Chapter I

Introduction

AQUATIC MICROBIAL FOOD WEBS

Viruses, archaea, bacteria, phytoplankton and zooplankton that comprise the microbial loop are the smallest but most abundant and diverse organisms on Earth. These members of the aquatic food web have been quietly controlling global biogeochemical cycles and the evolution of life on Earth since the first microbial organisms appeared in the ocean (Fuhrman 2009; Rohwer and Vega Thurber 2009). Their global importance and impact have, however, remained concealed until only recently (Karl 2007; Falkowski *et al.* 2008). Understanding the complex connectivity, relationships, controls and feedbacks between the different members of the microbial food web is of utmost importance to understanding the impact of increasing anthropogenic activities (Karl 2007). Failure to understand how the microbial food web responds to change leaves us unable to mitigate the effects of anthropogenic activities.

The oceans cover 71% of the Earth's surface (Miller and Spoolman 2009) and harbour a diverse and abundant microbial community (Fuhrman 2009). As an easily accessible and vital human resource, a large proportion of microbial food web research has been conducted in marine environments. As a result, a great deal is known about the diversity, structure and processes occurring between members of the microbial community in marine environments (Fuhrman 1999; Suttle 2005; Azam and Malfatti 2007). In aquatic environments, phototrophic microorganisms form the basis of the food web. The energy, matter and elements they assimilate are conveyed to higher trophic levels through predation by zooplankton; retained in the microbial loop by utilisation of cellular exudates or viral lysis products by heterotrophic bacteria and archaea; or conveyed to the benthos through sedimentation. Exudates or lysis products consumed

by bacteria or archaea can also be passed to higher trophic levels via zooplankton predation or further recycled in the microbial loop by viral lysis and the sloppy feeding of zooplankton on bacteria and archaea. Zooplankton faecal pellets are also an important source of nutrients and energy for the microbial community (Suttle 2005; Azam and Malfatti 2007).

"TOP-DOWN" VERSUS "BOTTOM-UP" CONTROL

Aquatic bacterial, phytoplankton and archaeal community abundance and diversity are affected by "top-down" and "bottom-up" processes; models established on extensive food web research. "Top-down" control can be defined as control of the community mainly by the actions of zooplankton grazing and viral lysis (Pernthaler 2005) and sedimentation (Crumpton and Wetzel 1982; Brussaard *et al.* 1995). While other factors such as programmed apoptosis and DNA damage resulting in cell death are also likely to impact upon the community but are much less understood and not as yet quantified (Sommaruga *et al.* 1997; Franklin *et al.* 2006). Physiochemical parameters such as salinity, temperature and nutrients exert strong "bottom-up" selective pressures on aquatic microbial community composition (Lozupone and Knight 2007; Allan and Froneman 2008) and are important determinants of the dominant species and metabolic processes present in an environment (Bouvier and del Giorgio 2002). So while the actions of the microbial community drive global biogeochemical cycles, microbial communities are themselves structured by "bottom-up" physiochemical factors. However, the relationship between "bottom-up" and "top-down" processes is more multifaceted for example; predation on a specific bacterial population will releases a certain amount of energy and potential nutrients which may in turn influence the

growth of another bacterial population (Weinbauer *et al.* 2011). With an unprecedented increase in anthropogenically induced physiochemical changes in ecosystems worldwide, investigating shifts in the microbial community structure and processes in response to these changes is of utmost importance.

PHYSIOCHEMICAL FACTORS CONTROLLING THE MICROBIAL COMMUNITY

Physiochemical changes occur along spatial and/or temporal scales. Studying processes in naturally occurring environmental gradients provides the ideal opportunity to determine the impact of an environmental variable on the community and the potential flow on effects for an entire system. Salinity is a major selective pressure structuring microbial communities and processes (Painchaud *et al.* 1995; Lozupone and Knight 2007), and alterations in the salinity of aquatic environments is an issue of concern worldwide as coastal environments are affected by and respond to climate change (Nielsen *et al.* 2003; Hilton *et al.* 2008). As salinity increases in aquatic environments, the dynamics of the microbial food web change; the microbial community becomes more abundant but less diverse, and organisms at higher trophic levels decline in abundance and diversity before eventually dropping out of the system (Guixa-Boixereu *et al.* 1996; Pedrós-Alió *et al.* 2000). This has been well documented in solar salterns (Pedrós-Alió *et al.* 2000); saline ponds of increasing salinity tightly controlled within specific physiochemical parameters. Data are lacking, however, from natural connected systems exhibiting large changes in salinity.

THE COORONG: A MODEL SYSTEM FOR INVESTIGATING SALINITY

The Coorong is a shallow estuary, lagoon and lake system emanating from the terminus of the River Murray, South Australia (Lamontagne *et al.* 2004). Along its 140 km length there exists a large natural gradient in physiochemical parameters, specifically salinity, nutrients and matter (Ford 2007), and an accompanying dramatic increase in microbial (viruses, bacteria and phytoplankton) abundance (Schapira *et al.* 2009; Schapira *et al.* 2010). The Coorong is a reverse estuary with, in contrast to most estuarine systems, salinity increasing with distance from the river mouth: from brackish in the North-West to hypersaline in the South-East (Geddes 2005). The increase salinity is the result of evaporation; wind and tidal action; decreased riverine flow; and rainfall; prolonged drought; increased water diversion; groundwater drainage; and seawater inflow (Webster 2005; Ford 2007; Lester and Fairweather 2009). The environmental quality of the River Murray, and the Coorong and Lower Lakes region is currently considered poor due to human impacts (Mudge and Moss 2008; Lester and Fairweather 2009; Kingsford *et al.* 2011).

Recent investigations of the Coorong's aquatic microbial community show that salinity is an important factor structuring the community (Schapira *et al.* 2009; Schapira *et al.* 2010; Jeffries *et al.* 2011), and predict that "top-down" community control plays an important role in shaping the bacterial and phytoplankton population of the Coorong (Schapira *et al.* 2009; Schapira *et al.* 2010). Nevertheless, data is still lacking as to how 'top-down" and "bottom-up" processes impact specifically upon the community. Additionally investigation into the aquatic microbial taxonomic diversity, metabolic potential, physical characteristics and energy conservation strategies is also lacking. As such, the Coorong provides an ideal environment in which to investigate the diversity,

structure and processes of the microbial community as salinity increases from marine at the Murray River Mouth to hypersaline in the upper-reaches of the lagoon.

AIMS OF THIS THESIS

In this thesis I use the natural continuous gradients of salinity, particulate matter and nutrients exhibited in the Coorong as a model environment to investigate aquatic microbial community diversity, structure, and specific processes that occur between the different community members.

Specifically I aim to:

Determine the contributions of microzooplankton grazing and viral lysis to aquatic bacterial and phytoplankton mortality along strong physiochemical gradients. Additionally, I aim to determine the contribution of grazing and lysis to different sub-populations of the bacterial community.

Determine the change in the taxonomic identity and metabolic potential of the aquatic bacterial community at two extremes of salinity to ascertain the dominating influences on the community composition and function in the Coorong.

Determine the impact of salinity on viral community structure and production at two extremes of salinity.

Determine bacterial community structure and physical diversity in order to establish how physical characteristics can contribute to cellular energetic budgets in natural bacterial communities.

This thesis has been prepared in the style of a suite of manuscripts for submission to scientific journals. Each chapter focuses on a specific aspect of the microbial community present in the Coorong and as such there is some redundancy in the introduction and methods sections of each chapter. This thesis investigates the microbial community present in the Coorong and addresses 3 distinct themes: Chapters II and III investigate microbial processes, how these are affected by salinity, and the potential flow-on effects for the aquatic community; Chapters IV and V investigate the diversity of the bacterial and viral community at the extremes of the salinity gradient; Chapters V and VI investigate the physical structure of the viral and bacterial community; Chapter VI also investigates the bacterial process of motility in a range of temperate coastal environments in addition to the Coorong. While change in microbial communities in response to salinity is the overriding theme of this thesis, this thesis also provides the first insight into the Coorong microbial community diversity and how this community interacts and functions.

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Chapter II

Environmental and Community Variability Shapes Top-Down Mortality Pressures on Aquatic Bacteria

ABSTRACT

The manner in which bacteria mediate the flow of organic matter throughout aquatic environments is constrained by nutrient availability, viral infection and microzooplankton grazing. The latter two processes determine trophic vectors, which control the balance between organic material retention and export in the microbial food web and community composition. Mortality pathways coexist, with dominance varying spatially and temporally or according to shifting environmental factors. Dilution experiments were used to determine the mortality rates of several flow-cytometrically defined bacterial sub-populations at four sites in the Coorong, a coastal lagoon characterized by strong salinity and nutrient gradients. Total mortality rates varied from 3.6 to 9.5 d^{-1}. The impact of grazing and lysis was heterogeneous, at both the total community and sub-population level. Furthermore, the influence of grazing and lysis varied with shifting environmental parameters along the gradient. The combined effects of sub-population and environmental variability on microzooplankton grazing and viral lysis observed here indicate that the processes driving bacterial mortality in aquatic ecosystems are highly complex and inter-related.

INTRODUCTION

Heterotrophic bacteria typically dominate aquatic ecosystems in terms of abundance, diversity and metabolic activity (Azam and Malfatti 2007), and play a vital role in recycling nutrients and organic matter (Strom 2008). Bacterial community structure is dependent on 'bottom-up' physicochemical factors such as temperature and salinity (Bouvier and del Giorgio 2002; Wu *et al*. 2006; Lozupone and Knight 2007; Allan and Froneman 2008; Schapira *et al*. 2009), as well as 'bottom-up' factors relating to the availability of organic and inorganic nutrients (Yokokawa *et al*. 2004). Additionally, the 'top-down' influence of microzooplankton, heterotrophic organisms ranging in size from 2 to 200 µm (Stelfox-Widdicombe *et al*. 2000), and viral lysis also regulates bacterial abundance and composition (Posch *et al*. 1999; Yokokawa *et al*. 2004; Judd *et al*. 2006).

Viruses and grazers are the principle agents of bacterial mortality (Fuhrman and Noble 1995; Choi *et al*. 2003) and exert a strong selective influence on community composition and growth (Šimek *et al*. 2001; Middelboe and Lyck 2002; Brussaard *et al*. 2008a; Strom 2008). Additionally, the relative contributions of microzooplankton grazing and viral lysis in causing bacterial mortality alter the transfer of organic matter throughout aquatic systems (Fuhrman 1999; Wells and Deming 2006). Essentially, microzooplankton grazing transfers organic matter to higher trophic levels, while viral lysis recycles organic matter in the microbial loop, reducing transfer to higher trophic levels (Fuhrman 1999; Middelboe and Lyck 2002; Choi *et al*. 2003).

In coastal environments, microzooplankton grazing and viral lysis rates have similar impacts (Fuhrman and Noble 1995; Choi *et al*. 2003). Viral lysis can account for between 2 to 77% of bacterial mortality in marine environments (Fuhrman 1999;

Wommack and Colwell 2000; Weinbauer 2004), but only occasionally exceeds the effect of microzooplankton grazing (Fuhrman 1999). This is also observed in environments characterized by strong environmental pressures, such as hypersaline environments where microzooplankton grazing dominates transfer processes until salinity exceeds 250 PSU. Under these elevated salinity levels microzooplankton do not survive and viral lysis becomes the dominant mortality factor (Pedrós-Alió *et al*. 2000a, b). Overall the relative contributions of viral lysis and microzooplankton grazing to bacterial mortality is environment-dependent and has previously been related to spatiotemporal shifts in physicochemical parameters which affect the bacterial, zooplankton and viral communities (Guixa-Boixereu *et al*. 1996; Weinbauer and Höfle 1998; Guixa-Boixereu *et al*. 1999; Pedrós-Alió *et al*. 2000a, b; Schallenberg *et al*. 2003; Allan and Froneman 2008).

In addition to the changes in mortality pressures, the composition and function of microbial communities shift along environmental gradients (Allan and Froneman 2008). Increasing salinity results in a decrease in eukaryotic abundance and species diversity at higher trophic levels and conversely an increase in prokaryotic abundance (Guixa-Boixereu *et al*. 1996; Pedrós-Alió *et al*. 2000b) and appears to be a major selective pressure for aquatic microbes (Painchaud *et al*. 1995). The influence of strong environmental gradients, such as salinity, on the composition and distribution of bacterioplankton has been widely investigated in many environments (Guixa-Boixereu *et al*. 1996; Pedrós-Alió *et al*. 2000a, b; Langenheder *et al*. 2003; Crump *et al*. 2004; Demergasso *et al*. 2004; Kan *et al*. 2006; Maturrano *et al*. 2006; Demergasso *et al*. 2008; Foti *et al*. 2008). The common view that bacteria respond physiologically or phylogenetically to environmental variability ignores the potential for environmental changes to alter the dynamics of mortality agents on the bacterial community. Little is

known about the relative impact of large physiochemical environmental changes on viral lysis and microzooplankton grazing of bacterial populations in natural systems, which have to our knowledge only been investigated in environments where physio-chemical properties are relatively stable (Fuhrman and Noble 1995; Tuomi *et al*. 1999; Pedrós-Alió *et al*. 2000a, b; Choi *et al*. 2003; Jacquet *et al*. 2005; Wells and Deming 2006; Boras *et al*. 2009). Furthermore, the extent of heterogeneous mortality due to grazing and lysis on different sub-populations of the bacterial community has rarely been considered. Bacterial sub-populations exhibiting differences in cellular size and DNA content can be defined using flow cytometry, these sub-population can reflect changes in system productivity and cellular function and activity (Bouvier and del Giorgio 2002; Judd *et al*. 2006; Wang *et al*. 2009). Determining the extent to which grazing and lysis impact upon different compartments or sub-populations within the bacterial community will be illuminating when determining overall shifts in mortality (Yokokawa *et al*. 2004; Yokokawa and Nagata 2010).

The objectives of the present work were to assess the relative contributions of microzooplankton grazing and viral lysis on bacterial mortality along strong environmental gradients of salinity and particulate matter within the Coorong coastal lagoon system using dilution assays (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007; Brussaard *et al*. 2008b). Specifically, the effects of viral lysis and microzooplankton grazing on different flow cytometrically-defined bacterial sub-populations were assessed.

MATERIALS AND METHODS

Study site

The Coorong is a coastal lagoon system extending 140 km from the termination of Australia's largest river, the Murray River. The Coorong exhibits an intense environmental gradient increasing in salinity, and organic and inorganic matter and nutrients with distance from the Murray River mouth to Salt Creek, approximately 98 km southeast of the river mouth (Figure 1). Spatial and seasonal variation in salinity and nutrient content are major driving forces in the Coorong, which have been shown to affect the microbial communities present along the Coorong (Ford 2007; Schapira *et al*. 2009; Schapira *et al*. 2010). Samples were obtained with permission from the Department of Environment and Natural Resources (DENR) South Australia (scientific permit number G25583-1).

Sampling strategy

Sub-surface samples were collected mid-morning in May (Austral Autumn) 2008 from 4 sites along a transect characterized by increasing salinity from seawater conditions (38 PSU) at the River Murray mouth to hypersaline conditions (131 PSU) near Salt Creek (Figure 1). At each site, salinity (PSU), temperature (°C), pH, dissolved oxygen (mg L^{-1}) and turbidity (NTU) were recorded using a 90FL-T (TPS) multiparameter probe. Triplicate 1 mL water samples were fixed with 2% glutaraldehyde (final concentration) in the dark at 4°C for 15 minutes, immediately snap frozen in liquid nitrogen and stored at -80°C prior to flow cytometric (FCM) enumeration of virus-like particles (VLP) and bacteria (Brussaard 2004).

Triplicate 50 mL filtered samples (0.45 µm porosity, Millipore), were collected for dissolved inorganic nutrient analysis and stored at -20°C in the dark. Nutrient concentrations were analyzed within 72 hours using a LF 2400 photometer

(Aquaspex®) and standard colorimetric methods for determining concentrations of ammonium (Indophenol Blue), nitrite (Naphthylethylene diamine), nitrate (Naphthylethylene diamine after zinc reduction), phosphate (Ascorbic acid reduction) and silica (Heteropolyblue).

Chlorophyll *a* (Chl *a*) concentration was determined from triplicate 60 mL samples filtered through glass-fibre filters (0.7 µm porosity, GF/F Whatman) and stored at -20°C in the dark until analysis. Chlorophyllous pigments were extracted from the filters for 24 hours in 5 mL of methanol at 4°C in the dark. Chlorophyll *a* concentration (µg L^{-1}) was determined using a Turner 450 fluorometer.

Total suspended matter (TSM), particulate inorganic matter (PIM) and particulate organic matter (POM) was determined from triplicate 60 mL samples filtered through pre-combusted and weighed 25 mm glass-fibre filters (GF/F Whatman; 400°C, 4 hours). Following sample filtration, 60 mL of MilliQ water was used to rinse the filters and they were stored at -20°C in the dark until analysis. Filters were dried (60°C, 24 hours) and weighed to determine the total suspended matter, then combusted (450°C, 1 hour) and weighed to determine particulate inorganic matter and particulate organic matter (Barillé-Boyer *et al*. 2003).

Dilution experiments

This experiment dilutes natural site water with either grazer or virus free diluents obtained from the same site to different percentages of natural water (20, 40, 70 and 100%), triplicate samples are incubated for 24 hours and bacterial abundance is determined at time 0 and 24 hours. The natural logarithm of bacterial abundance at

time 0 is subtracted from abundance at abundance at time 24 hours and is regressed against each dilution factor, resulting in two linear relationships. Mortality due to grazing and lysis was determined using the equations of the lines generated as described below in the section entitled bacterial mortality (Evans *et al*. 2003; Baudoux *et al*. 2006).

Water was collected from each site by submerging a 25 L acid-washed (0.1 N HCl) polyurethane carboy and allowing the water to carefully and slowly fill the vessel to avoid damage or bursting of any organisms. Mesozooplankton were then removed by gravity siphoning the sample water through a 200 µm mesh filter.

Two dilution series were immediately prepared using this water, one bottle each of grazer and virus free site water per site. The grazer-free diluent fraction was prepared by vacuum filtration through a 0.2 µm filter (Pall Corporation), clogged filters were regularly removed and replaced with fresh filters, minimum vacuum pressure was applied. The virus-free water fraction was prepared by filtering half of the grazer-free diluent through a 30 kDa tangential flow filtration system (Millipore). Diluents were then siphoned into acid washed (0.1 N HCl) 1 L glass bottles (Schott) to create 4 levels of natural site water concentration (20, 40, 70 and 100%). These bottles were subsequently filled to capacity with freshly collected 200 µm filtered natural site water, a 20% natural water dilution was comprised of 20% 200 µm filtered site water and 80% diluent while a 100% natural water dilution contained only 200µm filtered natural site water, as each bottle had slightly different capacities percentages of water were determined by pre weighing each bottle and calculating the weight of dilutent required. For each dilution bottle, 250 mL was siphoned into triplicate acid washed (0.1 N HCl) 250 mL glass incubation bottles (Schott) and triplicate samples were taken from each bottle for VLP and bacterial enumeration (t = 0 h, t_0). These bottles were then refilled to 250 mL with previously diluted water. The incubation bottles were sealed using

Parafilm® M (Pechiney Plastic Packaging Company) and incubated for 24 hours in a 4,000 L holding tank containing sea water on site. After 24 hours (t = 24 h, t_{24}), triplicate samples were taken from each incubation bottle for flow cytometric VLP and bacterial enumeration as described above.

Microbial enumeration

Multiple VLP and bacterial populations were defined and enumerated at each site using flow cytometry according to differences in side scatter (SSC, i.e. cell size) and SYBR I Green fluorescence (i.e. DNA content) (Marie *et al*. 1999a, b; Brussaard 2004). Prior to analysis, frozen samples were thawed in 50°C water, diluted (1:10 or 1:100) in 0.2 µm filtered and autoclaved TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8), to better differentiate between bacterial sub-populations (un-published data), stained with SYBR-I Green solution (Molecular Probes, Eugene, Oregon; 5:100,000 dilution) and incubated at 80°C in the dark for 10 minutes (Brussaard 2004; Schapira *et al*. 2009). Samples were then analyzed using a FACSCanto flow cytometer (Becton-Dickson) with 1 µm fluorescent beads (Molecular Probes, Eugene, Oregon) added to each sample at a final concentration of 10^5 beads mL^{-1} to determine viral and bacterial abundance (Gasol and del Giorgio 2000). Each sample was processed for 2 minutes at 200-600 events per second and forward-angle light scatter (FSC), right-angled light scatter (SSC) and green (SYBR-I) fluorescence were recorded. VLP and bacterial sub-populations were identified and enumerated using WinMDI 2.9 software (© Joseph Trotter) according to variations in SYBR-Green I fluorescence and SSC (Marie *et al*. 1999a, b; Brussaard 2004). Viral and bacterial sub-population lowest in SYBR-Green I fluorescence were assigned sub-population number 1 (i.e. V1/B1), sub-populations with

the second lowest fluorescence were assigned number 2 (i.e. V2/B2) and so forth. Using microbial abundance determined by flow cytometry the virus to bacteria ratio (VBR) was determined at each site. All samples were analysed within 2 months of initial storage date.

Bacterial mortality

Bacterial mortality was determined for the total bacterial community as well as for each flow cytometrically-defined sub-population. At each site the apparent growth rate of each bacterial sub-population (μ) was determined from the change in abundance over the incubation period as

$$\mu = \ln N_{t_{24}} - \ln N_{t_0} \quad (1),$$

where N_{t_0} and $N_{t_{24}}$ represent the abundance of bacteria at time t = 0 and 24 hours, respectively. The slope S_1 of the regression of apparent growth versus dilution factor for the 0.2 µm dilution series corresponds to the microzooplankton grazing rate, M_g (d^{-1}). In contrast, the slope S_2 of the regression of apparent growth versus dilution factor for the 30 kDa dilution series represents the sum of microzooplankton grazing rate and viral lysis, ($M_g + M_v$). The mortality rate due to viral lysis (M_v, d^{-1}) is then given by the difference

$$M_v = S_2 - S_1 \quad (2).$$

The viral lysis to microzooplankton grazing ratio (LGR) for each bacterial sub-population at each site was determined as

$$M_v/M_g \quad (3).$$

Statistical analysis

Due to the low number of samples, multiple comparisons between sampling salinities and between mortality rates at each site were conducted using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test was used to identify distinct groups of measurements (Zar 1996).

RESULTS

Environmental properties

Environmental properties exhibiting notable increases in concentration along the Coorong were; salinity, which exhibited the greatest change between sites increasing from 37.6 PSU at Site 1 to 131.4 PSU at Site 4; and chl *a*, which remained below 3.4 µg L^{-1} from Sites 1 to 3, and then exhibited a 3-fold increase to 12.2 µg L^{-1} at Site 4 (Table 1). Dissolved oxygen, TSM, PIM and POM, all increased consistently along the gradient. Inorganic nutrient concentrations were more variable between Sites 1 and 3, but were generally increased with distance from river mouth (Table 1).

Flow cytometric analysis of microbial communities

Four distinct sub-populations of VLPs and five sub-populations of heterotrophic bacteria were identified in the Coorong water samples (Figure 2, Table 2). Total VLP and bacterial abundance increased along the environmental gradient from 6.8×10^7 to 2.3×10^9 mL^{-1} and from 1.3×10^7 to 6.7×10^8 mL^{-1}, respectively. The VBR showed variability along the gradient, ranging from 5.0 at Site 1 to 2.7 at Site 3 (Table 1).

Bacterial Mortality

The rate of bacterial mortality (d^{-1}) due to viral lysis and microzooplankton grazing was determined for total bacterial community and for each flow cytometrically-defined sub-population, at each site along the gradient (Figure 3, Figure 4).

Total bacterial mortality rates ($M_g + M_v$) remained between 3.6 and 6.4 d^{-1} from Sites 1 to 3, and increased to 9.5 d^{-1} at Site 4 (Figure 3). Microzooplankton grazing rates ranged from 1.1 d^{-1} to 3.9 d^{-1}, whilst viral lysis rates varied from 1.7 d^{-1} to 7.2 d^{-1}. However, despite high levels of variability between sites, no coherent trend in the relative contribution of viral lysis or grazing to bacterial mortality was apparent along the gradient. Grazing rates were highest at Site 3, did not significantly differ between Sites 1 and 4 and were lowest at Site 2 ($p < 0.05$). Viral lysis was also not significantly different between Sites 1 and 3, where it contributed the least to bacterial mortality; the contribution increased at Site 2 and was greatest at Site 4 ($p < 0.05$). Grazing and lysis contributed equally to bacterial mortality at Site 1, virus-mediated mortality was significantly higher than microzooplankton grazing at Sites 2 and 4 ($p < 0.05$), whilst at Site 3 microzooplankton mediated mortality was significantly higher than virus-mediated mortality ($p < 0.05$).

More complex patterns in viral lysis and grazing rates were apparent when the bacterial population was considered as sub-populations, rather than treated as a whole. While microzooplankton grazing and viral lysis contributed to mortality in all bacterial sub-populations at all salinities, the contribution varied greatly between populations (Figure 4). Generally, at each site bacterial sub-populations with larger cell size and greater DNA content were more heavily predated upon by grazers; Site 1, B2 (M_g = 1.26); Site 2, B3 (M_g = 0.43) and B5 (M_g = 0.39); Site 3, B3 (M_g = 1.67); Site 4, B2 (M_g = 1.02). At Sites 1 and 4 sub-populations with high abundance were more heavily lysed by viruses; Site 1, B1 (M_v = 1.77, abundance = 0.69 × 10^7 cells mL^{-1}); Site 4, B3 (M_v = 2.90, abundance = 35.77 × 10^7 cells mL^{-1}) (Table 2, Figure 4). This trend was not observed at Site 2 where the most abundant population (B2) was only moderately lysed (M_v = 0.39) and at Site 3 where B3 was moderately lysed yet highly grazed upon (M_v = 0.47, M_g = 1.67) (Figure 4). Typically, viral lysis had the greatest impact on the majority of bacterial sub-populations, but the extent of this pattern varied between populations and along the gradient.

At Site 1, grazing and lysis each controlled a single sub-population (B2 and B1 respectively). At Site 2, bacterial mortality was dominated by viral lysis for 3 of 5 sub-populations (B1, B4 and B5). Viral lysis was also the dominant mortality agent at Site 4 where all 3 sub-populations were controlled by lysis. In contrast, at Site 3 microzooplankton grazing was the dominant mortality agent as 2 of the 3 bacterial sub-populations (B1 and B3) were significantly controlled by grazing. Overall 3 bacterial sub-populations were controlled by grazing, 7 by viral lysis, and 3 sub-populations being controlled equally by both grazing and lysis (Figure 4).

Using the different contributions of lysis and grazing to the mortality of each sub-population a lysis to grazing ratio (LGR) was determined for each sub-population. LGR showed the greatest variation between sub-populations present at Site 2 ranging from 0.03 to 22.94 and varied by 3 orders of magnitude between all populations (Table 3).

DISCUSSION

Bacterial and viral abundance increased along the length of the Coorong lagoon (Table 1), which is consistent with previous observations (Schapira *et al*. 2009). However, we found that the dominant cause of bacterial mortality was highly variable along the Coorong and among sub-populations (Figure 3, Figure 4). Thus, while microbial abundance and environmental gradients generally increased monotonically along the Coorong, mortality causes were not as predictable. This indicates that microbial communities should not be viewed as a single unit that responds uniformly to shifts in environmental parameters. Instead, the alteration in bacterial sub-population structure observed here is consistent with the idea that microbial populations and communities are highly dynamic and respond in a variety of ways to changes in their environment (Judd *et al*. 2006; Dinsdale *et al*. 2008). Our results indicate that these shifts in community structure can also be driven by changes in 'top-down' pressures which also change along environmental gradients. This indicates that bacterial mortality is subject to the complex effects of environmental variation on both the bacterial community and the grazers and viruses that cause bacterial mortality.

Environmental gradients and total bacterial mortality

Total bacterial mortality rates increased with increasing salinity. However, the influence of grazing and lysis did not exhibit any similar monotonic trend along the salinity gradient (Figure 3). Large variation in the relative importance of viral lysis was observed between the different sites (Figure 3). This is consistent with previous evidence that viral induced mortality is more variable than that caused by grazing (Bettarel *et al*. 2002; Boras *et al*. 2009). The equal contribution of microzooplankton grazing and viral lysis to bacterial mortality at Site 1 (Figure 3) is consistent with previous studies showing that mortality can be equally controlled by both factors in coastal environments (Steward *et al*. 1996; Fuhrman 1999; Šimek *et al*. 2001; Bettarel *et al*. 2002) and can be explained by the moderate productivity observed at this site, as indicated by chlorophyll *a* concentrations, allowing for successful co-existence of grazers and viruses (Bohannan and Lenski 2000).

Bacterial mortality was caused primarily by viral lysis at Site 2 with microzooplankton grazing having minimal impact on three out of the five bacterial sub-populations (Figure 4). This could indicate that the bacterial community was comprised largely of grazing-resistant cells leaving the community vulnerable to viral lysis or that grazer concentration was low at this site.

At Site 3, bacterial mortality was dominated by microzooplankton grazing, suggesting that increased productivity, moderate salinity and high bacterial abundance at this site lead to an increase in bacterial populations preferred by microzooplankton grazers. Whilst all sub-populations were grazed to some extent, the most abundant bacterial population, with greatest cell size and DNA content were the most heavily grazed sub-populations (Table 2, Figure 4) indicating this was the preferred prey population with microzooplankton known to choose prey based on their size and nutritional value (Anderson 1997; Stelfox-Widdicombe *et al*. 2000; Joint *et al*. 2002).

Bacterial mortality was also primarily caused by viral lysis at Site 4 with microzooplankton grazing again having little impact on the bacterial community (Figure 3). This contradicts previous studies that show bacterivory is the dominant loss factor at comparable salinities (Guixa-Boixereu *et al*. 1996; Pedrós-Alió *et al*. 2000a, b). The dominance of viral lysis in this instance could be attributed to the high host abundance at this site (Weinbauer and Höfle 1998; Weinbauer 2004; Hewson and Fuhrman 2007). Alternatively, the potential decrease in microzooplankton predators, as has been seen in high saline environments (Guixa-Boixereu *et al*. 1996), or the increased production and success of viruses in highly productive environments (Weinbauer 2004), may explain this imbalance. Organic and inorganic matter also peaked at this site (Table 1) indicating increased viral survival (Bitton and Mitchell 1974) and greater potential for lysis. Viral persistence at high abundances in aquatic environments suggests that removal and replacement are in equilibrium (Wilhelm *et al*. 2002), indicating high lysis rates in the Coorong are most likely the norm for this system.

The large shifts in the relative importance of grazing and viral lysis observed between sites indicates that these top-down processes can be influenced by environmental variability, which may either exert selective pressures on the microzooplankton and viral communities, or alter the bacterial assemblages in ways that affect their susceptibility to each mortality agent.

Differential bacterial mortality

Our study indicates that the identification of the dominant mortality agent for a community does not reflect the complexity of mortality throughout an entire assemblage. Instead, the relative importance of grazing or lysis varies according to the

features of different bacterial sub-populations. We show that different components of the bacterial community, differentiated using flow cytometry, are affected by grazing and lysis to a different extent. This reveals the intricate array of pressures on bacterial communities that are not detected when determining mortality on the community as a whole. These complex interactions can be successfully revealed by dilution experiments targeting flow cytometrically-defined sub-populations (Figure 4). Flow cytometry generally reveals the existence of at least two distinct bacterial populations in aquatic samples, consisting of cells with a high- or low-nucleic acid content (HNA/LNA; Lebaron *et al*. 2001). Different flow cytometrically defined sub-populations have been shown to reflect differences in activity, growth, life cycle stage and phylogenetic composition of the community (Judd *et al*. 2006; Bouvier *et al*. 2007; Wang *et al*. 2009). The relative number of flow cytometrically defined sub-populations and their cytometric characteristics vary across ecosystems and are dependent on system productivity (Bouvier *et al*. 2007). Each bacterial sub-population has been proposed to exhibit different functions, and to perform specific roles in the functioning of the ecosystem (Wang *et al*. 2009). Thus, differences in the mortality of each of these sub-populations is likely to have important flow-on effects in the microbial food-web, stressing the need to define the main contributors to the mortality of each sub-population.

Specifically, we found that the larger cell-sized bacterial sub-populations with greater DNA content experienced higher rates of microzooplankton predation. This is consistent with previous research in the marine environment indicating that medium to large sized bacteria, along with more active and dividing cells are most susceptible to grazing (Sherr *et al*. 1992; Jürgens and Güde 1994; Posch *et al*. 1999). Yokokawa and Nagata (2010) found that grazing has similar impacts on different bacterial phylogenetic groups,

indicating the relationship between flow cytometric characteristics, phylogenetic composition and cellular activity needs to be fully described and defined.

Our data indicates that viruses preferentially infected the most abundant bacterial sub-populations (Table 2; Figure 4). This is consistent with the idea that host separation distance is the most important factor in defining virus success (Hewson and Fuhrman 2007). No relationship was found regarding bacterial cell size and lysis rate, which contradicts the hypothesis that bacteria might exhibit a smaller cell size to avoid contact with viruses (Weinbauer and Höfle 1998). This highlights a crucial, but seldom made point in microbial ecology that grazing and infection create a dynamic balance between cell size and cell abundance, preventing either from being the continuously dominant strategy.

The complex dynamics of bacterial mortality is further highlighted here by the strong variability in the LGR between populations. The LGR increased by nearly 3 orders of magnitude between sub-populations. This dramatic increase in LGR indicates that whilst grazing is reported to be the dominant mortality agent in many systems, microzooplankton can have little to no impact on certain bacterial sub-populations (Figure 4, Table 3).

Finally, we note that whichever mortality process dominated, this domination was usually almost total, with only 3 out of 13 sub-populations showing similar contributions (Figure 4). As such, considering the different vectors of carbon flow associated with each form of mortality, the contribution of the different flow cytometrically-defined bacterial sub-populations to biogeochemical fluxes and nutrient transfer is likely to vary markedly. This highly selective mortality and dominance of viral lysis pressure may also

lead to considerable evolutionary divergence between bacterial sub-populations (Marie *et al*. 1999a; Hewson and Fuhrman 2007).

Caveats of interpretation

The dilution technique used in this study is widely and routinely used to determine both bacterial and phytoplankton mortality. There is evidence that dilution can stimulate the bacterial growth rate (Ferguson *et al*. 1984; Li and Dickie 1985), whilst this potential bias is unavoidable using this technique Tremaine and Mills (1987) examined this issue and found this potential bias to be insignificant. In addition we investigate relative shifts between treatments and there is no evidence to suggest there will be unbalanced effect between treatments.

Here, we focus on the impacts of grazing and lysis on bacterial community sub-population structure, our findings show that grazers predate mainly on larger bacteria and viruses have no specific size target, generally infecting the most abundant bacteria, this is consistent with other authors (Sherr *et al*. 1992; Jürgens and Güde 1994; Posch *et al*. 1999; Hewson and Fuhrman 2007). For this reason we believe that the growth stimulated dilution has had minimal impact on our consideration of relative contributions of grazing and lysis to community mortality.

SYNTHESIS

Here we have shown that the relative impacts of virus infection and microzooplankton grazing on aquatic bacterial communities can shift strongly between habitats distributed along strong environmental gradients of salinity and matter. This indicates that shifts in

top-down pressures occurring in association with environmental variability should also be considered when assessing the effects of fluctuations and gradients in physical parameters on microbial assemblages. We also show that the effects of viruses and grazers on the bacterial community can vary strongly between different components of the assemblage and that the variable role of these controlling processes should be considered at finer scales than whole community abundance. Taken together our findings suggest that the influence of viruses and grazers should not be considered as two clear-cut mortality causes but as a more complex set of varying factors, which while working in parallel, can quickly shift substantially in importance as a consequence of changes in both environmental and ecological parameters.

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Table 1 GPS position; VLP and bacterial abundance; environmental conditions; and particulate variables at each site (mean ± standard error).

Parameter	**Site 1**	**Site 2**	**Site 3**	**Site 4**
GPS position (South)	35° 32.974'	35° 47.766'	35° 56.224'	36° 09.806'
GPS position (East)	138° 52.977'	139° 19.081'	139° 29.390'	139° 38.963'
VLPs (mean × 10^7 mL^{-1})	6.82 ± 0.77	13.17 ± 0.43	31.18 ± 5.03	229.89 ± 44.74
Bacteria (mean × 10^7 mL^{-1})	1.35 ± 0.15	3.92 ± 0.01	11.66 ± 2.02	67.54 ± 7.99
VBR	5.05	3.36	2.67	3.40
Salinity (PSU)	37.6	54.8	112.3	131.4
Temperature (°C)	16.5	15.3	16.6	12.8
pH	8.2	8.5	8.1	8.0
$[O_2]_d$ (mg L^{-1})	10.6	11.4	11.5	13.5
Turbidity (NTU)	12.0	4.0	18.0	19.7

Chl *a* (µg L^{-1})	1.5 ± 0.1	3.4 ± 0.6	2.8 ± 0.5	12.2 ± 2.6
TSM (× 10^5µg L^{-1})	0.2 ± 0.0	0.4 ± 0.1	1.5 ± 0.5	1.8 ± 0.5
PIM (× 10^5 µg L^{-1})	0.1 ± 0.0	0.4 ± 0.1	1.2 ± 0.4	1.2 ± 0.4
POM (× 10^4 µg L^{-1})	0.5 ± 0.2	2.3 ± 0.4	3.6 ± 1.4	5.9 ± 1.8
Ammonium (µmol L^{-1})	5.5 ± 2.1	3.2 ± 1.4	BDL	10.7 ± 4.5
Nitrite (µmol L^{-1})	<1	0.1 ± 0.1	BDL	0.4 ± 0.2
Nitrate (µmol L^{-1})	BDL	0.5 ± 0.3	0.5 ± 0.5	0.5 ± 0.3
Phosphate (µmol L^{-1})	0.8 ± 0.2	3.5 ± 1.8	1.1 ± 0.3	8.4 ± 3.2
Silica (µmol L^{-1})	5.0 ± 0.9	1.4 ± 0.7	43.3 ± 3.1	48.9 ± 2.8

Table 2 Bacterial sub-population abundance at each site (mean abundance $mL^{-1} \times 10^7 \pm$ standard error). B1 denotes bacterial sub-population 1; B2 denotes bacterial sub-population 2 etc.

Sub-population	Site1	Site 2	Site 3	Site 4
B1	0.69 ($\pm$ 7.6 × 10^5)	0.23 ($\pm$ 6.4 × 10^2)	1.18 ($\pm$ 1.77 × 10^3)	7.71 ($\pm$ 4.3 × 10^3)
B2	0.66 ($\pm$ 1.0 × 10^3)	1.74 ($\pm$ 1.8 × 10^3)	2.45 ($\pm$ 2.72 × 10^3)	24.01 ($\pm$ 6.4 × 10^3)
B3	-	1.61($\pm$ 1.7 × 10^3)	8.03 ($\pm$ 4.08 × 10^3)	35.77 ($\pm$ 1.2 × 10^4)
B4	-	0.18 ($\pm$ 5.6 × 10^2)	-	-
B5	-	0.17($\pm$ 5.6 × 10^2)	-	-

Table 3 Lysis to grazing ratio (LGV) for each bacterial sub-population at each site.

Sub-population	Site1	Site 2	Site 3	Site 4
B1	4.09	17.09	0.43	2.44
B2	0.06	22.94	0.69	2.45
B3	-	0.03	0.28	5.60
B4	-	9.93	-	-
B5	-	4.48	-	-

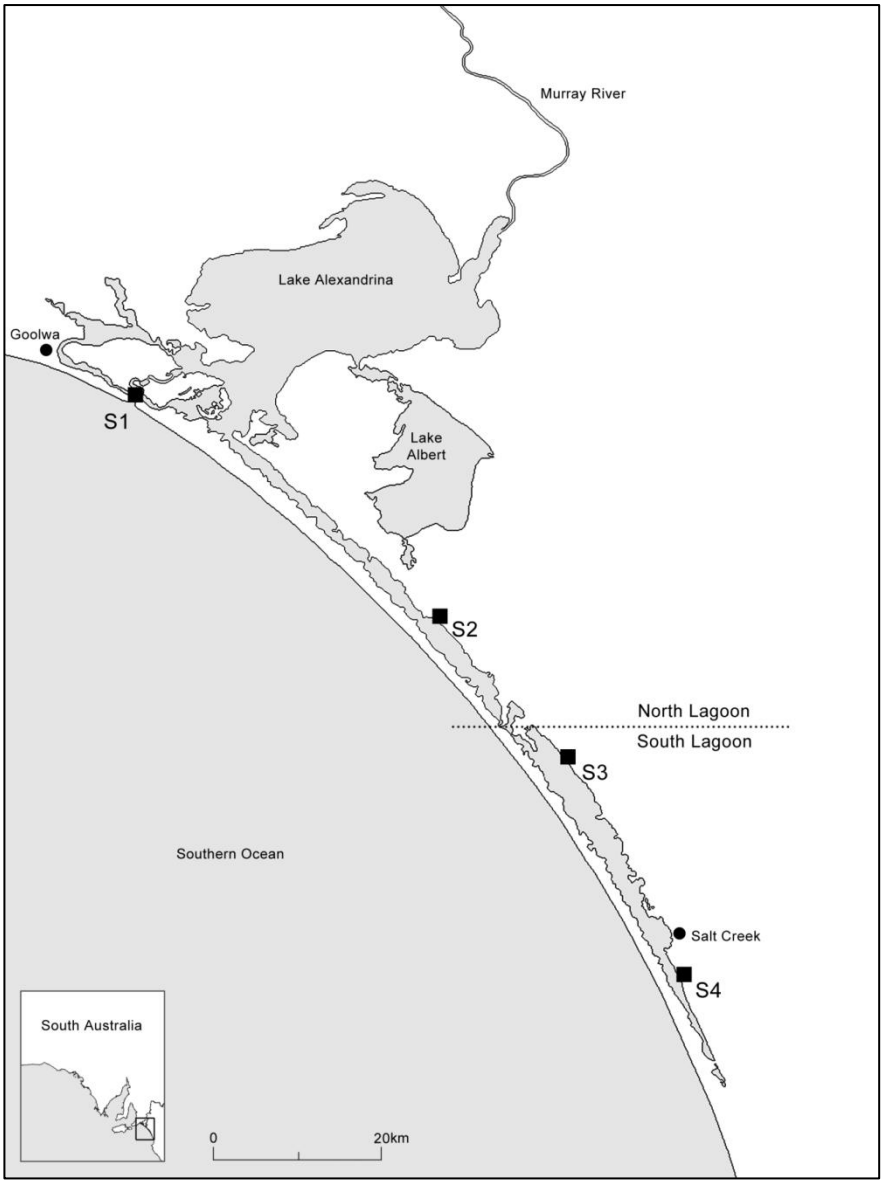

Figure 1 Study site; the Coorong (South Australia, Australia) showing the location of the 4 sampling sites (■).

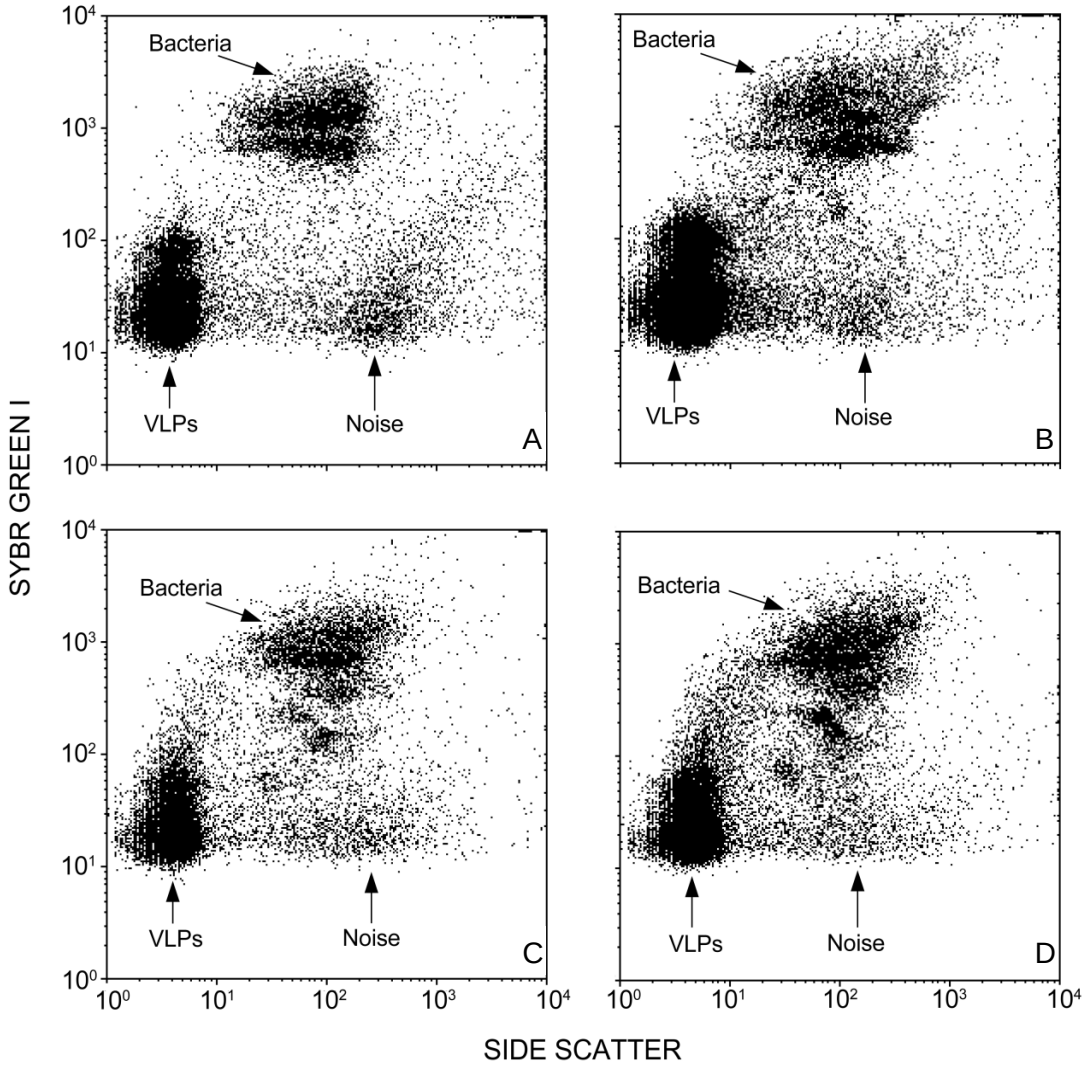

Figure 2 Cytograms showing virus-like particles (VLPs) and heterotrophic bacterial sub-populations found along the Coorong at Sites 1 (A) to 4 (D).

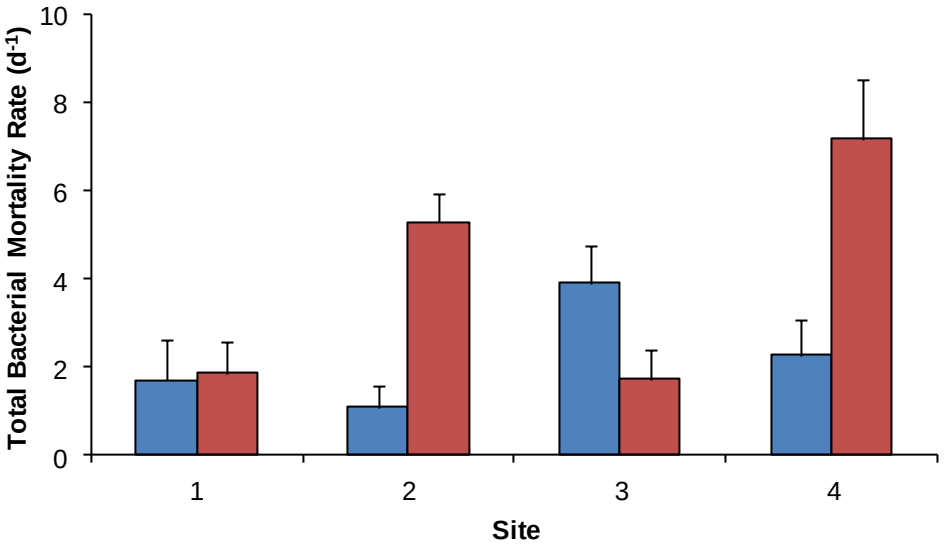

Figure 3 Total bacterial mortality rates (d^{-1}) due to microzooplankton grazing ( ) and viral lysis ( ). Error bars are standard error of the mean.

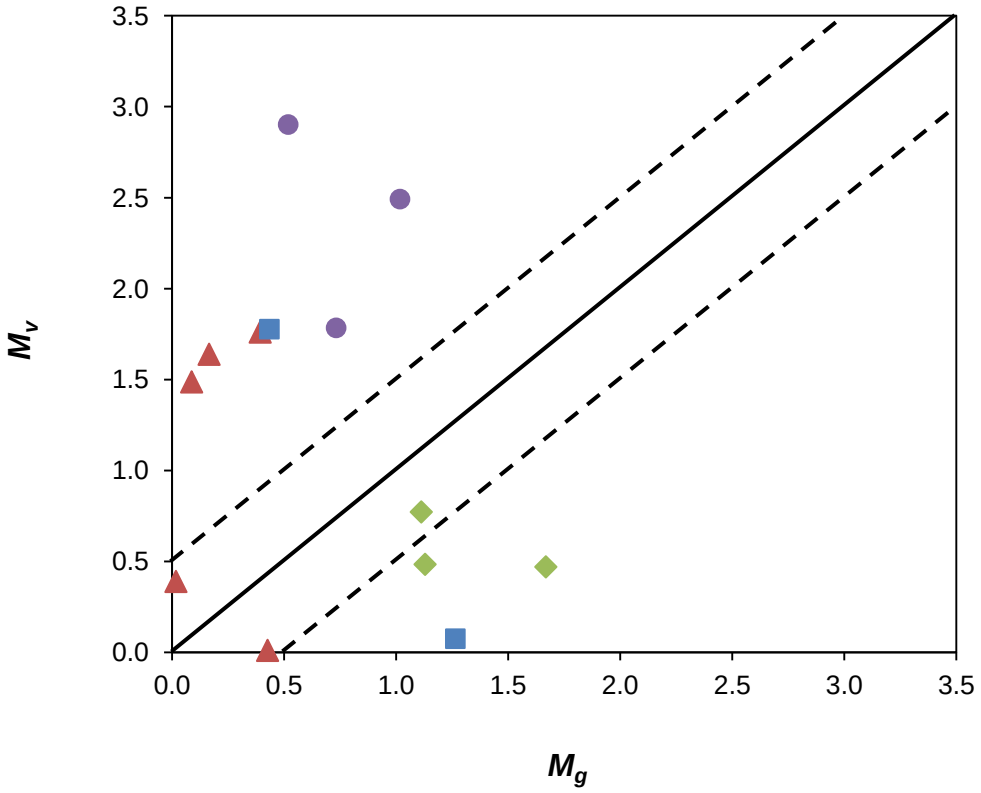

Figure 4 Microzooplankton grazing rates versus viral lysis rates (M_g; d^{-1} and M_v; d^{-1} respectively), for each bacterial sub-population. ■ Site 1; ▲Site 2; ◆ Site 3; ● Site 4. Each symbol represents an individual sub-population. The continuous line is the first bissectrix, i.e. $M_g = M_v$, and dashed lines indicates the 95% confidence intervals.

Chapter III

Differential Impacts of Microzooplankton and Viruses on Phytoplankton in a Hypersaline Lagoon

ABSTRACT

Salinity structures phytoplankton communities in estuarine environments and is predicted to alter with climate change. Phytoplankton communities are also controlled by mortality induced by microzooplankton grazing and viral lysis which determines transfer of organic matter though aquatic systems. However, the contribution of viral lysis to mortality is only beginning to be addressed. Modified dilution assays were conducted to determine the contribution of grazing and lysis to phytoplankton mortality as salinity increased at 4 sites from 38 to 131 PSU in a coastal estuary and lagoon system. Phytoplankton biomass and viral abundance increased with salinity, but total mortality was 0.3 d^{-1} at the extremes of the system and increased to 1.7 d^{-1} at 55 PSU and 4.1 d^{-1} at 112 PSU, with phytoplankton growth rate exhibiting the same trend. The high biomass recorded at locations of slow growth and low mortality suggests productivity has built up over time and current activity is minimal. A reduced but equal contribution of grazing and lysis was observed at the extremes of the gradient, lysis was increased at intermediate salinities and grazing was prominent at 112 PSU. This highlights that neglecting the contribution of lysis clouds interpretation of matter transfer. In addition, the unusually slow response to high productivity indicates energy balances and biomass control processes warrant further study. A potential consequence of this is that as climate changes and salinity increases the complex interactions between all microbial community members needs to be considered and even typically resilient microbial ecosystems may lose their recovery ability.

INTRODUCTION

Estuaries, wetlands and lakes are subject to salinisation due to the intrusion of saline waters (Najjar *et al*. 2000; Schallenberg *et al*. 2003). Salinity is likely to become a threat for these ecosystems as increased salinity alters the physical environment of water-bodies which, in turn, affects the resident organisms and the various processes they mediate (Nielsen *et al*. 2003; Hilton *et al*. 2008). This is especially true in Australian estuaries where salinity is expected to increase due to both climate change and the rise of salinity in groundwater caused by the clearing of native vegetation (Jolly *et al*. 2001), reduced frequency of riverine high-flow events, modification of water regime and increased evaporation (Nielsen *et al*. 2003; Ford 2007). Estuaries are productive ecosystems with strong environmental gradients and as such exhibit dynamic microbial communities (Ruiz *et al*. 1998; Henriques *et al*. 2006; Kan *et al*. 2007). However, despite global concern about increases in salinity, the effect of salinity on microbial community activity, specifically activity relating to biogeochemical cycling, has been largely overlooked.

Increasing salinity results in a decrease in eukaryotic abundance, increased prokaryotic abundance and decreased species diversity, and hence alters microbial processes in aquatic systems (Pedrós-Alió *et al*. 2000a, b; Ayadi *et al*. 2004; Estrada *et al*. 2004; Schapira *et al*., 2009; Schapira *et al*. 2010). As such salinity is a major selective pressure (Burić *et al*. 2007), and consequently its influence on the composition and distribution of phytoplankton has widely been investigated along estuaries (Burić *et al*. 2007; Muylaert *et al*. 2009), hypersaline solar salterns (Ayadi *et al*. 2004; Estrada *et al*. 2004; Elloumi *et al*. 2009) and hypersaline lakes (Gilabert 2001). Phytoplankton form the basis of the aquatic food web, their activities impact upon organisms and communities at higher trophic levels and influence biogeochemical cycles (Baudoux *et al*. 2006, Falkowski *et al*. 1998). Phytoplankton communities are structured by a range of abiotic factors (i.e. light, temperature,

nutrients and salinity (Burić *et al*. 2007) and biotic factors (i.e. predation by microzooplankton and viral infection and lysis; (Evans *et al*. 2003). The cause of phytoplankton mortality is of particular importance to the cycling of organic matter through aquatic systems as microzooplankton grazing transfers organic matter to higher trophic levels while viral lysis remineralises organic matter through the microbial loop (Fuhrman 1999; Brussaard *et al*. 2008a). As such the dominant mortality agent of phytoplankton exerts significant influence not only on phytoplankton communities but also on the structure and function of aquatic systems (Šimek *et al*. 2001; Brussaard *et al*. 2008a, b; Strom 2008).

Recent methodological improvements of the original dilution method (Landry and Hassett 1982) allow direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of phytoplankton (Evans *et al*. 2003; Baudoux *et al*. 2006; Brussaard *et al*. 2008b). Whilst there have been several studies on the impacts of grazing and lysis on marine phytoplankton mortality (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007 Brussaard *et al*. 2008b) this comparison is still overlooked in the literature (Stelfox-Widdicombe *et al*. 2004; Nejstgaard *et al*. 2007; Calbet *et al*. 2010; Grattepanche *et al*. 2010). The failure to compare different causes of phytoplankton mortality is based on the assumption that microzooplankton are the dominant consumers of phytoplankton (Calbet 2001; Calbet and Landry 2004). However, in aquatic systems, the relative contribution of microzooplankton grazing and viral lysis to phytoplankton mortality strongly depends on the environment considered, and varies spatially and temporally. Specifically there is a gap in the literature as to how grazing and lysis alter with increasing salinity in natural environments. Phytoplankton community composition and subsequent susceptibility to grazers and viruses is affected by salinity (Flöder and Burns 2004; Schapira *et al*. 2010). As the salinity of natural systems alters with climate change there is a pressing need to determine the effects

salinity have on the contributions of grazing and lysis. In this context, the objectives of the present work were to assess the relative contributions of microzooplankton grazing and viral lysis to phytoplankton mortality along a 38 to 131 PSU salinity gradient in the Coorong, a 140 km long South Australian lagoon that gradually becomes hypersaline.

MATERIALS AND METHODS

Study site

The Coorong is a 2 to 3 km wide estuarine and lagoon system extending 140 km from the termination of the Murray River in South Australia (Lamontagne *et al*. 2004; Geddes 2005) (Figure 1). It is considered a reverse estuary (Geddes 2005), exhibiting an increasing salinity gradient from a near-seawater salinity of 38 PSU at the Murray River mouth to a moderately hypersaline salinity of 131 PSU near Salt Creek in the upper reaches of the lagoon. The Coorong is separated from the Southern Ocean by the Younghusband Peninsula (Geddes 2003) and is divided into Northern and Southern Lagoons at Parnaka Point where water exchange and movement is restricted by a narrow 100 m wide, shallow channel (Webster 2005a) (Figure 1). Barrages separate the Coorong from the Lower Lakes and prevent the exchange of saline water from the Coorong to the Lower Lakes, Alexandrina and Albert, and the Murray River (Webster 2005a). Freshwater inflow into the Coorong is received from Lake Alexandrina, the Murray River and precipitation, while seawater is exchanged through the Murray Mouth (Geddes 2003; Geddes 2005). Interactions between sea level, tides, wind, rainfall, evaporation, and inflow cause variation in lagoon water level and salinity (Webster 2005a; Ford 2007), leading to the increase in salinity and water level observed along the Coorong. Water level is lowest from mid-summer to mid-autumn, ranging approximately from sea level to 0.2

m below sea level, and highest in the winter months, ranging from 0.4 to 0.8 m above sea level (Webster 2005b). Salinity is the major driving factor of phytoplankton abundance and growth within the Coorong and shows a strong seasonal gradient (Ford 2007). Variation in salinity and nutrient content leads to spatial and temporal differences in the microbial, planktonic and algal communities present and the various processes mediated by these communities (Ford 2007).

Sampling strategy

Samples were collected from 4 sites exhibiting increasing salinity from the Murray mouth to Salt Creek in May 2008. Sub-surface water was collected from each site using 25 L polyurethane carboys washed in 0.1 N HCl. Salinity (PSU), temperature (°C), pH, dissolved oxygen (mg L^{-1}) and turbidity (NTU) were recorded using a 90FL-T (TPS) multiparameter probe at each site. Dissolved inorganic nutrient concentrations were estimated from triplicate 50 mL filtered samples (0.45 µm porosity, Millipore), stored at -20°C in the dark and analysed within 72 h using a LF 2400 photometer (Aquaspex®). In the laboratory, analysis was performed according to standard colorimetric methods for ammonium (Indophenol Blue), nitrite (Naphtylethylene diamine), nitrate (Naphtylethylene diamine after zinc reduction), phosphate (Ascorbic acid reduction) and silica (Heteropolyblue). Triplicate 60 mL samples for chlorophyll *a* (Chl *a*) were filtered through 25 mm glass-fibre filters (0.7 µm porosity, GF/F Whatman) and stored at -20°C in the dark until analysis. In the laboratory chlorophyllous pigments were extracted from the filters in 5 mL of methanol at 4°C in the dark for 24 hours. Chl *a* concentration (µg L^{-1}) was then determined using a Turner 450 flurometer. Triplicate 60 mL samples for total suspended matter (TSM), particulate inorganic matter (PIM) and particulate organic matter (POM) were filtered through pre-combusted and weighed 25 mm glass-fibre

filters (GF/F Whatman; 400°C, 4 hours), filters were then rinsed with 60 mL of MilliQ® water and stored at -20°C in the dark until analysis. In the laboratory filters were dried (60°C, 24 hours) and weighed to determine the total suspended matter. Filters were then combusted (450°C, 1 hour) and weighed to determine particulate inorganic matter and particulate organic matter (Barillé-Boyer *et al*. 2003). Triplicate 1 mL samples collected for virus-like particle (VLP) enumeration were fixed in 2% gluteraldehyde (final concentration) at 4°C in the dark for 15 minutes, snap frozen in liquid nitrogen and stored at -80°C until flow cytometric analysis (Brussaard 2004).

Dilution experiments

The viral lysis dilution assay was used to determine phytoplankton mortality due to viral infection and microzooplankton grazing for each site (Evans *et al.* 2003; Baudoux *et al*. 2006; Brussaard *et al*. 2008b). Briefly, *in situ* water was collected by submerging 25 L acid-washed (0.1 N HCl) polyurethane carboys allowing the water to slowly fill the vessel. Sampled water was then immediately siphoned through a 200 µm mesh filter to remove mesozooplankton and two dilution series were prepared. Water was gravity filtered through a 0.2 µm filter (Pall Corporation) to create the grazer-free fraction. Half of this fraction was then filtered through a 30 kDa tangential flow filtration system (Millipore) to create the virus-free fraction. Four levels of natural site water concentration (20, 40, 70 and 100%) were then created by siphoning grazer-free or virus-free water (80, 60, 30 and 0%) into acid washed (0.1 N HCl) 1 L glass bottles (Schott). Freshly collected 200 µm filtered *in situ* water was then used to fill the bottles to capacity. For each dilution, water was siphoned in triplicate into acid washed (0.1 N HCl) 250 mL glass incubation bottles (Schott) and triplicate samples were taken from each bottle for chlorophyll *a* analysis (t = 0 h, t_0). Original dilution water was then used to refill each incubation bottle. Parafilm® M

(Pechiney Plastic Packaging Company) was used to seal the incubation bottles which were then incubated for 24 hours in a 4,000 L holding tank on site. After 24 hours (t = 24 h, t_{24}), triplicate samples were taken from each incubation bottle for chlorophyll *a* analysis.

Phytoplankton mortality

Phytoplankton mortality was determined using chlorophyll *a* as a proxy for total phytoplankton biomass, with measurements taken at t_0 and t_{24}. Briefly, the apparent growth rate of the phytoplankton population (μ; µg L^{-1} d^{-1}) at each site was determined from the change in abundance over the incubation period as

$$\mu = \ln N_{t_{24}} - \ln N_{t_0} \quad (1),$$

where Nt_0 and Nt_{24} are the chlorophyll *a* concentrations at time t = 0 and 24 hours, respectively. Specifically, the slope S_1 of the regression of apparent growth versus dilution factor for the 0.2 µm dilution series is an estimate of microzooplankton grazing rate, M_g (d^{-1}). In contrast, the slope S_2 of the regression of apparent growth versus dilution factor for the 30 kDa dilution series represents the sum of microzooplankton grazing rate and viral lysis, (M_g + M_v). The mortality rate due to viral lysis (M_v, d^{-1}) is then trivially returned by the difference

$$M_v = S_2 - S_1 \quad (2).$$

Phytoplankton growth, production and loss

The phytoplankton growth rate (k; µg L^{-1} d^{-1}) was determined, using chlorophyll *a* as a proxy for total phytoplankton biomass, from the general exponential model (Landry and Hassett 1982; Murrell and Hollibaugh 1998)

$$N_t = N_0 e^{(k-M_g)t} \quad (3)$$

for the 100% dilution series, where N_t and N_0 are the chlorophyll *a* concentrations at time t = 0 and 24 hours, respectively, k is the population growth rate and M_g is the mortality rate due to grazing, M_v in the case of lysis, t is time in days.

Potential production (P_p; µg L^{-1} d^{-1}) was determined from time averaged chlorophyll a concentration for the 100% dilution series (Chl *a*) multiplied by the phytoplankton growth rate

$$P_p = \text{Chl } a \times k \quad (4).$$

Production grazed (P_g; µg L^{-1} d^{-1}) and lysed (P_v) were determined from the grazing rate (M_g) and the lysis rate (M_v), and the averaged chlorophyll *a* concentration for the 100% dilution series (Chl *a*) as

$$P_g = M_g \times \text{Chl } a \quad (5)$$

and

$$P_v = M_v \times \text{Chl } a \quad (6).$$

Virus-like particle enumeration

Triplicate 1 mL water samples were fixed with a final concentration of 2% gluteraldehyde at 4°C for ten minutes, then frozen in liquid nitrogen and stored at -80°C prior to flow cytometric enumeration of VLPs. In the laboratory VLP concentration at each site was determined using a FACSCanto flow cytometer (Becton-Dickson). Frozen samples were thawed in 50°C water, diluted (1:10 or 1:100) in 0.2 µm filtered and autoclaved TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8), stained with SYBR-I Green solution (Sigma-Aldrich; 1:500 dilution with TE buffer) and incubated for 10 minutes in the dark at 80°C (Brussaard 2004). 1 µm fluorescent beads (Molecular Probes, Eugene, Oregon) were added to each sample before processing as an internal concentration and size standard. Samples were processed for 2 minutes and forward-angle light scatter (FSC, 285V), right-angled light scatter (SSC, 500V) and green (SYBR-I) fluorescence (500V) were recorded. VLP sub-populations were identified and enumerated using WinMDI 2.9 (© Joseph Trotter) according to variations in SYBR-Green fluorescence and SSC (Marie *et al*. 1999a, b; Brussaard *et al*. 2004).

Statistical analysis

Multiple comparisons between sampling sites and between mortality rates at each site were conducted using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test was used to identify distinct groups of measurements (Zar 1996).

RESULTS

Environmental properties

Environmental characteristics for each site are summarized in Table 1. Salinity increased along the Coorong from 38 PSU at Site 1 to 131 PSU at Site 4 (Figure 1). Dissolved oxygen increased along the gradient from 10.6 mg L^{-1} at 38 PSU to 13.5 mg L^{-1} at 131 PSU. Temperature, pH and turbidity all varied between sites. Mean temperature was 15°C, pH ranged from 8.0 at 131 PSU to 8.5 at 55 PSU and turbidity ranged from 4 NTU at Site 2 to 19.7 NTU at 131 PSU.

Chlorophyll *a* concentrations remained below 3.4 µg L^{-1} from 38 to 112 PSU, then increased to 12.2 µg L^{-1} at 131 PSU. VLP concentration increased by approximately 2 orders of magnitude from 6.8 × 10^7 mL^{-1} at 38 PSU to 229.9 10^7 mL^{-1} at 131 PSU. TSM, PIM and POM concentration increased along the gradient. Nutrient concentration varied between sites. Ammonium was the most abundant form of nitrogen and varied in concentration from below detection at 112 PSU to 10.7 µmol L^{-1} at 131 PSU. Nitrite was greatest at Site 4, 0.4 µmol L^{-1}, whilst remaining low from 38 to 112 PSU. Nitrate was below detection at 38 PSU yet remained constant from 55 to 131 PSU at 0.5 µmol L^{-1}. Phosphate concentrations were below 3.5 µmol L^{-1} from 38 to 112 PSU then increased to 8.4 µmol L^{-1} at 131 PSU. Silica concentrations were below 5.0 µmol L^{-1} at 38 and 55 PSU then significantly increased to 43.3 µmol L^{-1} and 48.9 µmol L^{-1} at 112 and 131 PSU respectively.

Phytoplankton mortality

Total mortality rate (M_g + M_v) was greatest at 112 PSU (4.1 d^{-1}) and least at 38 and 131 PSU (0.3 d^{-1}) with 55 PSU exhibiting a total mortality of 1.7 d^{-1} (Table 2). The rate of phytoplankton mortality (d^{-1}) due to microzooplankton grazing and viral lysis varied from 0.1 to 2.7 d^{-1} and from 0.1 to 1.5 d^{-1} respectively (Figure 2). Specifically, microzooplankton grazing and viral lysis rates were not significantly different at 38 and 131 PSU ($p > 0.05$, Figure 2). Virally-mediated mortality was significantly higher

($p < 0.05$) than microzooplankton grazing at 55 PSU, whilst microzooplankton-mediated mortality was significantly higher ($p < 0.05$) than viral lysis at 112 PSU (Figure 2).

The percentage of phytoplankton mortality respectively caused by grazing and lysis is shown in Table 2. Grazing was responsible for 61.3% of the total phytoplankton mortality at 38 PSU, 24% at 55PSU, 64.2% at 112 PSU and 48.8% at 131 PSU. Viral lysis caused 38.7% of total mortality at 38 PSU, 76% at 55 PSU, 35.8% at 112 PSU and 51.2% at 131 PSU.

Phytoplankton production lost to grazing and lysis is shown in Table 3. Typically phytoplankton loss is least at 38 PSU; 0.03 µg L^{-1} d^{-1} grazed and 0.02 µg L^{-1} d^{-1} lysed, greatest at 112 PSU; 12.11 µg L^{-1} d^{-1} grazed and 6.8 lysed µg L^{-1} d^{-1}, and similar at 55 and 131 PSU; 1.6 µg L^{-1} d^{-1} grazed, 5.2 µg L^{-1} d^{-1} lysed and 2.3 µg L^{-1} d^{-1} grazed and 2.4 µg L^{-1} d^{-1} lysed respectively (Table 3).

Phytoplankton growth and potential production

Phytoplankton growth rate (*k*) ranged from -0.2 µg L^{-1} d^{-1} at 38 PSU to 1.4 µg L^{-1} d^{-1} at 55 PSU and 3.5 µg L^{-1} d^{-1} at 112 PSU then fell substantially to 0.7 µg L^{-1} d^{-1} at 131 PSU (Table 3). Potential production (P_p) followed the same trend with potential production increasing from -0.03 µg L^{-1} d^{-1} at 38 PSU, to 5.4 µg L^{-1} d^{-1} at 55 PSU and 15.8 µg L^{-1} d^{-1} at 112 PSU, then decreasing to 10.4 µg L^{-1} d^{-1} at 131 PSU (Table 3).

DISCUSSION

Salinity and phytoplankton mortality

Although both phytoplankton and viral abundance increased with salinity (Table 1) there was no clear trend in phytoplankton mortality with salinity (Figure 2). Total phytoplankton mortality was the same (0.3 d^{-1}) at the extremes of the gradient of 38 and 131 PSU, respectively, and increased by approximately 6- and 13-fold at the intermediate salinities of 55 (1.7 d^{-1}) and 112 PSU (4.1 d^{-1}), respectively (Figure 2). The mortality rates observed at 38 and 131 PSU are similar to or smaller than those reported in studies only considering microzooplankton grazing, while at 55 and 112 PSU our estimates are generally higher (Calbet and Landry 2004; Grattepanche *et al*. 2011). More specifically, our total mortality estimates are similar to those reported in studies examining both grazing and lysis (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007; Brussaard *et al*. 2008b) at the extremes of the salinity gradient (38 and 112 PSU). In contrast, the mortality rates observed in the intermediate salinity range were larger, especially when compared to estimates obtained under bloom conditions (Evans *et al*. 2003; Baudoux *et al*. 2006).

When averaged across the Coorong grazing and lysis contributed almost equally to total phytoplankton mortality, with respectively 3.4 d^{-1} and 3.1 d^{-1} (Figure 2), indicating that viral lysis is an important phytoplankton loss factor in the Coorong. However, the relative contribution of grazing and lysis varied considerably with salinity (Figure 2), which was reflected in the amount of production lost to grazing and lysis at each site.

Specifically, at 38 PSU grazing and lysis were similar and mortality rates were consistent with values reported in the literature (Evans *et al*. 2003; Calbet and Landry 2004; Baudoux *et al*. 2006; Brussaard *et al*. 2008b; Grattepanche *et al*. 2011). At the intermediate salinities (55 and 112 PSU), virally-induced mortality did not significantly differ and were the highest of those observed in the Coorong (Figure 2). This is consistent with the highly productive nature of the Coorong and

the increased phytoplankton and viral abundance observed at theses salinities (Table 1).

Compared to a meta-analysis of the grazing literature (Calbet and Landry 2004), the mortality observed at 112 PSU due to grazing was strikingly high. While further work is needed to assess the the microzooplankton community composition and its relative contribution to phytoplankton mortality in the Coorong, we note, that phytoplankton mortality due to grazing is highly variable and mortality estimates exceeding 2 d^{-1} are not uncommon (Calbet and Landry 2004) especially in highly productive estuarine environments (Ruiz *et al*. 1998). In contrast to the high mortality rates observed at the intermediate salinity range, both 38 and 131 PSU showed a decrease in total mortality, with significant decreases in grazing and lysis (Figure 2). The non-significantly different contribution of grazing and lysis to phytoplankton mortality at 38 and 131 PSU (Figure 2) indicates that the activity of these three groups is tightly linked. The decrease in mortality at 131 PSU was unexpected as phytoplankton concentration was at a maximum and viruses were abundant (Table 1). The observed high phytoplankton abundance, hence the related small separation distances between cells, should have led to increased contact rates with both grazers and phytoplankton viruses. In turn this should have led to increased mortality, especially when compared to the lower salinities where phytoplankton concentration was much lower (Table 1).

The composition of the phytoplankton community at 131 PSU may hence have resulted in low mortality rates as blooming species can avoid grazing via cellular size alteration, colony formation or toxin production (Irigoien *et al*. 2005). Microzooplankton can also select specific preferred phytoplankton species which may not have been present at this site (Brussaard *et al*. 2008; Teixeira and Figueiras 2009), this site may have had an senescent phytoplankton community unappealing to grazers (Calbet *et al*. 2010) or some of the phytoplankton present

may have been resistant to viral infection (Lennon *et al*. 2007). As such the high biomass and low growth rate observed here is likely be the result of community of mortality resistant phytoplankton that has accumulated over a period of time.

Ford (2007) also speculated that high salinity may inhibit grazing in the Coorong and although grazing was reduced at 131 PSU, in comparison to 55 and 112 PSU, it was still an important loss factor contributing equally with lysis to mortality (Figure 2). This may indicate that while salinity might inhibit the growth and activity of some zooplankton other salinity tolerant species are actively predating upon phytoplankton at 131 PSU. Further investigation is, however, needed to determine the abundance and species of microzooplankton present at high salinities in the Coorong and their relationship to the phytoplankton community present in the Coorong.

More generally, the variability observed in mortality causes is related to complex interactions and behaviours between different members of the food web. These interactions lead to complexity in the feeding behaviour of grazers (Teixeira and Figueiras 2009) and potential shifts in the importance of grazing and lysis to phytoplankton mortality. As such, and as suggested from our observations, salinity cannot be used as a predictor for the fate of organic matter resulting from phytoplankton mortality, hence potential flow on effects throughout the food web resulting from changes in salinity are still difficult to determine.

Phytoplankton growth and production potential

Phytoplankton growth rates (*k*) reflected the trend observed in total mortality rates. Growth was the greatest in the intermediate salinity range, with the extremes in salinity showing little or negative growth (Figure 2, Table 3). The growth rates observed at the intermediate salinities exceeds the mean growth rate determined for

estuarine environments (Calbet and Landry 2004). However, these estimates were determined in waters of salinity lower than 55 PSU, hence as microbial abundance increases with salinity an increase in growth rate can be expected. Phytoplankton production lost due to grazing and lysis exceed the growth rate at all salinities with the greatest loss of biomass occurring at 112 PSU (Table 3), indicating that the phytoplankton communities present in the Coorong were in the end stages of a bloom. Whilst phytoplankton biomass is high, especially at greater salinities, and growth rate is in agreement with or exceeds estimates in other estuarine environments (Calbet and Landry 2004), dissolved nutrient concentrations were relatively low (Table 1) and appeared insufficient to support the high biomass and production potential (Table 1, Table 3). To sustain the high biomass observed, nutrients may be tightly cycled between their inorganic and organic forms or mortality may be consistently low in the Coorong due to reasons discussed above. The elevated potential phytoplankton production potential (P_p) observed at 112 and 131 PSU (Table 3) is a result of the high phytoplankton abundance observed at these salinities (Table 1) and in the case of 112 PSU a combined effect of abundance and elevated growth rate.

Microzooplankton vs. viral lysis

Traditional approaches to investigating phytoplankton mortality ignore the potential impact of viral lysis and focus only on microzooplankton grazing (Stelfox-Widdicombe *et al*. 2004; Nejstgaard *et al*. 2007; Calbet *et al*. 2010; Grattepanche *et al*. 2011). Whilst this issue is beginning to be addressed in the literature i.e. (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007; Kimmance *et al*. 2007; Brussaard *et al*. 2008b) there are still gaps in our knowledge as to the scale of viral action on phytoplankton communities, especially in natural ecologically important

and diverse environments such as the Coorong. As shown in the present work, viral lysis has a substantial impact on the phytoplankton community due to the fact that lysis often matches or exceeds mortality due to microzooplankton grazers (Figure 2, Table 3). This generalises to estuarine waters and hypersaline lagoons previous findings in marine waters (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007; Kimmance *et* al. 2007; Brussaard *et al*. 2008b). The present work also stresses the need to consider viral lysis in ecosystem studies to avoid systematic misunderstanding as to how the microbial community interacts with the herbivorous food chain, hence how aquatic ecosystems function. Neglecting the part of primary production that is not available to the herbivorous food chain through microzooplankton grazing would lead to (i) overestimate microzooplankton grazing, which in our work accounted for 24 to 64% of total phytoplankton mortality (Table 2), and (ii) underestimation of phytoplankton productivity (see Table 3). As such the omission of viral lysis in plankton dynamics would critically underestimate the role of primary production to the microbial loop, hence its impact on ecosystem energy and nutrient budget estimates (Evans *et al*. 2003).

SYNTHESIS

In summary, nutrient transfer in the environment through phytoplankton mortality is an integral part of global nutrient and energy cycling (Fuhrman 1999). As ecosystems over the world respond to climate change phytoplankton communities will be altered thus impacting upon the biogeochemical cycles they underpin. As such quantification and prediction of the fate of these communities are of vital importance. Although salinity is known to structure planktonic communities, in the present work we did not identify any direct link between salinity and the dominant phytoplankton mortality agent indicating that the mechanisms potentially linking

salinity and phytoplankton mortality may be more cryptic than initially thought, and require further investigation.

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Table 1 Environmental conditions, VLP abundance and particulate variables at each site (mean ± standard error).

	Site			
Parameter	**1**	**2**	**3**	**4**
Salinity (PSU)	37.6	54.8	112.3	131.4
pH	8.2	8.5	8.1	8.0
$[O_2]_d$ (mg L^{-1})	10.6	11.4	11.5	13.5
Turbidity (NTU)	12.0	4.0	18.0	19.7
Temperature (°C)	16.5	15.3	16.6	12.8
Chl *a* (µg L^{-1})	1.5 ± 0.1	3.4 ± 0.6	2.8 ± 0.5	12.2 ± 2.6
VLPs (× 10^7 mL^{-1})	6.8 ± 0.8	13.2 ± 0.4	31.2 ± 5.0	229.9 ± 44.7
Ammonium (µmol L^{-1})	5.5 ± 2.1	3.2 ± 1.4	<0.01 BDL	10.7 ± 4.5
Nitrite (µmol L^{-1})	0.04 ± 0.0	0.1 ± 0.1	<0.01 BDL	0.4 ±0.2

Nitrate (µmol L^{-1})	<0.01 BDL	0.5 ± 0.3	0.5 ± 0.5	0.5 ± 0.3
Phosphate (µmol L^{-1})	0.8 ± 0.2	3.5 ± 1.8	1.1 ± 0.3	8.4 ± 3.2
Silica (µmol L^{-1})	5.0 ± 0.9	1.4 ± 0.7	43.3 ± 3.1	48.9 ± 2.8
TSM (× 10^5µg L^{-1})	0.2 ± 0.0	0.6 ± 0.1	1.5 ± 0.5	1.8 ± 0.5
PIM (× 10^5 µg L^{-1})	0.1 ± 0.0	0.4 ± 0.1	1.2 ± 0.4	1.2 ± 0.4
POM (× 10^4 µg L^{-1})	0.5 ± 0.2	2.3 ± 0.4	3.6 ± 1.4	5.9 ± 1.8

Table 2 Total mortality rate (d^{-1}) and the relative percentage of mortality caused by grazing (M_g) and lysis (M_v).

Salinity (PSU)	Total M (d^{-1})	M_g (%)	M_v (%)
38	0.3	61.3	38.7
55	1.7	24.0	76.0
112	4.1	64.2	35.8
131	0.3	48.8	51.2

Table 3 Phytoplankton production loss due to microzooplankton grazing and viral lysis (P_g/P_v, µg L^{-1} d^{-1}), together with phytoplankton growth rate (k, µg L^{-1} d^{-1}) and potential production (P_p, µg L^{-1} d^{-1}).

	Production (µg L^{-1} d^{-1})			
Salinity (PSU)	**Grazed (P_g)**	**Lysed (P_v)**	***k* (µg L^{-1} d^{-1})**	***P_p* (µg L^{-1} d^{-1})**
38	0.03	0.02	-0.2	-0.03
55	1.6	5.2	1.4	5.4
112	12.1	6.8	3.45	15.8
131	2.3	2.4	0.7	10.4

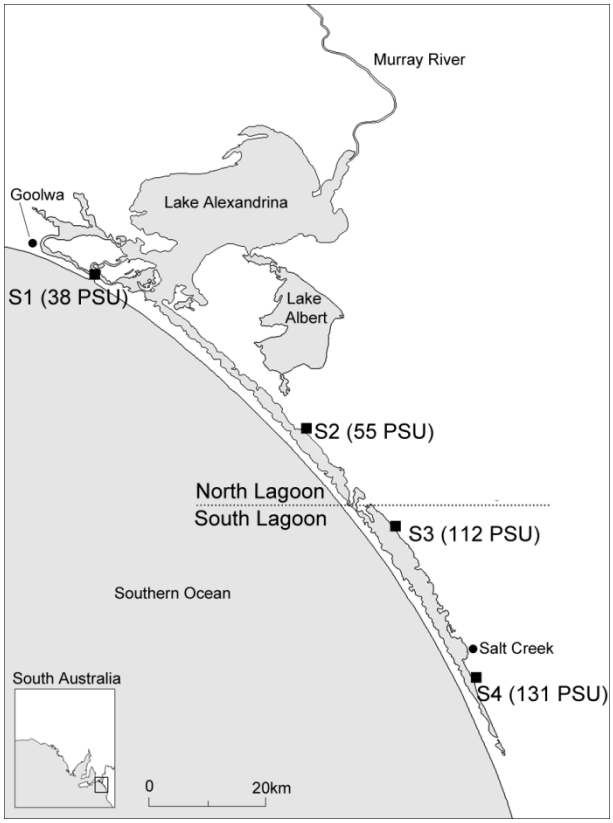

Figure 1 Study site; the Coorong (South Australia) showing the location and salinity (PSU) of the 4 sampling sites (■).

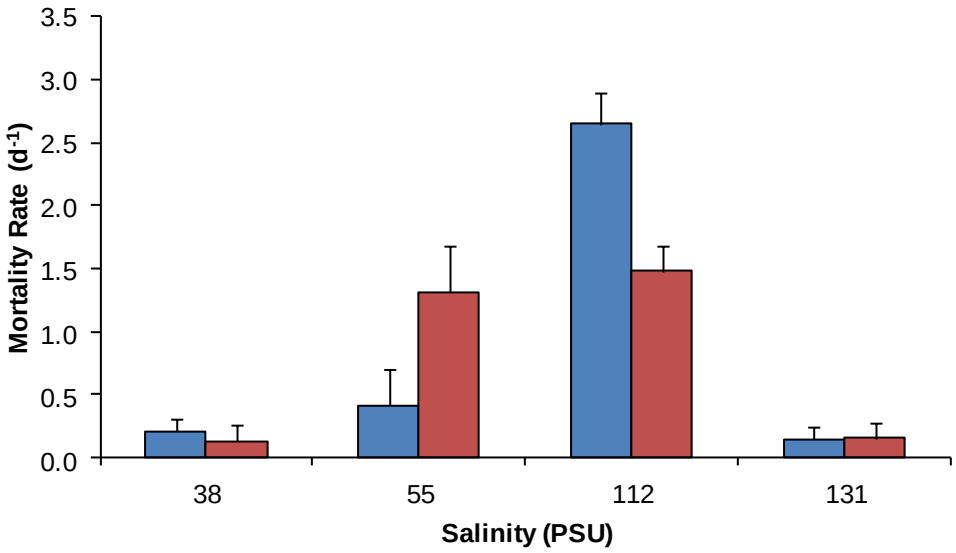

Figure 2 Phytoplankton mortality rates (d^{-1}); due to microzooplankton grazing ( ), viral lysis ( ); calculated using the modified dilution method of (Evans *et al*. 2003). Error bars are standard deviations of the mean.

Chapter IV

The Coorong Bacterial Metagenome Reveals Dramatic Shifts in Taxonomy and Function Driven by Riverine Input and Salinity

ABSTRACT

The Coorong estuary lies at the terminus of Australia's largest river system, the Murray-Darling; both are strongly influenced by human activity. We used metagenomic approaches to determine the planktonic bacterial community composition and potential function at two extremes in the Coorong, the river mouth which exhibits marine salinity, and the hypersaline upper-reaches of the estuary. Significant shifts in taxa and metabolic function were seen between the two sites. The river mouth exhibited an increase in abundance of *Rhodobacteriaceae* and *Alteromonadaceae*; families readily able to adapt to change in nutrient conditions; and the potentially pathogenic families *Brucellaceae*, *Enterobacteriaceae* and *Vibrionaceae*. Metabolisms over-represented include motility and chemotaxsis, RNA metabolism and membrane transport, all of which are involved in actively searching for and obtaining nutrients. Also over-represented were metabolisms involved in population succession and stress response. An over-representation of taxa and metabolisms indicative of environmental change is reflective of anthropogenically affected riverine input. In the hypersaline upper reaches of the estuary the halophilic family *Ectothiorhodospiraceae* was over-represented as were the families *Flavobacteriaceae*, *Cytophagaceae and Nocardioidaceae*, members of which are able to survive over a wide salinity range. Metabolisms over-represented here were reflective of increased bacterial growth, classic of hypersaline environments, and included DNA metabolism, nucleotide and nucleoside synthesis and cell cycle. When compared to other metagenomes the Coorong communities clustered with other marine environments, however they exhibited the least similarity both taxonomically and metabolically to the marine metagenomes they clustered with at 71 and 84 % similarity respectively. This indicates that Coorong exhibits a unique planktonic bacterial community that is influenced by riverine input at the river mouth and salinity in the upper-reaches.

INTRODUCTION

Aquatic ecosystems including estuaries, rivers, coastal embayments and wetlands are under increasing pressure due to anthropogenic stressors such as climate change, salinization, land clearing, decreased riverine flow and rising sea levels (Jolly *et al.* 2001; Kingsford *et al.* 2011). Estuaries in particular exhibit strong changes in physiochemical properties and are extremely productive ecosystems harbouring diverse wildlife and dynamic microbial communities (Ruiz *et al.* 1998; Henriques *et al.* 2006; Kan *et al.* 2007), as such the impact of anthropogenic changes on estuaries is of great importance. Aquatic bacterial communities present in estuaries underpin major biogeochemical cycles and influence the community at higher trophic levels (Azam and Malfatti 2007; Strom 2008) yet their taxonomic composition and metabolic function is structured by “bottom-up” physiochemical factors, such as salinity and nutrient concentration and availability (Yokokawa *et al.* 2004; Lozupone and Knight 2007). As such anthropogenic and natural processes resulting in changes in physiochemical dynamics impact greatly upon bacterial communities resulting in dramatic shifts in community structure and function which have flow on effects for aquatic environments.

The Coorong, South Australia, is an estuary, lagoon and lake system extending from the terminus of the Murray-Darling River system which exhibits large spatio-temporal shifts in salinity and nutrient concentrations along its length (Ford 2007). The Coorong is a Ramsar listed Wetland of International Importance and yet is in unprecedented poor condition (Mudge and Moss 2008). Previous research has demonstrated that aquatic and sediment bacterial community diversity and abundance in the Coorong is driven by salinity (Schapira *et al.* 2009; Jeffries *et al.* 2011; Jeffries *et al.* 2012), although for the sediment community substrate is the major structuring factor (Jeffries *et al.* 2011). This is consistent with previous research indicating salinity is a selective pressure governing global bacterial

distribution (Lozupone and Knight 2007). The identity and metabolic potential of the planktonic Coorong bacterial community has not yet been determined. As bacteria underpin major biogeochemical cycles, impact upon higher trophic levels and have flow-on effects for the entire ecosystem determining the identity and function of the community present and how anthropogenically riverine input and salinity effect the community is of upmost importance.

Recent advances in community profiling, specifically metagenomic approaches, have allowed for unprecedented characterisation of bacterial taxonomic composition and functional metabolic potential. In this context, the objectives of the present work were to determine shifts in bacterial taxonomic and metabolic diversity at two sites in the Coorong which exhibit marked difference in salinity from marine at the river mouth to hypersaline 100 kms to the south east in the upper reaches of the estuary.

MATERIALS AND METHODS

Study site

The Coorong is a shallow 2-3 km wide coastal estuarine and lagoon system extending 140 km South East from the terminus of the River Murray in South Australia (Lamontagne *et al.* 2004; Geddes 2005) (Figure 1). It is a reverse estuary and as such exhibits increasing salinity from marine at the River Mouth to hypersaline in the upper reaches of the southern lagoon. The large salinity gradient present is a result of interactions between sea level, tides, wind, rainfall, evaporation and riverine input (Webster 2005; Ford 2007). Temporal and spatial alterations in salinity and nutrient loading drive shifts in the abundance and flow-cytometric structure of the bacterial communities present (Ford 2007; Schapira *et al.* 2009).

Sampling strategy

Samples were collected from 2 sites exhibiting the extremes in salinity found in this system; marine (37 Practical Salinity Units, PSU) at the river mouth and hypersaline (106 PSU) near Salt Creek, in November 2009. At each site salinity, (PSU), temperature (°C), pH, dissolved oxygen (mg L^{-1}) and turbidity (Nephelometric Turbiditu Units, NTU) were recorded using a 90FL-T multiparameter probe (TPS Ltd.). Triplicate 60 mL samples to determine chlorophyll *a* (Chl *a*) concentration were collected and analysed as described by Schapira *et al*. (Schapira *et al.* 2009). Triplicate 120 mL samples were collected and analysed to determine total suspended matter (TSM), particulate inorganic matter (PIM) and particulate organic matter (POM) concentration as described by Barillé-Boyer *et al*. (Barillé-Boyer *et al.* 2003). Analysis of triplicate 100 mL samples for inorganic nutrient concentration was conducted using an LF 2400 photometer (Aquaspex®) and standard colorimetric methods for determining concentrations of ammonium (Indophenol Blue), Nitrite (Naphtylethylene diamine), nitrate (Naphtylethylene diamine after zinc reduction), phosphate (Ascorbic acid reduction) and silica (Heteropolyblue) as described by Schapira et al. (Schapira *et al.* 2009).

Microbial enumeration

Triplicate 1 mL samples for bacterial enumeration were fixed in 2% final concentration gluteraldehyde and stored as described by (Brussaard 2004). Prior to analysis samples were thawed in 50°C water, diluted in 0.2 µm filtered and autoclaved TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8) (1:10 dilution Site 1; and 1:100 Site 2), stained with SYBR-I Green solution (Molecular Probes, Eugene, Oregon; 1:20,000 dilution) and incubated at 80°C in the dark for 10 minutes (Brussaard 2004; Seymour *et al.* 2007). Samples were then processed using a FACSCanto flow cytometer (Becton-Dickson) with 1 µm fluorescent beads

(Molecular Probes, Eugene, Oregon) added to each sample at a final concentration of 10^5 beads mL^{-1} to determine bacterial and Virus-Like Particle (VLPs) abundance (Gasol and del Giorgio 2000). Bacteria and viruses were identified and enumerated using WinMDI 2.9 (© Joseph Trotter) according to variations in green fluorescence and side scatter (Marie *et al.* 1999a, b; Brussaard 2004).

Sample collection, microbial community DNA preparation and metagenomic sequencing

Microbial community DNA was extracted from 20 l of 5 µm gravity filtered and 100 kDa tangential flow filtration treated (Millipore™) sub-surface water using a bead beating and chemical lysis extraction procedure (UltraClean® Water DNA Isolation Kit; MO BIO laboratories, Inc) then further concentrated using ethanol precipitation. DNA was amplified using the strand-displacement Φ29 DNA polymerase (Genomiphi V2 kit; GE Healthcare Life Sciences, Inc) and further purified using a DNeasy blood and tissue kit (Qiagen) as described by Thurber *et al*. (2009). DNA quality and concentration was determined by agarose gel electrophoresis and spectrophotometry and approximately 5 µg of high molecular weight DNA from each site was then sequenced by the Australian Genome Research Facility (AGRF), St Lucia, Queensland. Sequencing was conducted on a GS-FLX pyrosequencing platform using Titanium series reagents (Roche).

Bioinformatics and statistical analysis

Unassembled DNA sequences were annotated using the MetaGenomics Rapid Annotation using Subsystem Technology (MG-RAST) pipeline version 2 (Meyer *et al.* 2008). BLASTX E-value cut-off was 1×10^{-5} and minimum alignment length was

50bp (Dinsdale *et al.* 2008). Taxonomic and metabolic profiles were generated using the normalised abundance of sequences matches to the SEED database (Overbeek *et al.* 2005). For this analysis taxonomic information was resolved to the genome level and metabolism was resolved to individual subsystems, groups of gene involved in a particular metabolic process (Overbeek *et al.* 2005).

To determine difference in bacterial taxonomic community structure rank abundance plots were generated. At each site the abundance of each family was normalised by overall number of sequences to give a proportion. Taxa rank was plotted on the *x*-axis and abundance on the *y*-axis, both $\log_{10}$ transformed. The data that produced the best fit then had a power law trend line regressed to those data, represented by the equation

$$y = ax^b \quad (1),$$

where *a* is the *y*-axis intercept and *b* is the slope. Significant difference between the slopes were then determined using the Student's *t* test (Zar 1996). The data not included in the power law regression, the noise/rare biosphere, is generally not included in analysis (Mitchell 2004) and a linear trend line was regressed to these data not included in the analysis.

Significant differences in taxonomic identity and metabolic function between the 37 and 106 PSU metagenomes were then determined by generating an abundance summary table of frequency of hits to individual taxa or subsystems for each metagenome. The abundance summary table was imported into the STastical Analysis of Metagenomic Profiles (STAMP) software package. STAMP normalises the data to remove bias in sequencing effort and read length by dividing by the total number of hits (Parks and Beiko 2010). Taxonomy was resolved to family level and metabolism was resolved to the second level of the MG-RAST metabolic hierarchy. Fisher's Exact test (Fisher 1958) was used to determine corrected P-values (q-

values), confidence intervals were calculated using the Newcombe-Wilson method (Newcombe 1998) and the false discovery rate was corrected for using the Benjamini-Hochberg FDR approach (Benjamini and Hochberg 1995). To ensure only the most significant differences are reported for taxonomy the data were filtered to a q-value cut-off value of $<1 \times 10^{-15}$.

Similarity between our metagenomes and 26 publically available metagenomes (Table 2) on the MG-RAST database was determined to organism and metabolic subsystem level 3 by first generating a heatmap of the frequency of MG-RAST hits to individual genomes or subsystems for each metagenome. This was normalised by dividing by the total number of hits to remove any potential bias created by sequencing effort or difference in read length. Hits were identified using an E-value cut-off of 1×10^{-3} to allow for difference in read length and sequencing effort. Statistical analysis was then determined using square root transformed frequency data using Primer 6 for Windows (Version 6.1.6, Primer-E Ltd. Plymouth) (Clarke and Gorley 2006). Bray-Curtis similarity relationships between our metagenomes and the 26 publically available metagenomes were analysed using hierarchical agglomerative clustering (CLUSTER) (Clarke 1993) and a dendogram obtained.

RESULTS

Coorong environmental properties

Sites were characterised by a strong difference in salinity from marine conditions of 37 PSU at the Murray Mouth to hypersaline conditions of 106 PSU at Salt Creek (Table 1). Microbial abundances were greatest at 106 PSU, heterotrophic bacterial abundance increased by 12 fold, VLPs increased by 10.6 fold and Chl *a* increased

by 12 fold (Table 1). TSM, PIM and POM were all increased at the hypersaline site while inorganic nutrient concentrations were low at both 37 and 106 PSU (Table 1).

Bacterial metagenomes

Metagenome libraries were obtained from 37 and 106 PSU in the Coorong. These libraries respectively yielded 70,353 and 47,910 reads with average read lengths of 324 and 323 bases, respectively. Both metagenomic libraries were dominated by bacteria with 99.65% and 98.75% hits to the SEED database, respectively (Table 1). Other domains represented in lower abundance were; archaea accounting for 0.06% and 0.39%, eukaryote 0.26% and 0.81% and viruses 0.03% and 0.05% of hits respectively. Within the 37 and 106 PSU metagenomic libraries, a total of 5% and 7% could not be assigned to any known sequence in the database.

Shift in taxonomic profile with salinity

At 37 PSU, *Proteobacteria* dominated, (84.42%) with a lesser contribution from *Bacteroidetes* (11.35%) (Figure 2). Of the *Proteobacteria*, *Alpha-* and *Gammaproteobacteria* contributed to 50.26% and 44.49% of sequences, respectively, while the *Bacteroidetes* were dominated by *Flavobacteria*, with 76.86% of sequences. At 106 PSU *Actinobacteria* dominated (37.09%), with lesser contributions from *Bacteroidetes* (30.16%) and *Proteobacteria* (25.88%) (Figure 2). Of the *Actinobacteria*, *Actinomycetales* represented 92.03% of sequences, *Proteobacteria* were dominated by *Alpha-* and *Gammaproteobacteria* contributing 26.8% and 52.8% of sequences respectively. *Bacteroidetes* were dominated by *Flavobacteria*, with 70.3% of database hits. Analysis of the slope of the power law fits to rank abundance of bacterial families revealed a significant change in bacterial

community structure between 37 and 106 PSU (Figure 3) ($p < 0.001$). The slope of the trend line at 37 PSU is steeper than that at 106 PSU indicating that there are a few dominant taxa at 37 PSU but overall richness and diversity are greatest at 106 PSU (Magurran 2004).

STAMP was used to investigate significant shifts in abundance of specific taxa between the bacterial communities present at 37 and 106 PSU. At 37 PSU there was an over-representation of *Proteobacteria* (Figure 4A), specifically *Alpha*- and *Gammaproteobacteria* (data not shown). The alphaproteobacterial family *Rhodobacteraceae* and the Gammaproteobacterial families *Enterobacteriaceae* and *Vibrionaceae* were over-represented by 24, 8 and 4.5% respectively (Figure 4B). Interestingly, there were numerous other families marginally over-represented by 2.3 ± 0.3% including the Alphaproteobacterial family *Brucellaceae* and the Gammaproteobacterial families *Pseudoalteromonadaceae* and *Alteromonadaceae*.

At 106 PSU there was an over-representation of *Bacteroidetes* and *Actinobacteria* (Figure 4A); specifically the families *Flavobacteriaceae* and *Microbacteriaceae* were over-represented by 13 and 8% respectively (Figure 4B). Again there was a small over-representation of other families of 1.7 ± 0.08%, including the *Bacteroidetes* families *Nocardioidaceae*, *Rhodothermaceae*, *Cytophagaceae* and the *Actinobacteria* families *Micrococcaceae* and an unclassified family (Figure 4B). Additionally, while the phyla *Gammaproteobacteria* was not over-represented at 106 PSU the gammaproteobacterial family *Ectothiorhodospiraceae* was.

Shifts in metabolic potential with salinity

Both metagenomes were dominated by the core metabolic functions of carbohydrate, amino acid and protein metabolism (Figure 5). Clustering based

subsystems, comprising groups of genes that perform related functions, were also predominant and comprised around 15% of all metabolic genes at both salinities. At 37 PSU these clustering based subsystems were dominated by fatty acid metabolism (6.6%) and bacterial cell division (5.0%) while at 106 PSU they were dominated by a clustering subsystems category (4.7%), which was in turn were dominated by DNA polymerase III alpha subunit (47%), and bacterial cell division (3.9%) (data not shown).

Significant shifts in the metabolic potential of each bacterial community were further investigated using STAMP to the first, second and third levels of MG-RAST metabolic hierarchy. Genes involved in cellular response to stress, specifically oxidative stress, were over-represented at 37 PSU as were processes involved in RNA processing and modification. Sequences responsible for searching, obtaining and utilisation of nutrients were also over-represented. These included the processes of metabolism of a variety of compounds including aromatic compounds, cofactors, vitamins, prosthetic groups and pigments, di- and oligosaccharides, organic acids, fatty acids, nitrogen and sulphur metabolism. Interestingly Motility, chemotaxis and membrane transport, specifically Ton and Tol transport systems, were also over-represented however the difference between proportions was approximately 0.2 % (Figure 6A-B).

At 106 PSU sequences related to increased bacterial growth and reproduction including the core metabolic functions of DNA metabolism; nucleoside and nucleotide synthesis; cell wall and capsule; protein metabolism; amino acid biosynthesis; carbohydrate metabolism; and clustering based subsystems involved in the biosynthesis of the cell wall components galactoglycans and lipopolysacharides were over-represented. Other sequences involved in bacterial growth and functioning over-represented by a smaller percentage included; cell division and cell cycle; and regulation and cell signalling. Sequences for bacterial

resistance to virulence, disease and defence, specifically resistance toxic compounds were also over-represented, while sequences for iron acquisition and metabolism; photosynthesis; and dormancy and sporulation genes were also over-represented however the difference between proportions was again reduced (Figure 6A-B).

Comparison of planktonic Coorong taxonomic and metabolic profiles to profiles from other habitats

The taxonomic and metabolic potential of our metagenomes were compared to 26 publically available metagenomes form a variety of aquatic habitats, including fresh, marine and hypersaline waters and Coorong sediment (Table 2). Coorong metagenomes were obtained from sites with vast differences in salinity, biomass and inorganic nutrient content (Table 1) and as such shared only 71 and 84% similarity to each other taxonomically and metabolically (Figure 7, Figure 8). Taxonomically and metabolically planktonic Coorong metagenomes from a discrete cluster with metagenomes from other marine, freshwater and estuarine environments with > 65 and 84 % similarity respectively (Figure 7, Figure 8). While metagenomes from solar salterns of varying salinity, Coorong sediment and anthropogenically affected environments clustered seperatly. Within the cluster that includes planktonic Coorong metagenomes, taxonomically the 106 PSU metagenome showed greatest similarity to the other marine, freshwater and estuarine metagenomes (Table 2) than the 37 PSU metagenome (Figure 7), while the converse was observed for metabolism (Figure 8). Metagenomes from salterns with similar salinities to our samples, an anthropogenically affected coral reef, an aquiculture pond and samples obtained from Coorong sediment (Table 2) do not

cluster with planktonic Coorong metagenomes either taxonomically or metabolically (Figure 7, Figure 8).

DISCUSSION

The Coorong is a dynamic ecosystem that exhibits large increases in physiochemical parameters, microbial abundance and productivity from the river mouth to the upper reaches (Ford 2007) (Table 1). Both the River Murray and the Coorong are currently threatened by anthropogenic and climate influences (Lester and Fairweather 2009; Kingsford *et al.* 2011). In the lower reaches of the river water quality is poor with irrigation return waters from dairy farms likely to be responsible for increased nutrient loads and faecal coliform abundance (Cugley *et al.* 2002). Inflows into the river have been greatly decreased by extended drought and water diversion resulting in the need for constant dredging since 2002 (Kingsford *et al.* 2011). In the upper-reaches of the Coorong hypersalinity is caused by reduced inflow, seawater exchange, rainfall, groundwater drains and barrages separating the Coorong from the Lower Lakes (Webster 2005; Ford 2007).

Planktonic Coorong taxa shift with salinity

Salinity drives bacterial community diversity (Bouvier and del Giorgio 2002; Wu *et al.* 2006; Lozupone and Knight 2007; Tamames *et al.* 2010) and governs global bacterial distributions (Lozupone and Knight 2007). Here, we show that both salinity and riverine input play a role in shaping the bacterial community and its functional potential at two extremes of salinity in a coastal lagoon/estuary. A shift in the dominant taxa was observed between 37 and 106 PSU confirming previous observations in the Coorong describing greater diversity in bacterial flow cytometric

signatures with increased salinity (Schapira *et al.* 2009). Analysis of the rank abundance of bacterial families at 37 and 106 PSU suggests that 37 PSU is dominated by a few highly abundant taxa while 106 PSU has increased bacterial richness and greater diversity. This is reflected in the STAMP analysis which shows a greater number of families over-represented marginally at 106 PSU while only a few families are greatly over-represented at 37 PSU (Figure 4B). Coarse grain analysis highlights that the phyla *Proteobacteria* and *Bacteroidetes* were highly abundant at both 37 and 106 PSU (Figure 2) however there were statistically significant shifts in abundance of specific families which drove dissimilarity between the metagenomes (Figure 4A-B). We highlight that generally it is only over-representation of a few families that drive over-representation of an entire phyla (Figure 4A-B). While many marine bacteria phyla are ubiquitous, some families are niche specialists (Reen *et al.* 2006) and as such, finer levels of taxonomic resolution should be investigated when examining taxonomic shifts in planktonic environments.

Several *Alpha-* and *Gammaproteobacteria* families were over-represented at 37 PSU (Figure 4B), these classes are ubiquitous in marine environments and contain many of the most abundant marine species (Biers *et al.* 2009; Brindefalk *et al.* 2011). Consequently many families over-represented at 37 PSU are ubiquitous in the marine environment and have been found in other marine metagenomic studies (Jones and Betaileb 1986; Ivanova *et al.* 2004; DeLong *et al.* 2006; Reen *et al.* 2006; Allers *et al.* 2007; Biers *et al.* 2009; Brindefalk *et al.* 2011). A strong riverine influence was observed at 37 PSU where the *Rhodobacteriaceae* and *Alteromonadaceae* were over-represented. These families are known to respond quickly to allochthonous nutrient input and their over-representation is likely be an indication of disturbance events in coastal surface waters (Allers *et al.* 2007). Members of these families plus the *Pseudoalteromonadaeceae* respond to increased nutrients regardless of concentration (Nogales *et al.* 2011). This coupled

with the over-representation of *Brucellaceae* which are generally associated with the soil microbiota (Garrity *et al.* 2005) and several potentially pathogenic families, *Brucellaceae*, *Enterobacteriaceae* and *Vibrionaceae* (Jones and Betaileb 1986; Garrity *et al.* 2005; Reen *et al.* 2006) that have previously been associated with anthropogenic stress (Dinsdale *et al.* 2008; Nogales *et al.* 2011) indicate that the community composition at 37 PSU is heavily influenced by the increase in faecal coliforms, nutrient loads and other pollutants present in terrestrial runoff (Cottrell *et al.* 2005; Hill *et al.* 2005) in the lower reaches of the River Murray (Cugley *et al.* 2002) and riverine input has a significant impact upon the community composition.

Increased salinity at 106 PSU resulted in the over-representation of halophilic families including: the mainly halophilic *Ectothiorhodospiraceae* (Imhoff and Süling 1996; Tourova *et al.* 2007); *Flavobacteriaceae*, *Cytophagaceae* and *Nocardioidaceae* which include known halophillic members (Dobson *et al.* 1993; Lee *et al.* 2008); and the *Rhodothermaceae* whose members are taxonomically similar to the hypersaline genera *Salinibacter* (Pășic *et al.* 2009) (Figure 4B). The *Bacteroidetes* were also over-represented at 106 PSU, this phyla is diverse and widespread in marine environments (Rusch *et al.* 2007; Stevens *et al.* 2007; Tamames *et al.* 2010), present in hypersaline salterns (Benlloch *et al.* 2002) and increase in productive systems (Alonso-Sáez *et al.* 2007) with *Flavobacteriaceae* major contributors to mineralisation of primary-produced organic matter (Bowman and Nichols 2005). As a result over-representation is a likely due to increased phytoplankton biomass at 106 PSU (Table 1).

Actinobacteria are a diverse group of degraders common in soil and marine sediment (Magarvey *et al.* 2004; Bull *et al.* 2005; Stach and Bull 2005) and were originally thought to translocate to marine environments via terrestrial run off (Bull *et al.* 2005). Over-representation at 106 PSU of the families *Microbacteriaceae*, *Micrococcaceae* and unclassified *Actinobacteria* confirms recent evidence that

Actinobacteria are resident members of aquatic communities (Han *et al.* 2003; Cottrell *et al.* 2005; Rusch *et al.* 2007; Stevens *et al.* 2007) and hypersaline environments (Ghai *et al.* 2011).

Metabolic function in the Coorong

Metabolisms over-represented at 37 PSU indicate the river mouth is highly competitive environment and bacteria must exhibit the ability to utilise many metabolic pathways in order to exploit changing nutrient conditions. Metabolic processes over-represented at 37 PSU related to the metabolism and degradation of a variety of nutrient sources including sugars, fatty-acids, urea and aromatic compounds (Figure 6A-B). Processes relating to prosthetic groups were also over-represented including, tetrapyrrole derivatives responsible for chlorophyll, niacin, cobalamin and coenzyme B12 synthesis (Figure 6A-B). Prosthetic groups perform a wide range of functions (Raux *et al.* 2000), B12 in particular is postulated to be associated in population succession (Starr *et al.* 1957) supporting evidence for the over-representation of taxa that respond quickly to and are able to exploit changes in nutrient concentrations seen at 37 PSU (Figure 4A-B).

Increased nutrient acquisition by highly responsive taxa at 37 PSU results in the creation of reactive oxygen species through the processes of nutrient oxidation, metabolism and cellular respiration (Cabiscol *et al.* 2000) and is likely to be responsible for the over-representation of cellular responses to oxidative stress, specifically glutathione regulation which is a major component of the oxidative stress response (Klatt and Lamas 2000). Polyhydroxybutyrate metabolism was also over-represented and has been associated with increased bacterial oxidative stress tolerance environments subject to change (Ayub *et al.* 2004). To compete successfully for nutrient resources efficient nutrient uptake is required, this is

achieved through over-representation of tRNA modification processes (Figure 4A-B) in bacteria and archaea, these processes increase both gene regulation, expression and metabolic control resulting in more efficient nutrients uptake (Persson 1993). Membrane transport processes including Ton and Tol transport systems over-represented also indicate active bacterial community nutrient uptake. Behavioural adaptations indicative of increased nutrient competition were also over-represented including flagellar production and chemotaxis related metabolisms (Figure 6A-B). Flagella and chemotaxis assist bacteria to successfully acquire nutrients, avoid predators and other potentially detrimental situations (Blackburn *et al.* 1998; Kiørboe and Jackson 2001; Pernthaler 2005).

Hypersaline aquatic environments are characterised by an increase in microbial abundance (Guixa-Boixereu *et al.* 1996) consequently the majority of course and fine grain metabolic processes over-represented at 106 PSU were related to bacterial reproduction, growth and cellular activity (Figure 4A-B). An increase in *Actinobacteria* may also be related to over-representation of genes related to dormancy and sporulation and resistance to toxic compounds (specifically cobalt zinc and cadmium). Marine *Actinobacteria* are known to form spores (Mincer *et al.* 2005) and halophilic heavy metal tolerant *Actinobacteria* able to adapt to extreme conditions have been described previously (Nieto *et al.* 1989; Sarikhan *et al.* 2011). Metabolic processes related to increased viral abundance including phage replication and phage plasmid machinery were also over-represented, consistent with increased viral abundance (Table 1, Figure 6A). Interestingly, it is important to note that while some bacterial families with halophilic members were over-represented at 106 PSU (Figure 4A-B) generalised metabolic processes related to osmotic stress were not over-represented and only a small over-representation in the synthesis of the osmoprotectant betaine was observed (Roberts 2005). This pathway was also over-represented in hypersaline Coorong sediment in an earlier

study (Jeffries *et al.* 2012) and indicates that as bacteria move into an environment they readily adapt to the prevalent conditions (Herlemann *et al.* 2011).

Taxonomic and metabolic similarity to other metagenomes

Although planktonic Coorong metagenomes were sourced from two very different salinities in the system, they were taxonomically and metabolically most similar to metagenomes from other marine, freshwater and estuarine environments. However taxonomically the 106 PSU metagenome was most similar to this cluster while metabolically the 37 PSU metagenome was most similar (Figure 7, Figure 8). Both metagenomes consistently had deeper branching nodes and generally were the least similar to other metagenomes in the cluster. This indicates that while the planktonic Coorong community is similar to marine, freshwater and estuarine communities it is a unique aquatic environment as a result of biogeochemical properties and as such selects for a divergent bacterial community structure which exhibits functions specific to survival in this system.

Notably metagenomes from steady state, unconnected, sediment or anthropogenically influenced environments consistently clustered together, separate from the marine, freshwater and estuarine clade containing planktonic Coorong metagenomes (Table 2). The exception being aquifer metagenomes which clustered taxonomically with this clade yet were most dissimilar (Figure 7, Figure 8). That planktonic and sediment Coorong metagenomes are dissimilar and cluster separately is expected given that substrate type is important in structuring microbial communities (Lozupone and Knight 2007; Jeffries *et al.* 2011) and indicates little transference of taxa between water and sediment and different metabolic potential. Lack of environmental connectivity also appears to be an important influence on microbial communities. Lower metabolic rates in aquifers are thought to be a result

of reduced connectivity (Chapelle and Lovley 1990), here aquifer and other unconnected metagenomes show least metabolic similarity to the marine/freshwater/estuarine metagenomes. Further evidence for this is seen in the dissimilarity of saltern and planktonic Coorong metagenomes which while similar in salinity, have markedly different environmental connectivity. The Coorong receives rainfall, riverine and marine input (Geddes 2005) while saltern are steady state systems (Guixa-Boixereu *et al.* 1996) controlled within strict biochemical limits (Rodriguez-Brito *et al.* 2010) whose communities decrease in diversity with increased salinity driving metagenomic dissimilarity (Benlloch *et al.* 2002). Anthropogenic influences have previously been shown to be important in structuring bacterial communities and their functions (Dinsdale *et al.* 2008; Nogales *et al.* 2011) and in conjunction with extremes in biogeochemical conditions in the Coorong likely explain why planktonic Coorong metagenomes consistently show the least similarity to other marine/freshwater/estuarine environments.

SYNTHESIS

Here we show the Coorong exhibits a unique bacterial consortium which is most similar taxonomically and metabolically to other marine communities despite the large differences in physio-chemical properties between the two sampling sites. Significant differences in taxonomic composition are driven by anthropogneically effected riverine input and salinity, while metabolic differences are reflective of a highly adaptive community able to exploit varied nutrient sources and salinity driven increased biomass in the upper reaches of the estuary.

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Table 1 GPS position; sequencing data; environmental conditions; bacterial and VLP abundance (mean ± standard error); and particulate variables (mean ± standard error) at each site.

Parameter	**Site 1**	**Site 2**
GPS position (South)	35° 32.974'	36° 09.806'
GPS position (East)	138° 52.977'	139° 38.963'
Number of reads	70,353	47,910
Average read length (bp)	324	323
% sequences matching SEED sub-systems	94.8	92.6
Salinity (PSU)	37.3	105.8
Temperature (°C)	23.4	19.2
pH	8.2	8.1
$[O_2]_d$ (mg L^{-1})	10	9.7
Bacteria (× 10^6 mL^{-1})	7.6 ± 0.7	90.2 ± 2.4
VLPs (× 10^7 mL^{-1})	2.9 ± 0.3	30.7 ± 8.9
Chl *a* (µg L^{-1})	0.1 ± 0.01	1.2 ± 0.2
TSM (× 10^4µg L^{-1})	1.0 ± 0.6	11.1 ± 10.4
PIM (× 10^4 µg L^{-1})	0.6 ± 0.5	8.5 ± 8.2
POM (× 10^4 µg L^{-1})	0.4 ± 0.1	2.7 ± 2.3
Ammonium (µmol L^{-1})	0.4 ± 0.2	0.4 ± 0.1
Nitrite (µmol L^{-1})	< 0.01BDL	0.003 ± 0.03
Nitrate (µmol L^{-1})	0.01± 0.01	0.003 ± 0.003

Phosphate (µmol L^{-1})	0.1 ± 0.1	0.1 ± 0.03
Silica (µmol L^{-1})	< 0.1 BDL	3.4 ± 0.4

Table 2 Summary of publically available metagenomes used in this study. All metagenomes are sourced from aquatic environments unless otherwise specified.

MG-RAST ID	Description/Reference	Environment	Reference
4446411.3	Coorong surface water, 37 PSU	Marine – Coorong	This study
4446410.3	Coorong surface water, 106 PSU	Hypersaline - Coorong	This study
4440984.3	Coorong sediment, 37 PSU	Marine sediment - Coorong	Jeffries *et al*. 2011
4441020.3	Coorong sediment, 109 PSU	Hypersaline sediment - Coorong	Jeffries *et al*. 2011
4441022.3	Coorong sediment, 132 PSU	Hypersaline sediment - Coorong	Jeffries *et al*. 2011
4441022.3	Coorong sediment, 136 PSU	Hypersaline sediment - Coorong	Jeffries *et al*. 2011
4453064.3	Unconfined aquifer	Fresh - Groundwater	Smith *et al*. 2012
4453083.3	Confined aquifer	Fresh - Groundwater	Smith *et al*. 2012
4441590.3	East Australian current 1(EAC1)	Marine	Seymour *et al*. 2012

4441593.3	East Australian current 2 (EAC2)	Marine	Seymour *et al*. 2012
4446407.3	East Australian current 3 (EAC3)	Marine	Seymour *et al*. 2012
4446707.3	East Australian current 4 (EAC4)	Marine	Seymour *et al*. 2012
4446457.3	Botany Bay 1	Marine	
4446406.3	Botany Bay 2	Marine	
4440437.3	Low Salinity Saltern 1	Low Salinity Saltern cf marine PSU	Rodriguez-Brito *et al*. 2010
4440324.3	Low Salinity Saltern 1	Low Salinity Saltern cf marine PSU	Rodriguez-Brito *et al*. 2010
4440426.3	Low Salinity Saltern 3	Low Salinity Saltern cf marine PSU	Rodriguez-Brito *et al*. 2010
4440435.3	Med Salinity Saltern	Medium Salinity Saltern cf 106 PSU	Rodriguez-Brito *et al*. 2010
4440041.3	Kiririmati	Marine	Dinsdale *et al*. 2008
4440440.3	Aquiculture pond	Fresh	
4441590.3	Lake GS020	Fresh	Rusch *et al*. 2007

4441584.3	Estuary GS012	Estuary	Rusch *et al*. 2007
4441599.3	Lagoon GS033	Marine cf 46 PSU	Rusch *et al*. 2007
4441598.3	Mangrove GS032	Marine	Rusch *et al*. 2007
4441613.3	Coastal GS117a	Marine	Rusch *et al*. 2007
4441595.3	Coastal GS027	Marine	Rusch *et al*. 2007
4441610.3	Coastal GS113	Marine	Rusch *et al*. 2007
4441606.3	Coastal GS108a	Marine	Rusch *et al*. 2007

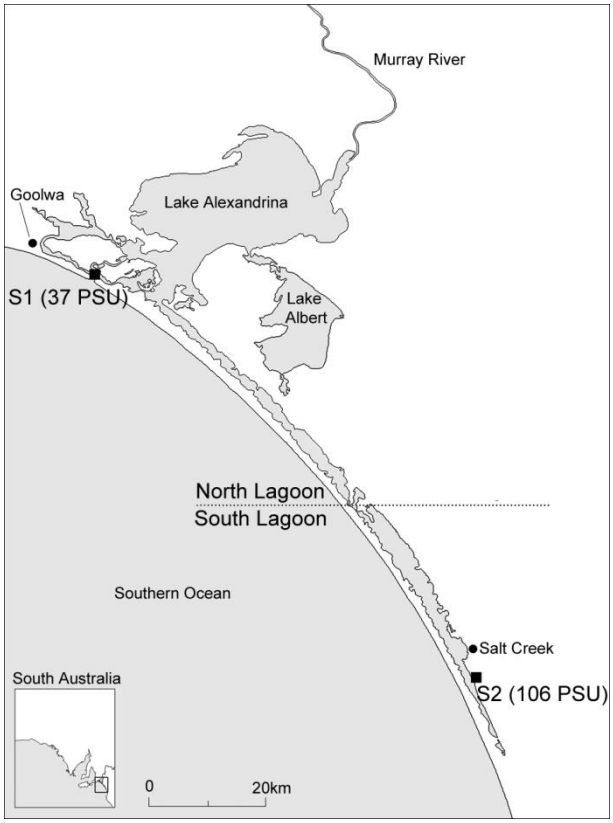
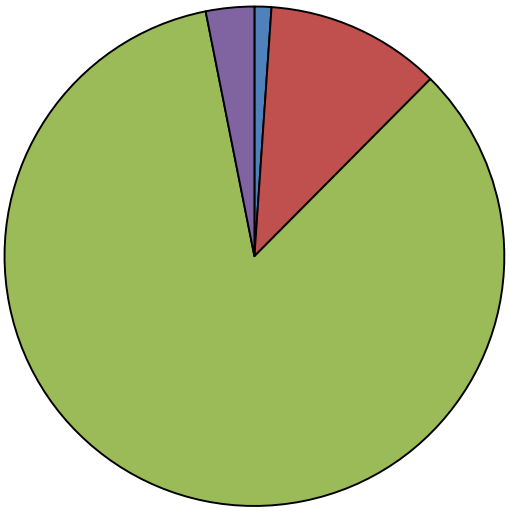
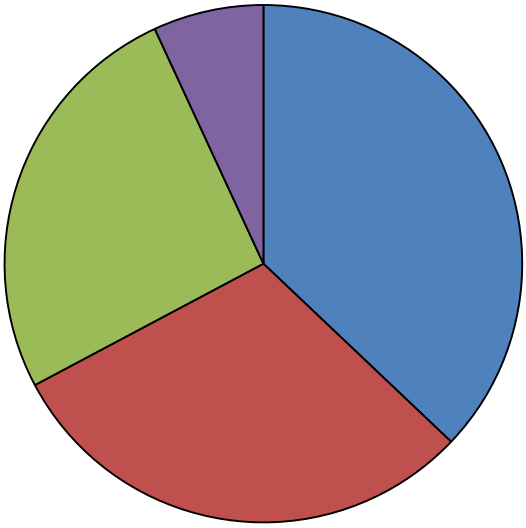


Figure 1 Study site; the Coorong (South Australia, Australia) showing the location and salinity of the 2 sampling sites (■).



A



B

Figure 2 Phylogenetic composition of bacterial metagenomes from 37 (A) and 106 (B) PSU. Normalised abundance of sequences matches to the SEED database.

Actinobacteria; *Bacteroidetes*; *Proteobacteria*; Other

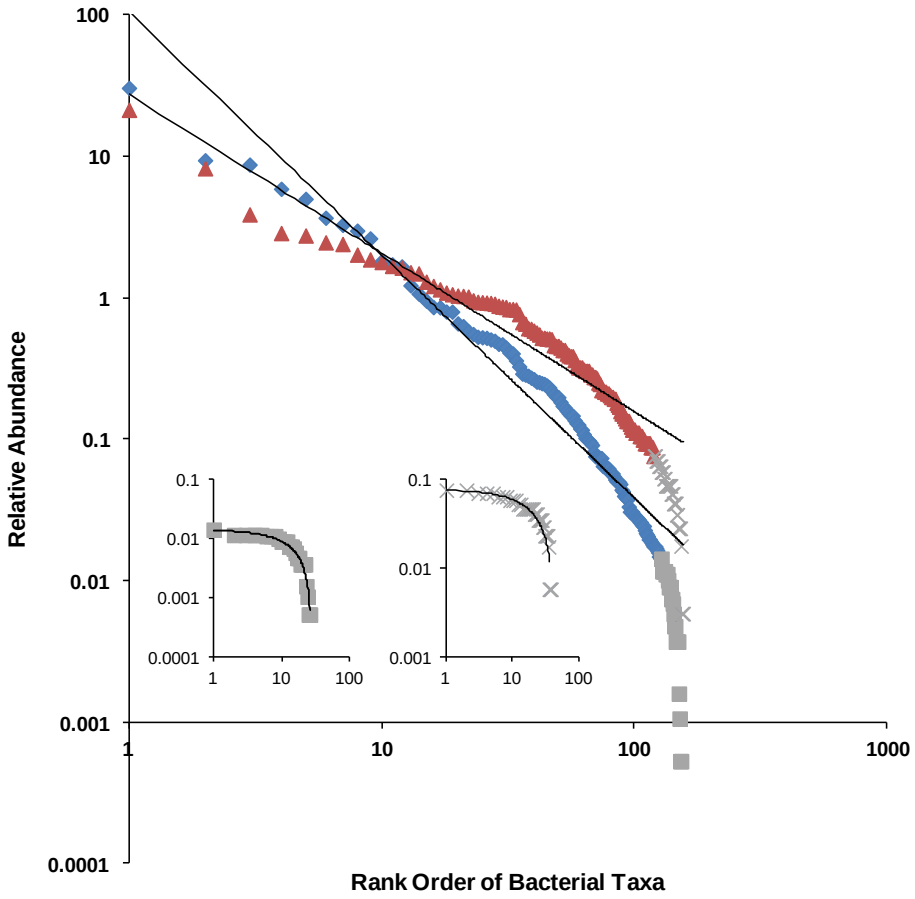
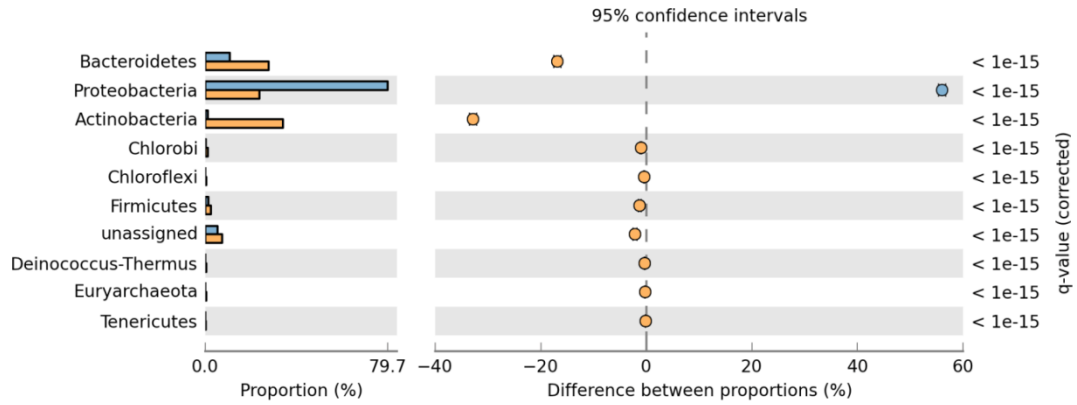
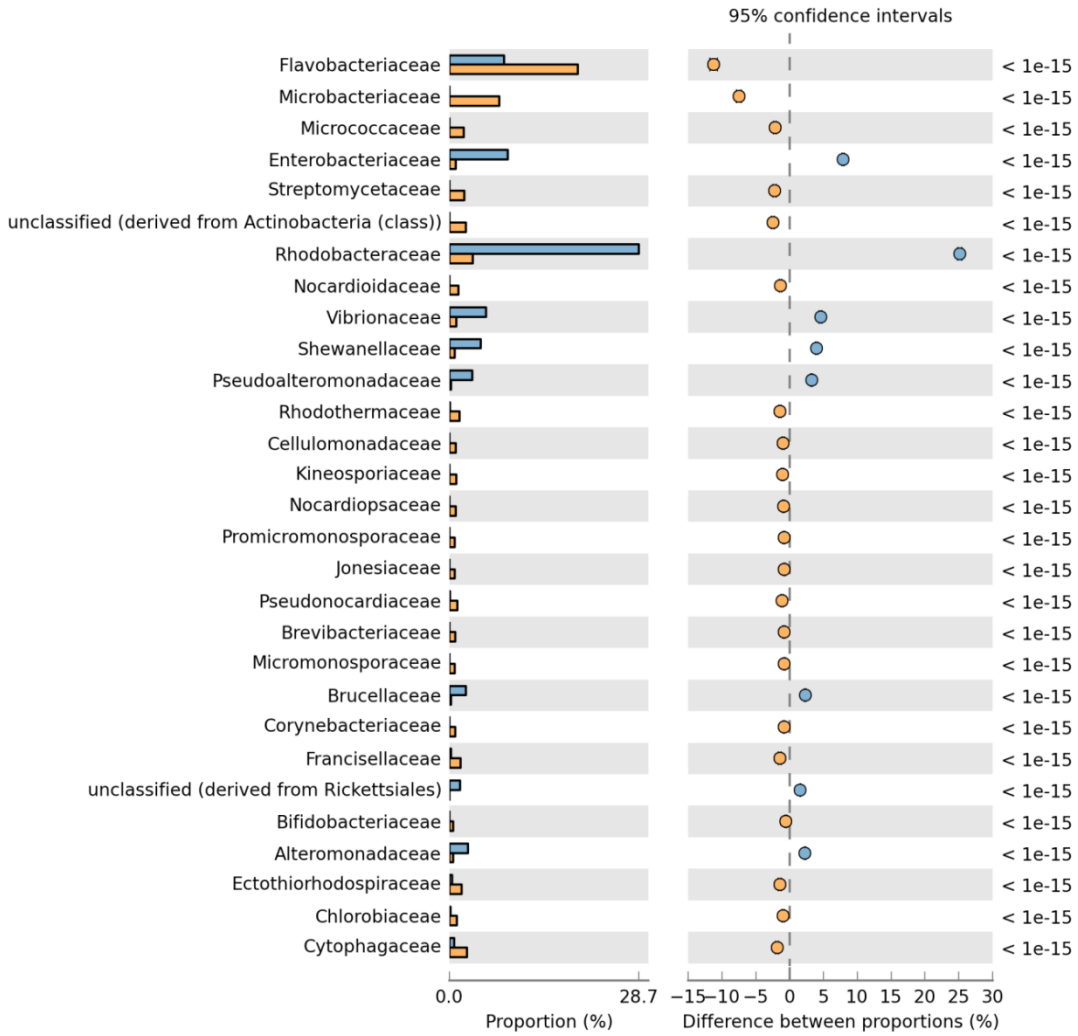


Figure 3 Rank abundance curves of bacterial community family structure showing power law trend lines at 37 PSU (◆; $y = 104.8x^{-1.7}$, $R^2 = 0.96$) and 106 PSU (▲ ; $y = 27.1x^{-1.1}$, $R^2 = 0.94$). Inserts show the rare biosphere with linear trend-lines, at 37 PSU (■; $y = -0.001x + 0.014$, $R^2 = 0.97$) and 106 PSU (X ; $y = -0.002x + 0.077$, $R^2 = 0.97$); y-axis shows relative abundance; x-axis shows rank order of bacterial taxa.


95% confidence intervals
Bacteroidetes
Proteobacteria
Actinobacteria
Chlorobi
Chloroflexi
Firmicutes
unassigned
Deinococcus-Thermus
Euryarchaeota
Tenericutes
< 1e-15
q-value (corrected)
0.0
79.7
Proportion (%)
−40
−20
0
20
40
60
Difference between proportions (%)

A



B

Figure 4 Phyla (A) and families (B), significantly different in abundance between 37 PSU and 106 PSU.

● taxa overrepresented at 37 PSU; ● taxa overrepresented at 106 PSU.

A further 43 families were over-represented at $<1 \times 10^{-15}$ however these families exhibited <1% difference between proportions and were removed for clarity.

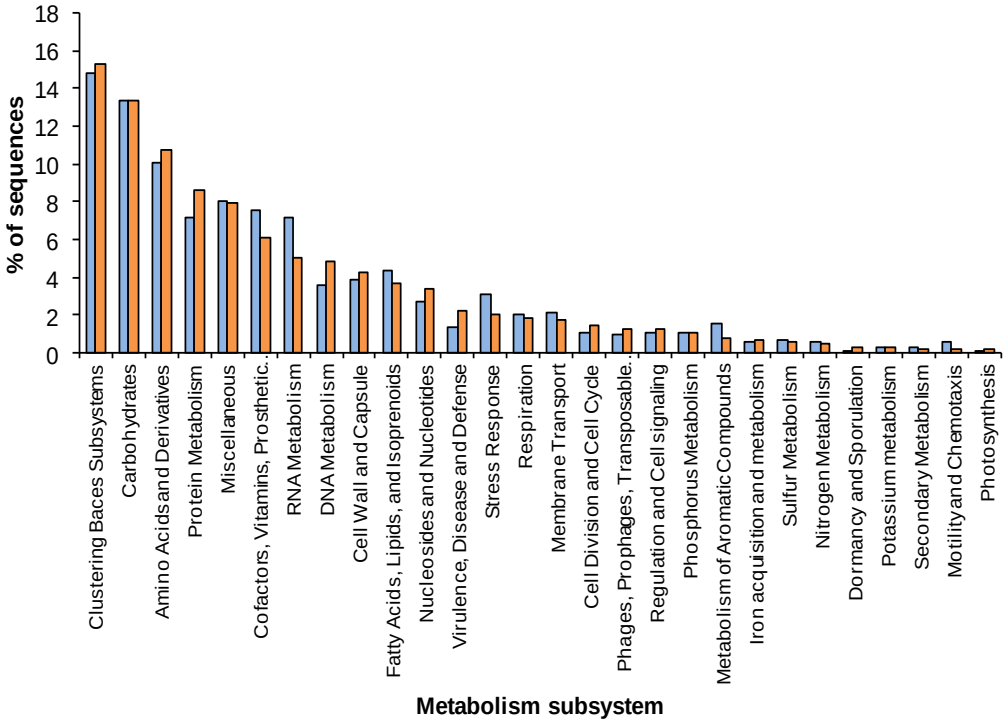
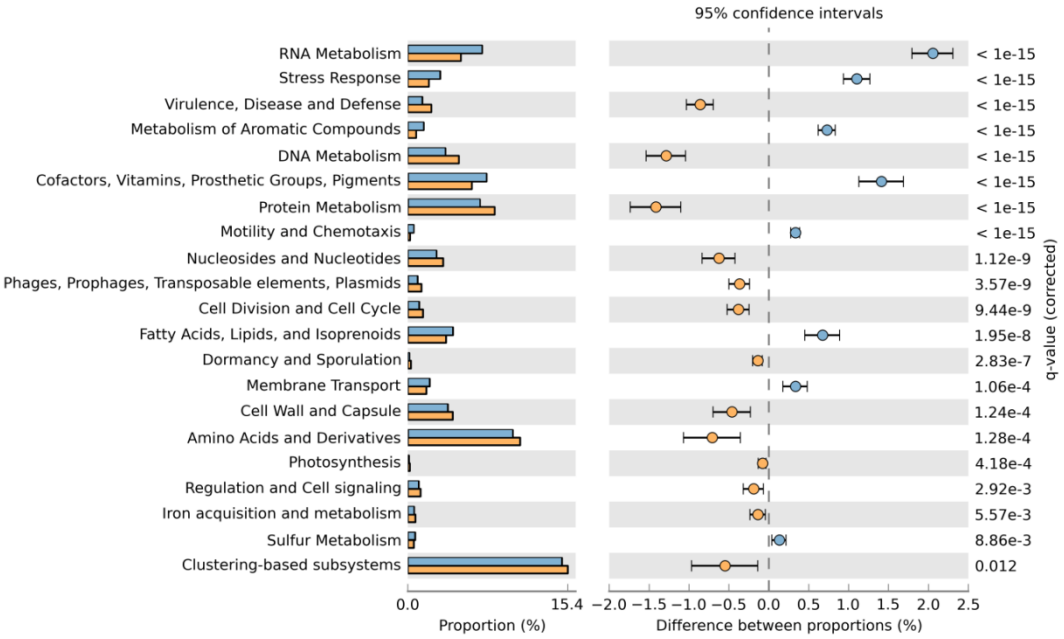
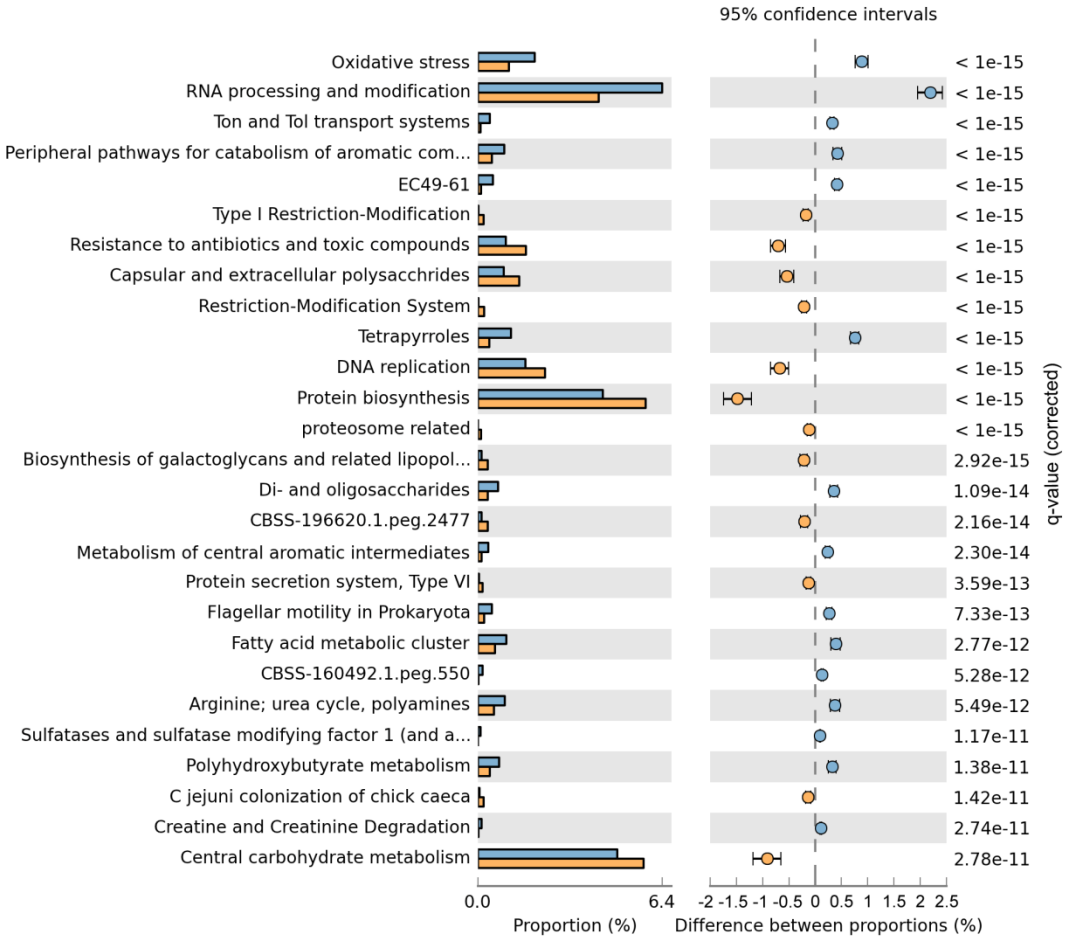


Figure 5 Metabolic potential of bacterial metagenomes from 37 ( ) and 106 ( ) PSU. Normalized abundance of sequences matches to the SEED database.


95% confidence intervals
RNA Metabolism
Stress Response
Virulence, Disease and Defense
Metabolism of Aromatic Compounds
DNA Metabolism
Cofactors, Vitamins, Prosthetic Groups, Pigments
Protein Metabolism
Motility and Chemotaxis
Nucleosides and Nucleotides
Phages, Prophages, Transposable elements, Plasmids
Cell Division and Cell Cycle
Fatty Acids, Lipids, and Isoprenoids
Dormancy and Sporulation
Membrane Transport
Cell Wall and Capsule
Amino Acids and Derivatives
Photosynthesis
Regulation and Cell signaling
Iron acquisition and metabolism
Sulfur Metabolism
Clustering-based subsystems
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
< 1e-15
1.12e-9
3.57e-9
9.44e-9
1.95e-8
2.83e-7
1.06e-4
1.24e-4
1.28e-4
4.18e-4
2.92e-3
5.57e-3
8.86e-3
0.012
q-value (corrected)
0.0
15.4
Proportion (%)
−2.0 −1.5 −1.0 −0.5 0.0 0.5 1.0 1.5 2.0 2.5
Difference between proportions (%)

A



B

Figure 6 Level 1 sub-system (A) and level 2 sub-system (B) metabolic processes. significantly different between 37 PSU and 106 PSU.

● processes overrepresented at 37 PSU; ● processes over-represented at 106 PSU. Only the most significant categories (q-value $<10^{-11}$) are displayed.

Other metabolisms over-represented at level 3 sub-system hierarchy not shown for clarity include: chlorophyll; niacin; cobalamin; and coenzyme B12 synthesis; glutathione regulation; and Polyhydroxybutyrate metabolism at 37 PSU; and: cobolt, zinc and cadmium resistance; phage replication and phage plasmid machinery; and betaine synthesis at 106 PSU.

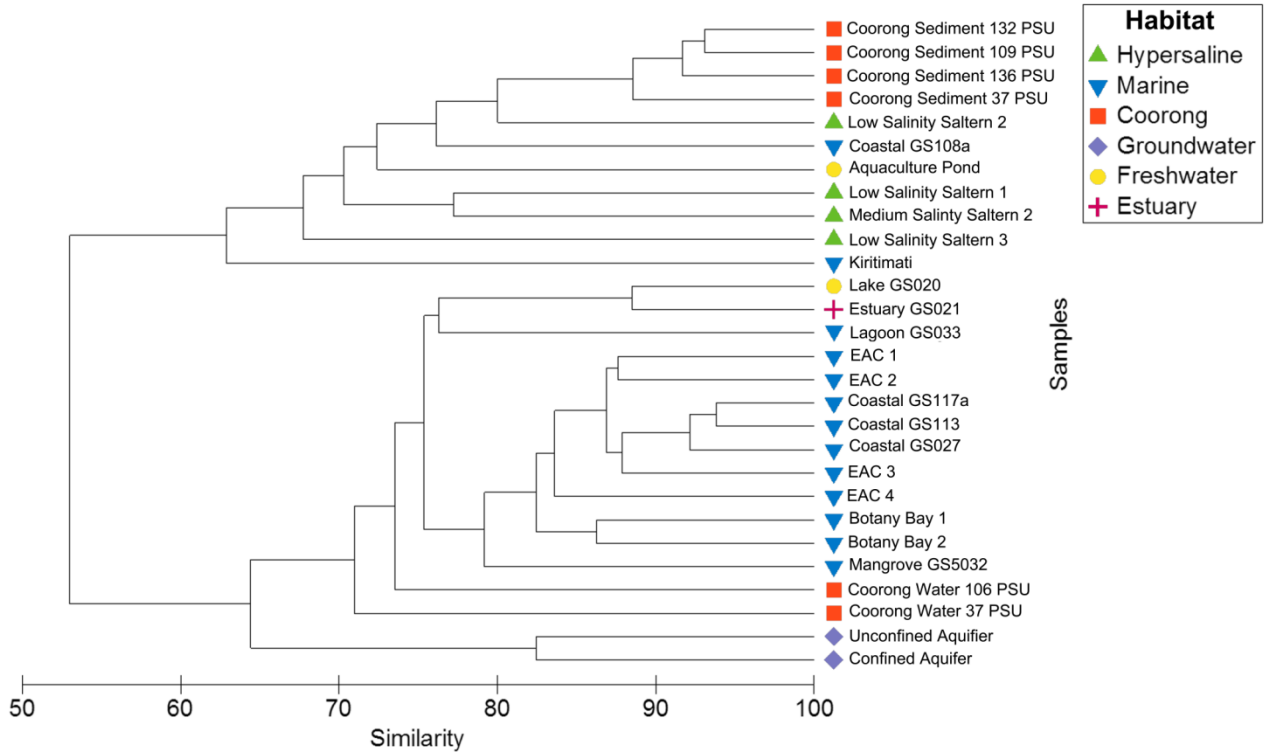


Figure 7 Comparison of taxonomic profiles derived from 37 PSU, 106 PSU and 26 selected metagenomes publically available on the MG-RASTdatabase.

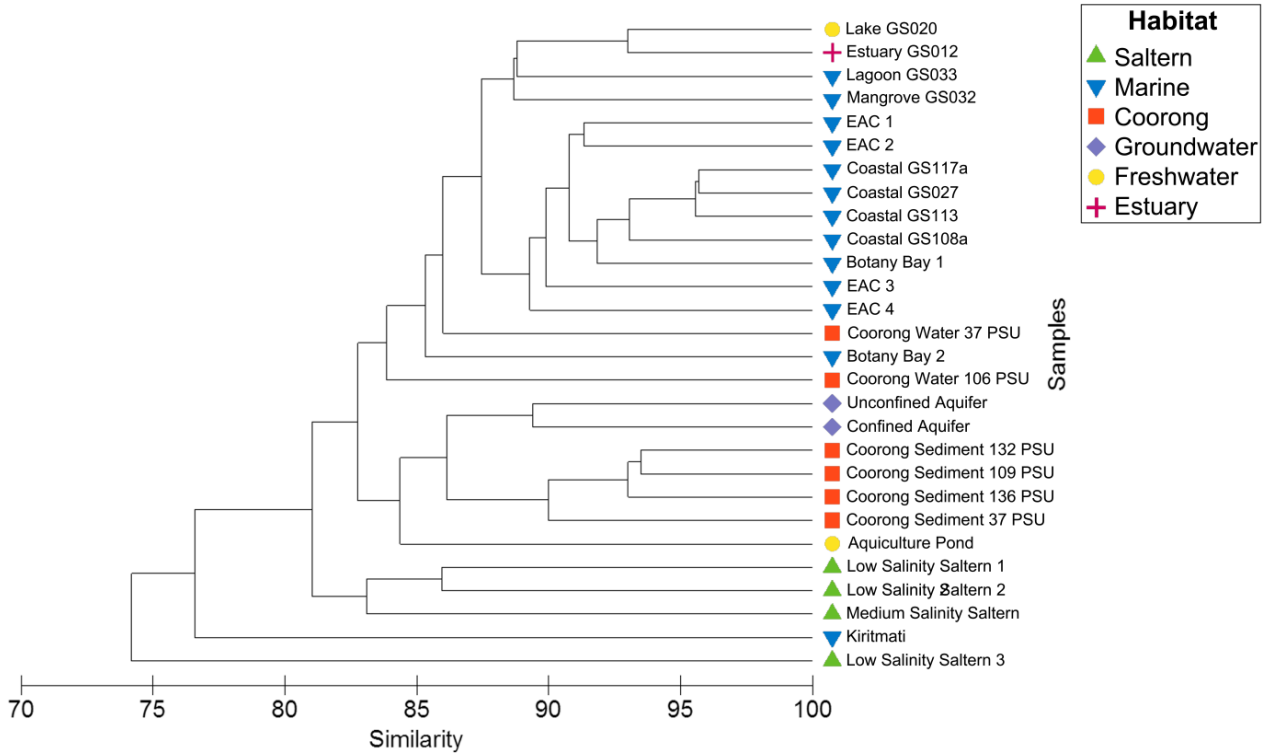


Figure 8 Comparison of metabolic profiles derived from 37 PSU, 106 PSU and 26 selected metagenomes publicly available on the MG-RAST database.

Chapter V

Viral Community Dynamics in a Hypersaline Lagoon

ABSTRACT

Viruses influence aquatic systems both on a local and global scale, however viral abundance, diversity and production are driven by physiochemical factors. Salinity has been shown to impact upon viral abundance, morphology and production, however studies from natural, connected environments exhibiting large changes in salinity are rare. Here we used transmission electron microscopy and modified dilution experiments to determine the morphological diversity and production of the community at 38 and 131 PSU in a productive coastal lagoon. Morphological diversity as determined by the Shannon index increased at 131 PSU along with the contribution of virus like particles (VLPs) characteristic of extreme environments, specifically lemon-shaped and short linear VLPs. VLP production increased from 3.6×10^7 VLPs mL^{-1} d^{-1} at 38 PSU to 14.0×10^7 VLPs mL^{-1} d^{-1} at 131 PSU. Here we confirm that salinity impacts strongly upon the viral community in natural connected productive environments.

INTRODUCTION

Viruses are abundant and diverse in aquatic environments; their activities influence genetic transfer, biogeochemical cycling of organic matter and nutrients, population dynamics and the activities of organisms at higher tropic levels (Fuhrman 1999: Suttle 2005). Viral abundance and community diversity can be influenced by chemical and environmental factors such as salinity, pH and temperature (Guixa-Boixereu *et al*. 1996; Geslin *et al*. 2003; Le Romancer *et al*. 2007). In environments exhibiting physiochemical extremes, viral abundance is high and many unique viral morphologies have only been observed in these extreme environments (Le Romancer *et al*. 2007; Sime-Ngando *et al*. 2011). In aquatic environments bacteria are major viral hosts and salinity is known to drive bacterial diversity (Lozupone and Knight 2007), however studies investigating viral diversity in relation to salinity are somewhat limited (Bettarel *et al*. 2011a). Previous work has shown that viral abundance increases with salinity (Guixa-Boixereu *et al*. 1996; Schapira *et al*; 2009); viral morphological diversity and the dominance of specific morphotypes alters as salinity increases (Guixa-Boixereu *et al*. 1996; Bettarel *et al*. 2011a; Helton *et al*. 2012); and the greatest genetic diversity is found where salinity ranges from 40 to 150‰ (Sandaa *et al*. 2003), demonstrating that salinity has an important influence on the viral community and in turn entire aquatic ecosystems.

Viral community diversity can be investigated using molecular methods such as Pulse Field Gel Electrophoresis (Diez *et al*. 2000; Sandaa *et al*. 2003), primers targeting specific viral components (e.g. the tail) (Filée *et al*. 2005), and more recently, next generation sequencing (Angly *et al*. 2006), however the lack of known viral sequence

data hinders these approaches (Brum and Steward 2010). Techniques such as transmission electron microscopy (TEM) can not only complement sequence information but can also be utilised to visualise the viral community, obtaining information about viral morphology, size, abundance and diversity. This technique is widely used to compare different aquatic viral communities (Guixa-Boixereu *et al*. 1996; Wommack and Colwell 2000; Davy and Patten 2007; Brum and Steward 2010; Bettarel *et al*. 2011a) and describe novel virus-like particles (VLPs) (Häring *et al*. 2005). As salinity increases, different VLP morphologies become more or less dominant in the community. Tailed and icosahedral VLPs dominate at lower salinities while filamentous, lemon-shaped, spherical and a variety of unique morphologies emerge as the dominant morphotypes as salinity increases (Guixa-Boixereu *et al*. 1996; Oren *et al*. 1997; Bath and Dyall-Smith 1998; Le Romancer *et al*. 2007; Bettarel *et al*. 2011a; Sime-Ngando *et al*. 2011). Most of these studies are conducted in steady state environments, except in the case of Bettarel *et al*. (2011a), and as such there is limited knowledge of VLP morphological change as salinity increases in natural environments.

Increased viral abundance in hypersaline environments is thought to be a result of the inability of both viral and host predators to survive at high salinities (Pedrós-Alió *et al*. 2000; Bettarel *et al*. 2011a), increased resistance to destructive agents (Bettarel *et al*. 2011a) and high contact rates between viruses and hosts (Hewson and Fuhrman 2007; Brum and Steward 2010). Increased abundance of viruses and hosts, and contact rates between the two may result in greater production of VLPs, however viral infection dynamics in hypersaline environments are variable. Adsorption rates of viruses to their hosts show a wide range of variability and depend on the host, virus and surrounding environment (Daniels and Wais 1998; Kukkaro and Bamford 2009); viral proliferation

generally corresponds with unfavourable host conditions (Oren *et al*. 1997); latency times can be prolonged (Guixa-Boixereu *et al*. 1996; Bettarel *et al*. 2011a); and persistent cellular infection can result in viral release with minimal host death (Stedman *et al*. 2006; Porter *et al*. 2007). High viral production rates have been observed over a range of salinities in solar salterns (Guixa-Boixereu *et al*. 1996) and many estimates exist for marine environments (Heldal and Bratbak 1991; Steward *et al*. 1991; Noble and Fuhrman 2000), however VLP production estimates from environments exhibiting natural extremes in salinity are lacking.

Generally studies investigating the impact of salinity on the viral community are carried out in solar salterns or hypersaline lakes (Guixa-Boixereu *et al*. 1996; Pedrós-Alió *et al*. 2000; Porter *et al*. 2007; Sime-Ngando *et al*. 2011), however research in environments exhibiting natural salinity gradients is somewhat limited (Schapira *et al*. 2009). In this context we determined VLP morphological diversity and production at two salinity extremes, marine and hypersaline, in a coastal lagoon, the Coorong, South Australia. The Coorong has previously been found to exhibit a highly abundant and flow cytomerically diverse VLP populations (Schapira *et al*. 2009); however VLP morphologies and production values are unknown. The Coorong is a estuary high in primary productivity and microbial abundance; bacteria, VLPs, chlorophyll *a* and phytoplankton increase with salinity (Schapira *et al*. 2009; Schapira *et al*. 2010). We found that the productive nature of the Coorong coupled with an increase in salinity lead to greater VLP morphological diversity and production in the hypersaline reaches of the lagoon.

MATERIALS AND METHODS

Study site

The Coorong is a coastal lagoon system at the termination of the Murray River which exhibits an intense environmental gradient of increasing salinity, organic and inorganic matter with increasing distance from the Murray mouth to Salt Creek, approximately 98 km south west of the river mouth (Figure 1). The microbial community is known to be structured by spatial and temporal variation in salinity and nutrient content (Ford 2007). Specifically, salinity structures the distribution of the bacterial community, which consequently contributes to viral abundance, and distribution (Schapira *et al*. 2009).

Sampling strategy

Samples were collected in May 2008 from 2 sites exhibiting extremes in salinity, from seawater (38 PSU) at the River Murray mouth (Site 1) to hypersaline (133 PSU) near Salt Creek (Site 2). Salinity (PSU), temperature (°C), pH, dissolved oxygen (mg L^{-1}) and turbidity (NTU) were recorded using a 90FL-T (TPS) multiparameter probe.

Triplicate 1 mL samples for flow cytometric VLP and bacterial enumeration were collected and fixed in 2% glutaraldehyde (final concentration) in the dark at 4°C for 15 minutes, snap frozen in liquid nitrogen and stored at -80°C until flow cytometric analysis (Brussaard 2004). Triplicate 60 mL samples for chlorophyll *a* (Chl *a*) determination were filtered through 25 mm glass-fibre filters (0.7 µm porosity, GF/F Whatman) and stored in the dark at -20°C until analysis. For analysis, chlorophyllous pigments were extracted from each filter in 5 mL of methanol at 4°C in the dark for 24 hours. Chl *a* concentration (µg L^{-1}) was then determined using a Turner 450 fluorometer.

Collection and pre-treatment of water

At each site 25 L of water was allowed into an acid-washed (0.1 N HCl) polyurethane carboy. Water was then pre-treated via 200 µm mesh gravity filtration followed by 0.8 µm gravity filtration (Millipore^{TM}). Viral-free and viral concentrate water fractions were then prepared by filtering the sample through a 30 kDa tangential flow filtration system (Millipore^{TM}). Virus-free water was then used immediately in production experiments and virus concentrate (~500 mL) was fixed in formaldehyde (2% final concentration) and stored at 4 °C in the dark until further sample preparation for transmission electron microscopy.

Collection and concentration of VLPs for TEM

Triplicate samples of fixed viral concentrations (17 mL) from Site 1 were further concentrated by centrifugation for 2 hours at 146,000 × g (Beckman Optima L-XP ultracentrifuge, SW 32.1 Ti rotor). The resulting viral pellet was then resuspended in 100 µL of supernatant. Samples of fixed viral concentrations from Site 2 were purified by centrifuging using caesium chloride (CsCl) step gradients to remove cellular material and detritus present (Angly *et al*. 2006), the viral fraction was extracted from the interface of the 1.5 and 1.7 CsCl layer using a 1 mL syringe and resuspended in 2 mL of supernatant, both samples were stored at 4 °C in the dark until analysis preparation for analysis by TEM.

Transmission electron microscopy

Copper or copper/aluminium TEM grids (3 mm, formvar coated and glow-discharged) were floated on 20 µL sub-samples of centrifuged viral concentrate for 1 hour in the dark. Grids were washed by floating on 20 µL droplets of MilliQ® for 2 seconds and excess water absorbed using filter paper (Whatman™). Grids were then negatively stained for 30 seconds using uranyl acetate (2%), excess stain absorbed using filter paper (Whatman™) and the grids air dried in the dark (Davy and Patten 2007). Grids were examined on a Phillips CM100 transmission electron microscope at 80 kVa (46,000-100,000x magnification). Eight bit digital images were captured using a SIS Megaview II CCD camera and AnalySIS software (Olympus). Morphology and size of a total 85 randomly selected individual VLPs from each site were measured using Motic Images 2.0 (Motic China Group Co., Ltd).

VLP morphological diversity

VLPs were assigned to one of 5 major morphological groups based upon previously described morphologies (Ackermann 2001; Ackermann 2007; Davy and Patten 2007; Bettarel *et al*. 2011a; Sime-Ngando *et al*. 2011); tailed, polyhedral/spherical, filamentous, lemon-shaped (also described as spindle) and short linear VLPs. Tailed VLPs were further distinguished into the families *Siphoviridae*, *Podoviridae* and *Myoviridae* on the basis of tail size and shape. VLP morphotype diversity at each site was determined using the Shannon index (Legendre and Legendre 2012):

$$H' = \frac{[n\log n - \sum_{i=1}^{k} f_i \log f_i]}{n} \quad (1),$$

where *n* is the total number of VLPs observed, *k* is the total number of morphologies observed, *i* is the morphology observed and f_i is the frequency of an observed morphology.

Evenness of viral morphological diversity at each salinity was determined using the Pielou index (Pielou 1966):

$$J' = \frac{H'}{H'_{max}} \quad (2).$$

where *H'* is the Shannon index and H'_{max} is the maximum value of *H'* in the community if all viral morphologies are equally frequent.

VLP production experiments

VLP production was determined at each study site using a modified dilution assay (Evans *et al*. 2003; Baudoux *et al*. 2006; Baudoux *et al*. 2007; Brussaard *et al*. 2008). Four levels of natural water dilution (20, 40, 70 and 100% natural sample) were created by siphoning virus-free water into acid washed (0.1 N HCl) 1 L glass bottles (Schott). These bottles were filled to capacity with freshly collected 200 µm filtered natural sample water. Replicates were created by siphoning 250 mL of sample into triplicate acid washed (0.1 N HCl) glass incubation bottles (Schott). Triplicate samples for flow cytometric VLP enumeration were taken from each bottle (t = 0 h, t_0). Bottles were then refilled to 250 mL with sample, sealed using Parafilm® M (Pechiney Plastic Packaging Company) and incubated for 24 hours in a 4,000 L holding tank on site. After 24 hours (t = 24 h, t_{24}), triplicate samples were taken from each incubation bottle for flow cytometric VLP enumeration.

VLP and bacterial enumeration and production

VLP and bacterial concentrations were determined via flow cytometry. Samples were thawed from frozen in hot water, diluted (1:10 Site 1; 1:100 Site 2) in 0.02 µm filtered TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8), stained with SYBR-I Green solution (Invitrogen; 5:100,000 dilution in TE buffer) then incubated in the dark at 80°C for 10 minutes (Brussaard 2004). Samples were then analysed using a FACSCanto flow cytometer (Becton-Dickson) with 1 µm fluorescent beads (Molecular Probes, Eugene, Oregon) added to each sample as an internal size standard and to determine viral concentration (Gasol and del Giorgio 2000). Each sample was processed for 2 minutes and forward-angle light scatter (FSC, 285V), right-angled light scatter (SSC, 500V) and green (SYBR-I) fluorescence (500V) were systematically recorded. VLP and bacterial populations were identified and enumerated using WinMDI 2.9 (© Joseph Trotter) according to alterations in SYBR-Green fluorescence and SSC (Marie *et al*. 1999a, b; Brussaard 2004).

Viral production for each population was directly estimated by the slope of the change in VLP abundance, *Δ*VLP versus the dilution factor

$$\Delta VLP = VLPt_{24} - VLPt_0 \quad (3).$$

Comparisons between VLP production at each site were performed using the Mann-Whitney Test, significance was determined when $p < 0.05$.

RESULTS

Environmental properties

Environmental properties generally increased between Site 1 and 2 (Table 1). Notably salinity increased from 37.6 PSU at Site 1 to 131.4 PSU at Site 2. Bacterial abundance increased from 1.4×10^7 mL^{-1} at Site 1 to 6.8×10^8 mL^{-1} at Site 2. Chlorophyll *a* increased from 1.5 µg L^{-1} at Site 1 to 12.2 µg L^{-1} at Site 2.

VLP abundance and production

VLP abundance increased from 6.8×10^7 mL^{-1} at Site 1 (hereafter referred to as 38 PSU) to 2.3×10^8 mL^{-1} at Site 2 (hereafter referred to as 131 PSU) (Table 1). VLP production increased from 3.6×10^7 VLPs mL^{-1} d^{-1} at 38 PSU to 1.4×10^8 VLPs mL^{-1} d^{-1} at 131 PSU (Figure 2), however, this increase was not significantly different.

VLP morphology and diversity

The number of VLP morphotypes observed in the Coorong increased from 3 at 38 PSU to 5 at 131 PSU (Figure 3A-B). Tailed phage, polyhedral/spherical and filamentous VLPs were common at both salinities while lemon-shaped and short linear VLPs were observed at 131 PSU only (Figure 3A-B). Tailed VLPs comprised approximately 35% of the VLP community at each site (Figure 3C-D), polyhedral/spherical VLPs comprised 56% of the VLP community at 38 PSU yet only 24% of VLPs at 131 PSU were polyhedral/spherical (Figure 3E, G, H). Filamentous VLPs increased with salinity, doubling from 6% at 38 PSU to 12% of the community at 131 PSU (Figure 3F, Figure 4). Of the VLPs exclusive to 131 PSU lemon-shaped comprised 24% of the community

while short linear phage comprised only 6% of the VLP community (Figure 3I-J Figure 4). Of the tailed VLPs *Siphovirus*-like VLPs were most abundant at both 38 and 131 PSU, comprising over 84% of phages of the tailed VLP community, *Podovirus*-like VLPs were second in abundance, comprising around 10% of each community, while *Myovirus*-like VLPs comprised only 6% of the VLP community at 38 PSU and were not found at 131 PSU (Figure 3C-D, Figure 5).

Greater VLP morphological diversity at 131 PSU was supported by the Shannon index which increased from 0.37 at 38 PSU to 0.61 at 131 PSU (Table 2). Increased diversity of tailed VLPs at 38 PSU was also confirmed by a decrease in Shannon index from 0.23 at 38 PSU to 0.16 at 131 PSU (Table 2). VLP community evenness as measured by Pielou's index increased with salinity from 0.34 at 38 PSU to 0.4 at 131 PSU. Tailed VLPs were also more evenly distributed in abundance between at 131 PSU, Pielou's index increasing from 0.21 at 38 PSU to 0.24 at 131 PSU (Table 2).

DISCUSSION

Viral morphological diversity

The Coorong is a reverse estuary exhibiting a 3.5 fold increase in salinity with increasing distance from the river mouth. Combined with enhanced productivity typical of estuarine environments (Kan *et al*. 2007), this results in a dramatic increase in bacterial and viral abundance (Table 1), with VLP morphological diversity and community evenness also greater in the hypersaline South East reaches of the Coorong (Table 2, Figure 3A-B). The increase in VLP morphological diversity at 131 PSU is consistent with a study which shows sediment VLP genetic diversity is greatest

around 131 PSU (Sandaa *et al*. 2003). When compared with morphological studies from other environments, the Coorong showed similar trends VLP morphology: Lemon-shaped morphologies and short linear morphologies characteristic of high saline environments (Guixa-Boixereu *et al*. 1996; Porter *et al*. 2007; Bettarel *et al*. 2011a; Sime-Ngando *et al*. 2011) were absent at 38 PSU then increased to comprise approximately 30% of the viral community at 131 PSU (Figure 4). Filamentous VLPs were less abundant at 38 and 131 PSU when compared to other studies (Davy and Patten 2007; Bettarel *et al*. 2011a); however, followed the same trend observed by Bettarel *et al*. (2011a) and showed increased abundance at higher salinity (Figure 4). Tailed phage comprised approximately 35% of the community at both salinities which is consistent with Bettarel *et al*. (2011a) yet inconsistent with Davy and Patten (2007) who found less than 2% of marine VLPs were tailed (Figure 4). While Guixa-Boixereu *et al*. (1996) reported a large contribution of icosahedral VLPs, it is not known if these were tailed or un-tailed VLPs. Polyhedral/spherical VLPs were lower in abundance at 38 PSU when compared to Davy and Patten (2007) yet followed the same trend seen by Bettarel *et al*. (2011a) decreasing in abundance at higher salinities (Figure 4).

Although bacterial diversity is richest at salinities in the range observed in this study (Benlloch *et al*. 2002; Schapira *et al*. 2009) and bacterial abundance increased by approximately 50 fold between 38 and 131 PSU (Table 1), the percentage of tailed VLPs, 96% of which are estimated to be bacteriophages (Wommack and Colwell 2000), did not increase with salinity and remained about 35 % in both cases (Figure 4). This was also reflected in a decrease in tailed phage diversity at 131 PSU (Table 2). While this is consistent with previous literature (Bettarel *et al*. 2011a) it is unexpected that the contribution of tailed phage does not increase given the substantial increase in bacterial

abundance at increased salinities, especially as a previous study in the Coorong theorised that most VLPs should be bacteriophage at 131 PSU (Schapira *et al*. 2009). However, this increase in bacterial abundance may allow the bacteriophage population to exploit a greater number of hosts and ecological niches resulting in the expression of a more diverse range of morphologies. Increased bacteriophage morphological diversity may also explain why tailed VLP diversity is lower at 131 PSU (Figure 4) and is supported by evidence suggesting viruses from other extreme environments are genetically distinct indicating niche adaptation (Le Romancer *et al*. 2007).

Recent work has shown that viruses exhibiting a membrane are more sensitive to changes in salinity (Kukkaro and Bamford 2009), as we grouped polyhedral and spherical VLP together, including VLPs that appeared to have a membrane and eukaryotic VLPs (Figure 3E, G, H), this may explain the 2 fold decrease in polyhedral/spherical VLP abundance. Decrease in un-tailed icosahedral VLPs has also been observed in highly hypersaline environments previously (Bettarel *et al*. 2011a) and it is important to note that un-tailed polyhedral VLPs may be tailed VLPs where the tails have been accidently removed during processing or filtering. As filtration using 0.2 – 0.22 µm filters potentially biases studies of viral diversity by removing larger viruses (Brum and Steward 2010) we used filters with a large 0.8 µm pore size to remove matter from our samples.

Filamentous VLPs are thought to occur rarely in the water column (Middelboe *et al*. 2003) yet have been described in numerous extreme environments including hot acidic springs, hot springs, deep fractured hard rock and hypersaline lakes (Häring *et al*. 2005;

Le Romancer *et al*. 2007; Kyle *et al*. 2008; Sime-Ngando *et al*. 2011). They are also important agents of bacterial mortality in alpine freshwater lakes (Hofer and Sommaruga 2001) and have only recently been described over a wide range of salinities from brackish to hypersaline (Davy and Patten 2007; Bettarel *et al*. 2011a). Filamentous VLPs increase in estuarine sediments, environments which are high in bacterial abundance and diversity (Middelboe *et al*. 2003), at 131 PSU the 2 fold increase in filamentous VLPs could also be the result of increased abundance of diverse hosts in this hypersaline environment. Our observations of filamentous VLPs (Figure 3F) at marine and hypersaline extremes of a coastal lagoon confirm that they are an important component of the aquatic viral population.

At 131 PSU we observed an increase in lemon-shaped and short linear morphotypes (Figure 3I-J) often associated with hypersaline environments and high archaeal abundance (Guixa-Boixereu *et al*. 1996; Bath and Dyall-Smith 1998; Porter *et al*. 2007; Bettarel *et al*. 2011a; Sime-Ngando *et al*. 2011), although these morphotypes have also been observed in numerous other aquatic environments at a range of salinities (Montanié *et al*. 2002; Davy and Patten 2007; Bettarel *et al*. 2011a, b). However, a dramatic increase in the abundance of lemon-shaped VLPs is in agreement with other literature associating increase of lemon-shaped VLPs with salinity ranging from 130 PSU to saturation (Guixa-Boixereu *et al*. 1996; Bettarel *et al*. 2011a).

The increase in lemon-shaped VLPs typical of hypersaline archaeal viruses observed at 131 PSU (Figure 4) and the 3.5 fold increase in salinity suggest that archaea comprise an important proportion of the microbial community in the Coorong. Viruses from high

saline environments generally exhibit slower adsorption to their host (Kukkaro and Bamford 2009) and archaea can remain persistently infected, continually releasing viral particles without cell lysis (Bath and Dyall-Smith 1998; Porter *et al*. 2007). Filamentous bacterial viruses can also cause chronic infections and are increased in abundance at 131 PSU (Figure 4). Increased bacterial and archael abundance along with a large proportion of these steadily releasing new virions may account for the increase in VLP production at 131 PSU (Figure 2). Burst sizes are larger in hypersaline environments over 150 PSU (Guixa-Boixereu *et al*. 1996) therefore it is reasonable to assume that burst sizes may be increased at 131 PSU relative to 38 PSU. If this is the case then lytic busts occurring at different times may explain the large variation in VLP production at 131 PSU.

VLPs were assigned to one of five distinct morphological groups; however, it should be noted that determination of morphology is somewhat subjective. For example, ambiguous particles which appear in micrographs only once or twice and have not previously been described as VLPs in the literature could easily be overlooked as VLPs, or variances in staining intensity could lead to confusion between polyhedral and spherical VLPs (Sime-Ngando *et al*. 2011). For these reasons morphologies were categorised into 5 distinctive and easily discernible groups. As such our diversity estimates are likely underestimates; however, since this bias is applied equally to both salinities the relationship between diversity at 38 and 131 PSU is unaffected. It is common practice to be conservative when determining VLP morphology and studies identifying more than 5 major groups are rare in the literature, with the exception of discrimination of tailed VLPs which are commonly identified to family level (Guixa-Boixereu *et al*. 1996; Ackermann 2007; Davy and Patten 2007; Bettarel *et al*. 2011a, b;

Sime-Ngando *et al*. 2011). Diversity indexes have previously been used to quantitatively assess viral and bacterial community diversity with increase in salinity (Benlloch *et al*. 2002; Sandaa *et al*. 2003; Helton *et al*. 2012); however, this is the first time to our knowledge that a diversity index has been used to investigate viral morphological diversity. We highlight that although molecular techniques are best to ascertain which viral species are present and their functional potential, determining community diversity from viral morphological descriptions enables a quick, relatively inexpensive and efficient observation of viral diversity in an ecosystem.

Viral production

While VLP production was not significantly different between 38 and 131 PSU, production at 38 PSU was greater than estimates observed at comparable salinities (Heldal and Bratbak 1991; Steward *et al*. 1991; Steward *et al*. 1996; Noble and Fuhrman 2000; Bettarel *et al*. 2002; Bongiorni *et al*. 2005; Hewson and Fuhrman 2007) while production at 131 PSU was similar to estimates in a solar saltern of comparable salinity (Guixa-Boixereu *et al*. 1996). High VLP production observed in the Coorong could be a result of larger burst sizes and smaller separation distances. This is consistent with the observed increase in viral abundance in hypersaline environments, where species diversity is decreased and the prokaryotic community dominates (Guixa-Boixereu *et al*. 1996). High viral production and low virus to bacteria ratio (VBR) (Figure 2, Table 1) suggest that viral turnover is high or that the bacterial community is not particularly diverse, the latter of which is possible at increased salinity (Guixa-Boixereu *et al*. 1996; Wommack and Colwell 2000; Schapira *et al*. 2009). The large estimates of viral production observed in the Coorong are consistent with the physical characteristics

of the Coorong, i.e. a productive estuary with an increasing salinity gradient (Schapira *et al*. 2009). Viral persistence at high abundances in aquatic environments suggests that removal and replacement are in equilibrium (Wilhelm *et al*. 2002) or could potentially be a result of ionic shielding preventing viral loss due to solar irradiation. In high saline environments, increased microbial abundance results in an increase in the number of microbial interactions leading to greater infection rates and subsequent viral production (Hewson and Fuhrman 2007; Brum and Steward 2010). Studies conducted in estuarine environments showed comparable viral production estimates to the values estimated here (Wilhelm *et al*. 2002; Helton *et al*. 2005). This indicates differences in viral production estimates between environments may be attributed to variation in microbial abundance and general system productivity (Weinbauer 2004), estuarine environments specifically are known for exhibiting increased productivity (Kan *et al*. 2007).

SYNTHESIS

Here we provide the first insight into viral morphological diversity and production at two extremes of a natural salinity gradient in the Coorong lagoon, South Australia. We confirm that salinity impacts strongly upon the morphology of viruses in the Coorong resulting in an increase in morphological diversity at 131 PSU. VLP production in the Coorong is higher at 38 PSU when compared to other marine estimates and production estimates at 131 PSU are similar to other observations in hypersaline salterns, however production between 38 and 131 PSU is not significantly different. As viral, bacterial and phytoplankton populations are closely linked the results here suggest a dynamic viral community that warrants further investigation.

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Table 1 VLP abundance and bacterial abundance (mean ± standard error); VBR; Chl *a* (mean ± standard error); and environmental conditions at each site.

Parameter	**Site 1**	**Site 2**
VLPs ($\times 10^7$ mL^{-1})	6.8 ± 0.8	229.9 ± 44.7
Bacteria ($\times 10^7$ mL^{-1})	1.4 ± 0.2	67.5 ± 8.0
VBR	5.1	3.4
Chl *a* (µg L^{-1})	1.5 ± 0.1	12.2 ± 2.6
Salinity (PSU)	37.6	131.4
Temperature (°C)	16.5	12.8
pH	8.2	8.0
$[O_2]_d$ (mg L^{-1})	10.6	13.5
Turbidity (NTU)	12.0	19.7

Table 2 Shannon and Pielou indexes for the VLP community and tailed VLPs at 38 and 131 PSU.

Site	VLP Community		Tailed VLPs	
	Shannon Index	**Pielou Index**	**Shannon Index**	**Pielou Index**
38 PSU	0.37	0.34	0.23	0.21
131 PSU	0.61	0.40	0.16	0.24

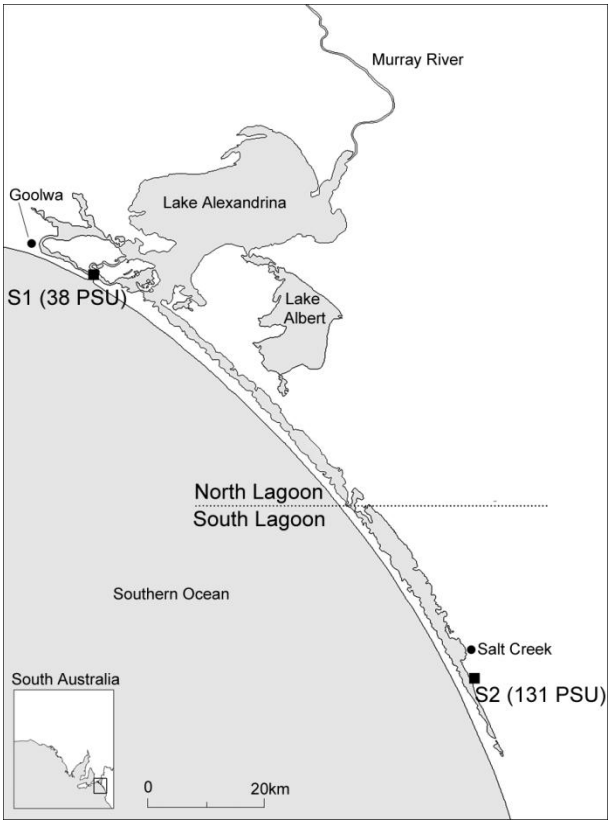

Figure 1 Study site; the Coorong (South Australia, Australia) showing the location and salinity (PSU) of the 2 sampling sites (■).

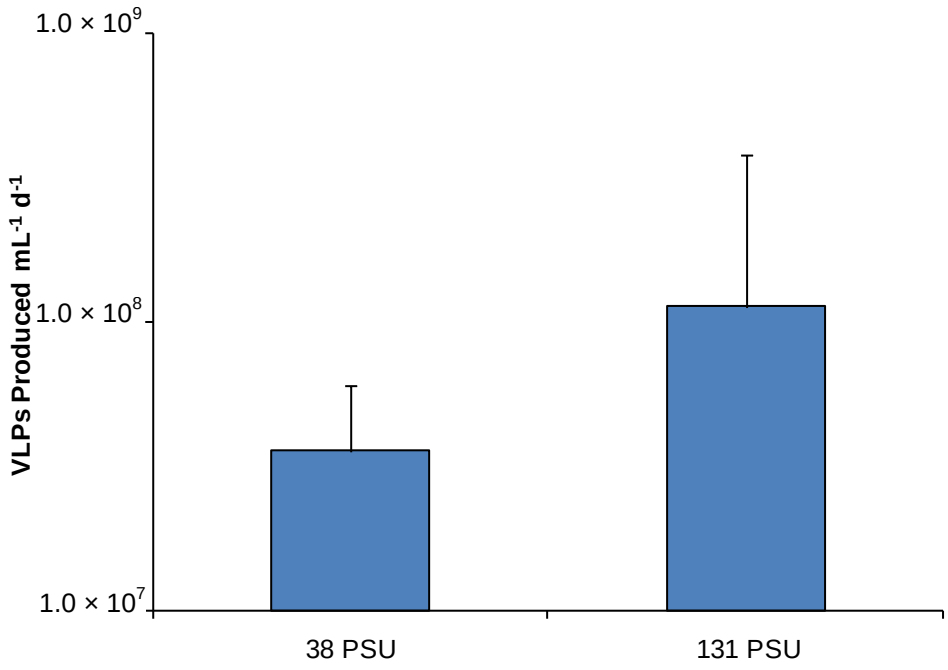

Figure 2 Virus-like Particles (VLP) produced (mL^{-1} d^{-1}) at 38 and 131 PSU. Error bars are standard error of the mean.

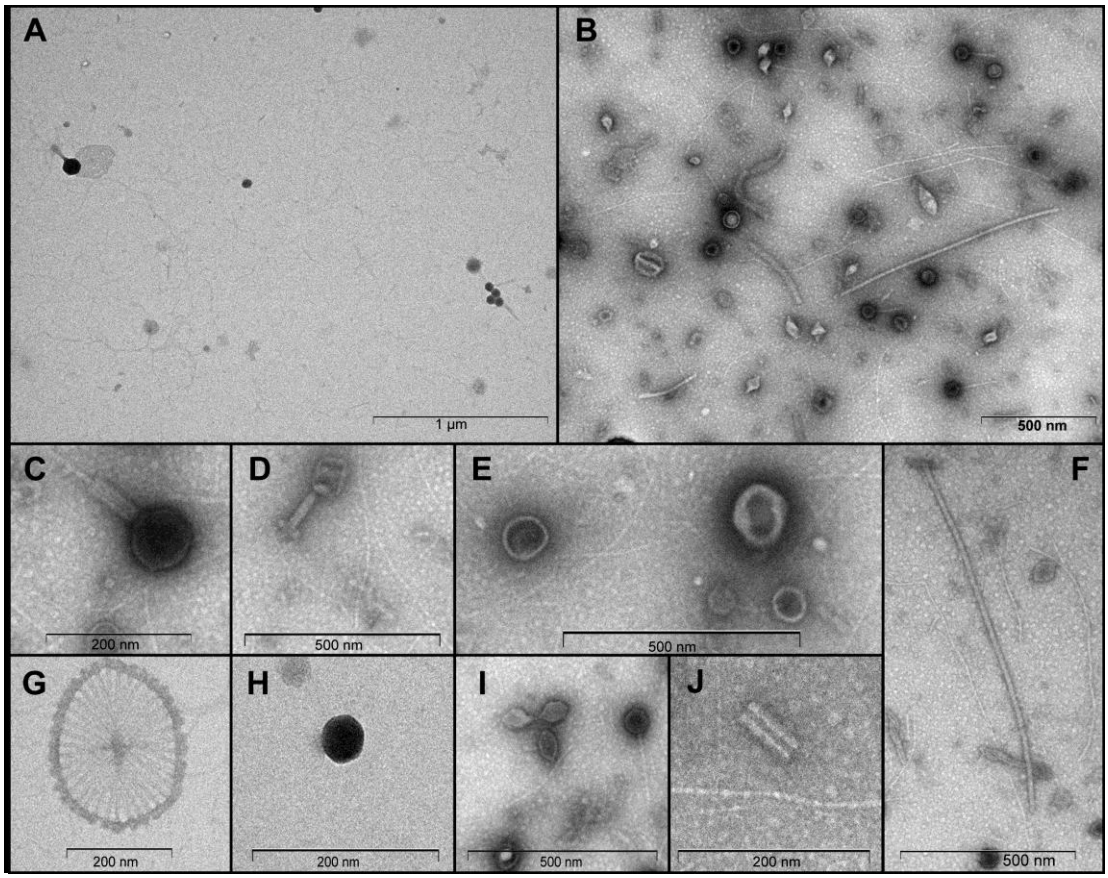

Figure 3 Overview of VLPs observed at 38 PSU (A) and 131 PSU (B) by TEM. (C, D) Tailed VLPs; (E, G, H) Spherical/polyhedral VLPs; (F) Filamentous VLP; (I) Lemon-shaped VLPs; (J) Short linear VLP.

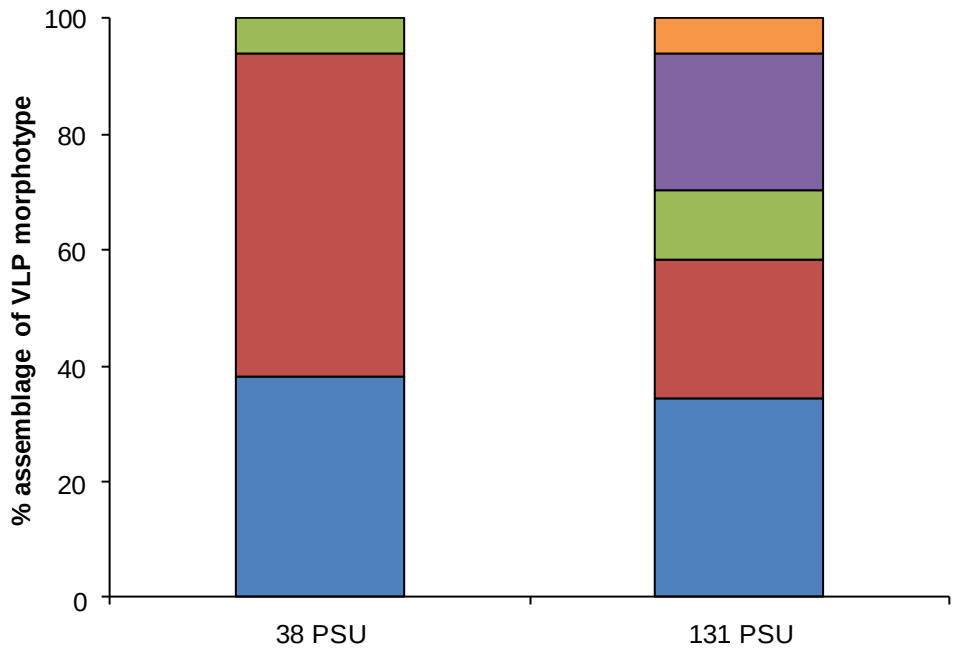

Figure 4 Percentage (%) abundance of each VLP morphological group at 38 and 131 PSU.

Tailed; Polyhedral/Spherical; Filamentous; Lemon-shaped;

Short linear.

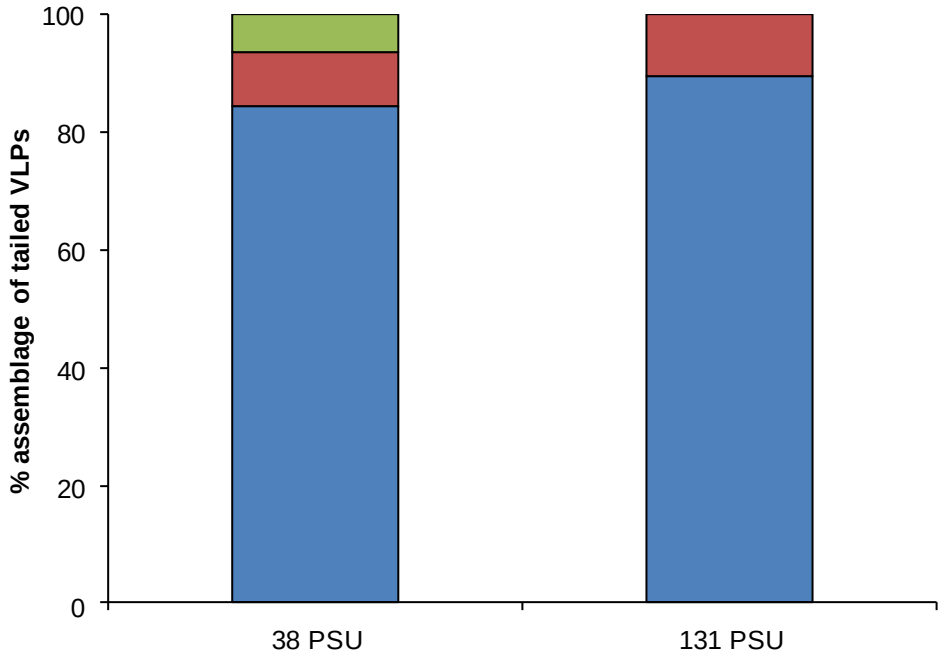

Figure 5 Percentage (%) abundance of different tailed VLP at 38 and 131 PSU.

Siphovirus-like; *Podovirus*-like; *Myovirus*-like.

Chapter VI

Energetic Benefits from Marine Bacterial Flagellar Stabilisation

ABSTRACT

Prokaryotes allocate energy and matter among the processes of finding nutrients, acquiring nutrients, growing and reproducing. Flagella-mediated chemotaxis is a potentially energetically costly step in this process, but its ubiquity indicates its importance in survival. Strategies for minimizing chemotactic cost have been little examined. Here, using morphological measurements, we examined how flagella reduce chemotactic cost by reducing Brownian rotation of cells. We found for flagella over 3.0 μm cost decreases 3 fold from 6×10^{-11} g $cm^2.s^{-3}$ to 2×10^{-11} g $cm^2.s^{-3}$. Additionally, we demonstrate that helicity of flagella and, when present, prosthecae reduce energetic cost by half and 2.5 times. Overall, flagella and prosthecae reduced cost 15 times, from 6×10^{-11} to 4×10^{-12} g $cm^2.s^{-3}$. This represents a decrease in use from 5% of organic matter taken up to less than 1% use of organic matter taken. However, considering the effect of a range of bacterial sizes the maximum effect would reduce cost from 165% of organic matter potentially taken up to less than 5% of overall organic matter uptake. Thus, subtle morphological changes, such as flagella length, helix amplitude and prosthecae significantly decrease the cost of finding nutrients and overall bacterial operating costs.

INTRODUCTION

The efficient acquisition, accumulation and use of organic matter are critical components of energy flow in aquatic food webs; through these crucial steps in energy management cells balance reproduction against survival. Among aquatic heterotrophs, bacteria play a crucial role in the creation and movement of organic matter through food webs (Azam and Malfatti 2007). Bacteria compete among themselves and with eukaryotic microbes by detecting and taking up dissolved nutrients, specifically monomeric compounds, to provide the necessary energy required for growth, reproduction and motility (Mitchell 2002; Yoshiyama and Klausmeier 2008). The success of bacterial cells in regards to these factors depends on the cell's ability to detect and respond to these nutrient sources (Dusenbery 1997).

Although chemotaxis is a non-obligatory contributor to cellular energetic costs, it allows chemotactic movement towards nutrient sources; sustaining bacterial growth and facilitating reproductive success (Blackburn *et al*. 1998). It is essentially a strategic optional cost that presumably when properly used confers advantage. The influence of bacterial movement on cell energy budgets has received little attention compared to predation and viral lysis (Mitchell *et al*. 1995; Fuhrman 1999; Middleboe and Lyck 2002). There is a lack of information on how the production and maintenance of motility-related structures such as flagella, receptors and motors influence food web dynamics (Mitchell 2002). Here, we partially redress this paucity of information by considering how the energetic expenditure for chemotaxis influences bacterial energy use.

To reduce energy use and conserve organic matter, bacteria employ strategies such as intermittent movement (Grossart *et al*. 2001) and speed reduction (Mitchell 1991; Mitchell 2002). The production of flagella is proposed to confer an energetic saving on

cells by decreasing rotational diffusion, which reduces the velocity necessary to perform chemotaxis (Mitchell 2002). The importance of controlling velocity is shown in the equation describing basic energy cost, which is given as (Berg 1993):

$$P = 6\pi\eta a v^2 \quad (1),$$

where *P* is the power required for movement, *η* is the dynamic viscosity, *a* is the cell radius, and *v* is the cell velocity of the movement. Since power expenditure increases with the square of the speed in equation (1), reducing speed saves energy. Mitchell (2002) found that chemotaxs could consume up to 50% of cellular energy, which equates to about 6 x 10^3 glucose molecules/s taken up. While moderate nutrient concentrations were used for the 50% estimates (Mitchell 2002), in oligotrophic environments or when nutrients have been exhausted in cultures, the cost of chemotaxis should exceed the available energy flux. So, by conserving energy and making chemotactic movement less demanding, there is more energy available for other respiratory processes or growth.

Past studies on the bacterial energetics of chemotaxis have considered a long, fixed hypothetical flagella (Mitchell 2002). The objectives of this work were to (i) determine the cell and flagella lengths in natural communities, (ii) to calculate the energetic savings cell and flagella lengths provide the cell during chemotaxis, and (iii) to determine the energetic savings that prosthecae confer on bacteria.

MATERIALS AND METHODS

Study locations and sampling strategy

Samples were collected from coastal locations in the Gulf St. Vincent and at the River Murray Mouth (South Australia). Gulf St. Vincent is an inverse estuary where evaporation exceeds precipitation and there is limited exchange to the open ocean (Käempf 2006). Samples were collected from sub-surface waters at 6 locations along the South Australian coastline in October 2006 (Figure 1); Brighton Beach (B), Glenelg marina (G), River Murray Mouth (M) Noarlunga, inside and outside reef (N1 and N2, respectively) and the Port River (P). All locations can be grouped into three distinct categories; metropolitan swimming beaches (B, N1 and N2) and marine (M) or industrial waterways (G and P).

Specifically, the Glenelg location was located at the Holdfast Shores Marina. The Noarlunga locations were located within 1 metre inside and outside the semi submerged rocky reef approximately 150m from the shoreline. The reef waters are oligotrophic and turbulence is created by wave action on the reef as opposed to the shore as in the case of all other locations (Seymour *et al*. 2004).

The Port River is a shallow estuarine waterway comprised of mud flats, mangroves and samphire marshes (Jones *et al*. 1996). The Port River is eutrophic, with high levels of industrial waste and intermittent fresh water flow (Seymour *et al*. 2004). The River Murray Mouth is located at the beginning of the Coorong; a lagoon and lake system extending from the termination of the River Murray. Though a river Mouth, this location exhibits physical and chemical properties characteristic of coastal marine environments (Lamontagne *et al*. 2004).

Sample processing

Water samples were obtained from all locations by submerging 500 ml sterile bottles and allowing them to fill slowly. Bottles were immediately transported to the laboratory to minimise disturbance to the bacteria. Samples were spiked with 10% tryptic soy broth in sterile sea water to a final concentration of 0.1%, to stimulate motility (Mitchell and Barbara 1999) and left at room temperature overnight.

Transmission Electron Microscopy (TEM)

Copper or copper/aluminium Transmission Electron Microscopy (TEM) grids (3 mm, formvar coated and glow-discharged) were inverted on 20 ml of water sample for three minutes, then stained with 7 % phosphotungstic acid for 30 s. Grids were examined using a Phillips CM100 Transmission Electron Microscope at 80 kv. Eight bit digital images were captured using an SIS Megaview II CCD camera and AnalySIS software (Olympus). A total of 219 randomly selected flagellated marine bacteria from the different locations were examined for cell lengthwise radius, flagellum length, flagellum major radii and flagellum helix radii, using Motic Images 2.0 (Motic China Group Co. Ltd). In addition to flagella, some bacteria possessed prosthecae-like cellular protrusions, differentiated based on width, length and straightness. Only the chemotactically competent (Brun *et al*. 1994) flagellated cells were assessed for cell and flagellum length.

Energy required for movement

From morphological data obtained, the minimum energetic power cost required for chemotactic swimming (P_{cs}) was calculated for the mean cell length from each environment using the following equation (Mitchell 2002).

$$P_{cs} = \frac{3kTD_m}{(a\theta)^2} \quad (2),$$

where k is Boltzmann's constant, T is absolute temperature, D_m is translational diffusion, a is the cell radius and θ is the mean turn angle in radians at the end of a run. The energetic power cost with flagellum stabilisation (P_{fs}) was calculated for the mean cell length, mean flagellum length, minimum and maximum flagellum length from each location using the following equation (Mitchell 2002).

$$P_{fs} = \frac{2 \times 10^{-19} a_c}{a_c^3 + a_f^3 \Big/ \{3Ln\left(\frac{2a_f}{b_f}\right) - 0.5\}} + 3a_c^3 \quad (3),$$

where a_c is the cell radius (lengthwise), a_f is the major radii of the flagellum and b_f is the minor radii of the flagellum (3×10^{-6}) cm (Mitchell *et al*. 1995). The mean lengthwise cellular radius (a_c) and flagellum major radius (a_f) was calculated for each location.

Additionally; flagellum minor radii (b_f) was substituted with the maximum radii of the flagella helix ($P_{fsHelix}$) observed at each site to determine any energetic saving conferred by the helical nature of the flagellum. Flagellum major radii (a_f) was substituted with the minimum and maximum flagellum lengths ($P_{fsMinFL}$ and $P_{fsMaxFL}$) observed at each site to determine the effect of flagellum length on energetic expenditure. Prosthecae were also assessed for their contribution to energetic savings (P_{ex}) because in a planktonic state, these structures could further stabilise the cell against rotational diffusion and reduce the speed necessary to perform chemotaxis.

Statistical analysis

Comparisons between mean cell length and mean flagellum length between sites were performed using an ANOVA with Tukey post-hoc analysis, significance was determined when $p < 0.05$ for all cases. Comparisons between mean prosthecae length between sites were performed using the Kruskal-Wallis Test, significance was determined when $p < 0.05$. Comparisons between mean energetic cost required for chemotactic swimming, mean energetic power cost with flagellum stabilisation, mean energetic power cost with flagellum and prosthecae stabilisation, mean energetic power cost with flagellum stabilisation using the minor radii of the flagellum helix, mean energetic power cost with flagellum stabilisation using the minimum and maximum observed flagellum length were performed using an ANOVA with Tamhane post-hoc analysis, significance was determined when $p < 0.05$ for all cases.

RESULTS

General bacterial characteristics

Mean bacterial cell length ranged from 1.6 µm (± 0.1 µm, n = 36) at the Port River to 1.9 µm (± 0.1 µm, n = 43) at Noarlunga outer reef and Brighton (Table 1), although shrinkage due to TEM preparation is likely to have resulted in an underestimation of cell length (Montesinos *et al*. 1983). Mean flagella length varied by 1.4 µm between locations, ranging from 3.9 µm (± 0.3 µm, n = 36) at the Port River to 5.3 µm (± 0.3 µm, n = 43) at Noarlunga Outer Reef (Table 1). A significant difference in mean flagellum length was observed between Noarlunga Outer Reef and the Port River, where flagellum lengths were 5.3 and 3.9 µm respectively ($p = 0.006$). Figure 2A shows a

typical example of a flagellated bacterium. Of the flagellated bacteria observed 81% were bacilli, 10% were coccoid, 6% were vibroid and 3% were other. At all locations, except Glenelg, between 12 to 47% of the flagellated bacterial population possessed prosthecae in addition to flagella (Figure 2B, Table 1). Mean prosthecae length ranged from 4.6 μm (± 1.3 μm, n = 6) at Noarlunga Inner Reef to 10.9 μm (± 3.5 μm, n = 5) at the Murray Mouth, but was not significantly different between locations (Table 1, $p = 0.152$).

Energy required for movement

The minimum energetic cost required for chemotactic swimming with and without flagellar stabilisation was determined for each location (Figure 3). The energetic cost required for chemotactic swimming ranged from 5×10^{-11} g $cm^2.s^{-3}$ at Brighton Beach to 8×10^{-11} g $cm^2.s^{-3}$ at the Port River. The energetic power cost with flagellar stabilisation always reduced the energetic cost of chemotaxis at all sites, and ranged from 2×10^{-11} g $cm^2.s^{-3}$ at Noarlunga Outer Reef to 3×10^{-11} g $cm^2.s^{-3}$ at the Port River (Figure 3). Comparing the mean values of cost required for chemotactic swimming without and with flagellar stabilisation for all locations, possession of a flagellum significantly reduced the energetic cost of chemotaxis from 6×10^{-11} g $cm^2.s^{-3}$ to 2×10^{-11} g $cm^2.s^{-3}$, $p < 0.001$ (Figure 4).

The cost with flagellar stabilisation was also determined using minimum and maximum flagellar lengths from each location (Figure 5), the maximum flagellar length always reduced cost. The shortest mean flagellar length (0.5 μm) actually significantly increased the mean cost of chemotaxis by 3.3 times, from 6×10^{-11} g $cm^2.s^{-3}$ to 2×10^{-10} g $cm^2.s^{-3}$ ($p = 0.002$, Figure 4), whilst mean maximum flagellum length significantly

decreased the cost by 12 times, from 6×10^{-11} g $cm^2.s^{-3}$ to 5×10^{-12} g $cm^2.s^{-3}$ ($p < 0.001$, Figure 4). The cost was high for flagella lengths of 0.2–0.9 µm compared to unstabilized chemotaxis (Figure 6). After the maximum at 0.9 µm, the cost decreased monotonically, becoming less expensive than unstabilized chemotaxis at 3.0 µm (Figure 6).

Previous work (Mitchell 2002) used the flagellar radius as the minor radii (b_f). We noted that the flagella must be helical to work as pointed out by Purcell (1977), and so instead used the helix radius (b_f) to determine the extent to which the helix height contributed to stabilization and cost savings. The helix height reduced the cost at each site, the maximum helix height, 1.1 µm observed at Noarlunga Inner Reef, resulted in the greatest cost reduction (Figure 7). Considering the flagellum helix in our analysis significantly reduced the mean cost from 2×10^{-11} g $cm^2.s^{-3}$ to 1×10^{-11} g $cm^2.s^{-3}$ ($p = 0.026$, Figure 4).

As a final modification of the cost estimates, the role of prosthecae in stabilising cells against rotation and the associated cost savings were calculated. Prosthecae reduced the cost at each site (Figure 8) and a prosthecae of mean length, in addition to a flagellum, significantly decreased cost 5 times from 2×10^{-11} g cm^2/s^3 to 4×10^{-12} g cm^2/s^3 ($p = 0.003$, Figure 4).

DISCUSSION

The production, maintenance and utilization of flagella, whilst energetically costly to an individual bacterial cell (Mitchell 1991; Berg 1993), provides numerous benefits, including the ability to move towards and encounter nutrient sources (Blackburn *et al*. 1998), avoid predators (Matz and Jürgen 2005; Pernthaler 2005) and move away from

repellent substances (Kiørboe and Jackson 2001; Englert *et al*. 2009). Through their activity in marine environments, motile bacteria play an important role in the cycling of organic and inorganic matter (Grossart *et al*. 2001; Fenchel 2002; Seymour *et al*. 2009) and can potentially impact upon global climate cycles (Seymour *et al*. 2010). Despite this crucial role in global biogeochemical cycling, insight into the direct energetic benefits of motility conferred to individual bacterial cells has remained only hypothetical (Mitchell 2002) until now. Here we showed, based on measurements from the marine environment that marine bacteria that expend energy in producing flagella over 3 μm in length are at a distinct cost advantage compared to cells with short or no flagella.

Flagella provide cost savings when moving chemotactically

The production of flagella and motility are estimated to require around 2% (Macnab 1996) to greater than 10% (Mitchell 1991) of the total bacterial energy expenditure. Flagellar genes are well conserved between bacterial species (Macnab 1996) and studies indicate that motility is so valued by marine bacteria that even when starved, bacteria will reduce their cellular size but still retain their ability to swim at high speed (Kjelleberg *et al*. 1982; Matz and Jürgens 2005). Additionally, the enteric bacterium *Escherichia coli* has been shown to increase motility and chemotactic movement when grown on poor quality carbon sources (Zhao *et al*. 2007). These studies indicate that the potential benefits that motility confers on bacteria outweigh the substantial energetic cost of producing and maintaining flagella.

Efficient movement allows for bacterial survival in the marine environment (Sibona 2007), where resources are heterogeneous and nutrient patches dissapate quickly (Blackburn *et al*. 1998). Specifically, motility enhances the encounter rate between

bacteria and nutrient resources and allows bacteria to colonise sinking aggregates and nutrient patches. In such heterogeneous environments we have shown that even a marine bacterium's single polar flagellum provides a significant cost saving during chemotactic movement (Figure 3, Figure 4). All marine bacterial communities investigated in this study followed an observed cost saving trend correlated to flagellum length, independent of environment type (Figure 3). This result, while unsurprising, was not the only possible outcome. A model of chemotactic cost suggested that there was a cost minimum as a function of cell size, flagella length and swimming speed, and that outside of this minimum the cost rose rapidly as a cubic or square power function (Mitchell 2002). The bacteria from the marine environments we sampled all fell in the cost minimum zone. This supports the concept of a cost minimum and suggests that the energetic advantages of possessing flagella of a particular length and helix amplitude is a general principle that applies to all bacterial communities.

Our comparison of flagellated swimming cost to non-flagellated swimming might explain why non-flagellated swimming is seldom encountered. Chemotactic movement towards nutrient sources without the aid of flagella occurs in the cyanobacteria *Synechococcus* strain WH8113 (Waterbury *et al*. 1985; Brahamsha 1996; Pitta *et al*. 1997), which is capable of chemotactic swimming using pulsed surface flow (Ehlers *et al*. 1996). However, this genus is phototrophic, so energy supplies are probably not limiting, at least during light periods. Self-electrophoresis driven locomotion is also theoretically possible by bacteria without flagella (Lammert *et al*. 1996), however, this is predicted to not be possible for marine bacteria, including *Synechococcus* strain WH8113, due to the conductivity of seawater (Pitta and Berg 1995). We note that motility via pulsed surface flow has only been observed in *Synechococcus* strain WH8113, which suggests that it is not a common chemotactic movement strategy. Whilst the cost of this is

unknown, its rarity suggests that flagella provide chemotactic bacteria with a distinct advantage over non-motile and non-flagellated, motile bacteria.

Coupling the ability to “turn” motility on and off (Mitchell *et al*. 1995; Grossart *et al*. 2001) with the flagellar stabilisation described here confers a significant energy savings. By actively seeking out nutrient sources motility allows cells to accumulate nutrients and gain a competitive edge over sessile bacteria that rely on nutrients diffusing to them, while stabilisation allows nutrient accumulation to be directed primarily to biomass and thus contributes to trophic transfer.

The disadvantage of motility is that it increases encounters with predators (Blackburn *et al*. 1997; Matz *et al*. 2002) and as such could potentially be detrimental to survival. However, motile bacteria have greater opportunity to escape predation and highly motile species exhibiting fast swimming speeds have been observed to increase under grazing pressure (Grossart *et al*. 2001; Jügens and Matz 2002; Matz and Jürgens 2005). It may also be useful to distinguish between random motility and directed chemotaxis, which by its nature explores a more restricted volume than random movement. As a result of these studies it remains unclear the extent to which bacteria are ever at a disadvantage in moving.

Importance of flagellar length in chemotactic movement

Our results indicate that control of flagellar length may be of greater importance to cells than previously appreciated. Also, as cells produce flagella there may be benefits in delaying use as cells with shorter flagella incur greater costs compared to cells with flagella of the lengths reported here (Figure 5). The mean minimum observed flagella

length ($P_{fsMinFL}$) resulted in significantly greater costs than the mean minimum cost required for unstabilized swimming (P_{cs}) (Figure 2). This demonstrates that the initial stages of flagellar growth are a cost rather than a benefit to a cell. Flagellar synthesis is a lengthy process, generally taking a generation or longer to produce a flagellum that is able to confer motility (Aizawa and Kubori 1998), however, motility can be used intermittently, thereby saving energy. One study observed that motile bacteria move less than 20% of the time (Grossart *et al*. 2001), therefore bacteria should encounter enough nutrient patches in surface waters (Kiørboe *et al*. 2002) to sustain them in the initial costly stages of flagellar growth.

The longest flagella, with a length of 12.2 µm at Brighton (Table 1), did not have the lowest cost. The lowest cost was incurred by bacteria with flagella length of 10.6 µm at Glenelg, where the greater cell length compensated for the shorter flagella (Table 1, Figure 5). This indicates that in the marine environment there is a combined effect of flagellar length and the cell length. There is a claim that there is no control for flagellar length (Macnab 2003). The narrow range of lengths found here suggest that there is some as yet undescribed control mechanism. To further investigate the impact of flagellar length on cellular cost, in Figure 6, flagellar length was extrapolated at lesser and greater than the minimum and maximum mean flagellar lengths observed at each location in this study. We determined that there are three distinct flagellar length ranges for cost. Initially, the minimum cost with stabilisation increases for flagellar lengths of 0.2 – 0.9 µm where cost reaches a maximum. In this initial range a flagellum is not long enough to substantially increase the drag on the cell; therefore there is minimal decrease in a cell's rotation and no stabilisation benefit (Mitchell 2002) and the production of flagella of these lengths is energetically disadvantageous to the cell. However, in the second region this trend is reversed and an increase in flagellar length

beyond 0.9 μm results in increased rotational drag on the cell, which causes rotational stabilization, which allows the cell to chemotax at slower speeds. In region three, as length passes 3.0 μm, the cost of stabilisation is less than the cost required for chemotactic swimming and the cell benefits from the effects of drag on the flagellum. Our results modify previous claims (Dusenbery 1997; Mitchell 2002), as it is from this point of 3.0 μm in flagella length that the combined benefits of stabilisation and potential acquisition of nutrients outweigh the energetic costs of production and maintenance.

Marine bacterial communities, at least in South Australia, exhibit a narrow range of mean flagellar lengths (Figure 6, Table 1), which are well below the theoretical maximum length of 20 μm for free swimming (Tanner *et al*. 2011), but long enough to exploit the cost saving benefits of flagellar stabilisation during chemotactic movement. As flagella lengthen beyond approximately 13 μm the cost savings becomes marginal and increases in flagellar length only have a small impact on cost saving (Figure 6).

The helix of a flagellum

The helical structure of the flagellum increases stabilisation and reduces cost (Figure 4). Intrinsic helicity has recently been shown to contribute to the smooth nature of flagellar movement (Clark and Prabhakar 2001). To this we have added that intrinsic helicity also contributes to cost savings (Figure 4, Figure 7).

Environmental bacterial communities

Eighty percent of motile cells were bacilli, which is consistent with previous environmental observations (La Ferla and Leonardi 2005), predictions that a bacilli shape makes swimming and detection of stimulus more efficient (Dusenbery 1998) and Young's (2006) prediction that predation pressure should favour the survival of small fast moving bacillus. Of the cells photographed and measured all but one were greater than Dusenbery's (1997) predicted minimum cell diameter of 0.6 µm for flagellated cells. Dusenbery (1997) estimated that cells below this size would not obtain any benefits from movement. The one observed cell less than 0.6 µm in size is likely to be smaller due to measurement error induced by shrinkage during sample preparation, although there is some debate as to the significance of shrinkage in relation to TEM sample preparation, (Montesinos *et al*. 1983; Fagerbakke *et al*. 1996; Gundersen *et al*. 2002) or shrinkage due to starvation. For the latter of these, some bacteria are known to reduce cell size, but retain the ability to swim after being starved (Matz and Jürgens 2005). Motility without chemotaxis has been shown in *Vibrio cholerae* (Butler and Camilli 2004) so there is a precedent for benefits from non-chemotactic motility. Given the caveats of the single sub-minimum cell, our measurements are consistent with Dusenbery's (1997) predicted minimum. Whilst some studies have measured flagella length in natural aquatic communities (e.g. Jannasch and Jones 1959, Hirsch and Pankratz 1970) to the authors' knowledge this is the first study to accurately measure each flagellum observed and investigate the impact of flagella length on chemotactic swimming natural marine communities.

Prosthecae stabilisation

Prosthecae are a prominent feature in 12 - 47% of this planktonic bacterial community, (Table 1, Figure 2). Prosthecae are often associated with *Caulobacter* (Bodenmiller *et al*. 2004), and are produced in nutrient poor environments, as they enhance nutrient uptake through increasing a cell's surface area (Gonin *et al*. 2000; Young 2006). They are also involved in reproduction, deterring predators, surface attachment, slowing settling and orientation of a cell near to the air water interface (Bodenmiller *et al*. 2004; Young 2006). To this list we can add rotational stabilisation as determined by our study. Most prosthecate cells observed were clearly in the process of dividing when photographed and as a result the prosthecae observed are likely to be present as a part of the cellular division process. In division, prosthecate producing bacteria, such as *Caulobacter* species, produce a motile flagellated cell and a sessile attached prosthecate cell (Bodenmiller *et al*. 2004) and while the holdfast in *Caulobacter* is strong (Tsang *et al*. 2006) it is possible that the prosthecate cells observed in this study were somehow dislodged. Although they have not previously been considered in cellular motility (Brun *et al*. 1994; Jacobs-Wagner 2004), and are reported to be used for attachment of dividing cells, prosthecae still provide a cost benefit when cells detach (Figure 4, Figure 8). As the total length is important in energy savings, examination of the role of prosthecae in stabilisation and energy savings is important.

Broad Implications

Flagellated chemotaxis cost 3 times less than non-flagellated chemotaxis. The presence of a helix in a flagellum further halved the cost. A prosthecae reduced cost by another 2.5 times. The overall effect is that cellular protrusions reduce cost by 15 times (6×10^{-11} g $cm^2.s^{-3}$ to 4×10^{-12} g $cm^2.s^{-3}$). Since the motility mechanisms are estimated

to be only 1% efficient (Berg 1983, Mitchell 1991), the energy output of 6×10^{-11} g $cm^2.s^{-3}$ to move the cell for unstabilized chemotaxis requires the consumption of enough dissolved organic matter to produce 6×10^{-9} g $cm^2.s^{-3}$. Converting this energy consumption to glucose equivalents allows for the fraction of uptake used for chemotaxis to be calculated (Mitchell 1991). Assuming a moderate dissolved organic monomer concentration of 0.1 µM glucose equivalents, which is probably high for standard marine bacteria in the ocean, gives a flux of 6×10^3 molecules/s (Mitchell 1991). At the optimal yield of 1.8×10^{-11} g $cm^2.s^{-2}.glucose^{-1}$ equivalents, 330 glucose molecules are required, or about 5% of the total flux. However, the bacteria measured here, at a radius of 1 µm, are at the high end of the size range for marine bacteria; for the common radii of 0.2 µm (Mitchell 1991) the cost is 30 times greater, or 165% of the total organic matter flux into the cell. Even the lower limit of 5% is significant considering that Lenski *et al*. (1991), using growth as a proxy for energy use, found that in a competitive environment a 0.01% difference in growth resulted in a 10% fitness difference and led to competitive exclusion. The results of our study combined with Lenski *et al*. (1991) suggest that without the stabilizing effect of flagella and the helix amplitude, chemotaxis would place at least all chemotactic bacteria at a selective disadvantage.

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Table 1 Mean bacterial cell length (µm ± standard error) and samples size, mean flagellum length (µm ± standard error), flagellum length range (µm), mean prosthecae length (µm ± standard error) and sample size and prosthecate length range (µm) at each location.

Location	Mean Cell Length (sample size)	Mean Flagellum Length	Flagella Length Range	Mean Prosthecate Length (sample size)	Prosthecate Length Range
Brighton (B)	1.9 ± 0.1 (31)	4.7 ± 0.3	2.1 - 12.2	10.2 ± 1.1 (17)	3.8 – 20.3
Glenelg*(G)	1.8 ± 0.1 (43)	4.9 ± 0.3	1.2 – 10.6	5.8 ± 0.0 (1)	NA
Murray Mouth (M)	1.7 ± 0.1 (34)	4.6 ± 0.3	1.0 – 9.4	10.9 ± 3.5 (5)	3.9 – 24.1
Noarlunga Inner Reef (N1)	1.8 ± 0.1 (32)	5.1 ± 0.8	0.6 – 9.5	4.6 ± 1.3 (6)	1.6 – 8.8
Noarlunga Outer Reef (N2)	1.9 ± 0.1 (43)	5.3 ± 0.3	1.1 – 9.8	7.0 ± 1.7 (5)	2.6 – 11.7
Port River (P)	1.6 ± 0.1 (36)	3.9 ± 0.3	0.5 – 9.5	10.6 ± 5.6 (4)	4.1 – 27.4

* 1 prosthecate cell observed

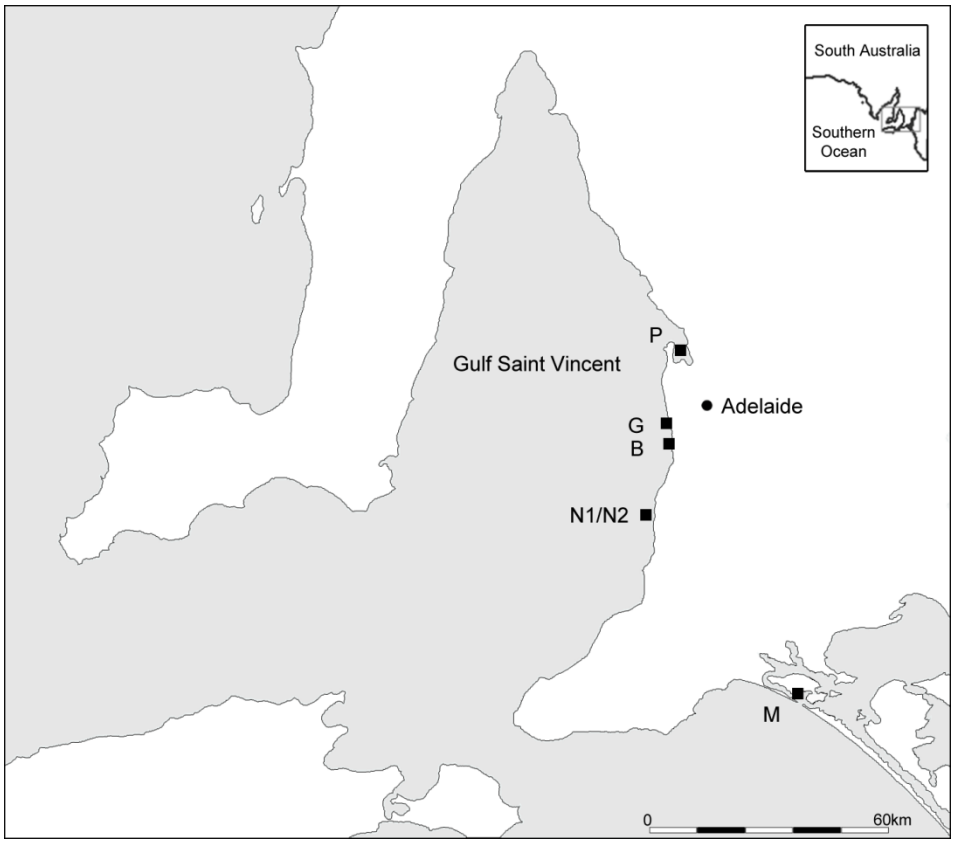

Figure 1 Study site; Gulf St. Vincent and River Murray Mouth (South Australia) showing the location of the 6 sampling sites. Brighton Beach (B), Glenelg marina (G), Murray Mouth (M) Noarlunga, inside and outside reef (N1 and N2, respectively) and the Port River (P).

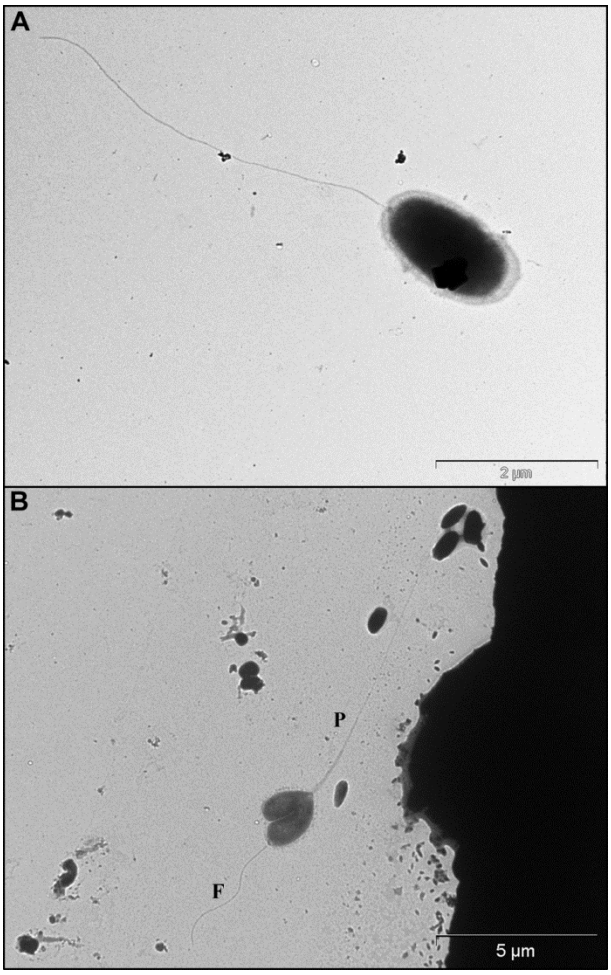

Figure 2 Electron micrograph of (A) typical flagellated bacterium sampled from the Port River and (B) dividing prosthecae bacterium sampled from the Murray Mouth. F denotes flagellum and P denotes prosthecae.

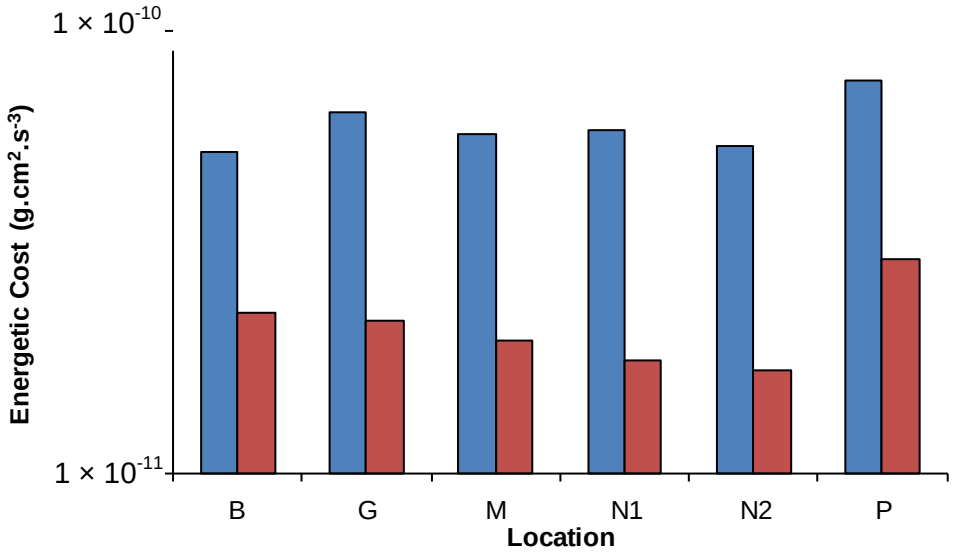

Figure 3 Minimum energetic power cost of chemotactic swimming (P_{cs};) and energetic power cost of swimming with flagella stabilisation (P_{fs};) at each location.

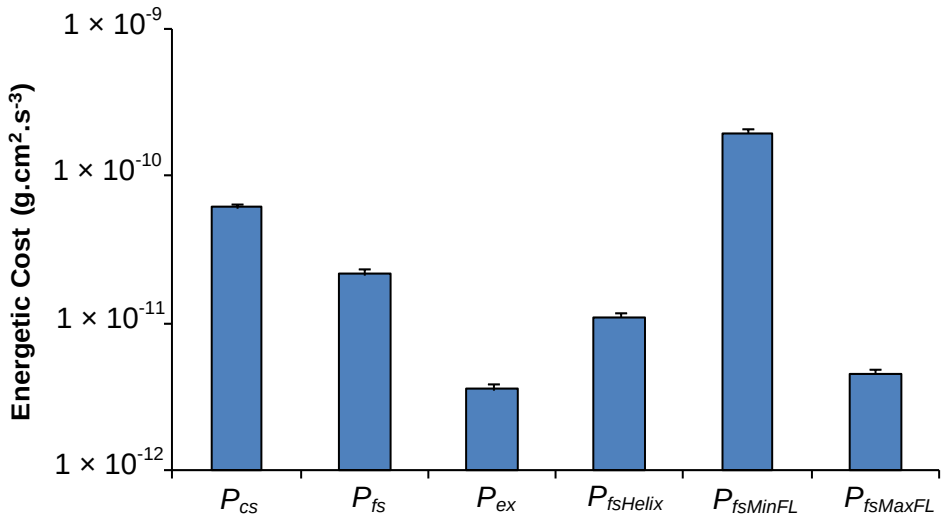

Figure 4 Mean minimum energetic power cost of chemotactic swimming (P_{cs}), energetic power cost of swimming with flagella stabilisation (P_{fs}), energetic power cost of swimming with flagella and *prosthecae* stabilisation (P_{ex}), energetic power cost with flagellum stabilisation using the major radii of flagellum helix ($P_{fsHelix}$), energetic power cost with flagellum stabilisation using minimum observed flagellum length ($P_{fsMinFL}$) and maximum observed flagellum length ($P_{fsMaxFL}$) of all locations, error bars are standard error of the mean.

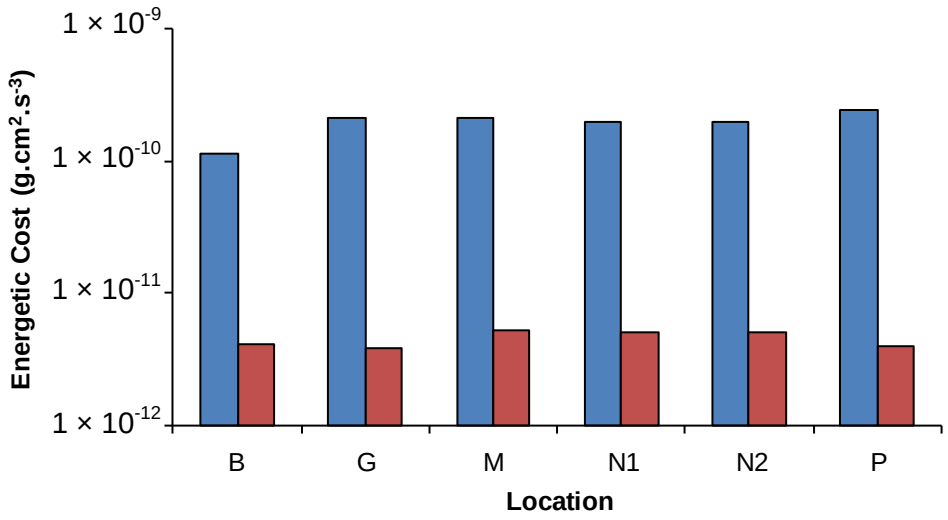

Figure 5 Energetic power cost with flagellum stabilisation (P_{fs}) of minimum (▭) and maximum (▭) flagellum lengths at each location.

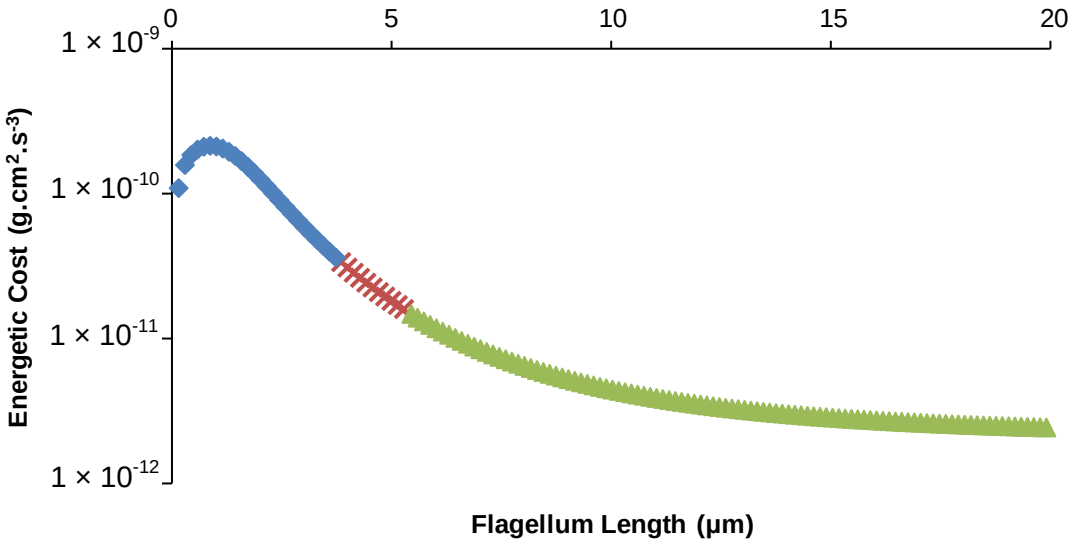

Figure 6 Energetic power cost with flagellum stabilisation (P_{fs}) predicted for a range of flagellum lengths lesser than (◆) and greater than (▲) the minimum and maximum of mean flagellum lengths observed at each site in this study (X).

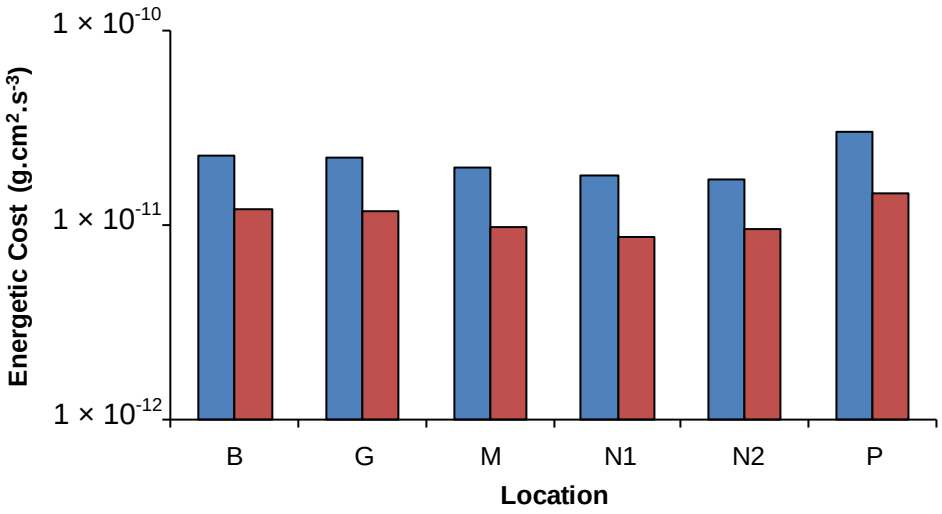

Figure 7 Energetic power cost with flagellum stabilisation (P_{fs}) using the minor radii of single flagellum (▭) and the major radii of flagellum helix (▭) at each location.

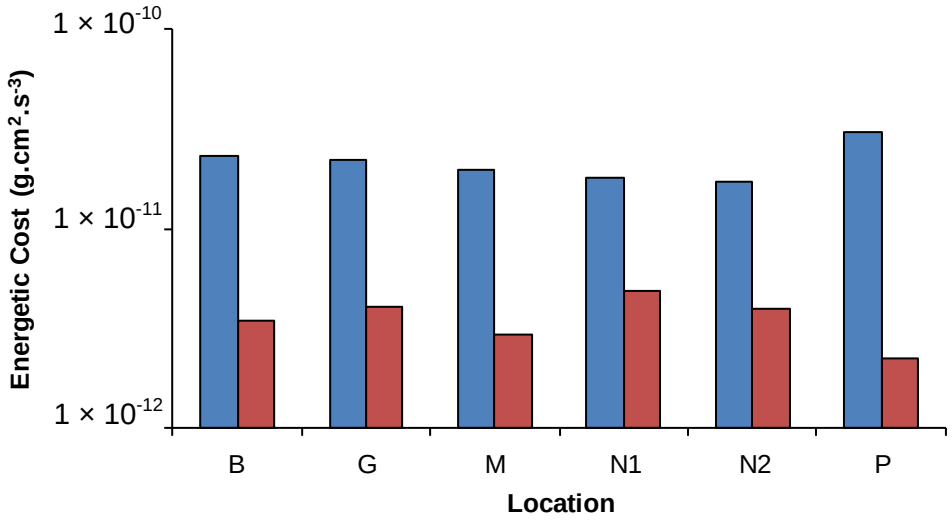

Figure 8 Energetic power cost of swimming with flagella stabilisation (P_{fs}; ▭) and energetic power cost of swimming with flagella and prosthecae stabilisation (P_{ex}; ▭) at each location.

Chapter VII

Discussion

The activities of the microbial members of the aquatic food web influence the cycling of energy, matter and elements, thereby determining the composition of the entire aquatic community (Fuhrman 1999; Suttle 2005; Rohwer and Thurber 2009). Interestingly, these communities are themselves influenced by “bottom-up” physiochemical parameters and “top-down” mortality pressures (Bouvier and del Giorgio 2002; Pernthaler 2005). The interplay of these factors controls the structure and function of the microbial community and how resources are allocated (Fuhrman 1999; Pernthaler 2005). As anthropogenic influences affect aquatic ecosystems, the elucidation and qualification of the microbial community’s response to environmental change is of vital importance. This thesis investigated microbial community processes, diversity and structure throughout the Coorong estuarine, lagoon and lake system in South Australia. The large natural salinity gradient present in the Coorong was exploited to examine the influence of salinity on bacterial and phytoplankton mortality; bacterial community diversity and metabolic potential; and viral community structure, diversity and production. Additionally, energetic conservation strategies and the physical characteristics of the bacterial community were investigated in the Coorong and other temperate coastal environments.

This thesis initially addressed the processes of microzooplankton grazing and viral lysis of bacteria over a large salinity gradient. As energy and matter derived from heterotrophic bacterial mortality can be transferred up the food web by grazing or recycled in the microbial loop by lysis, heterotrophic bacterial mortality impacts upon movement of energy through the system and is a selective force acting upon other organisms (Fuhrman 1999). As bacterial size affects the resulting energy and matter input into the system, the extent to which bacterial physiology, specifically cell size and

DNA content, influences a sub-population's specific mortality was also investigated (Chapter II). Results showed that an increase in salinity does not uniformly alter the contribution of grazing and lysis to bacterial mortality, however, it was found that viral lysis has a significant and important impact on the bacterial community at higher salinities. Grazers and viruses were observed to have dissimilar impacts upon different sub-populations of the bacterial community, with most bacterial sub-populations being significantly predated upon by either grazers or viruses and only a few attracting equal predation pressure. In general, larger bacteria with higher DNA content were favoured by grazers while viruses lysed the most abundant bacterial sub-populations. That one group of predators (i.e. grazers or viruses) may have little to no impact on the mortality of a specific bacterial sub-population, has not been considered previously and highlights that energy and matter flow in aquatic environments varies greatly depending on the bacterial prey population.

While heterotrophic bacteria recycle energy, matter and nutrients in aquatic systems, phytoplankton (including photosynthetic bacteria, diatoms etc.) are responsible for primary production. As such, the contribution of grazing and lysis to their mortality also affects biogeochemical cycling and aquatic community structure (Fuhrman 1999). Results from dilution experiments again indicated no clear link between the dominant "top-down" mortality agent and salinity (Chapter III). While salinity is a strong "bottom-up" selective influence on microbial communities, it is likely that the impact salinity of on the agents of mortality also plays a part. Therefore, this should be considered when investigating how physiochemical parameters affect microbial processes. Also noted is the importance of viruses to phytoplankton mortality. Although consideration of lysis is growing in the literature (Evans *et al.* 2003; Baudoux *et al.* 2006), some recent studies

still overlook the impact of viral lysis on the phytoplankton community (Grattepanche *et al.* 2011).

Though Chapters II and III showed the impact of salinity on the “top-down” processes of grazing and lysis was more complex than initially envisioned with no clear relationship between mortality with increasing salinity, salinity was found to have a significant “bottom-up” impact on the taxonomic and metabolic potential of the bacterial community at the extremes of salinity in the Coorong (Chapter IV). Analysis of bacterial metagenomes at the extremes of salinity in the Coorong revealed a significant increase in the number of halotollerant taxa at hypersaline conditions, due to an increase in biomass typical of highly saline aquatic environments (Guixa-Boixereu *et al.* 1996). A significant increase in metabolisms associated with increased cellular growth and activity was also observed. The river mouth community reflected a significant increase in taxa associated with anthropogenically affected aquatic environments and metabolisms reflective of varying nutrient input. This highlights that the community is able to exploit numerous potential nutrient sources no matter how small the concentration, and demonstrates a significant contribution from human activities on the water quality and microbial community of the Murray River. Comparison of the two Coorong metagenomes to other publicly available aquatic metagenomes revealed that taxonomically and metabolically the Coorong metagenomes were most similar to metagenomes from marine environments, as opposed to other aquatic environments. However, of the marine group the Coorong metagenomes showed the least similarity. These results indicate a highly adaptive marine-like bacterial community able to respond quickly to the specific physiochemical and anthropogenic stressors of the Coorong.

Bacterial and phytoplankton mortality experiments (Chapters II and III) revealed that viruses have considerable impact on the bacterial and phytoplankton community of the Coorong. Their actions reduce upwards trophic transfer of energy and matter and thus have flow-on implications for biogeochemical cycling in all aquatic environments (Suttle 2005). While the impacts of salinity on viral "top-down" action remain complex (Chapters II and III), it is well characterised that viral abundance increases with salinity and certain viral morphotypes are present in greater abundance in extreme environments (Le Romancer *et al.* 2007). However, the impact of salinity on the production of new viruses is less well characterized. To the author's knowledge the only estimates of viral production from hypersaline environments have been obtained from solar salterns, and these showed that viral production increases at higher salinities (Guixa-Boixereu *et al.* 1996). It appears that high bacterial abundance in hypersaline environments increases viral production (Chapter V). In the Coorong, increased salinity drove an increase in viral abundance, production, community morphological diversity and the occurrence of "extreme" viral morphotypes. Increased morphological diversity at higher salinities reflects the greater abundance of potential bacterial or archaeal hosts and allows viruses to exploit a wider range of morphologies (i.e. niche specialization).

Utilisation of energy moving through the microbial food web is as important to cellular survival as the trophic direction that it flows (Blackburn *et al.* 1998). This thesis investigated a crucial paradox of microbial survival: how expending energy and cellular resources on costly optional functions such as flagella, with no guarantee of energetic

gain, assist in bacterial cellular survival (Chapter VI). Results from natural bacterial communities sampled from the Coorong and other temperate coastal sites confirmed previous theoretical calculations (Mitchell 2002) and showed that not only can flagella provide a competitive edge by allowing bacterial cells to swim towards nutrient sources and away from disadvantageous situations, but also that flagella over 3.0 µm actually save bacteria energy when moving chemotactically. Results indicate that energy utilisation in bacterial populations has a substantial impact on marine energetic budgets and should be considered in future investigations.

This thesis aimed to investigate the diversity, structure and specific processes that occur between the different members of the aquatic microbial food web as salinity increases in the Coorong estuary, lagoon and lake system. In doing so, this thesis also provides the first comprehensive study of bacterial and phytoplankton mortality; taxonomic diversity and metabolic potential of the bacterial community; viral community morphological diversity, viral production and the energetic saving conferred to aquatic bacteria by flagellum in the Coorong.

As in other aquatic environments, this thesis finds that salinity is the major physiochemical factor structuring the diversity and abundance of the Coorong's unique marine-like bacterial and viral diversity and abundance, and bacterial metabolic potential. This thesis highlights that differential mortality on individual bacterial sub-populations impacts the flow of energy, matter and elements through the food web, however, the impact of salinity on these "top-down" processes was harder to elucidate. As salinity impacts and structures both the prey bacterial/phytoplankton community and

the predatory viral and microzooplankton communities then the impact of salinity on both prey and predator community structure must be assessed when determining bacterial or phytoplankton mortality as this can lead to differential mortality patterns. This thesis also confirmed that energy and nutrient uptake by bacterial cells used in chemotactic movement can reduce energetic expenditure and thereby place these bacteria at a competitive advantage

Many anthropogenic factors impact on the microbial community, one of the most important is salinity (Lozupone and Knight 2007). The “bottom-up” effect of increased salinity on bacterial and viral community diversity and function can be determined. However when the “bottom-up” effects of salinity on community diversity interact with the “top-down” effects of mortality the interplay between the impacts of salinity on both groups and the interactions between these groups then leads to the clouding of the true consequences of anthropogenic change. The complexity of the microbial food web is well known. More difficult to elucidate, however, is how interactions between different members of this web will change in response to anthropogenic modification of aquatic ecosystems. As the aquatic microbial community impacts upon global processes, evolution and indeed life on Earth determining the impact of salinity on the microbial food web are of critical importance.

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Rohwer F and Thurber RV (2009) Viruses manipulate the marine environment. Nature 459: 207-212

Suttle CA (2005) Viruses in the sea. Nature 437: 356-361

Chapter VIII

Appendices

The following publications are manuscripts the author contributed to during her candidature in which she developed techniques and concepts that contributed to her thesis.

Seymour JR, Doblin MA, Jeffries TC, Brown MV, **Newton K**, Ralph PJ, Baird M, and Mitchell JG (2012) Contrasting microbial assemblages in adjacent water-masses associated with the East Australian Current, Environmental Microbiology Reports. Published online 27.06.2012

Jeffries TC, Seymour JR, **Newton K**, Smith RJ, Seuront L and Mitchell JG (2012) Increases in the abundance of microbial genes encoding halotolerance and photosynthesis along a sediment salinity gradient. Biogeosciences. 9: 815-825

Smith RJ, Jeffries TC, Roudnew B, Fitch AJ, Seymour JR, Delpin MW, **Newton K**, Brown MH and Mitchell JG (2012) Metagenomic comparison of microbial communities inhabiting confined and unconfined aquifer ecosystems. Environmental Microbiology. 14: 240-253

Jeffries TC, Seymour JR, Gilbert JA, Dinsdale EA, **Newton K**, Leterme SC, Roudnew B, Smith RJ, Seuront L and Mitchell JG (2011) Substrate type determines metagenomic profiles from diverse chemical habitats. PloS One. 6 (9) e25173.doi

Seuront L, Lacheze C, Doubell MJ, Seymour JR, van Dongen-Vogels V, **Newton K**, Alderkamp AC and Mitchell JG (2007) The influence of *Phaeocystis globosa* on microscale spatial patterns of chlorophyll *a* and bulk-phase seawater viscosity. Biogeochemistry. 83:173–188