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Virally-Mediated Versus Grazer-Induced Mortality Rates in a Warm-Temperate Inverse Estuary (Spencer Gulf, South Australia)

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Abstract

We investigated the seasonal dynamics of flow cytometrically-defined populations of viruses, heterotrophic bacteria, and the picoeukaryotic and prokaryotic phytoplankton at three sites in the temperate oligotrophic inverse estuary of Spencer Gulf (South Australia). We consistently identified two sub-populations of viruses, three sub-populations of heterotrophic bacteria, one population of picoeukaryotic phytoplankton and two populations of prokaryotic phytoplankton (cyanobacteria *Prochlorococcus* and *Synechococcus*). Both the cytometric community composition and the abundance of viruses, heterotrophic bacteria and both prokaryotic (*Synechococcus* and *Prochlorococcus*) and eukaryotic picophytoplankton were consistent with previous observations conducted in South Australian continental shelf waters. Noticeably LDNA bacteria (*i.e.* inactive or dormant cells) were consistently significantly the most abundant group of heterotrophic bacteria (totaling from 29% to 68% of total bacterial abundance) and were up to 10-fold more abundant than that previously reported in South Australian continental shelf waters, including the nearby Saint Vincent Gulf. These results suggest an overall low activity of the microbial community, and are consistent with previous evidence that LDNA cells may play a greater role in heterotrophic processes than HDNA cells in oligotrophic waters. In an attempt to further assess the qualitative and quantitative nature of the mortality of these organisms, we used a specific dilution assay to assess the relative contribution of viruses and microzooplankton grazers to the mortality rates of heterotrophic bacteria, and picoeukaryotic and prokaryotic phytoplankton. We consistently reported site-specific, population specific and sea-son-specific viral lysis and grazing rates of heterotrophic bacteria and the picoeukaryotic and prokaryotic (cyanobacteria *Prochlorococcus* and

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***Synechococcus*) phytoplankton across sites and seasons. Specifically, both viral lysis and mi-crozooplankton grazing rates of heterotrophic bacteria were consistently relatively low across sites and seasons, even though their seasonality suggested an overall dominance of grazing over viral lysis in both summer and winter. In contrast, no seasonality is found in either lysis or grazing rates of prokaryotic and eukaryotic picophytoplankton, which are comparable to previous observations conducted in oligotrophic waters, suggesting the mortality dynamics of these populations is similar to those en-countered in other oligotrophic waters. The observed patterns of mortality rates of heterotrophic bacteria and both prokaryotic and eukaryotic picophytoplankton are consistent with the low chlorophyll concentration and production previously observed in the waters of the Spencer Gulf.**

Keywords

Lysis, Grazing, Bacteria, Viruses, Prokaryotic and Eukaryotic Picophytoplankton, Dilution Assay

1. Introduction

Viruses are ubiquitous in the world ocean, and by far the most abundant (typically in the range 10^5 to 10^8 ml^{-1}) biotic agent [1]-[3]. Viruses play a critical role in marine biogeochemical cycling through their role in the transformation of host cells into bio-available dissolved organic matter, hence the diversion of carbon away from the classical food web toward microbially-mediated recycling processes [1]-[4]; they are estimated to be responsible for *ca*. 10^{29} infections per day, which ultimately lead to the release of 10^8 to 10^9 tons of carbon per day from the biological pool [3]. Beyond the ongoing biogeochemical debate whether viruses 1) short-circuit the biological pump by releasing elements back to the dissolved phase [5]; 2) prime the biological pump by accelerating host export from the euphotic zone [6]; or 3) drive particle aggregation and transfer of car-bon into the deep sea through the release of sticky colloidal cellular components during viral lysis [7]. There is also compelling evidence that viral activity potentially affect population dynamics, community structure, diversity and genetic transfer [8] [9]. Viral activity is also expected to increase net community respiration (hence to decrease the production/respiration ratio), and accelerates the recycling of potentially growth-limiting nutrients [10]. Viruses may hence potentially either hinder or stimulate biological production, which is an issue of global significance, especially in oligotrophic waters.

The role of viral lysis is acknowledged in the mortality of marine bacteria and phytoplankton [2] [3] [11]-[13]. The study of relative contributions of viral lysis and microzooplankton grazing to bacteria and phytoplankton mortalities is, however, still overlooked [3] [14]-[16]. In contrast, there is a plethora of literature solely devoted to microzooplankton grazing in a range of environments; see [17]-[19] for re-views. As viral lysis may account for 2% to 77% of bacterial mortality in the ocean [1] [8] [9], a thorough assessment of the relative contribution of microzooplankton grazing and virally-mediated mortality is critically needed to improve our understanding of the transfer pathways of organic matter throughout marine ecosystems. Both microzooplankton grazing and viral lysis regenerate nutrients. However, there are fundamental differences in 1) the resulting physical (size classes of organic material) and chemical (organic *vs*. inorganic) speciation of released materials; and 2) the redistribution of nutrients to more organic species through viral lysis relative to the inorganic forms excreted by grazers that may respectively regulate community diversity and productivity and shift the competitive equilibrium for growth-limiting nutrients between phytoplankton and bacteria [1]-[3] [10].

A specific assay that allows a direct estimation of the relative contribution of virus-induced and grazer-induced algal mortality has been successfully applied for the picophytoplankter *Micromonas pusilla* during a mesocosm study [20], to the nanophytoplankter *Phaeocystis globosa* during a field study in temperate eutrophic coastal waters [14] and in oligotrophic waters of the open ocean to different groups of prokaryotes and eukaryotes in the subtropical northeastern Atlantic [15] and to the phytoplankton <200 μm in the Southern Ocean southeast of the Kerguelen [16]. To our knowledge, this dilution assay has, as yet, never been applied to assess the mortality rates of both heterotrophic bacteria and the eukaryotic and prokaryotic components of the picophytoplankton community. In particular, beyond the general paucity of information available in the literature, no study has yet focused on the microbial dynamics of inverse estuaries, known to seasonally alternate between

oligotrophic and mesotrophic/eutrophic conditions; see e.g. [21]. In this context, the present work investigates the nature of the viral, microbial and picophytoplanktonic communities of the Spencer Gulf, a South Australian warm-temperate inverse estuary considered oligotrophic due to the paucity of both riverine and terrigenous inputs and precipitation, and characterized by a relatively low primary productivity [22]-[24] that is unique at times phosphorus-limited [25], and subsequently low secondary productivity that does not exert a control on the phytoplankton community [22]-[25]. These results suggest a bottom-up control of the matter cycling in the Spencer Gulf, hence we stress the need to thoroughly assess the structure and function of the microbial food web in these waters.

Specifically, the objectives of the present work were 1) to assess the nature of the viral, microbial and picophytoplanktonic communities; and 2) to elucidate the relative contributions of virally-induced mortality and microzooplankton grazing in heterotrophic bacteria, and the picoeukaryotic and prokaryotic (cyanobacteria *Prochlorococcus* and *Synechococcus*) phytoplankton at three sites representative of the hydrographic conditions encountered from the oceanic mouth to the inland head of the Spencer Gulf (South Australia), in spring, summer, autumn and winter.

2. Materials and Methods

2.1. Study Site

Spencer Gulf is the largest marine embayment along the southern Australian coastline (**Figure 1**). It extends northwards *ca*. 320 km and is 130 km wide at its mouth, with an average width of 60 km (**Figure 1**). The coastal

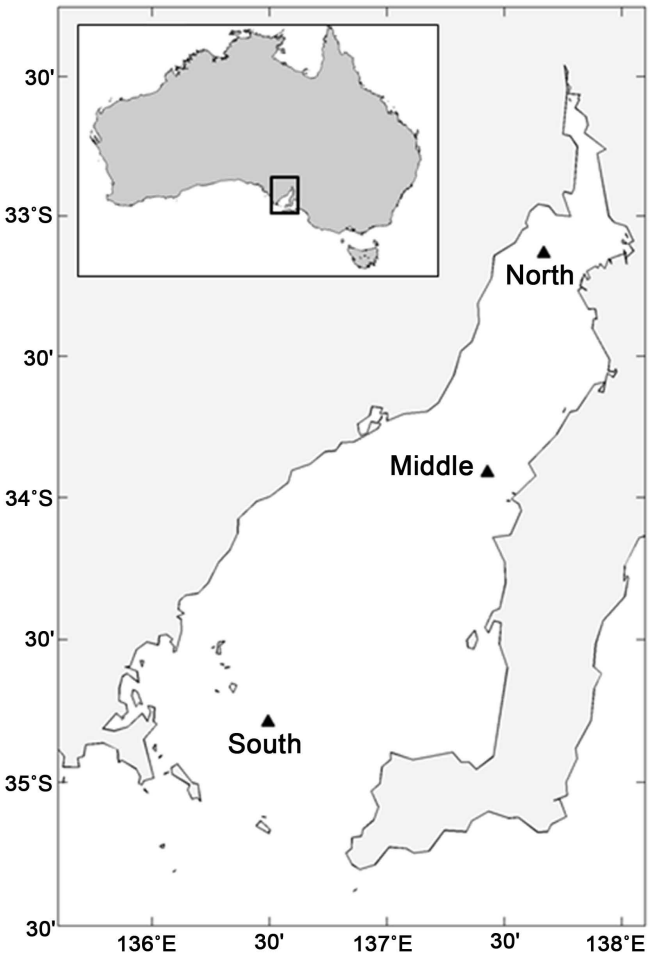


Figure 1. Study area and sampling sites within Spencer Gulf, South Australia.

geometry affords some shelter from predominantly southwest ocean-generated swell [26]-[28]. No permanent rivers bring freshwater or terrigenous sediments into the Gulf, and the semi-arid (annual rainfall typically <550 mm) [26] [29] warm-temperate climate results in seasonal heating and high evaporation. Salinity hence ranges from oceanic salinity at the Gulf mouth up to *ca.* 50 PSU at the inland head in late summer, leading to a pattern of inverse estuarine circulation with low productivity and little seasonal variation [30]-[32]. Specifically, this circulation pattern is imposed on an overall internal clockwise gyral circulation that brings marine waters into the Gulf from the west. These waters are modified therein and exit the Gulf in the east as a mesotrophic saline outflow. Whilst the gulf experiences outflow throughout the year, this outflow is enhanced during autumn and winter seasons as salty waters formed in northern regions of the gulf are cooled forming a gravity current, which exists the gulf along its eastern flank [33] [34]. With an average water depth of *ca*. 24 m (maximum depth is only 60 m), tidal current velocities within the gulf range between 0.5 to 1.5 $m \cdot s^{-1}$ [35] and are large relative to the velocities of *ca*. 0.1 to 0.2 $m \cdot s^{-1}$ generated by gravity currents [33] and wind [36]. As a consequence, the vertical structure of the water column is generally well mixed. The interface between Spencer Gulf and shelf water creates a front in summer [37] [38] where larval fish have been shown to aggregate [39], possibly as a result of convergent flows [40].

The Spencer Gulf is characterized by a variety of habitats including mangroves, saltmarshes and seagrass beds, tidal flats, dune systems, pebble beach ridges and submerged "sand waves" and reef systems [41]. It supports, as most of the southern Australian coast, a uniquely high degree of endemism and high floristic and faunistic diversity [42]-[44], and is home of iconic species such as the Giant Australian Cuttlefish [45] [46], Great White Sharks [47], Leafy and Weedy seadragons [48] [49], a variety of marine birds and mammals [50], and a relic population of Tiger Pipefish [51]. The Spencer Gulf is also an active area for an aquaculture industry that is worth $242 M for 2011/12 [52]. Key aquaculture species include southern Bluefin tuna, yellowtail kingfish, abalone and oysters. Despite the observed diversity and sustained fisheries and aquaculture activities, very limited information are still available on the structure and function of the planktonic ecosystem in Spencer Gulf waters that are generally considered oligotrophic, with 1) phytoplankton biomass consistently lower than 0.5 $\mu g \cdot l^{-1}$ across the Gulf, concentrations that are comparable to levels reported for oligotrophic waters off western and south eastern Australia (0.1-0.7 $\mu g \cdot l^{-1}$) [53]-[55] and offshore waters of the eastern and central Great Australian Bight (<0.1 - 0.4 $\mu g \cdot l^{-1}$) [56]; 2) relatively low primary productivity uniquely phosphorus-limited [25]; and 3) a subsequently low secondary productivity that is not considered strong enough for the mesozooplankton to exert a top-down control of phytoplankton community [25]. No information is yet available on the nature, abundance and diversity of the viral, microbial and picophytoplanktonic communities of the Spencer Gulf.

2.2. Sampling Sites and Sampling Strategy

Water samples were collected from three locations with-in Spencer Gulf using the FV Atlas during four (seasonal) surveys of the gulf on November 16-18, 2010 (Spring), February 16-18, 2011 (Summer), 17-19 April, 2011 (Autumn) and 14-16 August, 2011 (Winter). Site locations were based the spatial regions defined by Nunes-Vaz [57], with one site sampled in the southern (South; 34˚47'S, 136˚29'E), middle (Middle; 34˚54'S, 137˚25'E) and northern (North; 33˚08'S, 137˚40'E) basins of the gulf, respectively (**Figure 1**). At each site, two surface water samples were taken using a 30 L Niskin bottle before being transferred immediately for storage into 25 liter acid-washed (0.1 N HCl) polyurethane carboy's.

Water samples were then prepared for flow cytometric picophytoplankton, bacterial and viral enumeration and identification (see Sections 2.3 and 2.4) and micro-zooplankton grazing and viral lysis dilution assay experiments (see Section 2.4). Triplicate samples (1 ml) for flow cytometry were fixed 1) with glutaraldehyde (0.5% final concentration) and incubated for 15 min at 4˚C [58] for viruses and heterotrophic bacteria identification and enumeration; and 2) with paraformaldehyde (2% final concentration) for picophytoplankton identification and enumeration. All samples were subsequently quick frozen in liquid nitrogen, stored at −80˚C once re-turned to the laboratory and analyzed by flow cytometry (FCM) using a FAC Scanto flow cytometer (Becton Dickinson) within a month of collection.

Additional water samples were collected for determining macro-nutrient and chlorophyll *a* concentrations. For macro-nutrients analysis 100 ml was filtered through a 0.45 µm filter then frozen and stored at −22˚C prior to laboratory analysis. Dissolved ammonium (NH3, APHA-AWWA-WPCF 1998a, detection limit 0.071 µM), oxides of nitrogen (NO_x, APHA-AWWA-WPCF 1998b, detection limit 0.071 µM), phosphate (PO_4 APHA-

AWWA-WPCF 1998c, detection limit 0.032 µM) and silicate (SiO_2, APHA-AWWA-WPCF 1998d, detection limit 0.333 µM), were determined by flow injection analysis with a QuickChem 8500 Automated Ion Analyzer. The pigment composition of water samples was measured using High Pressure Liquid Chromatography (HPLC). Two liter water samples were filtered through Whatman GF/F filters (nominal pore size 0.4 µm) which were snap-frozen in liquid nitrogen and stored at −80˚C prior to analysis via the gradient elution procedure [58] on an Algilent 1200 series HPLC system in the environmental chemistry laboratory at SARDI Aquatic Sciences. Vertical profiles of temperature and salinity were measured using a Seabird SBE 19-plus conductivity, temperature and depth recorder (CTD) at each site prior to water sampling.

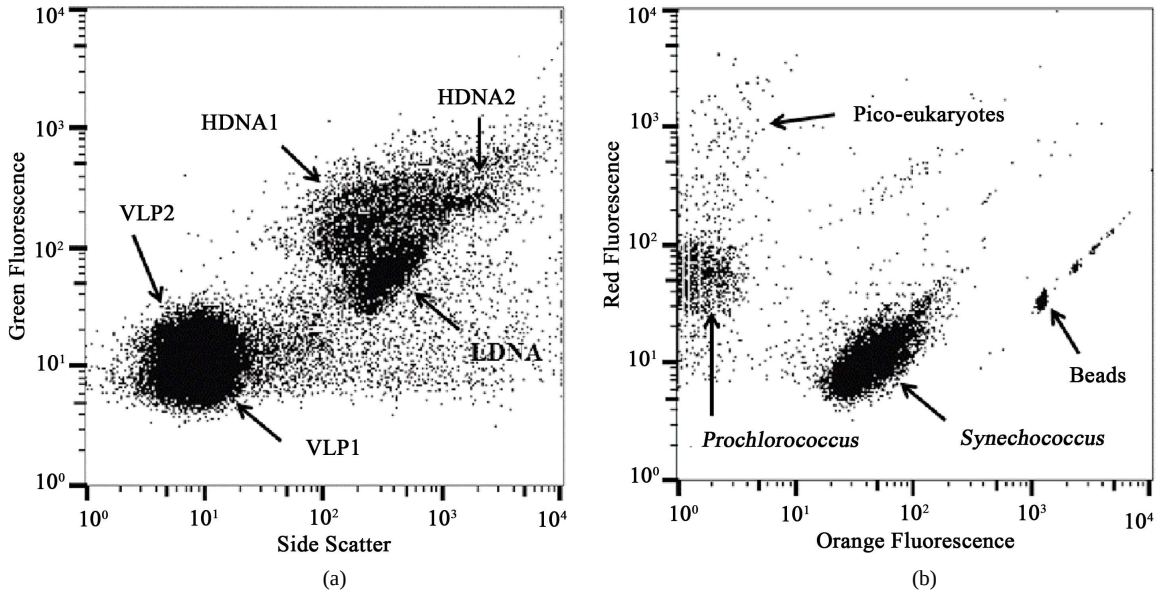
2.3. Viral and Bacterial Identification and Enumeration

Prior to FCM analysis, samples were quick thawed and diluted 1:10 with 0.2 mm filtered TE buffer (10 mM Tris, 1 mM EDTA), stained with SYBR-I Green solution (1:500 dilution; Molecular Probes) and finally incubated in the dark at 80˚C for 10 min [58]. Fluorescent beads with a diameter of 1 mm (Molecular Probes) were added to each sample as an internal size and concentration standard at a final concentration of approximately 10^5 beads ml^{-1} [59]. Phosphate-buffered saline (PBS) solution was used as sheath fluid, while forward-angle light scatter (FSC), side-angle light scatter (SSC) and green (SYBR-I) fluorescence were recorded for each sample. Data for each sample were collected in list-mode files and analyzed using Win MDI 2.8 software (© Joseph Trotter).

Viral and heterotrophic bacterial populations were discriminated based on their differences in cell side scatter, a proxy of cell size, and SYBR Green fluorescence, which indicates the amount of nucleic acid present in each cell [59]-[61]. Bacterial populations were then split into two high DNA (HDNA1 and HDNA2) and one low DNA (LDNA) groups using the differences in green fluorescence [62] [63]. Viral populations were split into two virus-like particle populations (VLP1 and VLP2) based on their differences in green fluorescence and SSC (**Figure 2(a)**). More specifically, VLP1 and VLP2 corresponded to populations widely observed in seawater samples and identified as bacteriophages e.g. [61] [64]-[67].

2.4. Picophytoplankton Identification and Enumeration

Prior to FCM analysis, samples were quick thawed and 1 mm fluorescent marker beads (Molecular Probes, Eugene, OR, USA) added [61]. Each sample was run for 5 min, and natural orange fluorescence from phycoerythrin and red fluorescence from chlorophyll, together with forward light scatter and side light scatter (SSC), were recorded. All cytograms were then analyzed using Win MDI 2.8 software (© Joseph Trotter) following [61].



(a) (b)

Figure 2. Representative cytograms used to determine viral and bacterial ((a): autumn, Southern site) and pico-phytoplankton ((b): summer, Northern site) abundance.

One population of picoeukaryotic phytoplankton and two populations of prokaryotic phytoplankton (cyanobacteria *Prochlorococcus* and *Synechococcus*) were then discriminated based on their distinct autofluorescence and light scatter properties (**Figure 2(b)**). Specifically, *Synechococcus* and *Prochlorococcus* mainly differed by their orange fluorescence (**Figure 2(b)**), while picoeukaryotes were identified by their distinct red fluorescence (**Figure 2(b)**).

2.5. Dilution Assay

This experiment aimed at assessing the mortality rates of heterotrophic bacteria and both eukaryotic and prokaryotic picophytoplankton due to microzooplankton grazing and viral lysis. Mortality rates due to grazing (M_g) and viral lysis (M_v) were estimated following [20]. Specifically, seawater was collected from each site with 25 liter acid-washed (0.1 N HCl) polyurethane carboy, and first mesozooplankton were removed by gravity siphoning the sample water through a 200 µm mesh filter. Two dilution series were subsequently prepared, using grazer-free and virus-free diluent fractions. The grazer-free diluent fraction was prepared by vacuum filtration through a 0.2 µm filter (Pall Corporation). The virus-free water fraction was prepared by filtering grazer-free diluent through a 30 kDa tangential flow filtration system (Millipore). Diluents were then siphoned into acid washed (0.1 N HCl) 2 L Nalgene bottles (Schott) to create 4 dilutions of natural seawater (20%, 40%, 70% and 100%). These bottles were subsequently filled to capacity with freshly collected 200 µm filtered natural site water, *i.e*. 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 200 µm filtered natural site water. 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 enumeration of heterotrophic bacteria and eukaryotic and prokaryotic picophytoplankton (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 4000 l holding tank containing seawater on site. After 24 hours (t = 24 h, t_{24}), triplicate samples were taken from each incubation bottle for flow cytometric enumeration of heterotrophic bacteria and eukaryotic and prokaryotic (*Synechococcus*, *Prochlorococcus*) picophytoplankton.

2.6. Statistical Analysis

Due to the low number of data points available, non-parametric statistics were used throughout this work. Specifically, multiple comparisons between sites and seasons were conducted using the Kruskal-Wallis test (KW test hereafter), and a subsequent multiple comparison procedure based on the Tukey test was used to identify distinct groups of measurements.

3. Results

3.1. Environmental Conditions

Samples were collected months that are representative of the Austral seasons and water temperatures and salinities measured by the CTD were consistent with seasonal oscillations and the inverse estuarine circulation of the Gulf (**Table 1**). Near surface water temperatures showed the greatest variability, ranging from 13.7˚C to 23.9˚C, at the northern site. Water temperatures adjacent to shelf at the southern site showed reduced seasonal variability and ranged between 15.3˚C to 21.5˚C. With the exception of winter, surface waters generally increased in temperature northwards along the gulf. Salinity showed a similar pattern to temperature and increased from south to north across the seasons. Salinity minimum and maximum values ranged from 35.9 PSU at the southern site in winter to a maximum of 39.6 PSU at the northern site in autumn.

The concentrations of macro-nutrients were typically low, with phosphate and nitrate concentrations typically below the detection limit (<0.032 µM; **Table 1**) for each site and season, with exception for winter at the southern site where both the concentration of phosphate (PO_4 = 0.035 µM) and nitrate (NO_x = 0.33 µM) peaked. Silica concentrations were measured above the detection limit for all sites and seasons and generally increased in concentration from the south to the north across the seasons. Similarly, chlorophyll *a* concentrations generally increased from the south to the north across the seasons with the majority of the phytoplankton biomass dominated by the small (<5 µm) size fraction.

Table 1. Seasonal abiotic and biotic properties of surface waters at sampling locations within Spencer Gulf. bd: below detection limit.

Northern site	Spring	Summer	Autumn	Winter
Bottom depth (m)	15	15	15	15
Temperature (˚C)	19.1	23.9	19.2	13.7
Salinity (PSU)	38.5	39.4	39.6	39.4
Chl. *a* (>5 µm; mg·m^{-3})	0.08	0.12	0.07	0.05
Chl. *a* (<5 µm; mg·m^{-3})	0.20	0.52	0.27	0.27
NO_x (µM)	bd	0.16	bd	bd
NH_3 (µM)	bd	0.13	0.27	bd
PO_4 (µM)	bd	bd	bd	bd
SiO_2 (µM)	1.32	1.25	1.04	0.66
Middle site	**Spring**	**Summer**	**Autumn**	**Winter**
Bottom depth (m)	25	25	25	25
Temperature (˚C)	18.1	23.2	18.6	13.8
Salinity (PSU)	37.7	38.5	37.9	37.9
Chl. *a* (>5 µm; mg·m^{-3})	0.03	0.04	0.07	0.03
Chl. *a* (<5 µm; mg·m^{-3})	0.18	0.27	0.39	0.19
NO_x (µM)	bd	0.08	bd	0.28
NH_3 (µM)	0.35	0.14	0.23	0.14
PO_4 (µM)	bd	bd	bd	bd
SiO_2 (µM)	0.45	1.07	0.46	1.12
Southern site	**Spring**	**Summer**	**Autumn**	**Winter**
Bottom depth (m)	45	45	45	45
Temperature (˚C)	16.5	21.5	18.6	15.3
Salinity (PSU)	36.2	36.8	36.8	35.9
Chl. *a* (>5 µm; mg·m^{-3})	0.03	0.02	0.05	0.03
Chl. *a* (<5 µm; mg·m^{-3})	0.15	0.19	0.35	0.13
NO_x (µM)	bd	bd	bd	bd
NH_3 (µM)	0.11	bd	0.20	bd
PO_4 (µM)	bd	bd	bd	0.03
SiO_2 (µM)	0.59	0.59	0.44	0.72

3.2. Viral and Bacterial Abundance

Two viral (VLP1, VLP2) and three bacterial (LDNA, HDNA1, HDNA2) sub-populations were consistently observed at all sites over the four seasons (**Figure 2(a)**). Total viral abundance ranged from 3.04×10^5 to 5.26×10^6 ml^{-1}, while total bacterial abundance ranged from 3.18×10^5 to 2.58×10^6 cells·ml^{-1}. Total viral and bacterial abundances were significantly positively correlated ($p < 0.05$), and both exhibit significant spatial and temporal patterns of variability (KW test, $p < 0.05$; **Figure 3**, **Table 1**). Specifically, both viral and bacterial abundances significantly differed ($p < 0.05$) between sites for each season, and show an increasing trend from the

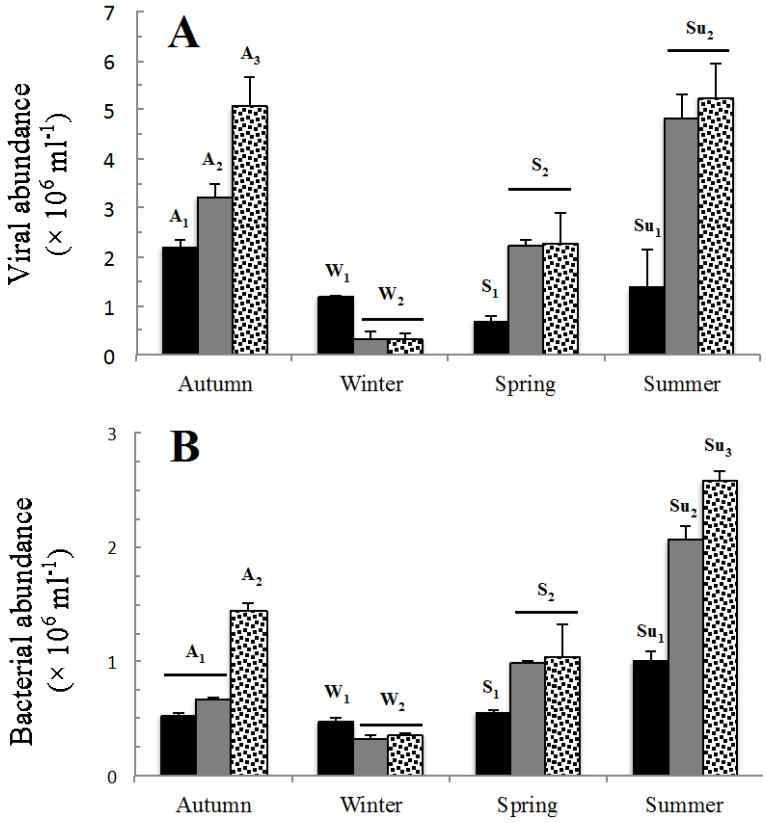


Figure 3. Total viral (A) and bacterial (B) abundance at the Southern (black bars), Middle (grey bars) and Northern (dotted bars) sites in autumn, winter, spring and summer. The letters "A_i", "W_i", "S_i" and "Su_i" identify significant differences ($p < 0.05$) inferred for each season using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test. Error bars are standard deviations.

southern site to the Northern site in autumn, spring and summer. In winter, significantly higher viral and bacterial abundances were, however, observed at the Southern site. Site-specific significant differences were also observed seasonally in viral and bacterial abundances with a general trend towards lower abundances in spring and winter and higher abundances in summer and autumn (**Table 2**).

VLP1 (**Figure 4(a)**) was by far the most abundant viral sub-population at all sites, especially in winter, spring and summer where it was respectively from 18 to 56-fold, 4 to 133-fold and 57 to 200-fold more abundant than VLP2 (**Figure 4(b)**). In autumn, VLP1 was only 1.2 to 2.4-fold more abundant than VLP2. VLP1 hence accounted for 94.7% to 98.2% of VLP in winter, 80.5% to 99.3% in spring, 98.3% to 99.5% in summer, and was nearly as abundant as VLP2 in autumn, especially at the middle and Northern sites where they represented 54% to 58% of VLP against nearly 70% at the Northern site. In contrast, VLP2 were significantly more abundant in autumn at all sites, reaching concentrations on average 7, 32 and 80-fold higher than in spring, summer, and winter (**Figure 4(b)**). VLP1 and VLP2 abundances were both highly significantly positively correlated ($p < 0.01$) to total virus abundances, but only VLP1 abundance correlated significantly with bacterial abundance ($p < 0.05$). VLP1 and VLP2 temporal patterns are overall similar to those reported for total viral and bacterial abundance (**Table 2**).

The abundance of LDNA, HDNA1 and HDNA2 sub-populations significantly differed between sites for each season ($p < 0.05$), and was consistently significantly higher in summer at the Northern site (**Figure 5**). LDNA was significantly more abundant at all sites for all seasons, except in summer at the Southern site. Specifically, the relative contribution of LDNA to total bacteria abundance ranged from 38.9% to 53.9% in spring, 47.3% to 52.0% in autumn, and 44.8% to 68.4% in winter, but only from 28.7% to 41.0% in summer. Both total viral and bacterial abundances were highly significantly positively correlated with LDNA, HDNA1 and HDNA2 abun-

Table 2. Results of the multiple comparisons conducted on the abundance of viral and bacterial subpopulations and both picoeukaryoteic (*Prochlorococcus* and *Synechococcus*) and prokaryotic phytoplankton using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test when the null hypothesis was rejected.

Northern site							
VLP	Winter	<	Spring	<	Autumn	=	Summer
VLP1	Winter	<	Spring	<	Autumn	=	Summer
VLP2	Winter	<	Summer	<	Spring	<	Autumn
Bacteria	Winter	<	Spring	<	Autumn	<	Summer
LDNA	Winter	<	Spring	<	Autumn	<	Summer
HDNA1	Winter	<	Spring	=	Autumn	<	Summer
HDNA2	Winter	<	Spring	=	Autumn	<	Summer
Synechococcus	Spring	=	Winter	<	Autumn	=	Summer
Prochlorococcus	Winter	=	Autumn	=	Spring	<	Summer
Picoeukaryotes	Winter	<	Autumn	<	Spring	<	Summer
Middle site							
VLP	Winter	<	Spring	<	Autumn	<	Summer
VLP1	Winter	<	Spring	=	Autumn	<	Summer
VLP2	Winter	<	Summer	<	Spring	<	Autumn
Bacteria	Winter	<	Autumn	<	Spring	<	Summer
LDNA	Winter	<	Autumn	=	Spring	<	Summer
HDNA1	Winter	<	Autumn	<	Spring	<	Summer
HDNA2	Autumn	=	Winter	<	Spring	<	Summer
Synechococcus	Spring	=	Winter	<	Autumn	<	Summer
Prochlorococcus	Winter	<	Spring	<	Autumn	<	Summer
Picoeukaryotes	Winter	<	Autumn	<	Summer	<	Spring
Southern site							
VLP	Spring	<	Winter	=	Summer	<	Autumn
VLP1	Spring	<	Winter	=	Autumn	<	Summer
VLP2	Spring	<	Winter	=	Summer	<	Autumn
Bacteria	Spring	=	Winter	=	Autumn	<	Summer
LDNA	Autumn	=	Summer	=	Spring	=	Winter
HDNA1	Winter	<	Spring	<	Autumn	<	Summer
HDNA2	Autumn	=	Winter	<	Spring	<	Summer
Synechococcus	Spring	<	Summer	<	Winter	<	Autumn
Prochlorococcus	Spring	=	Winter	<	Summer	<	Autumn
Picoeukaryotes	Winter	<	Autumn	<	Summer	<	Spring

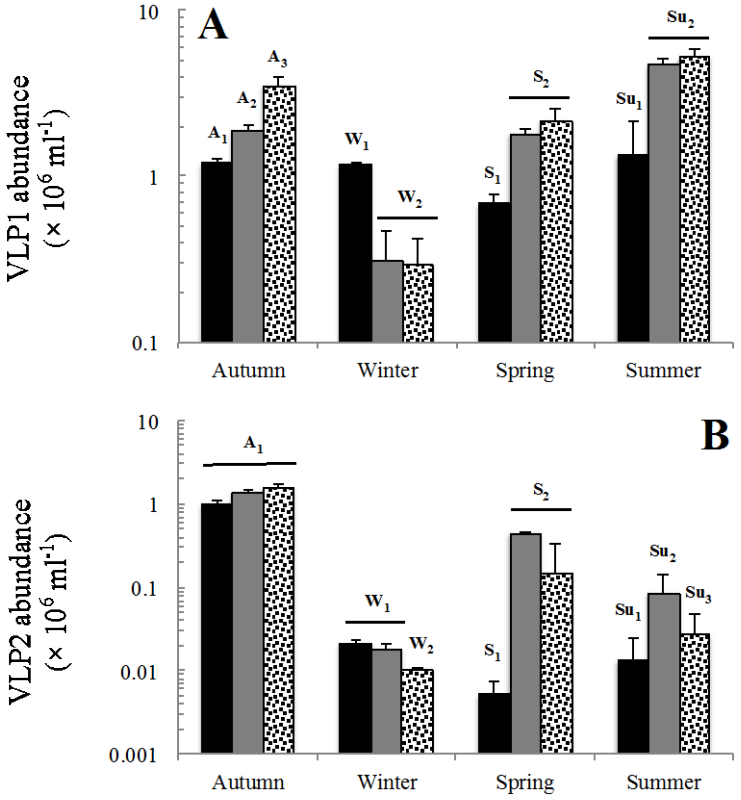


Figure 4. Abundance of viral sub-populations VLP1 (A) and VLP2 (B) at the Southern (black bars), Middle (grey bars) and Northern sites (dotted bars) in autumn, winter, spring and summer. The letters "A_i", "W_i", "S_i" and "Su_i" identify significant differences ($p < 0.05$) inferred for each season using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test. Error bars are standard deviations.

dance ($p < 0.01$). VLP1 abundance was also significantly correlated with LDNA, HDNA1 and HDNA2 abundances ($p < 0.01$). In contrast, VLP2 abundance did not exhibit any significant correlation with LDNA, HDNA1 or HDNA2 abundance ($p > 0.05$). While site-specific temporal patterns exist, LDNA, HDNA1 and HDNA2 abundances are overall significantly less abundant in winter than in summer at all sites (**Table 2**).

3.3. Picophytoplankton Abundance

Two populations of prokaryotic phytoplankton (*Synechococcus* and *Prochlorococcus*) and one population of picoeukaryotic phytoplankton were consistently identified at all sites over the four seasons (**Figure 2(b)**). *Synechococcus*, *Prochlorococcus* and picoeukaryote abundance ranged from 4.25×10^2 to 7.67×10^4 cells ml^{-1}, 1.56×10^3 to 2.91×10^4 cells ml^{-1} and 2.07×10^2 to 1.68×10^3 cells ml^{-1}, respectively. Picoeukaryotes were significantly less abundant than *Synechococcus* and *Prochlorococcus* at all sites and seasons ($p > 0.05$); they hence accounted for 0.5% to 6.5% of picophytoplankton abundance, except in spring at the Southern site where they represented between 39.7% and 44.3% of the picophytoplankton community. All three picophytoplankton populations reached their peak abundance at the Northern site in summer (**Figure 6**), but exhibit significant spatial and temporal patterns of variability (KW test, $p < 0.05$). Specifically, their abundance significantly ($p < 0.05$) differed between sites for each season (**Figure 6**), and show complex temporal patterns of variability that are both population- and site-dependent (**Figure 6**, **Table 2**). Highly significant correlation was found between *Synechococcus* and *Prochlorococcus* ($p < 0.01$), and their abundance was significantly positively correlated with most viral and bacterial sub-populations. In contrast, the abundance of picoeukaryotes did not exhibit any significant correlation with either *Synechococcus* or *Prochlorococcus* ($p > 0.05$), and were only significantly correlated with total bacterial abundance, HDNA1 and HDNA2 (**Table 2**).

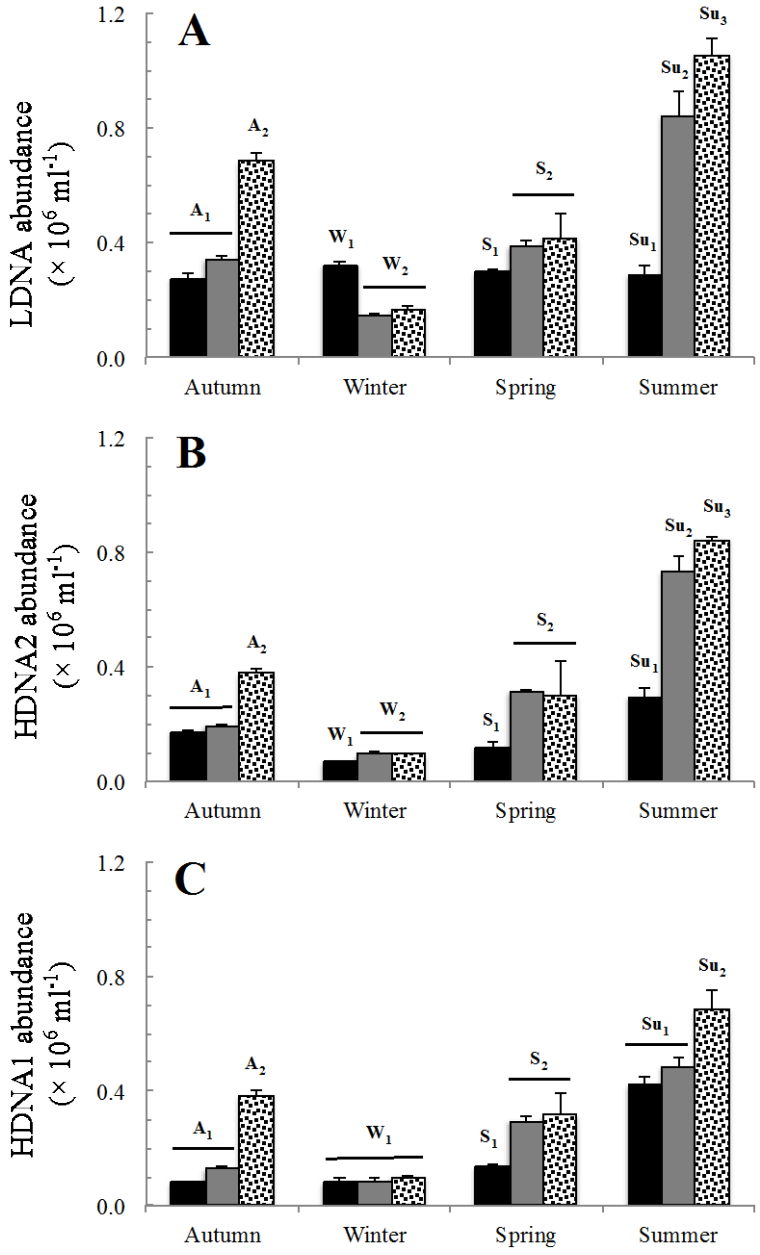


Figure 5. Abundance of heterotrophic bacteria sub-populations LDNA (A), HDNA1 (B) and HDNA2 (C) at the Southern (black bars), Middle (grey bars) and Northern (dotted bars) sites in autumn, winter, spring and summer. The letters "A_i", "W_i", "S_i" and "Su_i" identify significant differences ($p < 0.05$) inferred for each season using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test. Error bars are standard deviations.

3.4. Mortality Rates

3.4.1. Bacterial Mortality

The rates of viral lysis and microzooplankton grazing of heterotrophic bacteria were estimated for each site in spring, summer, autumn and winter. No samples were available for the Southern site in autumn. Bacterial mortality rates were highly variable both between sites and between seasons (**Figure 7**). Specifically, no microzooplankton grazing was observed in spring across all sites, and in winter at the Southern site. Microzooplank

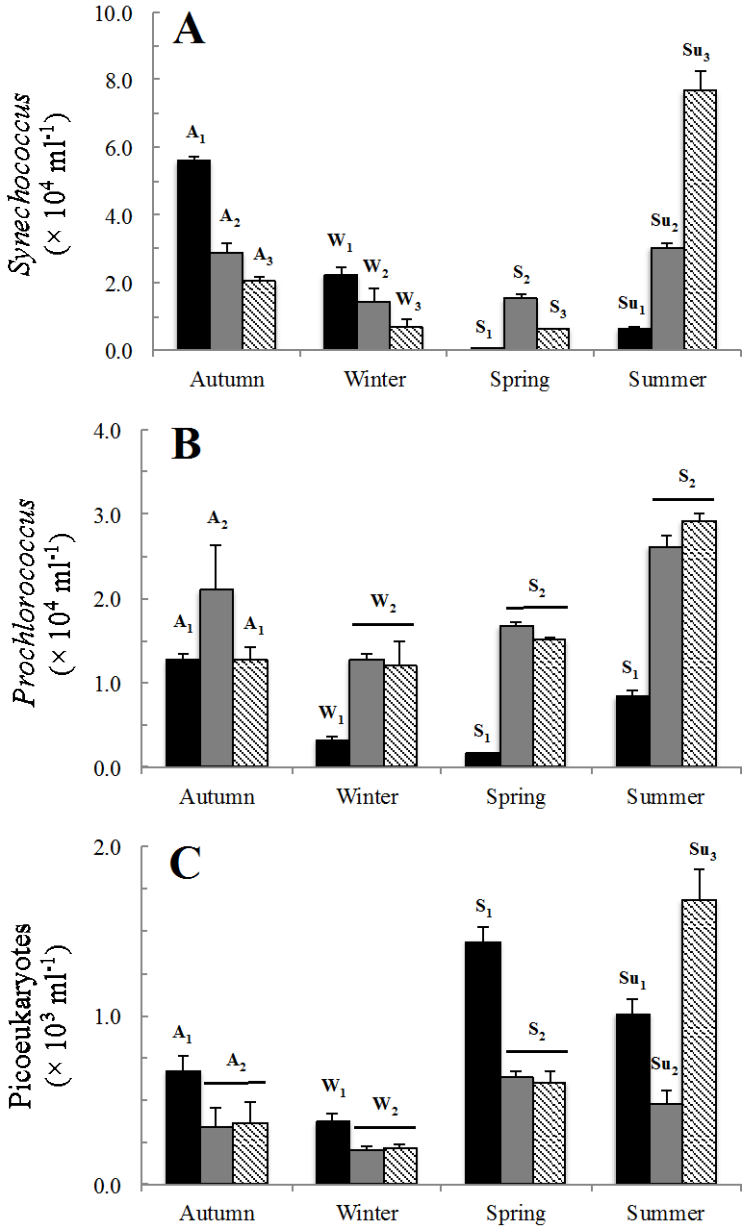


Figure 6. Abundance of prokaryotic (Synechococcus, (A); Prochlorococcus, (B)) and eukaryotic (C) picophytoplankton at the Southern (black bars), Middle (grey bars) and Northern (dotted bars) sites in autumn, winter, spring and summer. The letters "A_i", "W_i", "S_i" and "Su_i" identify significant differences ($p < 0.05$) inferred for each season using the Kruskal-Wallis test, and a subsequent multiple comparison procedure based on the Tukey test. Error bars are standard deviations.

ton grazing rates, however, ranged from 0.37 to 0.57 d^{-1} in summer, 0.24 to 0.40 d^{-1} in autumn and 0.00 and 0.28 d^{-1} in winter. No microzooplankton grazing was observed in spring. Bacterial losses due to viral lysis were observed at all sites in spring, summer and autumn; no viral lysis was, however, observed in winter. Viral lysis ranged from 0.13 to 0.44 d^{-1} in spring, 0.02 to 0.06 d^{-1} in summer and 0.33 and 0.58 d^{-1} in autumn. Bacterial mortality was markedly dominated by microzooplankton grazing in summer at all sites and in winter at sites B and C (**Figure 7**). In contrast, mortality was essentially due to viral lysis in spring at all sites and in autumn at sites B and C (**Figure 7**).

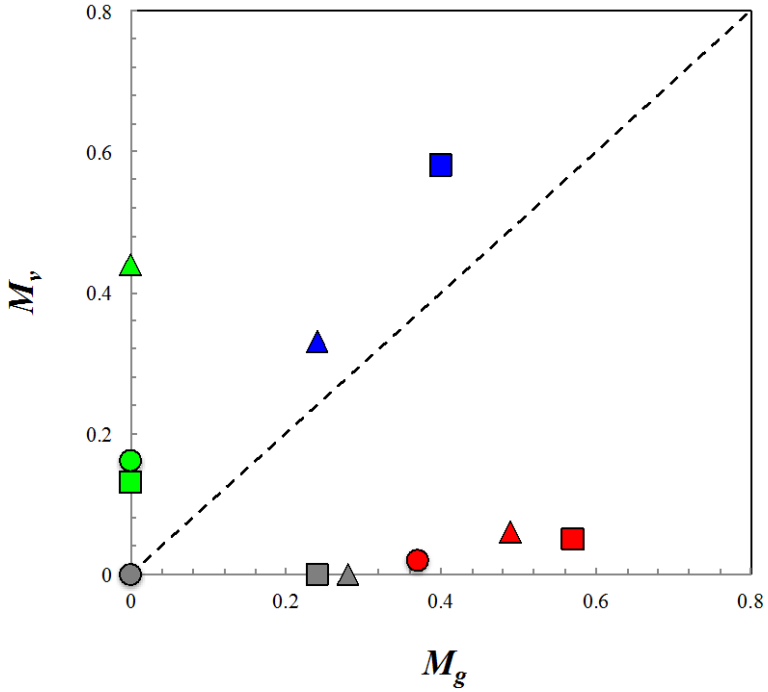


Figure 7. Mortality rates of heterotrophic bacteria due to microzooplankton grazing (M_g, d^{-1}) and viral lysis (M_v, d^{-1}) at the Southern (circles), Middle (triangles) and Northern (squares) sites in spring (green), summer (red), autumn (blue) and winter (grey). The dashed line indicates the situation where $M_g = M_v$.

3.4.2. Picophytoplankton Mortality

The rates of grazing by microzooplankton and viral lysis of *Synechococcus*, *Prochlorococcus* and picoeukaryotes were estimated for each site in spring, summer, autumn and winter (**Table 3**). No samples were available at the Southern site in autumn, and for viral lysis for the Northern site in winter. As observed for the mortality rates of heterotrophic bacteria (**Figure 7**), the mortality rates due to microzooplankton grazing and viral lysis were highly variable between sites and season for a given population, but also between populations (**Table 3**). More specifically, the mortality due to microzooplankton grazing ranged from 0.00 to 0.28 d^{-1} for *Synechococcus*, 0.00 and 0.16 d^{-1} for *Prochlorococcus*, and 0.00 and 0.12 d^{-1} for picoeukaryotes (**Table 3**). Viral lyses were also very variable, ranging from 0.00 to 0.09 d^{-1} for *Synechococcus*, 0.00 and 0.14 d^{-1} for *Prochlorococcus*, and 0.00 and 0.08 d^{-1} for picoeukaryotes (**Table 3**). In contrast to the observations conducted on bacterial mortality rates (**Figure 7**), no clear seasonality was found in the dominance of mortality due to grazing and viral lysis at any of the sites (**Table 3**).

4. Discussion

The cytometric community composition and the abundance of viruses, heterotrophic bacteria and both prokaryotic (*Synechococcus* and *Prochlorococcus*) and eukaryotic picophytoplankton were consistent with previous observations conducted in South Australian continental shelf waters, and more generally in eutrophic and oligotrophic waters; see [66]-[69] and references within.

4.1. Community Composition and Abundance

4.1.1. Viruses

Two populations of viral populations were identified based on their differences in green fluorescence and SSC (**Figure 2(a)**). More specifically, the cytometric signature of the two viral populations VLP1 and VLP2 consistently observed across the Spencer Gulf has also commonly been reported in a variety of environments and consistently considered as bacteriophages [61] [64]-[67]. Note that no viral sub-group characterized by either

Table 3. Viral lysis and microzooplankton grazing (d^{-1}) for picoeukaryoteic (Euk) and prokaryotic (*Synechococcus* and *Prochlorococcus*; *Syn* and *Proc*) phytoplankton.

SPRING	*Syn*		*Proc*		Euk	
	M_g	M_v	M_g	M_v	M_g	M_v
Northern site	0	0	0.041	0.015	0.045	0
Middle site	0	0.087	0	0	0	0
Southern site	0.276	0	0	0.061	0	0.056
SUMMER	***Syn***		***Proc***		**Euk**	
	M_g	M_v	M_g	M_v	M_g	M_v
Northern site	0	0.044	0.031	0.004	0.022	0.026
Middle site	0.073	0.046	0	0.049	0	0
Southern site	0.103	0.073	0.048	0.069	0.097	0.069
AUTUMN	***Syn***		***Proc***		**Euk**	
	M_g	M_v	M_g	M_v	M_g	M_v
Northern site	-	-	-	-	-	-
Middle site	0.087	0.016	0	0.136	0	0.084
Southern site	0.086	0	0.159	0	0.125	0
WINTER	***Syn***		***Proc***		**Euk**	
	M_g	M_v	M_g	M_v	M_g	M_v
Northern site	0	0	0.035	0	0.124	0.040
Middle site	0	0	0	0	0	0
Southern site	0.084	-	0	-	0	-

similar levels of SYBR Green fluorescence than VLP2 but a greater size, or similar size but greater levels of SYBR Green fluorescence were observed, suggesting the absence of phytoplankton viruses that are generally characterized by higher side scatter and/or green fluorescence signatures [14]-[16] [70]-[72]. Noticeably, VLP1 abundance was significantly correlated with LDNA, HDNA1 and HDNA2 abundances. VLP2 abundance, however, did not exhibit any significant correlation with LDNA, HDNA1 or HDNA2 abundance. It was, however, significantly correlated to the abundance of *Synechococcus* and *Prochlorococcus*. These results hence suggest that while commonly considered as bacteriophages, the viral populations VLP1 and VLP2 may instead be, at least in the specific case of the Spencer Gulf, be respectively composed of bacteriophages and viruses infecting prokaryotic phytoplankton. Further work based on e.g. virus PFGE fingerprinting is, however, needed to confirm this theory.

4.1.2. Heterotrophic Bacteria

The sub-populations of heterotrophic bacteria identified through flow cytometry are consistently relatively low; we consistently identified three sub-populations of heterotrophic bacteria (LDNA, HDNA1, HDNA2) at all sites over the four seasons (**Figure 2(a)**). This result is comparable to observations conducted at various coastal locations around the south-west of the Spencer Gulf where the microbial community was limited to one LDNA and one HDNA sub-populations [73], and in the coastal waters of the nearby Saint Vincent Gulf, where heterotrophic bacteria were consistently composed of three sub-populations [73] [74] comparable to the LDNA, HDNA1 and HDNA2 sub-populations described here (**Figure 2(a)**). In contrast to our observations, the cytometric diversity of heterotrophic bacteria previously reported in South Australian coastal waters seems to vary seasonally [75] [76]. For instance, the cytometric diversity of the bacterial community observed in a mangrove estuary drastically increase from summer to autumn [75]. In summer, the bacterial community was divided into three

discrete groups, based on their increasing SYBR Green fluorescence. The low DNA (LDNA) and high DNA (HDNA) populations generally evident in most aquatic systems [63] were clearly present in all samples. The HDNA population also exhibited two well defined peaks on histogram plots of SYBR Green fluorescence as observed here (see **Figure 2(a)**) and could subsequently be divided further into HDNA I and HDNA II categories. In autumn, however, the bacterial community was characterized by a substantially different "cytometric structure". While the LDNA, HDNA I and HDNA II populations were all still present, at least two more sub-populations were evident within all samples. Two additional populations were hence defined based respectively on their much higher SYBR Green fluorescence and higher levels of side scatter than the other bacterial subpopulations [75]. The low cytometric diversity reported in the coastal waters of both the Spencer Gulf [73], present work] and the Saint Vincent Gulf [73]-[75] sharply contrast, however, with other observations conducted in South Australian water bodies. For instance, up to seven sub-populations of heterotrophic bacteria of increasing size and DNA content were identified in the Coorong, a hypersaline coastal lagoon [76]. While further work is needed to confirm this hypothesis, these observations suggest that the dynamics of the microbial communities of the Spencer Gulf and the Saint Vincent Gulf may be relatively different, and both significantly limited. Noticeably LDNA bacteria were consistently significantly the most abundant group of heterotrophic bacteria (totaling from 29% to 68% of total bacterial abundance) and were up to 10-fold more abundant than previously reported in South Australian continental shelf waters [66] [67], including the nearby Saint Vincent Gulf [73] [74] [77]. Specifically, sub-populations of heterotrophic bacteria characterized by a low DNA content have been suggested to be either inactive or dormant cells; sub-populations with DNA content represent, in turn, active cells [62] [63] [78] [79]. This has, however, been challenged and LDNA cells may also be active [80]-[83]. In addition, while having lower metabolic rates than HDNA cells, LDNA cells have been suggested to play a greater role in heterotrophic processes than HDNA cells in upwelling-driven eutrophic systems [81]. As such, the high relative abundance of LDNA cells consistently observed in the Spencer Gulf may also indicate a prominent role of these cells in oligotrophic waters.

4.1.3. Picophytoplankton

To our knowledge, the space-time dynamics of prokaryotic (*Synechococcus* and *Prochlorococcus*) and eukaryotic picophytoplankton has only been investigated in South Australian waters along a salinity gradient occurring along a hypersaline coastal lagoon [84], and from a few stations scattered along the 100 m isobaths on the South Australian continental shelf near the mouth of the Spencer Gulf [68] [69]. Specifically, six picophytoplankton populations were identified by flow cytometry, including two *Synechococcus* populations, two *Prochlorococcus* populations, and two groups of small and large picoeukaryotes. The abundance of each of these populations was controlled by a range of physical processes, including downwelling and dense waters outflowing from the Spencer Gulf in winter, early spring, upwelling in summer and early spring and eddy formation in summer. These processes resulted in up to four orders of magnitude changes in their abundances, and population-specific hotspots [69]. Note that the unexpected high abundances and local dominance of *Prochlorococcus* in summer was hypothesized to result from eastward and westward current transports and the presence of a High-Light and Low-Light ecotypes [69]. Our results are consistent with the seasonality in physical forcing characterizing the Spencer Gulf, with a clear dichotomy between winter and summer in the abundance of *Synechococcus*, *Prochlorococcus* and picoeukaryotes at the Northern and Middle sites, while the distinct temporal patterns observed from the Southern site is likely the result of the influence of the complex physical processes occurring on continental shelf waters (**Table 2**).

4.2. Viruses and Microzooplankton as Mortality Agents

There is a plethora of published papers quantifying the role of microzooplankton grazing rates in the ocean, see [19] for a review, and a vivid debate around the reliability of their estimates [85]-[87]. In contrast, studies reporting mortality rates due to both microzooplankton grazing and viral lysis on planktonic organisms are still very scarce [14]-[16] [20], with so far only one study which investigated the impact of grazing and lysis on heterotrophic bacteria [88]. Microzooplankton grazing pressures on total chlorophyll generally reported for coastal and estuarine ecosystems typically range from 0.08 and 2.19 d^{-1}; see [18] [19] for a review. More specifically, the grazing and viral lysis of a *Micromonas* spp. population estimated from a mesocosm experiment were respectively in the range 0.26 - 0.65 d^{-1} and 0.10 - 0.29 d^{-1} [20]. A study investigating the rates of viral lysis and grazing by microzooplankton of *Phaeocystis globosa* over the course of a spring bloom in the Southern Bight of

the North Sea [14] reported highly variable virally mediated mortality rates (0.01 - 0.35 d^{-1}) and grazing rates (0.05 - 0.40 d^{-1}). Similar conclusions were reached by a study conducted in the Southern Ocean that showed both viral lysis (0.00 - 0.11 d^{-1}) and grazing rates (0.00 - 1.78 d^{-1}) being highly variable for four phytoplankton groups differing in size [16]. In the Coorong, a hypersaline coastal lagoon, grazing and lysis rates for phytoplankton standing stock averaged respectively 3.4 d^{-1} and 3.1 d^{-1}, and for heterotrophic bacteria ranging from 0.02 to 1.67 d^{-1} and from 0.01 to 2.9 d^{-1}, respectively [89]. A study that may be more comparable to the present one, though conducted in the oligotrophic waters of the Atlantic Ocean, found grazing and lyses rates bounded between 0.00 and 0.81 d^{-1} and 0.00 and 0.32 d^{-1} for picoeukaryotes, 0.00 and 0.12 d^{-1} and 0.07 and 0.25 d^{-1} for *Synechococcus*, and between 0.00 and 0.06 d^{-1} and 0.00 and 0.8 d^{-1} for *Prochlorococcus* [15].

These observations show that the microzooplankton grazing and viral lysis rates reported in the present work for heterotrophic bacteria are in the lower range of values previously reported in the literature, hence suggest 1) a weak transfer of microbially-mediated matter and energy towards the classical food chain, and 2) a weak transformation of this microbial materials as bioavailable dissolved organic matter. This low viral activity and the related low recycling of potentially growth-limiting nutrients [10] may hence be one explanation for the low chlorophyll concentration consistently observed across the Spencer Gulf ([22]-[25]; present study). The bacterial losses due to both microzooplankton grazing and viral losses then seem minimal in the coastal waters of the Spencer Gulf, even though their seasonality (**Figure 7**) suggests an overall dominance of grazing over viral lysis in summer and winter; the opposite holds true in spring, while microzooplankton grazers and viruses are equivalent mortality agents in autumn. In contrast, the microzooplankton grazing and viral lysis rates of *Synechococcus*, *Prochlorococcus* and picoeukaryotes are comparable to previous observations conducted in the oligotrophic waters of the Southern Ocean, which suggests that the mortality dynamics of these populations is similar to those encountered in other oligotrophic waters.

4.3. Methodological Considerations

The dilution assay used in this study has the advantage of excluding the use of a conversion factor and of minimizing the handling of the sample. Its use has been validated across different heterotrophic bacteria and algal host taxa [14]-[16] [20] [88]. Besides, the consistency between the viral lysis rates obtained using this approach and other methods assessing cell lysis in a range of phytoplankton [14] [15] and heterotrophic bacteria [88] guarantees the reliability of this assay to infer the effects of viruses on bacteria and phytoplankton mortality. We nevertheless recall hereafter a few potential limitations of the methods that need to be kept in mind when interpreting its results.

First, this assay only detects the lysis of hosts that are newly produced, hence favors lytic infection in fast growing species [20], which hence may lead to overestimate viral lysis; see also [14] for a detailed discussion. In turn, viral lysis is likely to be underestimated when viral infections occur in cells with reduced metabolism [89], which might be the case in environments dominated by low DNA bacteria such as the Spencer Gulf. In addition, recent results dissecting the effect of viruses on the mortality of distinct bacterial sub-populations showed that viruses preferentially infect the most abundant bacterial sub-populations [88]. This is consistent with the idea that host separation distance is the most important factor in defining virus success [90]. This also highlights the possibility that estimates of global mortality rates may only provide partial information on the "game" at play between viruses and their bacterial hosts.

Second, a fundamental assumption of the dilution assay is that host losses are proportional to the dilution effect on the abundance of the mortality agents. This implies that a single round of infection should be detected, hence that the host's cell lysis must occur later than 12 h after infection but within the 24-h incubation. Studies of bacterial and algal host-virus model systems (including prokaryotic algal hosts) indeed suggest that the time to cell lysis upon viral infection ranges within 24 h [91] [92]. A fundamental limitation of the dilution assay that has, however, to be stressed is related to the balance existing between lytic and lysogenic viral infection. A prophage can be induced into the lytic pathway either spontaneously or by physical or chemical factors [93], but little is still known on the relative importance of the two modes of infection [94]. Prophage not induced into the lytic pathway within 24 h would then intrinsically lead to underestimate viral lysis.

The dilution assay also assumed that there is no preferential grazing by microzooplankton. For instance, in case of preferential grazing upon infected cells, viral lysis would be underestimated, as suggested earlier [4]. A recent study dissecting the effect of microzooplankton grazing on the mortality of distinct bacterial sub-populations showed that the larger cell-sized bacterial sub-populations with greater DNA content experienced higher

rates of microzooplankton predation [89]. While this is consistent with previous results indicating that medium to large sized bacteria, along with more active and dividing cells, are most susceptible to grazing [17], this is likely to bias bacterial mortality rates towards microzooplankton grazing.

Finally, reliable results of the dilution assay also rely on the absence of substantial loss of virus during the incubation. Grazing by heterotrophic nanoflagellates (HNFs) may be a source of viral loss, but reported rates of viral removal by HNFs are low enough, *i.e.* ≤0.3% d^{-1} [95], to be considered negligible.

5. Conclusion

We have shown that the relative impacts of viral lysis and microzooplankton grazing on heterotrophic bacteria and both prokaryotic and picoeukaryotic phytoplankton in the oligotrophic waters of the Spencer Gulf were very heterogeneous in space and time, specifically, viral lysis fuels primary and secondary productivity through the release of organic matter and nutrients, which shunts substrates to uninfected microbes. The relatively low mortality rates reported here for heterotrophic bacteria—in particular the low viral lysis rates and the related low recycling of potentially growth-limiting nutrients—are consistent with a bottom-up limitation of phytoplankton biomass and production. In turn, the phytoplankton mortality rates are similar to those reported in other oligotrophic waters, hence we suggest a top-down control of phytoplankton communities. More generally, our results—and in particular the observed space-time heterogeneity of the impact of mortality agents on heterotrophic bacteria and prokaryotic and picoeukaryotic phytoplankton—indicate that shifts in top-down and bottom-up pressures occurring in association with environmental variability should be carefully considered when assessing the effects of fluctuations and gradients in physical parameters on microbial assemblages. Taken together our findings, we suggest that the influence of viruses and microzooplankton 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 substantially shift in importance as a consequence of changes in both environmental and ecological parameters.

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