmospheric CO<sub>2</sub> solubilisation. Referencing again the work of Moss (1977), the authors observed that gross photosynthetic activity actually increased during the first 3 days of experimentation for both 'sealed' and 'unsealed' illuminated flasks, after which the rate of photosynthetic oxygen production dramatically declined to zero by Day 10 for the sealed treatment only, and whereafter photosynthesis remained inactive for the rest of the 22 day experimental duration. The observations made during the current research reflect those of Moss (1977) in that after 'Day 7' of illumination, the sealed 'light / low D.O.' treatment cultures did not appear to be suffering significant ill-effects as a result of their atmospheric isolation. By the next sampling interval on 'Day 14', however, a significant proportion of both *C. vulgaris* and *C. reinhardtii* cultures were already presenting as 'non-viable' and continued to die at an accelerated rate for the remainder of the 65 day experimental duration. It is possible, therefore, that Moss' original conclusions about the relative importance of DO concentration on algal survival under light conditions may have been somewhat skewed by the compounding effects of



inorganic carbon limitation or indeed starvation. Having said this, the previously discussed observation by Moss (1977) of diminishing algal survival under 'dark–anaerobic' conditions still stands, as it can be appreciated that there is no photosynthetic requirement for DIC under dark conditions. The poor dark-survivorship observed by Moss under those particular conditions, therefore, would more likely have been a result of respiratory hypoxia and oxygen starvation due to the large microbial oxygen demand in their non-axenic incubations.

The curious 'light / low D.O.' treatment observations during the current work could also be linked to those of Bartosh *et al.* (2002), where it was reported that *Chlorella* cultures—when continuously illuminated and hermetically-sealed—displayed an ever-decreasing rate of photosynthetic oxygen production during the first 30 hours, after which no further photosynthetic oxygen evolution was observed during the following 3 days. Whilst Bartosh *et al.* (2002) offered no explanation for this strange behaviour, it is again possible that gradual exhaustion of DIC reserves (as was assumed to be the case for the current research) could have been responsible for the cessation of photosynthetic oxygen production in Bartosh and co-workers' *Chlorella* cultures, and at the same time, explain the lack of photosynthetic re-oxygenation in 'light / low D.O.' treatment cultures reported here. Additional support for the 'DIC starvation' hypothesis proposed here comes from the work of Pearson *et al.* (1987). Pearson and co-workers actually observed an increase in relative growth rates for *Chlorella* and *Chlamydomonas* species when under simulated 'light / anaerobic' conditions, but notably, this result was achieved in the presence of additional substrate DIC (CO<sub>2</sub>). This again serves to suggest that the poor survival rates observed here and also by Moss (1977) above under simulated 'light / low D.O.' conditions, were more likely a manifestation of substrate DIC limitation rather than oxygen starvation.

Further backing for the idea of DIC starvation-induced algal cell death comes from the work of Pieterse and Cloot (1997). The authors state that there is a net mass uptake of CO<sub>2</sub> in algal (*Chlamydomonas*) cells during photosynthesis (i.e. there is more CO<sub>2</sub> 'going in' than there is O<sub>2</sub> 'coming out' at a ratio of 1.063:1 CO<sub>2</sub>:O<sub>2</sub>). This could imply that that in a sealed system, there is essentially a net loss of CO<sub>2</sub> for the relative amount of O<sub>2</sub> that the system recovers during algal photosynthesis. Under conditions of

atmospheric isolation and continuous illumination, this could confer a steady exhaustion of dissolved CO<sub>2</sub> (DIC) from these systems, such that the algal cultures could be expected to initially convert the available pool of CO<sub>2</sub> into O<sub>2</sub> and then, in the absence of both microbial and dark algal respiration, ultimately suffer DIC starvation and eventual culture senescence. This was in effect what was seen in 'light / low D.O.' treatments, whereby algal cultures slowly senesced over the course of two months of continuous illumination and DIC limitation, to the point where both *C. vulgaris* and *C. reinhardtii* cultures were 98% dead after 65 days; although apparently not to the point where re-growth was unachievable for both species (see Section 9.91).

Despite there being evidence to suggest that endogenously-respired CO<sub>2</sub> is re-assimilated during photosynthesis (Raven, 1972a; Raven, 1972b), others have demonstrated that cellular respiration in some green algae is photoinhibited in the light (Brown and Tregunna, 1966; Scherer *et al.*, 1984) and also that the rate of respiratory CO<sub>2</sub> production declines with increasing irradiance (Brown and Weis, 1959); something probably linked to the recognised light-restriction or 'photodepression' of O<sub>2</sub> uptake in some algal species including *Chlorella* (Govindjee *et al.*, 1963) and *Chlamydomonas* (Healey and Myers, 1971). This could, therefore, imply that there might have potentially been less opportunity for algal CO<sub>2</sub> production (and endogenous photosynthetic re-assimilation) under the 'light / low D.O.' treatment conditions of continuous mid-level-intensity (60 μmol photons m<sup>-2</sup> s<sup>-1</sup>) irradiance. Since there were thought to have been no means by which to generate significant amounts of new DIC through these respiratory pathways, algal cells were again thought to have suffered ultimately from DIC starvation. Although the level of CO<sub>2</sub> saturation for photosynthesis is recognised to be in the order of 0.1% for species of *Chlorella* (Myers, 1944) and 2.0–2.2 mmol L<sup>-1</sup> (or ≈130 mg L<sup>-1</sup> as HCO<sub>3</sub><sup>-</sup>) for marine phytoplankton (at pH≈8; Raven, 1991), and despite the recognised high-level affinity of phytoplankton for DIC in general (Goldman and Graham, 1981; Hein, 1997), algal cells were still assumed to have been DIC starved in these sealed 'low D.O.' treatments, especially at DIC levels some 500-fold lower than common saturation levels.

Scutt (1964) hypothesised that growth inhibition in ageing illuminated *Chlorella* cultures could be due to the accumulation of organic peroxides from photo-oxidation of

fatty acids; although it was not unequivocally demonstrated. The more recent conclusions of Vavilin *et al.* (1998) were in apparent agreement with the earlier suggestions of Scutt (1964), in that Vavilin *et al.* observed no peroxidation (oxidative degeneration) of cell membrane lipids in heat-stressed *Chlorella* cultures kept in the dark; instead observing an increase in lipid peroxidation only in stressed cells that were re-exposed to light. Vavilin *et al.* (1998) stated that whilst oxygen is essential for aerobic metabolic processes, its participation in cellular events results in the appearance of ever-present toxic 'reactive oxygen species' (ROS) (e.g. superoxide anion radical, hydroxyl radical, hydrogen peroxide, and singlet oxygen). These toxic forms of oxygen can then react with cellular biomolecules such as nucleic acids, proteins, lipids and pigments—causing their degradation. The authors then went on to say that an important example of this biomolecular degradation is the peroxidative degeneration of membrane lipids. Considering this information, it is possible that in the hermetically-sealed 'light / low D.O.' treatment flasks, these toxic ROS might have been able to accumulate within the isolated environment, potentially accelerating the rate of membrane lipid oxidation (degeneration) and cell death in those treatments. Backing for this particular degenerative membrane lipid cell death hypothesis is found in the results of previously discussed PI membrane integrity assessments (Section 9.8.1), where it was shown that algal cells in this particular treatment had significantly higher rates of cell membrane disruption and subsequent cell death than did the remaining treatments.

Vavilin *et al.* (1998) also found that *Chlorella* were far more resilient to environmental and cytotoxic stressors (heat stress 50–70°C; and 1.6µM Cu<sup>2+</sup> exposure) when maintained in darkness compared with illuminated cultures. They reported that PS-II activity reduced to zero after several hours of illumination with phytotoxic copper exposure, whereas cells maintained in darkness retained 80% of their initial PS-II activity even after 24 hours of copper exposure. This trend was again mirrored with respect to heat stress in their work, whereby cellular lipid peroxidation (measured as high-temperature chlorophyll thermoluminescence) was markedly lower in heat-stressed (50–70°C) cells maintained in the dark in comparison to those illuminated at 60µmol photons m<sup>-2</sup> s<sup>-1</sup>. The final conclusions of Vavilin *et al.* (1998) suggested that lipid peroxidation occurs primarily in dead cells kept in the light. Strikingly, this was a condition experienced by both 'light' treatments during the current research, and one

that could perhaps account for some of the elevated rates phytoplankton cell death in these treatments over the 65 day experiment. Having said this, however, the true significance of oxidative cellular degeneration for cell death in both light treatments in the current work remains unclear. Considering that there is a generally positive association between rates of cellular (microbial) attenuation and DO concentration (Curtis *et al.*, 1992), and based loosely on the data of Curtis *et al.* (1992), for high light- and high DO-mediated photo-oxidative cellular destruction (by ROS) to have contributed significantly to the observed high rates of phytoplankton death in 'light / low D.O.' treatments, it is likely that DO levels would have had to have been super-saturated ( $\geq 12 \text{mg L}^{-1}$ ). It does remain possible, however, that the hermetic sealing in this particular treatment promoted the slow but *chronic* accumulation of ROS, such that they were able to exert a less intensive but ultimately destructive effect over the extended 65 day incubation period (far greater than the 4 hour duration employed by Curtis *et al.*, 1992).

Interestingly, Mandalam and Palsson (1995) concluded that algal senescence in ageing *C. vulgaris* cultures was due to the accumulation of high levels ( $>10 \text{mM}$ ) of  $\text{HCO}_3^-$  and the associated increase in pH. This was conclusively shown not to have been the cause of cell death in ageing 'light' treatment cultures during the current experiments, given that Day 64 DIC measurements for the 'light / low D.O.' treatment revealed a total DIC of  $<0.5 \text{mg L}^{-1}$  (or  $\approx 0.008 \text{mM HCO}_3^-$  assuming the total DIC pool exists predominantly as  $\text{HCO}_3^-$  at a mean pH of 7.5). The well buffered growth media used here would have also presumably guarded against the development of highly alkaline pH (as was recorded by Mandalam and Palsson, 1995). The work of Mandalam and Palsson (1995) was, therefore, not considered to be important with respect to interpreting the current research findings.

Further to the above-discussed concepts, algal 'photorespiration' could also go some way toward explaining the advanced rates of cell death recorded in the 'light / low D.O.' treatment. Photorespiration is a process that involves cellular metabolism (synthesis) of reducing power for photosynthesis at low  $\text{CO}_2$  concentrations and is a process that incurs a significant energetic cost through an altered ATP balance (Reynolds, 2006). Photorespiration is known to occur under  $\text{CO}_2$ -limiting conditions and, under continued

photosynthesis, the CO<sub>2</sub>:O<sub>2</sub> ratio can get so low that RUBISCO starts to combine RuBP with O<sub>2</sub> instead of CO<sub>2</sub>—creating toxic phosphoglycolates. If produced, these toxic substances would have obviously accumulated within the air-tight 'light / low D.O.' treatment flasks, such that the resident phytoplankton must try and de-toxify them. This is done firstly through dephosphorylation (removing the phosphate group) and converting the molecule to glycolic acid. The glycolic acid is then further oxidised (in chlorophyte algae) to glyoxalate and then glycerate 1,3-biphosphate (GP3). The full sequence of these reactions is termed the 'photosynthetic carbon oxidation cycle'. All of the above chemical conversions come at a significant energetic cost and results in the net loss of already limiting CO<sub>2</sub>.

In natural environments, these leached organic compounds (glycolate, monosaccharides, carboxylic acid, amino acids) are readily taken up and metabolised by resident microorganisms (Reynolds, 2006); however, due to the axenicity of the algal cultures used here, there would have been no opportunity for further microbial oxidation of these unwanted algal by-products. Therefore, it is possible that the net loss of already scarce CO<sub>2</sub>, combined with the accumulation of undesirable intermediates of photorespiration, could go toward explaining the high levels of cell death in the 'light / low D.O.' treatment cultures for both *C. vulgaris* and *C. reinhardtii*. The inclusion of photorespiration as a likely contributing factor in the observed high rates of cell death for 'light / low D.O.' treatments is, however, done with due caution. This phenomenon is known to occur under simultaneous conditions of high-intensity irradiance and high DO concentration (commonly during periods of high photosynthetic productivity) and is most notably prevalent at DO concentrations in the order of 10mg L<sup>-1</sup> (Simpson and Eaton, 1986; Graham *et al.*, 1995; Sakshaug *et al.*, 1997). Although there was indeed exposure to a continuous intermediary PFD, oxygen concentration was much lower than that which might be expected to have induced such photorespiratory behaviour ( $\approx 2.2\text{mgL}^{-1}$ ; see Figures 9.2 and 9.3).

At first glance, the observation of catastrophic algal cell death under light conditions with DIC limitation seems somewhat elementary and not especially revelatory. It is nonetheless interesting that, when illuminated, these two phytoplankton species do not appear to be able to recognise that an essential 'ingredient' (in this case inorganic

carbon) for photosynthesis is missing and cease photosynthetic processes; rather, the cells appear not to adjust accordingly and proceed instead to wholesale culture senescence. At the same time, however, when another essential and indeed primary photosynthetic ingredient is absent (i.e. light energy), both species are able to 'recognise' its absence and adjust accordingly, and under these conditions, are able to effectively retain complete culture viability even after some two months of light-starvation. This observation can be likened to an earlier reference made by Anita (1976). The author, in referencing the work of Jitts *et al.* (1964), raised a very compelling point regarding an unexpected effect of light intensity on the growth of the cold-water diatom *Thalassiosira nordenskiöldi*. This particular organism showed growth capability over a wide temperature range (2–18°C) at low light intensity, but could not tolerate the lower temperatures when exposed to *high* light intensities. Perhaps of even greater relevance to the above is the work of Burrell *et al.* (1985), whereby *C. vulgaris* was found to actually be capable of photoheterotrophic growth over a period of 11 days in the presence of glucose (0.5%) under constant illumination ( $30\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and in the absence of CO<sub>2</sub>. Although the authors did not control for growth in the absence of substrate glucose, it is possible that the presence of a suitable organic carbon source under conditions of DIC starvation negated the large-scale culture senescence like that which was observed during the current research. It is possible also that the 11 day experimental duration of Burrell *et al.* (1985) may have been too short to replicate the high-level population decline seen here, given that 'light / low D.O.' treatment *C. vulgaris* cultures really only started to show significant ill-effects from Day 14 onwards (see Figure 9.36).

In the context of the current 'light / low D.O.' treatment observations, the above data again suggests an ability to recognise the absence of the primary raw material (i.e. light energy), but a failure to make the same physiological 'realisation' when presented with the absence of other possibly lesser, but still entirely essential, raw materials (in this case inorganic carbon). It should also be noted that the ability to adjust photosynthetic rate in accordance with the availability of DIC substrate has been shown to be light-dependent in some phytoplankton species. Bartual and Galvez (2003), for example, reported that two marine diatoms (*Skeletonema costatum* and *Phaeodactylum tricorutum*) could indeed adapt to reduced CO<sub>2</sub> availability; however, the ability to do so was observed to be light-dependent (i.e. under *limiting* irradiance, both species were able to adapt

accordingly to the low CO<sub>2</sub> conditions by reducing photosynthetic rates; however, under *high* irradiance, neither species was seen to have reduced its photosynthetic rate to suit the low DIC availability). This could again go toward explaining the relatively quick cellular ‘burn-out’ of ‘light / low D.O.’ treatment algal cells during the current work under the simultaneous DIC-limited and high PFD conditions, and suggests again an inability of both species to recognise conditions of DIC limitation under a favourable light climate.

In conclusion, and considering the evidence for DIC starvation, results from the current experimentation suggest that there are no significant negative effects imposed by ‘low D.O.’ conditions for the survival of *C. vulgaris* and *C. reinhardtii* under either light or dark conditions. This conclusion was in apparent agreement with the discussion of Dor *et al.* (1987, p. 237) whereby it was hypothesised that “*Chlorella vulgaris*... must have some selective advantages over other algae, like resistance to wide fluctuations in DO”. It is possible then, that this wide-ranging tolerance for DO concentration may have in some way aided long-term *Chlorella* dark-survival during the current work. Similar suggestions can also be made here for *C. reinhardtii* based on the known tolerance of this species to anoxic conditions within WSP environments (Pearson *et al.*, 1987; Almasi and Pescod, 1996). Interestingly, although inadvertently, results presented here also showed that algal cells exposed to conditions of DIC limitation (probable starvation) displayed an accelerated and ultimately catastrophic population decline for both species.

Although phytoplankton species were considered to have succumbed to inorganic carbon starvation *in vitro* during these experiments, the practical *in situ* implications of these findings remain unclear. As reported by Hein (1997), there is a general disinterest in DIC limitation in phytoplankton research based on a number of factors. For example, the ubiquitous nature of DIC (in some form or another) in aquatic environments means that it is almost never the rate-limiting substrate. Furthermore, the innate high-level affinity of most phytoplankton for DIC (due to their high surface area-to-volume ratios) means that even at low concentrations, passive cellular DIC uptake will generally be sufficiently adequate to be non-rate-limiting. What this means is that phytoplankton in the natural environment—with the exception of some acidic softwater lakes (Hein,

1997)—will almost certainly never experience severe DIC limitation, or even less likely, DIC starvation. Although it has been suggested previously that within hypereutrophic aquatic environments such as a WSP, inorganic carbon is more likely to be the growth-limiting substrate for phytoplankton (Pipes, 1962; Goldman *et al.*, 1972) than either N or P (Talbot and de la Noüe, 1993; Bartosh *et al.*, 2002), the wider implications of these findings remain unknown. Whilst DIC could potentially be the most limiting inorganic nutrient in WSP environments (e.g. under conditions of photosynthetically-elevated pH and reduced free CO<sub>2</sub> availability), it is thought that the persistence of such low levels of inorganic carbon (< 0.2–0.3 mgL<sup>-1</sup>) would be unlikely to occur *in situ*, such that algal DIC starvation within a WSP environment would be considered highly unlikely. The applied consequences of observing high rates of population death under conditions of continuous illumination and DIC starvation, therefore, remain unconsolidated.

As a final point of note, there is more recent evidence to suggest the existence of an ‘apoptotic-like’ programmed cell death pathway in *C. reinhardtii* (Moharikar *et al.*, 2006). Moharikar and co-workers identified cell death in *C. reinhardtii* based on morphological cellular characteristics, whereby dying cells were categorised as either apoptotic (shrinkage of cells surrounded by an intact plasma membrane) or necrotic (cellular swelling and complete degradation of the plasma membrane). Based on the available data (i.e. from physical light scattering and PI fluorescence), the likelihood of programmed cell death having occurred during the research reported here is small; with cell volume and membrane integrity results instead suggesting that cell death in both species most likely came about via a necrotic-type pathway.

### **9.11 Light versus dark survival—light controls for dark treatments?**

The primary objective of research reported in this Chapter was to investigate the survival of two ubiquitous WSP algal species during and following prolonged darkness under varying DO availability. When the current experiments were conceived, the necessity and indeed practicality of implementing ‘light’ controls for ‘dark’ treatments was, from the outset, a challenging concept. In the absence of varying *degrees* of darkness, light incubations were implemented to serve more as additional treatments rather than true



experimental controls for the respective dark treatments *per se*. It was envisaged that comparisons between long-term light and dark-survivorship could then be made in order to identify potential differences between the two, in terms of: the effects of ‘light’ and ‘oxygen concentration’ on overall population fitness; the kinetics of growth and/or death; and any other factors that may have become apparent following data collection.

Chapter results from ‘light’ treatments, whilst they cannot be strictly seen as controls for ‘dark’ ones, may actually serve to provide insights into the ecophysiology of WSP phytoplankton populations *in situ*. Information on phytoplankton survival obtained from these light treatments might, therefore, allow for an examination of factors that affect natural senescence of algae in the light and under a variety of DO conditions. Given the large daily flux in DO (and also DIC) concentration found within WSPs, this information might also contribute toward an increased understanding of the physiological ecology of phytoplankton within these environments. Whilst the potentially confounding factors discussed in Section 9.10 above have made it difficult to isolate and distill the actual effects that a variable DO regime have on overall phytoplankton survivorship in the light, it does appear that these conditions have generally promoted both a more heterogeneous and less viable population structure. Regrettably, it is beyond the scope of this work to attempt to identify the likely practical implications of these findings to WSP plankton ecophysiology; however, it would be suggested that this constitutes a topic worthy of future investigation.

### **9.12 Darkness, organic substrates and heterotrophic nutrition: was the advanced dark-survival purely inorganic?**

Obligate photolithotrophy is common amongst the phytoplankton. Despite this being the overwhelming norm with respect to their energetic lifecycle, and following on from prior indications (see Section 6.3.1), there exists a substantial body of research describing other trophic states within the phytoplankton (i.e. phago-, mixo-, osmo- and heterotrophy). It is beyond the scope of this thesis to describe this literature in any great detail; however, it is considered relevant within the current research context to enter into a brief discussion here.

The potential exists for complex interactions between photosynthesis and respiration when dissolved organic substrates are taken up by phytoplankton in the light (Lewitus and Kana, 1994), interactions that to the present time appear far from being fully defined. In species capable of utilising them, organic substrates can supply the same metabolic requirements for growth as the photosynthetic process (i.e. biosynthetic material, metabolic energy and reducing power), serving either as alternatives or supplemental additions to the material and energy normally sourced through cellular photosynthesis (Lewitus and Kana, 1994). It has been recognised for some time that whilst heterotrophic nutrition is thought to be unimportant for the vast majority of planktonic algal populations, heterotrophy may be of particular importance to algae inhabiting areas rich in organic substrates (Bennett and Hobbie, 1972); such as some WSPs for example.

In the presence of a suitable organic substrate, algal species that are capable of metabolising exogenous organic compounds exhibit a range of responses that can affect cellular photosynthesis and growth. At one extreme are algae that totally repress chloroplast development and change to heterotrophic nutrition exclusively (these include *Euglena gracilis* and some *Chlorella* species), whilst other responses involve varying degrees of reduction of pigmentation or photosynthetic activity (e.g. some *Chlamydomonas* and *Scenedesmus* species) (Eichenberger, 1976; Burrell *et al.*, 1985; Laliberté and de la Noüe, 1993; Lewitus and Kana, 1994; Reynolds, 2006). According to Neilson and Lewin (1974) it is generally not possible to predict which organic substrates can be used by any given algal species. This diversity in physiological responses among various phytoplankton species suggests a complex variability in the regulation of algal heterotrophy, even in closely related species; complexity that has so far hampered the development of unifying principles that might aid in predicting organic substrate use by phytoplankton in nature (Lewitus and Kana, 1994).

According to Uhlmann (1980) and also Ogawa and Aiba (1981), most of the phytoplankton species predominating wastewater environments are ‘mixotrophic’ and can at times, therefore, utilise dissolved organic materials for non-autotrophic growth. What precisely defined ‘mixotrophy’ some 30 years ago is dubious (presumably some mixture of auto- and heterotrophic nutrition including photoassimilation); however, a

more recent qualification of this overall viewpoint comes from the work of Reynolds (2006). The current definition of ‘mixotrophy’ (according to Reynolds, 2006) confers a facultative capability possessed by some nominally photosynthetic algae for the ingestion of particulate matter as a typical feature of their lifecycle. This kind of mixotrophic nutrition is seen among the dinoflagellates and certain chrysophytes; however, mixotrophy is generally regarded as the facultative ability to supplement limiting nutrients *other than carbon* (chiefly N or P) (Reynolds, 2006). Alternatively, the bacterium-like ability for direct absorption of selected dissolved organic compounds across the cell surface is nowadays referred to as *osmotrophy* (Reynolds, 2006) and is a recognised feature of some Chlorophyceae (Chlorococcales; to which the genus *Chlorella* belongs), Euglenophyceae and cryptomonads (Lewitus and Kana, 1994).

Accordingly, the issue of there being some form(s) of readily oxidisable organic nutrients in the MBL culture medium used during this research, and the subsequent effect (if any) of their presence on the observed capacities for long-term dark-survival of both *C. vulgaris* and *C. reinhardtii*, must be addressed. Pillay (1990) classified Tris-[hydroxymethyl] aminomethane (TRIS) and B-vitamins as “organic micronutrients” and, following the discussion of Anita and Cheng (1970), it is possible that Na<sub>2</sub>EDTA may also be included in this category. The culture-growth medium used here (Woods Hole MBL; Appendix G) had a total DOC content of  $\approx 215 \text{ mg L}^{-1}$ . This DOC comes from a combination of TRIS (used for buffering), Na<sub>2</sub>EDTA (used for chelation of metals) and also a small amount of added vitamins, with the vast molecular majority ( $\approx 94\%$ ) of total DOC relating to the presence of TRIS buffer.

Wiedeman and Bold (1965) reported no capacity for dark growth in WSP-isolated *Chlorella* and *Chlamydomonas* species in TRIS-buffered inorganic medium at final TRIS concentrations 20% greater than that of the current MBL medium. Anita and Cheng (1970), following dark-survival assessment of 31 marine phytoplankton species, deemed it “unlikely” that either TRIS or EDTA from the culture medium would be suitable organic substrates for cellular respiration or assimilation; however, they did stop short of ruling out all possibility of it having some “unknown supportive role” in maintaining long-term dark-viability. Further to this, the findings of Ademoroti (1990) would also suggest that sodium EDTA is not a suitably respirable carbonaceous

substrate (for microbial oxidation at least) following no signs of an increased BOD<sub>5</sub> in wastewater with added Na<sub>2</sub>EDTA at concentrations up to 85 times higher than that used in the current growth medium. Additionally, and even if the relatively high molecular weight ( $\approx 372 \text{ g mol}^{-1}$ ) Na<sub>2</sub>EDTA was able to be degraded by bacteria into smaller and perhaps more palatable organic substrates for algal cell assimilation, the axenic status of the algal cultures excluded this as a realistic possibility. Following this, it is relatively safe to conclude that the presence of low concentrations of Na<sub>2</sub>EDTA had no significant effect on phytoplankton dark-survival measured during the current research.

Furusato *et al.* (2004) hypothesised that the 20 day dark-survival (not growth) of *Scenedesmus* was thought to have been enhanced or fuelled by the utilisation of TRIS; although this also remained unconfirmed for their research. At the same time, some researchers have even reported that TRIS can actually inhibit growth in some phytoplankton species (*Cryptomonas rostratiformis*; Gervais, 1997). The possibility of vitamins serving as a suitable metabolic substrate is unlikely given their extremely low final concentrations ( $\leq 0.15 \text{ nM}$ ) and also the probability that the vitamins were quite likely to have been destroyed by the high temperature autoclaving process in the basal (i.e. without the presence of protective reducing agents) MBL medium (Hendlin and Soars, 1951; Anderson *et al.*, 1986). Additionally, Smayda and Mitchell-Innes (1974) recorded no enhancement of marine diatom (*Skeletonema* and *Ditylum* species) dark-survival following vitamin enrichment.

It still remains possible, however, that *C. vulgaris* and *C. reinhardtii* could have been utilising some organic carbon sources in the MBL culture medium to sustain their dark-viability (particularly the lower molecular weight ( $\approx 121 \text{ g mol}^{-1}$ ) TRIS at relatively high (4.13mM) concentrations). It must be reiterated, however, that no dark *growth* was observed, and so whilst there was no evidence of heterotrophic algal growth *per se*, it remains a possibility that the presence of organic substrates may have in some way enhanced or augmented their dormant dark-survival capacity (e.g. through osmotrophy). This is a point previously highlighted by Richardson and Fogg (1982), whereby a lack of dark growth in the presence of DOC does not necessarily imply an inability to metabolise DOC; instead, and under conditions of light-limitation, phytoplankton might be expected to direct any energy derived from extracellular organic substrates into

meeting immediate metabolic demands rather than into cellular division. Osmotrophic cellular assimilation of dissolved TRIS seems unlikely, however, given that Furusato *et al.* (2004) reported that TRIS cannot permeate algal cell (*Scenedesmus*) membranes.

Further evidence that culture medium DOC played no significant role in mixo- or heterotrophic nutrition during the current dark-survival experiments, comes from the observation that no growth (rather, catastrophic cell death) was observed for 'light / low D.O.' treatments of both *C. vulgaris* and *C. reinhardtii* in the absence of an inorganic carbon source (i.e. CO<sub>2</sub> or NaHCO<sub>3</sub>). Interestingly, the ability of an alga to photoassimilate acetate or glucose as a carbon source for or photoheterotrophic growth in the light, does not necessarily imply that it will grow in the dark (Neilson and Lewin, 1974); something demonstrated previously for species of *Chlamydomonas*, *Chlorella* and *Scenedesmus* (Eppley and MaciasR, 1962; Wiedeman and Bold, 1965; Vincent and Goldman, 1980; Laliberté and de la Noüe, 1993). Although photohetero- and chemoheterotrophic modes of nutrition have been described for species of *Chlorella* (Killam and Myers, 1956; Syrett, 1956; Pipes and Gotaas, 1960; Wiedeman and Bold, 1965; Ukeles and Rose, 1976; Endo *et al.*, 1977; Ogawa and Aiba, 1981; Burrell *et al.*, 1985) and *Chlamydomonas* (Eppley and MaciasR, 1962; Anderson, 1975; Laliberté and de la Noüe, 1993; Heifetz *et al.*, 2000), the lack of any suitable DOC source for heterotrophic nutrition in the MBL medium precludes this as a realistic possibility for having augmented the long-term dark-survival during of these algae during this research.

In an applied WSP context, however, this relative uncertainty surrounding the supportive role of DOC for phytoplankton dark-survival is of lesser importance, due to the ever-present nature of DOC in wastewater systems (some of which might be of greater suitability in terms of direct substrate bio-availability for cellular uptake and respiration) and also due to the vast numbers of heterotrophic WSP microbes (microbes that may further enhance the bioavailability of such high molecular weight DOC through the degradation of complex organic molecules into smaller and more easily assimilated forms). Therefore, the presence of some DOC (bio-available or not) in the MBL culture medium here could arguably be seen as providing a more realistic reflection of applied algal dark-survival capacities *in situ* within a WSP environment. Richardson and Fogg (1982), reporting on the growth and dark-survival of six species of marine

dinoflagellates in the presence of several organic carbon (glycerol, glucose and acetate) substrates, showed that in no case did the presence of DOC affect the growth or dark-survival of *axenic* phytoplankton cultures. The authors did report, however, that bacterially-contaminated cultures of one species (*Amphidinium carterae*) did exhibit some growth stimulation in the presence of dissolved organic substrates, and that this stimulatory outcome was lost when the same strain was axenified.

Similar trends have also been reported elsewhere, whereby there are apparent trophic interactions (competitive and synergistic) between heterotrophic microbes and phototrophic phytoplankton that can impact on algal survival and population dynamics under certain conditions (Grover, 2000). Richardson and Fogg (1982) concluded from their work that the assimilation of organic carbon by autotrophic phytoplankton was not likely to be an important factor for their survival in low-light environments. At the same time, Amblard *et al.* (1992) suggested that organic carbon was likely to play an important role in the dark-survival of some phytoplankton species and/or in competitive community interactions, despite the authors recording some 1000-fold lower production rates under heterotrophic compared to phototrophic nutritional pathways. Similarly, Berman *et al.* (1977) also suggested that organic substrates may actually be of some importance in maintaining the low-light or dark-survival of some phytoplankton. Even further uncertainty is cast over the true ecological significance of heterotrophic nutritional pathways for phytoplankton in the natural environment, given that such experiments are commonly carried out in batch culture situations, in artificial media, and with unnaturally high concentrations of added organic substrates (Cheng and Anita, 1970; Berman *et al.*, 1977; Tsavalos and Day, 1994)—conditions that may not adequately reflect those experienced *in situ*.

At the same time, it could be argued that the apparent lack of a suitable DOC source for supplementing algal dark-survival (or indeed promoting dark-growth) in the current MBL culture medium was not representative of a classical WSP environment. Whilst this may be the case for a normal facultative WSP, in respect of the current Bolivar situation, however, it was thought to have very much reflected both the low *in situ* DOC concentration ( $\approx 20\text{--}30\text{mg L}^{-1}$ ) and also the highly-refined nature (i.e. complex and non-bioavailable; soluble  $\text{BOD}_5 \ll 1\text{mgL}^{-1}$  at those DOC levels) of the final maturation pond

effluent. This means that within a Bolivar-based advanced WSP upgrade, algae would not be expected to be able to (directly) utilise any of the aqueous DOC for supplementing their dark-survival, such that the DOC scenario seen within the dark-survival experiments reported here remains valid. Although the capacity for uptake and utilisation of organic carbon have been unequivocally established for a number of phytoplankton species, since substrate concentrations and cellular affinities have been shown to be generally low *in situ*, such modes of nutrition are deemed to be relatively unimportant for phytoplankton growth in the natural environment (Sloan and Strickland, 1966; Neilson and Lewin, 1974; Berman *et al.*, 1977; Gibson and Smith, 1982), with production rates commonly orders of magnitude slower than normal photolithotrophic nutritional pathways (Wright and Hobbie, 1966; Bennett and Hobbie, 1972; Vincent and Goldman, 1980; Ogawa and Aiba, 1981; Richardson and Fogg, 1982; Amblard *et al.*, 1992; Tsavalos and Day, 1994).

Even in highly polluted waterways, it has been argued (e.g. Hobbie and Wright, 1965 cited in Bennett and Hobbie, 1972) that heterotrophic microbes are so efficient at metabolising small organic substrates, that glucose levels *in situ* rarely exceed  $0.1\mu\text{M}$ . Even in raw domestic sewage, Abeliovich and Weisman (1978) reported total carbohydrate concentrations of only  $80\text{--}100\text{mg L}^{-1}$  ( $0.5\text{mM}$  of equivalent glucose), with measured levels of ‘available glucose’ in high-rate oxidation ponds peaking at  $8.3\mu\text{M}$ . Abeliovich and Weisman (1978) went on to suggest that perhaps bacterial populations within this environment were more successful at competing with algae for the low levels of substrate glucose. This is despite the more recent reporting of Pearson *et al* (1987) that *Chlamydomonas* is able to compete with heterotrophic bacteria for acetate in WSPs—albeit at far greater substrate concentrations of  $10\text{mM}$ . As discussed by Pearson *et al.* (1987), it is likely that because of the low substrate availability *in situ*, the apparent capacity of some phytoplankton to utilise sugars for heterotrophic dark growth may be of limited relevance to WSP environments, with their heterotrophic affinity for organic acids (such as acetate) possibly of greater practical significance in these environments.

Even if some species have the capacity for DOC assimilation *in situ*, the work of Bouarab *et al.* (2004) suggests that acetate incorporation in the WSP-isolated green alga *Micractinium pusillum* Fresenius (Chlorophyceae) is an energy-consuming ‘active

uptake' process that is dependent on the processes of both anabolic (photosynthesis) and catabolic (respiration) metabolism, such that acetate uptake was deemed to be more important in *light* conditions than in darkness; sentiments echoed by the work of Berman *et al.* (1977) for leucine uptake in *Pediastrum* and also Burrell *et al.* (1985) for glucose uptake in *Chlorella*. Similarly, and despite Hellebust and Guillard (1967) observing similar rates of amino acid uptake in *Melosira nummuloides* to that of CO<sub>2</sub> photoassimilation, substrate concentrations used by these authors were in the order of 100 $\mu$ M—orders of magnitude greater than natural levels (Bennett and Hobbie, 1972). It follows then, that few phytoplankton species have been shown to be truly chemoheterotrophic at naturally occurring substrate concentrations (Ellis and Stanford, 1982).

It should also be pointed out that the addition of organic nutrients may not necessarily enhance dark-survival of other phytoplankton species with heterotrophic capabilities. *Scenedesmus acuminatus* has previously been shown to be able to take up acetate and other organic compounds in the dark (Neilson and Lewin, 1974); however, Dehning and Tilzer (1989) did not find high uptake rates of acetate in *S. acuminatus* stored in complete darkness and there was no reported increase in dark-survival of algal cells stored in acetate-amended medium. Popels and Hutchins (2002) observed that dark-survival of the marine brown tide alga *Aureococcus anophagefferens* (Pelagophyceae) was not significantly enhanced by the addition of organic substrates (1–3 $\mu$ M) to the culture medium, despite there being a recognised capacity for heterotrophy in that particular species. This trend has also been reported by others for numerous species of marine diatoms (Sloan and Strickland, 1966; Bunt and Lee, 1972; Smayda and Mitchell-Innes, 1974).

Finally, Jochem (1999) reported no apparent heterotrophy or osmotrophy of supplemented organics (5 $\mu$ M glucose and leucin) during 12 day dark-survival experiments for the chlorophyte *Brachiomonas submarina*. Jochem (1999, p. 726) did caution, however, that his failure to record heterotrophic nutrition did not definitively rule out its potential influence under other conditions, stating instead that the dark duration might have been “*too short to yield a physiological state of deprivation dire enough to initiate heterotrophy*”. Similar conclusions are made regarding results from



the current research, in that although the presence of some DOC was thought to have not significantly influenced long-term dark-survival, this by no means rules it out as a potentially influential factor under different substrate conditions.

Results from the current long-term dark-survival experiments clearly demonstrate that there was no detectable heterotrophic growth in either *C. vulgaris* or *C. reinhardtii*. Given the relative uncertainties surrounding the biological (and ecological) significance of phytoplankton DOC utilisation, however, it remains possible that these algal cells derived some form of energetic assistance as a direct result of the inclusion of some DOC in the present dark-survival experiments; although the extent to which this DOC influenced overall dark survivorship does appear minimal. Considering the applied context surrounding this research, whether or not some phytoplankton can indeed use a finite number of low molecular weight organic substrates to facilitate growth in dark and/or light conditions, is—regarding the Bolivar WSPs—of limited significance given the highly refined complex nature of the pond DOC. Because there are unlikely to be any significant amounts of glucose, sucrose, acetate, or any other simple organics or amino acids present toward the end of the Bolivar pond system, chemoheterotrophy as a dark-survival strategy within an advanced pond upgrade is considered to be of limited practical importance.

### **9.13 Prior light history and dark-survival**

Prior light history is known to influence numerous phytoplankton cellular characteristics (e.g. dark respiration, chlorophyll *a* concentration and dark-growth) which can then potentially affect cellular physiology during prolonged darkness (Hellebust and Terborgh, 1967; Ganf, 1974; Yallop, 1982; Gervais, 1997). Prior light climate can have a direct and marked influence on algal cell processes, with the simple addition of a ‘dark cycle’ to a laboratory culture for example shown to have a dramatic effect on photosynthetic light response (Hellebust and Terborgh, 1967; Knoechel and Kalff, 1978). Considering this, it is likely that prior light history could have a real influence on measured dark-survivorships in phytoplankton, or in the very least, upon cellular processes following the initial onset of prolonged dark-exposure.

As an example of this, Hellebust and Terborgh (1967) observed that prior light history in dark incubated *D. tertiolecta* had a striking influence on culture behaviour following transfer to dark conditions. Cultures of *D. tertiolecta* grown under continuous irradiance exhibited a rapid increase in photosynthetic enzyme activities and dark respiration during the first day of darkness, followed by a rapid decrease in these rates and activities. In contrast, cultures grown on 12:12 hour light–dark cycle reduced their photosynthetic capacity and enzyme activities gradually from the beginning of darkness. Gervais (1997) also observed a similarly evident effect of prior light history on the post-darkness (8 day) re-growth potential in species of the deep-living *Cryptomonas* (Cryptophyceae), with cultures grown under low ( $30\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) PFD displaying a reduced re-growth potential compared to cultures grown under higher ( $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) irradiance.

Recalling that algal cultures used for dark-survival experiments here were grown under continuous irradiance of  $60\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Section 7.1), it is possible that there may be some distortion of measured dark-survivorships in terms of their practical implications for WSPs (given that within such an environment, algae would normally be exposed to a sinusoidal 12:12 hour light–dark cycle). According to the reporting of Post *et al.* (1984, cited in Falkowski and LaRoche, 1991), cellular adjustment to this diel periodicity in light climate is, strictly speaking, not a form of ‘photoacclimation’. Instead, cells are said to be simply entrained within a 12:12 hour light–dark cycle and undergo *diel periodicity* with respect to photophysiological adjustments (such as cellular chlorophyll *a* content). In other words, cells under a constant 12:12 hour light–dark cycle do not ‘photoacclimate’ to the period of nocturnal darkness, but merely oscillate around the average irradiance encountered during the daily photoperiod (Falkowski and LaRoche, 1991; Prézelin, 1992). Considering this, the fact that algal cultures used here were grown under a continuous mid- to high-range PFD should, theoretically, have had no bearing on their long-term photoacclimation response capabilities or their measured physiological dark-survival outcomes. If anything, the fact that algal cultures were continuously illuminated prior to long-term dark-exposure should have meant that all measured dark-survival capacities would represent conservative estimates of their maximum dark-survival capabilities, given that any and all forms of prior ‘dark-conditioning’ were fundamentally excluded.

Interestingly, the two phytoplankton species used during this research were grown for multitudes of generations under continuously high levels of PAR, yet they appeared to have suffered no significant adverse effects from sudden and prolonged dark-exposure (remembering that this was most likely performed *without* a suitable organic carbon source). This was considered to be an interesting result in itself, and implies that there is not necessarily a need for any prior low-light or dark ‘photo-conditioning’ of phytoplankton in order for them to be able to survive extended periods of darkness. Ultimately, it is not known what effect, if any, the prior light history of experimental cultures had on the measured dark-survival capacities of *C. vulgaris* and *C. reinhardtii*. It is possible that the exclusion of a dark cycle during the maintenance and growth of all algal stock cultures may have had a direct influence on the observed dark-survivorships in both phytoplankton species; however, without verification of this, it must be ruled just as likely that prior light history had little or no influence on measured dark-survival potential for both species. This can only be recommended as a topic for future investigation.

#### **9.14 Timescales for phytoplankton acclimation during prolonged darkness—kinetics of dark-survival**

The ability of dark-exposed phytoplankton to recognise and then quickly adjust their cellular processes has obvious implications for their overall dark-survivorship (recalling the previously discussed ‘Type I’ and ‘Type II’ survival strategies; Section 9.8.2) and ecological competitiveness. Extreme instances of phytoplankton dark-survival have been reported in the literature: in the order of 6 years for *Ankistrodesmus*; 15 years for zygospores of *Pandorina* (Chlorophyceae; Coleman, 1975); and in excess of 9 years for cysts of the marine dinoflagellate *Lingulodinium polyedrum* (Lewis *et al.*, 1999). Notwithstanding these extreme cases, the importance of phytoplankton dark-survival from an ecological perspective, is a well recognised driving factor in the regulation of natural species succession (Anita and Cheng, 1970; Smayda and Mitchell-Innes, 1974) such that the competitive need for phytoplankton to be able to withstand extended periods of darkness (over whatever timescale) remains the same.

One of the original aims of this thesis was to distill the likely timescale for photoacclimation in WSP algae in response to a sudden and prolonged dark transition. Results from the long-term 65 day dark-survival experiment showed that a dramatic loss of cellular FDA metabolic activity occurred in both algal species over the course of the first 7 days of darkness (Figures 9.38 to 9.41)—a reduction above and beyond that which could be reasonably attributed to the observed reduction in mean cell volume. This trend was repeated in the follow-up 7 day experiment, with results showing that cellular FDA metabolic activity actually decreased even more rapidly than initially identified; declining sharply within the first two days of dark-exposure and then remaining relatively stable thereafter (Figure 9.47). It is suggested that perhaps this reduced Day 2 level of metabolic activity was close to the dark-induced basal ‘maintenance’ metabolic activity state for these algal cells, such that the initial sharp rate of decline observed from Day zero to Day 2 (ignoring the likely contribution of wholesale population metabolic rate reduction coming from the cessation of normal phototrophic culture growth) was not able to be sustained beyond Day 2. Based on this, it is suggested that general ‘metabolic photoacclimation’ (based on the relative activity of non-specific cellular enzymes) in both *C. vulgaris* and *C. reinhardtii* is likely to occur over a period of  $\leq 2$  days following initial dark-exposure.

During prolonged dark-exposure, phytoplankton may invoke specific physiological–biochemical mechanisms to aid their dark-survivorship; controlling energy expenditure from endogenous metabolism (including respiration) to the bare minimum required for long-term maintenance of cellular viability (see Section 6.3.1). According to Geider and Osborne (1989), this ‘maintenance metabolism’ during darkness is independent of normal cellular growth and biomass synthesis processes, with large-scale (5- to 10-fold) respiratory ‘wind-back’ occurring very soon following transfer from light to dark conditions. Furthermore, whilst respiration rates are often observed to be stable for several hours following initial dark transition, in some instances a rapid decline is observed directly after the start of the dark period (Grobbelaar and Soeder, 1985). It is highly likely then, that even the shortest time interval of two days post-darkness was many times too lengthy a period to accurately trace the true time kinetics of dark-induced metabolic decline in the two algal species reported here.

According to Vincent (1980), many algal species require several hours to measurably adjust their inter-photosystem electron flow capacity according to changes in environmental conditions—a timescale deemed to be relatively slow compared with many other photosynthetic acclimation responses such as photosystem electron spillover (between PS-I and II) and chloroplast contraction. Kroon *et al.* (1992) have showed that *Chlorella pyrenoidosa* was capable of photoacclimating to changes in light intensity within a relatively short 8 hour period, with detectable changes in the quantity of cellular chlorophylls having been reported to occur within a few hours as part of the low-light adaptational response in some algae (Prézelin and Matlick, 1980). According to Nultsch and Pfau (1979, cited in Ferris and Christian, 1991), photophysiological adaptation (chloroplast orientation) can take place on timescales of 1–2 hours. MacIntyre *et al.* (1997) also define the photoacclimation timescale for RUBISCO activation–deactivation following light–dark transition to be in the order of <10 minutes for the chlorophyte *D. tertiolecta*. Prézelin *et al.* (1991) also stated that, in response to changes in light climate (intensity and/or spectra), individual phytoplankton photosynthetic process response-times can vary by several orders of magnitude, whilst changes in photosynthetic pigment concentrations are evident on timescales in the order of hours to several days.

Regardless of the *precise* timeframe for photoacclimation in response to a reduced light intensity or darkness, these photophysiological modifications are recognised to occur on timescales less than or comparable to a cell’s generation time (Falkowski and LaRoche, 1991). Recognising then that phytoplankton take in the order of hours to adjust or ‘wind-back’ photosystem infrastructure in response to changing light conditions, it is reasonable to assume that the adjustment of cellular metabolic activity in response to darkness is also likely to be performed on a similar timescale of hours rather than days (far sooner than even the shortest sampling interval of two days investigated here). Based on the above information, it is likely that photoacclimation or metabolic acclimation to dark conditions here would have occurred at least within the first day and possibly even earlier; thereby assigning both *C. vulgaris* and *C. reinhardtii* to the relatively advanced ‘Type I’ photoacclimation and dark-survival response category. It should be re-emphasized, however, that it was not the specific aim of this research to define the short-term dark-adaptational response kinetics; rather, the research presented

here was aimed specifically at assessing the long-term dark-survival capability and physiological viability of algal cells following a *prolonged* dark-exposure event.

### **9.15 Darkness and physiological vitality—implications for phytoplankton sinking velocity and advanced WSP upgrade process efficiency**

In general, the regulation of phytoplankton motility and buoyancy in the natural environment can have a significant bearing on the degree of light to which phytoplankton are exposed (Ferris and Christian, 1991). Early work has already established that the sinking velocity of phytoplankton is not a species-specific constant (Smayda and Boleyn, 1965; Eppley *et al.*, 1967; Smayda, 1970; Smayda, 1974; Titman and Kilham, 1976) and there is evidence to suggest that sinking rates, particularly of non-motile phytoplankton, are influenced by physiological as well as morphological factors (Bienfang *et al.*, 1983; Heaney and Butterwick, 1985). The sinking velocity of some diatoms, for example, is predominantly influenced by their physiological state (Horn and Horn, 1993), with an increase in sinking rate thought to be associated with declining nutritional status and reduced physiological activity (Eppley *et al.*, 1967; Smayda, 1974; Titman and Kilham, 1976; Gibson, 1984). Some diatoms, for example, have been observed to sink more rapidly under conditions of nutritional and physiological stress (Jaworski *et al.*, 1981; Gibson, 1984) and also sink some 2–7 times faster during stationary-phase or senescence than during active growth (Smayda and Boleyn, 1965; Eppley *et al.*, 1967; Smayda, 1974; Titman and Kilham, 1976; Heaney and Butterwick, 1985).

Dead (heat-killed) phytoplankton (*Asterionella* and *Tabellaria* species) have also been shown to sink more rapidly than viable ones (Smayda, 1974); again suggesting physiological involvement in buoyancy regulation. This increased sinking velocity under conditions of physiological adversity is no doubt related to the fact that it is energetically expensive to regulate intracellular density and cellular buoyancy. For example, maintaining the right balance of ions (i.e. heavier and lighter) within the cell vacuole is necessary for maintenance of a low sinking rate; however, this process can only occur at the expense of cellular energy reserves and is thus dictated by a cell's energetic status (Heaney and Butterwick, 1985; Ferris and Christian, 1991; Fisher *et al.*, 1996).

As hypothesised some time ago by Stutz-McDonald and Williamson (1979), it is highly likely that the relative sinking rates of phytoplankton will be different in light versus dark conditions. This probable variability in light versus dark sinking velocity could be due to relative differences in: physiological growth rate (Titman and Kilham, 1976); pigment composition and photosynthetic activity (Moss, 1977; Fisher *et al.*, 1996); or differences in the ability to perform the necessary cellular adjustments involved in density and buoyancy regulation (Smayda, 1970; Anderson and Sweeney, 1977; Bienfang *et al.*, 1983; Fisher *et al.*, 1996). Given that prolonged darkness was seen to have been associated with decreased physiological activity during the current research, it is possible that algal cells subjected to dark conditions within an advanced WSP upgrade may suffer from an increased sinking velocity, especially for the motile flagellate *C. reinhardtii*; although the effects of prolonged darkness on motility were not directly assessed.

While a slight increase in sinking velocity may not be sufficient to overcome normal vertical mixing and turbulent resuspension processes within universally shallow WSP environments, this factor might be of increased importance within a duckweed-covered pond environment. Under this protective duckweed surface mat, the water column would be expected to be far less susceptible to wind-induced mixing, such that suspended algal populations would be more likely to be influenced by quiescent or 'advective' rather than turbulent settling processes. This, combined with a small physiological increase in settling velocity, might then be sufficient to further accelerate the rates of physical sedimentation and algal solids removal within these environments, especially when physiologically-mediated increases in sinking velocity have been reported to occur over timescales of less than 24 hours (Jaworski *et al.*, 1981; Gibson, 1984).

Meiring and Oellermann (1995) have suggested that algal cells passing through a shaded WSP environment could be expected to lose physiological vitality as a result of low-light or dark-exposure. The authors went on to suggest that this reduction in cellular vitality could potentially make the algal cells more susceptible to being adsorbed onto a biofilm (potentially like that of a rock filter or AGM system). The effects of darkness on phytoplankton cell surface charge interactions and their likely follow-on impacts on adsorptive processes were, however, not investigated here. Whilst it is possible that the

reduction in cellular vitality brought about by prolonged darkness may somehow result in an increased likelihood of substrate attachment, this concept remains purely speculative. The reader is instead directed to some topical reviews on the general subject for extracurricular insight (McDowell-Boyer *et al.*, 1986; Stevik *et al.*, 2004).

On the other hand, and as discussed earlier in Section 9.6.3.3, long-term dark-exposure also resulted in an apparent decrease in mean cell volume for both species, such that average sinking velocity was thought to have decreased by some 7–8% based on their implied empirical Stokes settling velocities. Whilst dark-exposed cells ‘shrunk’, they were also observed to have more capably maintained their discrete unicellular distributions and so were thought to have been less likely to suffer from increased settling rates resulting from cellular aggregation or ‘clumping’. Additionally, the differential dark respiration of cellular constituents during prolonged darkness could also have implications for phytoplankton settling velocity *in situ*. For example, some phytoplankton are known to preferentially catabolise carbohydrates (Geider and Osborne, 1989) followed by lipids and protein (Handa, 1969) in order to satisfy their energy requirements during prolonged dark respiration, and since cellular carbohydrates are relatively more dense than other energy reserves such as lipids (Fisher *et al.*, 1996), dark-exposed phytoplankton might be expected to become less dense and ‘more buoyant’ as a result of the prolonged dark event.

Published results from light versus dark sinking rate investigations have been highly variable. Some authors have reported lower phytoplankton sinking velocities under dark conditions (Anderson and Sweeney, 1977), others have reported somewhat reduced rates of dark sedimentation compared to those when illuminated (Bienfang, 1985; Johnson and Smith Jr., 1986), and there have also been reports of comparable settling velocities under both dark and light conditions (Boleyn, 1972); although the general consensus from this work appears to be a slight reduction in sinking velocity during dark-exposure. The precise implications of prolonged darkness for phytoplankton sinking velocity *in situ* are further complicated by species-specific factors such as cellular size, morphology and motility. Relative differences in sinking rates between algal species have been observed elsewhere to be large enough such that pond upgrade process performance at any given time could even be dictated by the taxonomic structure of the resident



phytoplankton community (Stutz-McDonald and Williamson, 1979; Bienfang, 1981; Johnson and Smith, 1985). Interestingly, Stutz-McDonald and Williamson (1979), following investigations into algal (*Chlorella*, *Scenedesmus* and *Microcystis*) settling rates in darkness under aerobic and anaerobic conditions, also found that there were no significant differences between the relative dark settling velocities of any of these species under either aerobic or anoxic conditions, suggesting instead that the presence of such conditions within the confines of a rock filter would be unlikely to alter the sedimentation rate of infiltrating algal cells.

Results from the current research, combined with those of Stutz-McDonald and Williamson (1979) above, could go toward answering the earlier (and unanswered) question posed by Martin (1970) regarding the mechanisms governing effective rock filter treatment. Martin theorised that the absence of light could lead directly to a reduction in algal cell vitality, such that infiltrating algal cells were thought to be less capable of maintaining themselves in a suspended ‘planktonic’ state (presumably through a reduced capacity for active motility and/or buoyancy regulation) and would, therefore, be more susceptible to becoming attached to, or settled onto, internal rock surfaces. Results from this Chapter have demonstrated a general reduction in algal cell vitality as a result of simulated *in situ* rock filter dark-exposure; although the follow-on effects of this reduction in cellular vitality with respect to *in situ* sinking velocity and/or the cell’s susceptibility to rock filter biofilm attachment/entrainment remain unclear. Following this, the overall effects of darkness on the sinking rate of both *C. vulgaris* and *C. reinhardtii* within the applied context of this thesis remain purely speculative. Nevertheless, it is hypothesised that there would be a likely sinking velocity trade-off as a result of concomitant reductions in both physiological activity and also cell volume—the exact balance of which remains unknown.

As introduced in Section 6.5, the earlier hypothesis of Zirschky and Reed (1988) was that most WSP phytoplankton would not sink until they were dead and that the precise duration of dark-exposure required to achieve this was not well defined. Zirschky and Reed (1988) did suggest—based on the earlier work of Wolverton (1980)—that a timeframe in the order of 20 days would be required for algal cell death within a duckweed pond environment; although the level of experimental rigor employed to

arrive at this figure remains unclear. Results from this Chapter have shown that some common WSP algal species are extraordinarily resilient to even prolonged (65 day) dark-exposure, with algal cells remaining largely viable following this extended dark period. Although results from laboratory experiments are difficult to translate directly to *in situ* dark-survivorship, they do suggest that the 20 day dark period suggested by Zirschky and Reed (1988) would, by itself, be unlikely to result in the higher algal solids removals commonly seen in duckweed WSPs. Instead, it is suggested that the enhanced rates of algal removal commonly reported within duckweed ponds are likely to come about from a combination of: the dark conditions suppressing phytoplankton growth and vitality; enhanced quiescent settlement; anaerobic exposure within the substratum; and also probable biological influences from allelopathy, competition, herbivorous grazing and/or microbial attack. Once again, however, the precise ratios of influence for each of the above factors remain unknown, such that this can only be suggested as an area for future investigations.

### **9.16 The conundrum of clinical manipulations—application of laboratory results to real life scenarios**

In performing laboratory-based experiments to derive information about natural environmental situations, one accepts that a whole host of applied limitations will inherently be attached to all subsequent findings. At the most basic level, it could be argued that the physical detention of phytoplankton within a laboratory setting, regardless of experimental hypothesis, might result in somewhat skewed or uncharacteristic experimental outcomes. Lewis *et al.* (1999) discuss the likely influence or bias exerted upon recorded dark-survival capacities of marine phytoplankton resulting from laboratory-based long-term storage, with the authors concluding that the physical act of laboratory-isolation and dark-storage in a controlled environment was an influential factor for the long-term dark-survivorship of phytoplankton quantified in the laboratory. In the case of Lewis *et al.* (1999), the fact that phytoplankton were also refrigerated during the long-term dark-storage programme could have potentially lead to an artificial extension of ‘natural’ survival times by lowering of cellular metabolic rates. Whilst this might be an acceptable reflection of dark-survival in phytoplankton from low latitudes, any attempt to apply results from Lewis *et al.* (1999) directly to what might be

expected in the temperate Bolivar WSPs would be difficult to justify. It is reiterated that whilst temperature has been shown elsewhere to have a defining role on dark-survival potential (Anita, 1976; Dehning and Tilzer, 1989; Popels and Hutchins, 2002; Bartosh and Banks, 2007), the temperature at which the current dark-survival experiments were performed (20°C) was specifically selected in order to reflect annual average *in situ* field temperatures in the Bolivar WSPs (see Figures 3.8 and 4.4) so as to maximise the environmental relevance of experimental findings.

The axenic status of the current algal cultures used for the dark-survival experiments is also not directly representative of *in situ* conditions. The work of Richardson and Fogg (1982) identified differential dark-survivorship potentials under axenic and non-axenic conditions (Section 9.12), with the authors suggesting that phytoplankton may benefit from the presence of bacteria during darkness (possibly through increased metabolite availability). Similarly, Brussaard and Riegman (1998) reported that algal (*Ditylum brightwellii*) survival under conditions of nitrogen starvation can actually be enhanced by the presence of bacteria; supposedly through the provision of remineralised ammonium to N-starved phytoplankton. In this respect, the axenic status of the current dark-survival experiments may have potentially *underestimated* the true dark-survival potential in terms of what might be expected *in situ*. On the other hand, the absence of resident microbial populations (through the absence of competition) might have also potentially altered the dark-survival outcomes in a favourable manner. Bunt and Lee (1972), commenting on the earlier findings of Wright and Hobbie (1968), for example, reported that phytoplankton are generally poorly equipped to compete with bacteria for organic substrates at low concentrations. The same trend was observed by Brussaard and Riegman (1998) under conditions of phosphorous limitation for *D. brightwellii*, whereby cultures displayed increased rates of cell death in bacterially-contaminated cultures—a supposed consequence of poor competitive capabilities for growth-limiting phosphorous. In this sense, the axenic nature of laboratory cultures used during this study may have potentially *overestimated* the true dark-survival potential compared to what might reasonably be expected in highly-competitive WSP environments. Regardless of these unknowns, axenicity in current work served to isolate the true dark-survival capacities of the chosen phytoplankton species without interference (or support) from foreign biological entities.

Like the absence of microbial competition, uni-algal experiments are invariably performed in an environment that is intrinsically devoid of natural predators (e.g. zooplankton and protozoa), viral (Agustí *et al.*, 1998; Brussaard *et al.*, 2001) and bacterial pathogens (by way of bacterially-mediated algal-cell-lysis; Cole, 1982; Berges and Falkowski, 1998). These foreign organisms would normally be expected to impart some additional (and likely adverse) effects on cellular survival during prolonged darkness within a WSP environment; particularly considering the demonstrated reduction in metabolic vigour following dark-exposure. Additionally, the absence of other chemical contaminants or cytotoxic substances in optimal culture media (e.g. ROS and humic acids)—substances that would normally be expected to be present in a WSP environment (Curtis *et al.*, 1992)—might also result in an artificial extension of the true *in situ* dark-survival period. In this way, dark-survival capacities measured via laboratory-based assessments involving buffered, nutrient-replete, sterile, optimal growth media, could potentially represent the ‘best case’ dark-survival scenario, such that phytoplankton dark-survival might reasonably be expected to be of a lesser duration *in situ*.

Dark-survival times derived from laboratory research can, therefore, only relate directly to the particular storage conditions under which the experiments were performed. Scheffer (2004) emphasized the difficulties involved with extrapolating laboratory-derived results (of algal productivity versus aquatic light climate) to predictions about phytoplankton growth in the field. Others have echoed similar cautions regarding physiological investigations of phytoplankton, suggesting that conclusions drawn from work under laboratory conditions should only be applied to natural situations with prudence (Sand-Jensen, 1989). The same cautions must, therefore, be made regarding the findings presented during this Chapter.

### **9.17 Research findings and experimental conclusions**

The ongoing uncertainty surrounding both the contributing factors, and also the very nature of cell death in phytoplankton, is compounded by the unpredictable and immeasurable natural environmental variability to which these organisms are exposed *in situ*. Recent work suggested that the specific environmental conditions experienced

during growth can affect the kinetics of phytoplankton cell mortality (Lee and Rhee, 1997; Jochem, 1999), with the dynamics of algal cell death varying also according to species (Agustí and Carmen Sánchez, 2002). More recently, some research has also brought into question the very means by which algal cell death might be instigated; coming about through the identification of internally-mediated, autocatalysed ‘apoptotic’ cell death processes in some phytoplankton.

As has been conclusively demonstrated throughout this Chapter, prolonged darkness resulted almost exclusively in greater overall population fitness and considerably lower levels of algal mortality (relative to ‘Day zero’ levels) than did continuous culture illumination. For some phytoplankton, it appears that being ‘under the cover of darkness’ can actually provide enhanced long-term cellular protection, especially in instances of cytotoxic stress (see Section 9.10). Whilst *population* growth may not be achievable during darkness, the original cell population may be significantly more likely to survive a given stressor if dark-exposed. Furthermore, and following the results from re-growth experiments, dark-exposed phytoplankton may also not be competitively disadvantaged following re-exposure to a more favourable light climate. Such are the apparent ‘protective’ qualities of darkness, Hargraves and French (1983, cited in Lewis *et al.*, 1999) stated that darkness generally prolonged the survival of diatom species studied to that time. Those findings were apparently very similar to those of the current research, and they highlight the apparent ‘dark-conservation’ of cellular photosynthetic pigments and photosystem machinery in some species of Chlorophyceae.

The stalwart dark-survival capabilities of both *C. vulgaris* and *C. reinhardtii* under the tested experimental conditions have been comprehensively demonstrated during this Chapter, with algal cells under both ‘aerobic’ and ‘low D.O.’ treatments able to successfully endure extended periods of dark-exposure without any significant loss of population viability. On the other hand, algal cells in both ‘light’ treatments were observed to have suffered significantly greater ill-effects as a result of prolonged illuminated culture stagnation than did either of the prolonged dark-exposed treatments. Additionally, and upon re-exposure to pre-dark irradiance, ‘dark’ treatments for both phytoplankton species were able to resume rapid population re-growth to the point

where stationary-phase cell densities were achieved within 10 days of re-illumination. Overall, prolonged darkness was observed to have resulted in:

- No significant change to long-term population cell density for *C. vulgaris* and a small ( $\approx 0.5\text{-log}_{10}$ ) reduction in culture density for *C. reinhardtii*; although it was suggested that surface biofilm attachment may have influenced this observation for *C. reinhardtii*;
- Significant cell volume reductions in the order of 35 and 41% for *C. vulgaris* and *C. reinhardtii* respectively;
- Conservation of cellular FSC:SSC ‘size-to-density’ ratios (an observation that was thought to suggest an effective long-term maintenance of pre-dark cellular constitution);
- No significant change in the long-term levels of cellular chlorophyll *a* pigment for both *C. vulgaris* and *C. reinhardtii*;
- No significant loss of *in vivo* cellular chlorophyll *a* fluorescence activity for either algal species (when fluorescence signals were normalised to diminishing cell volumes);
- No discernable loss of cellular membrane integrity and hence full retention of pre-dark viability status for both *C. vulgaris* and *C. reinhardtii*;
- Significant long-term reductions in general cellular FDA metabolic activities for both phytoplankton species;
- No apparent interference with the algal cell population’s ability to re-grow upon return to a more favourable light climate.

Initial results from these two month dark-survival experiments showed that both algal species were capable of adjusting cellular metabolism within the first 7 days of dark-exposure; something considered to have been a possible ‘photoacclimation-type’ response. Following these initial findings, a more detailed analysis of the initial 7 day dark period revealed that apparent cellular photoacclimation occurred within the first two days of dark-exposure. No subsequent attempts were made to further distill the kinetics of this dark acclimation response during the current research.

Experimental results conclusively showed that a reduced dissolved oxygen concentration ( $\approx 25\%$  saturation) had no bearing on the ability of either algal species to withstand long-term dark-exposure. In an applied context, it is suggested that subjecting algal cells to conditions of simultaneous darkness and reduced oxygen availability would be expected to impose no significant adverse effects on population survival within an advanced in-pond upgrade such as a duckweed pond, a rock filter or attached-growth media system. Despite algal cells of both species being observed to display accelerated and wholesale population decline when exposed to continuous light and DIC limitation, the practical implications of these findings for algal removal in WSP upgrade systems remain unclear.

### **9.18 Suggested experimental improvements and future research questions**

Throughout the current Chapter, several suggestions as to ways in which the original experimental design might have been improved or indeed supplemented have been alluded to. Below is a summary of these concepts in addition to several new research ideas which could form the basis of future research efforts into this area.

#### **9.18.1 Destructive sampling for absolute dark-control**

As discussed previously (Sections 7.2.2 and 9.9.1), periodic exposure to even very low-level light intensities can prolong dark-survival in some phytoplankton. It is possible, therefore, that in spite of employing utmost operator diligence during all sampling manipulations, there may have been short-term re-exposure to extremely low-intensity illumination during dark treatment sampling intervals; something that may then have unduly enhanced the measured dark-survival capacity of both algal species. It should be re-emphasized here that the desire for continuous sub-sampling of the *same cell population* during prolonged darkness was initially perceived to be of greater importance than was the very low-level light re-exposure the cultures may receive during sampling intervals. Also, and as referenced in Section 7.2.2, space limitations within the single illuminated orbital incubator largely precluded the use of a destructive sampling protocol during these dark-survival experiments.

As mentioned in Section 9.9.1, other researchers have reported conducting periodic sampling intervals during prolonged dark-survival experimentation under dim green ‘safe-lighting’ at irradiances  $< 0.1 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Ferroni *et al.*, 2007); however, the potential implications of this periodic low-intensity light exposure for subsequent dark-survivorship remain unknown and potentially significant. Further to this, Tuchman *et al.* (2006) actually referred to experimental conditions of continuous  $3 \mu\text{mol m}^{-2} \text{ s}^{-1}$  illumination as being “dark” experimental treatments. The sentiments of Tuchman and co-workers, however, would be difficult to justify in the context of dark-survival research. In all future work, it is suggested that the only way of being absolutely confident that there is no re-exposure to light during similar experiments, would be to adopt a destructive sampling regime, whereby individual dark-exposed cultures are sampled once only and then discarded. Whilst this method would be more resource-intensive, it would successfully remove this underlying uncertainty surrounding culture re-illumination (in terms of both the received light intensity and also the subsequent physiological effects) during periodic experimental sampling of ‘dark’ cultures.

### **9.18.2 Alternate trophic states, environmental media and dark-survival**

Although concerns surrounding alternate modes of nutrition were largely rationalised (see Section 9.12), there remained some unanswered questions regarding the potential role of such modes of nutrition in the ultimate long-term dark-survival of the two tested algal species. Whether or not there was some supportive role served by DOC toward the dark-survival capabilities of *C. vulgaris* and *C. reinhardtii*, although considered to be unlikely, remained ultimately unconfirmed. Whilst there is significant evidence within the relevant literature to support facultative heterotrophy (or ‘mixotrophy’) in both *C. vulgaris* and *C. reinhardtii*, this ‘dark-growth’ has been achieved using relatively simple carbon sources (i.e. glucose and acetate) and so was considered to be of limited practical relevance to the Bolivar WSP environment.

In order to negate future concerns surrounding the presence and/or availability of DOC within laboratory culture media, it would be suggested that further research effort incorporate the use of ‘environmental medium’ such as a filter-sterilised WSP effluent.



This notion was actually discussed by Anita (1976), who cautioned that the culture test-medium utilised for their experiments was possibly inadequate to reveal the ‘true ecological dark-survival potential’ of some phytoplankton species. At the same time, it is also possible that the culture medium used during assessments of phytoplankton dark-survival here (being highly filtered, sterile, adequately buffered and nutrient-replete) might have provided an unduly enhanced reflection of the true ecological capacity for dark-survival *in situ*. Talling (1955), for example, found that late spring populations of *Asterionella* suspended in environmental medium (lake water) died, whilst those maintained in optimal culture medium continued to grow. Regardless of these unknowns, the use of an environmental medium (preferably in parallel with optimal culture media) would serve to provide a more applied setting to algal dark-survival assessments and would also serve to provide greater practical relevance to the idea of non-autotrophic nutrition during darkness based on existing *in situ* carbon sources.

### **9.18.3 Strict anaerobiosis and dark-survival**

Although it was the aim of this research to investigate dark-survival under conditions of ‘low’ DO concentration (as it applied to the Bolivar pilot WSP upgrade systems), it might also be relevant to other WSP upgrade scenarios to look at algal dark-survival under strictly anoxic conditions. Rock filters, for example, are widely recognised to develop anaerobic conditions, particularly at night or under high loading events, such that it could be of greater relevance to those systems to look at algal dark-survival under strict anoxia, or even more ideally, under an oscillating ‘aerobic–hypoxic–anoxic’ diel cycle. Future experiments concerned with assessing prolonged (i.e.  $\geq 6$  days) dark-survival under strict anoxia could possibly incorporate the use of oxygen scavengers (e.g. sodium sulphite) for complete and sustained oxygen-stripping, thereby allowing for true ‘anaerobic’ assessment of phytoplankton dark-survival.

### **9.18.4 Axenic versus non-axenic, and uni-algal versus mixed dark-survival**

In light of the intrinsic synergism between heterotrophic microbes and phytoplankton in WSPs (Section 1.2.3), and probably also during dark-exposure (see Section 9.12), it would be desirable for future work to assess dark-survival potential in axenic versus

non-axenic algal cultures in order to further investigate possible influence of resident microbes on phytoplankton dark-survival. Whilst it is possible that there may be a supportive role provided by microbes during darkness, it is equally possible that there may also be some competitive or antagonistic interactions which could have adverse consequences for prolonged dark-survivorship—an issue presented during the discussion of Furusato *et al.* (2004).

Further weight to this concept comes from the work of Patil (1991), who observed greatest culture growth and photosynthetic capacity and in uni-algal (*Scenedesmus* and *Ankistrodesmus*) monocultures, with overall culture photosynthetic capacity being hampered by the presence of native bacteria and other algae present in culture medium seeded with an environmental ‘pond community’ inoculum. Similar reports were again given by Mara and Pearson (1986), who were of the opinion that the photosynthetic efficiency of algae (in terms of photosynthetic O<sub>2</sub> production) is greatest when they are grown as monocultures and is reduced somewhat during mixed culture situations. It seems likely, therefore, that an individual algal species’ dark-survival potential in a monoculture situation would be somewhat different to that which could be expected in a mixed culture situation, especially for axenic versus non-axenic cultures. Similarly, it is also possible that the reduction in general cellular metabolic vigour during prolonged dark-exposure as reported here, might also increase phytoplankton susceptibility to viral infection—a ‘loss factor’ that has gained increasing importance in recent times.

Additionally, future work could incorporate the use of locally-relevant ratios of mixed algal cultures in order to isolate any interspecific factors (e.g. symbiotic, allelopathic or antagonistic) that might influence long-term dark-survival; something which has not been previously investigated. It is suggested, therefore, that future work should investigate algal dark-survival in mixed cultures in order to provide a more applied setting to the measured outcomes, as well as to more closely replicate the native biological interactions which would be expected to occur during *in situ* dark conditions. Future research effort could also involve the use of both axenic and non-axenic algal cultures and possibly also include investigations into algal pathogens and their relative virulence under corresponding ‘light’ and ‘dark’ conditions.

### 9.18.5 Grazer interactions and dark-survival

To the best of the author's knowledge, there has been no prior research effort concerned with parallel assessments of phytoplankton dark-survival along side that of a resident grazer population (either in direct or indirect contact). Griffis and Chapman (1988) have come closest to doing so through their discussion about the parallel survival capacity of phytoplankton and zooplankton in darkness and whether there might be some selective grazing pressure under such conditions. Work by Duval and Geen (1976) and also Taguchi (1976) showed evidence for nocturnal zooplankton grazing of phytoplankton during laboratory incubations, and so it is reasonable to assume that there would be a sustained grazing pressure during dark conditions within a WSP upgrade system (especially for non-selective grazers that may not be so reliant on 'visual' feeding cues).

Alongside the internally-driven selective pressures associated with discrete physiological dark-survival, there could also be externally-selective grazing pressures exerted upon particular phytoplankton species during prolonged dark conditions; given the recognised capacity for species-specific selective feeding in some zooplankton species (Porter, 1973; Merrick and Ganf, 1988) and the documented species-specific nature of phytoplankton–zooplankton interactions in general (Elser *et al.*, 1990). Additionally, and given that a number of studies have demonstrated a capacity for food selectivity in zooplankton according to the relative 'palatability' or nutritional status of the algal food resource (e.g. DeMott, 1986; Butler *et al.*, 1989; van Donk and Hessen, 1993; van Donk *et al.*, 1997), it is possible that dark-exposed phytoplankton may have a reduced likelihood of being grazed and/or digested as a direct consequence of their depressed physiological state post-darkness. At the same time, prolonged darkness might confer some selective pressure upon the grazer community themselves, presumably through differential capacities for sustained dark nutrition and subsequent dark-survival. This begins to delve into the realms of zooplankton autecology (a notoriously complex area in its own right) and so the reader is instead directed to a tantalising discussion paper by Starkweather (1983) for further insights. It is proposed that further research effort should be directed toward obtaining a more unified understanding of this area; something that could be achieved through the incorporation of 'predator–prey' interactions into phytoplankton dark-survival investigations.

### 9.18.6 Additional research suggestions

- Future work into the illuminated ‘DIC starvation’ issue (given that there is some debate about the ability of some algae to use  $\text{HCO}_3^-$ ).
- Work with *Chlamydomonas* cultures could involve the use of ‘non-stick’ flasks (e.g. silicone coated) in order to negate the potential problems associated with cellular adherence.
- Future dark-survival work could include tests on cellular constituents and reserves (protein and carbohydrate) during and following darkness to see which internal resources are consumed and in what order/quantity.
- Real-time quantitative measures of photosynthetic activity ( $\text{C}^{14}$  or photosynthetic  $\text{O}_2$  evolution) in order to compare FCM results with actual *in vivo* photosynthetic capacity.
- Scanning electron microscopy to look at cellular ultrastructure and morphological changes (such as vacuolisation, cytoplasmic recession or granularisation) during prolonged darkness.
- Future work could assess the settlement rates of phytoplankton in darkness compared with light conditions (this would be of particular relevance to inherently shallow WSP upgrade environments).
- Additional research to define any dark-induced cellular behaviour (e.g. promotion of cellular aggregation/dispersion, biofilm adsorption/entrapment in dark versus light conditions, motility and buoyancy regulation during dark versus light conditions).

## 10 General discussion

As outlined in Chapter 1 (Section 1.3.1), the final effluent from the Bolivar WSP network has historically been characterised by high levels of algal suspended solids as well as a largely unpredictable and highly variable plankton ecology. Following the 1999 commissioning of the Bolivar DAF/F plant, a number of operational problems relating specifically to the periodic presence of high levels of algal biomass as well as some identified “problem” zooplankton species have been identified as threats to overall DAF/F process efficiency. The primary aim of research presented in this thesis was to investigate several ‘advanced in-pond treatment processes’ for upgrading of the final Bolivar WSP effluent prior to DAF/F treatment and horticultural reuse. Research presented was also aimed at distilling some of the factors involved in effective performance of the candidate in-pond effluent upgrade technologies, with specific emphasis on the *in situ* dark-survival potential of algal populations.

Results from pilot plant performance monitoring presented in Chapters 3 and 4 have demonstrated that of the four pilot upgrade series, a rock filter and an attached-growth media system would be expected to offer the greatest potential—in terms of the magnitude and reliability of SS and algal biomass removals—for producing the best quality WSP effluent for processing by the Bolivar DAF/F plant. Duckweed coverage was also shown to be at least as effective and in some instances significantly more advanced than an uncovered ‘Open Pond’ system in terms of its ability to significantly improve the final effluent quality of the Bolivar WSPs. There were, however, some issues relating to the likely influence of duckweed plant tissue on measured performance parameters (BOD<sub>5</sub>, SS and chlorophyll *a*) in the pilot DW pond system, with senescent duckweed biomass deemed likely to have directly contributed toward the elevation of these measured parameters during performance monitoring.

Throughout Chapters 3 and 4, there was a trend for a more advanced ‘rate’ of treatment performance down the pond series within the RF and AGM systems relative to the OP series. This resulted in what was a frequently observed pattern of the majority of parameter removals occurring within the first pond in series for the RF and AGM

systems, compared with a more gradual removal trend down the pond series in the parallel OPs. This general performance pattern often resulted in the observation of more advanced RF and AGM treatment performance by ‘Pond 1’ and statistically similar removal efficiencies for all pilot upgrade systems by ‘Pond 3’ of the three-pond series, and most notably for the more physical parameters such as SS, turbidity and chlorophyll *a*. It is important to emphasize that this trend for equivalent overall (three-pond) treatment train performance in the OP system was likely to have been a manifestation of the pilot-scale nature of the experimental setup and was not necessarily indicative of the performance trends one could expect from a full-scale ‘open WSP’. In this sense, it must be remembered that the pilot-scale OPs were not behaving like small-scale WSPs; rather, they were operated as parallel ‘non-interventional’ reactors to control for the effects of temporary quiescent impoundment during pilot plant passage (see Section 2.1.1.2 for initial description). Because of the reduced size of the pilot-scale reactors, there was an inherently large-scale reduction in wind fetch and therefore much less opportunity for classical *in situ* resuspension mechanisms (i.e. wind resuspension) to impact negatively on system performance compared with what would be expected in a full-scale pond. The above, combined with the 25% shallower hydraulic depth (compared with the Bolivar ponds), would have led to greater potential for SS abatement in the pilot-scale OPs than would be anticipated from an actual in-pond Bolivar upgrade system consisting of no intervention whatsoever (remembering that solids removal is first and foremost a function of water column depth; see Section 4.3.5).

At the same time, however, it should also be emphasized that the abovementioned limitations do not apply to results from the pilot RF and AGM upgrade systems, since these issues surrounding wind-induced resuspension and variable particulate settlement depth do not apply to these ‘fixed-bed’ systems. The same could also be argued for the DW system, with respect to wind resuspension, in that a thick duckweed surface cover would largely protect a full-scale pond from the effects of wind-induced resuspension. A thick duckweed cover would also be likely to restrict the severity of thermal stratification (e.g. Dale and Gillespie, 1976) and hence limit diel turnover (and mixing) of the water column; although the same issues regarding the shallower sedimentation depth do apply to results of the DW ponds as for the OP series above. All of this essentially means that there would have been an effective narrowing of the true

‘performance gap’ between the RF, the AGM and to a lesser degree the DW system, relative to the performance of the parallel Open Ponds. Because all three advanced upgrade systems performed at least as well as and commonly significantly better than the parallel OPs, this means that the WSP upgrade potential of each system presented in this thesis is likely to represent a performance minimum in terms of the difference between an upgraded Bolivar WSP effluent and the effluent quality one could expect in the absence of any upgrade intervention.

Work presented in Chapter 5 showed again that the RF and AGM series were highly effective at reducing the levels of both total and also problem zooplankton biomass in the Bolivar WSP effluent. Results also showed, in respect of zooplankton populations, that the DW upgrade system was equally efficient at reducing the levels of total biomass as well as the numbers of problem zooplankton in the final Bolivar effluent. Changes in phytoplankton communities, however, were not so apparent, with no proportionate change in the levels of problem algae being evident following effluent passage through any of the pilot treatment series; although total algal biomass levels were invariably reduced down the pond series in all treatments. As highlighted in Chapter 1 (Section 1.3.1), the effective removal of unwanted algal and zooplankton biomass from the final Bolivar WSP effluent would have significant down-stream implications for DAF/F plant process efficiency; translating to significant cost savings in the form of a more reliable DAF/F influent quality, less volatile operational protocols and reduced inputs of chemical flocculants. Work by Buisine and Oemcke (2003) identified that active management of Bolivar WSP ecology was likely to offer the best and most cost-effective long-term solution to the operational problems currently plaguing the DAF/F plant. Results presented in this thesis suggest that rock filtration, attached-growth media addition and even duckweed surface coverage could all constitute such ecological solutions for effective management of the Bolivar pond effluent. Aside from these more site-specific findings, monitoring data offered in Chapter 5 also constituted the first reported investigation into the temporal ecology of the three major freshwater zooplankton groups (rotifers, cladocerans and copepods) in a WSP environment, as well as offering the first quantitative insights into the zooplankton ecology of a rock filter, a duckweed-covered pond, or an attached-growth media system.

Further to the more immediate on-site advantages outlined above, effective upgrading of the final Bolivar effluent would also be expected to have flow-on benefits further downstream. Reducing the load on the DAF/F plant would be expected to effectively reduce the extent of algal SS and turbidity breakthrough during DAF/F treatment, thereby limiting the potential for re-growth of algal populations in horticultural storage reservoirs as well as minimising the potential for clogging of irrigation distribution networks (see Section 1.3.1 for more information). In addition to solids removal potential, the nitrifying capacity of a rock filter upgrade (possibly also an AGM system) would also be expected to result in small-scale  $\text{NH}_4^+\text{-N}$  ( $\approx 0.5\text{mg L}^{-1}$ ) and  $\text{NO}_2^-\text{-N}$  ( $0.1\text{--}0.2\text{mg L}^{-1}$ ) removals from the Bolivar WSP effluent; something that could directly translate to reduced chlorine demand and improved disinfection efficiency of the reclaimed wastewater. This would be of particular relevance to Bolivar operations in terms of potentially significant capital savings from reduced chlorine consumption, given that  $0.5\text{mg}$  of  $\text{NH}_4^+\text{-N}$  exerts a chlorine demand of  $5\text{mg Cl}_2$  and  $0.15\text{mg}$  of  $\text{NO}_2^-\text{-N}$  has a chlorine demand of  $1.1\text{mg Cl}_2$  (White, 1999). Since the cost of chlorine is in the order of AU\$1,300 per tonne, this would represent a cost saving of approximately \$8,400 per week for the Bolivar DAF/F plant when operating at full treatment capacity ( $150\text{ ML d}^{-1}$ ).

In addition to the potential for operational cost savings, a more nitrified final WSP effluent could also help improve the reliability of biological disinfection during the post-treatment chlorination process. Given that the Bolivar DAF/F plant was designed to deliver a ‘Class B’ reclaimed effluent (i.e.  $<100\text{ MPN FC }100\text{ml}^{-1}$ ; Buisine and Oemcke, 2003), more complete and/or reliable post-treatment disinfection could potentially offer additional cost benefits to Bolivar operators in terms increased revenue from the production and sale of a microbiologically higher grade ‘Class A’ reclaimed effluent (i.e.  $<10\text{ MPN FC }100\text{ml}^{-1}$ ; SAEPA, 1999); although the outcomes of in-pond effluent upgrading for protozoan (e.g. *Cryptosporidium* oocysts) pathogen control remain unclear. Furthermore, more complete and/or reliable DAF/F plant disinfection could even contribute toward minimising the public and products liability insurance premiums paid by both Bolivar WWTP operators as well as down-stream irrigators against claims made with respect to the use of reclaimed wastewater (e.g. Huijbregsen *et al.*, 1999).



Whilst much of the research into in-pond WSP upgrade technologies has been firmly focused on determining the potential advantages of the technology (e.g. BOD<sub>5</sub>, SS or nutrient sequestration potential), equally important regarding the focus of the current research were the potential *disadvantages* of the investigated pond upgrade methodologies. Because the final Bolivar effluent is already of a generally high quality with respect to parameters like BOD<sub>5</sub>, NH<sub>4</sub><sup>+</sup>-N and *E. coli*, of primary concern is whether or not the operational shift imposed by a given in-pond upgrade system would be likely to result in any significantly adverse changes in WSP ecology and final effluent quality. As highlighted by Sweeney *et al.* (2005a), retrospective WWTP process alterations have the potential to bring about unforeseen (and potentially adverse) changes in WSP ecology. This unfortunate situation has already been realised at the Bolivar site, whereby the 2001 up-stream activated sludge plant installation resulted in significant improvements in WSP water quality and a subsequently large increase in midge fly (*Chironomidae*) populations at the site during summer months. This unforeseen midge problem has in itself imposed additional process ramifications for WWTP operators at Bolivar; ultimately resulting in significant capital expenditure for on-site midge control strategies during summer. As defined by Sweeney *et al.* (2005a), the annual costs associated with midge fly management at Bolivar have so far been of a similar order of magnitude to the cost savings coming from a reduced load on the down-stream DAF/F plant, such that the full range of benefits coming from activated sludge treatment have not yet been realised.

Regarding any prospective treatment train upgrades at Bolivar, Sweeney *et al.* (2005a, pp. 21–22) made a point of emphasizing that “*The impact of further operational changes on algal and zooplankton ecology, and pathogen levels in the WSPs, will have to be clearly understood prior to implementation... to ensure that they will be benign.*” In respect of these concerns, results from this thesis suggest that implementation of either a rock filter or a horizontal-flow AGM upgrade system would not be expected to result in any foreseen operational adversities. Due to the ‘rear-end’ *in situ* location of a rock filter and a horizontal-flow AGM system, it is considered unlikely that such upgrade installations would have any real influence on the ecological function of the unmodified up-stream regions of the pond. The above concerns of Sweeney and co-workers would, however, become more of an issue for a duckweed-covered system, since duckweed

would be expected to cover a larger pond area than would a rock filter or AGM upgrade (which would be situated near the outlet only). There are also some additional unresolved concerns regarding the contribution of duckweed plant biomass to the final effluent SS. It can be imagined that achieving effective removal of algal and zooplankton SS from the final effluent, only to have these solids replaced by senescent duckweed tissue, may result in the effective nullification of any improvements in DAF/F plant efficiency coming from the reduced plankton loads; although large, sessile fragments of duckweed biomass would almost certainly be easier to remove during DAF/F treatment than would so-called problem algae and zooplankton. Additionally, it is unknown to what extent organic acids coming from decaying duckweed tissue could contribute to the formation of toxic halogenated disinfection by-products during DAF/F plant post-treatment chlorination. This issue would require thorough investigation prior to the adoption of a duckweed-based pond system at Bolivar, given that the reclaimed effluent is largely destined for horticultural reuse including spray irrigation on produce to be eaten raw (Bosher *et al.*, 1998; Huijbregsen *et al.*, 1999).

Whilst the above issues remain unconsolidated, there are some additional and potentially beneficial elements that could also arise from the installation of a duckweed pond cover at Bolivar. A dense duckweed surface coverage could, for example, have the capacity to impede the reproductive cycle of midge flies within the lagoons. According to Culley Jr. and Epps (1973), emerging insect larvae, such as those of mosquito for example, are unlikely to be able to penetrate a thick duckweed surface mat so long as adequate plant biomass density is maintained. It is possible then that the same could be true for midge flies, given that they too have a similar emergent stage in their life cycle. Similarly, other researchers have reported that mosquito (*Culex* species) populations are unable to colonise wastewater covered with duckweed (*Lemna*) as a result of both physical and insecticidal duckweed properties (Eid *et al.*, 1992b; Eid *et al.*, 1992a). As demonstrated in Section 3.3.4, *Lemna disperma* was able to develop and maintain a very thick (2–3cm) and dense ( $\approx 8.3\text{kg m}^{-2}$  fresh weight) surface coverage on the Bolivar WSP effluent; with this duckweed species also able to maintain active growth during the South Australian winter, when the rates of growth and biomass production would be expected to be at their lowest. It is, therefore, considered highly unlikely that midge fly larvae (or even egg-laying adults for that matter) would be able to penetrate such a dense

floating biomass layer, such that the potential for ecological control of midge flies at Bolivar could be significant.

In addition to its potential role in midge fly management, a dense duckweed cover might also help to minimise evaporative water losses from the Bolivar WSP network. Water losses in duckweed- (*Lemna*) covered water bodies have been reported elsewhere to be lower than that from an open water body under similar environmental conditions (Bonomo *et al.*, 1997; Baldizón *et al.*, 2002). This is especially the case at night, with stomatal pore closure acting as an insulating ‘blanket’ to further restrict water loss via transpiration (Dale and Gillespie, 1976; Oron *et al.*, 1987). As shown in Chapters 3 and 4 (Figures 3.6 and 4.2), local evaporation rates can be extreme during the summer months (300–450mm month<sup>-1</sup>). Reduced rates of evaporation from a duckweed pond could help minimise the potential loss of earnings during periods of peak demand, given that 100% of Bolivar WSP flow is on-sold during summer. Additionally, reduced evaporative water losses could also contribute toward the production of a ‘less concentrated’ final effluent (e.g. Baldizón *et al.*, 2002). In particular, this could help minimise the salinity of the final WSP effluent, given that the Bolivar DAF/F plant is designed to produce an effluent with 1500mg TDS L<sup>-1</sup> ( $\approx 2300\mu\text{S cm}^{-1}$ ) and potable water is in fact added to the reclaimed effluent during times of increased salinity (Buisine and Oemcke, 2003). It is possible that a rock filter and even an AGM system could also reduce evaporative water losses; however, this remained uninvestigated.

A summary of the proposed advantages and disadvantages of the investigated WSP upgrade methodologies, in addition to some issues referenced elsewhere in this thesis, are provided in Table 10.1.

**Table 10.1.** Executive summary of selected advantages and disadvantages for the three investigated advanced WSP upgrade systems.

	<b>Rock filtration</b>	<b>Duckweed surface coverage</b>	<b>Horizontal-flow attached-growth media</b>
<b>Advantages</b>	<p>≈25,000 m<sup>3</sup> of available media on-site                      Excellent SS, BOD<sub>5</sub> performance potential                      Good algal / zooplankton removal potential                      Relatively inexpensive technology                      Established design criteria                      More surface area for treatment activities                      Nitrification potential for NH<sub>4</sub>-N removal</p>	<p>Relatively inexpensive &amp; well researched                      Good SS, BOD<sub>5</sub> performance potential                      Good algal / zooplankton removal potential                      Less permanent system c.f. a rock filter                      Potential for saleable by-products                      Potential for N &amp; P removal (if harvested)                      Reduced evaporative water losses</p>	<p>Greater serviceable life estimate                      Easier decommissioning                      Less permanent installation c.f. a rock filter                      More ideal flow hydraulics c.f. a rock filter                      Excellent SS, BOD<sub>5</sub> performance potential                      Good algal / zooplankton removal potential                      Multiple sediment–water interfaces</p>
<b>Disadvantages</b>	<p>Much more permanent system                      Painful decommissioning                      Cleaning protocols not established                      Aeration of effluent may be required                      Reduced serviceable life estimate                      Possible hydraulic short-circuiting                      Potential for anoxia under high loading</p>	<p>Aeration of effluent may be required                      Plant biomass contributions to sludge                      Need for floating containment grid                      Susceptibility to wind-dispersion                      Issues of avian grazing &amp; aphid infestation                      Less permanent installation than rock filter                      Potential for anoxia under high loading</p>	<p>Very expensive technology                      Aeration of effluent may be required                      Little prior research (design criteria unknown)                      Cleaning protocols not established                      Large surface area for sediment BOD<sub>5</sub>                      Longevity of synthetic media unknown                      Potential for anoxia under high loading</p>

Prior to beginning this research, several questions were posed regarding the physiological capacity of common WSP algae to survive the adverse environmental conditions prevailing within a rock filter or duckweed pond. Long-term laboratory experiments were conducted under conditions aimed at simulating those of the selected advanced in-pond upgrades (i.e. simultaneous darkness and low oxygen availability at 20°C), with detailed physiological assessments of cellular fitness routinely performed throughout the two month incubation period. Results from this work suggest that algal species common to WSPs are likely to be highly resilient to prolonged exposure to dark conditions under either a 'low' or 'ambient' DO environments. Monitoring of cellular size during prolonged dark-exposure showed trends for a slight reduction in cell volume during dark conditions, with the potential effects of this observation on sinking rate *in situ* unclear, particularly in light of additional uncertainties surrounding the physiological effects of dark-exposure on buoyancy regulation.

Results of the dark-survival work suggested that so-called 'problem' algal genera like *Chlorella* and *Chlamydomonas* are unlikely to be adversely affected by extended exposure to the simulated 'dark / low D.O.' conditions of an advanced in-pond upgrade system. Practical support for these *in vitro* observations was provided by the findings of Chapter 5, whereby there was no apparent change in the numbers of problem algal species following pilot plant passage through any upgrade system. There were at the same time, however, some remaining questions regarding the predictability of algal dark-survivorship *in situ* based on the results obtained *in vitro*, as well as some additional factors relating to the effects that other biological entities (e.g. competitors, predators and microbial/viral pathogens) may have on phytoplankton dark-survival in an actual WSP setting. These uncertainties, along with some additional unanswered questions were raised at the end of Chapter 9 as areas for future investigation.

### **10.1 Logistics of upgrading the Bolivar WSPs**

Rock filters are well recognised as being a relatively inexpensive method for upgrading WSP effluent, with the cost of a rock filter said to be approximately half that of competing algal removal technologies (USEPA, 1983; Middlebrooks, 1995). In the case of the Bolivar WWTP, the cost of the rock media would be expected to be negligible,

since there are already large volumes (estimated to be in the order of 25,000m<sup>3</sup>) of suitable rock media on site as a result of earlier trickling filter decommissioning. Consequently, the most significant cost associated with rock filter construction at Bolivar would be that associated with physical relocation of the filter media on-site. Ongoing maintenance costs would be expected to be nil and post-aeration of the rock filter effluent should not be required, especially considering that large volumes of wastewater would be sufficiently aerated during DAF/F treatment prior to reuse.

Duckweed pond systems are also known to be a relatively simple and inexpensive advanced treatment technology, with the costs of physical duckweed containment infrastructure and routine biomass harvest constituting the major capital outlays of the technology. For a duckweed pond system, the costs would be expected to be more than for a rock filter in spite of the negligible cost associated with obtaining and cultivating the necessary duckweed biomass stock. The majority of this cost would be expected to come from the large area of containment network that would be required to prevent wind-dispersal of the small floating plants within the expansive Bolivar ponds (see Plates 1.1 and 1.2). There are commercial systems available within Australia (e.g. Bio-Tech Waste Management Pty Ltd., Armidale, NSW) with prices per hectare in the order of AU\$120,000; however, no attempts were made here to perform the necessary cost-benefit analysis for such a system installation at Bolivar. Additionally, and since it would not be recommended that duckweed be applied to all ponds within the Bolivar network, the likelihood of unwanted transfer and proliferation of duckweed in unwanted locations would be very high. Whether or not this would pose a significant problem in ponds without a floating containment system is unclear; although it is considered likely that wind-dispersal would prevent the duckweed from establishing a complete surface coverage in unwanted areas. Ongoing maintenance costs of a duckweed pond would be expected to be negligible, since routine harvesting of the duckweed plant biomass would most likely not be performed. Should such a system be installed at Bolivar, future work could investigate the economic viability of a duckweed harvesting regime for protein (animal feed) and/or energy (biogas) production. Foreseeable future benefits could also include emissions off-setting from on-site energy production; although this would need more detailed investigations to assess its likely feasibility.

Finally, a horizontal-flow attached-growth media upgrade system, like that reported here, would almost certainly be the most expensive of the three upgrade methodologies investigated. Initial capital expenditure for obtaining the horizontal-flow AGM would be in the order of AU\$250 m<sup>-3</sup> and this figure would be in addition to the costs of engineering a suitable media containment system *in situ*. Based on an equivalent available rock volume of 25,000m<sup>3</sup>, the cost of installing a similar sized attached-growth media upgrade system would be substantial (see Section 10.1.1). Shin and Polprasert (1988) commented that although natural sloughing of attached-growth biomass did occur as part of normal AGWSP operation, it was envisaged that periodic removal and cleansing of AGM would be necessary. Whilst this would also be the case for a Bolivar-based AGM upgrade installation, it can be appreciated that this process of decommissioning and systematic cleaning would be significantly less painful, less costly and more easily managed for an AGM system than it would be for an equivalent volume rock filter. The light-weight polypropylene media could feasibly be removed from the pond with heavy machinery, be rinsed in some way to remove the accumulated sludge and then returned to the pond. The proposed relative ease with which this could be achieved would also be likely reduce the period of downtime required during cleaning operations (compared with a rock filter); something which may then go toward offsetting the higher cost of initial installation.

### **10.1.1 Operational scale-up factors for Bolivar**

Work presented in both Chapters 3 and 4 was obtained under hydraulic loading rates that were toward the ‘high-end’ relative to those reported in the relevant literature for equivalent WSP upgrade methodologies (i.e. maximum of 1.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>). At the same time, however, the expansive Bolivar WSP network is loaded at a greatly reduced HLR of around 0.035m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>. Given that an in-pond effluent upgrade system may not be expected to occupy large areas of the pond *in situ*, the carrying out of pilot-scale upgrade investigations under elevated HLRs allows for ‘high-flow’ performance assessments; something that may then enable the final upgrade systems to be as small as possible in terms of the pond surface area and/or volume occupied. In the case of the Bolivar WWTP, a smaller in-pond effluent upgrade system would effectively minimise the area of the WSPs not exposed to incident sunlight; something that is very important for

achieving optimal UV disinfection in the ponds prior to DAF/F treatment and horticultural reuse applications (Sweeney *et al.*, 2005a).

If all of the available 25,000m<sup>3</sup> of rock media was to be used for a Bolivar rock filter, the HLR of the filter would be in the order of 5.5m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> at the mean daily flow rate of 145ML d<sup>-1</sup>. Although Mara *et al.* (2001) tested rock filter hydraulic loading rates of up to 2.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> and obtained reasonable performance results using a significantly more concentrated wastewater at 8-fold greater SS and 15-fold higher BOD<sub>5</sub> mass loadings, it is unknown whether at an HLR of 5.5m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>, interstitial fluid velocities ( $\approx 21.4\text{m d}^{-1}$ ) would exceed that required for effective discrete settlement of suspended particulates. If a Bolivar rock filter was to be loaded at 2.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> (the maximum HLR reported in the literature), the rock media volume required would be  $\approx 72,000\text{m}^3$ ; three times the volume of rock media available on site. Based on an arbitrary value in the order of AU\$25 per tonne of rock media, the approximate material cost of a Bolivar rock filter would be in the order of AU\$1.2 million.

An AGM upgrade system of equal volume ( $\approx 72,000\text{m}^3$ ) would be loaded at a similar hydraulic loading as for a rock filter above (2.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup>); however, owing to the  $\approx 40\%$  greater void volume of the artificial media, it would be expected that an AGM system would only have to be approximately 60% the volume of an equivalent rock filter (i.e. 43,000m<sup>3</sup> or  $\approx 1\%$  of the total Bolivar WSP surface area) to achieve the same interstitial fluid velocities as an *in situ* rock filter. This HLR of 2.0m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> would be considered feasible for such an upgrade system at Bolivar, given the very short interstitial settling distance within the media void spaces ( $\approx 20\text{mm}$ ) and the characteristically low BOD<sub>5</sub> of the final WSP effluent. Based on a volume of 43,000m<sup>3</sup>, the approximate material cost of an in-pond AGM upgrade for the Bolivar WSP network would be in the order of AU\$10 million.

Based on the mean daily volumetric throughput of the Bolivar WSPs (145ML d<sup>-1</sup>) and an HLR of 0.73m<sup>3</sup> m<sup>-3</sup> d<sup>-1</sup> (as applied to performance assessments during Chapter 3), a Bolivar duckweed upgrade system would be expected to cover an area in the order of 16 ha (i.e.  $\approx 5\%$  of the total WSP area or roughly 25% the area of WSP number 3 situated adjacent to the experimental pilot plant site; see Plate 2.1). It would be



anticipated, however, that this figure could be cut by some 25% to approximately 12 ha, by the duckweed-covered pond region being loaded at an HLR of  $1.0\text{m}^3\text{ m}^{-3}\text{ d}^{-1}$ , putting the approximate material cost of a Bolivar duckweed cover in the range of AU\$1.5 million. While the above-proposed costs of upgrading the Bolivar pond network seem high, it must be remembered that the total WSP network volume is in the order of  $4.5\times 10^6\text{ m}^3$ , such that the relative pond area occupied by any of the above upgrade systems is between only 1–5% of the total lagoon system. Furthermore, potential annual cost savings from reduced DAF/F plant chlorine usage alone would be roughly AU\$430,000 and likely savings from reduced dosing of aluminium sulphate and chemical polymers would also be expected to also be in the hundreds of thousands per year. With this in mind then, and taking into consideration the potential for additional revenue coming from the production of a higher grade ‘Class A’ effluent, these large initial capital outlays could potentially be recovered in <5 years for a rock filter, <15 years for an AGM upgrade and approximately 3 years for a duckweed system.

### **10.2 Multiple installations of advanced WSP upgrades—a cumulative treatment effect?**

The idea of ‘advanced integrated’ WSP treatment systems was originally introduced to the field of wastewater treatment by Oswald and co-workers in the early 1990’s (Oswald, 1991). It describes a treatment series that brings together a number of well known and lesser known treatment processes in an optimal sequence (Green *et al.*, 1996). Whilst advanced integrated WSP systems have so far consisted of linking a number of discrete treatment processes in separate reactor basins (e.g. Green *et al.*, 1996; Tadesse *et al.*, 2004), it is suggested that there could also be potential for multiple installations of advanced in-pond upgrade methodologies within a single pond as a means of obtaining a ‘cumulative treatment effect’.

Given that both floating macrophyte systems and rock filters have each been the subject of extensive research, and have also been shown to be highly effective advanced in-pond upgrades in their own right, it seems a logical progression to propose that a combination of the two technologies may be more efficient than the performance of each system alone. One could feasibly envisage combining a duckweed pond system with rock

filtration to give a 'dual-stage' treatment step (i.e. an effluent rock filter to remove any duckweed SS/BOD<sub>5</sub> generated during plant biomass turnover). Incidentally, this concept of combining a macrophyte pond and rock filtration was actually proposed somewhat earlier by Dinges (1978) and later by Ellis (1983), where it was suggested that installing a rock filter at the outlet of water hyacinth and duckweed ponds could serve as a physical barrier to prevent plant 'escapees'. Ignoring these isolated instances, there have been almost no prior suggestions made within the relevant literature regarding the potential for cumulative treatment benefits in combined or integrated systems involving any combination of duckweed, rock filtration and attached-growth media. Tanner *et al.* (2005, p. 313) perhaps provide the only known reporting of such a suggestion, by concluding that a hybrid system between conventional maturation ponds and constructed wetlands, "*where wastewaters pass through alternating zones of wetland vegetation (20–30% of area) interspersed with extensive open-water areas*" may benefit overall aquatic community stability and treatment performance.

In a similar nature to the suggestions of Tanner *et al.* (2005), one could possibly imagine having rock beds or horizontal-flow attached-growth media arranged in a series of 'non-continuous-beds' along the pond length. This sort of staggered system configuration would allow for periodic re-exposure of the infiltrating effluent to sunlight and to a lesser extent to the prevailing wind; elements that may assist in the maintenance of aerobic conditions (for optimal BOD<sub>5</sub>/NH<sub>4</sub><sup>+</sup>-N removal) whilst at the same time maximising UV disinfection. As mentioned by Tanner *et al.* (2005), this sort of 'non-continuous-bed' type arrangement could also lead to the promotion of more optimal flow conditions *in situ* by acting as a sequence of permeable hydraulic baffles to minimise the extent of short-circuiting or recirculation within the pond. At the same time, having a rock filter or attached-growth media system arranged in a non-continuous-bed could help reduce the likelihood of non-ideal flow conditions within the upgrade system itself, given that other authors have reported on instances of hydraulic short-circuiting in full-scale rock filters (Hirse Korn, 1974; Swanson and Williamson, 1980; Middlebrooks, 1988).

In a similar vein to that above, an arrangement of non-continuous rock filter beds in particular, could also feasibly assist in the prevention of thermal stratification during

warmer weather; something that has been shown to affect flow hydraulics within the Bolivar ponds (Sweeney, 2004). Intermittent, but ultimately finite and controllable regions of open water in between the enclosed rock or AGM beds could also promote grazing activities by zooplankton in these illuminated zones—further reducing algal SS and maximising biological stabilisation potential. Observations made during the course of the current research suggest that high-density zooplankton populations can indeed develop within these intermittent open areas not occupied by rock media (i.e. pilot reactor mixing chambers; see Plate 2.8). The same concept could also be extended to having duckweed surface coverage in the intermittent ‘open zones’ should excessive zooplankton development become problematic.

Results from this thesis suggest that there could be real potential for some form of hybrid in-pond upgrade system at Bolivar, and with the proper execution of careful pre-planning and management protocols, such a system could offer significant potential for final WSP effluent polishing prior to DAF/F treatment.

### **10.3 Problems with in-pond effluent upgrades**

Several confounding factors with regard to in-pond upgrade technologies for the removal of algal SS were outlined by Middlebrooks *et al* (1974) and USEPA (1983).

These were:

1. The decay and microbial degradation of in-pond settled material, resulting in the release of dissolved BOD<sub>5</sub> which could then have an effect on receiving waters;
2. The possibility for resuspension of settled materials;
3. The lack of positive control of effluent SS;
4. The problem of eventually filling the pond; and
5. The possibility of anaerobic reactions within the settled material producing malodors.

With any chosen technology, there will always be a list of accompanying advantages and disadvantages, such that choosing one method over another is commonly a case of accepting the lesser of the evils. Furthermore, some of the above drawbacks specifically

associated with in-pond algal removal techniques will occur as part of conventional WSP operation. For example, Factor 1 would be expected to occur as part of normal pond function, albeit at a potentially lower rate than for in-pond based technologies. Some in-pond techniques may, however, be equipped to re-assimilate this dissolved BOD<sub>5</sub> into biomass for removal from the system, either by sedimentation and anaerobic digestion of sloughed aggregates (e.g. within a rock filter or AGM system) or as harvestable plant biomass (e.g. in duckweed ponds). Furthermore, disintegration of algal solids back into their dissolved inorganic forms would arguably be seen as successful effluent upgrading (at least in the eyes of Bolivar WWTP operators) such that the initial goal of effective algal SS removal from final pond effluents would still be satisfied.

Factor 2—the resuspension of settled particulates—would also be expected to take place in standard WSP systems, particularly in larger ponds where greater wind-fetch commonly results in wave-induced turbulent resuspension. In this instance, an in-pond technology such as AGM addition, a rock filter or floating macrophyte cover, would promote hydraulic quiescence, thereby enhancing physical sedimentation of suspended materials whilst at the same time reducing their potential for resuspension. Factor 2 would, therefore, not be of significant concern to prevent the above in-pond technologies from being considered as potential WSP upgrade solutions at Bolivar.

Factor 3 above is an inherent feature of all conventional WSP installations in the first instance and indeed this confounding factor of a “lack of positive control” over effluent quality simply serves to provide further backing for the use of advanced in-pond WSP upgrades—as was the case for this research. The fact that generic WSP systems have no existing means of positive effluent control is indeed the primary reason behind them periodically producing a poor quality final effluent. One can appreciate that any wastewater treatment facility already capable of installing and operating effective and economically-viable positive effluent control technologies to a level that meets their requirements, would—from the outset—not be a candidate of consideration for these relatively ‘low-tech’ in-pond solutions. It is suggested that so long as the chosen in-pond upgrades are carefully selected and properly implemented, they should be capable of serving as effective positive effluent control strategies.

Factor 4, the issue of eventually filling the pond, is once again an issue affecting all WSP systems, given that the serviceable life of any and every WSP is recognised as being finite (Lawty *et al.*, 1996). Periodic desludging (every few years according to Pearson, 1996) is an inevitable and widely recognised maintenance requirement of WSP technology. According to Sweeney (2004), primary facultative WSPs at the local Bolivar treatment plant are able to function well at desludging intervals in the order of 15 years. Incidentally, this figure given by Sweeney (2004) is very similar to the 15–20 year practical serviceable lifespan estimated for a full-scale Bolivar rock filter (see Section 4.3.8). The accumulation of organic solids in the sediments of a WSP is both an unavoidable phenomenon, and at the same time, a keystone treatment outcome. Physical solids retention is a primary performance outcome of WSP technology, whilst accumulated sediments represent a reservoir of organic substances that can either be anaerobically digested and released as methane from the pond surface and/or solubilised and recycled back into the water column to serve as substrates for further biological stabilisation (Saraiva *et al.*, 2005).

The final concern listed in Factor 5 above would only be an issue of suitable merit under conditions of high organic loading and specifically in metropolitan locations. In instances where the pond was far enough removed from urban municipalities, or indeed small enough not to generate sufficient quantities of undesirable gases, short-term pond anoxia may not be of significant concern. Depending on the chosen method and the degree of importance associated with pond odours, some abatement of short-term anaerobic conditions may actually be possible. For example, rock filters can be designed with aeration capabilities so that they may be aerated under conditions of high loading (Shelef and Azov, 2000; Johnson, 2005) to reduce H<sub>2</sub>S production. With respect to floating macrophytes, and as described previously (Section 1.2.8.5.2), a thick duckweed surface mat might actually be capable of minimising the release of both malodours (e.g. H<sub>2</sub>S) and greenhouse gases (e.g. CH<sub>4</sub>, N<sub>2</sub>O) from underlying waters by acting as a physical barrier against normal gas exchange processes at the water–air interface. In respect of the Bolivar system in particular, the organic loading applied to the tertiary-level ponds is sufficiently low such that anaerobiosis and the subsequent production of malodors would be much less of a concern, with performance data (Figures 3.10 and

4.6–4.8) suggesting that anoxic operation of an in-pond Bolivar upgrade system would be unlikely.

#### **10.4 WSP ecology—a management tool?**

Understanding and being able to interpret WSP biology and its ecological dynamics can not only do much for the tailoring of actual physical pond design aspects to suit particular needs and conditions, but is also vital for the efficient trouble-shooting of pond systems (Pearson, 1990). According to Pearson (1990) a sound knowledge of the biological principles behind WSP operation will also contribute significantly toward ensuring that WSP systems meet specific specialist requirements (such as that of a down-stream DAF/F plant, for example). Historically, pond design and management approaches have been largely empirically-based, resulting in frequent overloading and mismanagement of pond systems (Mitchell, 1980). Indeed it was the viewpoint of Mitchell (1980, p. 7) that “*The failure of pond effluents to meet current quality requirements is the result of mismanagement due to poor understanding of pond function, rather than limitations of ponds as a treatment technique.*” Based on the inherent complexities of WSP function, and combining them with our sub-optimal understanding of ecological pond ecology, Mitchell’s viewpoint of some 30 years ago seems hard to refute even by today’s standards.

Given that pond biology (and hence ecology) is the ‘backbone’ of the treatment framework, it seems plausible to suggest that any technique(s) capable of effectively managing—indeed controlling—pond ecology, could represent an efficient a means of enhancing or upgrading WSP performance. In essence, this is a similar concept to the more thoroughly researched food web “biomanipulation” approach of Shapiro *et al.* (1975) which has been widely implemented for the ecological management of eutrophication in other freshwater environments (most commonly lakes). This same ecological treatment philosophy led Craggs *et al.* (1996, p. 150) to suggest that “*The use of controlled ecology, promoting the mechanisms of self-purification in natural ecosystems, can provide efficient, cost effective and environmentally sound technologies for treating wastewaters.*”

As outlined in Chapter 1 (Section 1.3.1.2), several strategies to actively ‘biomanipulate’ the Bolivar WSPs were proposed as ecological management solutions to the operational issues plaguing the DAF/F plant. Results from this thesis suggest that there is existing potential at the Bolivar WWTP for the implementation of ecological ‘in-pond’ solutions to manipulate and ultimately control the algal and zooplankton load entering the DAF/F plant. Work presented in Chapter 5 suggests that biomanipulation of zooplankton populations in the WSP system would be more plausible than active manipulation of phytoplankton populations. Zooplankton appeared to be much more susceptible to the altered environmental conditions within each of the pilot-scale upgrade systems; although the somewhat truncated hydraulic retention period of the Bolivar wastewater within the pilot plant itself (1.5–3.5 days) possibly underestimated the true capacity of each upgrade system to alter the phytoplankton community structure.

Conceptually, the notoriously variable chemistry, hypertrophic status, very shallow depth and flow through nature of WSPs makes it difficult—if not impossible (Uhlmann, 1980)—to achieve a stable state of ecological operation. Permanent alterations to the physical environment (i.e. a reduction in particulate settlement depth and the exclusion of light and wind) achieved via duckweed shading, rock or artificial AGM addition could, however, offer a means of achieving a more permanent ‘biomanipulated’ ecological state. In line with the above views of Pearson (1990), only through a comprehensive understanding of the operational, environmental and biological parameters surrounding effective upgrade system performance can we hope to achieve effective, economically-viable and ultimately sustainable pond management and effluent control. Therefore, while this thesis has contributed toward achieving a more comprehensive understanding of WSP function under a range of altered conditions, more work is needed before controlled ecology can be adopted as a viable management approach.

### ***10.5 Wider applications of research***

Whilst work presented in this thesis has demonstrated the capacity for good treatment potential by each of the selected in-pond effluent upgrade technologies, it is important to keep the performance results of these natural treatment systems in the correct context.

As detailed in Chapter 1, the particular WSP upgrade methodologies investigated as part of this research are recognised to be relatively low-cost, low-intensity systems and as such cannot be directly compared to more highly engineered, tertiary- or quaternary-level treatment processes. To this end, and as highlighted by Green *et al.* (1996), regardless of the performance efficiency of ecological treatment technologies, they rely exclusively on natural treatment processes, and consequently, will never achieve the degree of algal removal and final effluent polishing achieved by other more intensive treatment interventions like DAF/F or sand filtration.

In spite of their inherently lower-level treatment capacity, it is important to look at the performance efficacy of in-pond systems in terms of their relative ‘cost–benefit’ ratio. Furthermore, and as was also outlined in Chapter 1 (Section 1.2.6), it may not be economically feasible for some communities to upgrade their WSP with more expensive and highly-engineered effluent polishing processes. In these instances, natural upgrade alternatives provide the most feasible option for WSP effluent compliance. This situation has particular local relevance for South Australia, where in addition to the large-scale centralised wastewater treatment operations that incorporate WSPs for final treatment (e.g. Bolivar WWTP), there are a significant number (>180) of smaller decentralised CWM treatment facilities also reliant upon WSP treatment to serve the needs of regional communities (see Section 1.3.2). Given that a number of more recently developed CWM schemes now recycle 100% of the treated effluent for purposes such as irrigation and wetland development, and considering that overall State wastewater reuse is currently in the order of 29% for South Australia (Fallowfield, 2008; pers. comm.), the upgrading of small-scale WSPs could offer additional cost benefits to operators by reducing the load on solids removal and disinfection processes—ultimately minimising capital outlays whilst maximising revenues from the production of a higher grade recycled effluent.

## **10.6 Final impact of thesis findings**

This thesis has incorporated the first parallel assessment of three discrete advanced in-pond technologies for WSP effluent upgrading. This unique side-by-side mode of experimental operation has allowed for more direct comparative insights into the relative



treatment efficiency of each upgrade methodology for the polishing of a final WSP effluent. This thesis, in addition to the more conventional water quality analyses, also applied a unique and quantitative *ecological* monitoring approach to pilot-scale performance assessments of each advanced WSP upgrade system; something so far unreported in the literature. Periodic assessment of both phyto- and zooplankton communities across all pilot-scale experimental treatments gave an extra applied dimension to the classical water quality data; something that both complemented and further enhanced the overall interpretation of wastewater treatment performance.

The laboratory work investigating algal dark-survival attempted to bring together the dichotomy of field- and laboratory-based research in an applied experimental setting. This part of the thesis used a powerful analytical tool (flow cytometry) to gain new and detailed physiological insights into the likely survival potential of common WSP algal species under environmental conditions designed to simulate those to which algal cells would be exposed during passage through an advanced in-pond upgrade process. Work presented in Chapter 8 highlighted the need for proper and detailed pre-assessments of algal cell staining protocols, and results from 9 also emphasized the importance of factoring in cell volume changes when interpreting cellular fluorescence signals from flow cytometric analyses. It is suggested that work from Chapter 9 also contributed toward the general interpretation and further understanding of light scatter signals in phytoplankton research involving FCM. Results from both the prolonged dark-survival experiments and also from the monitoring of plankton community dynamics within the candidate upgrade interventions have provided new insights into the operational ecology of these advanced wastewater treatment technologies. It is hoped that this information will not only contribute toward a greater overall understanding of treatment function in these upgrade systems, but will also go toward promoting a wider recognition of the importance of ecological monitoring approaches in WSP research.

## Appendices

### Appendix A. Previously published data from Chapter 3:

Short, M. D., Nixon, J. B., Cromar, N. J. and Fallowfield, H. J. (2007). Relative performance of duckweed ponds and rock filtration as advanced in-pond wastewater treatment processes for upgrading waste stabilisation pond effluent: a pilot study. *Water Science and Technology* **55**(11): 111–119.





















## Appendix B. Correlation matrices for pilot plant performance data—Chapter 3

**Table B.1.** Pilot plant influent correlation matrix table for a selection of the monitored physicochemical water quality performance parameters.

<i>Pilot plant influent</i>		DO (mg L <sup>-1</sup> )	Temperature (°C)	Sp. Cond. (μS cm <sup>-1</sup> )	pH	NH <sub>4</sub> <sup>+</sup> -N (mg L <sup>-1</sup> )	NO <sub>2</sub> <sup>-</sup> -N (mg L <sup>-1</sup> )	NO <sub>3</sub> <sup>-</sup> -N (mg L <sup>-1</sup> )	PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> )
DO (mg L <sup>-1</sup> )	Spearman $r_s$								
	α Sig. level (2-tailed)								
	<i>n</i>								
Temperature (°C)	Spearman $r_s$	0.110							
	α Sig. level (2-tailed)	0.542							
	<i>n</i>	33							
Sp. Cond. (μS cm <sup>-1</sup> )	Spearman $r_s$	0.212	.888(***)						
	α Sig. level (2-tailed)	0.235	0.000						
	<i>n</i>	33	33						
pH	Spearman $r_s$	.557(**)	0.319	.379(*)					
	α Sig. level (2-tailed)	0.001	0.070	0.030					
	<i>n</i>	33	33	33					
NH <sub>4</sub> <sup>+</sup> -N (mg L <sup>-1</sup> )	Spearman $r_s$	-.673(*)	-.610(*)	-.736(**)	-.647(*)				
	α Sig. level (2-tailed)	0.023	0.046	0.010	0.031				
	<i>n</i>	11	11	11	11				
NO <sub>2</sub> <sup>-</sup> -N (mg L <sup>-1</sup> )	Spearman $r_s$	-.986(***)	0.319	0.029	-0.580	0.203			
	α Sig. level (2-tailed)	0.000	0.538	0.957	0.228	0.700			
	<i>n</i>	6	6	6	6	6			
NO <sub>3</sub> <sup>-</sup> -N (mg L <sup>-1</sup> )	Spearman $r_s$	0.714	-0.086	0.086	0.771	-0.714	-0.754		
	α Sig. level (2-tailed)	0.111	0.872	0.872	0.072	0.111	0.084		
	<i>n</i>	6	6	6	6	6	6		
PO <sub>4</sub> <sup>3-</sup> -P (mg L <sup>-1</sup> )	Spearman $r_s$	0.667	-0.638	-0.406	.812(*)	-0.145	-0.721	0.725	
	α Sig. level (2-tailed)	0.148	0.173	0.425	0.050	0.784	0.106	0.103	
	<i>n</i>	6	6	6	6	6	6	6	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.2.** Duckweed pond treatment correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Duckweed pond train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.467(***) 0.000 90							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.564(***) 0.000 90	.914(***) 0.000 90						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.444(***) 0.000 90	.402(***) 0.000 90	.338(**) 0.001 90					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.044 0.861 18	0.069 0.785 18	-0.220 0.381 18	0.126 0.618 18				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.389 0.111 18	0.096 0.705 18	-0.150 0.553 18	-0.237 0.344 18	.831(***) 0.000 18			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.554(*) 0.017 18	-0.366 0.135 18	-0.053 0.836 18	.530(*) 0.024 18	-.488(*) 0.040 18	-.647(**) 0.004 18		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.028 0.913 18	-0.063 0.804 18	0.307 0.216 18	-0.087 0.732 18	-0.245 0.328 18	-0.282 0.257 18	-0.036 0.887 18	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.3.** Open pond treatment correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Open pond train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.166 0.101 99							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.148 0.145 99	.907(***) 0.000						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.904(***) 0.000 99	-.203(*) 0.044	-0.190 0.060 99					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.752(***) 0.000 18	0.280 0.261 18	0.146 0.565 18	-.740(***) 0.000 18				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.336 0.173 18	0.072 0.776 18	-0.273 0.274 18	-0.296 0.234 18	.674(**) 0.002 18			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.579(*) 0.012 18	-0.395 0.104 18	-0.098 0.699 18	.618(**) 0.006 18	-.690(**) 0.002 18	-.482(*) 0.043 18		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.240 0.336 18	-.529(*) 0.024 18	-0.203 0.418 18	0.346 0.159 18	-0.162 0.521 18	-0.200 0.425 18	0.096 0.705 18	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.4.** Rock filter treatment correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Rock filter train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.395(***) 0.000 99							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.300(**) 0.003 99	.876(***) 0.000 99						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.445(***) 0.000 99	.413(***) 0.000 99	.514(***) 0.000 99					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.219 0.383 18	0.138 0.584 18	-0.072 0.776 18	-0.102 0.686 18				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.307 0.216 18	0.268 0.281 18	-0.178 0.481 18	-0.300 0.227 18	.867(***) 0.000 18			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.595(**) 0.009 18	-0.193 0.443 18	0.168 0.505 18	.590(*) 0.010 18	-0.124 0.624 18	-0.374 0.127 18		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.389 0.111 18	-.488(*) 0.040 18	-0.152 0.548 18	0.318 0.198 18	-0.414 0.088 18	-.500(*) 0.035 18	0.292 0.240 18	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.5.** Pilot plant influent correlation matrix table for a selection of the monitored biophysical water quality performance parameters.

<i>Pilot plant influent</i>		SS (mg L <sup>-1</sup> )	Turbidity (NTU)	VSS (%)	BOD <sub>5</sub> (mg L <sup>-1</sup> )	Chlorophyll a (μg L <sup>-1</sup> )
SS (mg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
Turbidity (NTU)	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.982(***) 0.000 24				
VSS (%)	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.531(**) 0.008 24	-.475(*) 0.019 24			
BOD <sub>5</sub> (mg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.524(*) 0.045 15	.538(*) 0.039 15	-.527(*) 0.043 15		
Chlorophyll a (μg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.657(**) 0.001 21	.774(***) 0.000 21	0.187 0.417 21	0.081 0.782 14	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.6.** Duckweed treatment correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<i>Duckweed pond train</i>		SS (mg L <sup>-1</sup> )	Turbidity (NTU)	VSS (%)	BOD <sub>5</sub> (mg L <sup>-1</sup> )	Chlorophyll a (μg L <sup>-1</sup> )
SS (mg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
Turbidity (NTU)	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.865(***) 0.000 63				
VSS (%)	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.546(***) 0.000 72	-.482(***) 0.000 63			
BOD <sub>5</sub> (mg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	0.251 0.206 27	0.295 0.136 27	-0.144 0.473 27		
Chlorophyll a (μg L <sup>-1</sup> )	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.486(***) 0.000 54	.506(***) 0.000 54	-0.068 0.624 54	-0.126 0.548 25	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).



**Table B.7.** Open pond correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Open pond train</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (µg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.770(***) 0.000 72				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.316(**) 0.006 75	-.378(**) 0.001 72			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	0.167 0.323 37	0.102 0.548 37	0.047 0.784 37		
<b>Chlorophyll a (µg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.502(***) 0.000 63	.607(***) 0.000 63	0.056 0.664 63	0.109 0.533 35	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table B.8.** Rock filter correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Rock filter train</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (µg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.775(***) 0.000 75				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.497(***) 0.000 75	-.440(***) 0.000 75			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-0.050 0.778 34	0.099 0.577 34	-0.110 0.535 34		
<b>Chlorophyll a (µg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.652(***) 0.000 60	.530(***) 0.000 60	-0.110 0.404 60	0.018 0.922 32	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

## Appendix C. Correlation matrices for pilot plant performance data—Chapter 4

**Table C.1.** Pilot plant influent correlation matrix table for a selection of the monitored physicochemical water quality performance parameters.

<b>Pilot plant influent</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.054 0.755 36							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.165 0.360 33	.552(**) 0.001 33						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.142 0.423 34	.800(**) 0.000 34	.476(**) 0.005 33					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.132 0.699 11	-.633(*) 0.036 11	-.433 0.184 11	-0.538 0.088 11				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.564 0.071 11	0.382 0.247 11	0.464 0.151 11	0.518 0.102 11	0.150 0.659 11			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.427 0.190 11	-.809(**) 0.003 11	-.627(*) 0.039 11	-.636(*) 0.035 11	.911(***) 0.000 11	-0.155 0.650 11		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.152 0.676 10	-.685(*) 0.029 10	-.491 0.150 10	-.794(**) 0.006 10	.760(*) 0.011 10	-0.176 0.627 10	.733(*) 0.016 10	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.2.** Rock filter correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Rock filter train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.091 0.354 105							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.396(***) 0.000 99	.637(***) 0.000 99						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.043 0.670 99	.734(***) 0.000 99	.507(***) 0.000 99					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.213 0.233 33	-.620(***) 0.000 33	-.394(*) 0.023 33	-.362(*) 0.039 33				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.519(**) 0.002 33	.589(***) 0.000 33	.646(***) 0.000 33	.390(*) 0.025 33	-0.240 0.178 33			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.300 0.090 33	-.824(***) 0.000 33	-.584(***) 0.000 33	-.611(***) 0.000 33	.423(*) 0.014 33	-.534(**) 0.001 33		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.303 0.104 30	-.666(**) 0.000 30	-.689(***) 0.000 30	-.741(***) 0.000 30	.363(*) 0.048 30	-.594(**) 0.001 30	.756(***) 0.000 30	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.3.** Open pond treatment correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Open pond train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.403(***) 0.000 105							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.439(***) 0.000 98	.679(***) 0.000 98						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.562(***) 0.000 98	.789(***) 0.000 98	.702(***) 0.000 98					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.489(**) 0.004 33	-.704(***) 0.000 33	-.741(***) 0.000 33	-.844(***) 0.000 33				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.117 0.516 33	0.188 0.296 33	.390(*) 0.025 33	0.325 0.065 33	-0.081 0.654 33			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.335 0.057 33	-.881(***) 0.000 33	-.654(***) 0.000 33	-.717(***) 0.000 33	.739(***) 0.000 33	-0.133 0.461 33		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.484(**) 0.007 30	-.819(***) 0.000 30	-.608(***) 0.000 30	-.818(***) 0.000 30	.836(***) 0.000 30	-0.155 0.413 30	.811(***) 0.000 30	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.4.** Attached-growth media treatment correlation matrix table for a selection of the monitored physicochemical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Attached-growth media train</b>		<b>DO (mg L<sup>-1</sup>)</b>	<b>Temperature (°C)</b>	<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	<b>pH</b>	<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>
<b>DO (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$								
<b>Temperature (°C)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.514(***) 0.000 102							
<b>Sp. Cond. (μS cm<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.365(***) 0.000 96	.624(***) 0.000 96						
<b>pH</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.461(***) 0.000 96	.841(***) 0.000 96	.590(***) 0.000 96					
<b>NH<sub>4</sub><sup>+</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.149 0.406 33	-.533(***) 0.001 33	-0.164 0.363 33	-.486(**) 0.004 33				
<b>NO<sub>2</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.052 0.781 31	0.202 0.276 31	.553(**) 0.001 31	0.104 0.577 31	-0.114 0.541 31			
<b>NO<sub>3</sub><sup>-</sup>-N (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.136 0.452 33	-.896(***) 0.000 33	-.645(***) 0.000 33	-.694(***) 0.000 33	.371(*) 0.034 33	-0.251 0.173 31		
<b>PO<sub>4</sub><sup>3-</sup>-P (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-.395(*) 0.031 30	-.822(***) 0.000 30	-.636(***) 0.000 30	-.804(***) 0.000 30	.392(*) 0.032 30	-0.233 0.233 28	.805(***) 0.000 30	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);

\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.5.** Pilot plant influent correlation matrix table for a selection of the monitored biophysical water quality performance parameters.

<b>Pilot plant influent</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (μg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.842(***) 0.000 36				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.411(*) 0.013 36	-0.301 0.074 36			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	0.302 0.172 22	-0.021 0.926 22	-.614(***) 0.002 22		
<b>Chlorophyll a (μg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.760(***) 0.000 36	.741(***) 0.000 37	-0.062 0.719 36	-0.085 0.707 22	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.6.** Rock filter correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Rock filter train</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (μg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.530(***) 0.000 105				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	-.329(**) 0.001 103	-.233(*) 0.018 103			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.258(*) 0.037 66	0.115 0.360 66	0.010 0.939 64		
<b>Chlorophyll a (μg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) <i>n</i>	.488(***) 0.000 105	.489(***) 0.000 108	-0.062 0.533 103	-0.015 0.908 66	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.7.** Open pond correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Open pond train</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (µg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.692(***) 0.000 105				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.115 0.244 105	0.080 0.415 105			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	0.039 0.760 64	-.250(*) 0.046 64	-0.165 0.192 64		
<b>Chlorophyll a (µg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.496(***) 0.000 105	.660(***) 0.000 108	.195(*) 0.046 105	-0.193 0.126 64	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Table C.8.** Attached-growth media treatment correlation matrix table for a selection of the monitored biophysical water quality performance parameters (data pooled from all ponds in each treatment series).

<b>Attached-growth media train</b>		<b>SS (mg L<sup>-1</sup>)</b>	<b>Turbidity (NTU)</b>	<b>VSS (%)</b>	<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	<b>Chlorophyll a (µg L<sup>-1</sup>)</b>
<b>SS (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$					
<b>Turbidity (NTU)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.552(***) 0.000 102				
<b>VSS (%)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.111 0.267 101	0.122 0.225 101			
<b>BOD<sub>5</sub> (mg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	-0.078 0.554 60	-0.172 0.189 60	-.440(***) 0.000 59		
<b>Chlorophyll a (µg L<sup>-1</sup>)</b>	Spearman $r_s$ α Sig. level (2-tailed) $n$	.399(***) 0.000 102	.433(***) 0.000 105	0.046 0.649 101	-0.091 0.489 60	

\* Correlation is significant at the  $p < 0.05$  level (2-tailed); \*\* Correlation is significant at the  $p < 0.01$  level (2-tailed);  
\*\*\* Correlation is significant at the  $p < 0.001$  level (2-tailed).

**Appendix D. Zooplankton taxa most commonly observed during pilot plant operation: 2005–2006.**

**D.1. Rotifera.**



*Brachionus novaezealandiae* (Morris, 1912; adult).



*Brachionus novaezealandiae* (Morris, 1912; hatchling).



*Brachionus quadridentatus* Hermann, 1783.





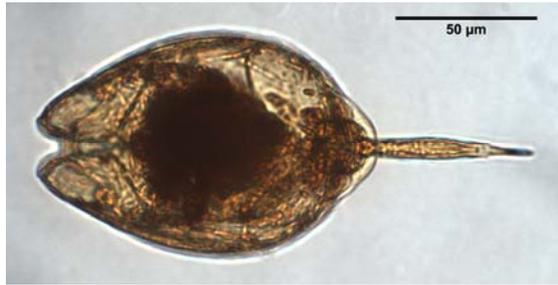
*Keratella australis* (Berzins, 1963).



*Keratella procurva* (Thorpe, 1891).



*Keratella slacki* (Berzins, 1963).



*Lecane bulla* Gosse, 1951.



*Lecane ludwigii* (Eckstein, 1883).



Unidentified Bdelloid rotifer (Bdelloidea; Habrotrochidae?).

## D.2. Cladocera.



*Daphnia carinata* King, 1853 s.l.



Daphniidae – *Simocephalus* species (juvenile).



*Moina micrura* cf. Kurz, 1874.



Chydoridae – *Pleuroxus* species.

### **D.3. Copepoda.**



*Boeckella triarticulatu* (Thompson, 1883).



*Mesocyclops cf. nothius* Kiefer, 1981.

#### D.4. Ostracoda.



*Bennelongia cf. barangaroo* De Dekker, 1981.

**Appendix E. Mean zooplankton body lengths, length–weight regression equations and biomass estimates for the dominant taxa observed from July 2005–August 2006. Individual dry weights were estimated either from length–weight equations or published biomass values of individuals from the same genus or species.**

<b>Taxon</b>	<b>Mean length (<math>\mu\text{m}</math>)</b>	<b>Length–weight equation (&amp; units)</b>	<b>Individual dry weight (<math>\mu\text{g}</math>)</b>
<b>Cladocera</b>			
<i>Daphnia carinata</i>	1800	$\ln W(\mu\text{g}) = 1.3877 + 2.8335 \times \ln L(\text{mm})$	21.20 <sup>B</sup>
<i>Moina micrura</i>	1000	$W(\mu\text{g}) = 6.61 L(\text{mm})^{2.57}$	6.61 <sup>A</sup>
<i>Plexorus</i> sp.	500	$W(\mu\text{g}) = 35.6 L(\text{mm})^{4.03}$	2.18 <sup>A</sup>
<i>Simocephalus</i> sp.	1100	$W(\mu\text{g}) = 4.00 L(\text{mm})^{3.81}$	5.75 <sup>A</sup>
<b>Copepoda</b>			
<i>Boeckella triarticulata</i>	1500	$W(\mu\text{g}) = 7.9 \times 10^{-7} L(\mu\text{m})^{2.33}$	19.86 <sup>A</sup>
<i>Mesocyclops nothius</i>	900	$W(\mu\text{g}) = 4.9 \times 10^{-8} L(\mu\text{m})^{2.75}$	6.52 <sup>A</sup>
Nauplii	160	$W(\mu\text{g}) = 1.1 \times 10^{-5} L(\mu\text{m})^{1.89}$	0.16 <sup>A</sup>
<b>Ostracoda</b>			
<i>Bennelongia barangaroo</i>	1300	-	25 <sup>C</sup>
<b>Rotifera</b>			
<i>Brachionus</i> sp.	-	-	0.42 <sup>A,D</sup>
<i>Keratella australis</i>	-	-	0.58 <sup>D</sup>
<i>Keratella procurva</i>	-	-	0.21 <sup>D</sup>
<i>Keratella slacki</i>	-	-	0.21 <sup>D</sup>
<i>Lecane bulla</i>	-	-	0.005 <sup>D</sup>
<i>Lecane ludwigii</i>	-	-	0.005 <sup>D</sup>
Unidentified bdelloid rotifer	-	-	0.25 <sup>D</sup>

<sup>A</sup>Dumont *et al.* (1975); <sup>B</sup>Mitchell and Williams (1982b); <sup>C</sup>Ikeda (1990); <sup>D</sup>Kobayashi *et al.* (1996).

**Appendix F. Executive summary of the existing phytoplankton dark-survival literature (taxonomic classifications sourced from AlgaeBase v.3.0 <http://www.algaebase.org> as at 30/06/2007).**

**Prokaryotes**

**Bacteria**

**Cyanobacteria**

<i>Phlum</i>	<i>Class</i>	<i>Order</i>	<i>Genus (and investigator)</i>
Cyanobacteria	Not assigned	Chroococcales Oscillatoriales	Agmenellum <sup>7,15</sup> , Anacystis <sup>7,15</sup> , Microcystis <sup>56</sup> , Synechococcus <sup>34,41</sup> Oscillatoria <sup>25</sup> , Phormidium <sup>59,60</sup>

**Eukaryotes**

**Chromista**

**Phytoplankton**

<i>Phlum</i>	<i>Class</i>	<i>Order</i>	<i>Genus (and investigator)</i>	
Cryptophyta	Cryptophyceae	Cryptomonadales	Cryptomonas <sup>14,40</sup> , Hemiselmis <sup>7,15</sup>	
		Pyrenomonadales	Chroomonas <sup>7,15</sup> , Rhodomonas <sup>7,15</sup>	
Haptophyta	Haptophyceae	Isochrysidales	Hymenomonas <sup>24</sup> , Isochrysis <sup>7,15</sup>	
		Pavlovales	Pavlova <sup>15,45</sup>	
		Prymnesiales	Chrysochromulina <sup>45</sup> , Emiliana <sup>15,51</sup> , Prymnesium <sup>7,15,45</sup>	
	Prymnesiophyceae	Coccolithophoridales	Coccolithus <sup>7,15,27</sup>	
Ochrophyta	Coscinodiscophyceae	Anaulales	Anaulus <sup>33</sup>	
		Chaetocerotales	Bacteriastrum <sup>45</sup> , Chaetoceros <sup>7,9,10,15,46,53</sup>	
		Coscinodiscales	Coscinosidcus <sup>11</sup>	
		Cymatosirales	Bellerochea <sup>15</sup>	
		Fragilariales	Asterionella <sup>10</sup> , Fragilaria <sup>9,15,36</sup>	
		Lithodesmidales	Ditylum <sup>10,27,37</sup> , Lithodesmium <sup>10</sup>	
		Meloseirales	Melosira <sup>7,56</sup> , Stephanopyxis <sup>10</sup>	
		Rhizosoleniales	Proboscia <sup>38</sup> , Rhizosolenia <sup>8,37</sup>	
		Synurophyceae	Monochrysis <sup>7,15</sup>	
		Thalassiosirales	Bacteriosira <sup>53</sup> , Cyclotella <sup>7,11,15</sup> , Porosira <sup>38</sup> , Skeletonema <sup>7,10,15,46</sup> , Thalassiosira <sup>7,10,15,24,34,37,38,39,41,42,46</sup>	
		Chrysophyceae	Chromalinales	Actinomonas <sup>53</sup>
		Pelagophyceae	Pelagomonadales	Aureococcus <sup>50,62</sup>
		Xanthophyceae	Mischococcales	Monallantus <sup>15</sup>
			Tribonematales	Heterothrix <sup>15</sup> , Xanthonema <sup>57</sup>

## Eukaryotes

### *Plantae*

#### Phytoplankton

<i>Phylum</i>	<i>Class</i>	<i>Order</i>	<i>Genus (and investigator)</i>
Bacillariophyta	Bacillariophyceae	Acnanthales	<i>Achnanthes</i> <sup>7,15</sup>
		Bacillariales	<i>Cylindrotheca</i> <sup>7,15,48</sup> , <i>Fragilariopsis</i> <sup>38</sup> , <i>Nitzschia</i> <sup>7,15,19,23,48,53</sup> , <i>Phaeodactylum</i> <sup>7,15</sup>
		Naviculales	<i>Amphiprora</i> <sup>7,15</sup> , <i>Navicula</i> <sup>7,15,19,48,53</sup>
		Surirellales	<i>Surirella</i> <sup>48</sup>
		Thalassiophysales	<i>Amphora</i> <sup>12</sup>
Chlorophyta	Chlorophyceae	Chlorococcales	<i>Chlorella</i> <sup>1,2,4,5,54,58</sup> , <i>Nannochloris</i> <sup>15</sup> , <i>Scenedesmus</i> <sup>16,17,18,31,36,54,56,58</sup>
	Trebouxiophyceae	Volvocales	<i>Brachiomonas</i> <sup>7,15,45</sup> , <i>Pandorina</i> <sup>13</sup>
		Microthamniales	<i>Koliella</i> <sup>61</sup>
	Ulvophyceae	Dasycladales	† <i>Acetabularia</i> <sup>30</sup>
		Ulvales	‡ <i>Ulva</i> <sup>26</sup>
Prasinophyta	Praisinophyceae	Dunaliellales	<i>Dunaliella</i> <sup>3,6,7,15,44,52</sup> , <i>Tetraselmis</i> <sup>7,15,35</sup>
		Chlorodendrales	<i>Prasinocladus</i> <sup>15</sup>
		n/a	Ω48-23 (unidentified) <sup>32</sup>
Rhodophyta	Bangiophyceae	Bangiales	‡ <i>Porphyra</i> <sup>20</sup>
	Florideophyceae	Batrachospermales	‡ <i>Batrachospermum</i> <sup>21</sup>
		Ceramiales	‡ <i>Delesseria</i> <sup>28</sup>
		Gigartinales	‡ <i>Iridaea</i> <sup>43</sup>
		Palmariales	‡ <i>Palmaria</i> <sup>43,49</sup>
	Rhodellophyceae	Porphyridiales	<i>Porphyridium</i> <sup>7,15</sup> , <i>Rhodella</i> <sup>15</sup>

## Eukaryotes

### *Protozoa*

#### Phytoplankton

<i>Phylum</i>	<i>Class</i>	<i>Order</i>	<i>Genus (and investigator)</i>
Dinophyta	Dinophyceae	Gonyaulacales	<i>Alexandrium</i> <sup>29,51</sup>
		Gymnodiniales	<i>Amphidinium</i> <sup>7,22,55</sup> , <i>Gymnodinium</i> <sup>29,22</sup>
		Peridinales	<i>Ensiculifera</i> <sup>27</sup> , <i>Gonyaulax</i> <sup>27</sup> , <i>Scrippsiella</i> <sup>22,27,46</sup> , <i>Thoracosphaera</i> <sup>27</sup>
		Prorocentrales	<i>Prorocentrum</i> <sup>22,29,47</sup>



\* The original sources for the information contained within this table are referenced according to the corresponding numerical superscripts above as follows:

<sup>1</sup>Finkle *et al.* (1950); <sup>2</sup>Killiam and Myers (1956); <sup>3</sup>Yentsch and Reichert (1963); <sup>4</sup>Karlander and Krauss (1966a); <sup>5</sup>Karlander and Krauss (1966b); <sup>6</sup>Hellebust and Terborgh (1967); <sup>7</sup>Anita and Cheng (1970); <sup>8</sup>Ignatiades and Smayda (1970); <sup>9</sup>Bunt and Lee (1972); <sup>10</sup>Smayda and Mitchell-Innes (1974); <sup>11</sup>White (1974); <sup>12</sup>Anderson (1975); <sup>13</sup>Coleman (1975); <sup>14</sup>Morgan and Kalff (1975); <sup>15</sup>Anita (1976); <sup>16</sup>Kulandaivelu and Senger (1976a); <sup>17</sup>Kulandaivelu and Senger (1976b); <sup>18</sup>Kulandaivelu and Senger (1976c); <sup>19</sup>Moss (1977); <sup>20</sup>Sheath *et al.* (1977); <sup>21</sup>Sheath *et al.* (1979); <sup>22</sup>Richardson and Fogg (1982); <sup>23</sup>Palmisano and Sullivan (1983); <sup>24</sup>Vaulot *et al.* (1986); <sup>25</sup>Richardson and Castenholz (1987); <sup>26</sup>Vermaat and Sand-Jensen (1987); <sup>27</sup>Griffis and Chapman (1988); <sup>28</sup>Lüning and Schmitz (1988); <sup>29</sup>Selvin *et al.* (1988/89); <sup>30</sup>Dazy *et al.* (1989); <sup>31</sup>Dehning and Tilzer (1989); <sup>32</sup>Dorsey *et al.* (1989); <sup>33</sup>du Preez and Bate (1992); <sup>34</sup>Harvey *et al.* (1995); <sup>35</sup>Montaini *et al.* (1995); <sup>36</sup>Deventer and Heckman (1996); <sup>37</sup>Peters (1996); <sup>38</sup>Peters and Thomas (1996a); <sup>39</sup>Peters and Thomas (1996b); <sup>40</sup>Gervais (1997); <sup>41</sup>Harvey and Macko (1997); <sup>42</sup>Murphy and Cowles (1997); <sup>43</sup>Wekyam *et al.* (1997); <sup>44</sup>Berges and Falkowski (1998); <sup>45</sup>Jochem (1999); <sup>46</sup>Lewis *et al.* (1999); <sup>47</sup>Manoharan *et al.* (1999); <sup>48</sup>Smith and Underwood (2000); <sup>49</sup>Lüder *et al.* (2002); <sup>50</sup>Popels and Hutchins (2002); <sup>51</sup>Wolfe *et al.* (2002); <sup>52</sup>Segovia *et al.* (2003); <sup>53</sup>Qing *et al.* (2003); <sup>54</sup>Bartosh and Banks (2004); <sup>55</sup>Franklin and Berges (2004); <sup>56</sup>Furusato *et al.* (2004); <sup>57</sup>Baldisserotto *et al.* (2005); <sup>58</sup>Montechiaro *et al.* (2006); <sup>59</sup>Montechiaro and Giordano (2006); <sup>60</sup>Bartosh and Banks (2007); <sup>61</sup>Ferroni *et al.* (2007); <sup>62</sup>Popels *et al.* (2007).

† Denotes unicellular macrophytoplankton species (≥2mm)

‡ Denotes multicellular macroalgal species

**Appendix G. Chemical constituents of the modified Woods Hole MBL growth medium (modified from Nichols, 1973).**

<b>Chemical component</b>	<b>Concentration (mg L<sup>-1</sup>)</b>
CaCl <sub>2</sub> ·2H <sub>2</sub> O	36.76
MgSO <sub>4</sub> ·7H <sub>2</sub> O	36.97
NaHCO <sub>3</sub>	12.6
K <sub>2</sub> HPO <sub>4</sub>	8.71
NaNO <sub>3</sub>	85.01
Na <sub>2</sub> EDTA	4.36
FeCl <sub>3</sub> ·6H <sub>2</sub> O	3.15
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.01
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.022
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.01
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.18
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.006
NH <sub>4</sub> Cl	5.40
Tris[hydroxymethyl]aminomethane	500
Cyanocobalamin (Vitamin B12)	2.5x10 <sup>-7</sup>
Thiamine HCl (Vitamin B1)	5x10 <sup>-5</sup>
Biotin	2.5x10 <sup>-7</sup>

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