

# Distribution of heterotrophic bacteria and virus-like particles along a salinity gradient in a hypersaline coastal lagoon

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**ABSTRACT:** The abundance and community structure of viruses and heterotrophic bacteria were investigated along a natural continuous salinity gradient (18 to 155 PSU) in a South Australian temperate coastal lagoon using flow cytometry. The brackish waters of the lagoon (18 to 25 PSU) were characterised by high viral concentrations ( $1.3 \times 10^8$  to  $1.5 \times 10^8$  ml<sup>-1</sup>) and elevated virus to bacteria ratios (11.7 to 15.5), suggesting high viral infection rates and long persistence of viruses in the water. The increase in salinity above 25 PSU resulted in a decrease in viral abundance without a significant modification of bacterial abundance. A concomitant increase in viral and bacterial abundances with salinity was observed from 50 to 150 PSU, where the highest abundances were observed ( $1.4 \times 10^8$  and  $2.5 \times 10^8$  ml<sup>-1</sup>, respectively). Prokaryote cytometric richness also varied greatly along the salinity gradient, suggesting a modification of the phylogenetic composition and/or variability in the activity level of the bacterioplankton community along the salinity gradient. The most cytometrically diversified bacterial community (6 to 7 subpopulations) was observed between 50 and 150 PSU. BIOENV analysis identified salinity as the main factor structuring the distribution of bacteria throughout the lagoon. The effect of salinity on viral populations appeared to be more indirect, as the distribution of viruses was mainly driven by host abundance (i.e. bacteria) along the salinity gradient. The complex patterns described here represent the first observation of viral and microbial dynamics along a continuous salinity gradient from 18 to 155 PSU.

**KEY WORDS:** Salinity · Virus-like particles · Heterotrophic bacteria · Hypersaline lagoon

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## INTRODUCTION

Temperature, nutrient and salinity gradients are likely to be important areas of highly dynamic compositional and functional changes in marine systems (Bouvier & del Giorgio 2002). In particular, important ecological changes such as decreasing biodiversity and increasing dominance of microbes occur along salinity gradients (Rodriguez-Valera 1988, 1993, Javor 1989). Salinity gradients are found over a wide range of marine environments, especially within estuaries and associated wetlands, salt marshes and coastal

lagoons (Kaiser et al. 2005). Most of these ecosystems constitute vital nurseries for fish and feeding grounds for birds (Ayadi et al. 2004, Kaiser et al. 2005). Long-term management of these ecosystems, often heavily threatened by anthropogenic and climatic changes (e.g. Dauvin et al. 2006), relies on an improved understanding of the effect of salinity on the structure and functioning of the organisms at the base of food webs.

Although viruses and heterotrophic bacteria are known to play a critical role in biogeochemical cycles and ecosystem functioning (Azam et al. 1983, Fuhrman 1999, Azam & Malfatti 2007, Suttle 2007), their dynam-

ics along natural salinity gradients still need to be documented. Several studies have been performed along salinity gradients, especially within estuaries, to investigate the influence of salinity on the composition and distribution of virioplankton and bacterioplankton communities (e.g. Cunha et al. 2000, Bouvier & del Giorgio 2002, del Giorgio & Bouvier 2002, Langenheder et al. 2003, Crump et al. 2004, Kan et al. 2006). However, in these studies estuarine salinities did not exceed 50 PSU, and the dynamics of these organisms under high salinity conditions (i.e. above 50 PSU) has been mainly conducted in crystallizer ponds from solar salterns (e.g. Guixa-Boixareu et al. 1996, Pedrós-Alió et al. 2000a,b, Benlloch et al. 2002, Santos et al. 2007) or in hypersaline lakes (i.e. 0.2 to 364 PSU) (e.g. Demergasso et al. 2004, Jiang et al. 2006, Wu et al. 2006, Demergasso et al. 2008, Foti et al. 2008).

In this context, the present study investigates the effects of the strong salinity gradient (i.e. 18 to 155 PSU) occurring in a temperate coastal lagoon on viral and bacterial communities. The Coorong lagoon is one of Australia's most significant wetlands, especially as a waterbird habitat. It has been designated a wetland of international importance under the Ramsar Convention in 1985. Beginning in the 1940s, the Coorong was impacted by the building of barrages that favored the flow of seawater into the wetlands over the usual freshwater flow from the Murray River. In addition, climate variability (lower freshwater inputs and higher evaporation processes) also led to increased salinity of the lagoon. A better understanding of the shifts in microbial communities with increasing salinity is thus critical—both locally, for the management of this threatened ecosystem, and globally, as it provides a convenient and unique model system—to rapidly and comprehensively study the potential effects of environmental gradients and perturbations on community shifts.

More specifically, given the lack of information related to the dynamics of viral and microbial communities along continuous natural hypersaline gradients, our objectives were to (1) investigate the changes in the abundance and diversity of flow cytometrically defined populations of virus-like particles (VLP) and heterotrophic bacteria observed from brackish to hypersaline waters; (2) identify the main structuring factors driving this distribution; and (3) discuss the potential consequences of these changes on food web structure and function.

## MATERIALS AND METHODS

**Study site.** The Coorong is a shallow coastal lagoon in South Australia, more than 140 km in length and characterised by a strong salinity gradient with values

ranging from ca. 20 PSU close to the mouth of the Murray River (estuarine habitat) to more than 150 PSU near Salt Creek (hypersaline habitat) (Fig. 1). This lagoon, parallel to the coast, is separated from the open ocean by a network of sand dunes and forms the Murray Mouth with the Lower Lakes (Lake Alexandrina and Lake Albert), which is the terminal lake system of the Murray River (Webster et al. 2004). The saline waters of the Coorong receive inputs from the ocean through the Murray River mouth and are separated from the lower lakes by a series of barrages. The Coorong is divided into the northern and southern lagoons by a narrow constriction near Parnka Point (Fig. 1). The freshwater inputs through the barrages lead to lower salinities in the northwest part of the Coorong, whereas the excess in evaporation over precipitation increases salinity along its north–south axis, especially during the summer period characterised by low water levels (ranging from 0.5 m in Goolwa to 0.9 m in the southern lagoon) and weak tidal impact (Webster et al. 2004).

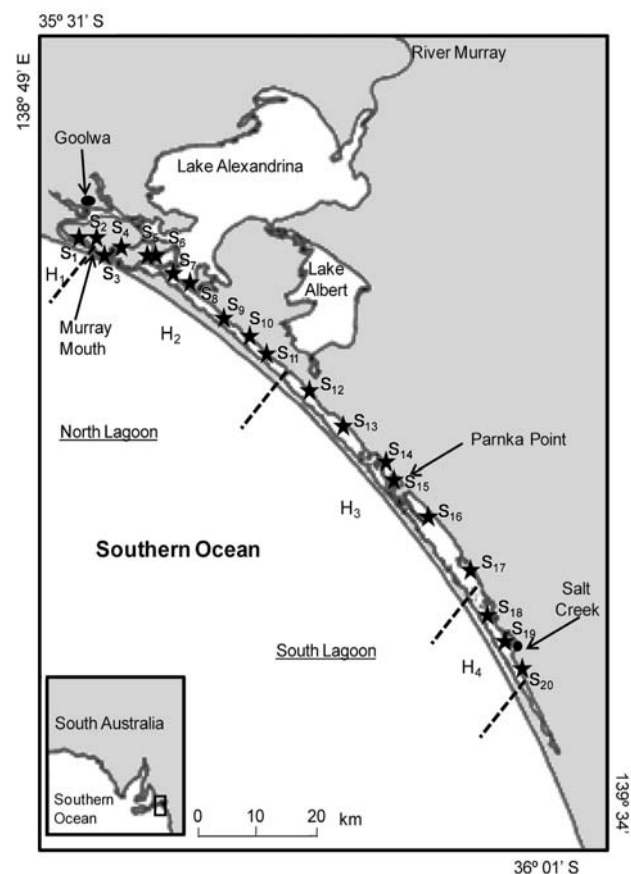


Fig. 1. The Coorong lagoon, South Australia, and 20 sampling sites (★). H<sub>1</sub>: brackish (<25 PSU); H<sub>2</sub>: low salinity (30–50 PSU); H<sub>3</sub>: high salinity (50–150 PSU); H<sub>4</sub>: hypersaline (>150 PSU) habitats. Black dots indicate location of cities

**Sampling strategy.** Samples were collected at 20 locations along the Coorong, from the brackish waters near Goolwa (18 PSU) to the hypersaline waters near Salt Creek (155 PSU; Fig. 1) on 3 to 4 February 2007. At each sampling site (Stns  $S_1$  to  $S_{20}$ ) (Fig. 1), measurements and water sample collection were performed in 50 cm of water from the subsurface waters and at the water–sediment interface (WSI). Temperature ( $^{\circ}\text{C}$ ), conductivity ( $\text{mS cm}^{-1}$ ) and dissolved oxygen (DO) concentrations ( $\text{mg l}^{-1}$ ) were recorded using a YSI 85 multiparameter probe (Fondriest). Salinity was calculated from temperature and conductivity following Fofonoff & Millard (1983). Water samples were collected using acid-washed 1 l borosilicate bottles with special care to avoid sediment resuspension.

Dissolved inorganic nutrient concentrations were determined from 12 ml filtered (Whatman GF/C) water samples. Analyses were performed in the field using a portable LF 2400 photometer (AquaspeX) according to standard colorimetric methods for  $\text{NH}_4^+$  (Indophenol blue),  $\text{NO}_2^-$  (naphthylethylene diamine),  $\text{NO}_3^-$  (naphthylethylene diamine after zinc reduction) and  $\text{PO}_4^{3-}$  (ascorbic acid reduction). The instrument measured values for ammonium, nitrite, nitrate and phosphate from 0.6 to 110, 0.2 to 160, 1.6 to 160 and 1.1 to 50  $\mu\text{mol l}^{-1}$ , respectively.

Samples (50 to 100 ml) for suspended particulate material (SPM) concentration ( $\text{mg l}^{-1}$ ) were filtered through pre-combusted ( $400^{\circ}\text{C}$ , 4 h) and pre-weighed glass-fibre filters (Whatman GF/C), and immediately deep frozen in liquid nitrogen until analysis. In the laboratory, filters were rinsed with Milli-Q water, dried at  $60^{\circ}\text{C}$  for 24 h, and reweighed to determine the mass of suspended solid retained on the filter (Hewson et al. 2001).

Samples for chlorophyll *a* (chl *a*) extraction (50 to 100 ml) were filtered through glass-fibre filters (Whatman GF/C) and immediately deep frozen in liquid nitrogen until analysis. Chlorophyllous pigments were then extracted in 5 ml of methanol in the dark at  $4^{\circ}\text{C}$  over 24 h, and chl *a* concentration ( $\mu\text{g l}^{-1}$ ) was determined following Strickland & Parsons (1972) using a Turner 450 fluorometer previously calibrated with a pure chl *a* solution (*Anacystis nidulans* extract, Sigma Chemicals).

**Flow cytometry analysis.** For the identification and enumeration of VLP and heterotrophic bacteria, samples were collected in triplicate (1 ml) at each sampling depth, fixed with 0.5% (final concentration) glutaraldehyde in the dark at  $4^{\circ}\text{C}$  for 15 min, quick frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until analysis (Brussaard 2004). All samples were processed within 1 mo to minimize storage loss (Marchant et al. 2000). VLP and heterotrophic bacteria were enumerated using a FACScanto flow cytometer (Becton-Dickinson). After being quick thawed, samples were diluted (1:10) in 0.2  $\mu\text{m}$  filtered TE Buffer (10 mM Tris, 1 mM EDTA,

[pH 8]), stained with SYBR-I Green solution (1:5000 dilution) and incubated at  $80^{\circ}\text{C}$  in the dark for 10 min (Brussaard 2004). For each sample, forward-angle light scatter (FSC; 285 V), side-angle light scatter (SSC; 550 V), green (SYBR-I) fluorescence (500 V), red fluorescence (500 V) and orange fluorescence (500 V) were acquired. Fluorescent beads 1  $\mu\text{m}$  in diameter (Molecular Probes) were added to all samples as an internal size and concentration standard. Working bead concentrations were estimated after each flow cytometry (FCM) session under epifluorescent microscopy to ensure their reliability (Gasol & del Giorgio 2000), and all FCM parameters were normalized to bead concentration and fluorescence. VLP and heterotrophic bacteria populations were identified and quantified using the FCM analysis software WinMDI 2.9 (Scripps Research Institute). Subpopulations of VLP and bacteria were discriminated based on their differences in SYBR-I Green fluorescence and SSC, i.e. as non-overlapping classes of size and green fluorescence, according to Marie et al. (1997, 1999a) and Brussaard (2004).

The correspondence between FCM counts and epifluorescence microscopy (EM) counts was investigated using samples collected from 4 stations characterized by increasing salinities, i.e. 26.7, 83.7, 134.0 and 154.2 PSU at Stns  $S_3$ ,  $S_{13}$ ,  $S_{16}$  and  $S_{20}$ , respectively (Fig. 1). Bacteria and viruses were counted by EM following the SYBR-I Green staining method described in Noble & Fuhrman (1998). Five samples were considered for each site, and for each sample 10 to 20 fields of view were selected randomly; a total of >500 viruses and >500 bacteria were counted on a Leitz dialux 20 EB microscope attached to a Leitz 12 V Quartz lamp at 1000 $\times$  magnification under blue excitation.

**Data analysis.** Comparisons between the 2 sampling depths were conducted using the Wilcoxon-Mann-Whitney *U*-test. Multiple comparisons between sampling sites 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).

The BIOENV procedure (PRIMER version 5; Clarke & Warwick 1994, 2001) was used to identify the environmental variables that best explained variation in VLP and heterotrophic bacteria. BIOENV is a non-parametric method that calculates Spearman's rank correlation coefficients between the matrices of VLP and bacteria (hereafter the biotic matrix) and the abiotic similarity matrix of environmental variables based on the Bray-Curtis similarity index. Environmental variables considered in the BIOENV analysis were: salinity, [DO], [SPM], [chl *a*], [ $\text{NH}_4^+$ ], [ $\text{NO}_3^- + \text{NO}_2^-$ ] and [ $\text{PO}_4^{3-}$ ]. For VLP, abundances of the 7 subpopulations of bacteria ( $B_1$  to  $B_7$ ) were considered as environmental variables. As temperature variability between stations

was mainly related to the time of the day when the sampling occurred, this parameter was not considered in the analyses. Combinations of environmental variables were considered at steadily increasing levels of complexity, i.e.  $k$  variables at a time ( $k = 1, 2, 3$ ), to yield the best matches of biotic and abiotic similarity matrices for each  $k$  as measured by Spearman's rank correlation. This method then selects the environmental variables that best explain the VLP and bacteria patterns by maximizing a Spearman's rank correlation between the biotic and abiotic matrices.

Similarities between stations for bacterioplankton communities along the salinity gradient were inferred through a cluster analysis (i.e. hierarchical agglomeration using complete linkage cluster analysis performed on Euclidian distances) performed on the log (abundance + 1) data matrix (Legendre & Legendre 2003).

## RESULTS

No significant differences were found between the subsurface and WSI for any abiotic or biotic parameters (Mann-Whitney  $U$ -test,  $p > 0.05$ ). This suggests that the water column was well-mixed throughout the lagoon, in accordance with previous results (Webster et al. 2004). Subsurface and WSI data were then pooled for further analysis.

### Environmental parameters

Mean ( $\pm$ SD) temperature was  $26.1 \pm 2.0^\circ\text{C}$ . Salinity increased from 17.7 PSU at Stn S<sub>1</sub> to 154.8 PSU at Stn S<sub>20</sub> (Fig.1), and allowed the identification of 4 distinct habitats (H<sub>1</sub> to H<sub>4</sub>, Table 1). At Habitat H<sub>1</sub> (Stns S<sub>1</sub> and S<sub>2</sub>), salinity levels remained below 25 PSU. Salinity then slowly increased from 27.5 to 50.3 PSU at Habitat H<sub>2</sub> (between Stns S<sub>3</sub> and S<sub>11</sub>). In contrast, salinity sharply increased at Habitat H<sub>3</sub> (from Stn S<sub>12</sub> to Stn S<sub>17</sub>), reaching 150 PSU. Finally, the hypersaline Habitat H<sub>4</sub> (Stns S<sub>18</sub>, S<sub>19</sub> and S<sub>20</sub>) exhibited salinity ranging between 150.1 and 155.8 PSU.

Ammonium was by far the most abundant form of nitrogen, as  $\text{NO}_3^- + \text{NO}_2^-$  concentrations were very low at most locations (i.e.  $<1.6 \mu\text{mol l}^{-1}$ ; Table 1).  $\text{NH}_4^+$  concentrations increased with salinity and Habitats H<sub>3</sub> and H<sub>4</sub> were characterised by high concentrations (i.e.  $>110 \mu\text{mol l}^{-1}$  at most stations; Table 1).  $\text{PO}_4^{3-}$  concentrations were highly vari-

able with local high ( $>50 \mu\text{mol l}^{-1}$ ) and low ( $<1.1 \mu\text{mol l}^{-1}$ ) concentrations without any specific pattern along the salinity gradient (Table 1).

DO concentrations ranged between 1.4 and  $5.5 \text{ mg l}^{-1}$  at Stns S<sub>20</sub> and S<sub>9</sub>, respectively (Table 1), and varied significantly among the 4 habitats (Kruskal-Wallis test,  $p < 0.05$ ). DO concentrations were significantly ( $p < 0.05$ ) lower in Habitats H<sub>1</sub> ( $1.9 \pm 0.3 \text{ mg l}^{-1}$ ) and H<sub>4</sub> ( $2.1 \pm 0.6 \text{ mg l}^{-1}$ ) than in H<sub>2</sub> ( $4.7 \pm 0.8 \text{ mg l}^{-1}$ ) and H<sub>3</sub> ( $4.4 \pm 0.9 \text{ mg l}^{-1}$ ).

SPM concentrations exponentially increased from  $30 \text{ mg l}^{-1}$  at Stn S<sub>1</sub> to  $967 \text{ mg l}^{-1}$  at Stn S<sub>17</sub>, and decreased to  $538 \text{ mg l}^{-1}$  at Stn S<sub>20</sub> (Table 1). Concentrations varied significantly among the 4 defined habitats (Kruskal-Wallis test,  $p < 0.05$ ), and were significantly higher in Habitats H<sub>3</sub> and H<sub>4</sub> than in H<sub>1</sub> and H<sub>2</sub> ( $p < 0.05$ ).

Chl *a* concentrations remained lower than  $6 \mu\text{g l}^{-1}$  in Habitats H<sub>1</sub> and H<sub>2</sub> (Table 1), and increased in Habitat H<sub>3</sub> from  $3.6 \mu\text{g l}^{-1}$  at Stn S<sub>11</sub> to  $14.1 \mu\text{g l}^{-1}$  at Stn S<sub>16</sub>. Concentrations sharply decreased from Stns S<sub>16</sub> to S<sub>17</sub> to lower than  $4 \mu\text{g l}^{-1}$ . Chl *a* concentrations varied significantly among the 4 habitats (Kruskal-Wallis test,  $p < 0.05$ ), and were significantly higher in Habitat H<sub>3</sub> than in the 3 other habitats ( $p < 0.05$ ), which were not statistically different from one another.

### Viral and bacterial abundances

No significant differences were found between viral and bacterial counts obtained from FCM and EM (Mann-Whitney  $U$ -test,  $n = 5$ ,  $p > 0.05$ ) at each of the 4 stations sampled (i.e. S<sub>3</sub>, S<sub>13</sub>, S<sub>16</sub> and S<sub>20</sub>). In addition, EM observations did not reveal any specific changes in filamentous bacteria abundances, nor specific changes in bacterial morphologies along the salinity gradient.

Viral abundance was highly variable, ranging between  $9.0 \times 10^6$  and  $2.5 \times 10^8 \text{ ml}^{-1}$  (Fig. 2a). Signifi-

Table 1. Variation ranges (min–max) of ammonium ( $[\text{NH}_4^+]$ ), nitrite + nitrate ( $[\text{NO}_3^- + \text{NO}_2^-]$ ), and phosphate ( $[\text{PO}_4^{3-}]$ ) concentrations, and mean  $\pm$  SD of dissolved oxygen (DO), suspended particular matter (SPM) and chl *a* concentrations measured in the 4 habitats. H<sub>1</sub> (Stns S<sub>1</sub> and S<sub>2</sub>): brackish ( $<25$  PSU); H<sub>2</sub> (Stns S<sub>3</sub> to S<sub>11</sub>): low salinity (30–50 PSU); H<sub>3</sub> (Stns S<sub>12</sub> to S<sub>17</sub>): high salinity (50–150 PSU); H<sub>4</sub> (Stns S<sub>18</sub> to S<sub>20</sub>): hypersaline ( $>150$  PSU)

	H <sub>1</sub> (S <sub>1</sub> –S <sub>2</sub> )	H <sub>2</sub> (S <sub>3</sub> –S <sub>11</sub> )	H <sub>3</sub> (S <sub>12</sub> –S <sub>17</sub> )	H <sub>4</sub> (S <sub>18</sub> –S <sub>20</sub> )
Salinity (PSU)	<25	30–50	50–150	>150
$[\text{NH}_4^+]$ ( $\mu\text{mol l}^{-1}$ )	18.7 – >110.0	3.3 – 70.0	20.0 – 110.0	60.0 – >110.0
$[\text{NO}_3^- + \text{NO}_2^-]$ ( $\mu\text{mol l}^{-1}$ )	<1.6	<1.6 – 9.7	<1.6 – 3.2	<1.6
$[\text{PO}_4^{3-}]$ ( $\mu\text{mol l}^{-1}$ )	4.2 – >50.0	<1.1 – >50.0	<1.1 – 31.6	<1.1 – >50.0
DO ( $\text{mg l}^{-1}$ )	$1.9 \pm 0.3$	$4.7 \pm 0.8$	$4.4 \pm 0.9$	$2.1 \pm 0.6$
SPM ( $\text{mg l}^{-1}$ )	$40.0 \pm 14.0$	$74.0 \pm 31.0$	$381.0 \pm 70.0$	$588.0 \pm 64.0$
Chl <i>a</i> ( $\mu\text{g l}^{-1}$ )	$1.7 \pm 0.2$	$2.5 \pm 1.7$	$7.6 \pm 4.2$	$2.8 \pm 1.0$

cantly higher viral concentrations were observed in Habitats H<sub>1</sub> and H<sub>4</sub> ( $1.3 \times 10^8$  to  $2.5 \times 10^8$  ml<sup>-1</sup>) than in H<sub>2</sub> and H<sub>3</sub>, where VLP abundances remained less than  $7.5 \times 10^7$  ml<sup>-1</sup> ( $p < 0.05$ ).

Bacterial abundances ranged from  $2.1 \times 10^6$  ml<sup>-1</sup> at Stn S<sub>3</sub> to  $2.4 \times 10^8$  ml<sup>-1</sup> at Stn S<sub>20</sub> (Fig. 2b). Abundances significantly differed between habitats (Kruskal-Wallis test,  $p < 0.05$ ) and were significantly higher in Habitat H<sub>4</sub> ( $7.7 \times 10^7$  to  $1.4 \times 10^8$  ml<sup>-1</sup>) than the other 3 habitats. The relatively low bacterial abundances observed in Habitats H<sub>1</sub>, H<sub>2</sub> and H<sub>3</sub> were not significantly different from one another ( $p < 0.05$ ).

The ratio of VLP to bacteria (VBR) ranged between 1.0 and 15.5 at Stns S<sub>18</sub> and S<sub>1</sub>, respectively (Fig. 2c) and varied significantly among the 4 habitats (Kruskal-Wallis test,  $p < 0.05$ ). VBRs were significantly higher at Stns S<sub>1</sub> and S<sub>2</sub> in Habitat H<sub>1</sub> (15.5 and 11.7, respectively) than at stations in Habitats H<sub>2</sub>, H<sub>3</sub> and H<sub>4</sub> ( $p < 0.05$ ), where values remained lower than 10 (Fig. 2c).

### Microbial communities identified using FCM

Samples were characterised by a highly complex community structure with multiple subpopulations of VLP and bacteria throughout the salinity gradient. FCM analysis revealed 3 distinct populations of viruses and 7 distinct populations of bacteria. These different subpopulations were identified according to their differences in size (SSC) and SYBR Green fluorescence.

Three VLP populations were identified as discrete peaks on histogram plots of SYBR Green fluorescence and cytograms (Fig. 3a). Two populations, defined as VLP1 and VLP2, corresponded to populations observed previously in seawater samples (Marie et al. 1999a). The third population, VLP3, exhibited the same SYBR Green fluorescence level as VLP2 but was characterised by a higher SSC (Fig. 3b). Recent work has indicated that most phytoplankton viruses are generally characterised by higher side scatter and/or green fluorescence (Brussaard et al. 1999, 2005, 2008). This suggests that the VLP3 population, characterised by higher side scatter and green fluorescence, represents a group of phytoplankton viruses. The VLP1 population was observed in all samples throughout the lagoon (Fig. 4a). In contrast, VLP2 and VLP3 populations were restricted to particular habitats (Fig. 4a). VLP2 was only present in Habitats H<sub>1</sub> and H<sub>2</sub>, while VLP3 was only observed from Stns S<sub>11</sub> to S<sub>14</sub> in Habitats H<sub>2</sub> and H<sub>3</sub>. From Stns S<sub>1</sub> to S<sub>14</sub>, where the 3 VLP populations were present, VLP1 was by far the most abundant population and always accounted for more than 66% of the viral community (Fig. 4a).

Seven different populations of heterotrophic bacteria were discriminated (Fig. 3). These populations consis-

tently exhibited different side scatter and fluorescence signatures (Fig. 3b). As such, they were not classified into the high and low DNA subpopulations previously observed in marine and freshwater systems (Gasol et al. 1999, Lebaron et al. 2002, Servais et al. 2003, Seymour et al. 2004, 2005), but defined as 7 discrete populations (Bouvier et al. 2007). These populations were identified as non-phototrophic bacterial populations as their size and nucleic acid content were consistent with bacterial-sized organisms, and because they lacked red or orange fluorescence (indicative of chlorophyll or photo-pigment content; Marie et al. 1999b). Three populations, B<sub>1</sub>, B<sub>3</sub> and B<sub>4</sub>, were by far the most abundant and contributed to more than 70% of the total heterotrophic bacteria abundances (Fig. 4b). Whilst B<sub>1</sub> and B<sub>3</sub> were observed in

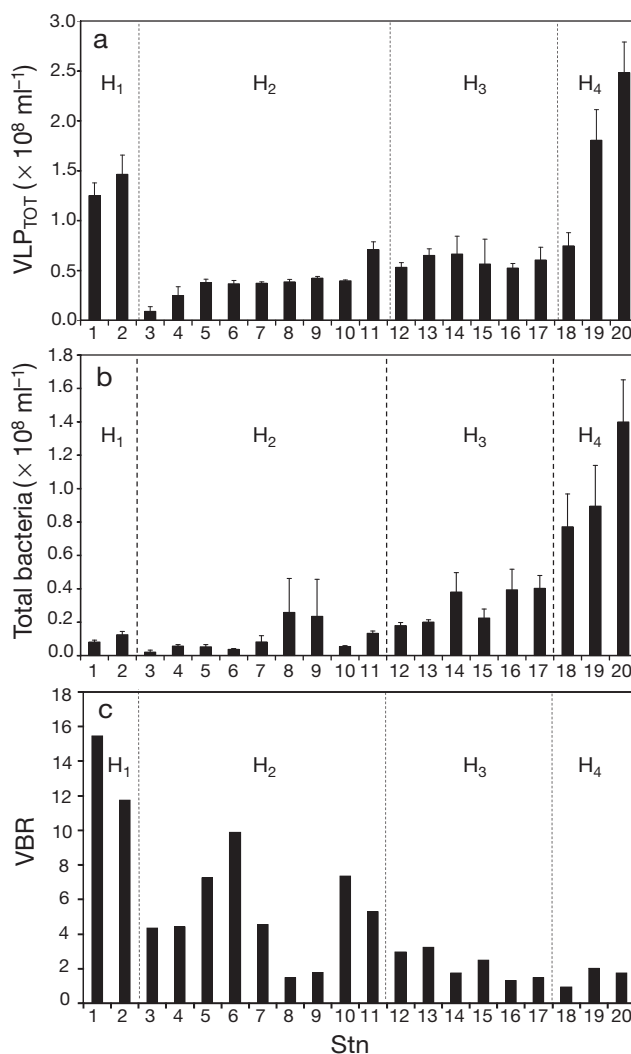


Fig. 2. Abundances of (a) total virus-like particles (VLP,  $\times 10^8$  ml<sup>-1</sup>) and (b) heterotrophic bacteria (total bacteria  $\times 10^8$  ml<sup>-1</sup>) along the salinity gradient. Error bars are SD. (c) Virus to bacteria ratio (VBR) along the salinity gradient. H<sub>1</sub>: brackish (<25 PSU); H<sub>2</sub>: low salinity (30–50 PSU); H<sub>3</sub>: high salinity (50–150 PSU); H<sub>4</sub>: hypersaline (>150 PSU) habitats

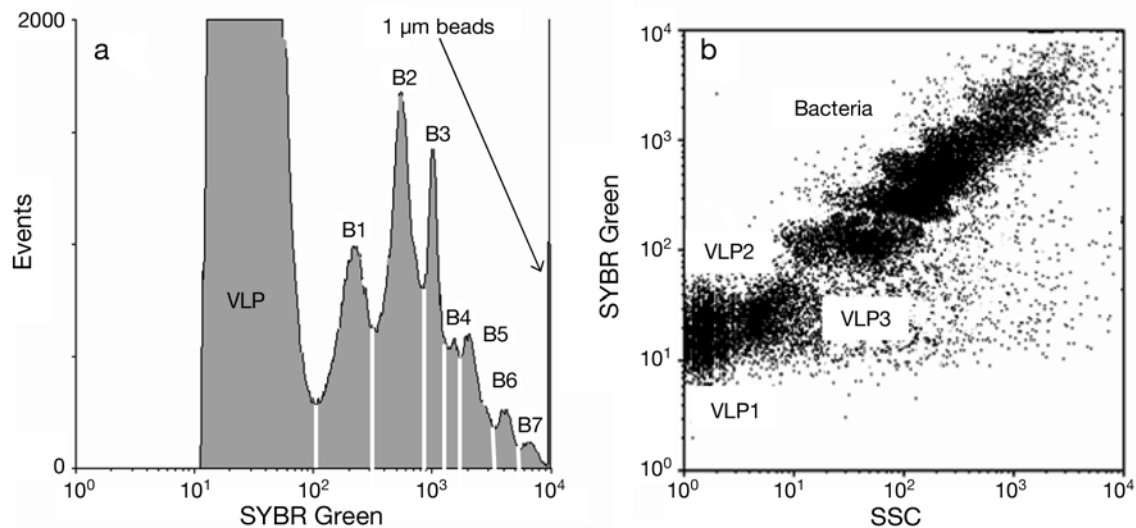


Fig. 3. Cytometric differentiation of heterotrophic bacteria populations identified along the salinity gradient. (a) Histogram of green fluorescence showing peaks relating to 7 populations of increasing DNA content (B1, B2, B3, B4, B5, B6 and B7). (b) Scatterplot of side-angle light scatter (SSC) versus green fluorescence (SYBR Green) showing 3 viral populations (VLP1, VLP2 and VLP3). Heterotrophic bacteria subpopulations differed through their green fluorescence and side scatter, and therefore were not classified into high and low DNA-subpopulations, but as different discrete populations as described by Bouvier et al. (2007)

all samples, Population B<sub>4</sub> was only observed in Habitats H<sub>2</sub> to H<sub>4</sub> (where salinity was >25 PSU; Fig. 4b). In contrast, Populations B<sub>2</sub>, B<sub>5</sub>, B<sub>6</sub> and B<sub>7</sub> were restricted to particular habitats and their relative abundance remained <25% (Fig. 4b). Population B<sub>5</sub> was observed locally over a large range of salinity conditions from Stns S<sub>3</sub> to S<sub>18</sub>. Populations B<sub>2</sub>, B<sub>6</sub> and B<sub>7</sub> were mainly observed in Habitat H<sub>3</sub> from Stns S<sub>10</sub> to S<sub>18</sub> (Fig. 4b). Population B<sub>2</sub> exhibited a particularly restricted distribution within Habitat H<sub>3</sub>; this population was only observed at Stns S<sub>15</sub>, S<sub>16</sub> and S<sub>17</sub> (Fig. 4b).

The cluster analysis performed on bacterial abundance discriminated 2 main groups of stations based on their population richness (defined here as flow cytometrically defined richness, i.e. FCM richness): (1) a high FCM richness group (i.e. richness > 4) mainly occurred in Habitat H<sub>3</sub>, and (2) a low FCM richness group (i.e. richness ≤ 4) comprised stations from Habitats H<sub>1</sub>, H<sub>2</sub> and H<sub>4</sub> (Fig. 5). FCM richness was highly variable between salinity habitats (Kruskal-Wallis test,  $p < 0.05$ ), and was significantly ( $p < 0.05$ ) higher in Habitat H<sub>3</sub> ( $6.5 \pm 0.5$  populations) than in Habitats H<sub>1</sub> ( $2.0 \pm 0.0$ ) and H<sub>2</sub> ( $4.0 \pm 1.1$ ).

#### Microbial communities and environmental variables

The results of the BIOENV analysis showed that the environmental variables that best explained the VLP abundance pattern along the salinity gradient were: (1) bacterial abundance of subpopulation B<sub>1</sub>, (2) bacte-

rial abundances of subpopulations B<sub>3</sub>, B<sub>4</sub> and B<sub>5</sub>, (3) salinity, (4) DO concentrations and (5) phosphate concentrations (Table 2). The Spearman's rank correlation coefficient for this analysis was 0.545 (Table 2).

Concerning heterotrophic bacteria, the BIOENV analysis revealed that the environmental variables that best explained the bacterial abundance pattern along the salinity gradient were: (1) salinity and DO concentrations, (2) SPM concentrations and (3) ammonium concentrations (Table 2). The Spearman's rank correlation coefficient for this analysis was 0.693 (Table 2).

#### DISCUSSION

Physical and chemical factors are important selective forces for bacterial and viral communities (Bouvier & del Giorgio 2002, del Giorgio & Bouvier 2002). The effect of high salinity (>50 PSU) on the distribution of heterotrophic bacteria and virus populations has been mainly described in multi-pond solar salterns where salinity ranged from seawater up to sodium chloride saturation and beyond (e.g. Rodriguez-Valera 1988, Javor 1989). Each pond can be considered at equilibrium and the related biota as a well-adapted and established community for that particular salinity (Pedrós-Alió et al. 2000b). In addition, limited species diversity, high cell abundance and short food chains make the system relatively simple (Pedrós-Alió et al. 2000b). Therefore, the biological dynamics of these systems is likely to be different from those observed along a continuous natural salinity gradient.

Most previous studies focusing on the distribution pattern or activity of bacterioplankton and VLP along salinity gradients have been conducted either in estuaries where maximum salinity did not exceed 50 PSU (e.g. Cunha et al. 2000, Bouvier & del Giorgio 2002, del Giorgio & Bouvier 2002, Langenheder et al. 2003, Crump et al. 2004, Kan et al. 2006) or by comparing the bacterial community composition observed in different lakes exhibiting a wide range of salinity values (i.e. 0.2 to 364 PSU; e.g. Demergasso et al. 2004, Jiang et al. 2006, Wu et al. 2006, Demergasso et al. 2008, Foti et al.

2008). The present study is, to our knowledge, the first related to the dynamics of heterotrophic bacteria and VLP along a natural and continuous salinity gradient with salinities ranging from 18 to 155 PSU. While the spatial dynamics of viruses and bacteria observed here is in accordance with trends previously demonstrated in different systems (i.e. estuaries, solar salterns and saline lakes), specific abundance patterns were observed which set this hypersaline lagoon apart.

### Viral and bacterial abundances along the salinity gradient

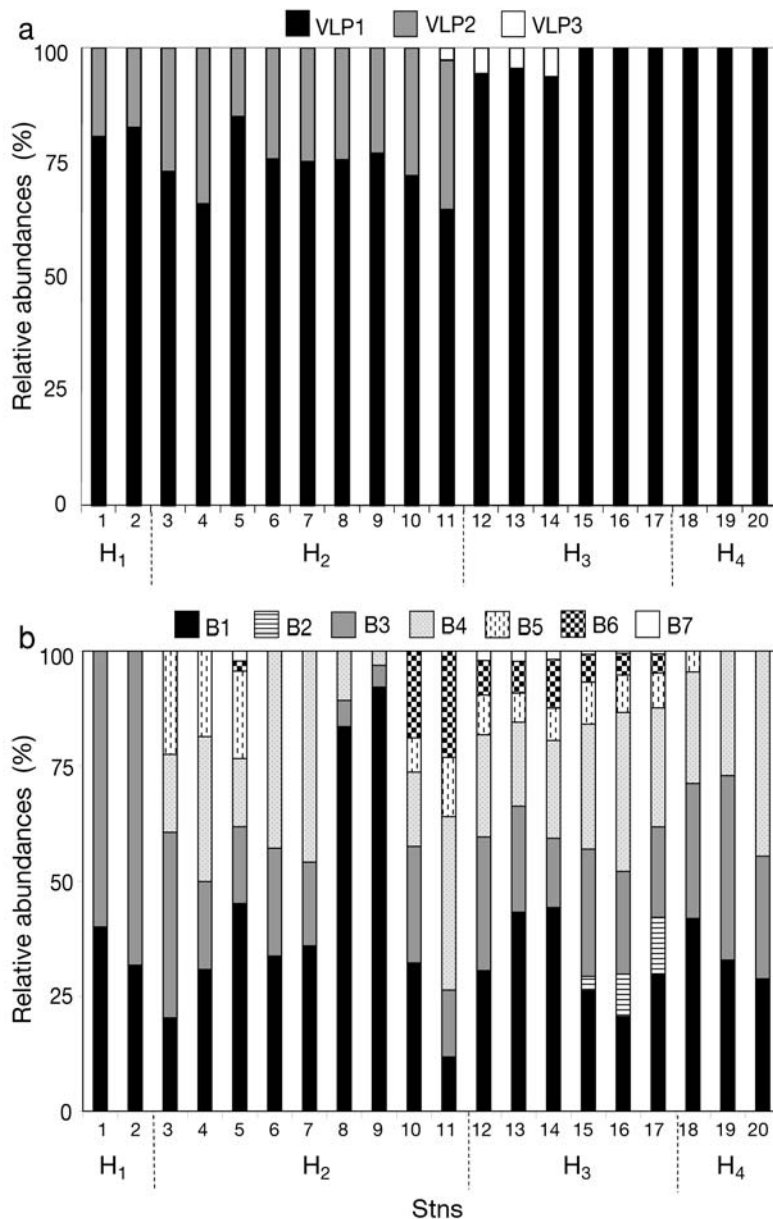


Fig. 4. Relative abundances of cytotmetrically different (a) virus-like particles and (b) heterotrophic bacteria subpopulations along the salinity gradient. H<sub>1</sub>: brackish (<25 PSU); H<sub>2</sub>: low salinity (30–50 PSU); H<sub>3</sub>: high salinity (50–150 PSU); H<sub>4</sub>: hypersaline (>150 PSU) habitats

A decrease in VLP abundances with increasing salinity from freshwater to marine environment has previously been found in estuaries (Jiang & Paul 1994, Hewson et al. 2001). This is consistent with the significant decrease in VLP abundance in the lagoon when salinity increased above 25 PSU (Fig. 2a); this occurred without a significant decrease in bacterial abundance (Fig. 2b). However, in the brackish zone of the lagoon (H<sub>1</sub>), viral abundance ranged between  $1.3 \times 10^8$  and  $1.5 \times 10^8$  ml<sup>-1</sup>, whereas at comparable salinities in the Brisbane estuary (Hewson et al. 2001), Tampa Bay (Jiang & Paul 1994) or saline Antarctic lakes (Laybourn-Parry et al. 2007), the maximum VLP concentration did not exceed  $1.26 \times 10^8$  ml<sup>-1</sup>. This observation suggests that salinity may not be the only factor that plays a part in determining VLP abundance in brackish waters. This hypothesis is supported by the results of the BIOENV analysis (Table 2). Limitation of nutrients such as carbon, oxygen, nitrogen and phosphorus has been shown to indirectly affect viral proliferation through its effects on host metabolism (Weinbauer 2004); additionally, changes in the phosphate status of water could directly affect viral production (Lymer & Vrede 2006).

One of the main features of the spatial dynamics of VLP and heterotrophic bacteria along the Coorong lagoon was the very high abundances observed under high salinity conditions, i.e.  $2.5 \times 10^8$  and  $1.4 \times 10^8$  ml<sup>-1</sup> above 150 PSU, respectively (Fig. 2a,b). This increase in VLP and bacterial abundances with increasing salinity is congruent with previous observations conducted in salterns for comparable salinity ranges. The maximum abundances observed in the present study were, however, well above values observed in these semi-artificial systems, which never exceeded  $4.6 \times 10^7$  ml<sup>-1</sup> for bacteria and  $5.0 \times 10^7$  ml<sup>-1</sup> for viruses, under similar

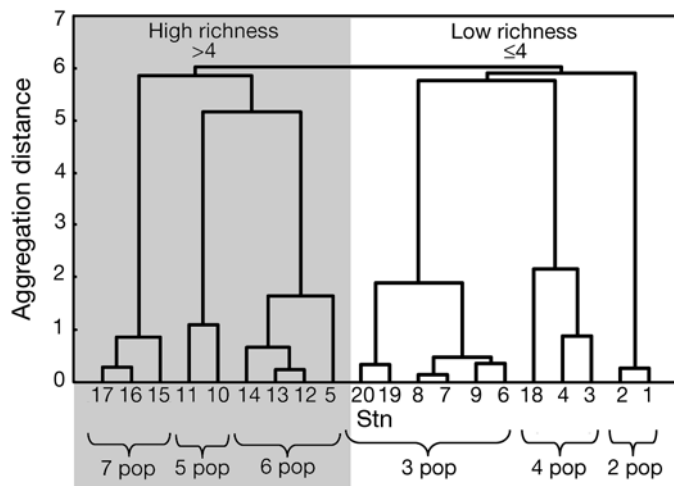


Fig. 5. Cluster analysis of heterotrophic bacteria communities along the salinity gradient, performed on the log (abundance + 1) data matrix. The x-axis shows station numbers and the number of bacterial sub-populations observed ( $x$  pop) at different groups of stations. Two main groups of stations were discriminated based on their population richness: high richness (grey,  $>4$  populations), and low richness (white,  $\leq 4$  populations)

salinity conditions (Guixa-Boixareu et al. 1996, Pedrós-Alió et al. 2000a, Joint et al. 2002). These strong differences in bacterial and viral abundances (5.4 and 2.8 times higher, respectively) highlight the unique properties of these communities found along a strong continuous salinity gradient.

The increase in bacterial abundance from 50 PSU was accompanied by an increase in SPM and chl *a* concentrations. While we did not measure the concentration of organic matter throughout the lagoon, high

Table 2. Results of the BIOENV analysis, showing the combination of the best environmental variables that predict the patterns of virus-like particles (VLP) and heterotrophic bacteria (HB) abundance along the salinity gradient. n: number of abiotic variables used in the analysis; Spearman's  $\rho$ : highest Spearman's rank correlation coefficient; k: number of corresponding significant environmental variables; S: salinity. For VLP, abundances of the 7 subpopulations of bacteria (B1 to B7) were entered as environmental variables in the BIOENV analysis. Significant contributing environmental variables were ordered according to the degree of match

	VLP	Bacteria
n	14	7
Spearman's $\rho$	0.545	0.693
k	7	4
Environmental variables	B1	S
	B3, B4, B5	SPM
	S	DO
	DO	[NH <sub>4</sub> <sup>+</sup> ]
	[PO <sub>4</sub> <sup>3-</sup> ]	

SPM concentrations may be indicative of a potential increase in organic matter, as previously observed in this part of the lagoon (Geddes 2005, Ford 2007). As bacterial growth is mainly influenced by organic matter availability (del Giorgio & Scarborough 1995, del Giorgio et al. 1997), this is consistent with the increase in bacterial abundances observed from 50 PSU. This hypothesis is supported by the results of the BIOENV analysis, underlying the role of SPM concentrations, and factors implicated in remineralisation processes (i.e. ammonium and DO concentrations), on the distribution of bacteria along the salinity gradient (Table 2). A positive correlation is generally observed between VLP and bacterioplankton in aquatic ecosystems (Wommack & Colwell 2000). The BIOENV analysis indeed highlighted a strong relationship between VLP and the abundances of the 4 dominant populations of bacteria (Table 2). The high abundances of VLP observed above 150 PSU could be largely explained by the high abundances of prokaryotic hosts found in the same area. In addition, this association between VLP and bacterial abundances, as well as the high abundances of bacteria compared to other potential planktonic hosts (i.e. phytoplankton) above 150 PSU, implies that the majority of viruses are likely to be bacteriophages in this part of the lagoon.

VBR has been used in numerous studies to define the relationship between VLP and bacteria populations (Wommack & Colwell 2000). The decrease in VBR from brackish waters ( $>10$ ) to hypersaline waters of the lagoon ( $<2$ ; Fig. 2c) suggests a weak contribution of viral lysis to prokaryotic loss in the hypersaline waters of the lagoon as previously shown in solar salters for the same range of salinity (105 to 150 PSU; Guixa-Boixareu et al. 1996). The decrease in VBR values may also indicate a short persistence of viruses in the water column in this part of the lagoon. In contrast, the elevated VBR (11.7 to 15.5) found in brackish waters suggests a longer viral persistence; viral infection of the bacterioplankton might be responsible for a large fraction of bacterial mortality in this area. These VBR values are, however, lower than those observed in saline Antarctic lakes (17.9 to 70.6) at comparable salinities (i.e. 16 to 34 PSU; Laybourn-Parry et al. 2007), suggesting that salinity is not the only abiotic factor controlling VBR. Consequently, the degree of bacterial mortality linked to viral lysis as well as the variability of abiotic factors involved in triggering viral persistence throughout the lagoon demand further research to fully assess the role of salinity on virally mediated processes. In particular, various abiotic variables such as nutrient limitation and solar radiation (particularly UV-B), known to be responsible for viral decay rates in the water column (e.g. Weinbauer 2004, Madan et al. 2005), could have played a role in deter-



mining the observed pattern of VBR along the salinity gradient.

Grazing processes could also contribute to the observed pattern of bacterial abundances along the salinity gradient. However, data on grazing patterns along salinity gradients are still scarce. Guixa-Boixareu et al. (1996) observed a strong decrease in the abundance of heterotrophic nanoflagellates and ciliates from 150 PSU, leading to a strong limitation of bacterivory processes which become negligible above 200 PSU. The increase in heterotrophic bacteria observed in hypersaline waters (>150 PSU) may thus also be the result of a decrease in grazing pressure.

### Salinity and viral and bacterial FCM richness

The increase in viral and bacterial abundances at salinities higher than 150 PSU ( $H_4$ ) was concomitant to a decrease in the number of FCM subpopulations of heterotrophic bacteria and VLP. This habitat was characterised by only 1 VLP population (VLP1) and 2 to 3 populations of heterotrophic bacteria (Fig. 4). In contrast, the number of FCM subpopulations was higher under less saline conditions, and particularly for salinity ranging between 50 and 150 PSU ( $H_3$ ; see Fig. 5).

Flow cytometry allows differentiation of subpopulations of bacteria via light scattering properties (related to size) and DNA content of individual cells (e.g. Li et al. 1995, Gasol & del Giorgio 2000, Lebaron et al. 2002, Servais et al. 2003, Bouvier et al. 2007). However, the ecological and functional roles of these different subpopulations within bacterioplankton communities remain uncertain (e.g. Bouvier et al. 2007). Variations in DNA content have been related to single cell activity with high DNA and low DNA content cells constituting, respectively, the active cells and the dormant or dead cells (Li et al. 1995, Gasol & del Giorgio 2000, Lebaron et al. 2002, Servais et al. 2003). However, there is now evidence that low DNA cells may also be active (Zubkov et al. 2001, Jochem et al. 2004, Longnecker et al. 2005). In addition there are conflicting results concerning the phylogenetic composition of these different fractions. While some studies conclude that these fractions are phylogenetically different (e.g. Longnecker et al. 2005), others suggest that the composition varies between different fractions (e.g. Zubkov et al. 2001). A recent study does not support any of these scenarios, and suggests that the existence of different FCM subpopulations within bacterioplankton assemblages is the result of complex processes that involve (1) the passage of cells from one subpopulation to another, through activation and growth, inactivation, damage and death; and (2) the existence of com-

ponents characteristic of each subpopulation (Bouvier et al. 2007). Consequently, the variability of bacterial cytometric richness observed here along the salinity gradient likely reflects a modification of the phylogenetic composition of bacterial populations as well as their activity level.

The most diversified bacterial community was observed between 50 and 150 PSU in Habitat  $H_3$  (Fig. 5). This coincided with the maxima of phytoplankton biomass and SPM, DO and ammonium concentrations observed in this habitat (Table 1). The related variety of resources and ecological niches may then have favoured the development of highly specialised populations of bacteria (e.g. Population  $B_2$ ; Fig. 4) and, therefore, the high bacteria richness observed in this part of the lagoon. However, this cytometric richness could also reflect a high activity level within the bacterial community. The abundance and diversity of resources present in this area could have favoured activation and growth of bacterial groups present in this part of the lagoon, leading to the observation of additional subpopulations exhibiting high DNA content (e.g. Populations  $B_5$ ,  $B_6$  and  $B_7$ ). With the increase in salinity above 150 PSU, the decrease in phytoplankton biomass (from  $7.6 \pm 4.2 \mu\text{g l}^{-1}$  in Habitat  $H_3$  to  $2.8 \pm 1.0 \mu\text{g l}^{-1}$  in Habitat  $H_4$ ; Table 1) and the resulting decrease in the diversity of ecological niches may have triggered both the loss of the more specialized group of bacteria observed previously throughout the lagoon and the decrease of bacterial activity, leading to the low cytometric bacterial richness observed in this area. Considering that both the phylogenetic composition and the metabolic activity of aquatic bacterioplankton determines the extent to which they contribute as potential hosts for virioplankton (Lenski 1988, Gasol & del Giorgio 2000), the decrease in viral FCM population richness could be explained by the limitation of heterotrophic bacteria richness and activity under high salinity conditions. These observations are consistent with the hypothesis that the diversity of metabolic prokaryotes would decrease with salinity (Oren 1999). A decrease in prokaryotic and viral diversity has been previously shown in solar salterns (Guixa-Boixareu et al. 1996, Diez et al. 2000, Benlloch et al. 2002, Sandaa et al. 2003, Santos et al. 2007) and in hypersaline lakes (e.g. Humayoun et al. 2003, Wu et al. 2006, Foti et al. 2008) for salinities >150 PSU. However, using fluorescent *in situ* hybridization (FISH) and/or denaturing gel gradient electrophoresis (DGGE) with subsequent DNA sequencing, these previous studies did not show a negative correlation between the number of DGGE bands with increasing salinity. Instead, they showed a decrease in the bacterial richness of genera, suggesting an increase in the microdiversity within the

remaining phylogenetic groups at higher salinities (Benlloch et al. 2002, Wu et al. 2006, Foti et al. 2008).

The succession of viral and bacterial populations was only defined in the present through their flow cytometric signature. Further work is therefore needed to confirm the identity and activity level of the different bacterial subpopulations identified through flow cytometry along the salinity gradient. However, our results provide new insight into the effects of salinity on prokaryotic communities.

### Effect of salinity on bacterial assemblages

Salinity has been identified as the main factor structuring the distribution of bacterial assemblages throughout the Coorong lagoon (BIOENV analysis, Table 2). Salinity could play a direct role in bacterial assemblage structure by selecting prokaryotic groups adapted to life at a particular salt concentration. Three FCM subpopulations of heterotrophic bacteria ( $B_1$ ,  $B_3$  and  $B_4$ ) were observed in all samples throughout the lagoon and numerically dominated the bacterial community along the gradient (Fig. 4). The occurrence and predominance of subpopulations  $B_1$  and  $B_3$  from 18 to 155 PSU also suggest that these subpopulations may represent halotolerant bacteria, as they are known to dominate up to ca. 100 PSU (Rodríguez-Valera 1988, Pedrós-Alió et al. 2000b, Benlloch et al. 2002). Halotolerant bacteria occur throughout the bacterial domain and vary widely in their physiological properties and tolerances (Oren 1999).

Independent of their intrinsic salinity tolerance, the succession of heterotrophic bacteria subpopulations along the salinity gradient could be indirectly controlled by salinity acting on the functional performance of bacterial communities. Changes in the ionic strength can affect the activity of extracellular enzymes and the structure of the substrate molecules (Langenheder et al. 2003); this could impact the ability of some phylogenetic groups to utilize organic matter as changes in salinity would decrease their cell-specific activities and growth efficiencies (del Giorgio & Bouvier 2002). In controlling bacterial physiology processes, salinity could then act as a filter on bacterial community, resulting in the loss of some taxa and the activation of others best adapted to the local salinity conditions.

More generally, salinity acts as a stress factor in aquatic ecosystems, favouring (directly or indirectly) the establishment of different taxa, from phytoplankton to macroinvertebrates. The composition of organic matter associated with the biological activities of these different organisms is susceptible to strong change along the salinity gradient. As organic matter composi-

tion gradients are known to strongly influence the composition and structure of the prokaryotic assemblages (Bouvier & del Giorgio 2002), these processes could indirectly have played a role in controlling the composition of bacterial assemblages along the salinity gradient. The bioavailability of organic matter can also differ strongly under different salt concentrations, as salinity influences aggregation and flocculation processes (Forsgren et al. 1996, Hedges & Keil 1999, Benner & Opsahl 2001). By controlling the composition and bioavailability of the organic matter pool, salinity could indirectly stimulate certain groups of bacteria while repressing others, leading to the variability of bacterial subpopulations observed throughout the Coorong lagoon. In addition, by controlling the diversity and abundance of protozoan bacterivores and VLP, salinity could exert control on top-down processes including grazing and viral lysis, which are known to play an important role in structuring bacterioplankton communities (Fuhrman 1999, Pedrós-Alió et al. 2000b). In particular, the recent finding of large numbers of heterotrophic nanoflagellates actively grazing on prokaryotes in Korean salterns up to the highest salinities (Park et al. 2003, Choi & Cho 2005) underlines the necessity to investigate the role of bacterivory in regulating the community structure of prokaryotes along this salinity gradient.

### CONCLUSIONS

In the literature, microbial community dynamics have been mainly described along discontinuous (e.g. solar salterns, hypersaline lakes) or weak salinity gradients (e.g. estuaries). The present study constitutes the first observation of bacterial and viral community dynamics in a system where salinity continuously ranged from brackish to hypersaline. The spatial dynamics of microbial communities observed here is in accordance with the trends demonstrated previously in different systems (e.g. Hewson et al. 2001, Joint et al. 2002, Laybourn-Parry et al. 2007); however, the high bacterial and viral abundances observed in the present study set this hypersaline lagoon apart from the saline systems studied previously. The Coorong lagoon thus constitutes a unique model system, and our results provide new insight into the potential effects of salinity gradients and perturbations on microbial community shifts.

Salinity was identified as the main factor structuring bacterioplankton communities along the salinity gradient. However, the variability of the composition and bioavailability of organic matter, as well as the intensity of top-down processes, including grazing by bacterivores and viral lysis, demand further study, as

these factors may also play a large role in structuring the community and trophic dynamics of the microbial populations inhabiting this lagoon. Our results also highlight a significant decrease in the number of flow cytometrically defined subpopulations of bacteria and VLP with increasing salinity, which could reflect both a modification of phylogenetic composition and activity level within the bacterial community. As these shifts may ultimately have considerable implications in biogeochemical cycling dynamics along the salinity gradient, the signification of this decrease in FCM richness with increasing salinity warrant further focused consideration.

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