Use of microcosm and in-situ studies for the estimation of exposure risk from recreational coastal waters and sediments

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SUMMARY

The interaction of microorganisms with sediments can enhance their survival by reducing exposure to various stressors and thus marine sediments may act as reservoirs for pathogenic microorganisms. In coastal waters there can be an increased risk of infection to humans due to the possible re-suspension of these microorganisms during recreational activities. This research attempts to more accurately identify environmental exposure in the first stage of a health risk assessment in recreational coastal waters.

Techniques were developed to successfully separate microorganisms from sediment particles. Of the methods investigated, subjecting diluted sediment samples to a sonication bath for 10 minutes was found to be the most efficient separation technique over a range of sediment types. This method was therefore used in the subsequent studies to enumerate organisms from the surface sediment layer, as distinct from the water column.

Faecal coliforms were enumerated by membrane filtration in both water and sediment from three Adelaide metropolitan recreational coastal sites, chosen to represent different physical sediment characteristics, over a 12-month period. All sites investigated met current National Health and Medical Research Council Guidelines for primary contact recreation. Faecal coliform concentrations were generally greater in sediment compared with overlying water for all samples. This was most evident in sediment consisting of greater silt/clay and organic carbon content (with up to 1000 times higher concentrations in the surface sediment layer compared with overlying water). For coastal recreational sites impacted by stormwater or river discharges, high faecal coliform concentrations were found to be associated with rainfall.

A laboratory-based microcosm study utilising intact sediment cores was undertaken to determine the decay rates of faecal indicator organisms (*E. coli*, enterococci and somatic coliphage) and pathogens (*Salmonella derby* and *S.*

typhimurium) in both overlying water and in various sediment types. For all organisms tested, temperature had an inverse relationship with survival. Greater decay was observed in the overlying water compared to the surface sediment layer. Small particle size and high organic carbon content was found to be more conducive to microbial survival. In general, decay rates of *E. coli* were significantly greater than enterococci and coliphage. Although no significant correlations were observed between decay rates of the pathogens and indicator organisms, decay of *Salmonella* spp. in overlying water more closely resembled that of *E. coli* than that of other indicators.

Using decay rates measured in the microcosm study and available dose-response data, a quantitative microbial risk assessment (QMRA) utilising Monte Carlo simulation was undertaken to estimate the risk of infection to *Salmonella* spp. and rotavirus following exposure to recreational coastal water subject to a range of faecal contamination levels. For modelling purposes, the assumption was made that rotavirus decay was equivalent to coliphage decay. The probability of infection from rotavirus due to exposure to contaminated recreational coastal water was greater than that for *Salmonella* spp. under all scenarios. This increased probability of infection is linked to the high infectivity of rotavirus compared to *Salmonella* spp.

Results of this research highlight the limited effectiveness of using prescribed faecal coliform concentrations in the water column alone to estimate the risk of exposure to pathogenic microorganisms during recreational activity at coastal areas. It demonstrated that coastal sediments act as a reservoir for both indicator and pathogenic organisms released into the coastal environment. This suggests an increased exposure risk if these organisms are resuspended back into the water column during recreational activity. A combined risk-based monitoring program would provide a more robust and reliable estimate of health risk associated with coastal recreational areas.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma at any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Duncan Craig

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ABBREVIATIONS

AFR	Accidental faecal release
AFRI	Acute febrile respiratory illness
AGI	Acute gastrointestinal illness
ANOVA	Analysis of variance
APHA	American Public Health Association
CFU	Colony forming units
d	Days
DVC	Direct viable count
FISH	Fluorescent in-situ hybridisation
FITC	Fluorescein isothiocyanate
h	Hours
k	Decay rate
min	Minutes
MPN	Most probable number
N_0	Initial number of bacteria
N_t	Bacterial survival after time t
NHMRC	National Health and Medical Research Council
NLV	Norwalk-like virus
P	Significance compared to control
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
QMRA	Quantitative microbial risk assessment
rev	Revolutions
SD	Standard deviation
s	Seconds
TDS	Total dissolved solids
UV	Ultra violet
VBNC	Viable but not culturable
WHO	World Health Organisation
WWTP	Wastewater treatment plant

CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Potential exposure to human pathogens in recreational coastal waters

In any recreational water both pathogenic and non-pathogenic microorganisms are present. The source of these microorganisms may originate from sewage effluent, native fauna, livestock, industry and even bathers themselves via accidental faecal release (AFR) and/or shedding. Indigenous microorganisms are also present. The potential health hazard associated with exposure to these microorganisms is dependent on a number of factors including their infectivity to humans and the number of microorganisms to which an individual is exposed. Exposure can occur via ingestion of water, inhalation or contact with ears, nasal passage, mucous membranes, or through cuts in the skin (WHO, 2001).

Illnesses associated with recreational contact with contaminated water were, until recently, thought to be primarily enteric diseases such as gastroenteritis. It has been identified, however, that non-enteric illnesses are also important and under certain circumstances may be more prevalent than gastroenteritis (Fleisher *et al.*, 1996). In most circumstances, ill-health effects from exposure to contaminated water are relatively minor and self-limiting. There is a potential health risk, however, for more serious diseases such as hepatitis A and amoebic meningoencephalitis (Cabanes *et al.*, 2001; Gammie and Wyn-Jones, 1997).

Very little information is available on the prevalence of waterborne disease associated with recreational water in Australia. From United States data it is apparent that the most common adverse health outcome following contact with faecally contaminated recreational water (from bacteria, viruses and for parasites) is gastroenteritis (Table 1.1). The majority of reported waterborne disease outbreaks have been associated with recreational contact with polluted freshwaters.

Table 1.1 Outbreaks associated with exposure to fresh and marine recreational water (non-treated, natural water bodies) in the USA, 1991-2000 (Lee *et al.*, 2002; Barwick *et al.*, 2000; Levy *et al.*, 1998; Kramer *et al.*, 1996; Moore *et al.*, 1993b).

Etiological Agent	**Illness**	**Number of Cases**	**Number of Outbreaks**
Giardia	Gastroenteritis	65	4
Shigella flexneri	Gastroenteritis	277	2
Cryptosporidium	Gastroenteritis	424	3
E. coli O157:H7	Gastroenteritis	326	10
E. coli O121:H19	Gastroenteritis	11	1
Shigella sonnei	Gastroenteritis	616	8
Naegleria	Meningoencephalitis	14	14
NLV	Gastroenteritis	216	3
Leptospira	Leptospirosis	381	2
Schistosoma sp.	Dermatitis	165	7
Adenovirus 3	Pharyngitis	595	1
AGI	Gastroenteritis	1082	12
	Total	**4393**	**68**

NLV=norwalk like virus
AGI=acute gastrointestinal illness of unknown etiology

Epidemiological studies

Epidemiological studies may be used to establish a causal relationship between health-risk and exposure to faecally contaminated recreational sites. Since the early 1980s studies of various design have been undertaken to attempt to establish this relationship for exposure to marine waters, and to a lesser extent fresh waters (Dufour, 1984). Depending on the study type/design, epidemiological studies are susceptible to many biases (WHO, 1998). Prüss (1998) performed a detailed review of epidemiological studies and concluded that 19 of 22 selected studies established significant relationships between rates of certain symptoms and faecal indicator organism concentrations in the recreational water body. Again the most commonly reported adverse health outcome was gastroenteritis.

Of all the epidemiological studies undertaken, it could be argued that the randomised controlled trials of Kay *et al*. (1994) and Fleisher *et al*. (1996) are the most rigorous and well defined. The major benefits of randomised controlled trials over other study designs are that exposure can be more accurately determined (ie faecal indicator organism concentration at time of exposure) and similarities between exposed and non-exposed groups can be maximised (Prüss, 1998). The study by Kay *et al*. (1994) compared the increased risk of gastroenteritis for individuals exposed to seawater (head immersion; n = 548) to that of non-exposed individuals (n = 668). Total coliforms, faecal coliforms, faecal streptococci, total streptococci and *Pseudomonas aeruginosa* concentrations were measured at time of exposure. Of the microorganisms analysed, a significant exposure-response relationship was only identified between faecal streptococci concentration and gastroenteritis. As noted by the authors, this does not mean that faecal streptococci was the cause of symptoms in the exposed population but they may be a better indicator of the presence of pathogenic microorganisms than the traditional faecal coliform bacteria. A threshold of 32 faecal streptococci 100 mL^{-1} was identified for increased risk of gastroenteritis.

Wyer *et al*. (1999) compared the increased risk of gastroenteritis from swimming in faecally contaminated marine waters with that of non-water related risk factors and person-to-person transmission (PPT) (Figure 1.1). Using the exposure-

response relationship identified by Kay *et al*. (1994) it was estimated that the risk of gastroenteritis from bathing in marine waters with a concentration of below 32 faecal streptococci 100 mL^{-1} was equivalent to not bathing (ie no increased risk). The associated risk then increased until a concentration of 158 faecal streptococci 100 mL^{-1} was reached. This was the highest exposure measured in the Kay *et al*. (1994) study. The model was not extrapolated beyond this concentration of faecal streptococci and it was assumed (conservatively) that the probability of developing gastroenteritis remained constant above this value.

Fleisher *et al*. (1996) investigated the relationship between marine water quality and increased risk of acute febrile respiratory illness (AFRI), and eye, ear and skin ailments using the same study population, design and results as Kay *et al*. (1994). A significant exposure-response relationship was identified between water faecal streptococci concentration and increased risk of acquiring AFRI. A threshold of 60 faecal streptococci was estimated for increased risk of AFRI. For ear ailments, however, a exposure-response relationship was identified with faecal coliform concentration. No other relationships were identified for the other illnesses.

Limitations of these epidemiological studies include the use of healthy adults volunteers (> 18 years of age), and they may therefore not be representative of susceptible individuals (the very young, old or immuno-compromised). To include individuals from these groups in a randomised controlled trial would be very difficult due to ethical considerations (ie exposure to recreational waters known to be faecally polluted). Also, the ability to detect increased risk of illness at faecal indicator organism concentrations below the identified thresholds may be limited due to lack of sufficient statistical power of the studies. In addition, given the known prevalence of disease associated with freshwaters, a convincing argument could be presented for the need to perform a randomised controlled trial at a freshwater recreational location. However, despite these limitations, the two randomised controlled studies of Kay *et al*. (1994) and Fleisher *et al*. (1996) provide the most accurate estimates of increased risk of illness due to exposure to faecally contaminated recreational water to date.

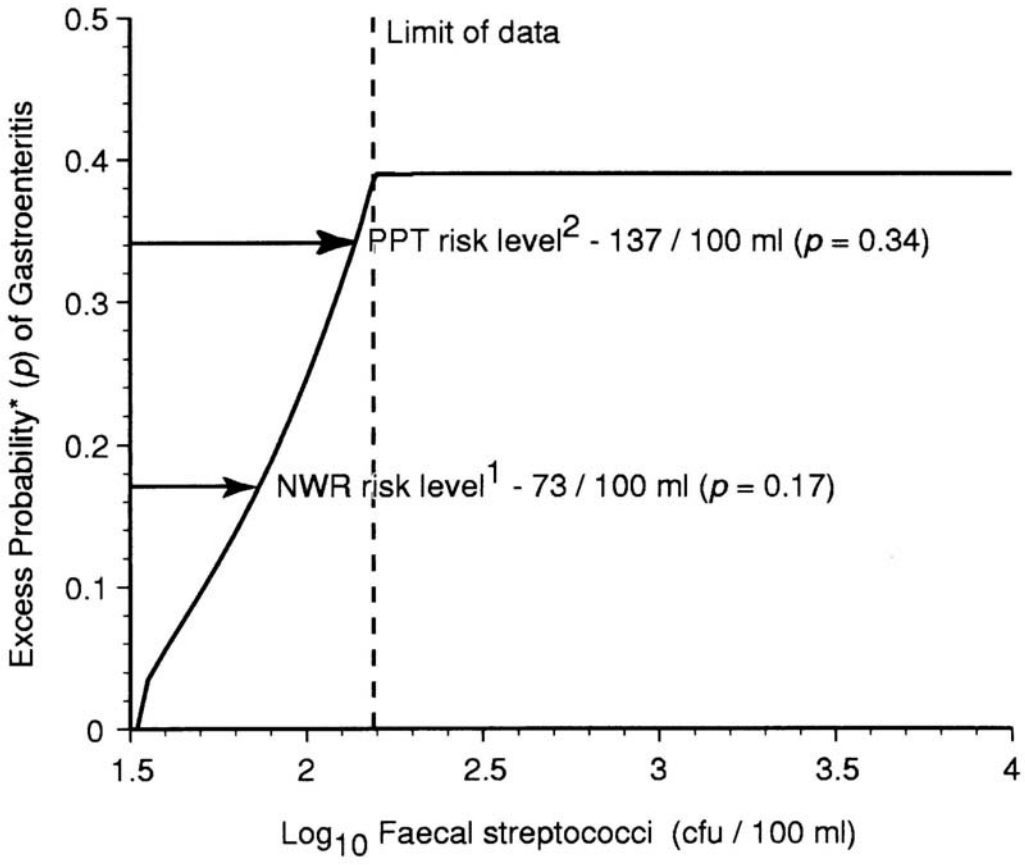

Figure 1.1 Relationship between exposure to faecal streptococci (CFU/100mL) and the probability (*p*) of developing gastroenteritis (Wyer *et al*. 1999 – adapted from Kay *et al*. 1994)

[1]Non water related risk factor – equivalent to everyday activities

[2]Person to person transmission factor – equivalent to contact with ill family members

[*]excess in relation to the probability of developing gastroenteritis and not bathing.

1.1.2 Quantitative microbial risk assessment (QMRA)

Quantitative Microbiological Risk Assessment (QMRA) is the application of risk assessment principles to the estimation of the probability of a negative event occurring following exposure to a microbial pathogen. This paradigm has been used in a number of published studies to estimate infection or illness risk based on dose-response relationships for pathogenic organisms, the distribution of such organisms in the aquatic environment and assumed exposure levels (López-Pila and Szewzyk, 2000; Ashbolt *et al.*, 1997; Crabtree *et al.*, 1997; Gerba *et al.*, 1996b). A benefit of QMRA is its ability to estimate (albeit crudely) the probability of infection or disease at very low concentrations of pathogenic organisms; levels at which epidemiological studies are unable to detect difference. The general risk assessment framework developed by the US National Academy of Science has been further refined for the use in estimating microbial risks by Haas *et al*. (1999) and others. An Australian version of the generic framework includes the following stages (enHealth, 2002; summarised in Figure 1.2).

Issue identification/problem formation

Identifies issues for which risk assessment is useful and establishes a context for the risk assessment by a process of identifying the concerns that the risk assessment needs to address. Need to consider what is of concern, why is it a concern, how urgent is it and how do stakeholders perceive the concern?

Health assessment

Needs to identify what types of adverse health effects might be caused by the organism (health outcomes). This information generally originates from epidemiological studies. Symptomatic and asymptomatic infections, severity and duration of infection or disease, hospitalisation or degree of medical care, mortality and host immune status are examples of factors which need to be considered when determining health outcomes.

The dose-response assessment is one of the most important steps in any risk assessment. Dose response models need to be clearly defined, with all assumptions and uncertainties identified and end-points stated (ie. infection or disease). For example, it needs to be stated whether threshold or non-threshold

mechanisms have been assumed. The conceptual framework for a non-threshold model is the determination of the probability of any one organism to survive and reach a site suitable for infection (Teunis and Havelaar, 2000). There are also issues such as independent action or synergistic action in the probability of pathogens causing infection or disease. There is general scientific agreement that dose-response models for most microorganisms are best described by the non-threshold (single-hit) and independent action assumptions (Haas *et al.*, 1999). For this reason, exponential or Beta-Poisson mathematical models are generally applied to dose response data, although other models are available. Most dose-response data are determined by exposing human volunteers to relatively high doses of pathogenic organisms. The model chosen, therefore, has a significant impact on the estimation of infection or disease following exposure to low doses of pathogenic microorganisms (as is usually the case with environmental exposures) due to uncertainties associated with interpolation. It also needs to be recognised that most dose-response data are obtained using healthy adult subjects. Issues such as immune status and previous exposure therefore need to be considered.

Exposure assessment

Exposure to a specific organism is determined by numerous factors including, but not limited to:

- The occurrence (and distribution) of the organism of interest
- Exposure pathways
- Frequency and extent of exposure
- Size and nature of the population exposed (including sensitive sub-populations)

For example, wastewater released from a sewage treatment plant is generally associated with high concentrations of potential human microbial pathogens. Depending on treatment and disposal practices and site-specific issues such as proximity to bathing beaches or receiving waters, there may be a significant risk of exposure to individuals (or populations) via direct contact. Issues to be considered in any exposure assessment include, although are not limited to,

methodologies used to determine pathogen concentration (ie has viability been determined), survival under various environmental conditions (temperature, exposure to UV light, salinity etc.) and transport within the medium.

For estimation of risk due to exposure to faecally contaminated recreational water via ingestion/inhalation/direct contact, the frequency and duration of exposure for any individual needs to be estimated. For each of the factors noted there is variability and uncertainty associated with their estimation. This variation needs to be considered in the exposure assessment. Any assessment requires the determination of both the occurrence and distribution (eg normal, log-normal) of the input variable.

Risk characterisation

Provides a quantitative (or qualitative) estimate, including uncertainties, of the nature, severity and potential incidence of effects in a given population. This often uses the process of Monte-Carlo simulation which involves the simultaneous generation of random numbers for each input variable (depending on the assigned occurrence and distribution frequencies). This process is repeated many times and determines the effect of uncertainty on the risk assessment model.

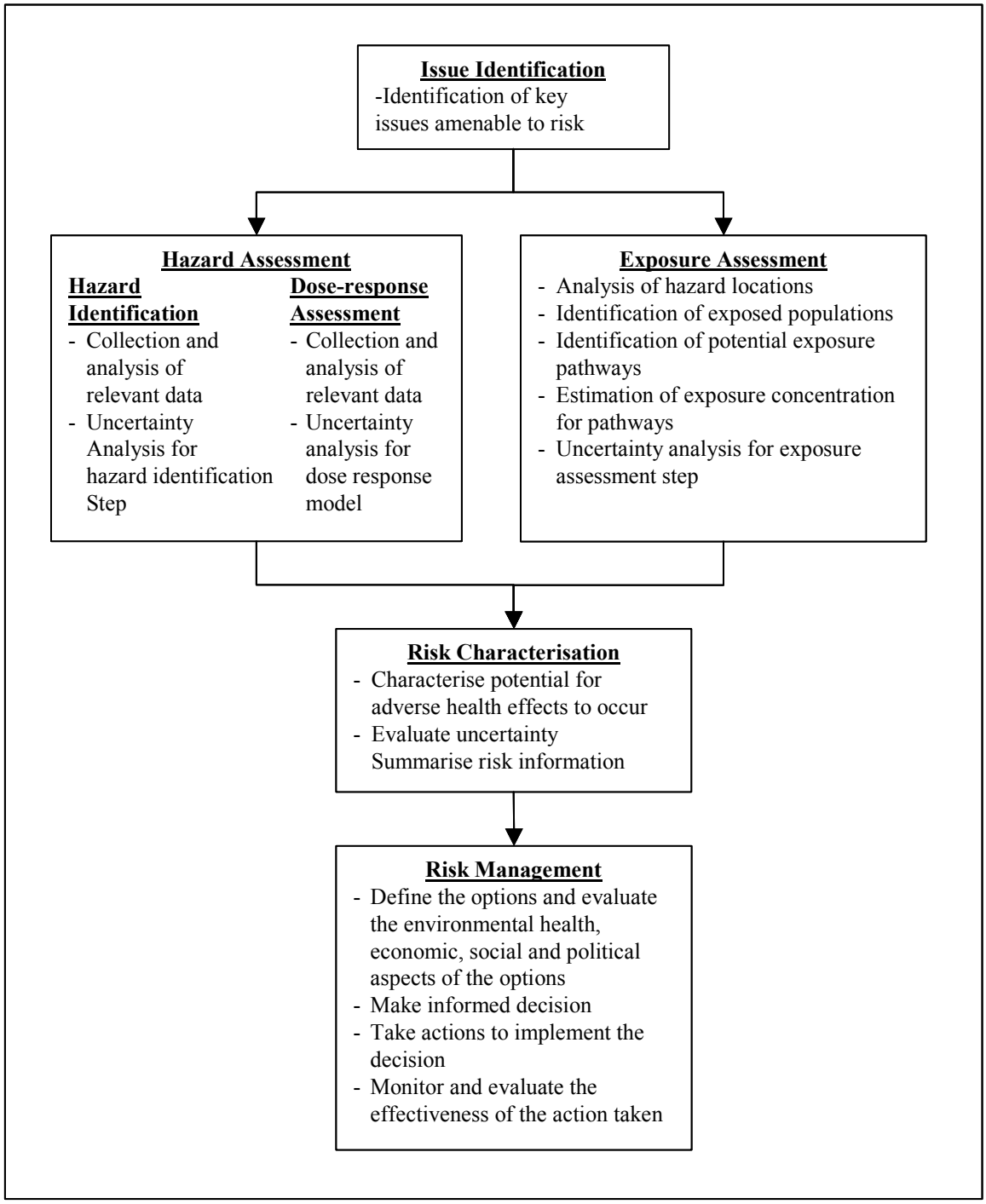

Figure 1.2 Summary of risk assessment paradigm (EnHealth Council, 2002).

1.1.3 Recreational water guidelines

To limit exposure risk in recreational water bodies, microbiological water quality guidelines are commonly employed. Traditionally, these guidelines were primarily based on compliance with prescribed faecal indicator counts during the bathing season (NHMRC, 1990). These types of management mechanisms have many limitations for minimising health risk at recreational water sites such as:

- management actions can only be taken retrospectively (after human exposure to faecally polluted water);
- results from microbiological analysis do not take into account the different degree of risk associated with human and non-human faecal sources;
- beaches are classified as either "safe" or "un-safe" without reflecting the gradient of increased health risk with increasing faecal contamination.

The World Health Organisation (WHO) released a Guidelines for Safe Recreational-water Environment: Coastal and Fresh-waters in 2004 (WHO, 2004). In the process of developing the guidelines over a number of years concerns were raised regarding existing approaches to monitoring and assessment of recreational waters. This lead to the formation of an expert consultation supported by WHO and co-sponsored by the US Environmental Protection Agency (USEPA) in Annapolis USA (WHO, 1999). This consultation aimed to develop guidelines for recreational waters (coastal and freshwaters) that better reflected health risk and provided more effective management and intervention options when necessary. The outcome of the expert consultation became known as the "*Annapolis Protocol*" and was further incorporated into the WHO report *Bathing Water Quality and Human Health* (WHO, 2001) which was the basis for the current WHO guidelines.

This new approach incorporates risk assessment and risk management frameworks and is described as a "harmonised approach" (Bartram and Rees, 2000). The underlying principle is the development of a health-risk based classification scheme combining long-term microbiological data with information collected on potential sources of faecal pollution (WHO, 2001). The suitability of

a water body used for human recreation is classified using the matrix described in Table 1.2.

Table 1.2 Suitability of recreation grade matrix.

Susceptibility to faecal influence		**Microbiological Assessment Category** (intestinal enterococci/100 mL)				**Exceptional circumstances**
		A ≤40	**B** 41-200	**C** 201-500	**D** > 500	
Sanitary Inspection Category	**very low**	Very Good	Very Good	Follow up[1]	Follow Up[1]	ACTION
	low	Very Good	Good	Fair	Follow Up[1]	
	moderate	Good[2]	Good	Fair	Poor	
	high	Good[2]	Fair[2]	Poor	Very Poor	
	very high	Follow Up[2]	Fair[2]	Poor	Very Poor	
Exceptional circumstances		ACTION				

From WHO (2001)

Notes:

- [1]Implies non-sewage sources of faecal indicators (e.g. livestock) which needs to be verified.
- [2]Indicates possible discontinuous/sporadic contamination (often driven by results such as rainfall). Requires further investigation (reassess SIC and MAC). If after reassessment the SFRG is still "follow up" then assign a conservative grade (ie the first grade to the right of the "follow up" in the SIC row).
- Exceptional circumstances relate to known periods of higher risk, such as during an outbreak with a pathogen that may be waterborne, rupture of sewer in a recreational water catchment etc. Under such circumstances, the classification matrix may not fairly represent risk/safety and would be superseded until the episode has abated.
- In certain circumstances, there may be a risk of transmission of pathogens associated with more severe health effects through recreational water use. The human health risk depends greatly on specific (often local) conditions. Public Health authorities should be engaged in the identification and interpretation of such conditions.

1.1.4 Use of indicator organisms to estimate risk of infection

Current methods used to estimate the risk of infection due to exposure to waters containing pathogenic microorganisms involve the detection of indicator organisms. Methods for detecting and identifying many human viral or protozoan pathogens from environmental samples are, at present, either extremely difficult to perform or do not exist at all. Bacterial pathogens can often be detected, but the methodologies used tend to be highly variable and susceptible to environmental stress. The most common indicator organism used currently is the coliform group of bacteria. Coliforms are common inhabitants of the intestinal tract of warm-blooded animals, including humans, and are generally excreted in large numbers. Not all of these organisms are pathogenic but as a group they are used to estimate the presence of other organisms of faecal origin being present in the water body (Fleisher *et al.*, 1993). The coliform group of organisms is defined as facultative

aerobic, Gram-negative, non spore-forming, rod shaped bacteria that ferment lactose with gas and acid production within 48 h at 35ºC (APHA *et al.*, 1998). This group is further characterised into faecal (or thermotolerant) coliforms and *Escherichia coli*. Faecal coliforms are a subset of total coliforms that grows and ferments lactose at elevated temperature (44.5ºC) and are therefore also referred to as thermotolerant coliforms. The faecal coliform group consists mainly of *E. coli*, but also some other enteric microorganisms such as *Klebsiella*. Tests for *E. coli* are considered a more specific indicator of faecal contamination and the possible presence of enteric pathogens (APHA *et al.*, 1998).

Borrego and Figueras (1997) describe that the ideal faecal indicator organism should fulfil the following criteria:

- Indicators should be present in faeces, sewage and sewage-polluted waters whenever the pathogens are also present, and absent in waters without faecal pollution.
- The levels of indicators should have some direct relationship to the density of pathogens in aquatic environments, and be proportional to the extent of the pollution.
- Indicators survival rate in water must be at least similar to that of pathogens. In addition, the resistance of indicators to depuration factors and disinfectants should be at least similar to that of the pathogens.
- Indicators must be detectable by a simple, inexpensive, accurate, rapid laboratory methodology
- Indicators should be non-pathogenic and applicable to all types of water samples that require a monitoring program.
- Their characteristics must be constant.
- Indicators must not grow and multiply in water.

The use of coliform bacteria for the estimation of other pathogenic microorganisms being present has many limitations. Studies have demonstrated differential survival rates of coliforms compared with that for pathogens (Lund, 1996; Koh *et al.*, 1994; Rhodes and Kator, 1988; Hood and Ness, 1982). For example, Rhodes and Kator (1988) studied the survival of *E. coli* and *Salmonella*

spp. using in-situ diffusion chambers placed in estuarine waters. It was found that *Salmonella* spp. survived significantly longer than *E. coli* when the water temperature was below 10ºC. After one week of exposure to estuarine water at this temperature, between 100% - 86% of *Salmonella* spp survived compared with only ≤ 6% of *E. coli*. Results from in-situ monitoring of coastal waters has identified, in some cases, no correlation between faecal coliform concentration and the presence of enteroviruses or adenoviruses (Vantarakis and Papepetropoulou, 1998). It has also been demonstrated that, under certain nutrient conditions, *E. coli* has the ability to grow in tropical soil environments (Byappanahalli and Fujioka, 1998).

It is not only important to determine the concentration of pathogenic bacteria in water, since the presence of viruses such as norwalk-like virus (NLV), adenovirus and poliovirus in a water body can also lead to significant morbidity (Wyn-Jones and Sellwood, 2001; Haas *et al.*, 1999). A number of studies have shown no significant correlation between the presence of faecal indicator organisms and viruses (Vantarakis and Papepetropoulou, 1998; LaBelle *et al.*, 1980).

A wide range of alternative faecal indicator organisms have been described for use in recreational water management such as enterococci, sulphite-reducing clostridia, bacteroides fragilis somatic coliphage, and male-specific coliphage (WHO, 1999). Enterococci is the group of bacteria which have more recently been accepted as an indicator of faecal contamination in recreational waters (WHO, 2001). Some confusion in the literature exists over the use of the terms enterococci and faecal streptococci. The term “enterococci” includes some organisms of the genus *Streptococcus* and all organisms from the genus *Enterococcus* which fulfil the following criteria: growth at 10°C and 45°C, resistance to 60°C for 30 min, growth at pH 9.6 and at 6.5% NaCl and the ability to reduce 0.1% methylene blue (Borrego and Figueras, 1997). In practice, the terms faecal streptococci, enterococci, intestinal enterococci and Enterococcus group may refer to the same bacteria (WHO, 2001). Enterococci are less ubiquitous than coliforms, always present in the faeces of warm-blooded animals and are believed to be unable to multiply in sewage-contaminated waters

(Borrego and Figueras, 1997). As previously discussed in this chapter, a relationship between adverse health outcomes following recreational contact to marine waters and enterococci concentration has been identified. In addition, survival of enterococci in coastal water has been determined to be greater than for coliforms (Bordalo *et al.*, 2002; Davies *et al.*, 1995; Vasconcelos and Swartz, 1976).

A study by Koh *et al.* (1994) investigated the relationship between the concentration of *E. coli*, enterococci, faecal coliforms and total coliforms with *Vibrio cholera* in various water samples. The results of this study found either no correlation, or negative correlation between the concentration of these indicator organisms with that of *V. cholera*.

It must be remembered, however, that no one single indicator organism is likely to accurately estimate risk of exposure to pathogenic microorganisms of faecal origin under all environmental conditions. Each group of indicator organisms has strengths and weaknesses associated with their use (WHO, 1999). Based on epidemiological data, enterococci are currently the most reliable indicator for infection risk in recreational coastal waters.

1.1.5 Determining microbial viability

Conventional methods for the detection of viable microorganisms depend on the ability of the organism to grow on laboratory media. There is evidence, however, that a number of metabolically active microorganisms may not grow on some selective media (Whitesides and Oliver, 1997; Oliver *et al.*, 1995; Roszak *et al.*, 1984) . As discussed detail in Section 1.1.7, these organisms have been described as being viable, but non-culturable (VBNC). These organisms require direct enumeration and often involve the use of viability dyes and epifluorescent microscopy.

A number of direct in-vitro assays utilising fluorescent dyes have been developed to estimate microbial viability. Fluorescent dyes such as acridine orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) can penetrate intact cell walls and bind to nucleic acid to give total cell counts (Porter and Feig, 1980). Acridine orange has

the ability to simultaneously stain DNA and RNA. When intercalated with DNA the complex emits green light (emission wavelength 530nm) whereas when bound to RNA the complex emits red light with an emission wavelength of 640nm (Mason and Lloyd, 1997). Active and rapidly growing bacteria have a high proportion of RNA compared to DNA and therefore fluoresce red (Hobbie *et al.*, 1977). Relatively inactive cells contain mainly DNA and therefore fluoresce green. DAPI is a highly specific dye for staining DNA and easily penetrates cellular membranes (Porter and Feig, 1980). When excited by UV light (wavelength of 350nm) DAPI bound to DNA emits bright blue light (wavelength of 450nm).

The direct viable count (DVC) method involves incubation of microorganisms in the presence of yeast extract and nalidixic acid (Kogure *et al.*, 1979). Nalidixic acid prevents the division of bacteria (by inhibiting DNA synthesis). Under these conditions viable bacteria do not divide but grow to form elongated cells. This makes it easier to count cell numbers by epifluorescence microscopy when used in conjunction with AO.

A problem with using AO is a high incidence of background fluorescence. It is often difficult to distinguish AO stained bacteria from autofluorescing non-living particles such as clays, detritus or colloids (Porter and Feig, 1980). This problem compounded because when cells contain similar proportions of RNA and DNA, the combination of red and green fluorescence looks yellow when viewed under the microscope.

Table 1.3 Spectral properties of common bacterial viability dyes.

Type	Dye	Max Excitation (nm)	Max. Emission (nm)
Nucleic acid dyes	Acridine orange	500 (DNA)	526 (DNA)
		460 (RNA)	650 (RNA)
	DAPI	358	461
	Ethidium Bromide	518	605
	Propidium iodide	536	617
	SYBR Green I	498	522
	SYTO 9	480	500
	SYTO 13, 16	494	515
	SYTO 11	515	543
	SYTOX green	504	523
Potentiometric dyes	Rhodamine 123	507	529
	$DiBAC_4(3)$	493	516
Redox probes	CTC	400 – 450	602

Fluorescent dyes that behave similarly to DAPI have been developed which are excited by blue light (as opposed to UV) and are used for the enumeration of aquatic microorganisms. Of a long list of blue-excitable dyes the difference usually lies in their affinity to DNA compared with RNA. This assists in the discrimination of actively growing cells, which generally contain greater proportions of RNA, from dormant cells. For example, of the SYTO dyes, SYTO-16 has a high affinity for DNA whereas SYTO-13 and SYTO-11 have higher affinities to RNA (LeBaron *et al.*, 1998). In marine samples the dye SYBR Green I (SYBR-I) has been found to bind to double-stranded DNA but it also binds with single-stranded DNA and RNA with lower affinities (Maire *et al.*, 1997).

A number of fluorescent dyes have been developed which use cell wall integrity as an estimate of microbial viability. Roth *et al*. (1997) compared the two nucleic acid stains propidium iodide and SYTOX Green. These dyes only bind to nucleic acid when the cell membrane is permeabilised (indicating a lack of viability).

When bound to DNA or RNA there is an enhancement in dye fluorescence as well as a shift in emission wavelength when viewed by epifluorescence microscopy enabling differentiation from non-bound cells. Cells stained with propidium iodide fluoresce red (wavelength >600nm) whereas those stained with SYTOX Green fluoresce at a wavelength of 523nm (Roth *et al.*, 1997).

Roth *et al*. (1997) found a direct relationship between the proportion of viable cells as determined by staining with SYTOX Green and detected by epifluorescent microscopy and flow cytometry, compared with the known proportion of permeabilised cells. The cells were permeabilised by exposure to 70% isopropyl alcohol. Results of the study found that the shift in fluorescent intensity between viable and non-viable cells was greater for SYTOX green than propidium iodide indicating greater efficiency of SYTOX green to stain DNA of cells with permeabilised membranes.

Propidium iodide has also been used to determine the viability of protozoan *Giardia* cysts and *Cryptosporidium* oocysts (Campbell *et al.*, 1992; Sauch *et al.*, 1991; Schupp and Erlandsen, 1987). Campbell *et al*. (1992) developed a viability assay for *Cryptosporidium* oocysts utilising both DAPI and propidium iodide. The inclusion or exclusion of the two dyes were compared with in-vitro excystation (the ability to excyst indicates oocyst viability). Propidium iodide only binds to nucleic acid of cells with compromised cell walls. Therefore when viewed by epifluorescent microscopy, oocysts that fluoresce red (bound to propidium iodide) were described as non-viable (see Table 1.4). These oocysts were not able to excyst in vitro. Oocyst which exclude PI but include DAPI were considered viable. Some oocysts exclude both PI and DAPI. The authors suggest that these oocysts were not viable at time of assay but may become viable after further external trigger (such as acidification). As demonstrated in Table 1.4, the inclusion/exclusion of propidium iodide is what discriminates between viable and non-viable oocysts.

Table 1.4 Oocyst viability determined by inclusion/exclusion of vital dyes (Campbell *et al*., 1992).

Type of oocyst	Viability
PI+	Non-viable
DAPI+ PI-	Viable at assay
DAPI- PI-	Viable after further trigger

DAPI- PI- oocysts can be converted to DAPI+ PI- and vice versa

Another group of fluorescent dyes used to estimate microbial viability are membrane potential dyes. Some examples of these are cyanide dyes such as carbocyanine and rhodamine 123 which are a lipophilic, cationic, fluorescent dyes (Kaprelyants and Kell, 1992). Actively metabolising bacteria accumulate the dyes into the cytoplasm due to an inside negative transmembrane electrochemical potential (Mason *et al.*, 1995). These dyes therefore only stain viable microorganisms. Oxonol dyes such as bis-(1,3-dibutylbarbituric acid) trimethine oxonol ((DiBAC$_4$(3)) and calcafluor white are lipophilic anions (Mason *et al.*, 1995). These negatively charged oxonol dyes enter depolarised cells and bind to lipid rich intracellular components (Jepras *et al.*, 1995). The fluorescence of these dyes decreases with increasing transmembrane potential and therefore only stains non-viable cells.

Studies have shown that rhodamine 123 does not accumulate well in actively metabolising gram-negative bacteria (Kaprelyants and Kell, 1992; Matsuyama, 1984). This may reflect differences in the outer membrane of these organisms compared to gram-positive organisms. Kaprelyants and Kell (1992) found that treating Gram negative *E. coli* cells with Tris-EDTA results in the permeabilisation of their outer membrane allowing the passage of rhodamine 123 into cells with negative membrane potentials.

Redox probes are fluorescent dyes that are capable of determining cellular respiratory activity (López-Amorós *et al.*, 1997). An example of a redox dye is the tetrazolium salt CTC (5-cyao-2,3-ditolyl tetrazolium chloride) which is reduced to a fluorescent insoluble formazan by cellular dehydrogenases in actively respiring organisms and detected by epifluorescence microscopy (Lisle *et*

al., 1998). Another redox probe commonly used to estimate microbial viability is 2-para (indo-phenyl)-3(nitrophenyl)-5(phenyl)tetrazolium chloride (INT). Again actively respiring organisms reduce INT to an insoluble formazan but unlike for CTC determinations the response is determined either microscopically with bright-field optics, or the INT-formazan is extracted with ethanol from filtered cells and measured spectrophotometrically (Maurines-Carboneill *et al.*, 1998; Rodriguez *et al.*, 1992).

López-Amorós (1997) used CTC to determine the respiratory activity of *E. coli* and *Salmonella* in response to starvation in seawater. Results of the study indicated that as the starvation time of cells in seawater increased the respiratory activity as measured by CTC reduction decreased. Starvation also lead to an increased incorporation of the oxonol dye $DiBAC_4(3)$. There was a negative correlation coefficient between CTC reduction and oxonol dye incorporation of 0.98. This study demonstrates the benefits of utilising double staining protocols when measuring microbial viability. In this case viable cells which reduced CTC fluoresced red whereas non-viable cells which incorporate $DiBAC_4(3)$ fluoresced green. The authors of this paper noted that reduced CTC accumulation in the cell is relatively toxic and therefore is usually used independently of $DiBAC_4(3)$.

Viability kits utilising dual staining protocols are commercially available. The LIVE/DEAD *Bac*Light bacterial viability kit from Molecular Probes has been used in a number of studies (LeBaron *et al.*, 1998; Lisle *et al.*, 1998; Langsrud and Sundheim, 1996). This kit utilises the SYTO-9 dye which stains nucleic acid and can penetrate intact as well as compromised cell membranes and propidium iodide which can only penetrate damaged membranes. Therefore all bacteria fluoresce green due to uptake of SYTO-9 and non-viable cells fluoresce red from uptake of propidium iodide.

Langsrud and Sundheim (1996) compared ability of rhodamine 123, SYTOX Green and LIVE/DEAD stains to determine the viability of *Staphylococcus* spp. and *Pseudomonas* spp. Results of the study indicated that both rhodamine 123 and the LIVE/DEAD dyes could not stain all strains of *Pseudomonas* tested. As *Pseudomonas* is a gram-negative bacteria this may again reflect differences in cell

wall constituents. A study by Lisle *et al*. (1998) used the LIVE/DEAD *Bac*Light kit to determine the viability of *E. coli* after starvation in seawater. This study found that there was a correlation between results using the LIVE/DEAD *Bac*Light assay and those using rhodamine 123.

In summary there are many different fluorescent dyes available to enable the estimation of cell viability. The decision of which viability dye to use depends on a number of different factors. There is not one dye that will be effective for all microorganisms under all conditions. As discussed, dyes which label nucleic acid in cells with damaged cell membranes often are less specific than other viability dyes. It needs to be determined whether the presence of a permeabilised membrane is an adequate indicator of cell viability. Redox probes such as CTC, INT and membrane potential dye Rhodamine 123 are incorporated into actively respiring cells. These dyes may be more specific than dyes such as propidium iodide or SYTOX green, but are unable to accumulate in some gram negative cells (Kaprelyants and Kell, 1992; Matsuyama, 1984). It is not only necessary to determine the relative effectiveness of the viability dye but also compare the wavelength at which it fluoresces. The emission spectrum needs to be significantly different from other probes/fluorescent antibodies being used. As demonstrated, it is important to trial different viability dyes with the organism being tested to identify the most effective method to estimate cell viability.

1.1.6 Molecular techniques for the enumeration of indicator organisms

An alternative technique for directly enumerating waterborne microorganisms is the fluorescence *in situ* hybridisation (FISH) technique (Amann *et al.*, 1990). This process utilises fluorescently labelled oligonucleotide probes targeted for specific ribosomal RNA (rRNA). Molecules of rRNA are ideal candidates for fluorescently labelled oligonucleotide probes for the following reasons (Vesey *et al.*, 1998; Amann *et al.*, 1990):

- The target region is often at least partially single stranded and therefore the procedure does not require a denaturing step.
- Targeted molecules may be present in very high numbers (between 10^4 and 10^5 ribosomes per cell), resulting in increased sensitivity.

- rRNA has a short half-life and should only be present in a high copy number in viable or recently viable cells.

Commonly, the larger rRNA molecules (16S and 23S-like rRNA) are targeted when designing oligonucleotide probes (Moter and Göbel, 2000). These molecules contain both conserved and variable regions which can be used to adjust the probe specificity (Amann *et al.*, 1990). This allows probes for each taxonomic level (from bacterial and archael down to genus-specific and species-specific) to be designed according to the rRNA region targeted (Amann *et al.*, 1995).

Once the oligonucleotide probe has been developed and labelled with a fluorescent dye (similar to the dyes used for the antibody detection methods described earlier) the processes involved with the FISH technique include fixation of the bacterial cells to allow penetration of the probes into the cell. The sample can be prepared in a number of ways, for example the sample investigated can be prepared on a glass slide or in a suspension. The hybridisation step, where the oligonucleotide probe binds with cell, is the most crucial step in the process. The conditions required are dependent on the organism being enumerated. Following hybridisation the sample is washed to remove excess fluorescence probe and then mounting for visualisation (by epifluorescence microscopy or flow cytometry). Lepeuple *et al*. (2003) combined the FISH technique with the direct viable count (DVC) method to more accurately enumerate viable *E. coli* cells by microscopy.

FISH has been used for the detection and enumeration of many human pathogenic microorganisms from a variety of sources (Moter and Göbel, 2000). Enumeration of pathogens from recreational coastal waters is often difficult due to usually low numbers being present. Oligonucleotide probes for the specific enumeration of coliforms from water is extremely difficult, since the coliform group is a group of bacteria from genera that are phylogenetically different (Rompre *et al.*, 2002). However, probes have been developed for the enumeration of the Enterobacteriaceae family from wastewater samples (Loge *et al.*, 1999).

A limitation with the conventional FISH method is the diminished ability to enumerate starved or stressed organisms from such sources as environmental water and treated drinking water (Lepeuple *et al.*, 2003; Rompre *et al.*, 2002; Lebaron *et al.*, 1997). Organisms under starvation conditions are associated with low ribosomal content and therefore result in reduced hybridisation with the oligonucleotide probe and subsequent weak fluorescence (Amann *et al.*, 1995). Fluorescence amplification methods have been developed using multi-probing techniques to enhance detection of such cells. This involves the direct labelling of the probe with horseradish peroxidase (HRP) and addition of the fluorescein tyramide, which is used as a substrate for the probes labelled with HRP (Lepeuple *et al.*, 2003; Lebaron *et al.*, 1997).

Highly sensitive methods based on the polymerase chain reaction (PCR) have also been developed to identify a large range of indicator and pathogenic microorganisms from a variety of sources. This method involves the recovery of DNA from the sample, amplification of target nucleotide sequences (using oligonucleotide primers) specific for the microorganism of interest using the PCR (DNA polymerase), and detection of the amplified DNA with gene probes (Toze, 1999; Waage *et al.*, 1999a; Bej *et al.*, 1990). The specificity of these primers can be designed for individual organisms or for a group of organisms (Toze, 1999).

PCR-based methods for the detection of specific pathogenic and indicator organisms from water samples has commonly been used as a qualitative detection method (Noble and Fuhrman, 2001; Griffin *et al.*, 1999; Waage *et al.*, 1999c; Waage *et al.*, 1999b; Waage *et al.*, 1999a; Kaucner and Stinear, 1998; Reynolds *et al.*, 1998; Vantarakis and Papepetropoulou, 1998; Rochelle *et al.*, 1997; Mayer and Palmer, 1996; Johnson *et al.*, 1995; Kirk and Rowe, 1994; Bej *et al.*, 1991a; Bej *et al.*, 1991b; Bej *et al.*, 1990). Semi-quantitative methods combining PCR methods with traditional most-probable-number (MPN) techniques have also been developed for enumeration of pathogenic microorganisms from food, soil and water (Fredslund *et al.*, 2001; Reynolds *et al.*, 2001; Mäntyen *et al.*, 1997). A more recent advance for the direct quantification of specific microorganisms is the process of real-time PCR which consists of monitoring the fluorescent PCR products as they are amplified (Toze, 1999). Real-time PCR has been used

successfully enumerate bacteria, protozoa and viruses in waters (Bellin *et al.*, 2001; Higgins *et al.*, 2001; Monpoeho *et al.*, 2000).

For the detection of *E. coli*, probes specific for a portion of the *uidA* gene, which encodes for the β-D-glucuronidase enzyme, have been developed (Farnleitner *et al.*, 2000; Bej *et al.*, 1991b). Monitoring of *E. coli* from many water sources requires a concentration step due low numbers of organism often present. Bej *et al*. (1991b) used membrane filtration methods to concentrate water samples followed by a freeze-thaw cycle to release the DNA prior to PCR amplification using the *uidA* gene probe. This qualitative method reported a detection limit of 1 cell 100 mL^{-1}.

The use of PCR-based methods for the enumeration of faecal indicator organisms, at present, has many limitations. The majority of studies have involved the detection of cultured *E. coli* in spiked water samples and therefore do not take into account environmentally stressed organisms (Waage *et al.*, 1999b; Waage *et al.*, 1999a; Bej *et al.*, 1991a). The analysis of environmental samples has also been associated with the presence of PCR enzyme inhibitors such as humic substances and colloidal material (Higgins *et al.*, 2001; Toze, 1999). A major drawback with PCR methods is that nucleic acid from viable and culturable, viable and non-culturable or non-viable cells is amplified and therefore this technique cannot be used to accurately estimate organism viability (Rompre *et al.*, 2002). PCR methods require highly specialised, expensive equipment and skilled operators. Despite these limitations, PCR remains a valuable tool for the highly specific detection of individual organisms and is extremely useful in identification and tracking of contamination sources.

1.1.7 Use of microcosms for survival studies

One method used to determine the survival of microorganisms in aquatic environments is the use of in-situ diffusion chambers. These usually involve placing the microorganisms in a container which has membrane filters at each end into the environment under study. This allows passage of nutrients and salts into and out of the chamber but prevents the passage of organisms between the

environment and the diffusion chamber (Vasconcelos and Swartz, 1976). In-situ diffusion chambers have been used to determine the survival of microorganisms in coastal sediments (Davies *et al.*, 1995). The authors of this paper found a combination of harsh environmental conditions and vandalism varied the duration of the experiments. The membrane filters are relatively fragile and susceptible to damage. Unlike the laboratory based microcosms, when using diffusion chambers, environmental factors such as light, temperature and nutrients cannot be controlled. The advantage of diffusion chambers is that in some circumstances they more closely represent environmental conditions.

Another mechanism for simulating environmental conditions involves the use of microcosms. A microcosm is described as a laboratory based system which attempts to simulate as far as possible conditions in the environment (or part of the environment) under study (Wimpenny, 1988). Microcosms are, in general, physically enclosed and not in contact with the natural ecosystem. Microcosms are compact subsets of the natural system from which they came and can be used to determine survival of microorganisms.

Microcosms vary greatly in their size and complexity. Simple systems involve placing sediment and/or water into Erlenmeyer flasks and incubating under varying conditions (Fish and Pettibone, 1995; Hood and Ness, 1982; Gerba and McLeod, 1976). In an effort to simulate *in-situ* sediment stratification, composites of previously collected sediment are sometimes used. In some cases, sterile sediment is used to control the effect of predation on survival of the microorganism under study. One problem with autoclaving sediments is that it can result in an increased availability of sediment-bound nutrients leading to enhanced microbial survival (Hood and Ness, 1982). It can be argued such simplistic microcosm systems are limited in representing actual environmental conditions, however they do allow the investigation of one parameter at a time without the confounding of all other environmental parameters.

More elaborate microcosms involve the collection of intact sediment cores and overlying water from the environment (Wagner-Döbler *et al.*, 1992). The microcosms are then inoculated and incubated at specific temperatures over a

period of time. Wagner-Döbler *et al*. (1992) collected samples by inserting perspex tubes (70mm diameter, length 310mm) into sediment, then capping the ends for transport back into the laboratory. The microcosms were inoculated with a test organism and incubated in a water bath for up to 28 days. To enhance the diffusion of oxygen into the microcosms over the sampling period the samples were mechanically stirred at a rate that did not disturb the surface sediment layer. This system also allowed for the inflow and outflow of water to and from the microcosms. Samples were destructively sampled on days 1, 6, 13, 20 and 28. Therefore for each condition tested there needed to be five identical microcosms.

Wagner-Döbler *et al*. (1992) investigated the variability between cores, the stability of ecosystem parameters and differences between laboratory microcosms and environmental samples. Visual inspection of the microcosms revealed an intact surface layer and vertical zonation of colour that did not change over the 28 day sampling period. The water overlying the sediment contained zooplankton while the sediment contained benthic invertebrates. This indicates that the sediment microcosms maintained an intact ecosystem with most of the complexity of a natural system over the sampling period. The microcosms were analysed over the course of the experiment for nitrate, phosphate, chlorophyll a, total CFU, bacterial community structure (cellulose degraders, enterobacteria, denitrifying bacteria, pseudomonads and fluorescent bacteria), thymidine incorporation rate, oxygen uptake rate and oxygen penetration depth. There was little difference observed between sediment in the microcosms compared to environmental samples after two weeks incubation, however, between two to four weeks incubation, there were slight differences in a nutrient levels, community structure of heterotrophic bacteria and thymidine-uptake rate, but overall the microcosms reflected the microbial community structure and microbial activities present in the target population for the full 28 days.

1.1.8 Survival of microorganisms in aquatic environments

Organisms released into coastal or freshwater environments are exposed to numerous physico-chemical and biological factors which cause stress including temperature change, salinity, nutrient deficiencies, predation and sunlight (Sinton *et al.*, 2002; Thomas *et al.*, 1999; Özkanca and Flint, 1997; Davies *et al.*, 1995). It

is important to note that survival is dependent on the response of the organism to the combination of these stressors rather than one stressor in isolation (McCambridge and McMeekin, 1981).

A number of studies have investigated the response of pathogenic microorganisms to changes in temperature (Thomas *et al.*, 1999; Terzieva and McFeters, 1991; Pérez-Rosas and Hazen, 1988; Flint, 1987; Huq *et al.*, 1984; Anderson *et al.*, 1983; Vasconcelos and Swartz, 1976). Of these Anderson *et al.* (1983) found a direct correlation between the survival of *E. coli* and estuarine water temperature in the absence of predators (water passed through 0.2µm filter). The authors also found that at temperatures of between 13ºC - 24ºC *E. coli* was capable of growth over a five-day exposure period. In contrast *E. coli* survival declined with increasing temperature in the presence of microbial predators. The decrease in the number of *E. coli* cells over the exposure period coincided with an increase in eukaryote densities. At lower temperatures the decline of *E. coli* cells was constant regardless of the presence of predators, presumably associated with the decreased activity of predators at low temperatures.

The study by Vasconcelos and Swartz (1976) discovered an inverse relationship between *E. coli* survival in seawater and temperature using diffusion chambers. The highest fatalities of *E. coli* occurred at a temperature of 14.5ºC compared with the lowest fatalities at 8.9ºC. Terzieva and McFeters (1991) also found that survival of *E. coli*, as well as *C. jejuni* and *Y. enterocolitica*, was greater at 6ºC compared with 16ºC.

Flint (1987) investigated the survival of *E. coli* in river water at various temperatures. Water was either untreated, passed through glass fibre (GF/C) filters (to remove protozoa and suspended solids), 0.45µm membrane filters (to remove most bacteria) or autoclaved to remove ultramicrobacteria and viruses. The author described the decrease of *E. coli* over the sampling period as "disappearance" rather than "die off". The disappearance of *E. coli* was expressed as the decay constant (k) which was the slope of the linear portion of the plot of log viable count against time. Differences in *E. coli* survival between water types

could be identified by comparing respective *k* values. Results of the study by Flint (1987) demonstrated that in autoclaved river water *E. coli* survived (as measured by plate count) for up to 260 days at temperatures between 4ºC to 20ºC. Autoclaved river water gave the longest survival times at all temperatures tested (4ºC, 15ºC, 25ºC and 37ºC). As previously discussed, autoclaving not only has the effect of inactivating microorganisms but may also increase the availability of nutrients (Hood and Ness, 1982). Survival in all water types (untreated and treated) was greater at lower temperatures. There was little difference between survival in untreated water and GF/C filtered water. It was concluded that the presence of protozoa and attachment to particles appeared to have little effect on the disappearance of *E. coli* in the water tested. There was, however greater survival at temperatures of < 25ºC in water which had been passed through a membrane filter (0.45 μm). It was suggested that the increase in survival may have been due to the removal of those microorganisms which would otherwise be competing for nutrients with *E. coli*.

Microcosm studies using pathogenic microorganisms have been undertaken by Thomas *et al*. (1999) to determine the survival of *Campylobacter* spp. in water. Survival was determined in ionised water and river water with and without sediment at temperatures ranging from 5ºC to 37ºC. The microcosms comprised 150mL of sterile ionised or river water in conical flasks. For samples containing sediment, 50g of river sediment previously sterilised by autoclaving was added. Decay rate constants (*k*) were determined by the same method as Flint (1987). As demonstrated by Flint (1987), there was a direct and inverse relationship between water temperature and both the persistence and decay rates of culturable organisms. Decay of *Campylobacter* spp. in deionised water was greater compared with river water, particularly at environmental temperatures (5ºC and 15ºC). It was suggested that the increased amount of nutrients present in river water compared with deionised water contributed to greater survival of *Campylobacter* spp. Maximum persistence was identified at 5ºC, with *Campylobacter jejuni* concentration exceeding 10^4 CFU/mL after 60 days. Unlike other studies, the addition of sediment did not result in significant decreases in decay rates in the water column, however, this may be explained by the use of

sterile water and sediment which removes the interaction of competing microorganisms and protozoa (Brenner *et al.*, 1999; Burton *et al.*, 1987).

Microorganisms in coastal waters are exposed to both visible and UV light. There is general agreement that exposure to light reduces the survival of pathogenic microorganisms in water (Aas *et al.*, 1996; Gourmelon *et al.*, 1994; Curtis *et al.*, 1992; Davies and Evison, 1991; Barcina *et al.*, 1989; Fujioka *et al.*, 1981; Kapuscinski and Mitchell, 1981; McCambridge and McMeekin, 1981). Studies have investigated the individual effects of exposure to either UV and/or visible light on microbial survival. A study by Fujioka *et al*. (1981) found that survival of faecal coliforms and faecal streptococci was significantly reduced when exposed to natural sunlight. In the absence of sunlight these bacteria survived for between one to three days in seawater (T_{90} 21 – 48 h and 36 – 84 h for faecal coliforms and faecal streptococci respectively). In the presence of sunlight the T_{90} were reduced to 30-90 minutes and 60-180 minutes for faecal coliforms and faecal streptococci respectively.

In an effort to determine the relative effects of both visible and UV light, Davies and Evison (1991) investigated the response of *E. coli* and *Salmonella* after exposure to various light sources. The decay rate constant (k) was calculated as the slope of the line of best fit when $\log_{10}$ (N_t/N_0) was plotted against time. The results indicated that there was a greater decrease in the number of culturable bacteria in seawater than in freshwater when exposed to natural sunlight (UV + visible light). There was no significant difference between the decrease of culturable bacteria in seawater and those in freshwater when exposed to visible light only at the three temperatures tested (5, 15 and 25°C). It was concluded that the effect of salinity on loss of culturability was more significant in the presence of UV light.

It has been suggested that for light to be able to damage a microorganism it must be absorbed by a chemical, or sensitiser (Curtis *et al.*, 1992). Once light is absorbed, the sensitiser may enter an excited state and react with other molecules initiating cellular damage. A study by Curtis *et al*. (1992) found that humic

substances acted as sensitisers by absorbing UV light and allowing more penetrating longer wavelength light to damage the microorganisms. This in effect enhances the production of toxic forms of oxygen such as hydrogen peroxide (H_2O_2) and singlet oxygen. The presence of scavengers of reactive oxygen species has variable protective effects. Studies have found that the addition of catalase (eliminates hydrogen peroxide), and thiourea (a hydroxyl radical scavenger) results in a net protection of *Escherichia coli* (Gourmelon *et al.*, 1994). It has also been identified that under anaerobic conditions there is a decrease in the toxic effect of exposure to light (Gourmelon *et al.*, 1994). Curtis *et al*. (1992) found oxygen to be a key variable in determining the survival of faecal coliforms in response to exposure to light. Without oxygen exposure to light had no effect. These studies highlight the role of oxygen in the photosensitive reactions upon exposure of microorganisms to sunlight.

A study by Huq *et al*. (1984) found that survival *Vibrio cholerae* was greater at higher temperatures in association with live copepods. It was demonstrated that water temperature had a positive influence on the attachment, and multiplication of *V. cholerae* onto live copepods. The greatest survival of *V. cholerae* occurred at 30ºC, whereas the influence of copepods was not observed at water temperatures of less than 15ºC. Salinity was also observed to have positive an effect on the attachment of *V. cholerae* to copepods and growth, with greatest survival occurring at a salinity of 15‰ (compared with 5‰ and 10‰).

Studies have demonstrated that oocysts of the protozoan parasite *Cryptosporidium* can survive and remain infectious in seawater for prolonged periods (Fayer *et al.*, 1998; Johnson *et al.*, 1997; Medema *et al.*, 1997; Robertson *et al.*, 1992). In the study by Fayer *et al*. (1998) it was found that *C. parvum* oocysts were able to retain infectivity after being exposed to seawater at salinities of 10, 20 and 30 ppt at 10°C for 12 weeks. When incubated in seawater at 20 ppt and 30 ppt at 20°C oocysts were infectious for eight and four weeks respectively.

Results from a study by Johnson *et al*. (1997) indicated that *Cryptosporidium* oocysts survived longer in marine waters in the absence of light than when light

was present. When incubated in *in-situ* diffusion chambers in the presence of light, between 1.4% and 2.9% of oocysts remained viable (as determined by *in-vitro* excystation) after 72h. In the absence of light, however, between 17% and 19% of oocysts were viable after 72h exposure to seawater. Of most significance was that *Cryptosporidium* oocysts survived longer in marine waters, both in the presence and absence of sunlight, than either poliovirus, *Giardia muris* or *Salmonella typhimurium*.

These studies illustrate the complexity of the interaction between a microorganism and the environment, and the impact this has on survival. The survival and/or growth of the microorganisms is a result of the simultaneous effect of a range environmental factors such as those discussed here.

1.1.9 Viable but non-culturable states

To determine total viable counts of microorganisms the standard plate count is commonly used (Roszak and Colwell, 1987). This method determines the number of microorganisms capable of forming colonies on media at a given temperature. When exposed to environmental stressors some microbial cells lose the ability to be cultured yet retain viability as measured by direct microscopic methods (Oliver *et al.*, 1995). Cells that have entered this state are therefore termed viable but nonculturable (VBNC or VNC). Some studies have found that under certain conditions unculturable cells can be resuscitated (Whitesides and Oliver, 1997; Oliver *et al.*, 1995; Roszak *et al.*, 1984).

Survival studies investigating the development of VBNC states involve the comparison of counts made by standard culture methods with those using direct microscopic methods. A study by Whitesides and Oliver (1997) monitored the survival of *Vibrio vulnificus* in artificial seawater microcosms at various temperatures. Results indicated that when incubated at 5ºC total cell counts remained constant over a 14-day period while plate counts declined to undetectable levels (<0.1 CFU mL^{-1}) within four to six days. Viable counts as measured by the direct viable count (DVC) declined to 10^5 cells/mL and remained constant at this concentration (microcosms initially inoculated with 10^6 cells/mL). In an effort to determine if the loss of culturability was as a result of a lack of

nutrients, suspensions of VBNC cells were plated on heart infusion agar (high nutrient content) and incubated at room temperature (22°C) for 30 days. Under this condition there was no resulting colony formation. When the microcosms were subjected to a simple temperature upshift from 5°C to 22°C for 24h, the culturable number of bacteria returned to 10^6 cells/mL. The results of this study suggest that nutrient is in some way inhibitory to the resuscitation of *V. vulnificus,* with those organisms being enumerated following nutrient addition likely to be residual culturable cells rather than temperature-stressed VBNC cells.

A study by Pommepuy *et al*. (1996) investigated the survival of *E. coli* in estuarine waters using in-situ diffusion chambers. Results of the study indicated that *E. coli* cells entered a VBNC state when exposed to sunlight. Using the acridine orange direct count (AODC) and the direct viable count (DVC) the number of cells remained constant during exposure to sunlight over the sampling period (up to 57h). The number of viable *E. coli* cells determined using plate counts showed that the cells responded to the diurnal solar cycle by entering a VBNC state upon exposure to sunlight. After 26h of exposure to sunlight the number of culturable cells decreased from 2×10^6 cells/mL to no detectable culturable cells. When the diffusion chambers were incubated in the dark however, there was no decrease in culturable cells. This study also investigated the ability of VBNC cells to retain pathogenicity. This was achieved by using a gangliodide-enzyme immunosorbent assay (GM1-ELISA) which is a sensitive method for the detection of enterotoxin production (Pommepuy *et al.*, 1996). It was found that both culturable and VBNC *E. coli* cells were able to produce enterotoxin and were therefore potentially pathogenic.

In order to determine if VBNC organisms are capable of taking up amino acids and incorporating them into proteins, studies have investigated the rate of ^{35}S-labelled methionine uptake (Rahman *et al.*, 1994). This study investigated the survival of *Shigella dysenteriae* Type 1 in microcosms incubated at various temperatures. Results of the study showed that when incubated at 30ºC and 37ºC *S. dysenteriae* cells became non-culturable after 12 and 21 days respectively. Over this period the number of viable cells as measured by AODC and DVC

methods remained constant. However, when incubated at 4, 10, 15, 20ºC cells remained culturable over the observation period of one month. The study demonstrated that these VBNC cells retained the ability to both actively uptake ^{35}S-labelled methionine and incorporate methionine into protein (indicating viability). The study also demonstrated the ability of VBNC *S. dysenteriae* cells to retain viability for over six months.

Grimes and Colwell (1986) demonstrated the ability of *E. coli* to enter a VBNC state using in-situ diffusion chambers. It was demonstrated that *E. coli* became non-culturable after only 13 hours incubation at 25ºC(diffusion chambers initially inoculated with 8.7×10^4 *E. coli* per mL). The number of *E. coli* cells detected using fluorescent antibodies and the DVC remained relatively constant over the sampling period of 112 hours. These results highlight the problems associated with using faecal coliforms as indicators of risk of infection. Enteric pathogens such as *E. coli* have the ability to remain viable in seawater long after they become non-culturable using standard detection methods.

There is also evidence of the production of VBNC *Salmonella typhimurium* cells when inoculated into soil (Turpin *et al.*, 1993). This study compared the survival of *S. typhimurium* in sterile and non-sterile soil using conventional plate counts and the fluorescent antibody technique. The authors found that the DVC method could not be used with soil samples because of the adsorption of nalidixic acid to clay particles. In an effort to determine the likely viability of the organisms, soil microcosms were also inoculated with UV-killed *S. typhimurium*. When viewed using fluorescent antibodies and epifluorescence microscopy the UV-killed cells were intact and could not be cultured on standard media. The results of the study demonstrated that when incubated at 22ºC in non-sterile soil there was a dramatic decrease in culturable cells, however for sterile soil culturable numbers of *S. typhimurium* remained relatively constant. When using direct counting techniques (fluorescent antibodies) the number of cells in non-sterile soil remained constant, suggesting the development of a VBNC state. Over the same sampling period there was a 95% reduction in the number of UV-killed *S. typhimurium* cells in non-sterile soil, presumably due to cell lysis and breakdown by indigenous micro-

flora. The authors suggested that the constant numbers of *S. typhimurium* counted using fluorescent antibodies is therefore not simply a results of dead cells remaining in the soil microcosm but rather VBNC cells. It was noted that the *S. typhimurium* cells in the non-sterile soil became progressively smaller and rounder with time when viewed by microscopy.

There is suggestion that cells which have entered a VBNC state may become more resistant to environmental stressors (Weichart and Kjelleberg, 1996). Results from this study indicated that in a population of cold incubated *Vibrio vulnificus* there existed culturable, injured and non-culturable cells. Counts using soft agar plates gave between 1.1 to 8-fold higher CFU counts compared to standard plate counts. Of the non-culturable population (determined by direct counting methods) resuscitation could not be achieved by any combination of temperature or nutrient up-shifts. The resistance of VBNC cells to mechanical stress (sonication) was compared with that of growing and starved cells. Initially the resistance of VBNC cells to sonication was similar to that of growing cells but increased during prolonged cold incubation (six weeks). After a period of six weeks the resistance of VBNC cells to sonication was similar to that of starved cells (which was markedly greater than growing cells). The results of the study demonstrated that although VBNC cells could not be resuscitated under laboratory conditions, VBNC cells undergo changes at low temperatures which may allow them to persist for extended periods of time in the environment.

There have been a number of studies undertaken suggesting that the VBNC state does not exist for some microorganisms (Bogosian *et al.*, 1998; Watson *et al.*, 1998; Bogosian *et al.*, 1996). Bogosian *et al*. (1996) investigated the survival of *E. coli* K-12 strain in sterile and non-sterile soil and river water. As with other studies, results showed the number of culturable *E. coli* cells in non-sterile soil and water fell to below detectable limits over the sampling period of 12 days. The number of cells detected by acridine orange direct count (AODC) decreased initially but then levelled off to give the number of indigenous bacteria present in the soil or river water. Total viable counts using the DVC method however declined in accordance with plate counts suggesting that the *E. coli* cells were in fact non-viable. *E. coli* inoculated into sterile soil and incubated at 4, 20 and 37ºC

remained culturable over the observation period. The same was true for cells incubated in sterile river water at 4ºC and 20ºC; and in sterile seawater at 4ºC. At the other incubation temperatures the AODC remained constant at the initial level where-as plate counts and DVC decreased. The authors suggest that these results indicate that the non-culturable population of *E. coli* cells were intact, but non-viable (as measured by the DVC) and therefore the K-12 strain of *E. coli* does not enter a VBNC state. Attempts were made to resuscitate the non-culturable cells in a rich nutrient medium and by most probable number estimations but the cells remained non-culturable.

In a study investigating the survival of enteric bacteria in seawater microcosms, Bogosian *et al*. (1998) again suggested that the VBNC state did not exist. This study utilised a mixed culture recovery (MCR) method whereby two easily distinguishable strains of bacteria were mixed in such a way that large numbers of non-culturable cells of both strains were present together with a small number of culturable cells of only one strain. Nutrient addition and resuscitation procedures were performed on the mixture before the suspension is plated to determine whether both cells were recoverable. Subsequently, direct counting methods such as AODC, DVC, INT reduction and staining with the LIVE/DEAD *Bac*Light viability kit were utilised.

Results of this study (Bogosian *et al*., 1998) indicated that during the observation period (300 days) plate counts gradually declined to below detectable levels. The total number of cells detected using AODC method remained constant at the initial level of 3×10^8 cells. As with the authors' earlier study, the DVC paralleled the plated counts. However, over the entire sampling period cells stained with the *Bac*Light viability kit fluoresced green indicating cellular membrane integrity. Results from the MCR method illustrated that only cells of the culturable strain were recovered after application of various resuscitation techniques. The authors suggested that these results demonstrate that non-culturable cells are in fact dead and that the resuscitation observed in other studies was merely due to the growth of remaining culturable cells. It was pointed out that the staining of cells with the SYTO-9 dye (green fluorescence) and lack of staining with PI is interpreted in

other studies as an indication that non-culturable cells were still viable. The authors suggest that the fact that non-culturable cells may stain the same as culturable cells, it does not necessarily mean that they are viable. As evidence of a VBNC state, this study required non-culturable cells to be resuscitated. This demonstrates the lack of definition of the term "viability". In the case of this study viability was described as "the ability of a single cell to attain a population discernible by the observer". The nature of VBNC cells makes it difficult to determine viability using this definition. That is, cells which are termed VBNC are unable to be cultured using conventional methods. Methods such as those utilised by Pommepuy *et al*. (1996) involving the ability of the organism to produce toxin, may give more reliable results in terms of estimating microbial viability.

In summary, substantial research needs to be undertaken on the public health significance of VBNC organisms. To date there are conflicting views on the significance (or even existence) of the VBNC state. An extremely important issue which needs to be addressed with urgency is the development of a more accurate definition of the term "viable".

1.1.10 Association of microorganisms with suspended particles and sediment

Studies have indicated that microorganisms attached to suspended particles and sediment contribute greater numbers than those in the surface waters (Crump and Baross, 1996; Davies *et al.*, 1995; Fish and Pettibone, 1995; Shiaris *et al.*, 1987; Goulder, 1977). In a report on a study into the distribution of indicator organisms in intertidal sediments by Shiaris *et al*. (1987) it was demonstrated that the number of faecal coliforms present in sediments were two to four orders of magnitude higher than in the overlying water column. There is also evidence of the attachment of pathogenic *Vibrio* species to live copepods leading to enhanced survival (Binsztein *et al.*, 2004 ; Pruzzo *et al.*, 2003; Huq *et al.*, 1990; Tamplin *et al.*, 1990).

A study by Davies *et al*. (1995) investigated the survival of faecal microorganisms in marine and freshwater sediments using in-situ diffusion chambers. Results indicated that in the absence of predators, faecal coliforms and

faecal streptococci were able to grow in both marine and freshwater sediments. The presence of predators, however, resulted in a net die-off of the organisms. Over the duration of the sampling period (63 days) the differences in the number of organisms enumerated by plate counts compared with those detected by direct count methods did not change. This suggests that when associated with sediment both faecal coliforms and streptococci do not enter a VBNC state. The authors suggest that plentiful nutrients would be available to organisms exposed to sediment unlike those exposed solely to the water column (which is often associated with low nutrients). It is therefore unlikely that sediment-associated cells would enter a VBNC state under these conditions, suggesting a greater proportion of the total bacterial population being able to be cultured.

A similar result was observed by Fish and Pettibone (1995). When inoculated into sterile sediment, the number of *E. coli* present in the microcosm increased over the sampling period. It was also noted that the number of cells detected by plate counts were equal to those made by direct total and viable counts. When inoculated into water-only microcosms the number of *E. coli* as measured by all three methods declined over a two-week period. Results indicated that after two weeks incubation only 58% of the total number of *E. coli* present in the microcosm were viable as determined by INT reduction. Results of this study are limited in their comparison with actual environmental conditions. This is due to the sediment being used for the microcosms being sterilised by autoclaving. This not only prevents predation but may also lead to an increased release of nutrients from the sediment available to microorganisms.

A study by Crump and Baross (1996) demonstrated a positive relationship between heterotrophic bacterial activity as measured by ^{3}H-thymidine uptake and estuarine turbidity. The estuarine turbidity consisted of suspended detrital material which averaged 3% to 10% organic material by weight. These suspended particles undergo a cycle between the water column and the surface sediment layer. Goulder (1977) determined the concentrations and glucose mineralisation potentials (V_{max}) of attached and free bacteria in the Humber estuary. The Humber estuary had relatively high and variable levels of suspended solids (concentrations of between 48 to 763mg/L). Samples containing only free bacteria were obtained

by passing water samples through a 3.0μm pore size Nuclepore polycarbonate membrane. Results of the study demonstrated a significant ($p<0.001$) relationship between the number of attached bacteria and concentration of suspended solids. The number of attached bacteria in a whole sample was considerably greater than that of free bacteria. The V_{max} of attached bacteria was also greater than that of free bacteria. The author suggests that these results demonstrate that breakdown of organic matter is mainly carried out by attached bacteria.

Studies have indicated that survival of *Vibrio cholerae* in water is extended in the presence of live copepods (Tamplin *et al.*, 1990; Huq *et al.*, 1983; Kaneko and Colwell, 1978). A study by Huq *et al*. (1983) investigated the attachment of *V. cholerae* to live and dead copepods. Results demonstrated that both non-O1 and O1 serovars increased in number (up to 100-fold) in the presence of live copepods compared with cells incubated in the presence of cold-killed copepods. Examination of copepods by scanning electron microscopy (SEM) determined that the greatest concentration of cells occurred in the oral region and on the egg sac of the copepods. The authors suggest that an explanation for the attachment and multiplication on live compared to dead copepods may be that live copepods excrete growth-promoting or chemical attractant compounds specific to *V. cholerae*.

The role of zooplankton on the ecology of *V. cholerae* non-O1 and *Salmonella* spp. has been investigated by (Venkateswaran *et al.*, 1989). The attachment of bacteria to zooplankton was determined by comparing counts from water samples with those from homogenised zooplankton samples. Results indicated that the association of *Salmonella* spp. with zooplankton was below detectible levels throughout the sampling period even though it was isolated in water from all sampling sites. This study also indicated that temperature was the most influential factor in determining the distribution of *V. cholerae* non-O1, also with no significant association with zooplankton.

Spatial positioning in sediment can also be important in organism survival. An investigation into microbial growth rates and biomass production in marine

sediment was undertaken by Novitsky (1987), where microbial biomass as determined by ATP concentrations at the water-sediment interface, was measured as over twice that found in other sediment horizons (over a 100mm profile). Other measurements of growth rate such as biomass carbon production (determined by DNA synthesis) and the specific growth rate (determined from the kinetics of [^{3}H]ATP pool labelling) were also elevated at the interface. These results suggest that the greatest amount of microbial activity, growth and biosynthesis occurs within the first few millimetres of sediment. In a comparison between acridine orange direct counts (AODC) and adenine turnover it was demonstrated that over 90% of the sediment-water interface microorganisms were not actively growing. As suggested by Whitesides and Oliver (1997), non-viable microbial cells in marine sediments are very labile. Therefore, non-growing organisms identified in this study were likely to be dormant cells as opposed to intact, non-viable cells.

The association of pathogenic microorganisms with sediment and suspended particles is of particular public health significance. Sediment appears to be a potential reservoir for pathogenic microorganisms in the coastal environment. It is therefore necessary to determine the number of organisms from the surface sediment layer as well as the overlying water to better estimate environmental exposure to pathogenic microorganisms at recreational coastal sites.

1.2 RESEARCH OBJECTIVES

The primary objective of this research is to attempt to more accurately identify environmental exposure in the first stage of a health risk assessment in recreational coastal waters. This includes the specific aims of:

- Identifying efficient techniques for the separation of microorganisms from sediment particles.
- Investigating the use of methods to directly enumerate and determine viability of indicator microorganisms from aquatic environments.
- Determining the survival of *Escherichia coli*, enterococci, coliphage, *Salmonella typhimurium* and *S. derby* in coastal water and sediments using laboratory based microcosms.

- Determining the concentration of faecal indicator organisms in water and sediment from recreational coastal sites along the metropolitan Adelaide coastline.
- Undertaking a quantitative microbial risk assessment for recreational coastal waters, using results obtained form the microcosm and in-situ studies.

CHAPTER 2: GENERAL METHODOLOGY

2.1 INTRODUCTION

This chapter describes sampling sites and outlines methodologies used throughout the thesis including sediment characterisation, enumeration of microorganisms from coastal water and sediment and statistics used. Methodologies specific to individual research objectives are described in respective sections.

2.2 MATERIALS AND METHODS

2.2.1 Sampling sites

Sediment and water were sampled from coastal recreational sites situated along the greater Adelaide metropolitan area in South Australia (Fig. 2.1). These sites were Southport Beach, the Onkaparinga River (estuary), Glenelg North, Henley Beach South and the Pt Adelaide River. The key sites are shown in Plates 2.1 – 2.4. The Pt Adelaide River site was approximately 100 m from a wastewater treatment plant (WWTP) outlet. Water from this activated sludge plant undergoes chlorination prior to disposal. Samples were also taken from Normanville, a site that was not impacted by major development, approximately 60 kilometres south of Adelaide on the Fleurieu Peninsula (see Fig 2.1).

2.2.2 Sample collection

Triplicate water and sediment samples were taken at low tide from respective coastal sampling sites at a distance from the shore where the depth of water was approximately knee high (750 mm). Sterile 1 L plastic bottles were used to collect water samples. Intact sediment core samples for subsequent bacterial enumeration were taken using Perspex columns described in the laboratory based microcosm survival study (chapter 6). These Perspex columns (310 mm, 70 mm diameter) were inserted into the overlying water and sediment. The top of the column was capped with a rubber bung to aid the removal of the core from the sediment. The sediment core was kept in place by inserting neoprene (5 mm thick) and closed-cell foam (20 mm thick) bungs into the bottom of the core (Fig. 2.2).

2.2.3 Sediment characterisation

2.2.3.1 Sediment collection

Sediment samples for characterisation were taken using a sterile 50 mL polypropylene syringe with the narrow end removed. The sample was placed into a sterile container prior to being transported to the laboratory. Sediment samples were stored at 4ºC and analysed within 3 h of collection.

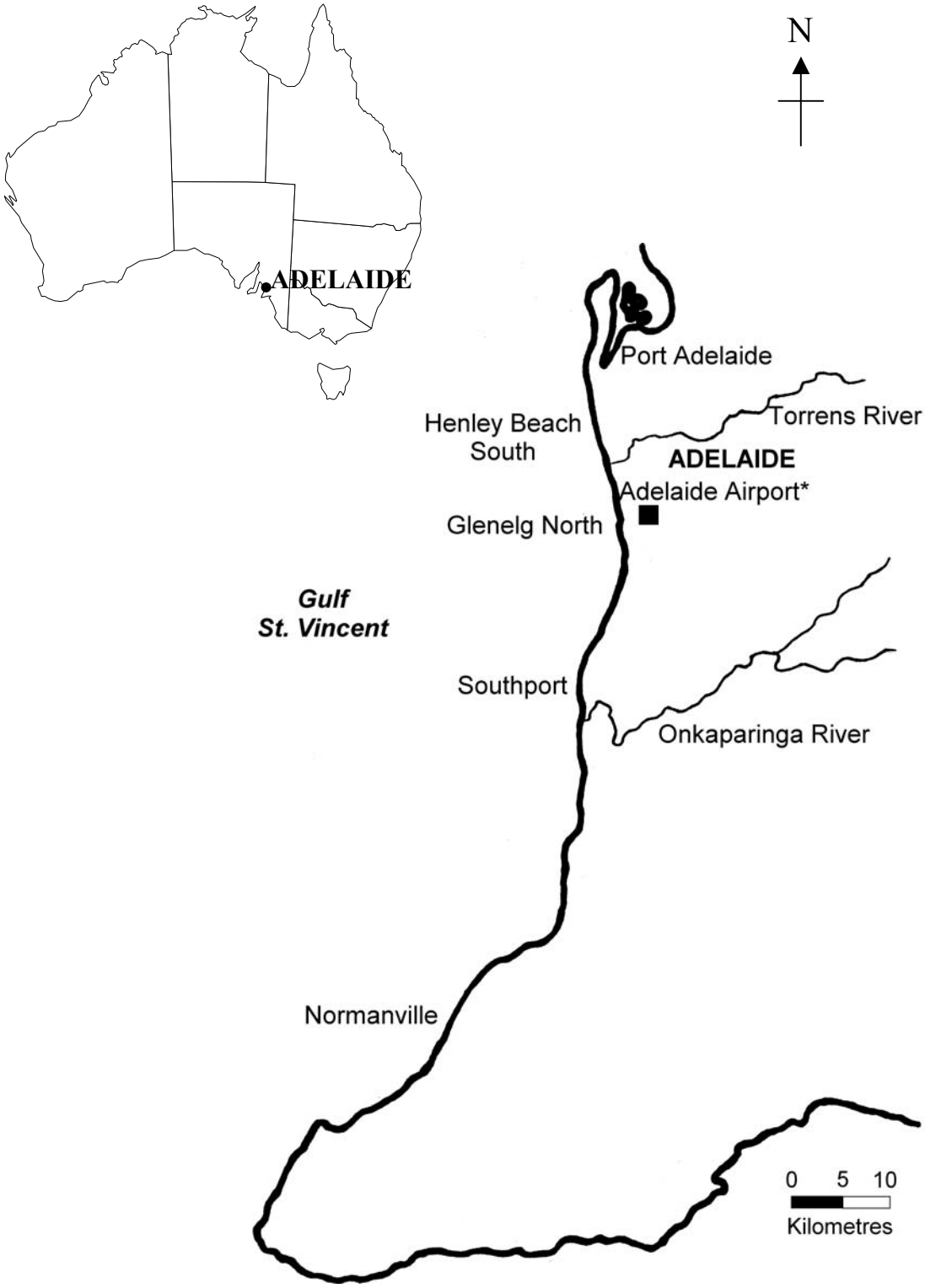

Figure 2.1 Location of sampling sites on the Adelaide metropolitan coastline, South Australia (*site for meteorological data).

Plate 2.1 Mouth of Onkaparinga River and Southport Beach.

Plate 2.2 Onkaparinga River being used for recreation.

Plate 2.3 Henley Beach South

Plate 2.4 Pt Adelaide River site. Note individual fishing at treated wastewater outlet.

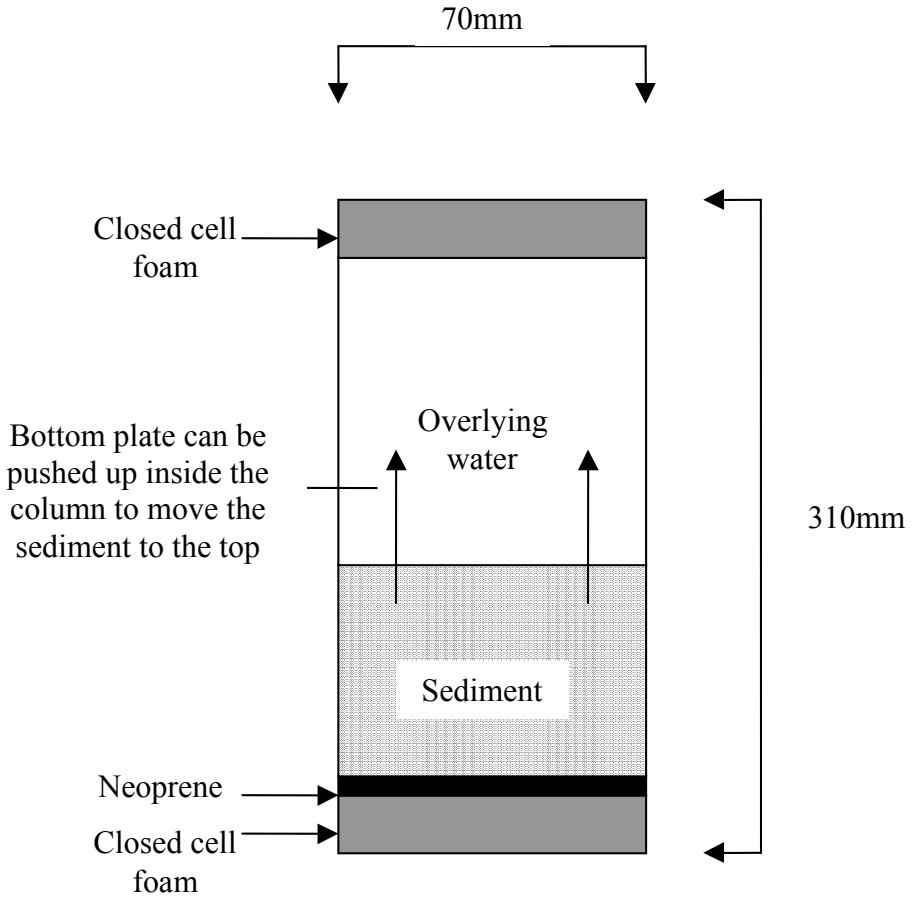

Figure 2.2 Column used for sampling intact sediment cores

2.2.3.2 Particle size analysis

The proportion of clay, silt and sand was determined using the pipette method (Sheldrick and Wang, 1993). The sediment was initially air dried and sieved (1.2 mm) to remover larger debris. To 10 g of this sediment, 10 mL of hydrogen peroxide (30% v/v) was added to remove any organic matter present. Excess hydrogen peroxide was removed by slowly heating the samples before adding 50 mL of dispersing agent (sodium hexametaphosphate, 0.06 mol L^{-1}). The samples were then placed in an orbital shaker (Ratek Instruments, Australia) at 100 rpm. for 24 h.

Sediment samples were transferred into one-litre cylinders and mixed with distilled water. The settling velocities, according to particle size, were calculated using a modified version of Stoke's Law and 20 mL of sediment suspension was removed at a depth of 8 cm from the surface at times corresponding to the particle sizes of interest (Sheldrick and Wang, 1993). The 20 mL sub-samples were placed into pre-weighed oven dried (105ºC for 24 h) glass vials and dried at 105ºC for 24 h. Results were expressed as a percentage of dry weight of the original sample.

2.2.3.3 Sediment organic carbon content

The dichromate method for the determination of oxidisable carbon and soil organic matter was used (Tiessen and Moir, 1993). An aliquot of 7.5 mL potassium dichromate (0.066 mol L^{-1}) solution was added to 1 g of air dried sediment and digested at 150ºC for 45 min. After cooling, the contents of the tubes were transferred into 250 mL conical flasks, rinsing the tubes with approximately 25 mL of distilled water to ensure that all of the dichromate was transferred. To this 7.5 mL of orthophosphoric acid (80%) and two drops of ferroin indicator were added. The solution was then titrated with approximately 0.2 mol L^{-1} ferrous ammonium sulphate solution to a brown end-point. Blanks of potassium dichromate were analysed in order to determine the exact molar concentration of ferrous ammonium sulphate. From this titration the amount of organic carbon present in the original sample was calculated.

2.2.4 Preparation of sediment samples for enumeration of microorganisms

The development of the technique used to separate microorganisms from sediment particles is detailed in Chapter 4. Sediment samples for microbiological analysis were obtained by first removing the remaining overlying water from the intact sediment core. The column was then placed on a coring device that extruded the sediment at controlled intervals (Plate 2.5). The top 10mm of sediment was removed and placed into a sterile beaker. Of this sediment, 25g (wet weight) was placed into 75 mL of sterile 0.1% peptone water (Oxoid, West Heidelberg, Australia). Sediment was sonicated in a sonication bath (700 W, 35 kHz, Cooper Vision Model 895, Irvine, CA, USA) for 10 min, stirred and sonicated a further 10 minutes to separate bacteria from sediment particles (Craig *et al.*, 2002).

2.2.5 Dry weight determination

To determine the dry weight content a known weight of wet sediment was placed in an oven at 105 ºC for 24 h and weighed. The percent dry weight was then calculated (by difference) from these results.

2.2.6 Enumeration of microorganisms

2.2.6.1 Faecal coliforms

Faecal coliforms in water and following extraction from sediment samples were enumerated by membrane filtration (47 mm diameter, 0.45 μm pore size, GN-6; Gellman, Pall Life Sciences, Lane Cove, Australia) and incubation on membrane lauryl sulphate agar (Oxoid) (Australian Standard AS 4276.7, 1995). Plates were incubated at 30°C for 4 hours, followed by 44ºC for 18 h. For *in-situ* sampling, a representative number of presumptive faecal coliform colonies (square root of the number of CFU) were incubated at 44°C for 24 h in EC broth (Oxoid) and confirmed as faecal coliforms by production of gas. The presence of *E. coli* was confirmed by sub-culturing positive faecal coliform colonies into tryptone water (Oxoid) and incubations at 44°C for 24 h with the subsequent production of indole detected using Kovac's reagent.

Plate 2.5 Coring device used to extrude sediment at controlled intervals.

2.2.6.2 Enterococci

Enterococci were enumerated by the Enterolert™ defined substrate method (IDEXX Laboratories, Maine, USA). Water or sediment samples (up to 10 mL) were added to sterile 120 mL containers and the volume made up to 100 mL with sterile distilled water. Enterolert reagent was added to the sample, mixed thoroughly and transferred to 97-well Quantitrays. Trays were heat sealed and incubated at 41°C for 24 h. Enterococci were enumerated by the presence of fluorescence under a UV lamp (365 nm) and expressed as most probable number (MPN) 100 mL^{-1}.

2.2.6.3 Somatic Coliphage

Host *E. coli* (FCC 84) cells were prepared by inoculation into 10 mL nutrient broth (Oxoid) and incubation overnight at 37°C and stored at 4°C until use. Coliphage was enumerated by a double-agar overlay method. Briefly, 4 mL of sample, or an appropriate dilution, was added to 4 mL molten nutrient agar (maintained at 50°C in a water bath) and 250 μL of host *E. coli* suspension, mixed and poured onto a petri dish containing a base layer of nutrient agar. Plates were incubated at 37°C for 24 h. Results were expressed as plaque forming units (PFU) 100 mL^{-1}.

2.2.6.4 Salmonella

Presumptive salmonellae were enumerated from water and sediment by membrane filtration (GN-6; Gellman) and incubation in XLD agar (Oxoid) at 37ºC for 24 h (Fish and Pettibone, 1995). A representative number of presumptive colonies were confirmed as *Salmonella* spp. using a salmonella latex agglutination test kit (Serobact; Medvet Science, Adelaide, Australia).

2.2.7 Determination of decay rate constants

The decay rate constant (k) was calculated as the slope of the line when $\log_{10}$ ($N_t.N_0^{-1}$) was regressed against time, where N_t is the number of bacteria at time t and N_0 is the number of bacteria at time 0 (Davies *et al.*, 1995). The more negative the decay rate, the greater is the disappearance of microorganism. The applicability of using the exponential decay model in the microcosm study was

determined by linear regression. The decay rate constant could then be used to calculate T_{90} values, which is the time required for a 1-$\log_{10}$ reduction in organism concentration (Pesaro *et al.*, 1995).

2.2.8 Meteorological data

Daily maximum and minimum temperature (ºC) and total rainfall (mm) data were obtained from a weather station located at the Adelaide Airport (Bureau of Meteorology, Adelaide).

2.2.9 Statistical analysis

Statistical analysis of data was undertaken using SPSS (Version 10.0, 1999; SPSS, Illinois, USA). Graphs were produced using Excel 97 (1996; Microsoft Corporation, USA) or Graphpad (Version 2, 1995; Graphpad software, California, USA). Monte-Carlo simulations were performed using @Risk (version 4.0.5, 2000; Palisade Corporation, Newfield, USA) which runs as an add-in to Microsoft Excel. Data were analysed using linear regression, bivariate correlation, ANOVA and Bonferroni post-hoc tests to determine whether results were significantly different as discussed in the following chapters. Statistical significance was expressed when $P \leq 0.05$. Results were generally expressed as the mean ± standard deviation (SD) of three determinations, unless otherwise stated.

CHAPTER 3: DIRECT COUNTING TECHNIQUES FOR THE ENUMERATION OF INDICATOR ORGANISMS

3.1 INTRODUCTION

Flow cytometry has been suggested as a rapid technique for the direct enumeration of organisms from aquatic environments (Diaper and Edwards, 1994; Porter *et al.*, 1993; Vesey *et al.*, 1993; Monfort and Baleux, 1992). Conventional microbial techniques for enumerating aquatic organisms often involve pre-enrichment and/or culturing on selective media at a specific temperature. These processes usually take a number of days to confirm the presence of specific microorganisms in the environment. As discussed in Chapter 1, pathogenic microorganisms released into the aquatic environment may also be able to enter a viable but non-culturable (VBNC) state (Whitesides and Oliver, 1997; Roszak *et al.*, 1984). Direct enumeration methods utilising fluorescent antibodies and viability dyes may potentially allow the detection of such microorganisms.

The underlying principle of flow cytometry is the passing of single particles in suspension through a light source (typically an argon laser) and the simultaneous measurement of degree of light scatter and/or fluorescence via detectors (Bernander *et al.*, 1998). A forward angle light scatter detector is used to give an indication of particle size whereas a side scatter detector (measures light scattered at 90° from the light source) gives an indication of particle surface and internal characteristics (Yamaguchi and Nasu, 1997; Vesey *et al.*, 1993). In addition to light scatter detectors, fluorescence detectors are used to detect intensities of fluorescence at specific wavelengths. For this study, two fluorescence detectors were used to detect particles stained with FITC-conjugated monoclonal antibodies and/or propidium iodide (PI).

The aim of this research question was to assess the ability of flow cytometry to detect viable bacteria, with the ultimate aim being the detection of these organisms in aquatic environments. *Escherichia coli* was used as the test organism due to its widespread use as a faecal indicator.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of *E. coli* cells

Escherichia coli (ATCC 25922) was inoculated into nutrient broth and incubated overnight at 37°C. Cells were harvested by centrifugation at 8000g for three minutes and resuspended in 1 mL of phosphate buffered saline (PBS; 0.1 mol L^{-1}; pH 7.2). For non-viable determinations, *E. coli* cells were permeabilised by resuspending the pelleted sample in 1 mL of 70% iso-propyl alcohol and incubating at room temperature for one hour (Roth *et al.*, 1997). After incubation, cells were washed twice in 0.1 mol L^{-1} PBS before being resuspended in 1mL PBS. All cell suspensions were stored at 4°C prior to use.

3.2.2 Staining of *E. coli* cells

Approximately 1×10^5 *E. coli* cells were stained with fluorescein isothiocyanate (FITC) conjugated *E. coli* antibodies (Biodesign International, Maine, USA; cat. no. B65003R). Antibody titres were undertaken to identify optimal antibody concentration for staining *E. coli* cells. Preliminary results suggested resuspension of *E. coli* cells in 50 μL of a 1:10 dilution of neat antibody provided adequate staining. Following incubation with antibody at room temperature for 30 min, cells were washed in PBS by centrifugation.

To determine cell viability, different volumes of 1 mg L^{-1} propidium iodide (PI; Sigma) in PBS solution was added to pure *E. coli* cell suspensions and incubated in the dark at 37°C for 30 min. The volumes of PI solution added were 10, 20, 30, 40 and 50 μL. Due to samples in this preliminary study containing only *E. coli*, the FITC-conjugated antibody was not added. After incubation, cells were washed by centrifugation prior to being resuspended in 50 μL PBS for detection by flow cytometry.

The proportion of viable to non-viable *E. coli* cells was varied (from 0% - 100%) to determine if propidium iodide staining could be accurately used to indicate the relative proportions of viable and non-viable *E. coli* cells in pure culture (the total number of cells remained constant). The estimated proportions of viable and non-

viable *E. coli* cells determined by plate count could then be compared with that enumerated by flow cytometry.

Dilutions of non-viable *E. coli* cells, from approximately 10^4 to 10^1 cells, were stained with PI and analysed to determine the limit of detection of *E. coli* in pure culture using flow cytometry.

3.2.3 Flow cytometry

A FACScan (Becton Dickinson, North Ryde, Australia) flow cytometer was used for the detection of *E. coli* cells. To enable the detection of cells stained with propidium iodide, results from the flow cytometer were expressed as a histogram of fluorescent intensities measured using a fluorescent detector consisting of a 585 ± 45 nm light filter and voltage setting of 700 V. Cells stained with FITC conjugated antibodies were detected using a fluorescent detector with a 530 ± 30 nm light filter and voltage setting of 620 V.

The volume of sample analysed by the flow cytometer (necessary to determine the concentration of bacterial cells) was determined using TruCount tubes (Becton Dickinson). These tubes contain a known number of fluorescent beads which can be detected as a separate peak on a histogram by flow cytometry. Stained *E. coli* cells were transferred into Tru-Count tubes, mixed with a vortex mixer and incubated at room temperature for 15 min (as described by manufacturer). Samples not analysed immediately prior to staining were stored at 4°C in the dark.

3.2.4 Microscopy

To visually determine the effectiveness of FITC conjugated antibody and propidium iodide to bind to *E. coli* cells, samples were filtered by syringe through black membrane filters (25mm diameter, 0.2 μm pore size; GTBP, Isopore®) and placed on a microscope slide. A drop of immersion oil and a cover-slip were placed over the filter for protection and to prevent drying. Slides were viewed using an Olympus Vanox epifluorescent microscope at a magnification between ×400 – ×1000. To view FITC-stained cells, an Olympus U filter block was used

with excite/dichroic/emit properties of 470-490/505/515-550 nm. For PI, a modified Olympus G filter block was used with excite/dichroic/emit properties of 530-550/570/>590.

3.3 RESULTS

3.3.1 Viability determination using propidium iodide

Initial results using pure *E. coli* cultures indicated that the addition of propidium iodide resulted in an increased fluorescent intensity of non-viable cells as measured at 580 nm, compared to samples with no PI added (Figure 3.1 a – c). This was confirmed by microscopy, where propidium iodide was visually confirmed to bind to non-viable cells (Plate 3.1). When propidium iodide was added to viable cells, only a relatively small proportion of cells were stained (Fig. 3.1c). The addition of more than 30 μL of propidium iodide solution did not result in an increase of fluorescent intensity as measured by flow cytometry (Figs. 3.2a – d). Therefore, 30 μL of propidium iodide was used as the working volume to stain non-viable *E. coli* cells.

Results for the enumeration of various proportions of non-viable *E. coli* cells (those cells bound with PI) by flow cytometry is summarised in Table 3.1. The intensity of fluorescence of the beads from the Tru-Count tube was significantly higher than stained *E. coli* cells, resulting in two separate peaks (Fig. 3.3), enabling the number of individual particles detected under both peaks to be determined. For a sample containing 100% non-viable *E. coli* cells (those treated with 75% iso-propyl alcohol), the total number of cells stained with PI was determined to be 44,904 ± 2167 mL^{-1}. For the sample containing only viable cells, the number of PI bound cells was 8,876 ± 624 mL^{-1}. No direct correlation was observed for decreasing proportions of non-viable *E. coli* cells as determined by plate count and flow cytometry.

The limit of detection was determined by analysing dilutions of non-viable *E. coli* cells stained with 30 μL PI. The histogram from the highest dilution (approximately 10^4 cells) indicated an adequate separation of peaks of those cells stained with propidium iodide from background fluorescence (Fig 3.3a). For samples containing lower numbers of cells, there was not a corresponding decrease at the same fluorescence peak. To determine the amount of non-specific binding of propidium iodide with other particles, samples containing only PBS were stained and analysed. There appeared to be very little background binding

(Fig 3.3 f). It was determined that 10^3 *E. coli* cells was the limit of detection using flow cytometry.

3.3.2 Detection of *E. coli* using FITC conjugated monoclonal antibodies

Preliminary results indicated that the addition of 50 μL of a 1:10 dilution of original antibody concentration resulted in optimum binding with *E. coli* cells in pure culture (Fig. 3.4). When dilutions of the original *E. coli* concentration (approximately 10^4 cells) were made, there was not a corresponding decrease in number cells bound with antibody as detected by flow cytometry. When antibody was added to samples containing no *E. coli* (only PBS) there was a significant amount of background binding. In an effort to overcome this problem, various washing techniques were investigated. As for propidium iodide staining, this resulted in a loss of fluorescence for samples containing greater than 10^4 *E. coli* cells (Fig. 3.5).

Problems associated with the binding of antibody to particles in the PBS media inhibited the detection of less than 10^4 *E. coli*. In addition to this, the degree of binding of antibody to *E. coli* was highly variable. This is considering that the methodology for preparing and staining of the cells remained constant throughout the experiment. Attempts were made to increase the efficiency of antibody labelling by using fresh *E. coli* cultures and changing incubation conditions but with limited success.

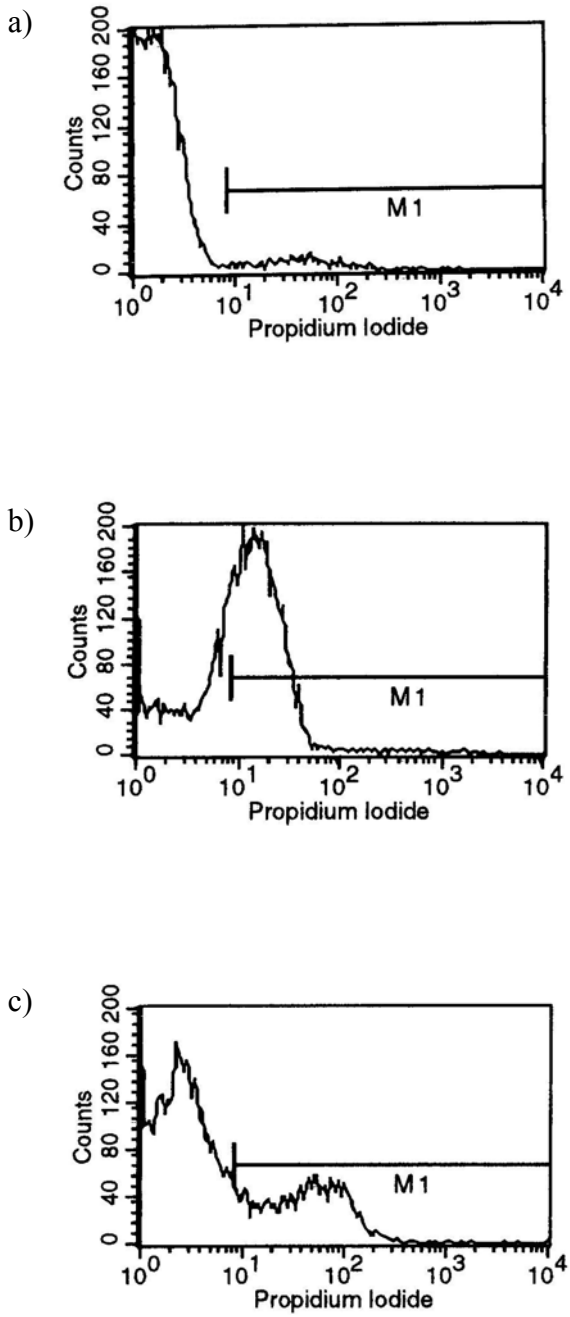

Figure 3.1 Histogram of fluorescent intensities measured at 580nm for samples stained with 30μL propidium iodide containing a) PBS only, b) 10^4 non-viable *E. coli* cells, c) 10^4 viable *E. coli*.

Plate 3.1 Non-viable *E. coli* cells stained with 30 μL of propidium iodide solution (×1000 magnification).

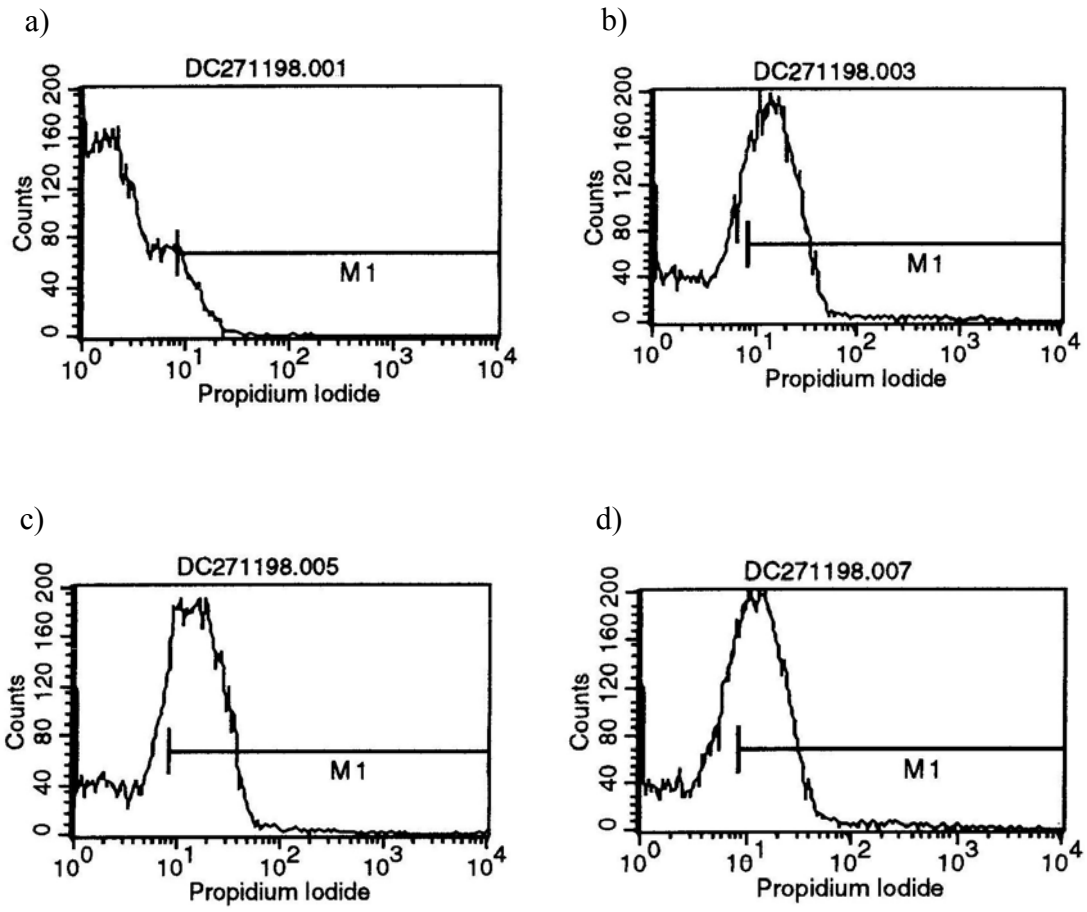

Figure 3.2 Histogram of fluorescent intensities measured at 580nm (to detect PI fluorescence) for samples containing 10^4 non-viable *E. coli* stained with a) 20 µL b) 30 µL, c) 40 µL and d) 50 µL PI solution.

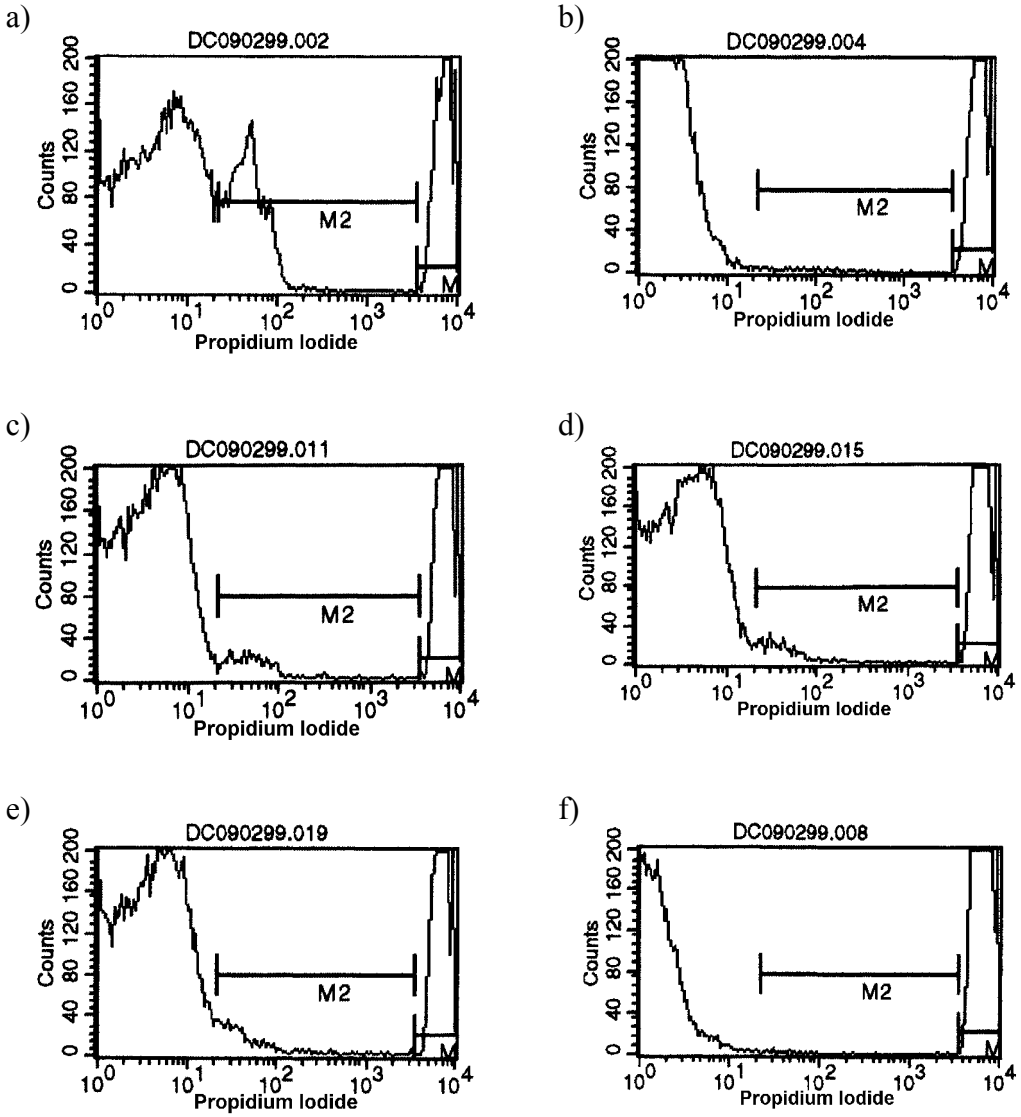

Figure 3.3 Histogram of fluorescent intensities measured at 580nm (to detect PI fluorescence) for samples containing approximately a) 10^4 non-viable *E. coli* cells + PI, b) 10^4 cells without PI added, c) 10^3 cells + PI, d) 10^2 cells + PI, e) 10 cells +PI and f) no cells (0.2 μm filtered PBS) + PI.

Table 3.1 Number of non-viable *E. coli* cells detected by flow cytometry stained with propidium iodide (mean ± SD; n=3).

Sample	**Number of cells mL^{-1} ± SD**
100% Non-viable (0% viable)	44904 ± 2167
75% Non-viable (25% viable)	20739 ± 851
50% Non-viable (50% viable)	23033 ± 804
25% Non-viable (75% viable)	16658 ± 1678
100% Viable (100% viable)	8876 ± 624

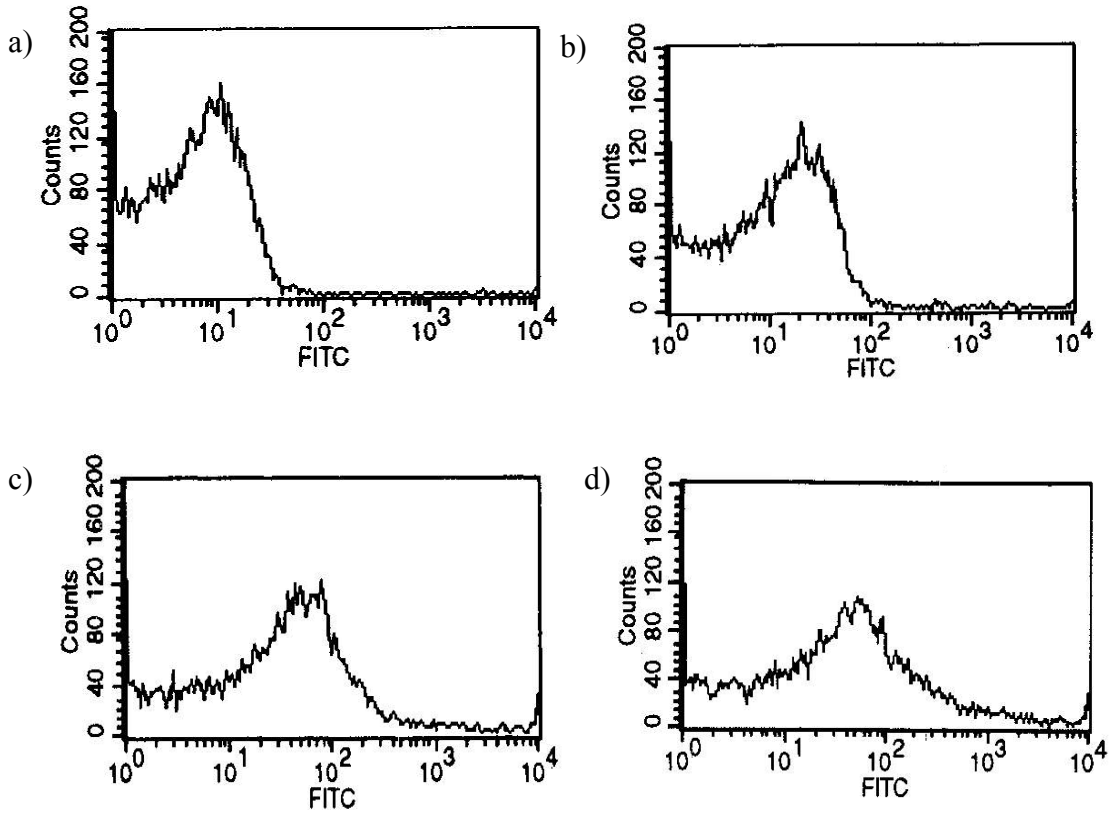

Figure 3.4 Histogram of fluorescent intensities measured at 530nm (to detect FITC fluorescence) for samples containing 10^4 *E. coli* stained with a) 10μL antibody, b) 25μL antibody, c) 50μL antibody and d) PBS only + 50μL antibody.

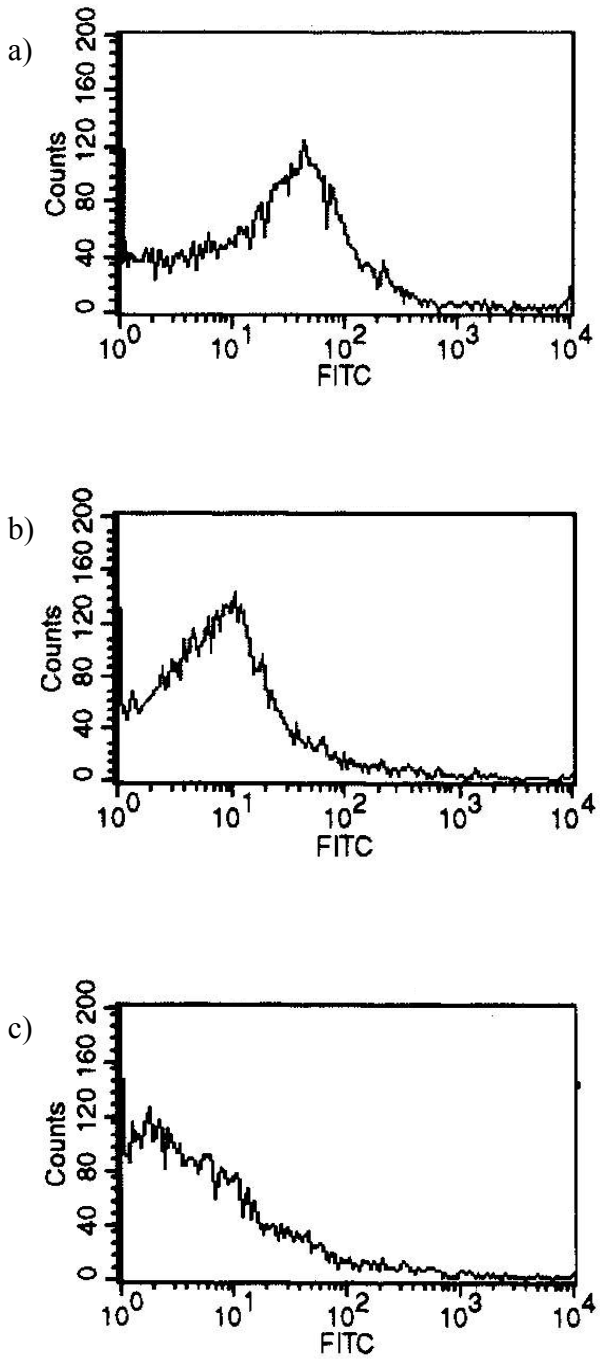

Figure 3.5 Histogram of fluorescent intensities measured at 530nm for samples containing 10^4 *E. coli* stained with 50µL antibody washed a) once, b) twice, c) three times in PBS prior to analysis.

3.4 DISCUSSION

Preliminary results of this research suggested that propidium iodide could be used to determine cell viability by indicating membrane permeability. When analysing non-viable *E. coli* cells, there was an increased intensity of fluorescence at 585 nm (Figs. 3.1 b and c), indicating non-viable *E. coli* cells were binding with propidium iodide compared with the reduced fluorescence observed for viable cells.

Further investigation involved varying the proportion of non-viable to viable cells. There was a demonstrated decrease in the number of cells stained with propidium iodide detected by flow cytometry in samples contained 100% non-viable compared with 100% viable cells (while maintaining a total cell concentration of approximately 10^4). For the sample containing 100% viable *E. coli* cells, as determined by standard plate count, approximately 20% may by non-viable as determined using direct counting methods. However, a direct correlation between the number of cells detected by flow cytometry with those estimated by standard plate counts was not observed in samples containing between 75% - 25% non-viable cells. This suggested that under the experimental conditions, propidium iodide may not enable the accurate determination of relative proportions of non-viable cells in a mixed population.

When analysing serial ten-fold dilutions of *E. coli* suspensions it was demonstrated that for samples containing less than 10^4 cells, it was difficult to separate the peak fluorescence from background fluorescence. When propidium iodide was added to samples containing no *E. coli* (only PBS) there was only a limited amount of fluorescence detected (Fig. 3.3 b). This suggests that the propidium iodide was not indiscriminately binding to particles present in the media. It is therefore likely that a significant proportion of cells with intact membranes (viable) were binding with propidium iodide. In an attempt to overcome this problem, stained samples were washed twice in PBS prior to analysis. This only resulted in a significant decease in fluorescence of non-viable cells.

The relatively poor limit of detection of 1×10^4 *E. coli* cells mL^{-1} may explain the problems associated with the determination of various concentrations of non-viable and viable cells described earlier. When the number of non-viable cells (those detected by flow cytometry) were decreased in proportion to viable cells, there were less non-viable cells present than the limit of detection.

The degree of background binding of FITC conjugated monoclonal antibody severely limited the ability to enumerate *E. coli* by flow cytometry. At concentrations of less than 10^4 *E. coli* ml^{-1} it was impossible to separate the peak fluorescence from the background fluorescence. The addition of antibody to samples containing only PBS resulted in significant peaks at 530 nm. This suggests that there was a significant level of background binding of antibody to particles present in the PBS. Including additional washing steps to remove non-specific antibody binding resulted in a decreased fluorescence of bound *E. coli* cells.

Other studies investigating the use of flow cytometry for the detection of bacterial cells have found similar results to those presented here (Clarke and Pinder, 1998; McClelland and Pinder, 1994). McClelland and Pinder (1994) used a combination of R-phycoerythrin (R-PE) conjugated monoclonal antibodies and the fluorescent nucleic acid dye ethidium bromide to detect *Salmonella* spp. The total number of cells was held constant while the number of specific salmonella species was reduced 10-fold from 3×10^8 to 3×10^3 cells mL^{-1}. Direct labelling of the sample with monoclonal antibodies resulted in inefficient staining. In comparison, an indirect labelling method was found to give both more effective labelling of cells and a decrease in the amount of background fluorescence. This method involves staining the sample with a primary antibody specific for the organism of interest, then adding a secondary antibody containing the fluorescent label which is specific to the primary antibody. It was found this method resulted in the ability to detect *Salmonella* spp. in mixed populations at levels below 10^4 cells mL^{-1}. McClellan and Pinder (1994) also described a method of sample enrichment (6 h) to lower the level of detection to <1 cell mL^{-1}. As an enrichment step is required,

this method only provides an indication of the presence/absence of *Salmonella* spp. at these low concentrations.

A study by Clarke and Pinder (1998) used a different combination of cell viability dye and fluorescent antibody label. The commercial viability dye Chemchrome fluoresces green when cleaved by specific enzymes present only in viable bacteria. The authors found that this viability dye resulted in lower levels of background binding compared with other dyes that label DNA. Due to the emission spectrum of Chemchrome being similar to that of FITC, monoclonal antibodies were labelled with the dye streptavidin-RED613, which fluoresces red. The number of viable *Salmonella typhimurium* cells analysed ranged from 10^7 to 10^2 cells mL^{-1}. The authors suggested the method used in the study was sensitive down to 10^2 viable cells mL^{-1}.

Flow cytometers exist which not only detect particles of interest but also sort these particles (based on measured properties) for further investigation while discarding background particles. This process, using a modified flow cytometer is often termed fluorescence-activated cell sorting (FACS) or flow cytometer cell sorting (FCCS) (Medema *et al.*, 1998; Vesey *et al.*, 1993). This is achieved by breaking the flow stream into droplets, charging the droplets containing the desired cells/particles and using voltage deflection to steer these droplets into separate containers or slides (Mendelsohn, 1980). The major benefit of this method is the ability to sort cells of interest from background particles and then confirm the presence of these organisms by epifluorescence microscopy (Vesey *et al.*, 1993). This method enables the rapid analysis of samples containing a large proportion of background particles which would provide extremely difficult if analysing using microscopy alone. At the time of undertaking this research, a cell sorting flow cytometer was not available, which prevented the visual confirmation of detected particles. Direct viewing of particles detected by the flow cytometry using epifluorescence microscopy would greatly facilitate the fine-tuning of the staining and detection methodology used in this study.

The benefit of flow cytometry for the detection of *E. coli* is that it counts cells directly and takes significantly less time to analyse samples compared with

conventional culture methods. The experiments described here used pure cultures. If this method was used to detect *E. coli* cells in mixed populations (for example from environmental samples) it would be anticipated that there would be even greater non-specific binding of antibody. As discussed in Chapter 1, recent advances in fluorescent in-situ hybridisation techniques may provide more specific binding properties than antibody techniques. However, issues still exists for the determination of bacterial viability and further research needs to be undertaken on the public health significance on the detection of such organisms.

The relatively high limit of detection would also prevent the effectiveness of this method for analysing environmental samples, such as recreational coastal water and sediment, with the limit of detection being greater than related guideline values. This method was therefore not continued in further research undertaken for this study.

CHAPTER 4: TECHNIQUES FOR THE SEPARATION OF MICROORGANISMS FROM SEDIMENT PARTICLES

The main content of this chapter has been published as:

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2002) Enumeration of faecal coliforms from recreational coastal waters and sediments: evaluation of techniques for the separation of bacteria from sediments. *Journal of Applied Microbiology*. 93(4): 557-565.

4.1 INTRODUCTION

The attachment of microorganisms to sediments poses many problems in regard to their subsequent detection and enumeration. If samples are viewed by microscopy, microorganisms may be obscured by sedimentary particles, or particles can effectively increase the working distance of the microscope objective causing a distortion of the image (McDaniel and Capone, 1985). Alternatively, when enumerated by conventional culture techniques such as spread plates or membrane filtration, microorganisms attached to sediment particles may not be randomly distributed across the surface of the media.

In most circumstances it is necessary to physically separate the microorganisms from the sediment particles. Methods other than manual shaking of samples include sonication and homogenisation but there is disagreement as to which method is most effective. Dale (1974) concluded that homogenisation of sandy sediment at 23,000 rev min^{-1} for five minutes yielded greater numbers of bacteria than shaking by hand or sonication. Other studies have determined treatment by an ultrasonic bath (40 - 50 kHz, 100 - 200 W) to be more effective than homogenisation (Ellery and Schleyer, 1984). Epstein and Rossel (1995) concluded for small sediment samples ($\leq$ 0.5 cm^3), optimal enumeration of bacteria was achieved using an ultrasonication probe (5 mm tip, 20 kHz, 100 W) for 180 s. As the results of these studies suggest, it is not the type of treatment alone that determines its effectiveness but also power outputs and frequencies of treatment. It is important that the separation technique is powerful enough the remove the microorganism from the particle without harming the organism (Epstein and Rossel, 1995; Ellery and Schleyer, 1984).

To enable estimation of the number of microorganisms in sediment, as distinct from the water column, it is necessary to develop methods to successfully separate these organisms from sediment particles. This chapter aims to identify the most efficient technique for the separation of bacteria from different types of coastal sediment found at recreational sites in the greater metropolitan Adelaide area.

4.2 MATERIALS AND METHODS

4.2.1 Sample collection

Sediment samples were taken from the six recreational coastal sites as described in Sections 2.2.1 and 2.2.3.1. Samples were transported and analysed within three hours of collection.

4.2.2 Separation techniques.

Approximately 1 g aliquots of well mixed wet sediment were each placed in 9 ml of 0.1% peptone water (Difco). These samples were then subjected to the various treatments, listed below. After treatment, the sample was left to settle for 10 minutes prior to aspirating the supernatant. Appropriate dilutions of this supernatant were made and 0.1 ml of each dilution was spread plated onto vibrio artificial seawater (VAS) agar and incubated for 48-72 hours at 20°C (Malmcrona-Frieberg *et al.*, 1990). Results were expressed as cfu g^{-1} (dry weight) of sediment.

The separation techniques investigated included hand shaking, use of a sonication bath (700 W, 35 kHz, Cooper Vision Model 895, Irvine, CA, USA) for six and ten min respectively and a sonication probe (100 W, 20 kHz, 19 mm probe, Branson Model B12, Danbury, CT, USA) for 15 s and one minute respectively. When using the sonication probe, samples were cooled in an ice slurry. Hand shaking involved vigorously shaking the sample by hand over a radius of approximately 300 mm for 1 min.

In order to determine the recovery efficiency for each treatment method, the original sedimented material (with supernatant removed) from each initial treatment was resuspended in 9 ml peptone water and separation treatment repeated. The resulting supernatant was again plated and counted as above.

4.2.3 Statistical analysis

Statistical analysis was undertaken using one-way ANOVA and significance expressed at $P \leq 0.05$ (SPSS version 10.0.5, 1999). The Bonferroni post hoc test

was used to compare means between test treatments to determine whether treatments were significantly different.

4.3 RESULTS

4.3.1 Sediment characterisation

Particle size analysis showed that the coastal sediments could be divided into two broad categories. Sediment samples from Normanville, Southport, Glenelg North and Henley Beach South consisted of mainly sand while the samples from the Onkaparinga River and the Pt Adelaide River had higher proportions of silt and clay (Table 4.1). Samples from these latter sites also comprised a higher organic carbon content. At the Pt Adelaide River the sediment was distinctly stratified (Plate 4.1), with the top 20 mm layer comprising anaerobic horizon with a high silt, clay and organic carbon content and below that a layer consisting of a mixture of sand and silt/clay. Only the surface layer (≤ 20 mm) of this sediment was evaluated as this would provide the main source of exposure to microorganisms in any recreational activity.

Table 4.1 Particle size analysis. Particles sizes for sand 2 – 0.2 mm; silt 0.2 mm – 2 μm; clay < 2 μm. All results normalised to 100%.

SITE	%SAND	%SILT	%CLAY	%ORGANIC C
Glenelg Nth	98.45	0.20	1.30	0.05
Henley Beach Sth	98.47	0.08	1.41	0.05
Southport	98.54	0.04	1.36	0.06
Normanville	98.88	0.11	0.93	0.07
Onkaparinga	95.48	1.26	2.91	0.35
Pt Adelaide (≤ 20 mm)	83.05	4.24	10.33	2.38
Pt Adelaide (≥ 20 mm)	90.43	3.20	5.57	0.80

Plate 4.1 Stratified sediment at Pt Adelaide.

Plate 4.2 10 g of sediment from each site added to 1 L distilled water and allowed to settle for six hours. From left to right, Normanville, Southport, Onkaparinga, Glenelg North, Port Adelaide and Henley Beach

4.3.2 Separation of microorganisms from sediment particles

For the sandy sites, all sonication techniques resulted in significantly greater number of organisms being enumerated from sediment compared with manual shaking (Fig. 4.1). However, treatment of the sample by a sonication bath for ten minutes provided significantly higher numbers of CFU's compared with other techniques investigated ($P < 0.05$). For the two sites with greater silt and clay contents (Onkaparinga River and Pt Adelaide River), hand shaking was the most effective separation technique ($P < 0.05$ for Onkaparinga sediment).

The initial recovery efficiency of using the sonication bath (10 min) was tested. The results illustrated that repeating the treatment in the sonication bath for 10 min resulted in only slightly improved recovery of microorganisms for the sandy sites, however it resulted in a great improvement for clay sediments (Fig. 4.2). The mean efficiency of initial treatment for sandy sediment types was 86% (range 81.2% - 90.8%). This suggests that treatment by sonication bath for ten minutes is a relatively efficient method for removing bacteria from coastal sediments with high sand content. In the first instance, this method was not as efficient for sediments from Pt Adelaide River and Onkaparinga River with a mean initial recovery efficiency of 62% (range 60.3% - 64.0%).

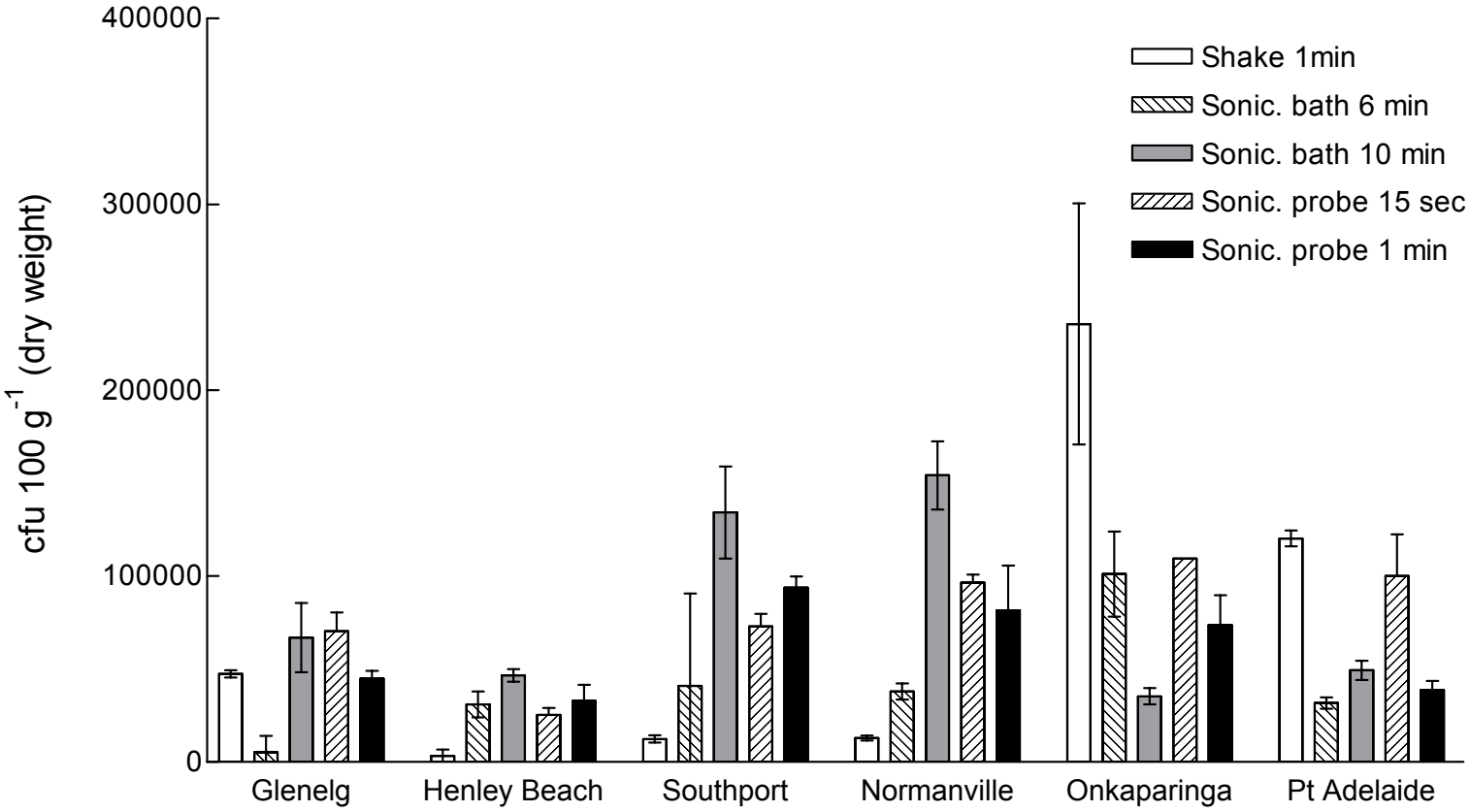

Figure 4.1 Comparison of separation techniques (mean ± standard deviation) for the enumeration of bacteria from coastal sediment. Sampling sites are ordered (from left to right) from greatest to least sand content (n=3).

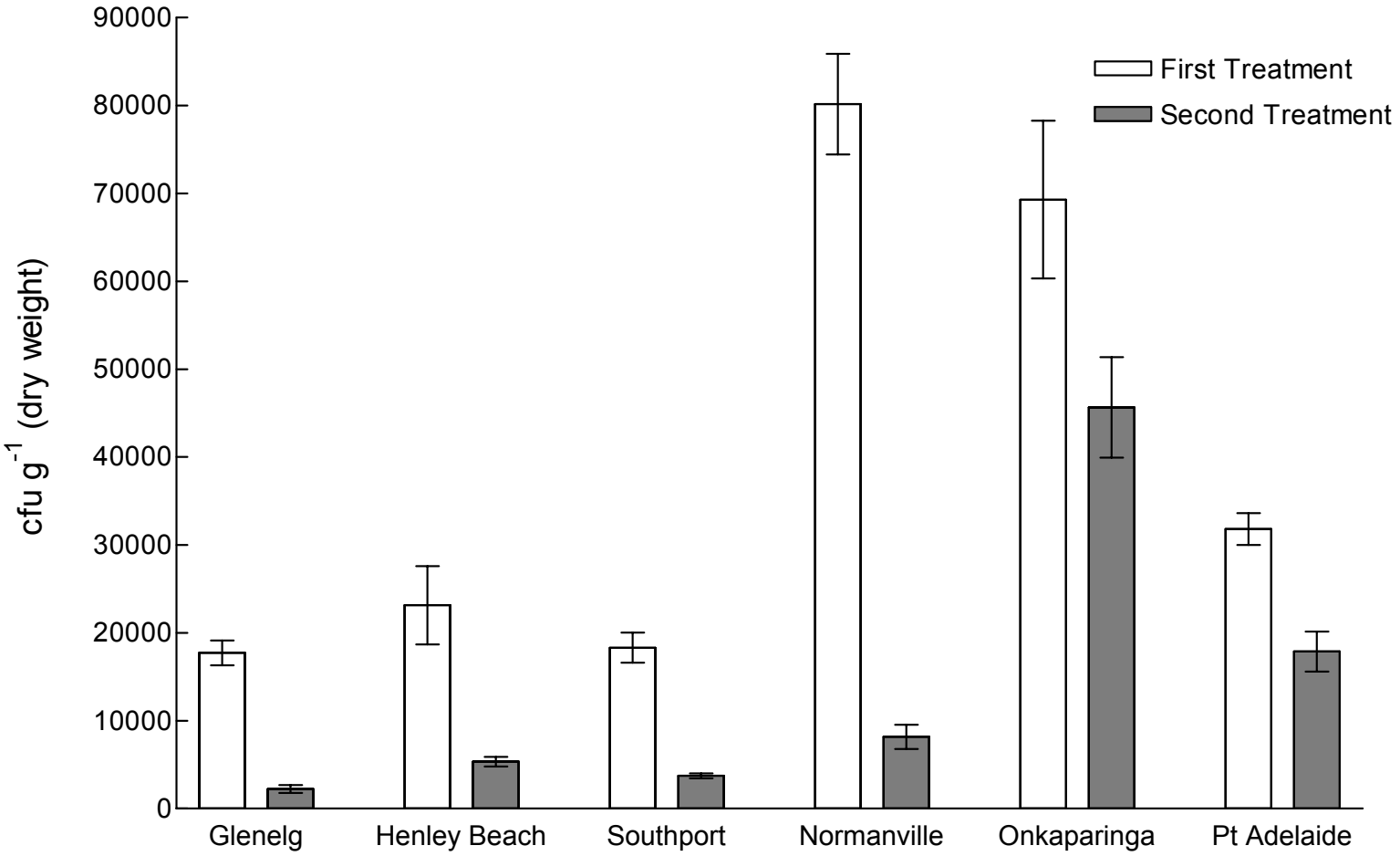

Figure 4.2 Recovery efficiencies using the sonication bath for 10 minutes. Sediment from the original sonication treatment was resuspended and treatment was repeated (n=3).

4.4 DISCUSSION

The coastal sediments investigated demonstrated significant variability in both particle size and organic carbon content. Results demonstrated for sediments consisting mainly of sand, treatment by sonication bath for 10 min resulted in the release of higher numbers of organisms compared with manual shaking. For sediments consisting of greater proportions of silt/clay, manual shaking resulted in higher numbers of organisms enumerated, however the sonication technique proved to be much more effective on the second treatment. The influence of sediment characteristics on the effectiveness of separation techniques suggests that the binding properties between the bacteria and the particle are highly dependent on the size and composition of the particles. This confirms findings of McDaniel and Capone (1985) who noted that there were significant differences in the effectiveness of separation techniques with the type of sediment being studied. Their results showed that when determining the most efficient treatment method, it is also necessary to establish the optimal treatment time. Cell death or injury may result due to vibration or heating of the sample over the course of the sonication treatment (McDaniel and Capone, 1985). In the current study it has been demonstrated that treatment time significantly influenced the recovery of bacteria from coastal sediment.

The relative efficiency of treatment by sonication bath for 10 min was greater for sediments with high sand content than for sediments containing greater proportions of silt, clay and organic carbon where the recovery efficiency was slightly less (62% compared with 86% for sandy sediments), indicating that a second treatment was required when this method was subsequently used. A study by Epstein *et al*. (1997) utilised radioisotope labelling of bacteria to quantitatively determine the efficiency of sonication treatment (using a sonication probe) for enumerating bacteria from sandy sediments. The radioactive labels used were [^{3}H]thymidine and [^{14}C]leucine which are incorporated into bacterial DNA or protein respectively. The efficiency of treatment was determined by comparing the amount of radioactive label in the supernatant (dislodged bacteria) compared with that remaining in the sediment (bacteria still particle bound). Comparisons between treatment methods were therefore considered quantitative rather than

relative. In that study it was demonstrated that treatment by sonication probe for 80-160 s resulted in 88 to 98 % of all bacteria present in the sediment being enumerated in supernatant which was similar to the recovery efficiencies observed in this current study using a sonication bath.

For this study, no attempt was made to use chemical treatment in combination with the physical dispersion techniques. In previous study by this group (Flint, unpublished) sodium pyrophosphate was shown to have no significant effect on recovery. This finding confirms that of other studies investigating the use of a chemical dispersants (0.01 mol l^{-1} sodium pyrophosphate) in combination with physical treatment (sonication) to remove bacteria from marine sediment (Epstein and Rossel, 1995; McDaniel and Capone, 1985). These studies found that the mean number of bacteria recovered between identical samples treated by a combination of chemical dispersion and sonication, and sonication alone were not significantly different. When compared with samples undergoing no physical treatment (no sonication), however, treatment by these methods resulted in significantly higher numbers of bacteria being recovered. Treatment by sonication also resulted in a lower coefficient of variation in comparison with untreated samples and resulted in a more even dispersion of bacteria over the filter surface when viewed microscopically (McDaniel and Capone, 1985).

It is likely that the number of bacteria enumerated in this study is an under-estimate of the total present, with the possibility of some bacteria being damaged by sonication and others not being removed from sediment particles. To achieve consistency for the subsequent enumeration of faecal coliforms from different recreational coastal sites it was deemed necessary to choose one separation technique which provided the greatest recovery of microorganisms across a wide range of sediment types. Treatment by sonication bath for 10 min on two occasions proved to be the most versatile and was therefore chosen as the primary separation method for subsequent analysis to provide comparison of relative, if not absolute numbers of organisms.

CHAPTER 5: DETERMINATION OF THE MICROBIAL QUALITY OF WATER AND SEDIMENT QUALITY AT COASTAL RECREATIONAL SITES

The main content of this chapter has been published as:

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2002) Enumeration of faecal coliforms from recreational coastal waters and sediments: evaluation of techniques for the separation of bacteria from sediments. *Journal of Applied Microbiology*. 93(4): 557-565.

5.1 INTRODUCTION

The interaction of microorganisms with sediments may enhance their survival by reducing exposure to stressors such as UV light and predation or by increasing the availability of nutrients (Davies *et al.*, 1995; Dale, 1974). In this way, coastal sediments may act as reservoirs for pathogenic microorganisms. The assessment of coastal recreational water quality is primarily undertaken by the enumeration of faecal coliforms and *Escherichia coli* from the water column (NHMRC 1990). In Australia, for a recreational water body to meet primary contact recreation guidelines the median concentration (from five samples) of faecal coliforms must be below 150 CFU 100 ml^{-1} (NHMRC 1990). As discussed in Chapter 1 (section 1.1.4) the National Health and Medical Research Council are currently drafting new recreational water quality guidelines based on the WHO framework which will result in a health-risk classification of recreational water sites based on combined long-term microbiological data (using enterococci as the faecal indicator organism) and results of sanitary surveys.

Studies have indicated however, that the number of faecal coliforms in coastal sediments can be 10-10,000 times greater than that in the overlying water column (Shiaris *et al.*, 1987). Dale (1974) demonstrated that sediment characteristics such as particle size and organic carbon and nitrogen content correlate with bacterial numbers. In coastal waters there may be an increased risk of infection to humans due to the re-suspension of potentially pathogenic microorganisms from the surface sediment layer during recreational activities.

This chapter forms part of an exposure assessment in the first stage of a health risk assessment for recreational coastal water. Faecal coliform concentrations in overlying water and sediment were monitored at four recreational coastal sites over a 12 month period. The influence of environmental conditions such as rainfall and temperature was also determined.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection

Triplicate sediment and water samples were collected from Henley Beach South, Onkaparinga River, West Lakes and Pt Adelaide River over a period of approximately 12 months. West Lakes is a large artificial lake, which was built as part of a large housing development and which acts as the focus for considerable Metropolitan aquatic recreational activity. Water is continually renewed with seawater entering at the southern end and being discharged at the northern end (into the Pt Adelaide River). Stormwater from the surrounding housing development is discharged into West Lakes. There have also been cases of accidental sewage release into the lake (via ruptured sewer pipes and transport by stormwater drains). West Lakes is used substantially for both primary and secondary contact recreation.

These four sites were chosen as they represented distinct sediment types, from mainly sandy sediment at Henley Beach to Pt Adelaide sediment which contained greater proportions of silt and clay (Table 5.1). Water and sediment samples were taken at low tide, at a distance from the shore where the depth of water was approximately knee height. Grab-samples of overlying water were collected in sterile containers (1 l). Intact sediment cores were taken as described in Section 2.2.2.

5.2.2 Faecal coliform enumeration

Sediment samples were prepared by extruding the top one centimetre of sediment from the intact core, of which 25 g was placed into 75 ml of 0.1% peptone. Using the results of the separation trial detailed in Chapter 4, sediment was sonicated in a sonication bath (700 W, 35 kHz, Cooper Vision Model 895, Irvine, CA, USA) for 10 min, stirred and sonicated a further 10 minutes to separate bacteria from sediment particles. Faecal coliforms in sediment and water samples were enumerated by membrane filtration (GN-6, Gellman) and incubation on membrane lauryl sulphate agar (Oxoid) as described in Section 2.2.6.1 (Australian Standard AS 4276.7, 1995).

5.2.3 Meteorological data

Total rainfall (mm) data was obtained from a weather station located at the Adelaide Airport (Bureau of Meteorology, Adelaide).

5.2.4 Statistical analysis

Bivariate relationships between faecal indicator organism concentration (log_{10} transformed) and rainfall totals over 2 preceding time intervals (2 d and 7 d) were examined using Pearson's correlation coefficient (*r*). All results were expressed as the mean ± standard deviation of three determinations.

5.3 RESULTS

In general, all sites investigated complied with the NHMRC guideline for primary contact recreational water quality over the sampling period, with median concentrations of < 150 faecal coliforms 100 ml^{-1} in the overlying water. The concentration of faecal coliforms was generally greater in sediment compared with overlying water at all sites (Figs. 5.1 – 5.4). This was most significant at the Pt Adelaide site (greatest silt/clay and organic carbon content) with a median concentration of faecal coliforms over the sampling period in the sediment of 2.1 $\times 10^4$ cfu 100 g^{-1} (range 2.3 $\times 10^3$ to 2.2 $\times 10^5$ cfu 100 g^{-1}) compared with 61 cfu 100 ml^{-1} (range < 1 to 695 cfu 100 ml^{-1}) in the overlying water. No correlation was identified between the number of faecal coliforms in the sediment with that in the overlying water for any site.

Seasonal variation of faecal coliform concentrations was only apparent at the Pt Adelaide River, with slightly elevated levels in both overlying water and sediment during the winter months. At Henley Beach and Onkaparinga, faecal coliform concentrations in both overlying water and sediment varied regardless of season, although more samples need to be taken during the summer months to confirm this.

A significant correlation between faecal coliform concentration in both overlying water and sediment and rainfall in the previous 2 d was identified at Henley Beach ($r^2 = 0.77$; $P < 0.001$) and West Lakes ($r^2 = 0.40$; $P < 0.05$). Correlations between sediment faecal coliform concentrations at Onkaparinga River, West Lakes and Pt Adelaide were noted with rainfall in the previous 7 d ($P < 0.001$ for the Onkaparinga River). See Figs. 5.5 – 5.7 for visual representation of some of these relationships.

Table 5.1 Particle size analysis. Particles sizes for sand 2 – 0.2 mm; silt 0.2 mm – 2 μm; clay < 2 μm. All results normalised to 100%.

SITE	%SAND	%SILT	%CLAY	%ORGANIC C
Henley Beach Sth	98.47	0.08	1.41	0.05
West Lakes	94.80	2.46	2.51	0.23
Onkaparinga	95.48	1.26	2.91	0.35
Pt Adelaide	83.05	4.24	10.33	2.38

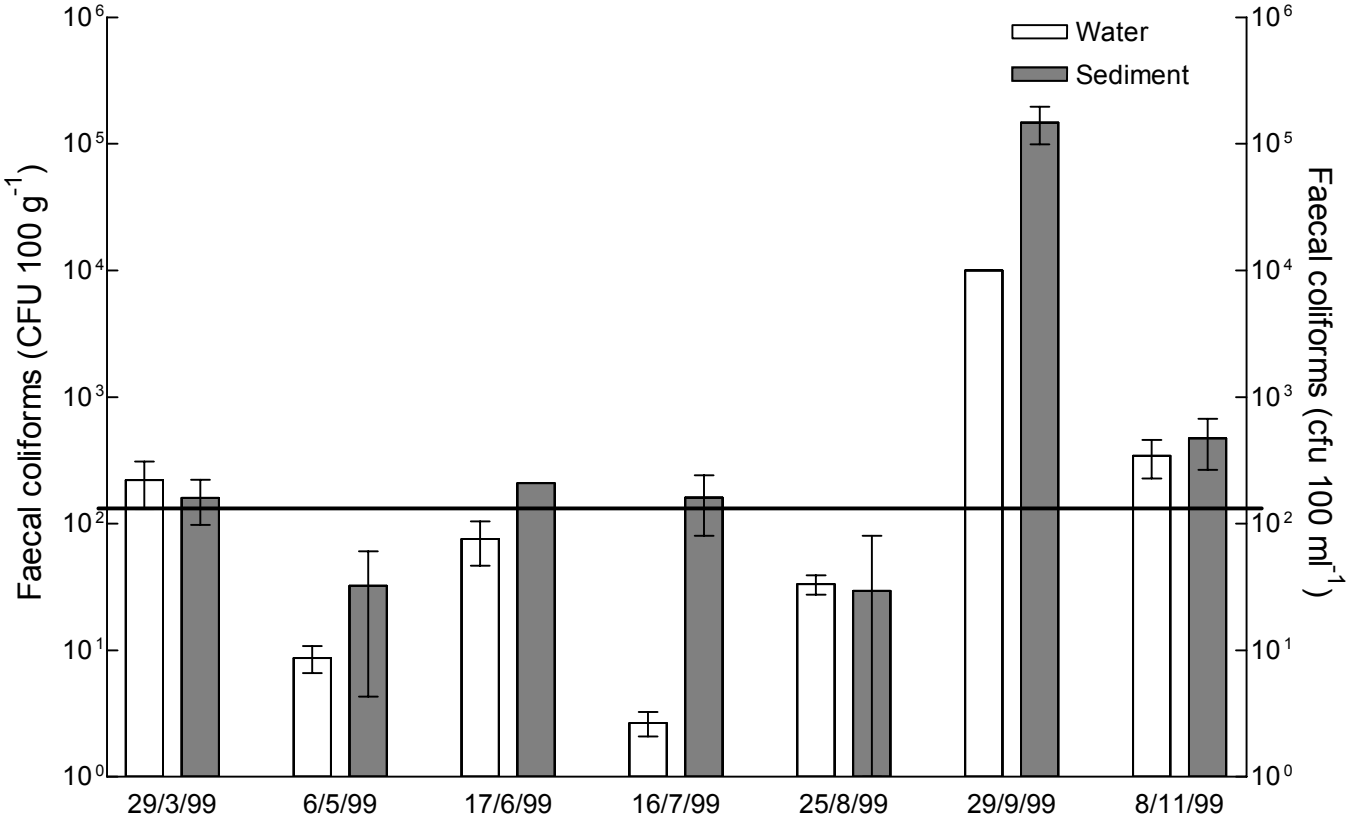

Figure 5.1 Concentration of faecal coliforms (mean ± standard deviation) in overlying water and sediment from Henley Beach South (n=3). Line represents NHMRC guideline for faecal coliform concentration in recreational coastal water of < 150 cfu 100 ml^{-1}.

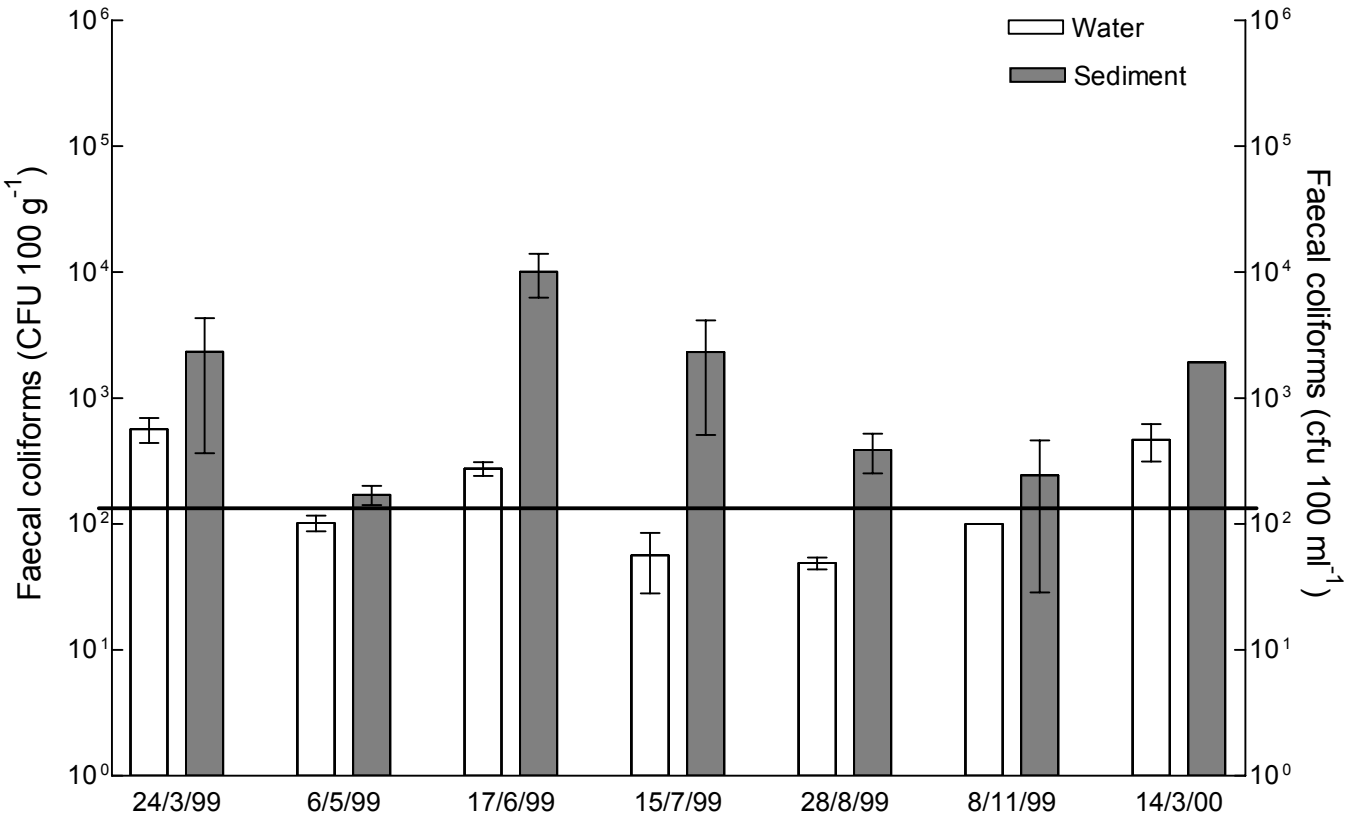

Figure 5.2 Concentration of faecal coliforms (mean ± standard deviation) in overlying water and sediment from Onkaparinga River (n=3). Line represents NHMRC guideline for faecal coliform concentration in recreational coastal water of < 150 cfu 100 ml^{-1}.

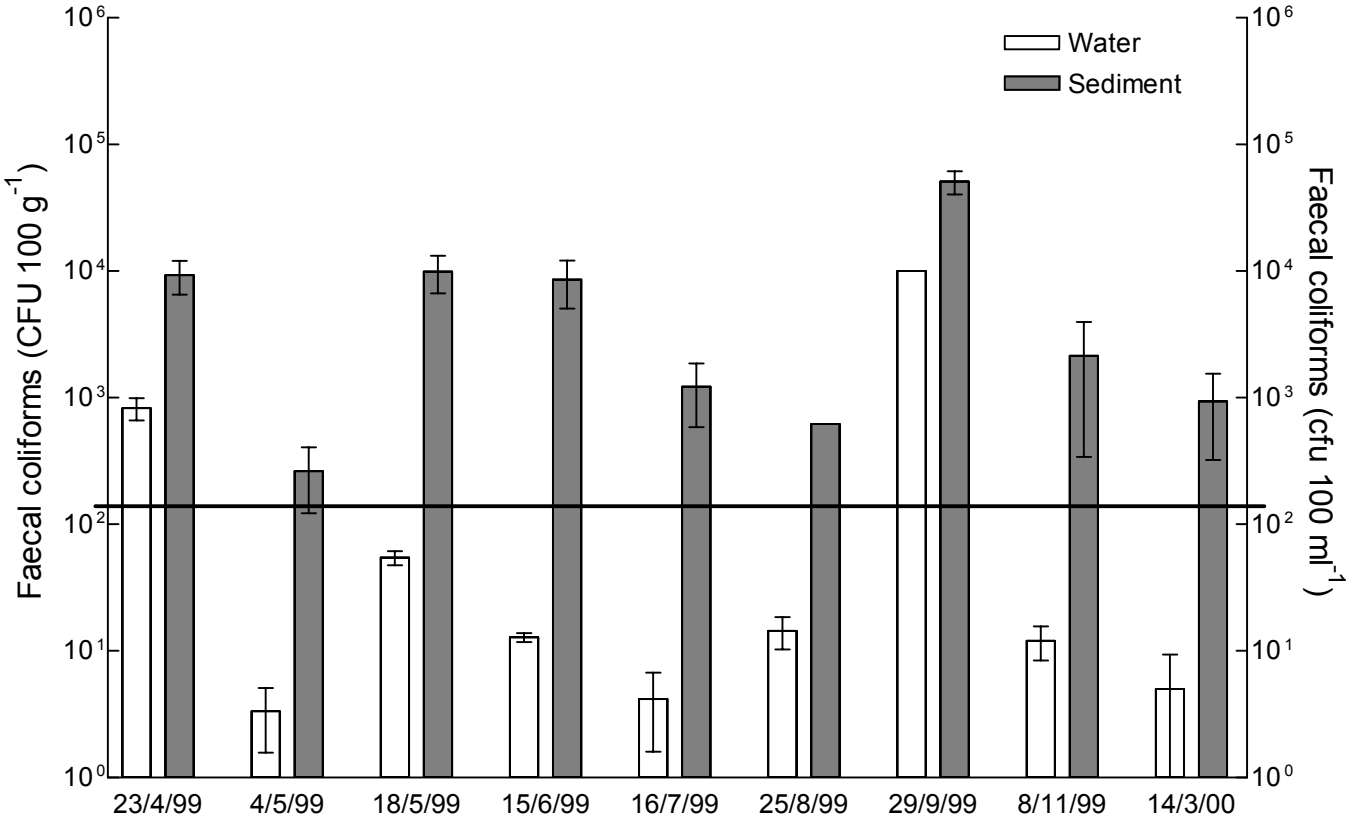

Figure 5.3 Concentration of faecal coliforms (mean ± standard deviation) in overlying water and sediment from West Lakes (n=3). Line represents NHMRC guideline for faecal coliform concentration in recreational coastal water of < 150 cfu 100 ml^{-1}.

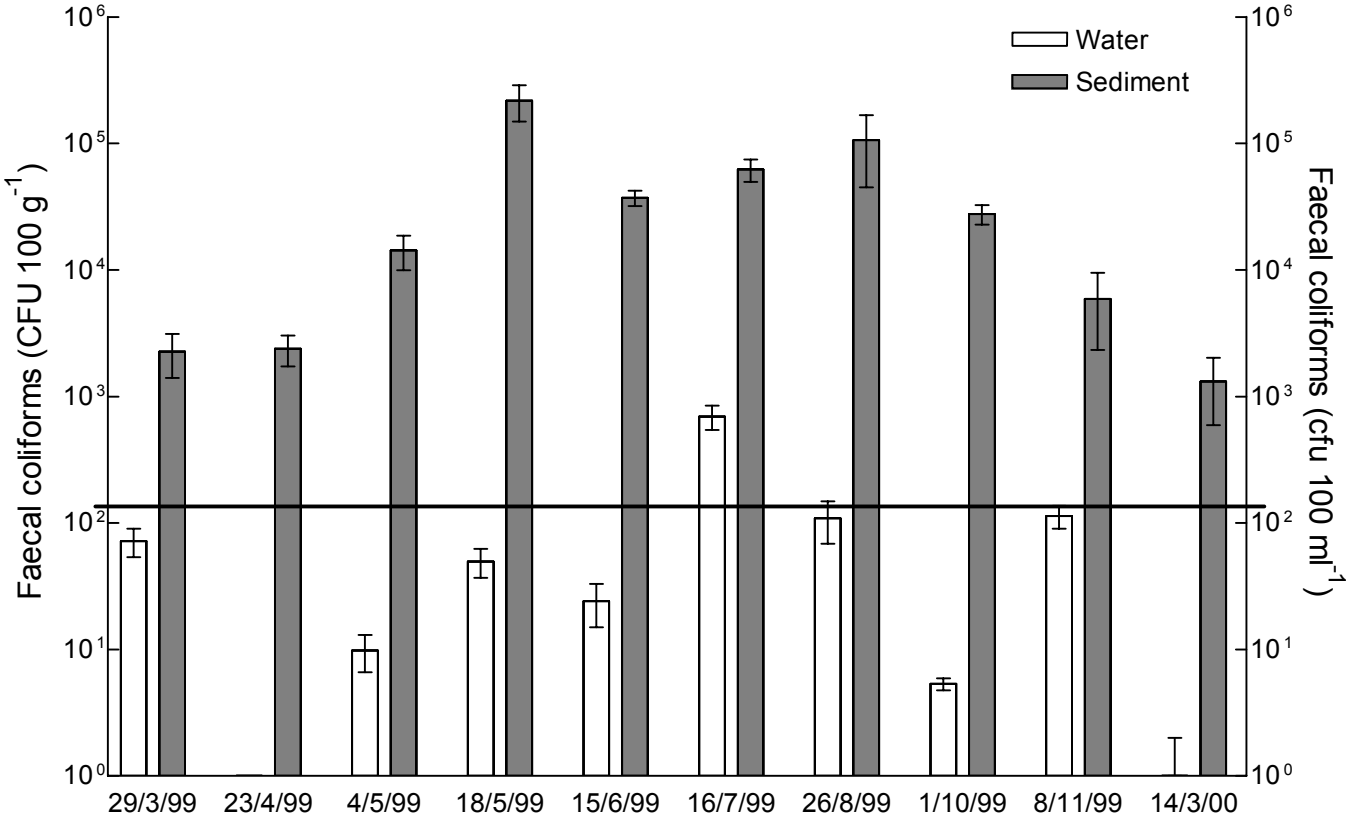

Figure 5.4 Concentration of faecal coliforms (mean ± standard deviation) in overlying water and sediment from Pt Adelaide (n=3). Line represents NHMRC guideline for faecal coliform concentration in recreational coastal water of < 150 cfu 100 ml^{-1}.

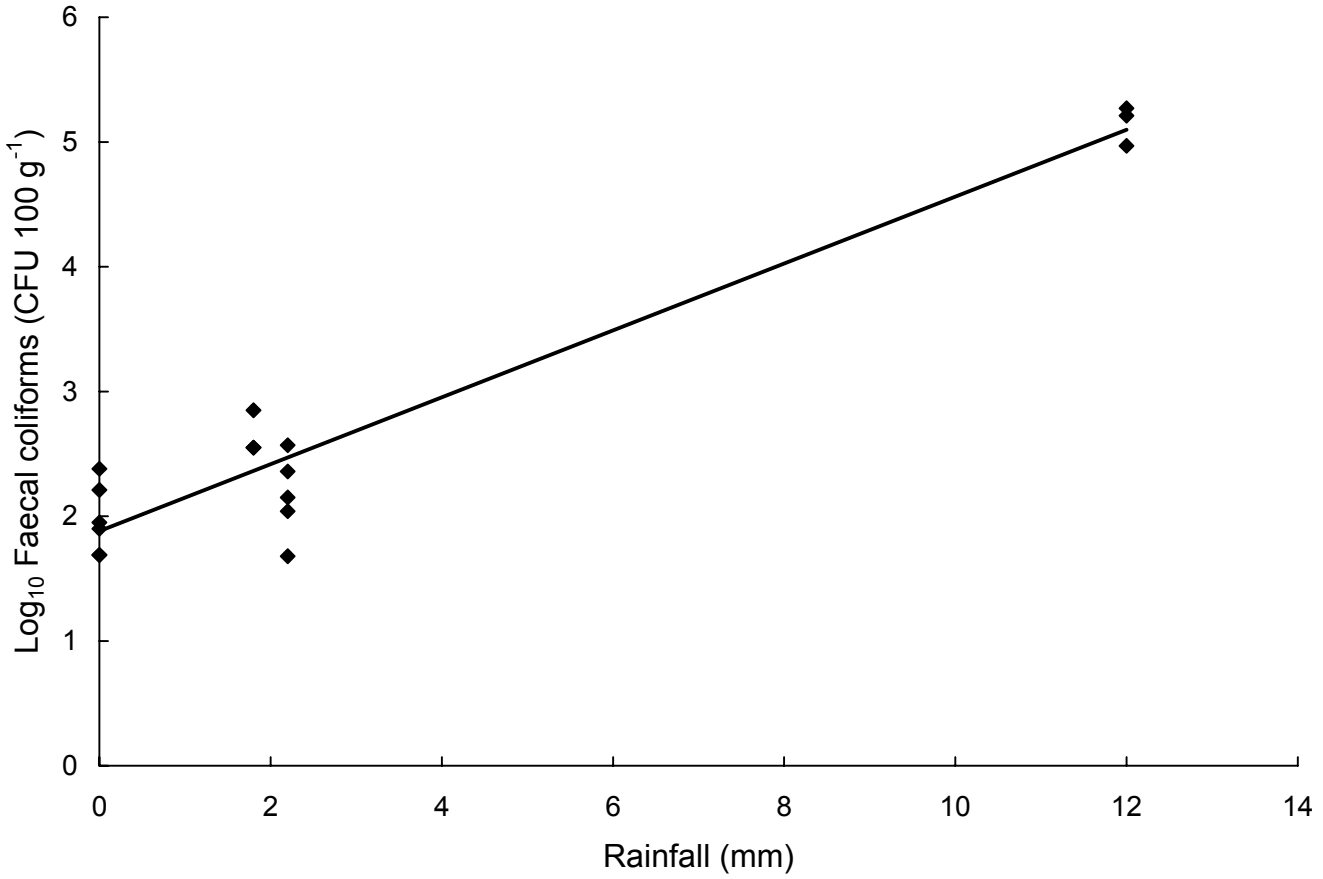

Figure 5.5 Correlation between faecal coliform concentration in Henley Beach sediment and rainfall in the previous 2 d ($y = 0.268x + 1.881$; $n = 21$; $r^2 = 0.93$; $P < 0.001$).

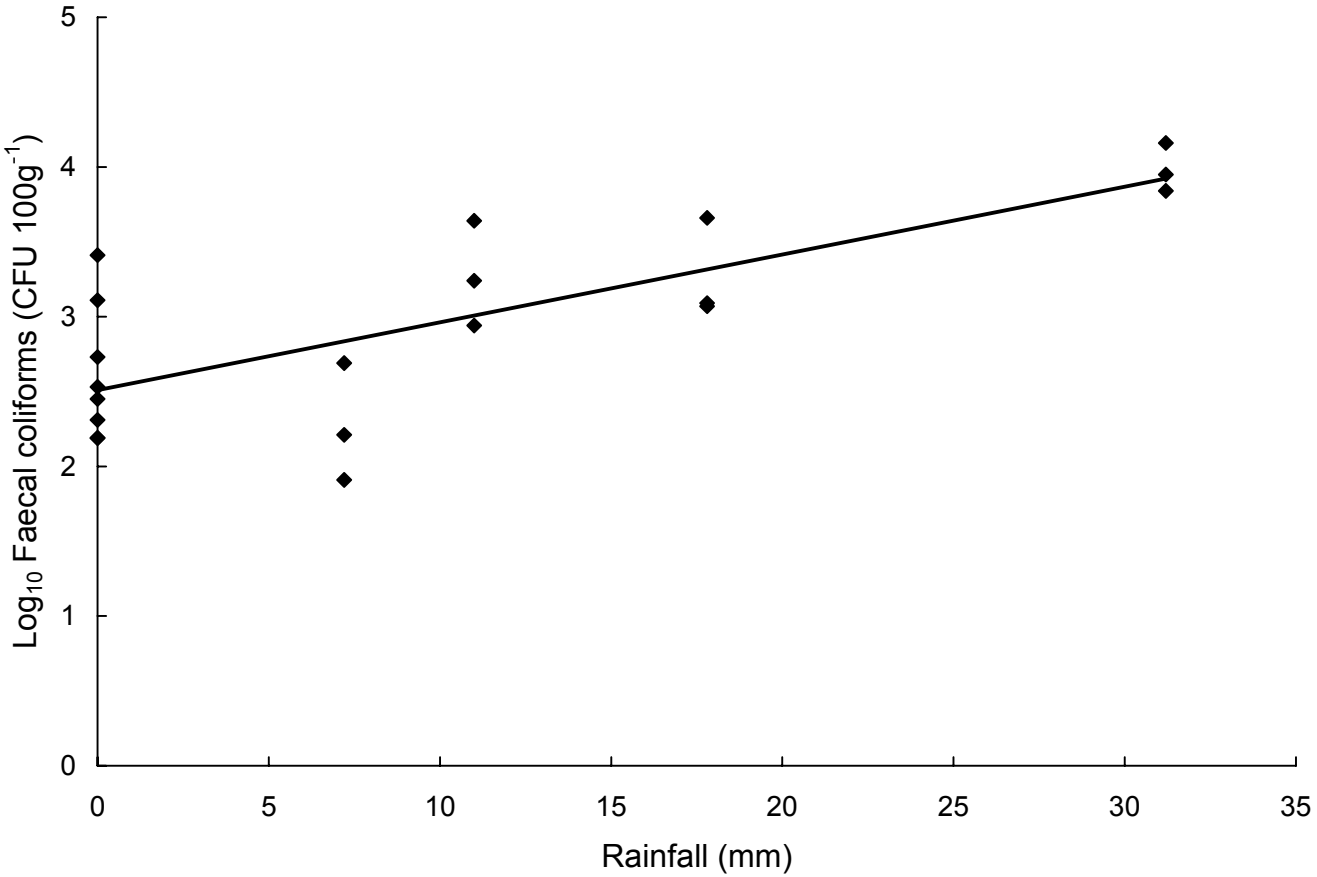

Figure 5.6 Correlation between faecal coliform concentration in Onkaparinga sediment and rainfall in the previous 7 d ($y = 0.045x + 2.510$; $n = 20$; $r^2 = 0.58$; $P < 0.001$).

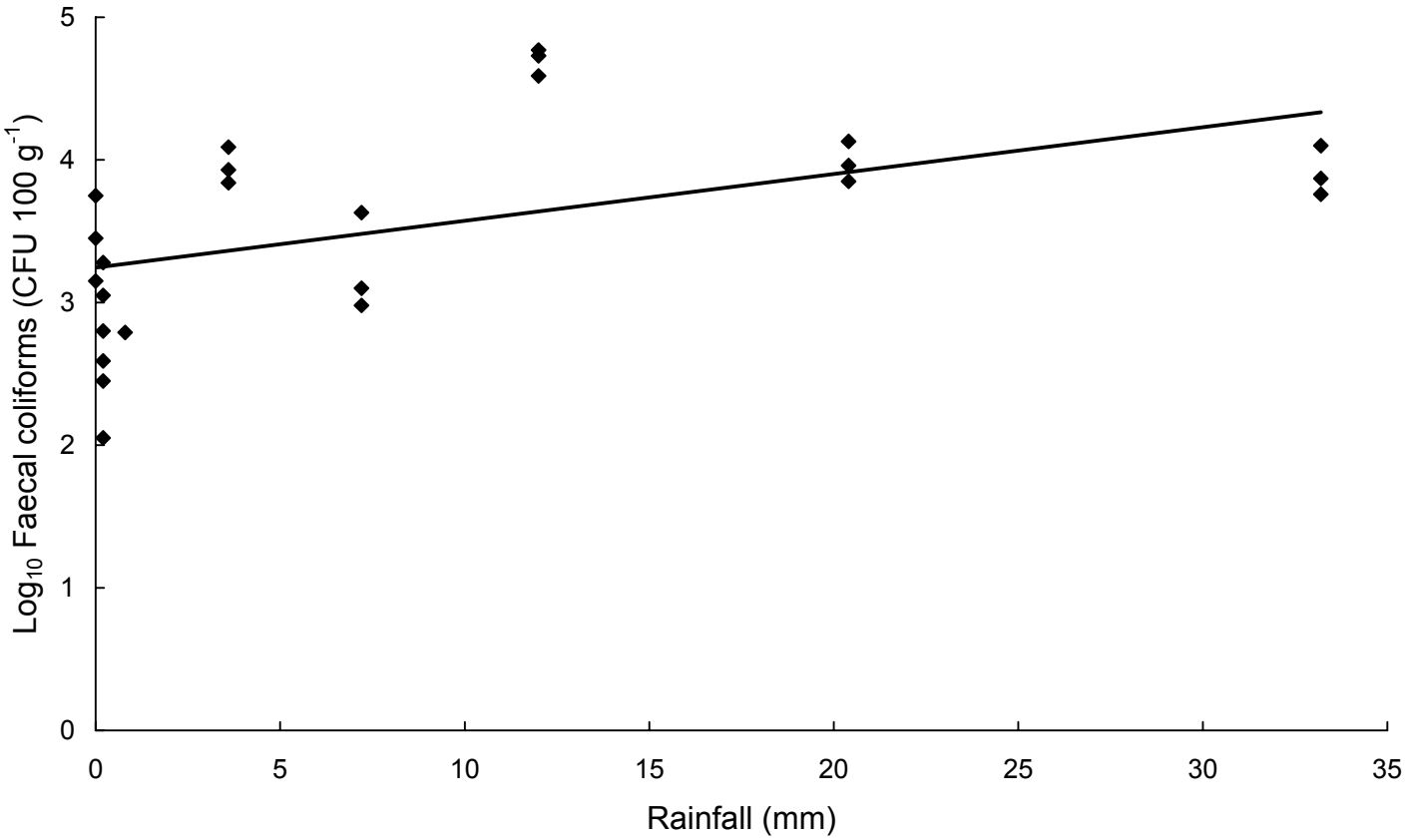

Figure 5.7 Correlation between faecal coliform concentration in West Lakes sediment and rainfall in the previous 7 d ($y = 0.033x + 3.244$; $n = 25$; $r^2 = 0.27$; $P < 0.05$).

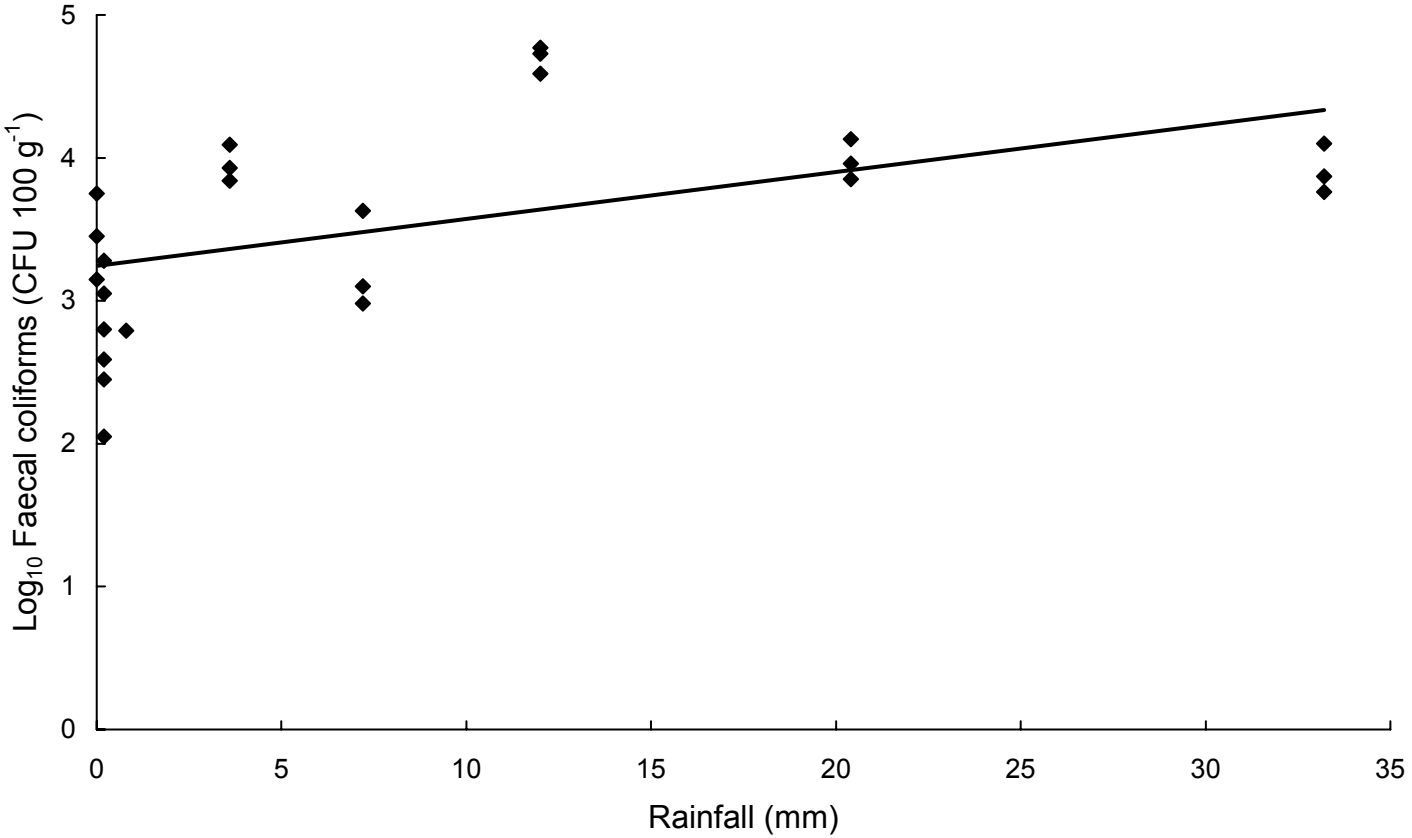

Figure 5.8 Correlation between faecal coliform concentration in West Lakes sediment and rainfall in the previous 7 d ($y = 0.033x + 3.244$; $n = 25$; $r^2 = 0.27$; $P < 0.01$).

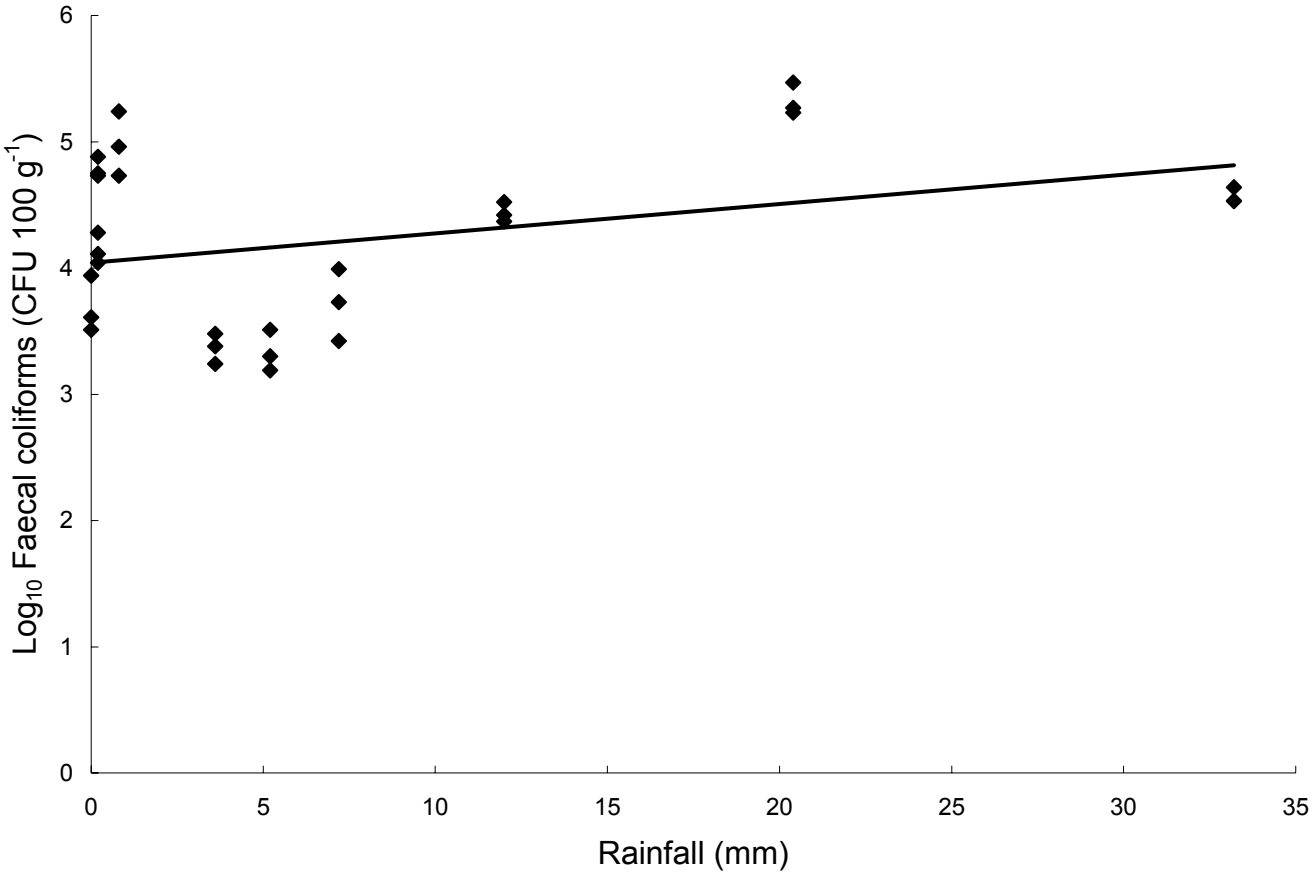

Figure 5.9 Correlation between faecal coliform concentration in Pt Adelaide sediment and rainfall in the previous 7 d ($y = 0.023x + 4.041$; $n = 30$; $r^2 = 0.13$; $P = 0.05$).

5.4 DISCUSSION

The year long investigation of these coastal sites demonstrated that the concentration of faecal coliforms was generally greater in the sediment compared with overlying water. This was most evident at Pt Adelaide, West Lakes and Onkaparinga River sites, suggesting sediment characteristics (particle size and organic carbon content) may have an influence on the persistence of faecal coliforms at recreational coastal sites. The concentration of faecal coliforms in Pt Adelaide River sediment was often 1,000 fold higher than in the overlying water (3.7×10^5 cfu $100g^{-1}$ compared with 2.0×10^1 cfu 100 ml^{-1} respectively on 15 June, 1999). On two occasions, no faecal coliforms were detected in the overlying water, however $> 1 \times 10^3$ 100 g^{-1} were detected in the sediment. These results confirm other studies which have identified greater survival of faecal coliforms (and other pathogenic microorganisms) in coastal sediment compared with water (Lipp *et al.*, 2001; Obiri-Danso and Jones, 2000; Davies *et al.*, 1995; Fish and Pettibone, 1995; Valiela *et al.*, 1991; Shiaris *et al.*, 1987; Goulder, 1977). Unlike other studies however, the number of faecal coliforms enumerated from the sediment did not correlate with those enumerated from the water column (Valiela *et al.*, 1991). This may suggest a more complex relationship between faecal coliform persistence in both overlying water and sediment and various environmental conditions. As discussed in Chapter 1, factors which may influence microbial survival in coastal environments include temperature, salinity, nutrient availability, sunlight, predation and presence of chemical sensitisers. The relative effect of each of these factors (and the relationships between them) may be significantly different in the surface sediment layer compared with overlying water and may therefore explain the lack of correlation between the concentration of faecal coliforms in the two matrices observed in this study.

Seasonal variation in faecal coliform concentration was only observed at Pt Adelaide, with higher concentrations in both overlying water and sediment during the colder months. A study by Obiri-Danso and Jones (2000) found the concentration of faecal coliform and faecal streptococci in coastal sediment did not vary seasonally, however, in contrast *Campylobacter* spp. were only enumerated during the colder months. In an earlier study, the same authors

identified higher concentrations of faecal coliforms in overlying water at a recreational coastal site during winter (Obiri-Danso and Jones, 1999). The increased concentration of faecal coliforms in both sediment and overlying water at the Pt Adelaide River site during winter may highlight the effects of temperature and UV light exposure on microbial survival. The influence of these environmental factors may be more significant at the Pt Adelaide site compared with other sites investigated due to it being relatively shallow and not affected by large flow or turbulence caused by wave action. Studies have identified decreased survival of faecal coliforms on exposure to UV light (Davies and Evison, 1991; Fujioka *et al.*, 1981). It has also been determined that survival of faecal coliforms in water is extended at lower temperatures (Terzieva and McFeters, 1991; Flint, 1987; Vasconcelos and Swartz, 1976). The most likely source of faecal indicator organisms at this site is a nearby WWTP outlet, with little immediate impact of stormwater associated with rainfall. Therefore, the lower temperature and UV light associated with winter may lead to increased survival of faecal coliforms in both water and sediment. The Pt Adelaide River is used throughout the year for recreation, primarily fishing (secondary contact recreation). The high numbers of faecal coliforms, especially in the sediments during the winter, may therefore pose a significant risk of exposure to faecal contamination due to secondary contact recreation.

The concentration of faecal coliforms enumerated from overlying water at Henley Beach was influenced by rainfall in the previous 2 d. The peak concentration of faecal coliforms which occurred on 29 September coincided with high rainfall (daily rainfall total 12mm, Bureau of Meteorology). This high rainfall resulted in significant flow of the Torrens River, the outlet of which is situated at Henley Beach. Rainfall also resulted in the concentration of faecal coliforms detected in the water column being similar to that in the sediment. The influence of high rainfall on faecal coliform concentrations in the water column at recreational coastal sites has also been identified by other studies (Crowther *et al.*, 2001; Lipp *et al.*, 2001; Rees *et al.*, 1998). Faecal coliform concentrations in the sediment, however, correlated with rainfall in the previous 7 d (at all sites). This may suggest detection of faecal coliforms in the water column is an indication of recent faecal contamination. The ability of sediments to act as a reservoir for

faecal coliforms suggests sediments may provide a more stable indicator of long-term faecal contamination.

In conclusion, greater numbers of faecal coliforms were enumerated in sediment compared with overlying water at the recreational coastal sites investigated. Environmental factors such as likely source of contamination, rainfall, temperature and sediment characteristics were identified to influence faecal coliform concentrations. The following chapter will discuss a laboratory-based microcosm study undertaken to determine the persistence of other faecal indicator as well as pathogenic organisms in coastal waters and sediments and to estimate the degree of resuspension from surface sediment layers into the overlying water.

CHAPTER 6: DETERMINING DECAY RATES OF FAECAL INDICATOR AND PATHOGENIC MICROORGANISMS USING LABORATORY-BASED MICROCOSMS

Parts of this chapter have been published in the following papers:

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2001) The effects of temperature and sediment characteristics on survival of *Escherichia coli* in coastal water and sediment. *Journal of Environmental Health*. 1(1): 43 – 51.

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2002) Comparison of decay rates of faecal indicator organisms in recreational coastal water and sediment. *Water Science and Technology: Water Supply*. 2(3): 131-138.

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2004) Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements. *Journal of Applied Microbiology*. 96(5): 922-930.

INTRODUCTION

Laboratory-based microcosms are used to study the influence of various environmental factors on the survival of microorganisms. This chapter investigates the influence of temperature and sediment characteristics on the survival of indicator and pathogenic microorganisms in recreational coastal water and sediments. This study utilised intact, non-sterile sediment cores taken from recreational coastal sites with distinctly different sediment characteristics (details of sediment characterisation in Chapter 4).

6.1 METHODOLOGY

6.1.1 Microcosm design

For each test condition, six intact sediment cores were collected from Henley Beach, Onkaparinga River (estuary) and Port Adelaide River in metropolitan Adelaide, South Australia using methods described in Chapter 4. In the laboratory the columns were placed in a water bath held at a constant temperature (10°C, 20°C or 30°C; Plate 6.1). Water in the water bath was constantly mixed to maintain even heat distribution. Overlying water was removed from columns and replaced with 500 mL of water from the Onkaparinga River to reduce the effect of differences between total dissolved solids (TDS) concentration and volume between cores. Columns were sparged with air at a rate which was determined not to disturb the upper layer of sediment. Overlying water pH (HACH, Colorado, USA), redox potential (HACH) and conductivity (Hanna) were measured with hand held meters when microcosms were sampled. The redox potential of the top 10 mm of sediment was also measured at time of sampling (HACH).

6.1.2 Preparation of faecal indicator and pathogenic microorganisms

Stock bacterial suspensions of *E. coli* (ATCC 25922), *Enterococcus faecium* (ATTC 19434), *Salmonella typhimurium* (ATCC 14028) and a clinical strain of *S. derby* (isolated from a human stool sample; Institute of Medical and Veterinary Science, Adelaide) were prepared by inoculation into 10 mL nutrient broth (Oxoid) and incubated overnight at 37°C. Cells were harvested by centrifugation at 2,500 g for 10 minutes. The pelleted samples were resuspended in phosphate

buffered saline (PBS; 0.1 mol l^{-1}, pH 7.2), centrifuged and resuspended in PBS to remove excess culture media.

Coliphage suspension was prepared using host *E. coli* (FCC 84) cells inoculated into nutrient broth (Oxoid) and incubated overnight at 37°C. A single coliphage plaque previously isolated from primary treated human sewage was added to this suspension and incubated for a further 24 h at 37°C. To remove host bacterial cells, the suspension was centrifuged and the supernatant (containing coliphage) was filtered (25 mm diameter, 0.2 μm pore size) to further remove any bacterial cells present. All stock suspensions were maintained at 4°C until use (within three weeks of preparation).

6.1.3 Determination of microorganism decay

Microcosms were inoculated by adding 500 μL of stock coliphage suspension or 50 μL of stock *E. coli*, *Enterococcus faecium*, *S. typhimurium* and *S. derby* suspension into the overlying water. Control columns containing water only were similarly inoculated. Microorganisms were enumerated from both overlying water and sediment on days 0 (1 h after inoculation), 1, 2, 7, 14 and 28 following inoculation (using the methods described in Chapter 2). Due to the rapid decay of *E. coli* and *Salmonella* spp. in overlying water at higher temperatures, organisms were enumerated on days 0, 1, 2, 4, 7 and 14 when incubated at 30°C. Sediment samples were prepared using the method described in section 2.2.4 to separate microorganisms from sediment particles. The decay rate constant (k) was calculated as the slope of semi-$\log_{10}$ plot of ($N_t.N_0^{-1}$) regressed against time, where N_t is the number of bacteria at time t and N_0 is the number of bacteria at time 0 (Davies and Evison 1991).

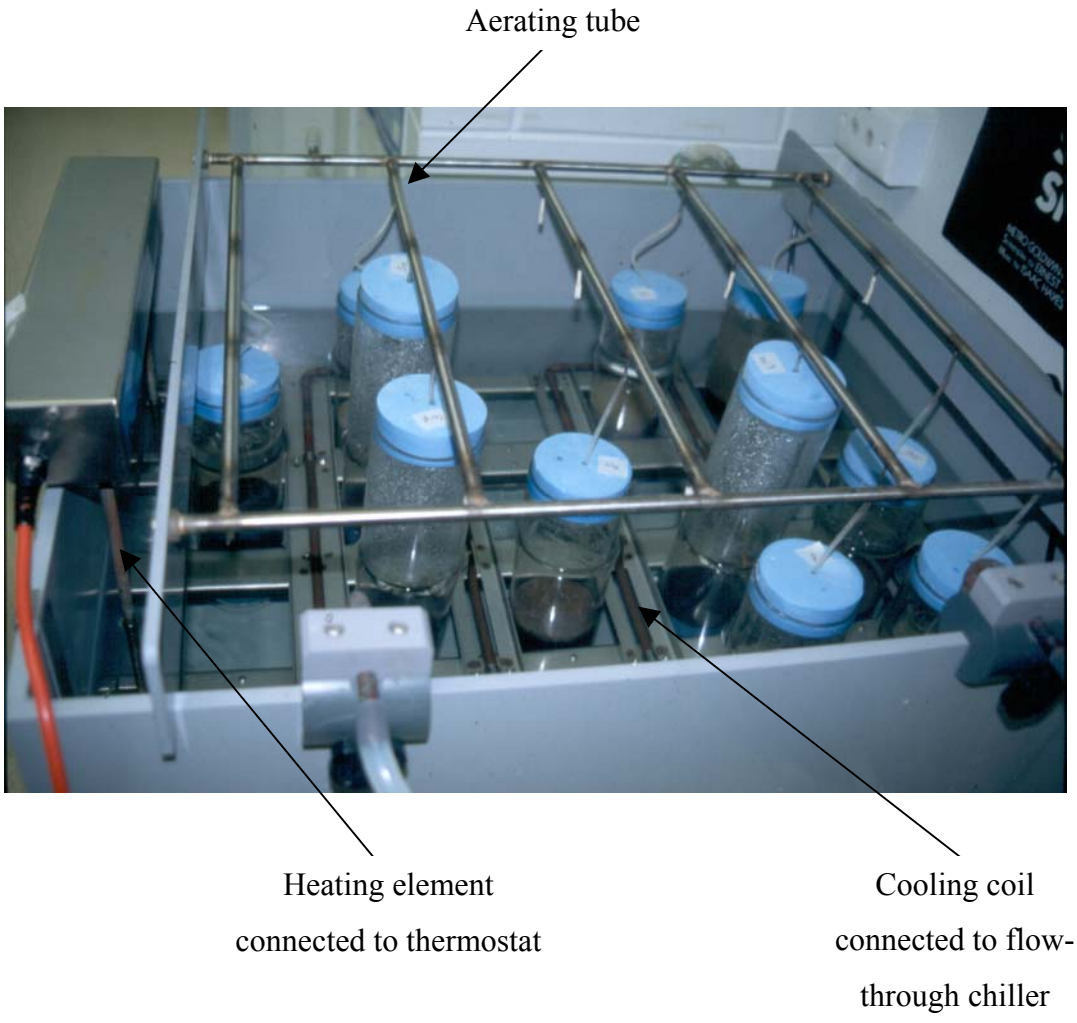

Plate 6.1 Microcosm columns held in constant temperature water bath.

6.1.4 Resuspension

In an effort to simulate disruption of sediment due to recreational activities, the microcosm experiment using faecal indicator organisms was repeated at all three temperatures with the top 10 – 20 mm of sediment being gently mixed using a sterile pipette immediately prior to sampling. To identify the possible resuspension of *E. coli*, enterococci and coliphage, results were expressed as percent partitioning between the sediment and water column under both static and stirred conditions.

6.1.5 Sampling of biofilm

To determine the possible loss of *E. coli* from the water column due to adhesion to biofilm on the surface walls of the microcosm column, this biofilm was sampled by scraping a marked 20 mm × 20 mm area on the inside wall of inoculated columns containing only overlying water with a sterile cotton swab on days 0, 1, 2, 7, 14 and 28. The swab was mixed vigorously in 9 mL of 0.1% peptone water (Oxoid) and organisms (except *E. coli*) were enumerated by methods previously described in Chapter 2. For this part of the experiment, *E. coli* were enumerated by the Colilert™ defined substrate method using 97-well Quantitrays (IDEXX Laboratories, Maine, USA), which is considered to give equivalent results to other standard methods for the enumeration of faecal coliforms and *E. coli* (Eckner, 1998).

6.1.6 Modelling of observed decay rate data

Decay rates of organisms were modelled against temperature using the following linear (Equation 6.1) or exponential (Equation 6.2) equations:

$$k = m\mathrm{T} + \mathrm{c} \qquad \text{Equation 6.1}$$

where,

k = decay rate (d^{-1})

T = temperature (°C)

m = slope of the regression line

$k = \alpha.e^{\beta T}$ Equation 6.2

where,

k = decay rate (d^{-1})

T = temperature (°C)

6.1.7 Statistical analysis

Slopes of the lines from linear regression (decay rates) were compared by analysis of co-variance using Graphpad Prism (version 3.0; Graphpad Software Inc) and significance expressed at $P \leq 0.05$.

6.2 RESULTS

Monitoring of overlying water demonstrated that conductivity, pH and redox potential remained relatively stable over the sampling period of 28 d (coefficient of variation < 5% - results not shown). In general, the redox potential of the top 10 mm of Pt Adelaide sediment was more negative than the other sediments investigated (mean = -185 mV), indicating more anaerobic conditions. Again, for all sediments redox potential remained relatively stable over the sampling period.

In control microcosms (not inoculated with test organisms) either zero, or very small, numbers of organisms of interest were detected in overlying water and sediment suggesting that the presence of indigenous faecal indicator or pathogenic organisms did not significantly influence results. Additionally, the incorporation of *E. coli* into biofilm attached to the inner surface, or walls, of the column was not significant (Tables 6.1 – 6.3). The disappearance of organisms observed from the water column could therefore be explained as either inactivation or partitioning into the surface sediment layer and not simply incorporation into biofilm.

6.2.1 Effect of temperature on microbial survival in water and sediment

When decay rates were plotted (eg. Fig 6.1) they largely followed first-order kinetics as illustrated by r^2 values in Tables 6.4 – 6.8. Thus, results could be expressed as decay rate constants (k), with more negative decay rates indicating a more rapid decline in the number of organisms enumerated.

The survival curves of *E. coli* in both sediment and water at 10°C, 20°C and 30°C are illustrated in Figs. 6.2 a, b and c respectively. Persistence of *E. coli* was greatly reduced when samples were incubated at 20°C and 30°C respectively compared with 10°C (not significant in overlying water containing sediment from Henley Beach). Even when incubated at 10°C, there was a relatively rapid decline in the concentration of *E. coli* in the overlying water. This was most pronounced in the microcosm containing water only, with no *E. coli* being detected after 7 d. The greatest rate of decay was observed in the column containing water only incubated at 30°C (k = -2.13 day^{-1}). In contrast, however, Onkaparinga and Pt

Adelaide sediments, greater than 5×10^3 CFU 100 ml^{-1} were enumerated after 28 d incubation.

Table 6.1 Concentration of *E. coli* in overlying water and biofilm present on column surface (mean ± SD; n=3).

Day	Overlying Water (MPN 100 ml^{-1})	Biofilm (MPN cm^{-2})
0	$5.9 \pm 4.3 \times 10^5$	2 ± 2.4
1	$1.4 \pm 0.5 \times 10^4$	$< 0.25^*$
2	$2.9 \pm 0.7 \times 10^3$	< 0.25
7	< 10	< 0.25
14	< 10	< 0.25

* <0.25 = limit of detection

Table 6.2 Concentration of enterococci in overlying water and biofilm present on column surface (mean ± SD; n=3).

Day	Overlying Water (MPN 100 ml^{-1})	Biofilm (MPN cm^{-2})
0	$5.2 \pm 0.8 \times 10^5$	0.7 ± 0.5
1	$3.4 \pm 0.5 \times 10^5$	$3.0 \pm 4.0 \times 10^1$
2	$1.1 \pm 0.1 \times 10^4$	$1.2 \pm 0.4 \times 10^2$
7	$1.3 \pm 0.6 \times 10^1$	$< 25^*$
14	< 10	< 25
28	< 10	< 25

* <25 = limit of detection

Table 6.3 Concentration of coliphage in overlying water and biofilm present on column surface (mean ± SD; n=3).

Day	Overlying Water (PFU 100 ml^{-1})	Biofilm (PFU cm^{-2})
0	$1.0 \pm 0.0 \times 10^6$	$< 0.6^*$
1	$5.1 \pm 0.6 \times 10^5$	< 0.6
2	$2.6 \pm 0.2 \times 10^5$	8.6 ± 2.8
7	$1.6 \pm 0.2 \times 10^5$	1.7 ± 0.6
14	$4.6 \pm 0.4 \times 10^2$	< 0.6
28	< 25	< 0.6

* <0.6 = limit of detection

In general, sediment from Henley Beach (mainly sand) was significantly less conducive to the survival of *E. coli* compared with Onkaparinga and Pt Adelaide sediment (higher silt, clay and organic carbon content) at all temperatures.

The decay of *E. coli* in both the water column and sediment was greater compared to the other faecal indicator organisms tested (enterococci and coliphage) at all temperatures (represented by more negative decay rates). This was also illustrated in the survival curves, with enterococci and coliphage persisting in the water column for longer periods of time compared with *E. coli* (Figs. 6.3 – 6.4).

Considering the pathogenic organisms, in the overlying water, the decay of *S. derby* was generally greater than *S. typhimurium* ($P < 0.05$ for columns containing Onkaparinga and Pt Adelaide sediments incubated at 20°C), with greatest decay rate of -1.36 (k; d^{-1}) occurring for *S. derby* in overlying water incubated at 30°C. No significant difference was observed, however, for decay rates of both pathogenic organisms in the sediment. Temperature did not have a significant effect on the decay rate of *S. typhimurium* in Henley Beach and Onkaparinga sediments, or *S. derby* in Onkaparinga sediment ($P > 0.05$).

No direct correlations were observed between decay rates in overlying water of *Salmonella* spp. and the faecal indicator organisms investigated ($P > 0.05$). Decay of the pathogenic microorganisms in overlying water closely resembled that of *E. coli* at 20C, however at 30C, decay of *Salmonella* spp. were generally less than *E. coli* although not significantly). In general, decay rates for *S. typhimurium* and *S. derby* were greater than enterococci and coliphage.

Decay rates of *Salmonella* spp. in the surface sediment layer were, however, generally lower than *E. coli*. This was most apparent at 30°C where in all sediment types, decay of *Salmonella* spp was significantly less than *E. coli* ($P < 0.01$). At 20°C the difference between decay rates was only significant in Pt Adelaide sediment ($P < 0.001$).

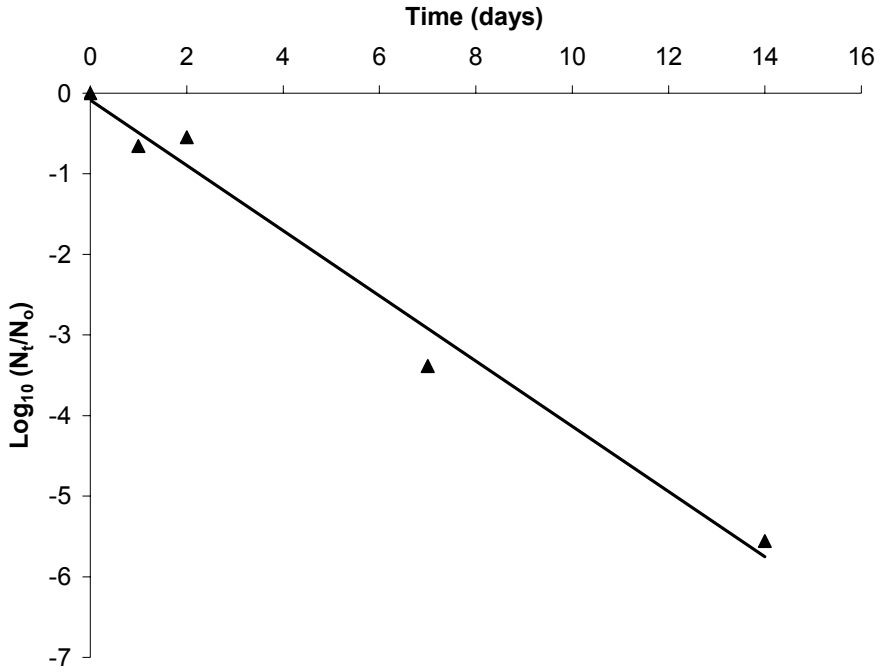

Figure 6.1 Example of exponential decay plot used to calculate decay rates (decay of coliphage in overlying water in microcosm containing Henley Beach sediment; $r^2 = 0.98$).

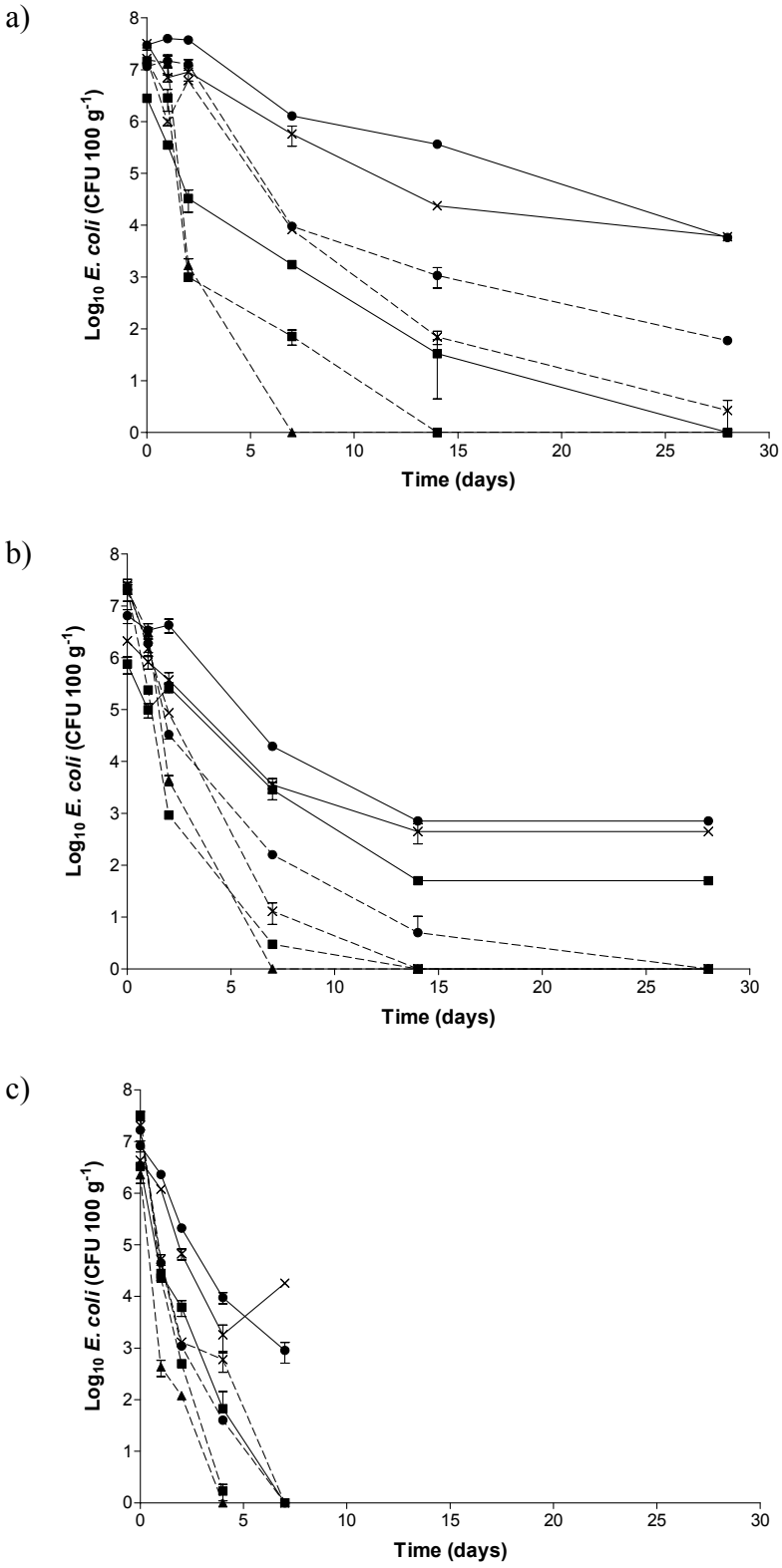

Figure 6.2 Survival of *E. coli* incubated at 10 °C (a), 20 °C (b), and 30 °C (c) in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (●) and column containing water only (▲).

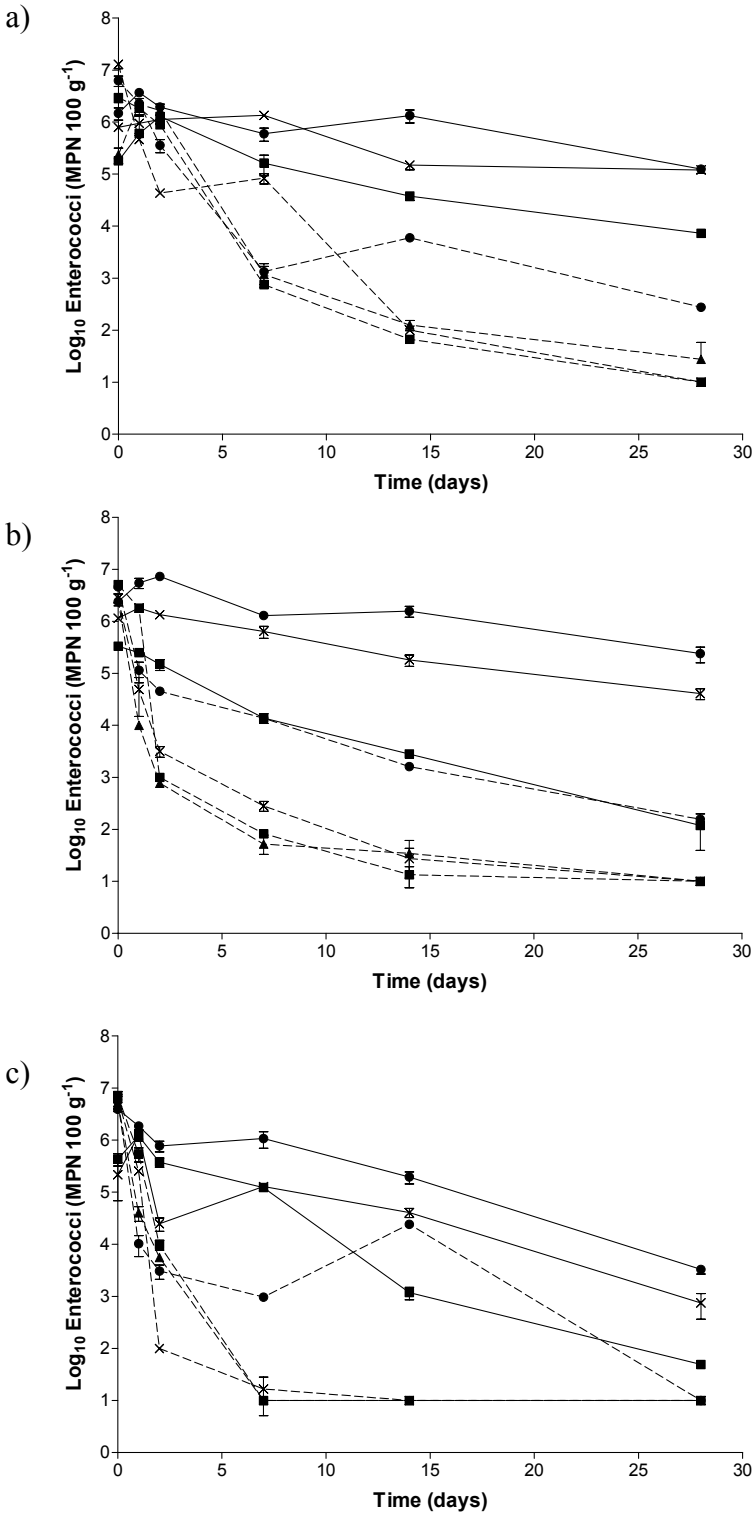

Figure 6.3 Survival of *Enterococcus faecium* incubated at 10 °C (a), 20 °C (b), and 30 °C (c) in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (●) and column containing water only (▲).

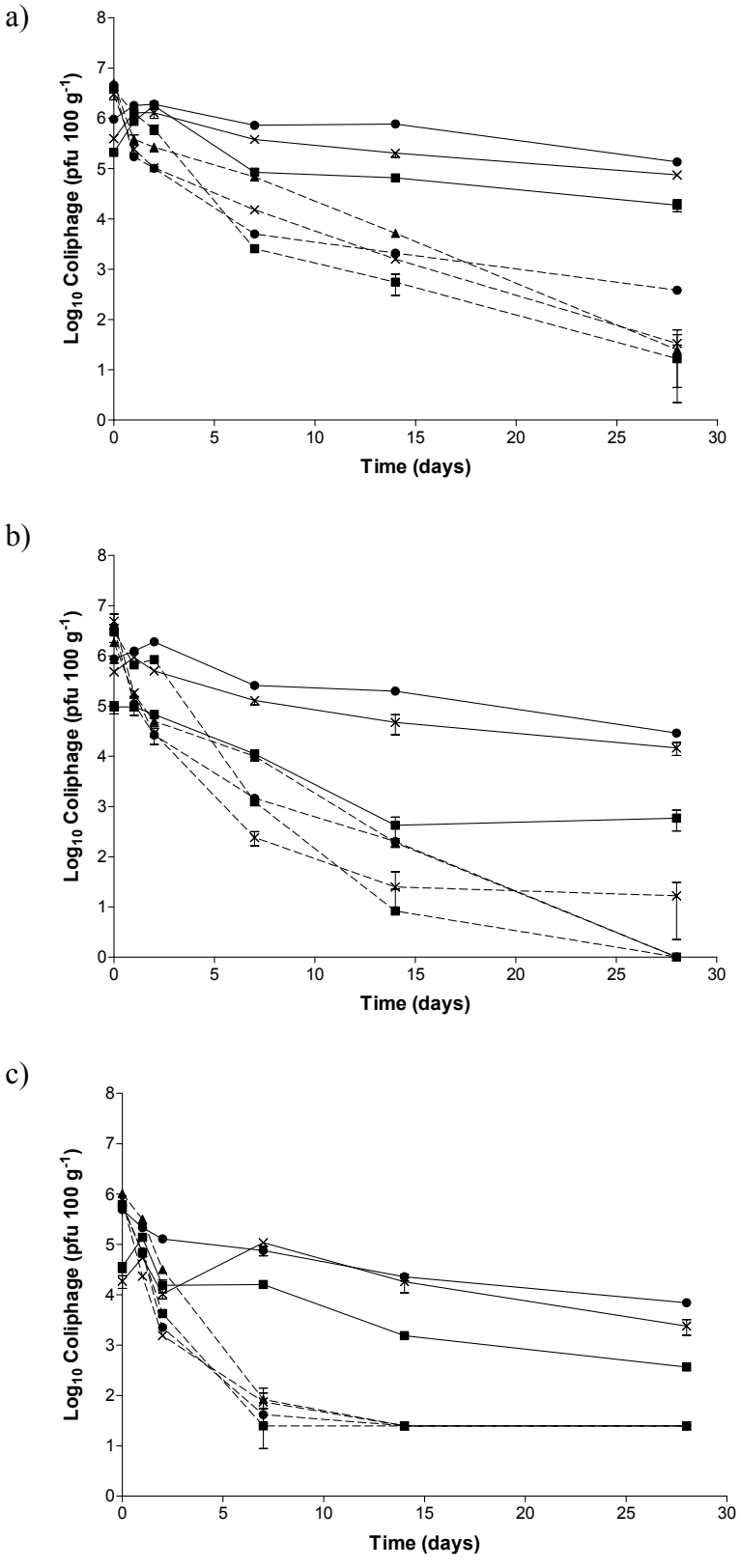

Figure 6.4 Survival of coliphage incubated at 10 °C (a), 20 °C (b), and 30 °C (c) in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (●) and column containing water only (▲).

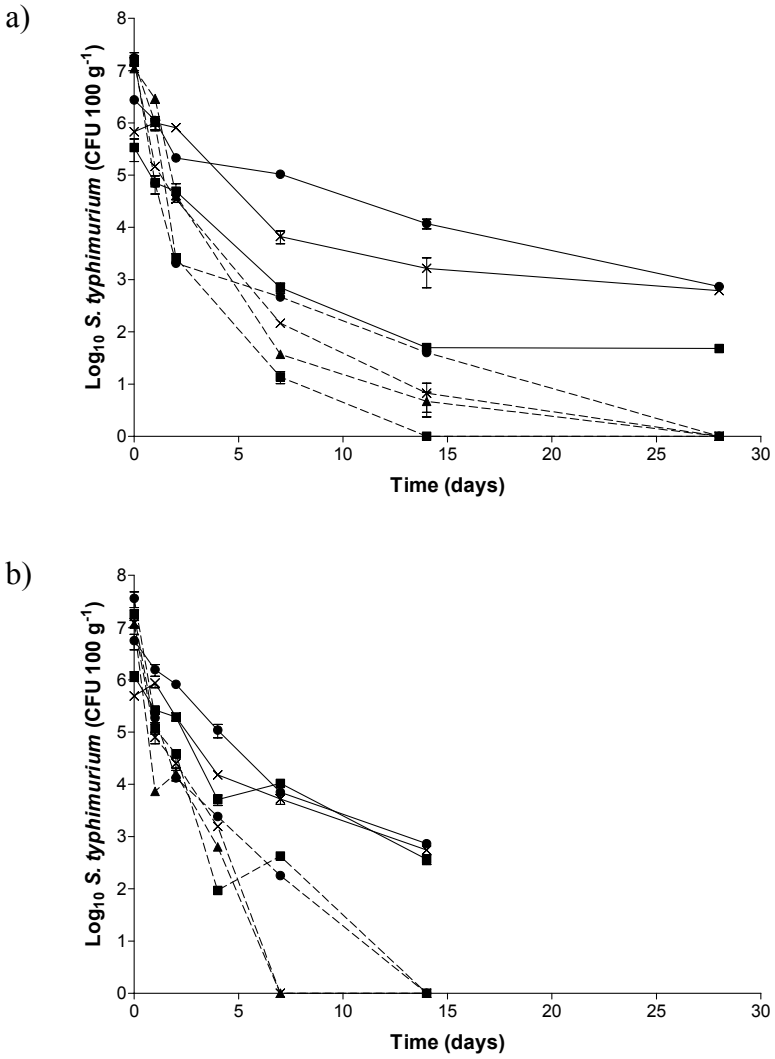

Figure 6.5 Survival of *S. typhimurium* incubated at 20 °C (a) and 30 °C (b) in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (●) and column containing water only (▲).

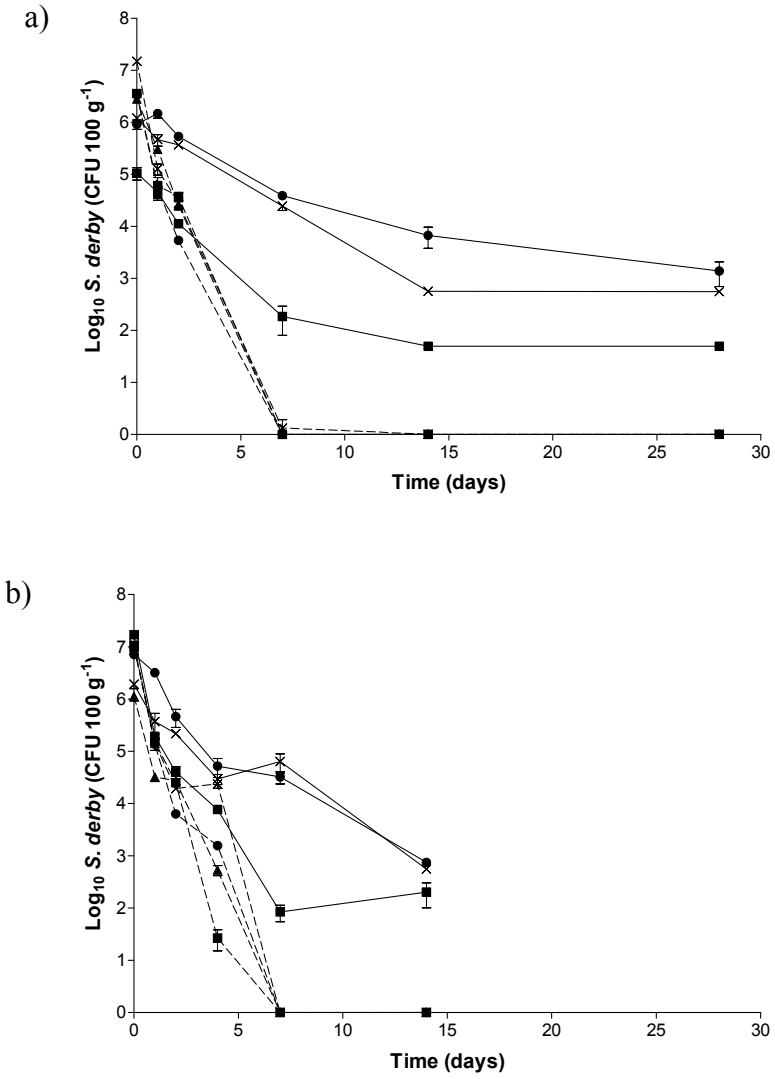

Figure 6.6 Survival of *S. derby* incubated at 20 °C (a) and 30 °C (b) in sediment (—) and water (----) from Henley Beach (■), Onkaparinga (×), Pt. Adelaide (●) and column containing water only (▲).

Table 6.4 Decay rate constants (k; d^{-1}) for *E. coli* in water and sediment; **P* <0.05.

	Water						**Sediment**					
	10 °C		20 °C		30 °C		10 °C		20 °C		30 °C	
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.47	0.80*	-0.89	0.86	-1.74	0.95*	-0.32	0.93*	-0.32	0.98*	-1.11	0.95*
Onkaparinga	-0.24	0.90*	-0.52	0.89*	-0.91	0.89*	-0.13	0.89*	-0.22	0.97*	-0.87	0.99*
Port Adelaide	-0.21	0.86*	-0.45	0.89*	-0.95	0.89*	-0.14	0.97*	-0.49	0.98*	-0.58	0.96*
Water only	-1.04	0.88	-1.03	0.94*	-2.13	0.84						

Table 6.5 . Decay rate constants (k; d^{-1}) for enterococcus in water and sediment; *P <0.05.

	Water						Sediment					
	10ºC		20ºC		30ºC		10ºC		20ºC		30ºC	
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.36	0.93*	-0.66	0.74	-0.80	0.95*	-0.08	0.92*	-0.12	0.97*	-0.16	0.95*
Onkaparinga	-0.20	0.85*	-0.30	0.78*	-2.40	0.94*	-0.04	0.75	-0.06	0.91*	-0.09	0.74*
Port Adelaide	-0.14	0.72*	-0.13	0.81*	-0.13	0.54	-0.04	0.74	-0.06	0.90*	-0.10	0.95*
Water only	-0.35	0.90*	-0.54	0.71	-0.73	0.91*						

Table 6.6 Decay rate constants (k; d^{-1}) for coliphage in water and sediment; *P <0.05.

	Water						Sediment					
	10ºC		20ºC		30ºC		10ºC		20ºC		30ºC	
	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.19	0.89*	-0.40	0.98*	-0.60	0.95*	-0.06	0.73	-0.18	1.00*	-0.08	0.83
Onkaparinga	-0.16	0.93*	-0.34	0.87*	-0.50	0.82	-0.04	0.92*	-0.06	0.91*	-0.04	0.52
Port Adelaide	-0.12	0.75*	-0.20	0.91*	-0.55	0.90	-0.04	0.92*	-0.06	0.90*	-0.06	0.92*
Water only	-0.17	0.97*	-0.21	0.96*	-0.58	0.99*						

Table 6.7 Decay rate constants (k; d^{-1}) for *S. typhimurium* in water and sediment; *P <0.05.

	Water				**Sediment**			
	20 °C		30 °C		20 °C		30 °C	
	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.81	0.87	-0.44	0.80*	-0.27	0.96*	-0.23	0.86*
Onkaparinga	-0.40	0.87	-0.92	0.96*	-0.22	0.90*	-0.23	0.90*
Port Adelaide	-0.20	0.74*	-0.46	0.87*	-0.12	0.94*	-0.28	0.94*
Water only	-0.78	0.96*	-0.87	0.89*				

Table 6.8 Decay rate constants (k; d^{-1}) for *S. derby* in water and sediment; *P <0.05.

	Water				Sediment			
	20 °C		30 °C		20 °C		30 °C	
	k	r^2	k	r^2	k	r^2	k	r^2
Henley Beach	-0.89	0.98*	-1.36	0.99*	-0.24	0.90*	-0.67	0.92*
Onkaparinga	-0.94	0.97*	-0.88	0.90*	-0.23	1.00*	-0.22	0.91*
Port Adelaide	-0.87	0.97*	-0.91	0.85	-0.11	0.88*	-0.27	0.93*
Water only	-0.92	1.00*	-0.77	0.93*				

An inverse relationship was identified between temperature and indicator organism survival in both overlying water and sediment (Tables 6.4 – 6.8). Temperature was found to have a more significant impact on *E. coli* survival compared with enterococci and coliphage. In particular, increased temperature had a relatively insignificant impact on survival of coliphage in sediment. Significant relationships between temperature and decay rate of faecal indicator organisms studied were observed in both overlying water and sediment (Figs. 6.7 – 6.17). In general, an exponential relationship between decay rate and temperature was observed for *E. coli* in the all sediment types ($P < 0.05$). No significant relationships were, however, observed for *E. coli* decay in the overlying water. Relationships between decay rates and temperature for enterococci was significantly linear in Henley Beach sediment, and exponential in Onkaparinga and Pt Adelaide sediment. Significant relationships for coliphage decay was only observed in overlying water.

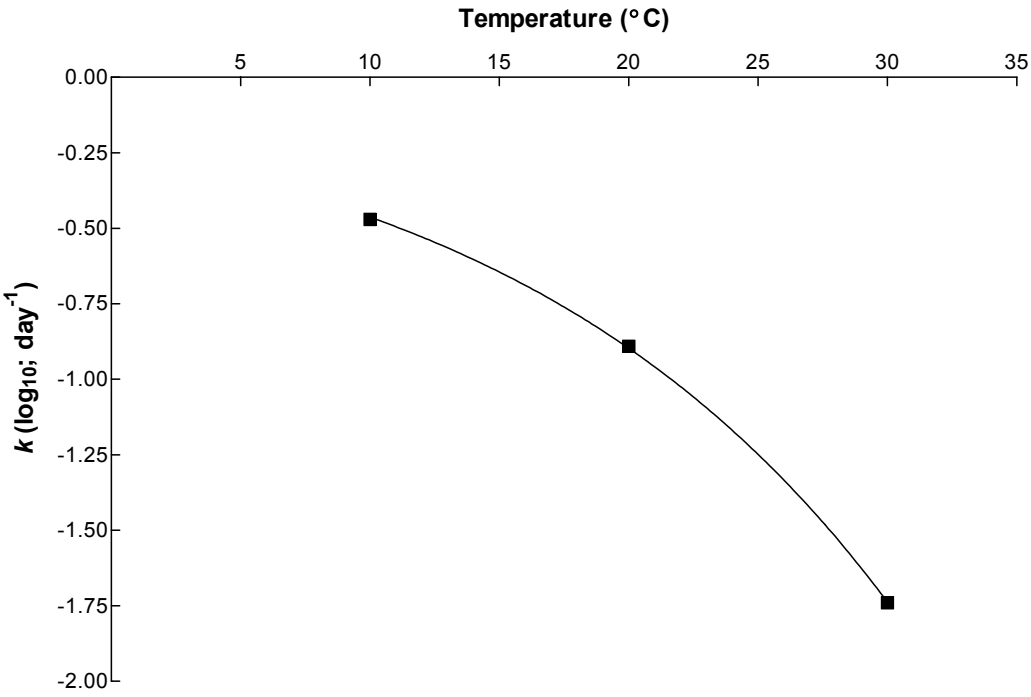

Figure 6.7 Relationship between decay rate (k; d^{-1}) of *E. coli* in overlying water of column containing Henley Beach sediment with temperature ($k = -0.239.e^{0.0661T}$; $r^2 = 1.000$).

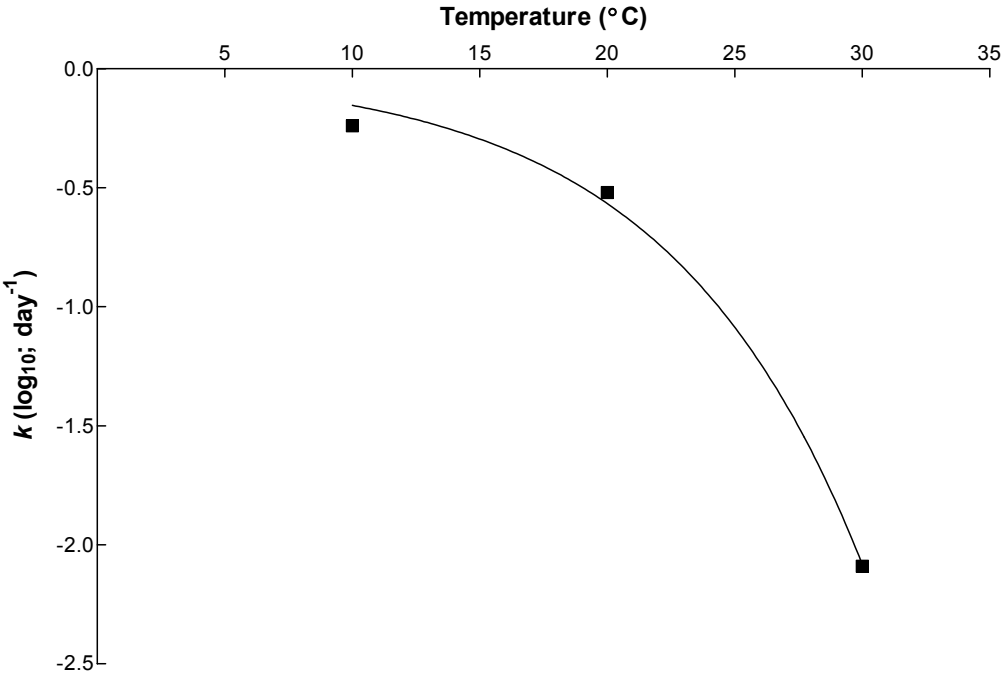

Figure 6.8 Relationship between decay rate (k; d^{-1}) of *E. coli* in overlying water of column containing Onkaparinga sediment with temperature ($k = -0.042.e^{0.995T}$; $r^2 = 0.995$).

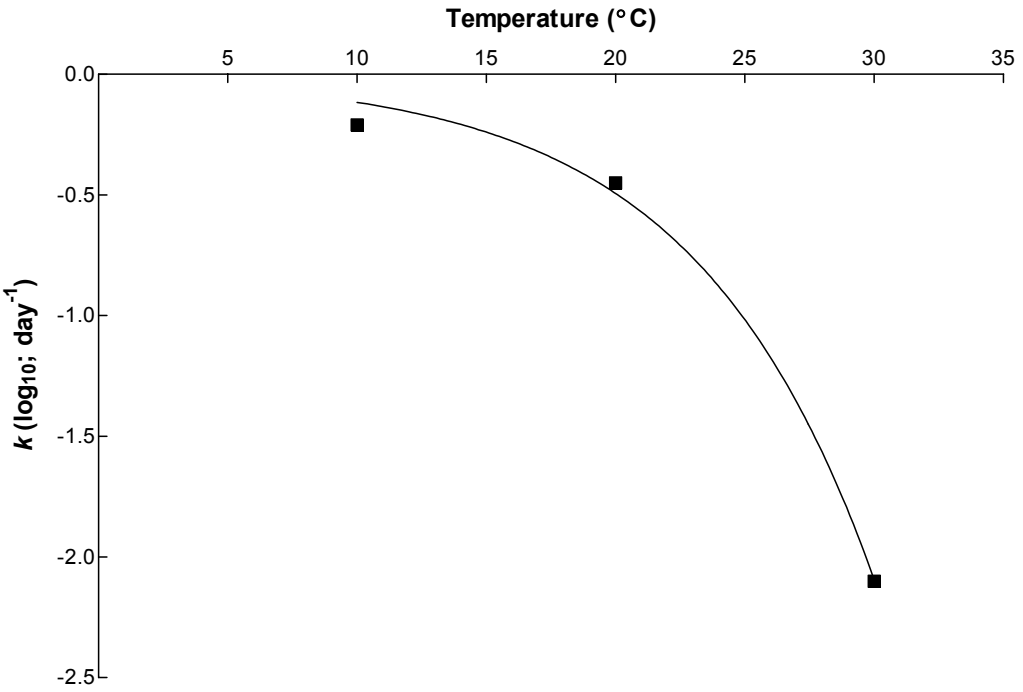

Figure 6.9 Relationship between decay rate (k; d^{-1}) of *E. coli* in overlying water of column containing Pt Adelaide sediment with temperature (k = -0.0275.$e^{0.1444T}$; r^2 = 0.995).

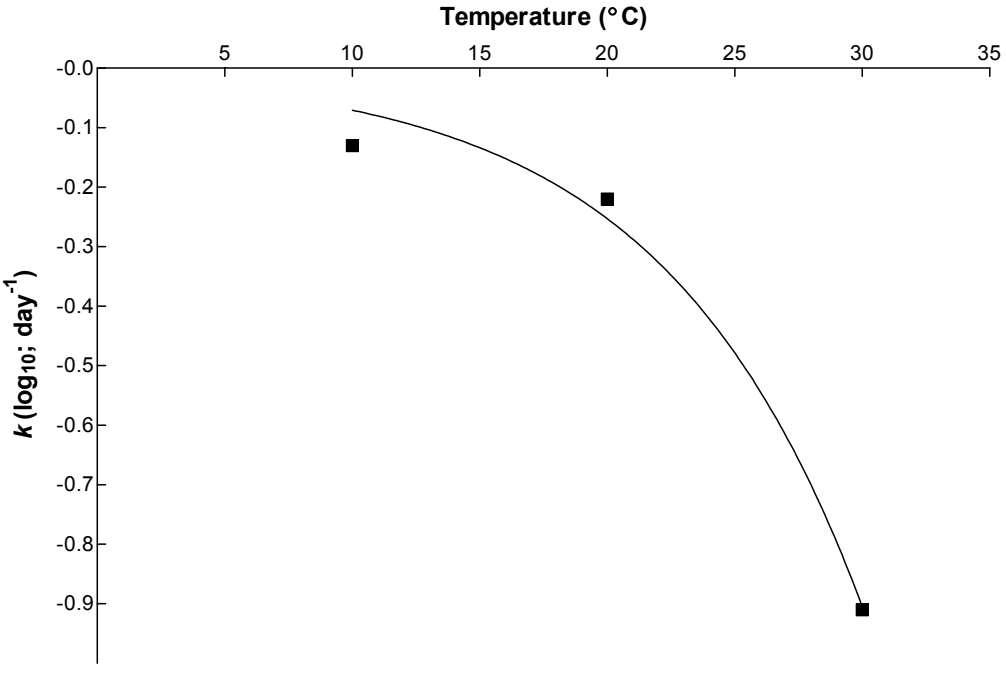

Figure 6.10 Relationship between decay rate (k; d^{-1}) of *E. coli* in surface layer of Onkaparinga sediment with temperature (k = -0.0198.$e^{0.1274T}$; r^2 = 0.987).

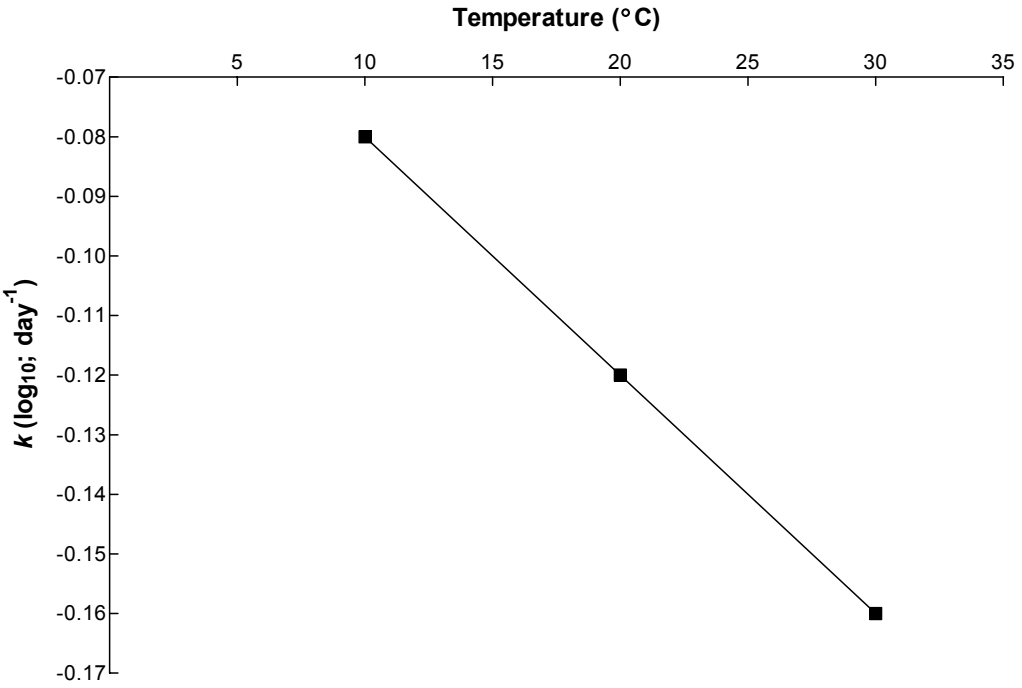

Figure 6.11 Relationship between decay rate (k; d^{-1}) of enterococci in the surface layer of Henley Beach sediment with temperature (k = -0.004T + 0.040; r^2 = 1.00).

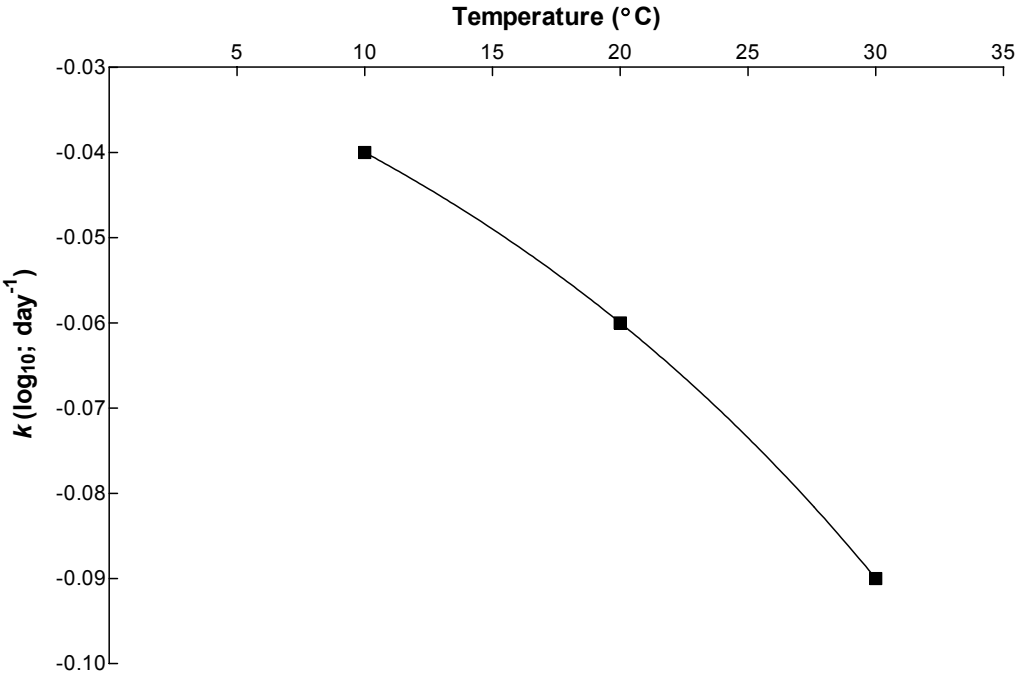

Figure 6.12 Relationship between decay rate (k; d^{-1}) of enterococci in the surface layer of Onkaparinga sediment with temperature (k = -0.0267.$e^{0.0406T}$; r^2 = 1.000).

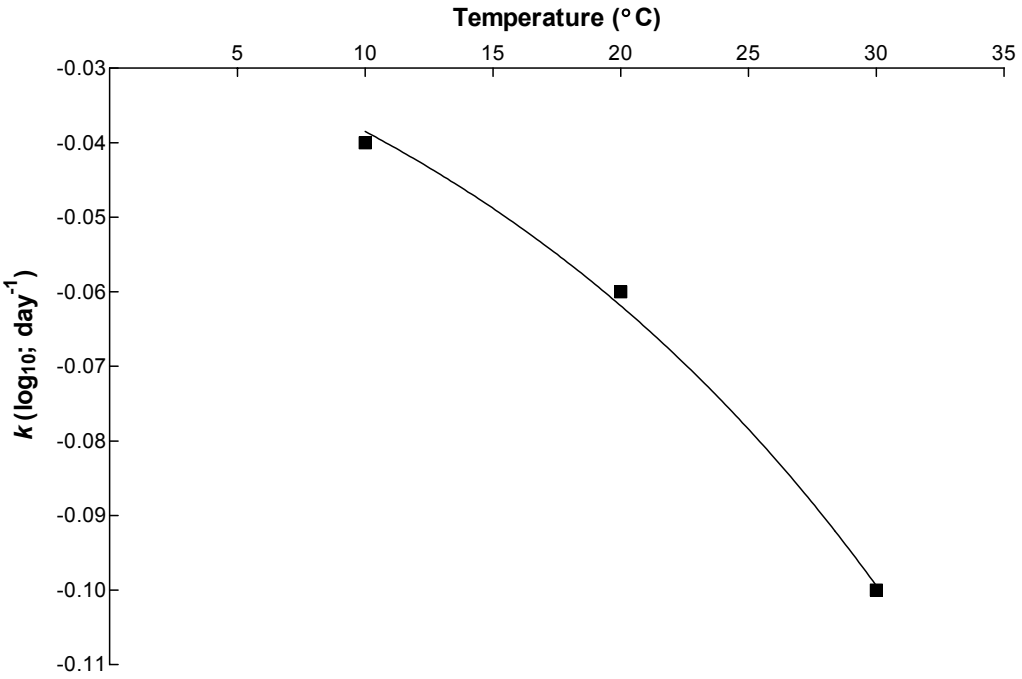

Figure 6.13 Relationship between decay rate (k; d^{-1}) of enterococci in surface layer of Pt Adelaide sediment with temperature (k = $-0.0240.e^{0.0.0474T}$; $r^2 = 0.997$).

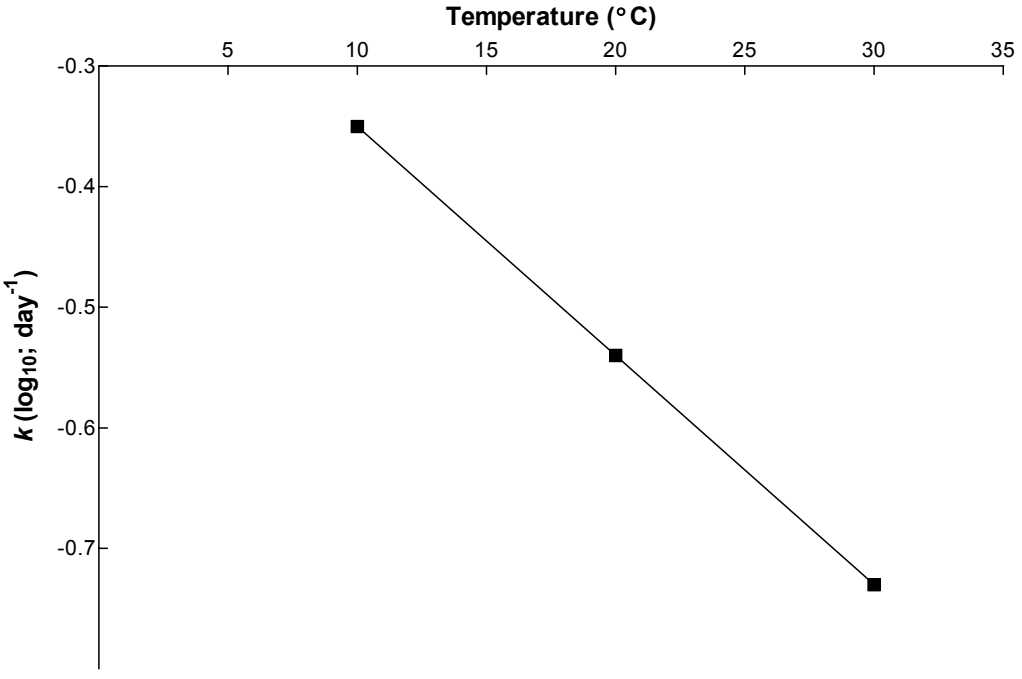

Figure 6.14 Relationship between decay rate (k; d^{-1}) of enterococci in overlying water of control column containing water only with temperature (k = $-0.0190T - 0.1600$; $r^2 = 1.000$).

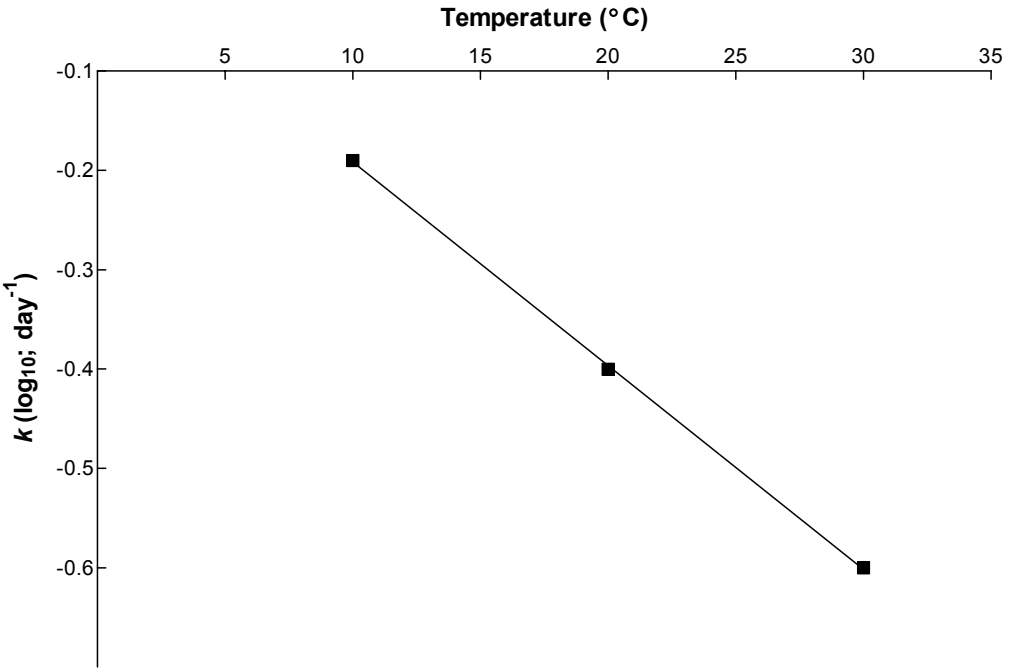

Figure 6.15 Relationship between decay rate (k; d^{-1}) of coliphage in overlying water of column containing Henley Beach sediment with temperature (k = -0.0205T + 0.0133; r^2 = 0.9998).

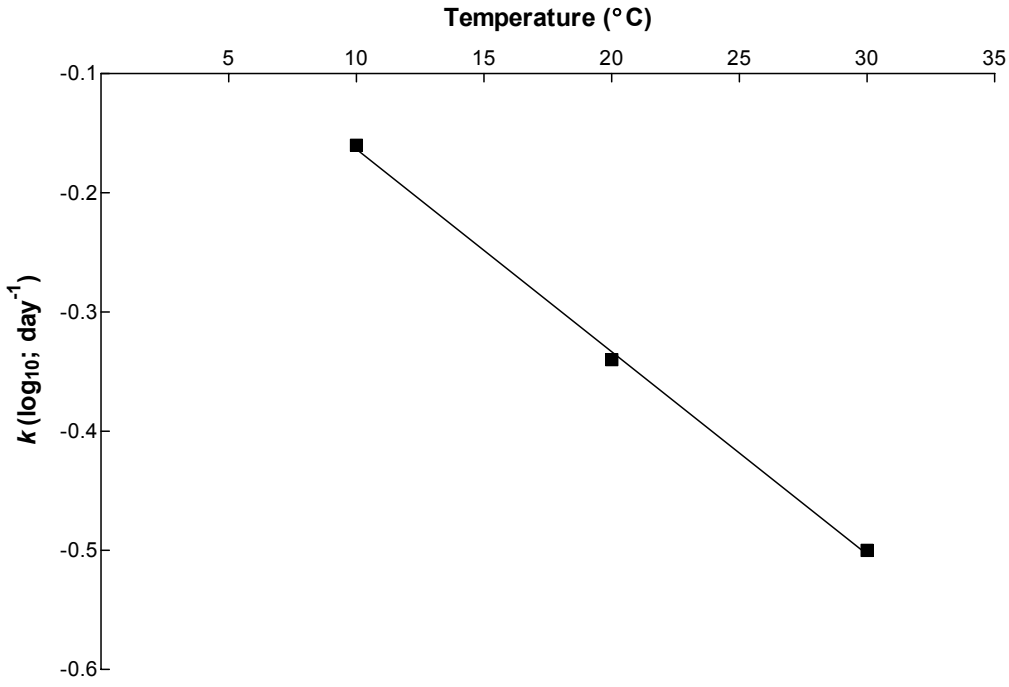

Figure 6.16 Relationship between decay rate (k; d^{-1}) of coliphage in overlying water of column containing Onkaparinga sediment with temperature (k = -0.0170T + 0.0067; r^2 = 0.998).

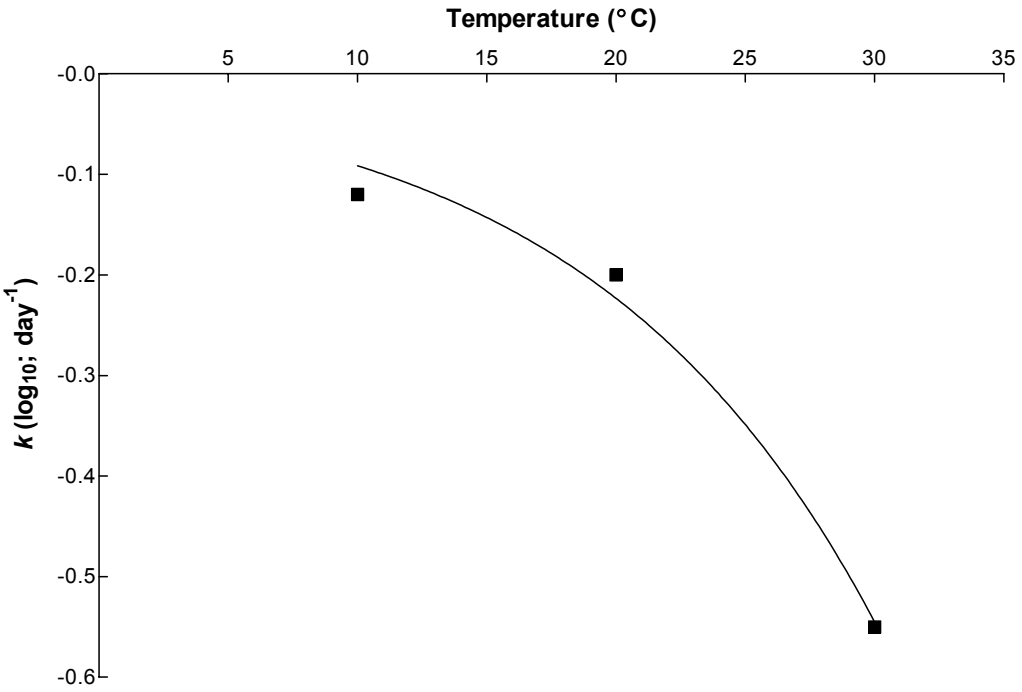

Figure 6.17 Relationship between decay rate (k; d^{-1}) of coliphage in overlying water of column containing Pt Adelaide sediment with temperature ($k = -0.0375.e^{0.0892T}$; $r^2 = 0.987$).

6.2.2 Resuspension studies

Resuspension of *E. coli* into the water column following disruption of the sediment was most noticeable for sediment from Henley Beach (high sand content) incubated at 10°C (Fig. 6.18). Under static conditions the concentration of *E. coli* in the water column contributed to only 3.0% of the total concentration (sediment and water) by Day 2. After the same period of time under stirred conditions, when the top two centimetres of sediment was mixed immediately prior to enumeration, the concentration of *E. coli* in the water column contributed 85.3% of the total load. The effect of stirring on resuspension was time dependent and thus concentration dependent, as by day 7 under stirred conditions the number of *E. coli* in the water column had declined to 25.9% of the total number (compared with 3.9% for static). Resuspension was also observed for Onkaparinga sediment at 10°C, particularly within the first 24 hours (Fig 6.19). At 30°C however, resuspension was not a significant factor from any of the sediments tested.

Enterococci appeared to be more readily resuspended from the surface sediment layer into the overlying water than *E. coli* under a variety of conditions tested. This was most apparent in Onkaparinga sediment (Fig. 6.20). At 10°C under static conditions, the concentration of enterococci in the overlying water had reached the limit of detection (< 10 MPN $100mL^{-1}$) by day 10. When the surface sediment layer was mixed immediately prior to sampling, the proportion of enterococci enumerated (of the total number) from the overlying water was 6.2% and 5.5% on Days 14 and 28 respectively (Fig. 6.20). A similar trend was observed for resuspension from sediment into overlying water when incubated at 30°C (Figs. 6.22 – 6.23). Resuspension of coliphage was also observed from Henley Beach and Onkaparinga sediment, particularly on the initial three days of sampling (Figs. 6.24 – 6.25). Resuspension of organisms was generally not observed from Pt Adelaide sediment (containing the greatest proportions of small particle size).

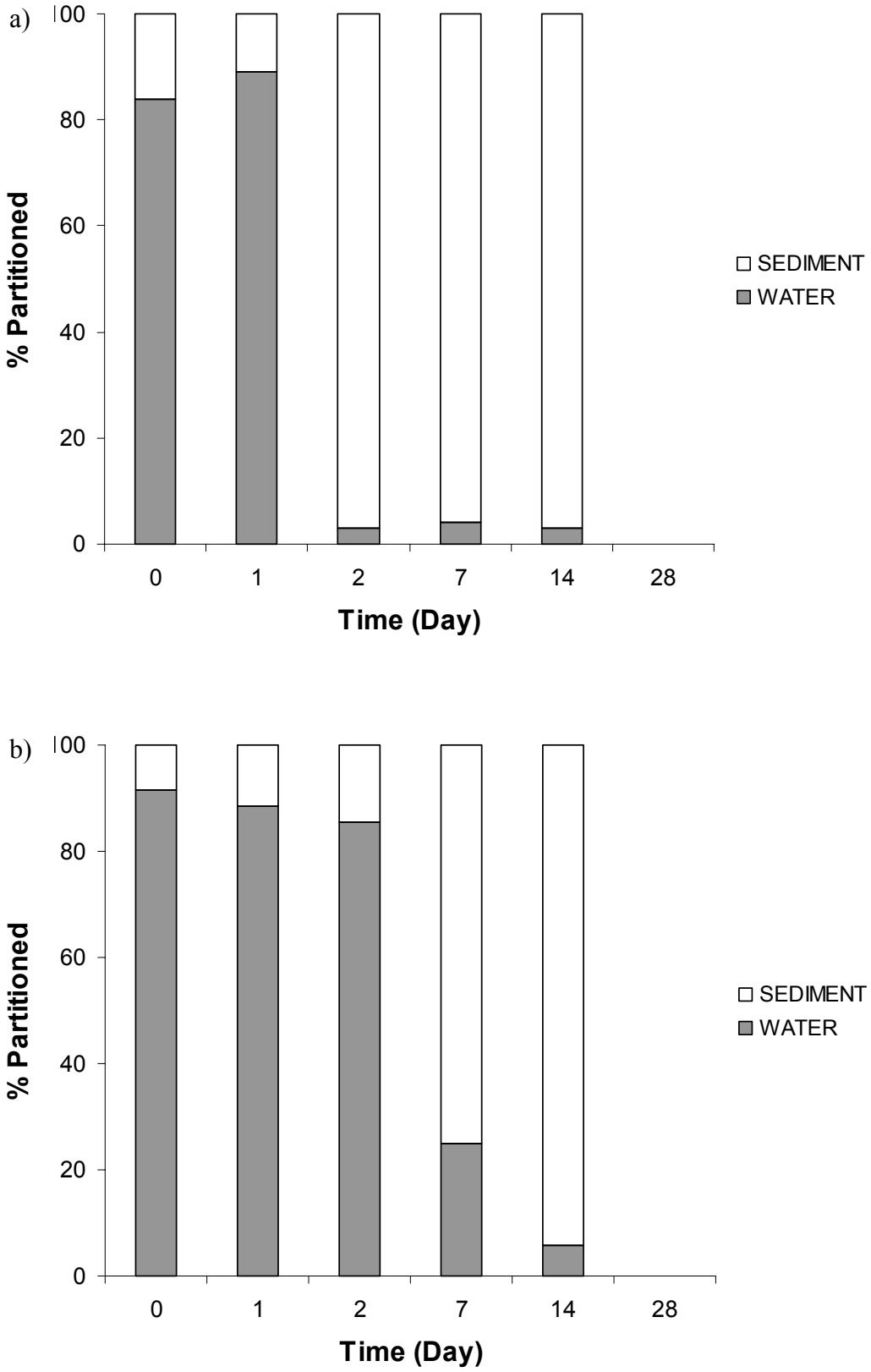

Figure 6.18 Partitioning of *E. coli* between Henley Beach sediment and water incubated at 10ºC under (a) static and (b) stirred conditions.

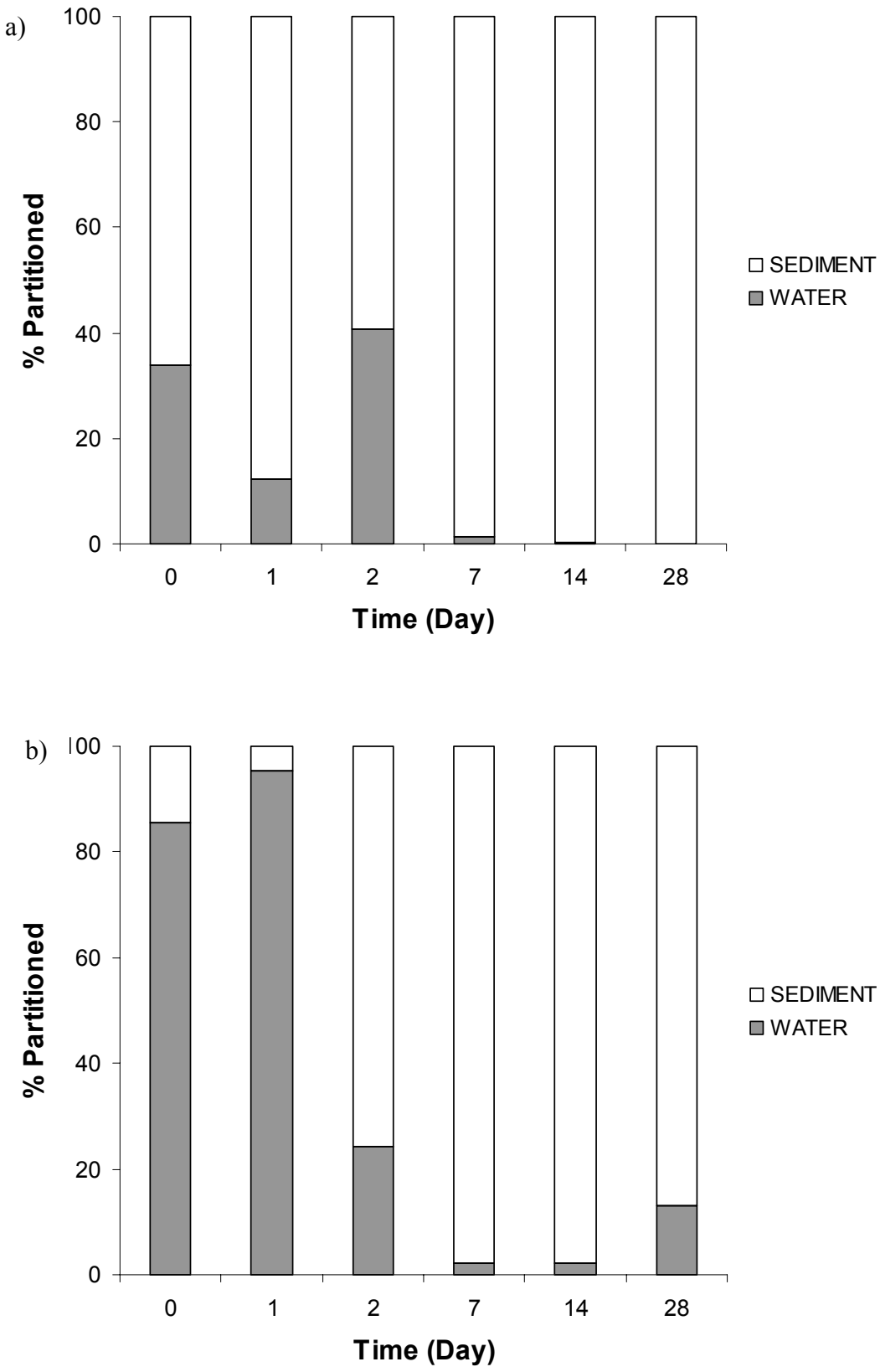

Figure 6.19 Partitioning of *E. coli* between Onkaparinga sediment and water incubated at 10ºC under (a) static and (b) stirred conditions.

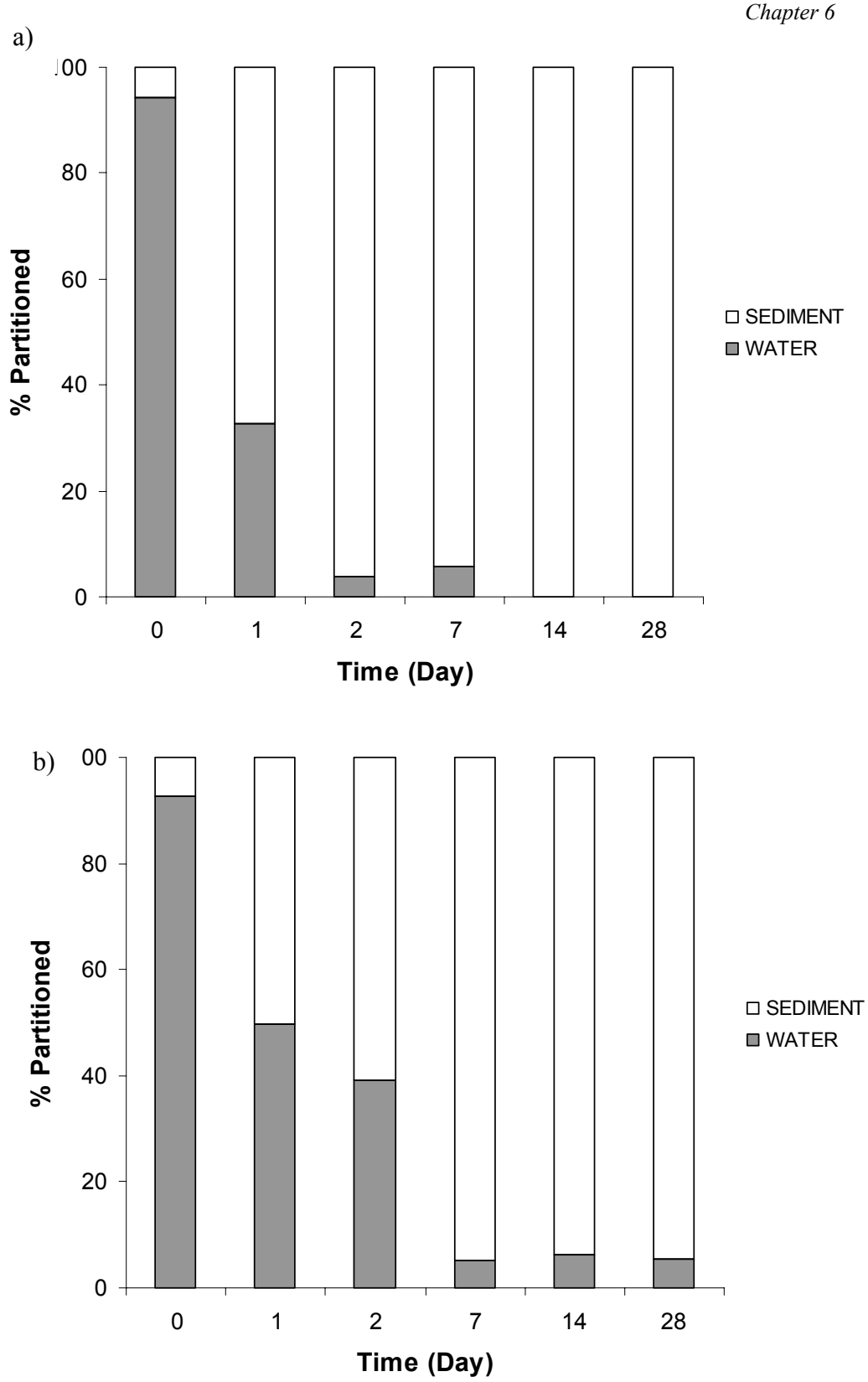

Figure 6.20 Partitioning of enterococci between Onkaparinga sediment and water incubated at 10ºC under (a) static and (b) stirred conditions.

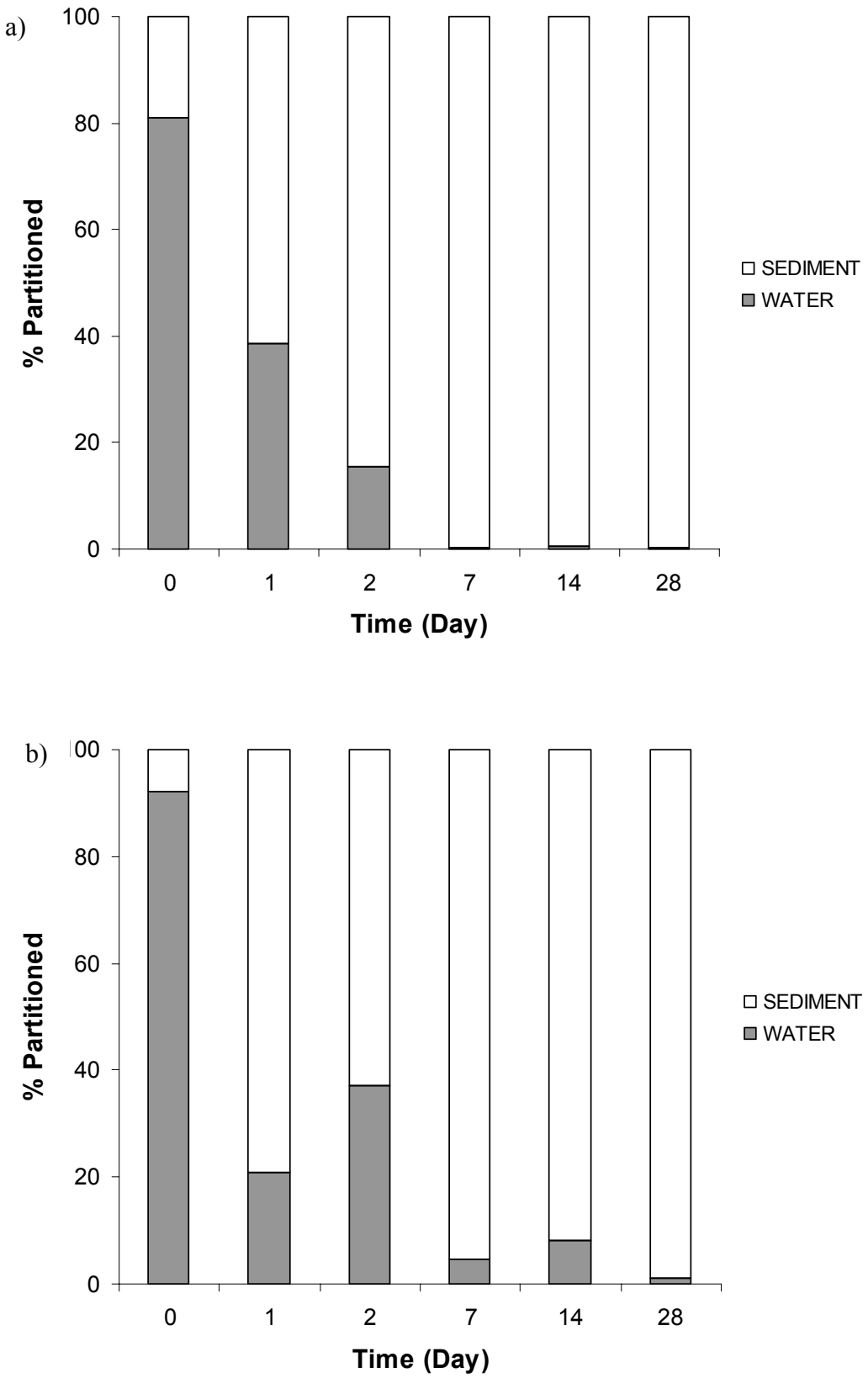

Figure 6.21 Partitioning of enterococci between Pt Adelaide sediment and water incubated at 10ºC under (a) static and (b) stirred conditions.

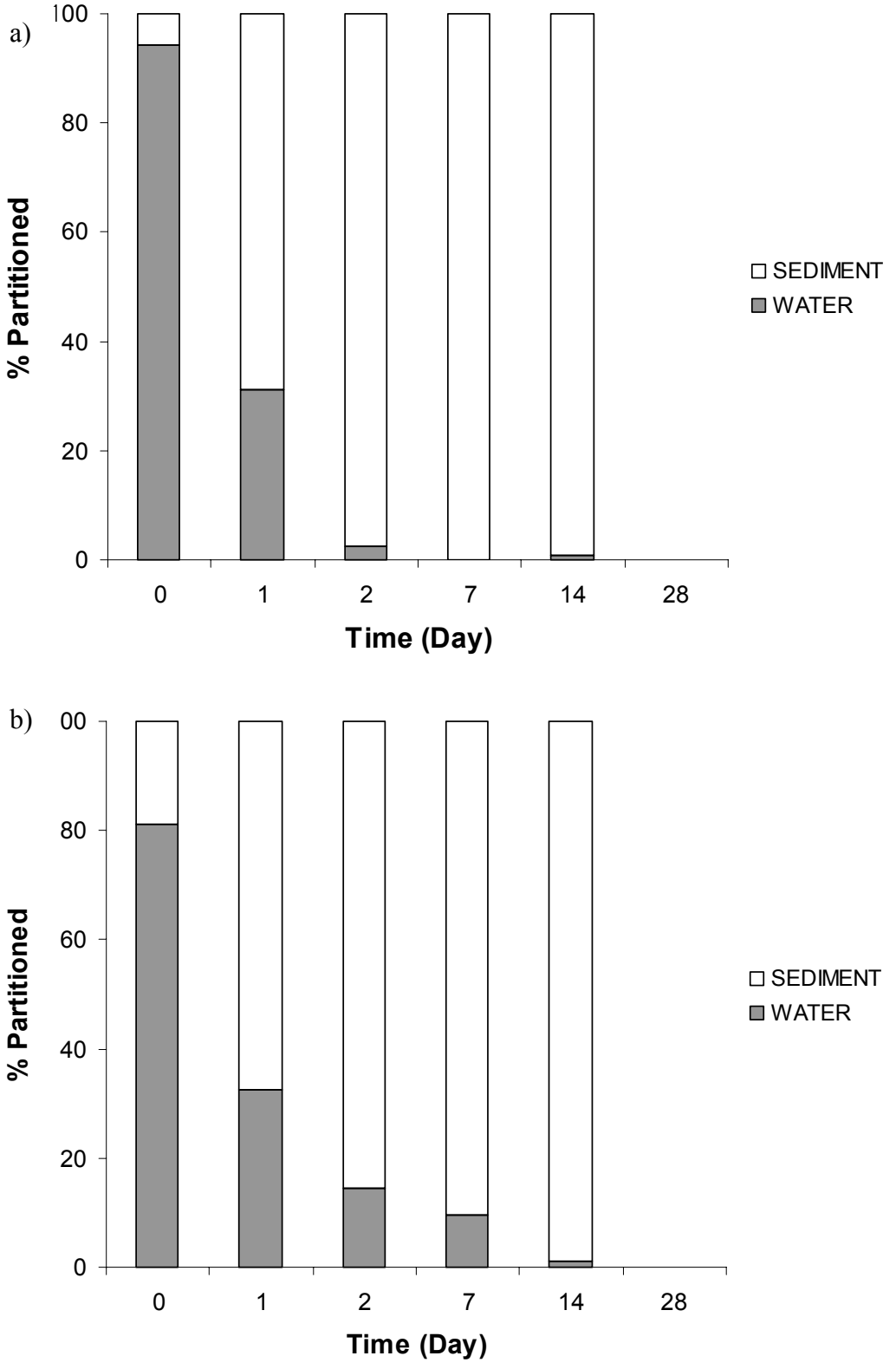

Figure 6.22 Partitioning of enterococci between Henley Beach sediment and water incubated at 30ºC under (a) static and (b) stirred conditions.

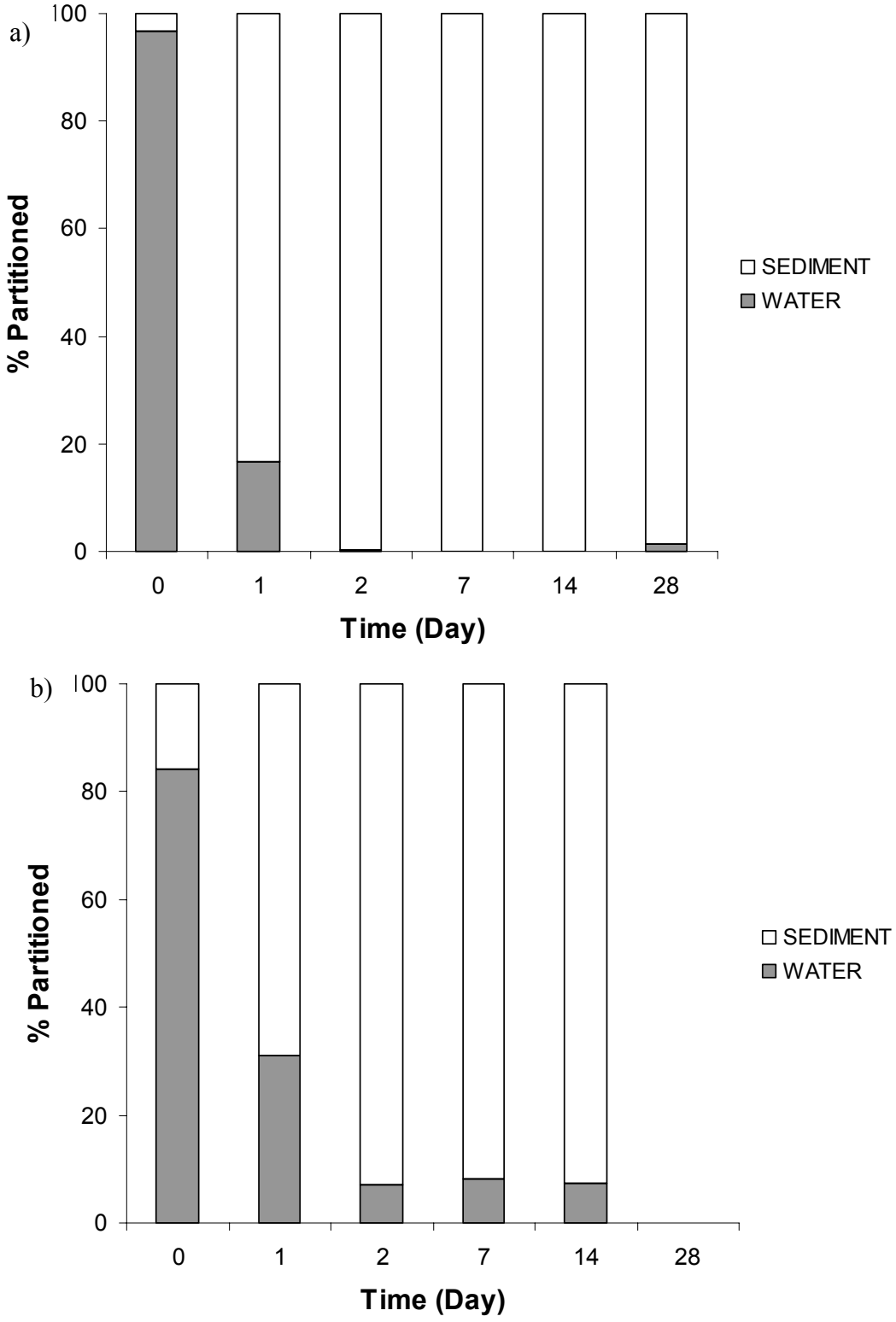

Figure 6.23 Partitioning of enterococci between Onkaparinga sediment and water incubated at 30ºC under (a) static and (b) stirred conditions.

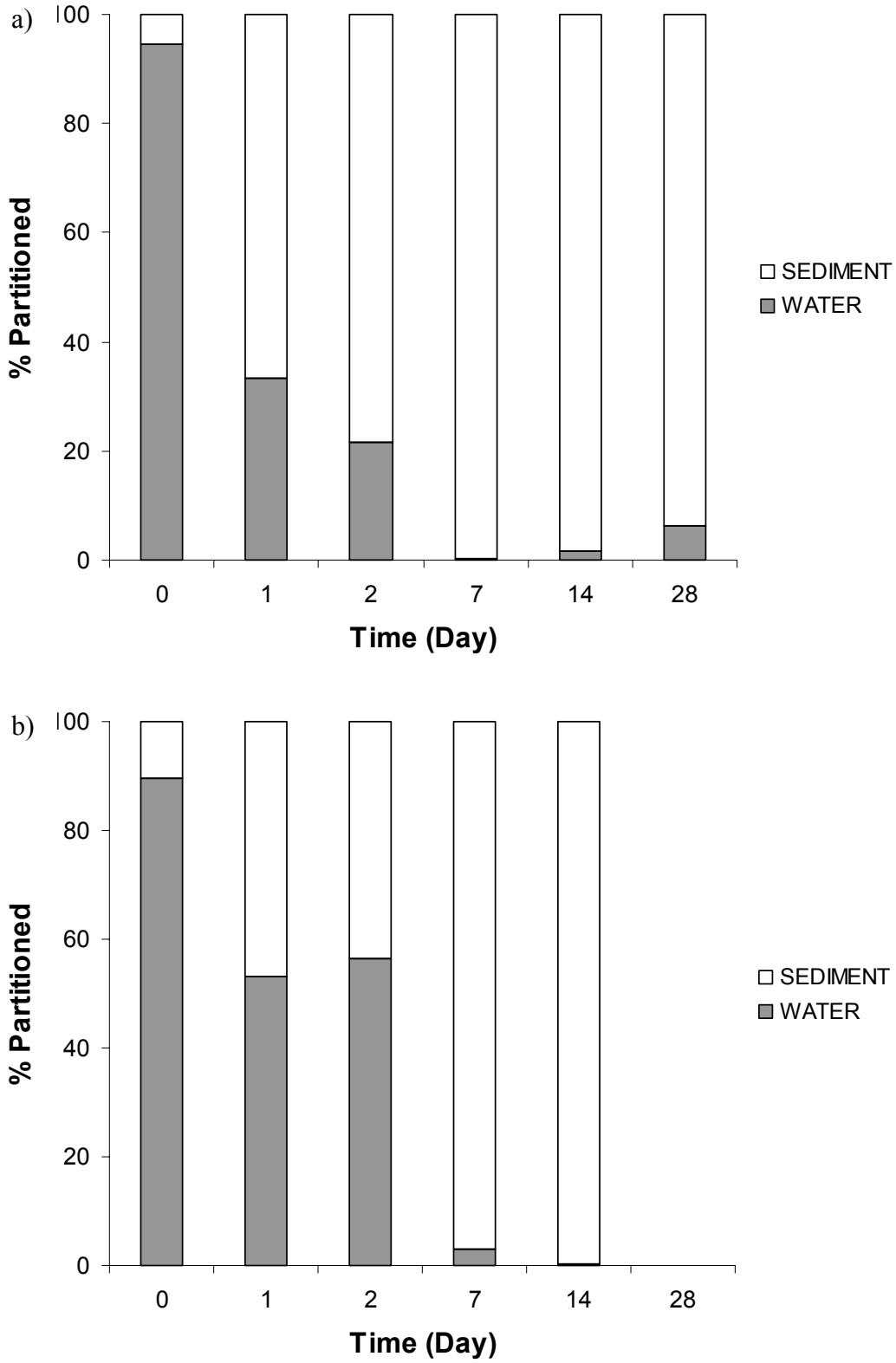

Figure 6.24 Partitioning of coliphage between Henley Beach sediment and water incubated at 30ºC under (a) static and (b) stirred conditions.

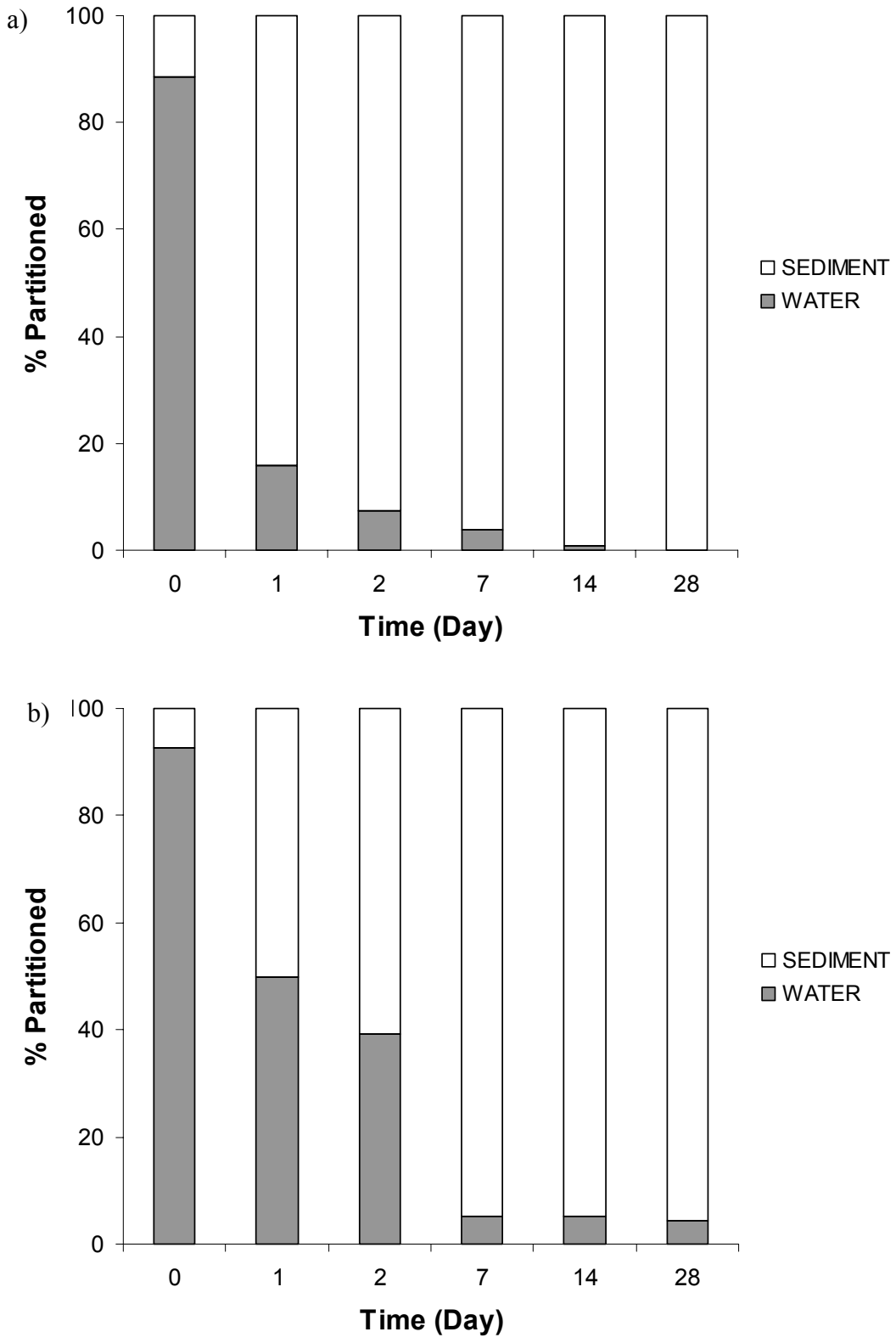

Figure 6.25 Partitioning of coliphage between Onkaparinga sediment and water incubated at 10ºC under (a) static and (b) stirred conditions.

6.3 DISCUSSION

In the non-sterile microcosms used in this study, the number of organisms enumerated in both overlying water and sediment declined more rapidly with increased temperature. Under many conditions, decay rates could be modelled against temperature using linear or exponential equations (Figs 6.7 – 6.17). Temperature was found to have a more significant impact on *E. coli* survival compared with enterococci and coliphage. In particular, increased temperature had a relatively insignificant impact on survival of coliphage in sediment ($P > 0.05$). As with the faecal indicator organisms, increased decay rates in the overlying water were observed for both *S. typhimurium* and *S. derby* at 30°C compared with 20°C. Similar results were observed in a study by Monfort *et al.* (2000) where decay rates for *S. panama* in both sterile and non-sterile seawater microcosms were found to be greater at higher temperatures.

Of the faecal indicator organisms tested, decay of *E. coli* was significantly greater than that of enterococci and coliphage. At 10°C and 20°C there was very minimal decay of enterococci and coliphage in sediments consisting of small particle size and high organic carbon over the 28 d sampling period (Figs. 6.3 – 6.4). The increased survival of enterococci compared with *E. coli* in seawater (using *in-situ* diffusion chambers) has previously been reported by Vasconcelos and Swartz, (1976). A study by Sinton *et al*. (1999) identified a greater survival of somatic coliphage in seawater compared with *E. coli*. This was most significant when organisms were exposed to sunlight, with the observed T_{90} for somatic coliphage being 51.3 h compared with 7.7 h for faecal coliforms during winter (8 – 10°C). Under dark conditions the T_{90} values were 102 h and 63 h for somatic coliphage and faecal coliforms respectively. This compares well with decay rates observed in this microcosm study (which was undertaken without exposure to sunlight) whereby the mean T_{90} for somatic coliphage and *E. coli* in overlying water was calculated as 150 h and 49 h respectively.

Other studies have demonstrated prolonged survival of *E. coli* in non-sterile water at lower temperatures (Özkanca and Flint, 1997; Rhodes and Kator, 1988; Flint,

1987). Of public health significance, the survival of enteropathogenic *E. coli* in freshwater has also been identified to be greater at lower temperatures (Terzieva and McFeters, 1991). A study by Özkanca and Flint (1997) investigating the activity of *E. coli* under starvation conditions identified that *E. coli* cells incubated at 37°C had reduced electron transport system and respiratory enzyme activity as well as exhibiting the largest reduction of cell size compared with *E. coli* cells incubated at lower temperatures which suggests greater organism stress at higher temperatures.

When directly comparing results with other studies, experimental differences must be recognised. This study utilised intact, non-sterile sediment cores which included added pressures on organism survival through competition with natural fauna, presence of predators and association with sediment nutrients and particles. It has been suggested that autoclaving sediment may increase the transfer of nutrients from the sediment into the water column, therefore influencing survival (Hood and Ness, 1982; Gerba and McLeod, 1976).

Decay rates of *E. coli*, and other pathogenic microorganisms, have been determined to be greater in unfiltered (non-sterile) water compared with filtered water, re-affirming the influence of predation on microbial survival (Rhodes and Kator, 1988; Flint, 1987; McCambridge and McMeekin, 1979). McCambridge and McMeekin (1979) concluded the role played by predacious bacteria was of secondary importance (compared with protozoan predation) in relation to the decay of *E. coli* in marine water. The results of the study indicated that the disappearance of *E. coli* was directly related to the concentration of protozoan predators. The presence of bacteriophage is also attributed to microbial decay in coastal and fresh waters (Roper and Marshall, 1974).

Under all test conditions, decay of *E. coli*, enterococci, coliphage, *S. derby* and *S. typhimurium* was greater in overlying water compared with the surface sediment layer. Sediment characteristics were found to influence survival. In general, of the columns containing sediment, greatest decay occurred in sediment from Henley Beach, which consisted mainly of sand (large particle size). Small particle size

and high organic carbon content in Pt Adelaide and Onkaparinga sediment were found to be more conducive to microbial survival. The lowest decay rates observed were for enterococci and coliphage in Pt Adelaide sediment incubated at 10°C (0.04 *k*; d^{-1}). Enterococci and coliphage persisted in all sediment types for more than 28 d at all temperatures. A study by Howell *et al*. (1996) also identified a significantly greater decay of both *E. coli* and enterococci in sediment consisting of large particle size, compared with sediments consisting of greater proportions of silt and clay. A weak correlation between small particle size and decreased bacterial decay has also been reported for freshwater sediments (Irvine and Pettibone, 1993; Burton *et al.*, 1987).

Roper and Marshall (1974) investigated the effect of sediment on the interaction between *E. coli* and bacteriophage. It was concluded that in clay sediments, *E. coli* is protected from bacteriophage attack due to a sorbed envelope of colloidal material around the cell, preventing attachment of the phage to its host. In saline sediments sorption of bacteriophage to colloidal material was also observed. This may explain the observed increased survival of *E. coli*, enterococci, *S. derby* and *S. typhimurium* in Pt Adelaide sediment (containing the greatest proportion of clay), with greater decay in Onkaparinga and Henley beach sediment respectively. LaBelle and Gerba (1979) studied the adsorption of enteric viruses to estuarine sediment consisting of 20.7% sand, 24.88% clay, 54.34% silt and 3.8% organic matter. Of the poliovirus, coxsackievirus, echovirus and rotavirus added, 99% became adsorbed to sediment. Roper and Marshall (1974) also demonstrated that organisms could be desorbed when salinity levels decreased below a critical value. This may have significant implications for estuarine environments where salinity levels are variable, especially following high rainfall events. This may result in the desorption of faecal indicator and pathogens into the water column during such events.

The inactivation of viruses in water has also been associated with increased temperature and sunlight (Davies-Coley *et al.*, 1999; Wommack *et al.*, 1996; Pesaro *et al.*, 1995). It has also been suggested that proteolytic bacterial enzymes have the ability to inactivate echovirus in fresh water by cleavage of viral

proteins, exposing viral RNA to nuclease digestion (Ward *et al.*, 1986b). Results of a study by Ward *et al*. (1986b) indicated that the virucidal activity of fresh water could be directly correlated with the presence of viable microorganisms. Survival of echovirus in filtered fresh water was substantially greater than in natural (unfiltered) fresh water.

In overlying water, the decay of *S. derby* was generally greater than *S. typhimurium* with greatest decay rate of -1.36 (k; d^{-1}) occurring for *S. derby* in overlying water incubated at 30°C. No significant difference was observed, however, for decay rates of both organisms in the sediment surface layer. Van Donsel and Geldreich (1979) also identified no significant difference between the decay of five species of salmonellae (including *S. typhimurium* and *S. derby*) in freshwater sediments.

Decay rates of *Salmonella* spp. in overlying water were similar to *E. coli* (although no significant correlation was observed). In Pt Adelaide sediment incubated at 20°C, decay of *Salmonella* spp. was significantly less than that observed for *E. coli* ($P < 0.001$). At 30°C, decay rates for *Salmonella* spp. was significantly less than *E. coli* in all sediments ($P < 0.01$). This suggests that under these conditions, the use of faecal coliforms and/or *E. coli* as an indicator of the presence of potential pathogenic bacteria (such as *Salmonella* spp.) in the surface sediment layer may not be appropriate. The use of bacterial indicators to indirectly estimate the presence of pathogens such as viruses and protozoa is increasingly being discouraged (Efstratiou *et al.*, 1998; Callahan *et al.*, 1995).

Using the relatively simple methods to simulate sediment disruption described in this chapter, evidence of resuspension of faecal indicator organisms from the surface sediment layer into the overlying water was observed at a range of temperatures. When the concentration of microorganisms is similar in both overlying water and sediment, the effect of resuspension is minimal due to the effect of dilution of resuspended sediment particles throughout the water column. Significant resuspension of microorganisms, increasing exposure risk during recreational activity, is most likely at recreational coastal sites where the decay

rate of microorganisms in the sediment is substantially less than in the water column. Under these conditions, the concentration of microorganisms in the water column decrease rapidly (from an initial peak), whereas in the surface sediment layer, numbers remain relatively high for a longer period of time. Therefore, as time increases, concentrations in the sediment are substantially greater than in the water column.

An example of this situation is the resuspension of *E. coli* when incubated at 10°C. Under static conditions, the number of *E. coli* present in the water column was 72 CFU 100 mL^{-1} by day seven, however, a concentration of 1.7×10^3 CFU $100mL^{-1}$ remained in the sediment (the number of organisms in the overlying water therefore represented 3% of the total number). When the surface sediment layer was disturbed immediately prior to sampling the microcosm on day 7 the concentration of *E. coli* in the overlying water represented 25% of the total number of *E. coli*). Immediately prior to stirring, only a low concentration of *E. coli* would have been present in the overlying water, with the effect of stirring resulting in the resuspension of sediment-associated bacteria being enumerated from the water column and thus being potentially available for ingestion.

Limited research has been undertaken to estimate resuspension of indicator or pathogenic microorganisms from sediment into the water column. A study by Pettibone *et al*. (1996) investigated the resuspension of bacteria due to turbulence caused by the passage of large ships in the Buffalo River, New York. Elevated concentrations of faecal coliforms and suspended solids were observed at a depth of one and four metres below the surface immediately after a ship passed, with a direct correlation ($P < 0.001$) between the two parameters. The authors noted that the nature of suspended particles will influence the degree of resuspension. Other studies have identified the resuspension of faecal coliforms from the surface sediment layer into the water column during rough weather and storm surges (Obiri-Danso and Jones, 2000; Crabill *et al.*, 1999). The resuspension of these organisms were found to have a significant impact on recreational water quality.

The results of these microcosm studies can be used to better estimate exposure during the first stage of a health risk assessment for recreational coastal areas. It has been demonstrated that temperature and sediment type play an important role in determining decay rates of faecal indicator and the pathogen *Salmonella* spp. in the coastal environment. Use of the modelled decay rates could be included in a quantitative microbial risk assessment to estimate risk of infection to pathogenic microorganisms over a period of time following an initial peak (such as that observed from a high rainfall event) during recreational contact with coastal waters and sediments.

Organisms enumerated from this laboratory study were not exposed to sunlight, which has been shown to adversely effect microbial survival (Fujioka and Yoneyama, 2002; Sinton *et al.*, 2002; Sinton *et al.*, 1999; Davies and Evison, 1991). To determine if results from this microcosm experiment can be representative of *in-situ* conditions, a study was undertaken to determine decay rates of faecal coliforms in water and sediment from a coastal site in metropolitan Adelaide following a high rainfall event. This study is discussed in Chapter 7.

CHAPTER 7: VALIDATION OF MICROCOSM STUDY WITH *IN-SITU* MEASUREMENTS AT A COASTAL SITE FOLLOWING HIGH RAINFALL

Sections of the following chapter have been published in the paper:

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2004) Use of microcosms to determine persistence of *Escherichia coli* in recreational coastal water and sediment and validation with *in situ* measurements. *Journal of Applied Microbiology*. 96(5): 922-930.

7.1 INTRODUCTION

This chapter investigates the persistence of faecal coliforms and *Escherichia coli* in both the water column and sediment at a recreational coastal site following a high rainfall event in mid-summer, February 2000 . Henley Beach is situated at the outlet of the Torrens River in metropolitan Adelaide and is used substantially for recreation during the summer months (at the time the study was undertaken). A high rainfall event occurred in mid February following an extended period of dry weather, providing an ideal opportunity to monitor *in-situ* decay rates for faecal coliforms and *E. coli* at a recreational coastal site and enabling validation of results obtained from laboratory based microcosm experiments.

7.2 MATERIALS AND METHODS

7.2.1 *In-situ* sediment and water samples

Triplicate sediment and water samples were taken from Henley Beach South, approximately 400 m north of the Torrens River outlet (same location as described in Chapter 5), each day following the rain event for a period of 10 days. The natural current along the Adelaide metropolitan coastline is north-ward, therefore areas of the beach are affected by the contaminated plume emanating from the Torrens River outlet. Intact sediment cores were sampled as described in Section 2.2.2 and prepared for enumeration of faecal coliforms by sonication as detailed in Section 2.2.4. Faecal coliforms were enumerated from overlying water and sediment by the membrane filtration method (Section 2.2.6). Water conductivity (Hanna Instruments, Keysborough, VIC, Australia), dissolved oxygen (WTW, Weilheim, Germany) and temperature were measured using hand-held meters at the time of sampling.

7.2.2 Laboratory-based microcosm experiment

Decay rates of *E. coli* in overlying water and sediment from Henley Beach were determined as detailed in Chapter 6.

7.2.3 Statistical analysis

All results were expressed as the mean ± standard deviation of three determinations. Statistical analysis was undertaken using one-way ANOVA and significance expressed at $P \leq 0.05$.

7.3 RESULTS

7.3.1 *In-situ* measurements

As illustrated in Figure 7.1, a significant rainfall event occurred in metropolitan Adelaide on 20 February 2000 with a total precipitation of 37.8 mm. This resulted in a large flow of water from the Torrens River outlet at Henley Beach South (Plate 7.1 – 7.2). In the previous 26 d a total of only 1 mm of rainfall was recorded. During this period of high rainfall, a large amount of debris originating from the Torrens River was deposited onto Henley Beach (Plates 7.3 – 7.4). The average water temperature over the sampling period was 26.1°C (range 24.1°C – 28.0°C) (Fig. 7.2). The mean total dissolved solids concentration of overlying water over the sampling period was 26,050 mg L^{-1}, with a minimum concentration of 20,600 mg L^{-1} recorded on the day of high rainfall (maximum 29,150 mg L^{-1}).

The baseline faecal coliform concentration in overlying water and sediment at Henley Beach prior to the high flow of the Torrens River was relatively low (Fig. 7.3). The concentration of faecal coliforms in the sediment were, however, greater than in the water column (143 ± 57 cfu 100 g^{-1} and 17 ± 11 cfu 100 ml^{-1} respectively). On 20 February, when the high rainfall occurred, there was a dramatic increase in the concentration of both faecal coliforms (Fig. 7.3) and *E. coli* (results not shown) in sediment and water, with > 1×10^6 cfu 100 g^{-1} being detected for both organisms. Two days following the initial high flow of the Torrens River, the concentration of faecal coliforms in the water at Henley Beach had decreased to 2.2×10^3 cfu 100 ml^{-1}. In contrast the concentration of faecal coliforms in sediment remained at 1.2×10^5 cfu 100 g^{-1}. Water at Henley Beach met the guideline for recreational water quality for primary contact recreation of < 150 faecal coliforms 100 ml^{-1} five days after the initial high bacterial load, but sediment did not meet the same value until six days had passed (NHMRC, 1990). The decay rate (k) for faecal coliforms was determined to be greater in water compared with sediment at Henley Beach during the sampling period (-0.78 and -0.60 k, d^{-1} respectively, Fig. 7.4).

It was noted that a large proportion of the debris deposited onto Henley Beach following the high rainfall consisted of horse faeces which may have contributed to this high concentration of faecal coliforms and *E. coli*. The source of this contamination is likely to be horses agisted along the banks of the Torrens River outlet (Plate 7.5).

Plate 7.1 Torrens River outlet at time of high flow.

Plate 7.2 Close-up of water from the Torrens River outlet.

Plate 7.3 Debris deposited onto Henley Beach immediately following the high rainfall event showing significant amount of horse faeces.

Plate 7.4 Local council cleaning up debris deposited on the beach from the Torrens River.

Plate 7.5 Horses agisted near Torrens River outlet.

Plate 7.6 Recreational fishers at Torrens River outlet during time of high flow.

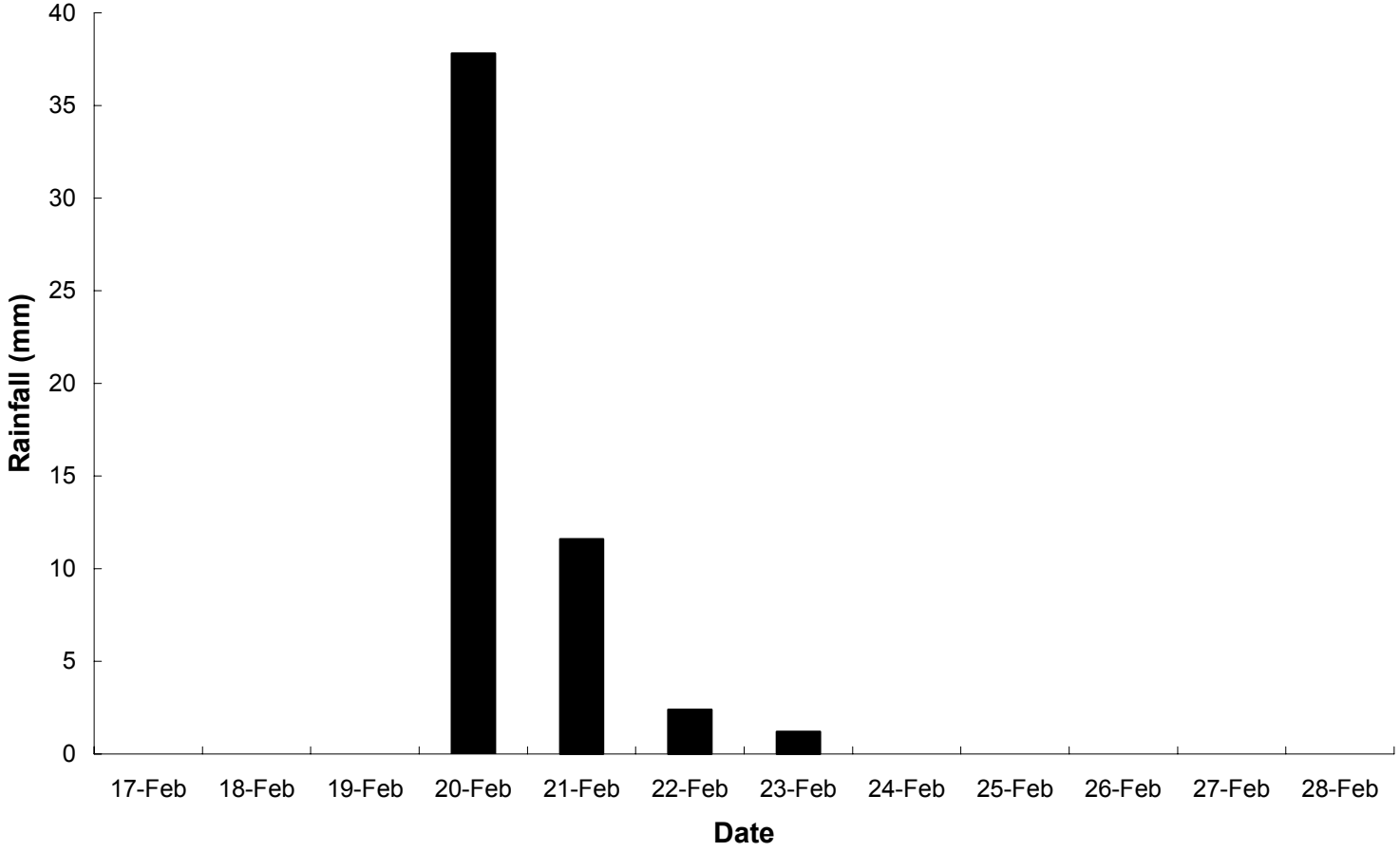

Figure 7.1 Daily rainfall totals during the sampling period (source: Bureau of Meteorology – Adelaide Airport).

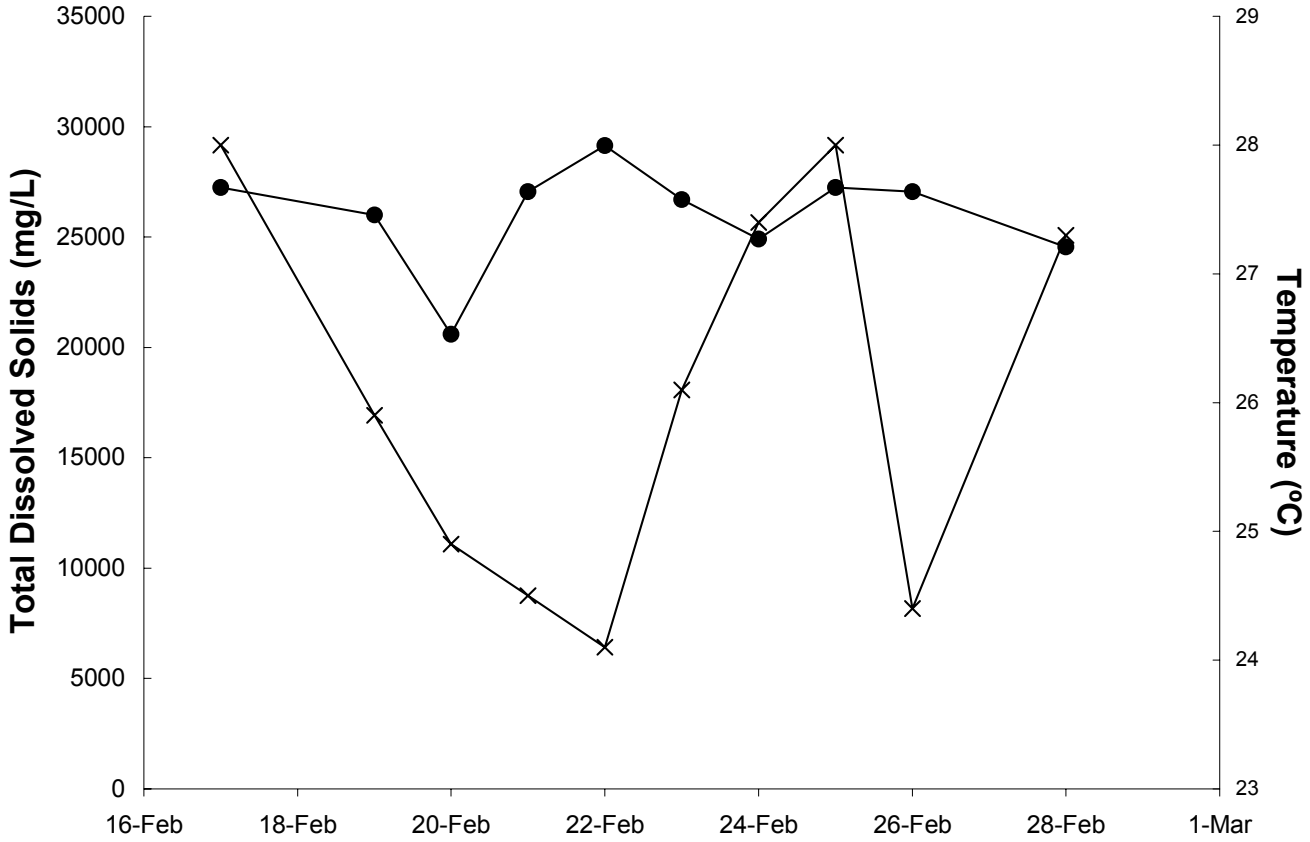

Figure 7.2 Temperature (×) and total dissolved solids (•) of overlying water at Henley Beach over sampling period.

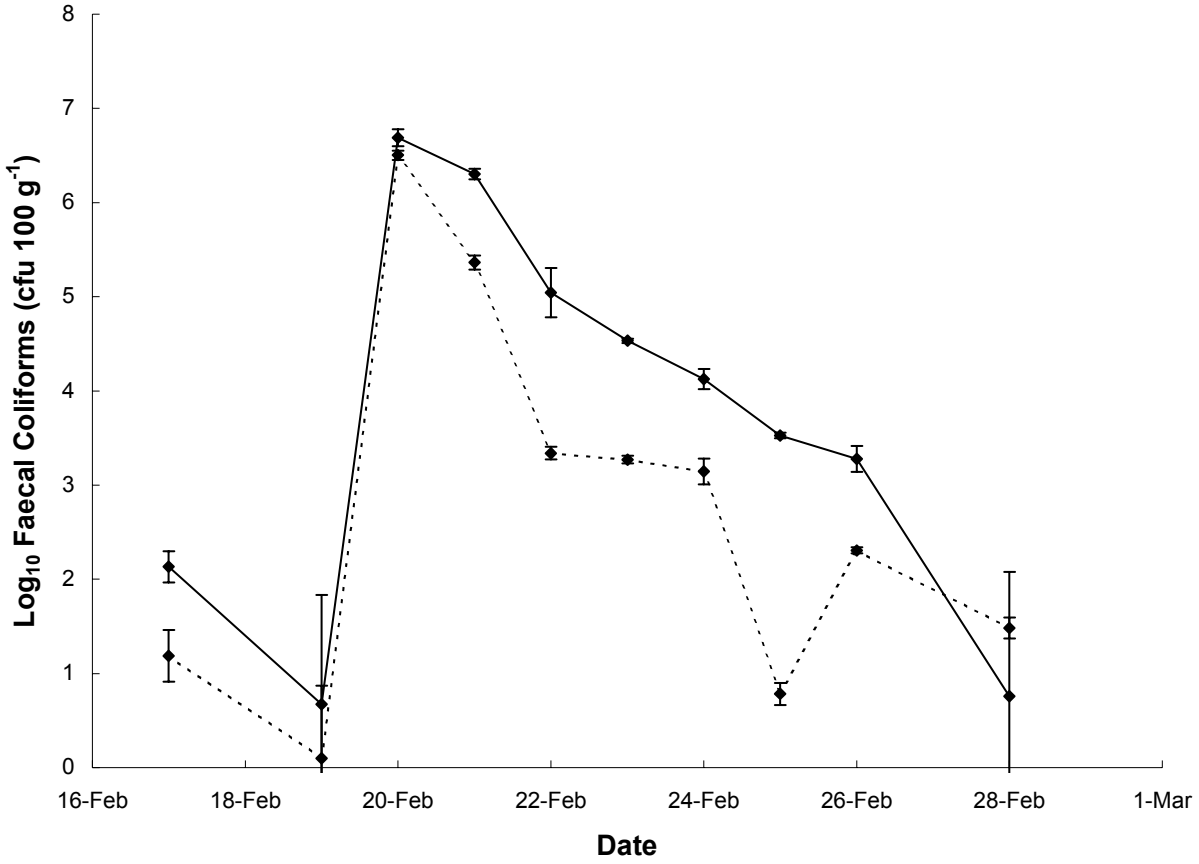

Figure 7.3 Concentration of confirmed faecal coliforms in sediment (—) and water (----) samples taken from Henley Beach (mean ± standard deviation; n=3).

7.3.2 Laboratory-based microcosm study

Results from the microcosm study have previously been discussed in Chapter 6 and are summarised in Table 7.1. Modelling the microcosm decay rate against temperature using an exponential relationship,

$k = \alpha.e^{\beta T}$ (Equation 6.1)

where,

k = decay rate constant

T = temperature (°C)

resulted in values for α and β in overlying water of –0.24 and 0.07 respectively. For microcosm sediment, α was –0.05 and β was 0.11. Using this exponential model, the estimated decay rate for *in situ* samples of overlying water and sediment at Henley Beach at 26.1 °C following the high rainfall event, was –1.34 and –0.86 respectively (compared with the measured *in situ* values of –0.78 and –0.60 for water and sediment respectively). The modelled estimate for *E. coli* decay rate in overlying water was somewhat lower than the measured value, whereas, the modelled estimate for sediment was much closer to the actual measured decay rate (Fig. 7.4).

Table 7.1 Decay rate constants (k; d^{-1}) for *E. coli* (microcosm) and faecal coliforms (*in situ*) in water and sediment determined by laboratory-based microcosm experiment and *in situ* measurements (*$P \leq 0.05$).

	10°C		20°C		26°C		30°C	
	k	r^2	k	r^2	k	r^2	k	r^2
Microcosm water	-0.47	0.80*	-0.89	0.86			-1.74	0.95*
Microcosm sediment	-0.32	0.93*	-0.32	0.98*			-1.11	0.95*
In situ water					-0.78	0.80*		
In situ sediment					-0.60	0.96*		

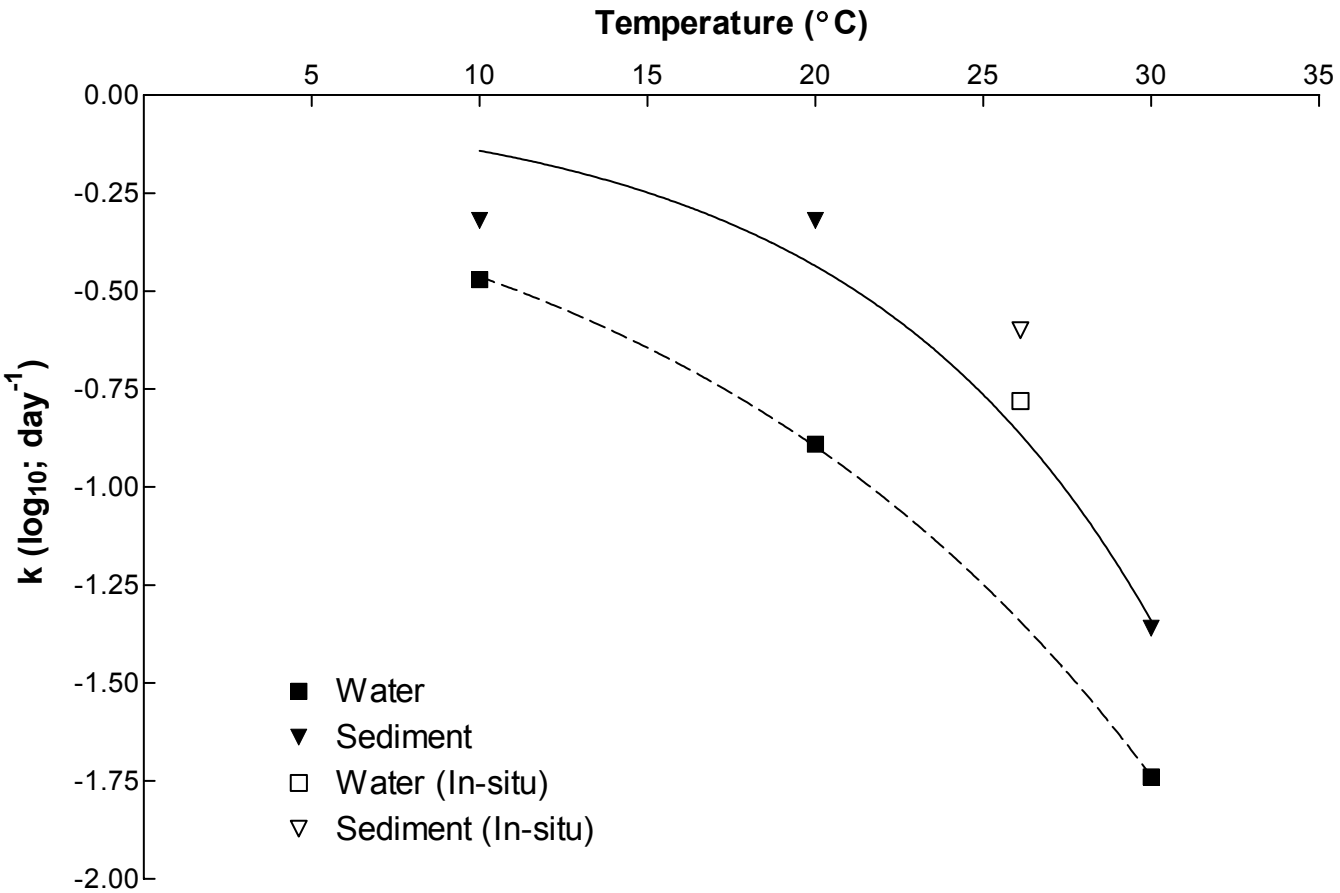

Figure 7.4 Relationship between decay rate (k; d^{-1}) of *E. coli* in Henley Beach microcosm sediment and water with temperature (r^2 = 0.94 and 1.00 respectively).

7.4 DISCUSSION

The average monthly rainfall total for Adelaide in February is less than 20 mm (Australian Bureau of Meteorology). Prior to this high rainfall event, there had been little or no flow from the Torrens River outlet for a considerable period. Therefore the significant amount of rainfall (37.8 mm) that occurred on February 20 resulted in what could be described as a "first flush" stormwater event. The effect of relatively high rainfall over a short period of time provided a unique opportunity to study the persistence of faecal coliforms at Henley Beach in both overlying water and sediment.

Prior to the high flow of the Torrens River, the concentration of faecal coliforms in both water and sediment were below the guideline value for recreational waters of <150 faecal coliforms 100 ml^{-1} (NHMRC, 1990). Following the high rainfall, however, there was a dramatic increase in faecal coliform concentration. At this time, the concentration of faecal coliforms were similar both in the water and sediment. Two days after this peak, the concentration of faecal indicator organisms were approximately 100 times greater in the sediment compared with the water. A study by Shiaris *et al*. (1987) also demonstrated the concentration of indicator bacteria 2 to 4 orders of magnitude higher in marine sediments compared with overlying water at a marine site subjected to sewage contamination. These results suggests indicator organisms released into the coastal environment can accumulate in sediment, leading to increased persistence.

Results from the microcosm experiment showed a similar decreased decay of *E. coli* in sediment from Henley Beach compared with overlying water. Considering the average water temperature at Henley Beach during the sampling period was 26.1 ± 1.6°C, the decay rates observed in sediment and water in the field were lower than those observed in the laboratory microcosm experiment. In a study by Wait and Sobsey (2001) decay rates observed for *E. coli* in water were greater under *in-situ* conditions (using diffusion chambers) compared with laboratory conditions. It should be noted, however, that unlike the microcosm experiment undertaken in the current study, the natural environment is not static. Factors such as turbulence and tidal movement, exposure to sunlight as well as the addition of

faecal coliforms during the sampling period will influence decay rates. Organisms associated with the surface sediment layer may be less influenced by exposure to sunlight (UV and longer wavelength light) and perturbation compared with organisms in the water column. This may partially explain the observed similarity between the measured *in-situ* decay rate in sediment with the modelled decay rate, whereas for the overlying water, a greater difference was observed between *in-situ* and modelled values.

After the initial peak, the concentration of faecal coliforms in the overlying water decreased dramatically in a relatively short period of time. The concentration of faecal coliforms in the sediment however, remained above those guidelines set for recreational water for a period of six days following the peak. These results demonstrated the ability of coastal sediment to act as a reservoir of faecal indicator organisms. This may indicate an increased risk of exposure to pathogenic organisms from the resuspension of sediment during human recreational activity. Since the likely source of faecal contamination experienced during this study is of animal origin, the risk of exposure may be significantly less than if the contamination was of human origin, however it is still an increased risk and thus warrants investigation.

This validation study suggests results from the laboratory-based microcosm experiments may be used to estimate *in-situ* decay rates of indicator and pathogenic microorganisms under a range of environmental conditions. The following chapter is an investigation in the use of quantitative microbial risk assessment for the estimation of infection risk following exposure to faecally contaminated recreational water using results from the microcosm experiments (Chapter 6) to estimate concentrations of pathogenic microorganisms over time.

CHAPTER 8: QUANTITATIVE MICROBIAL RISK ASSESSMENT FOR RECREATIONAL COASTAL WATER

Part of this chapter has been published as:

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8.1 INTRODUCTION

Numerous epidemiology studies have demonstrated that contact with bathing water subject to faecal contamination increases the risk of disease, in particular gastroenteritis, but also non-enteric diseases caused by respiratory, eye, ear and skin infections (Fleisher *et al.*, 1996; Kay *et al.*, 1994; Corbett *et al.*, 1993; Fleisher *et al.*, 1993; Alexander *et al.*, 1992; Fewtrell *et al.*, 1992; Cabelli *et al.*, 1979). Such studies have identified relationships between faecal indicator organism concentrations in the water column and probability of disease, which has been used to set guideline values for recreational waters. Another method used to estimate exposure and subsequent infection risk in recreational coastal waters is via the application of quantitative microbial risk assessment (QMRA). A number of studies using this paradigm have utilised published data on dose-response relationships for pathogenic organisms and the distribution of such organisms in the aquatic environment to estimate infection risk (López-Pila and Szewzyk, 2000; Crabtree *et al.*, 1997; Gerba *et al.*, 1996b). A benefit of QMRA is its ability to estimate (albeit crudely) the probability of infection or disease at very low concentrations of pathogenic organisms; levels at which epidemiological studies are unable to detect difference (Haas, 1983).

Information gained on survival rates of *Salmonella* spp. in the microcosm experiment (described in Chapter 6) facilitated the ability to perform a QMRA for recreational coastal waters. In Australia, salmonellosis is one of the most commonly reported causes of gastroenteritis with the mean number of notifications over the past five years being 7076 individuals per year (Communicable Diseases Network Australia – National Notifiable Diseases Surveillance System, personal communication 2002). While it is acknowledged that the majority of these cases are probably foodborne, the true incidence of those which are waterborne is unknown. Indeed, *S. derby* was chosen for the microcosm experiment because not only has it previously been isolated from recreational coastal waters in Sydney, Australia and in marine waters in Hong Kong (Yam *et al.*, 2000; Kueh and Grohmann, 1989), but in addition, dose-response studies have been undertaken on this organism in a human feeding study (McCullough and Eisele, 1951b).

Risk of disease from protozoan and viral pathogens following recreational exposure has been determined to be greater than for many bacterial pathogens, including *Salmonella* spp. and therefore these risks would need to be considered in any attempt to estimate total risk of gastrointestinal disease (Crabtree *et al.*, 1997; Gerba *et al.*, 1996b). Rotavirus is a common cause of gastroenteritis worldwide and was originally thought to be primarily associated with infection of young children (Hrdy, 1987). It has been demonstrated, however, that it has the ability to produce serious illness in adults and has been associated with waterborne outbreaks of disease (Fang *et al.*, 1989; Hopkins *et al.*, 1984). In addition to this, a dose-response relationship has been determined for the organism, enabling the application of QMRA (see Chapter 1 for a description of the QMRA framework). For the purposes of the QMRA in this study, the decay of rotavirus was assumed to be similar to coliphage (a viral faecal indicator organism). Thus, using information from the microcosm experiment (Chapter 6), the risk of infection by either rotavirus or *Salmonella* spp. following exposure to variety of recreational coastal waters subject to faecal contamination under different conditions could be estimated. The impact of sediment resuspension on risk of infection was also modelled.

8.2 MATERIALS AND METHODS

8.2.1 Exposure assessment

Exposure assessment was based on the initial concentration of either *Salmonella* spp. or rotavirus in the recreational coastal water (estimated using ratios with faecal coliform concentrations), the rate of decay at a specific temperature (20°C or 30°C) and the volume of water consumed per exposure as described in the following equation:

$$n = C_0 \times 10^{kT} \times V_i \qquad \text{(Equation 8.1)}$$

where,

n = the number of organisms ingested
C_0 = initial concentration of organisms in the overlying water (number of organisms mL^{-1})
k = decay rate (day^{-1}) as determined by the microcosm experiments
T = is time (d)
V_i = volume of water ingested (mL)

The volume of water ingested per exposure (one hour) was assumed to be 20 – 50 mL with a uniform distribution (Ashbolt *et al.*, 1997; Crabtree *et al.*, 1997).

8.2.2 Dose-response relationships

Human feeding studies have been undertaken to determine the pathogenicity of a number of different non-typhi *Salmonella* spp., including *S. derby* (McCullough and Eisele, 1951a; McCullough and Eisele, 1951b; McCullough and Eisele, 1951c). Fazil and Haas (1996) analysed the raw data from these initial human studies to determine the model which best described the dose-response relationship. Often, sample sizes for the human feeding studies of individual *Salmonella* spp. were small, therefore dose-response curves were determined for both individual species and pooled data (all of the *Salmonella* spp. data combined). It was concluded a single Beta-Poisson dose-response relationship for

the pooled data was statistically indistinguishable from the models used for individual species.

The following pooled Beta-Poisson dose-response relationship for non-typhi *Salmonella* spp. was used to estimate risk of infection for QMRA study (Fazil and Haas, 1996):

$$P_{inf} = 1 - (1 + [(n/N_{50}) \times (2^{1/\alpha} - 1)])^{-\alpha} \quad \text{(Equation 8.2)}$$

where,

P_{inf} = probability infection

n = number of *Salmonella* cells ingested

N_{50} = median infective dose

For *Salmonella* spp., α has previously be determined to be 0.3126 and N_{50} to be 23,600 (Fazil and Haas, 1996)(see Fig. 8.1).

The probability of infection (P_{inf}) following exposure to rotavirus was calculated again, using a beta-Poisson dose-response model:

$$P_{inf} = 1 - (1 + \frac{n}{\beta})^{-\alpha} \quad \text{(Equation 8.3)}$$

where,

P_{inf} = probability infection

n = number of viral particles ingested

The constants α (0.26) and β (0.42) describe the shape of the dose-response curve for rotavirus and are displayed in Fig. 8.2 (Gerba *et al.*, 1996b; Regli *et al.*, 1991; Ward *et al.*, 1986a).

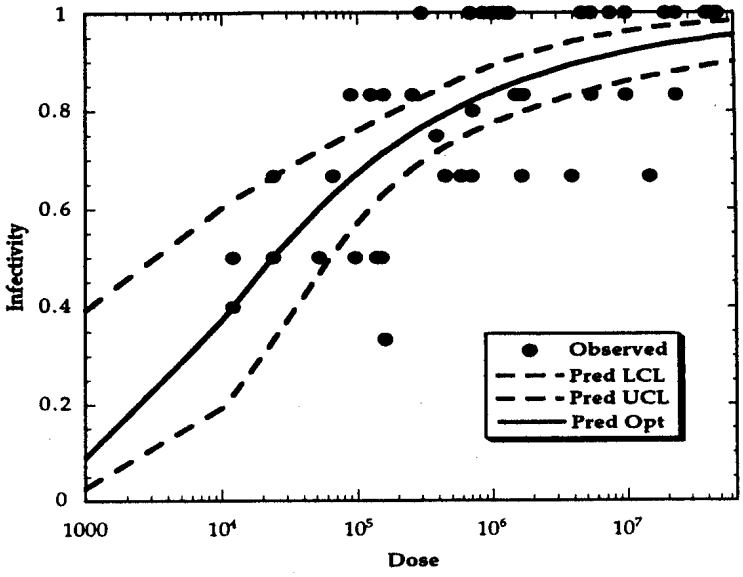

Figure 8.1 Dose-response curve for humans exposed to Salmonella spp. (Fazil and Haas, 1996).

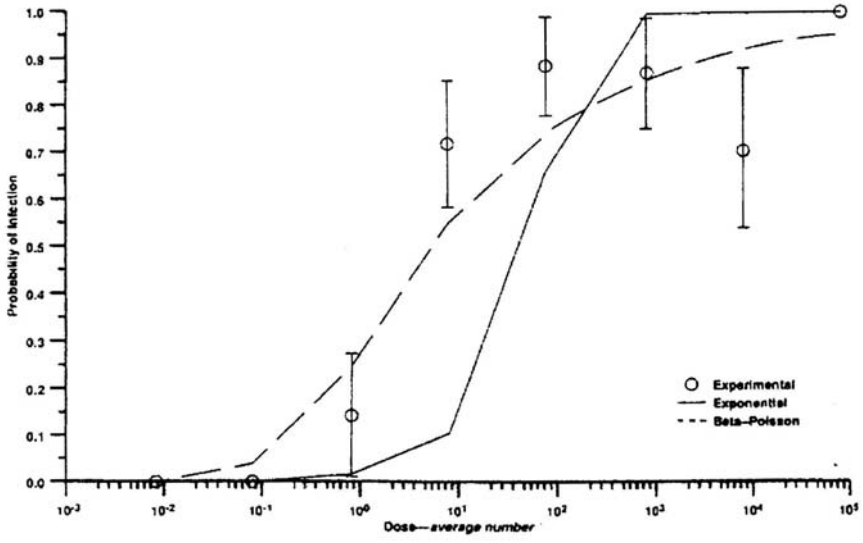

Figure 8.2 Dose-response curve for humans exposed to rotavirus (Regli *et al.*, 1991; Ward *et al.*, 1986a).

8.2.3 Uncertainty parameters

Rather than simply using fixed values for model inputs, uncertainty within various model inputs were addressed using probabilistic Monte Carlo simulation, with 10,000 iterations per scenario (@Risk, version 4.0.5, Palisade Corporation, Newfield, NY, USA, 2000). The values and distributions for the various inputs were selected from both previously published data and results from the laboratory-based microcosm study described in Chapter 6 (Table 8.1).

For estimation of exposure to rotavirus, a constant ratio of rotavirus concentration to faecal coliform concentration of 10^{-5} was used (López-Pila and Szewzyk, 2000). It was assumed that the decay rates of rotavirus in overlying water and sediment was the same as for somatic coliphage. Therefore, for estimation of rotavirus infection following exposure to contaminated recreational coastal water, the uncertainty parameters used were the original concentration of faecal coliforms (lognormal distribution), the decay rates determined from the microcosm experiment (described by a triangular distribution) and the volume of water ingested (same as for *Salmonella* spp. exposure).

Table 8.1 Summary of input variables and distributions for *Salmonella* spp. exposure.

Input Variable	Distribution	Value
[1]*Salmonella* spp:FC	Triangular	10^{-5} min 10^{-4} mode 10^{-3} max
[2]Decay rate *k* (in overlying water) at 20°C	Triangular	-0.94 min -0.91 mode -0.87 max
[2]Decay rate *k* (in overlying water) at 30°C	Triangular	-1.36 min -0.98 mode -0.77 max
[2]Decay rate *k* (in surface sediment layer) at 20°C	Fixed	-0.24 Henley Beach -0.23 Onkaparinga -0.11 Pt Adelaide
[2]Decay rate *k* (in surface sediment layer) at 30°C	Fixed	-0.67 Henley Beach -0.22 Onkaparinga -0.27 Pt Adelaide
[3]Volume of water ingested	Uniform	20 – 50 mL per exposure

[1]Range of reported Salmonella:FC ratios (Baudart *et al.*, 2000; Dionisio *et al.*, 2000; Van Donsel and Geldreich, 1971).

[2]Determined from laboratory based microcosm experiments for decay of *S. derby* in overlying water and/or sediment.

[3](Ashbolt *et al.*, 1997; Crabtree *et al.*, 1997).

Table 8.2 Summary of input variables and distributions for rotavirus exposure.

Input Variable	Distribution	Value
[1]Initial rotavirus:FC	Fixed	10^{-5}
[2]Decay rate *k* (in overlying water) at 20°C	Triangular	-0.40 min -0.29 mode -0.20 max
[2]Decay rate *k* (in overlying water) at 30°C	Triangular	-0.6 min -0.56 mode -0.50 max
[2]Decay rate *k* (in surface sediment layer) at 20°C	Fixed	-0.18 Henley Beach -0.06 Onkaparinga -0.06 Pt Adelaide
[2]Decay rate *k* (in surface sediment layer) at 30°C	Fixed	-0.08 Henley Beach -0.04 Onkaparinga -0.06 Pt Adelaide
[3]Volume of water ingested	Uniform	20 – 50 mL per exposure

1As described by López-Pila and Szewzyk (2000).

[2]Determined from laboratory based microcosm experiments for decay of rotavirus in overlying water.

[3](Ashbolt *et al.*, 1997; Crabtree *et al.*, 1997).

8.2.4 Recreational water quality scenarios

To describe a range of possible recreational water qualities, a range of faecal coliform concentrations, high, medium and low, were selected with lognormal distribution (parameters displayed in Table 8.2). A 'high' level of 1×10^6 CFU 100 mL^{-1} (poor water quality) was chosen to represent an incident of high faecal contamination due to stormwater pollution following a high rainfall event or an accidental release of untreated wastewater (as described in Chapter 4). "Average" water quality was defined as water which met current median guideline faecal coliform concentration for secondary contact recreation in Australia of 1,000 CFU 100 mL^{-1}(NHMRC 1990). The low level of 150 CFU 100 mL^{-1} (good quality) is the current median guideline faecal coliform concentration for primary contact recreation in (NHMRC 1990).

Table 8.3 Lognormal parameters for faecal coliform frequency.

Recreational Water Quality	Lognormal Distribution Parameters		
	μ	σ	Geometric mean (No. 100 mL^{-1})
Poor	6	0.8	1×10^6
Average	3	0.8	1,000
Good	2.18	0.5	150

μ= mean of log-transformed values; σ = std. deviation

An estimate of the likely resuspension of sediment due to human activity or environmental conditions was set as 100 g of sediment being resuspended per 1 m^3 of overlying water (Fearnley pers. com.)[1]. This would result in an addition of microorganisms into the water column. The risk of infection to either *Salmonella* spp. or rotavirus was therefore modelled both including and discounting the resuspension of sediment particles.

[1] Fearnley (2003) undertook research on a series of sediment types where sediment was resuspended by engaging in recreational type activity and then measured observed values for resuspension in both sandy and silty sediments.

8.2.5 Probability of infection from multiple exposure

To calculate the cumulative probability of infection due to numerous exposures the following equation was used (Haas *et al.*, 1993).

$$P = 1 - (1 - P_{inf})^{d} \qquad \text{(Equation 8.3)}$$

where d is the number of exposures.

8.3 RESULTS

In waters of poor quality (high faecal coliform concentrations) and an average water temperature of 20°C, the maximum probability of infection by *Salmonella* spp. (95^{th} percentile) on day 0 (the day of the contamination event) was above 2.0×10^{-1} and remained above 1×10^{-3} for three days following the initial high concentration. For the other two water quality scenarios, the probability of infection (95^{th} percentile) was below 1×10^{-3} on day 0 (Figure 8.1). The mean risk of infection by *S. derby* following exposure to coastal water that meets the guideline faecal coliform concentration was determined to be 6.27×10^{-6} (95^{th} percentile 1.85×10^{-5}).

The probability of infection from rotavirus due to exposure to faecally contaminated recreational water was greater than for *Salmonella* spp. in all scenarios investigated. Under modeled conditions, exposure to poor recreational water quality resulted in a probability of infection (95^{th} percentile) on Day 0 of > 70% (Figure 8.2). With an average water temperature of 20°C, the probability of infection remained above 10^{-3} for 19 days. In comparison, for an average water temperature of 30°C, the probability of infection from rotavirus was below 10^{-3} after 10 days, reflecting the greater rate of decay associated with higher temperatures.

Taking into account the likely resuspension of microorganisms from the surface sediment layer into the water column resulted in an increased probability of infection, from both organisms, in the later periods following the initial peak (Figures 8.3 – 8.4). This was most evident for rotavirus following exposure to poor recreational water quality at 30°C.

To illustrate the effect of different decay rates observed between sediment types, a comparison was made between the risk of infection following resuspension of Henley beach sediment compared with resuspension of Pt Adelaide sediment (Figure 8.5). Results suggested an increased risk of rotavirus infection due to resuspension of Pt Adelaide sediment compared with Henley beach sediment.

Again, the probability of infection for both sediment types was similar in the initial 10 day period but the difference between probabilities increased with time. This difference is likely due to a combination of greater survival of virus in Pt Adelaide sediment compared with Henley Beach sediment, as well as differences in the rate of sediment resuspension between the two sediment types.

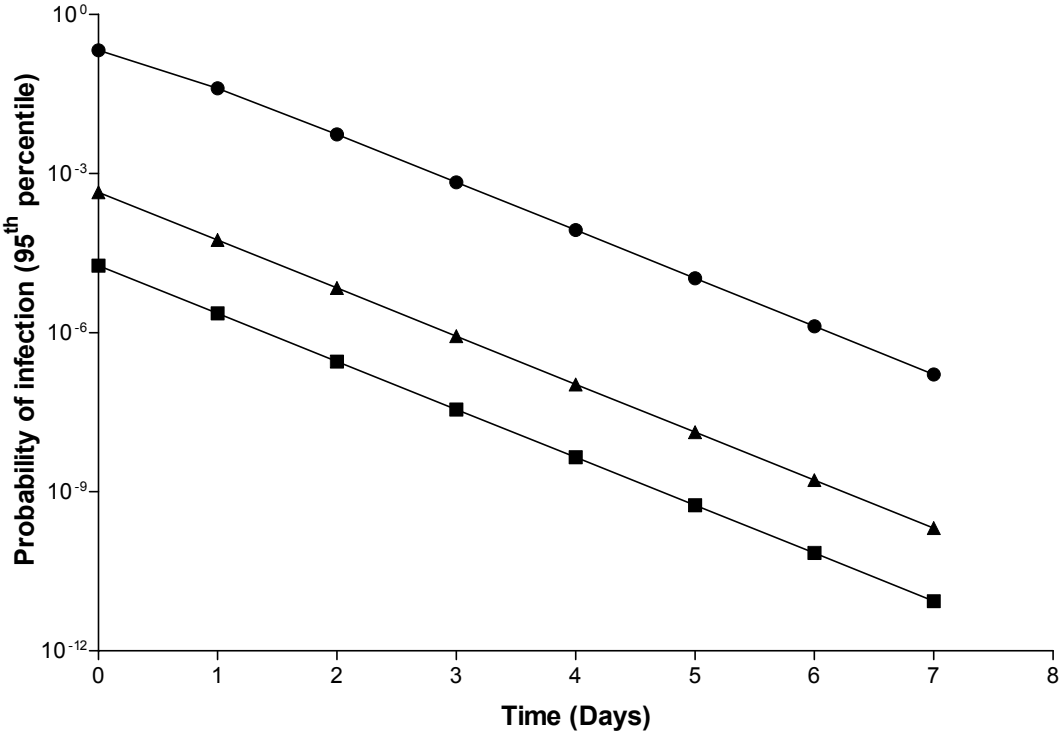

Figure 8.3 Probability of *Salmonella* spp. infection following exposure to good (■), average (▲) and poor (●) recreational water qualities.

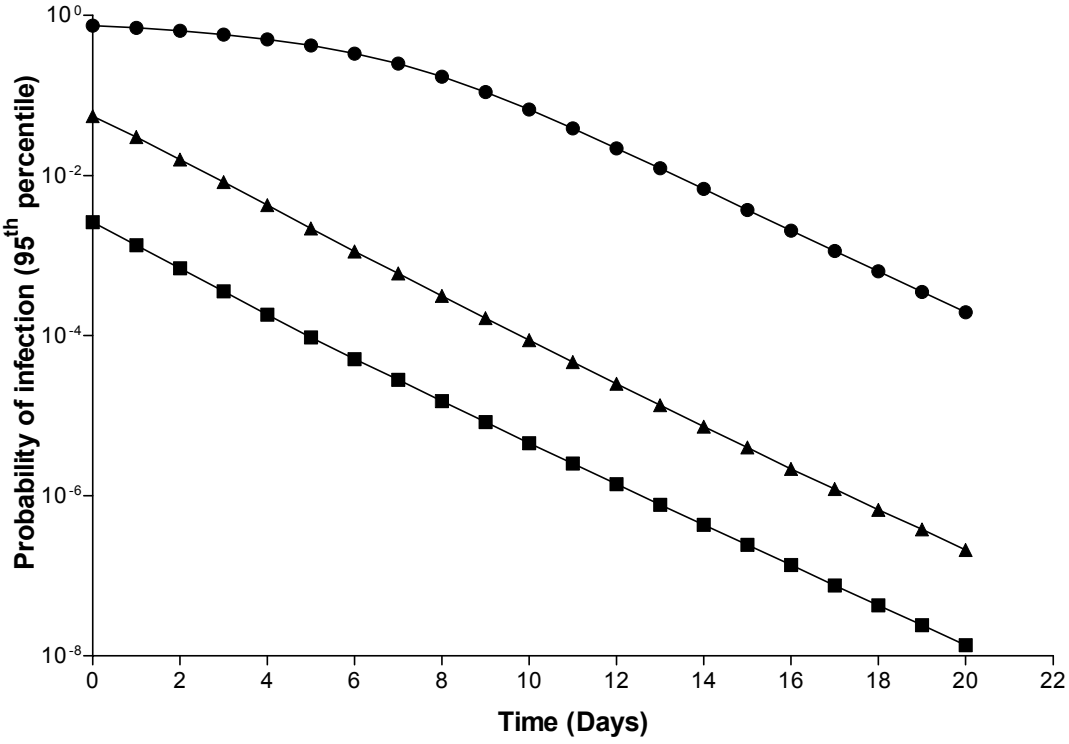

Figure 8.4 Probability of rotavirus infection following exposure to good (■), average (▲) and poor (●)recreational water quality.

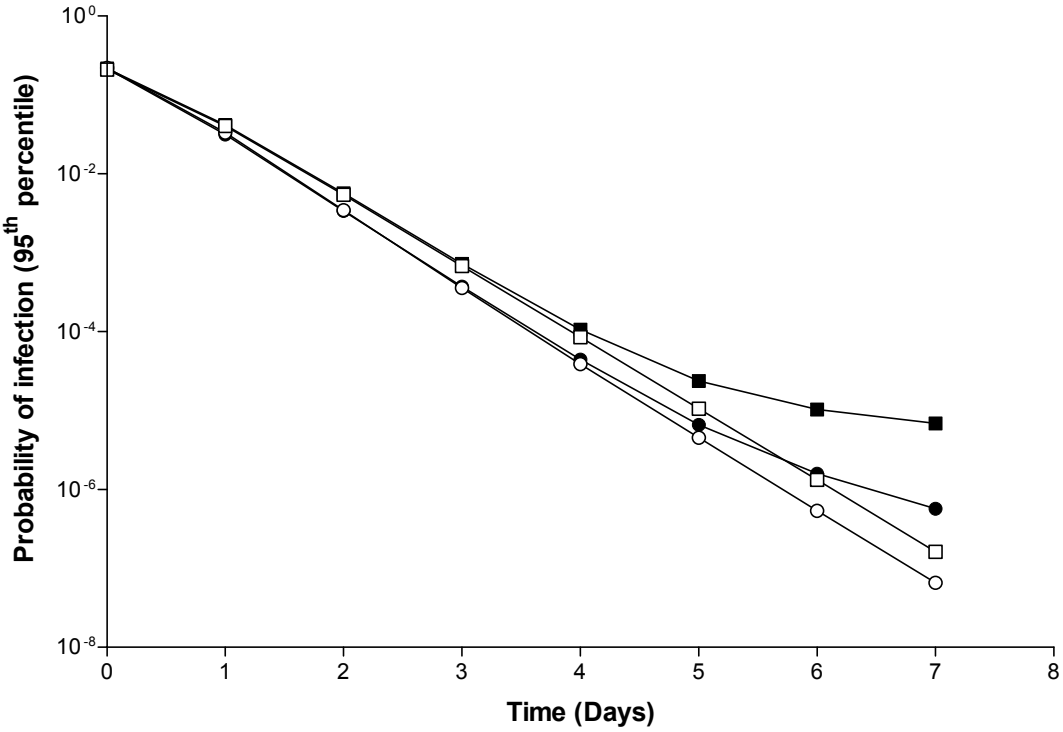

Figure 8.5 Probability of infection from *Salmonella* spp. following exposure to poor recreational water quality with average water temperatures of 20°C (○) and 30°C (□), with (closed symbols) and without (open symbols) taking into account resuspension of organisms from the surface layer of Pt Adelaide sediment.

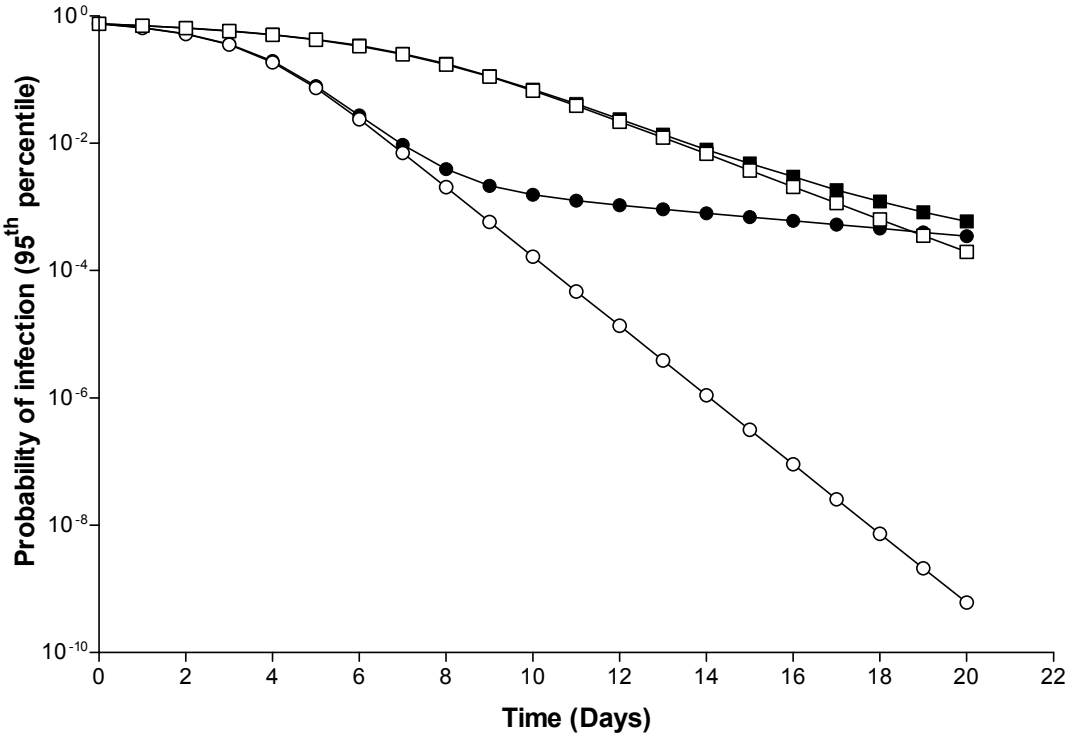

Figure 8.6 Probability of infection from rotavirus following exposure to poor recreational water quality with average water temperatures of 20°C (□) and 30°C (○), with (closed symbols) and without (open symbols) taking into account resuspension of organisms from the surface layer of Pt Adelaide sediment.

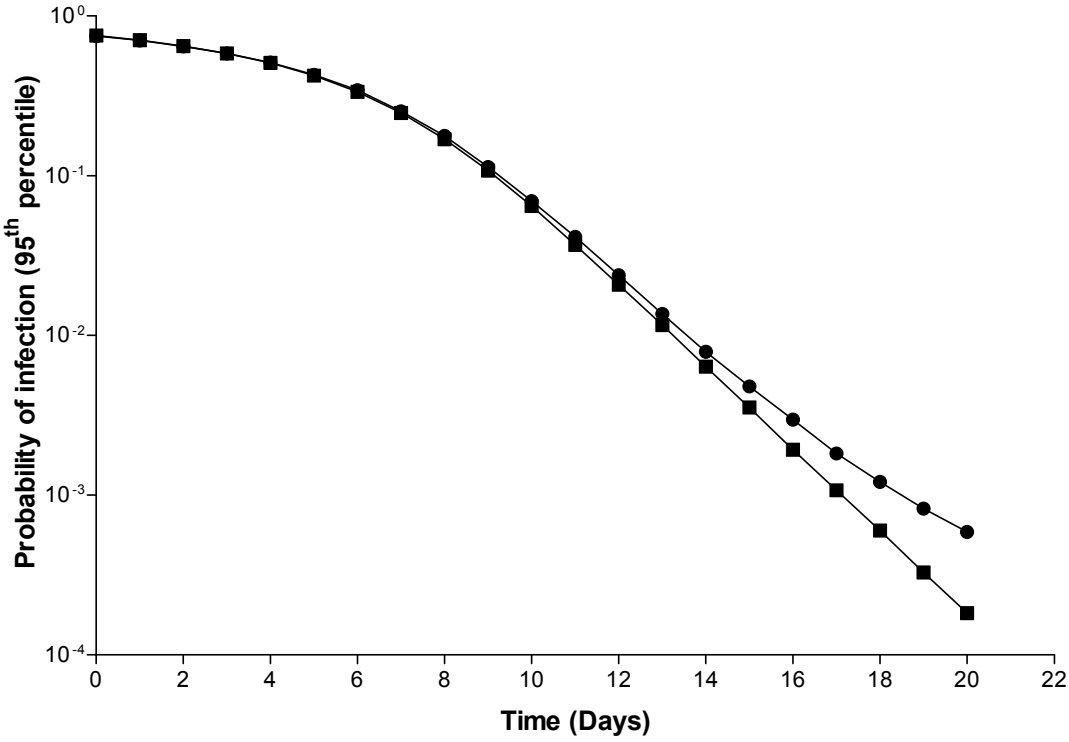

Figure 8.7 Probability of infection from rotavirus following exposure to poor quality recreational coastal water at 20C taking into account resuspension from either Henley Beach (■) or Pt Adelaide (●) sediment.

8.4 DISCUSSION

For potable water, the United States Environmental Protection Authority (USEPA) has suggested an acceptable level of risk of 1 in 100,000 excess incidence of cancer over a lifetime of exposure. For microbial pathogens, it has been suggested a risk of infection of 1 in 10,000 for a yearly exposure is acceptable for exposure via potable water (Regli *et al.*, 1991). Taking into account the usually less severe health outcomes and less frequent exposure, a generally accepted level of risk of disease from exposure to contaminated recreational water is 6×10^{-3} to 8×10^{-3} (Cabelli *et al.*, 1983).

The preliminary model described here was able to illustrate the change in risk of infection from *Salmonella* spp. and rotavirus over time following a faecal contamination event (assuming no further input) in recreational coastal waters following a single exposure. In waters of poor quality (high faecal coliform concentrations), the probability of infection (95^{th} percentile) on the day of the event was greater than 20% and remained above 1×10^{-3} for three days following the initial high concentration. The probability of infection for the other two water quality scenarios were relatively low on day 0 and reduced with time accordingly. These results suggest, for a single exposure to recreational coastal water, probability of infection from *Salmonella* spp. is relatively low under most circumstances. Only when a severe contamination event occurs does the probability of infection exceed acceptable levels.

The probability of infection from rotavirus due to exposure to contaminated recreational coastal water was greater than that for *Salmonella* spp. under all scenarios. This increased probability of infection is linked to the high infectivity of rotavirus compared to *Salmonella* spp. (Regli *et al.*, 1991; Ward *et al.*, 1986a). Under poor water quality conditions the risk of rotavirus infection was > 70% (Figure 8.2). Not only was the risk of infection greater than for *Salmonella* spp. on day 0, but the risk of infection over the following days decreased at a slower rate (due to lower decay rates observed in the microcosm survival experiment). As a result, the risk of infection remained above 10^{-3} for 19 days compared with 9 days for *Salmonella* spp. under the same conditions (with average water

temperatures of 20°C). For waters meeting the current Australian Recreational Water Quality Guidelines (NHMRC, 1990) the risk of infection was 2.62×10^{-3} and (with a mean concentration of 0.02 rotavirus L^{-1}) and remained above 1×10^{-3} for two days. In a study by Gerba *et al*. (1996), assuming a concentration of 0.24 rotavirus 100 mL^{-1} in recreational coastal water and ingestion of 100 mL^{-1} of water per exposure, the probability of infection was estimated to be 1.43×10^{-2}.

As demonstrated in the microcosm experiment (Chapter 6), decay rates of indicator and pathogenic microorganisms were greater in overlying water compared with sediment. This indicates that, following an initial peak, the concentration of microorganisms at any given time would be greater in sediment compared with overlying water. Estimation of the resuspension of *Salmonella* spp. from the surface sediment layer into the overlying water had a minimal impact on infection risk (Figure 8.3). Resuspension was only observed to increase risk of infection from approximately four days after the initial peak, at which time the risk of infection was below acceptable levels. For rotavirus infection risk, however, taking into account resuspension resulted in much greater infection risk at water temperatures of 30°C. Ignoring the role of sediment resuspension, the risk of rotavirus infection due to exposure to poor recreational water quality at 30°C decreased to below acceptable levels within nine days (Figure 8.4). Using the risk assessment model described in this chapter demonstrated the risk of infection from rotavirus when taking into account sediment resuspension remained above acceptable levels 13 days following the initial peak. The risk of infection also decreased at a much slower rate than in the overlying water only. Advice from health and/or environmental authorities following rain events is often that the site is safe for recreational activity 3 days later, which may not be adequate according to this quantitative model.

The effect of sediment resuspension on infection risk is significant when the rate of microorganism decay is significantly less in the surface sediment layer compared to the overlying water. In the days immediately following the peak microorganism concentration, the addition of microorganisms from the sediment into the water column is insignificant due to a number of factors. On day 0 (the

day of the initial peak concentration of microorganisms) the concentration of organisms in the overlying water and sediment is assumed to be equal. This assumption was based on previous research (Chapter 7) investigating faecal coliform concentrations at a recreational coastal site following a contamination event. On the day of high rainfall, the concentration of faecal coliforms was similar in both the sediment and overlying water, presumably because of the effect of physical disruption caused by the storm event turning over the sediment. The addition of sediment-associated microorganisms into the overlying water is, thus, relatively small when the effect of dilution is considered. The risk of infection in the first four to five days is therefore similar whether the effect of sediment resuspension is taken into account or not. In the following days, however, the number of microorganisms in the overlying water has decreased to much lower concentrations compared to that in the surface sediment layer (according to observed decay rates). In the case of rotavirus (based on coliphage decay), increased water temperature had a minimal effect on decay rates in the sediment compared with overlying water. The decay rate of coliphage in Pt Adelaide sediment was the same whether incubated at 20°C or 30°C (decay rate -0.06 day^{-1}). In the overlying water, however, observed decay rates increased from -0.21 day^{-1} when incubated at 20°C to -0.58 day^{-1} when incubated at 30°C. Therefore, even when taking into consideration dilution of resuspended sediment in overlying water (100g 1 m^{-3}) the high concentration of microorganisms in the surface sediment layer compared with overlying water at the same time (> 4 – 5 days following the initial event) results in an increased number of microorganisms in the water column and hence increases the probability of infection. To better estimate the risk of infection, taking into account sediment resuspension, there is a need to more accurately assess rates of sediment resuspension under various conditions. The rate of resuspension is dependant on a number of factors including particle size of the sediment and physical conditions (tidal influences, wind speed, currents and human influences such as ship traffic and recreation) As the results discussed here have demonstrated, sediment resuspension can have a dramatic impact on risk of infection (especially rotavirus) following exposure to faecally contaminated recreational water.

The probabilities of infection due to exposure to faecally contaminated recreational coastal water estimated using this QMRA model are based on single exposures. This may be an acceptable assumption for both "poor" and "average" water quality scenarios described in this chapter. Under these conditions, contamination is generally a result of either uncontrolled release of partially treated wastewater or from diffuse source pollution caused by high rainfall events. Human exposure to recreational waters subject to these conditions is likely to be a single event. For waters meeting current microbial recreational water quality guidelines (< 150 faecal coliforms 100 ml^{-1}) it is not only plausible, but also reasonable to assume that multiple exposures will occur. Using the equation discussed earlier (Equation 8.3) to calculate the cumulative probability of infection due to numerous exposures, the probability of rotavirus infection from 10 exposures to waters meeting current microbial recreational water quality guidelines would be 2.6×10^{-2} (95^{th} percentile). Given the likelihood, particularly for children, of spending numerous hours in the water on a summer's day this is considered to be a relatively conservative estimate of exposure and thus of risk.

The probabilities described by this model are for infection only. Infection from either *Salmonella* spp. or rotavirus has been observed to produce asymptomatic responses in humans (Ward *et al.*, 1986a; McCullough and Eisele, 1951b). It has been suggested the probability of illness can be estimated by multiplying the probability of infection by the morbidity rate of 0.5 (Haas *et al.*, 1993). However, sensitive sub-populations exist which are often at greater risk of infection from enteric pathogens (Gerba *et al.*, 1996a). These groups of individuals include the young, elderly, immuno-compromised and the pregnant. Not only are these individuals at greater risk of becoming infected, but often display greater rates of morbidity and mortality associated with infection compared with healthy individuals (Gerba *et al.*, 1996a).

In addition, it is acknowledged that the estimations determined by this model are exclusive to the risk of infection of *Salmonella* spp. or rotavirus and do not take into account exposure to other bacterial, protozoan or viral pathogens. Crabtree *et al*. (1997) concluded that the risk of infection by adenovirus following recreation

in freshwater, which had not been accidentally contaminated ranged from 1.48×10^{-5} to 1.48×10^{-4}. Adenovirus is also a major cause of gastrointestinal disease in humans but is also associated with non-enteric disease such as respiratory illness and has been linked to outbreaks following to exposure to contaminated recreational water (Crabtree *et al.*, 1997; Moore *et al.*, 1993a).

A limitation of the model developed here is lack of available data for a number of input parameters. More data is required on the occurrence and distribution of both rotavirus and *Salmonella* spp. in recreational coastal water. The dose-response data used in this study provides the probability of infection only for healthy adults and does not take into account previous exposure or immunity. Issues also exist regarding the determination of *Salmonella* spp. infectivity after exposure to low-nutrient conditions such as coastal waters (Caro *et al.*, 1999; Galdiereo *et al.*, 1994). Despite these limitations, QMRA is, nonetheless a useful tool for estimating the risk of infection under different scenarios and is extremely useful in the management of health risk for recreational coastal waters.

CHAPTER 9: GENERAL DISCUSSION

Epidemiological studies have demonstrated recreational exposure to faecally contaminated water (fresh and marine) is associated with an increased risk of illness, particularly gastroenteritis but also non-enteric diseases such as respiratory, eye, ear and skin infections (WHO, 2001). Very little information is available regarding waterborne disease outbreaks associated with recreational water contact in Australia. A number of outbreaks have occurred internationally, although largely associated with fresh water recreational water bodies (Lee *et al.*, 2002; Barwick *et al.*, 2000; Levy *et al.*, 1998; Kramer *et al.*, 1996; Moore *et al.*, 1993b). However, the true incidence of illness associated with recreational water contact is difficult to estimate, as illness is usually mild and self-limiting and therefore difficult to identify through routine disease surveillance networks. The primary objective of this research was to more accurately describe environmental exposure in the first stage of a health risk assessment from recreational waters.

A number of studies have identified higher concentrations of faecal indicator organisms and pathogens in the surface sediment layer compared with overlying water (Crump and Baross, 1996; Davies *et al.*, 1995; Fish and Pettibone, 1995; Shiaris *et al.*, 1987; Goulder, 1977). It is likely these sediment-associated microorganisms have the ability to be resuspended, either via natural turbulence or human activity. Therefore, to more accurately estimate exposure to pathogenic microorganisms during coastal recreational activity, it may be necessary to enumerate organisms from the surface sediment layer as well as from overlying water. The mechanism of attachment of microorganisms to sediment particles is complex, and is dependent on characteristics of the specific microorganism, physical characteristics of the sediment particle and environmental conditions (Crump and Baross, 1996).

Previous studies undertaken to enumerate microorganisms from the surface sediment layer have used a variety of physical and chemical techniques in an effort to separate organisms from sediment particles, although no conclusions have been made with regard to determining an overall most effective method (Crump and Baross, 1996; Davies *et al.*, 1995; Epstein and Rossel, 1995; Fish and Pettibone, 1995; Shiaris *et al.*, 1987; McDaniel and Capone, 1985; Ellery and Schleyer, 1984; Goulder, 1977; Dale, 1974). Investigation of methodologies for

enumeration of bacteria from a variety of sediment types collected from recreational coastal water sites along the greater Adelaide coastline, identified significant differences in the effectiveness of individual separation techniques depending on sediment type. For sediments consisting mainly of sand (large particle size), physical treatment by sonication resulted in significantly higher numbers of microorganisms enumerated than treatment by manual shaking. For sediments consisting of greater proportions of silt and clay, however, sonication was less effective than manual shaking. These results illustrate the importance of different sediment characteristics on the effectiveness of various techniques for the separation of microorganisms from sediment particles.

A 12-month *in-situ* study of the microbiological quality of a number of Adelaide recreational coastal sites identified consistently higher concentrations of faecal indicator organisms in the surface sediment layer compared with overlying water. This phenomenon was most apparent at coastal sites with sediments consisting of higher proportions of silt and clay (small particle size) and high organic content. At these sites, the concentration of faecal coliforms was often observed to be over 1000-fold higher in the sediment layer compared with overlying water. Mechanisms associated with increased survival of microorganisms in coastal sediments are not fully understood. They are known to be due to a combination of physical and chemical factors such as protection of the microorganism from exposure to sunlight, increased availability of nutrients and protection from predators such as bacteriophages (Davies and Evison, 1991; Gerba and McLeod, 1976; Roper and Marshall, 1974).

Results from a laboratory-based microcosm study demonstrated significant differences in decay rates of faecal indicator organisms and pathogens, with little or no correlation between them. Microcosms consisting of intact non-sterile sediment cores and overlying water were collected from three recreational coastal sites to represent a range of sediment types. Using these, it was demonstrated that temperature and sediment type played an important role in determining decay rates of faecal indicator organisms and *Salmonella* spp. in the coastal environment. Survival of *E. coli*, enterococci, coliphage and *Salmonella* spp. were observed to be greater in the surface sediment layer compared with overlying

water under laboratory-based microcosm conditions. This was demonstrated in all sediment types, but was most significant in sediments consisting of small particle size and high organic carbon content. Temperature had an inverse relationship to microorganism survival, with greatest decay occurring at 30°C.

In general, survival of enterococci and coliphage was greater than *E. coli* and *Salmonella* spp. Decay of *Salmonella* spp. in overlying water was similar that of *E. coli*, however, at high temperatures (20°C and 30°C), decay rates of *Salmonella* spp. in the surface sediment layer was significantly lower than *E. coli*. Therefore, the traditional practice of enumerating faecal coliforms and/or *E. coli* under these conditions may not adequately reflect the potential for other pathogenic microorganisms (such as *Salmonella* spp.) to be present.

Quantitative microbial risk assessment (QMRA) is a relatively newly developed tool to estimate risk of infection or illness from exposure to microbial pathogens. Based on similar principles to chemical risk assessment, QMRA considers the dynamic response (including inactivation, infectivity etc) of microorganisms in response to changes in environmental conditions to estimate exposure risk. QMRA also considers the response of any individual (the host) due the ingestion of specific number of organisms, which can vary significantly and depends on a number of factors, such as immune status and previous exposure of the individual, as well as the physiological state of the organism. When developing a QMRA model, probabilistic techniques can be used to incorporate variability and uncertainty associated with various model inputs. Unlike a deterministic QMRA model, which gives a single point estimate, results from a probabilistic QMRA model provide a distribution of possible outputs.

Decay rates of *Salmonella* spp. and coliphage observed in the microcosm studies were incorporated into a probabilistic QMRA model to estimate the risk of infection from exposure to coastal waters during recreation under various scenarios. Due to the availability of a quantitative dose-response model, risk of infection from rotavirus was estimated based on the decay of coliphage. Risk of infection from salmonella and rotavirus was estimated under three exposure

scenarios – good, average and poor recreational water quality. Results from the QMRA estimated the change of infection risk over time following a faecal contamination (such as a high rainfall event) event at a recreational coastal water site.

A combination of higher infectivity, and greater observed survival in overlying water and sediment, resulted in risk of infection from rotavirus being greater than that for *Salmonella* spp. under all conditions modelled. Resuspension of the surface sediment layer was shown to be associated with increased infection risk, particularly at higher temperatures. The impact of sediment resuspension on infection risk was most significant in the days (> 3 d) following the initial peak, where the concentration of microorganisms was significantly higher in the surface sediment layer compared with overlying water (due to differences in decay rates). This was associated with the significantly more rapid decline of microorganism concentration in overlying water compared with the surface sediment layer, particularly at higher temperatures.

Further research is required to reduce the level of uncertainty associated with a number of QMRA model inputs. Quantitative data on the concentration of human pathogens in recreational coastal areas (particularly in Australia) is limited. Enumeration of these organisms *in-situ* is often difficult, with concentrations of human pathogens in these environments usually low. Detection methods often require an enrichment step, therefore only providing qualitative data (presence/absence). In addition newly developed PCR techniques do not inform of the infective status of organisms detected. The availability of dose-response models for microbial pathogens is also limited. The models used in this research are based on laboratory-based human feeding trials using healthy adult volunteers. To be able to detect infection and/or illness in relatively small study populations, these studies often involve exposure to high numbers of microorganisms. As identified by epidemiological studies, illness associated with actual recreational water contact often involve the exposure of large populations to low numbers of pathogens (Kay *et al.*, 1994).

Survival of human enteric microorganisms in the recreational coastal environment is complex. Results of this research have significant implications towards understanding the estimation of exposure risk, and subsequent determination of possible risk management options for recreational coastal sites. Under both laboratory and *in-situ* conditions, survival of faecal indicator organisms and pathogens were observed to be greater in coastal sediments than overlying water. The use of prescribed faecal indicator organism concentrations in the water column alone may significantly underestimate potential exposure risk. Further research is required on the survival of other human pathogenic bacterial, viral and protozoan microorganisms to more accurately estimate risk of illness following recreational contact to coastal waters and sediments. Resuspension of sediment may present a significant increase in exposure risk under certain conditions, and requires further investigation.

APPENDIX 1

Craig, D. L., Fallowfield, H. J. and Cromar, N. J. (2000) Persistence of *Escherichia coli* in recreational coastal water and sediment. *In proceedings of 1st IWA World Congress, Paris, France, 3 – 7 July*. 4pp.

APPENDIX 2

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APPENDIX 3

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APPENDIX 4

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APPENDIX 5

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