Vol. 74: 263–277, 2015
doi: 10.3354/ame01740

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online March 25

Protist diversity along a salinity gradient in a coastal lagoon

Sergio Balzano[1,2,*], Elsa Abs[1], Sophie C. Leterme[1]

[1]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide 5001, South Australia
[2]*Present address:* Department of Marine Organic Biogeochemistry, Royal Netherlands Institute for Sea Research, PO Box 59, 1790AB Den Burg, The Netherlands

ABSTRACT: The importance of microbial eukaryotes to aquatic systems has been widely acknowledged in the last decade, and the application of high-throughput sequencing techniques has revealed an astonishing diversity and high proportions of novel taxa. Most studies have focused either on marine or freshwater ecosystems; thus, information on estuarine communities is either incomplete or missing. We assessed the composition of microbial eukaryotes along a South Australian coastal lagoon affected by a broad (7 to 65 PSU) salinity gradient, the Coorong Lagoon. This lagoon extends for over 170 km from the mouth of the River Murray (Murray Mouth) southwards, where the salinity increases up to hypersaline values. We sampled 5 stations during the austral summer and winter and sequenced the amplified V4 region of the 18S rRNA gene using Ion Torrent. Genetic libraries were mostly represented by reads from 5 phyla, with Chlorophyta prevailing in summer, diatoms in winter and Haptophyta in the southernmost sampling sites. In spite of the broad spatial and temporal salinity changes observed, the communities of small eukaryotes clustered in 2 groups reflecting the sample location. Moreover, dissimilarities between samples were unaffected by differences in salinity, but increased with increasing geographic distances. Microbial exchanges from the Coorong Lagoon towards both freshwater and seawater occur via the Murray Mouth and are likely to prevent the formation of communities adapted to local salinity conditions. However, such exchanges likely decrease with increasing distances from the Murray Mouth, resulting in distance-driven eukaryotic communities.

KEY WORDS: Microbial eukaryotes · V4 · 18S rRNA · Geographic distance · Weighted UniFrac · QIIME

INTRODUCTION

Microbial eukaryotes make up an important component of aquatic ecosystems, playing crucial roles in global primary production (Falkowski & Raven 2007) and carbon consumption (Sherr & Sherr 2002). PCR-based approaches on the diversity of the 18S rRNA gene followed by cloning/sequencing, and/or fingerprinting techniques have been widely applied in the last decade, revealing a massive diversity of aquatic protists (Lefranc et al. 2005, Vaulot et al. 2008, Logares et al. 2014). Recently developed high-throughput sequencing techniques applied on the V4 region of the 18S rRNA gene highlighted that a significant portion of taxa still remains to be uncovered (Cheung et al. 2010, Stoeck et al. 2010).

Estuarine systems are hotspots for a range of biogeochemical processes and harbour diverse microbial communities adapted to broad salinity shifts. In spite of that, molecular approaches have been poorly applied to investigate protist diversity in estuarine environments (Zinger et al. 2012), and no study focused on microbial eukaryotes has undertaken a deep sequencing effort based on high-throughput sequencing techniques. Small eukaryote communities can undergo great spatial and temporal changes

*Corresponding author: sergio.balzano@nioz.nl

in estuaries (Vigil et al. 2009) and include high proportions of undescribed taxa (Bazin et al. 2014). In inverse estuaries, evaporation rates can exceed freshwater and seawater flows, resulting in salinities above seawater values. Subsequently, microorganisms inhabiting inverse estuaries can tolerate greater salinity shifts compared to microbes from other estuaries. Similar to estuarine communities, hypersaline microbial eukaryotes are highly diverse and can include high proportions of novel taxa (Casamayor et al. 2013). For example, at least one-quarter of the 18S rRNA gene sequences recovered from Spanish coastal and inland ponds (Triadó-Margarit & Casamayor 2013) as well as Spanish (Triadó-Margarit & Casamayor 2012), Tibetan (Wu et al. 2009) and Romanian (Keresztes et al. 2012) hypersaline lakes shared <97% similarity with known sequences. Both estuarine and hypersaline environments can thus harbour diverse and partially unexplored microbial communities.

The Coorong Lagoon is an inverse estuary in South Australia, extending from the mouth of the Murray River (Murray Mouth) southwards for about 170 km (Fig. 1). It is connected with both freshwater and seawater only at its northern end, and salinity subsequently increases southwards until reaching hypersaline conditions at the southernmost points (Webster 2010). The Coorong Lagoon undergoes strong seasonal and interannual salinity changes (Jendyk et al. 2014, S. C. Leterme et al. unpubl.) that affect the local ecosystems. Microbial communities in the Coorong Lagoon tend to become increasingly abundant southward, and distinct populations can occur at different salinities for both bacteria (Schapira et al. 2009, Pollet et al. 2010) and photosynthetic picoeukaryotes (Schapira et al. 2010). The distribution of large (>5 µm) phytoplankton is also affected by salinity (Jendyk et al. 2014, S. C. Leterme et al. unpubl.), with Chlorophyta dominating brackish locations and diatoms prevailing in hypersaline conditions. In contrast, metagenome sequencing of hypersaline water and sediment samples from the Coorong Lagoon revealed highly similar prokaryotic compositions for sediment samples at different salinities (37 to 136 PSU) and greater differences between water and sediment samples from the same location (Jeffries et al. 2011). Protist diversity could not be investigated in details from that study because only 1.5% of reads were affiliated to eukaryotes (Jeffries et al. 2011). This is due to the fact that bacteria in aquatic systems usually outnumber eukaryotes, complicating metagenomic studies based on eukaryote diversity (Piganeau et al. 2008, Vaulot et al. 2012).

In the present study, we investigated summer and winter microbial eukaryotes in the Coorong Lagoon by high-throughput amplicon sequencing of the V4 region of the 18S rRNA gene in order to evaluate the composition of small eukaryotes at different salinities and to understand whether the community structure is controlled by salinity or other factors.

MATERIALS AND METHODS

Additional details on the methods are presented in Supplement 1 at www.int-res.com/articles/suppl/a074p263_supp.pdf.

Sampling

Water samples were collected near the surface (i.e. at 20 cm depth) from the Coorong Lagoon (Fig. 1) along a 96 km transect on 27 November 2012 and 23 June 2013. Water was collected at about 50 m from the shore at 5 sampling sites: Murray Mouth, Long Point, Bonney Reserve, Parnka Point, and Salt Creek. These sites correspond to sampling locations used in

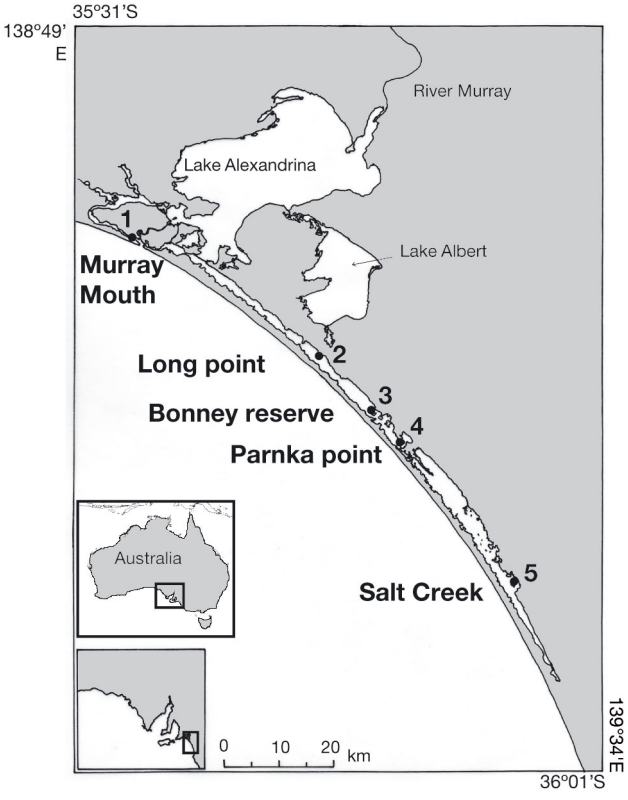

Fig. 1. Names and locations of the sampling sites within the Coorong Lagoon

previous studies (Schapira et al. 2009, Pollet et al. 2010, Leterme et al. 2013, Jendyk et al. 2014).

Ancillary data

Temperature, salinity, and pH were measured *in situ* at each sampling site using an AP-2000 multiparameter probe (Aquaread). Nutrients were analysed in triplicate using standard colorimetric techniques (Hansen & Koroleff 2007), whereas bacteria, virus-like particles (VLP), cyanobacteria and photosynthetic picoeukaryotes were enumerated in triplicate by flow cytometry (Marie et al. 1997). Small (<5 µm) and large (>5 µm) phytoplankton were counted by microscopy, and cells from the latter fraction were identified based on key taxonomic features (Tomas 1997, Hallegraef et al. 2010).

Sample collection, DNA extraction, PCR and sequencing

For molecular analyses, 1 l water samples were pre-filtered through 10 µm and then filtered through 0.45 µm Sterivex filters (Merck Millipore). Lysis buffer was added to the filters as described previously (Marie et al. 2010) and the filters were flash-frozen in liquid nitrogen and then stored at −80°C until analysis. For DNA extraction, the lysis buffer was removed from the Sterivex filters (see Supplement 1) and the DNA was extracted as described previously (Balzano et al. 2012a).

To amplify the eukaryotic V4 region of the 18S rRNA gene, we used the universal forward primer V4F (5′-CCA GCA SCY GCG GTA ATT CC-3′) (Stoeck et al. 2010) and a reverse primer V4RB (5′-ACT TTC GTT CTT GAT YRR-3′), differing from the V4R primer (Stoeck et al. 2010) by the last nucleotide sequence. These primers bind to a central region of the 18S rRNA gene; the binding sites are located between 565 and 584 bp for the forward primer and between 964 and 981 bp for the reverse primer, with respect to the 18S rRNA gene of *Saccharomyces cerevisiae* (Stoeck et al. 2010). The reverse primer used here was found to amplify a larger fraction of protists (85% of all classes), especially Haptophyta — compared to the V4R primer used previously (Stoeck et al. 2010) — using testprime (www.arb-silva.de/search/testprime/).

Primers were then modified for multiplex sequencing on Ion Torrent by adding an A-adaptor (5′-CCA TCT CAT CCC TGC GTG TCT CCG ACT- CAG-3′) and a sample-specific, 11 bp barcode (see Table S1 in Supplement 2 at www.int-res.com/articles/suppl/a074p263_supp.xls) to the 5 -end of the forward primer, and a P1 adaptor (5′-CCT CTC TAT GGG CAG TCG GTG AT-3′) to the 5′-end of the reverse primer as recommended by Life Technology (Mulgrave). PCR reactions were performed in 6 replicates for each sample, and each reaction included about 5 ng of template DNA, 0.35 µM of each primer, 2 U Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 1× Q5 reaction buffer (New England Biolabs), 3.5 µM dNTP (Promega) and MQ water for a total volume of 20 µl. Since the annealing temperature of the reverse primer was significantly lower than that of the forward primer, 2 subsequent thermal cycles with different annealing temperatures were used for the PCR as described previously (Stoeck et al. 2010, Logares et al. 2012). Specifically, PCR consisted of an initial denaturation at 98°C for 1 min, 14 cycles of 30 s at 98°C, 30 s at 53°C and 30 s at 72°C, followed by 21 cycles of 30 s at 98°C, 30 s at 2 distinct annealing temperatures each for 3 of the 6 replicates (48 and 50°C), 30 s at 72°C, and a final extension at 72°C for 1 min. Replicate amplicons were then pooled and purified using a UltraClean PCR kit (Mo-Bio Laboratories). Amplicons from different samples were pooled together in equimolar amounts and sequenced by Australian Genome Research Facility (AGRF) using an Ion Torrent Personal Genome Machine (PGM) provided with a 318 chip (Life Technology) and adapted for a maximum read length of 400 bp (see Supplement 1). The sequence data are available at National Centre for Biotechnology Information under submission ID 1746288. Although Ion Torrent technology is affected by higher error rates than other high-throughput sequencing platforms (Quail et al. 2012), it is still suitable for amplicon sequencing if each read used for further analyses is represented by at least 2 sequences (Bragg et al. 2013).

Bioinformatic analyses

Full details on bioinformatics analyses are provided in Supplement 1. About 2 500 000 raw reads were generated by 2 Ion Torrent runs, and sequence data were analysed using the Quantitative Insight In Microbial Ecology (QIIME) pipeline (Caporaso et al. 2010). Replicate sequencing of known bacterial genomes highlighted that the quality scores provided by the Ion Torrent run reports underestimated the real base accuracy (Bragg et al. 2013). Subsequently,

we used a phred quality threshold (20) lower than that typically used for other sequencing platform (i.e. 25) to filter our reads, similar to a previous study (Frank-Fahle et al. 2014).

Reads ≤230 bp (Logares et al. 2012), with a phred quality ≤20 over a 50 bp sliding window (Frank-Fahle et al. 2014), showing one or more nucleotide mismatches with the forward primer (Behnke et al. 2011) and >8 homopolymers were removed from the dataset. Barcodes and forward primer sequences were trimmed, and reads were then truncated to 230 bp length. Chimeric sequences were identified and removed using the UCHIME algorithm (Edgar et al. 2011) by comparison with the Protist Ribosomal Database (Guillou et al. 2013). Singletons (i.e. reads not sharing 100% identity with at least another read) were identified using the UCLUST algorithm (Edgar 2010) and removed. Using the UCLUST algorithm, we also clustered our reads into 1321 distinct Operational Taxonomic Units (OTUs) based on 97% sequence similarity (see Table 2). To compare the diversity of the different samples, the dataset was randomly subsampled to 16 855 reads per sample, which correspond to the number of sequences obtained from the less deeply sequenced sample (Murray Mouth, November). Subsequently, the number of OTUs recovered decreased to 1204. Representative reads (1 sequence per OTU) were then aligned using MUSCLE (Edgar 2004), and a phylogenetic tree was constructed using a FastTree algorithm (Price et al. 2010). The taxonomic affiliation of the OTUs was inferred by comparison with the Protist Ribosomal Database (Guillou et al. 2013) using the UCLUST algorithm (Edgar 2010). A heatmap reflecting the taxonomic distribution of the major taxa was then constructed using the R package 'gplots' (www.cran.r-project.org/web/packages/gplots/index.html).

For α-diversity, the Shannon (H) index (Shannon 1948), Simpson-Gini ($1-D$) index (Hurlbert 1971) and phylogenetic diversity (PD) (Faith 1992) were calculated. The proportion of OTUs shared between different samples was calculated using R (see Supplement 1). For β-diversity, Bray-Curtis dissimilarities and weighted UniFrac distances (Lozupone & Knight 2005) were calculated between our different samples based on their OTU distribution and phylogenetic differences, respectively. Bray-Curtis and weighted UniFrac distances were then explored using non-metric multidimensional scaling (NMDS) analyses, and the Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrograms. NMDS plots were generated using the R package 'vegan' (www.cran.r-project.org/web/packages/vegan/index.html). Jackknife support values for the UPGMA dendrograms were calculated from 1000 permutations using QIIME.

To investigate the influence of environmental conditions and geographic distance to the composition of small eukaryotes, Mantel tests were performed as described previously (Pagaling et al. 2009, Martiny et al. 2011, Lepère et al. 2013).

RESULTS

Water properties

The water temperature in the Coorong Lagoon ranged from 18.9 to 25.6°C in November and from 10.5 to 13°C in June, and increased southward as the sampling progressed during the day (Table 1). In November, salinity was well above seawater values (>50 PSU) at the 3 southernmost stations, whereas in June hypersaline waters were only found at Salt Creek (Table 1). Salinity was <22 PSU for all the other samples, and lower values were measured at the Murray Mouth compared to the other brackish sites (Table 1). The concentration of nutrients was also highly variable. In particular, high values of dissolved silica (>100 µM), ammonium (>850 µM) and nitrate/nitrite (>50 µM) were measured at Salt Creek, and to a lesser extent at Parnka Point (see Table S2 in Supplement 2 at www.int-res.com/articles/suppl/a074p263_supp.xls).

Table 1. Sample locations and main physico-chemical characteristics

Sampling site No.	Name	Latitude (°S)	Longitude (°E)	Salinity (PSU) Nov–12	Jun–13	Temperature (°C) Nov–12	Jun–13	pH Nov–12	Jun–13
1	Murray Mouth	35.55	138.89	7.09	8.5	18.9	10.5	7.9	8
2	Long Point	35.69	139.16	20.3	14.6	21.1	10.7	8.2	7.9
3	Bonney Reserve	35.80	139.32	51.3	17.3	23.7	11	8.1	8.2
4	Parnka Point	36.16	139.65	54.4	21.1	24.1	11.5	8.3	8.4
5	Salt Creek	35.90	139.40	58.1	65.4	25.6	13	8.1	8.4

Flow cytometry populations

The abundance of bacteria was up to one order of magnitude higher than typical seawater values and was greater at Parnka Point and Salt Creek during both seasons. Both bacteria and VLP were more abundant in November compared to June (Fig. 2). The photosynthetic picoplankton varied in abundance both between sites and between seasons, and was dominated by picoeukaryotes and *Prochlorococcus* (Fig. 2). Low abundances were measured at the Murray Mouth for both seasons, and abundances increased southward in June.

Microscopy

Small cells (<5 µm) increased in abundance southwards for both seasons, reaching about 10^7 cells ml^{-1} in Parnka Point and Salt Creek (Fig. 3). The abundance of large (>5 µm) phytoplankton ranged from 2.7×10^6 to 1.5×10^7. The highest values were generally found at Murray Mouth and at the 2 southernmost stations (Fig. 3). Overall, 56 taxa were identified to species level for large phytoplankton (see Table S3 in Supplement 2), and diatoms dominated all samples except at Long Point in June and at the Murray Mouth during both seasons (Fig. 3). Murray Mouth phytoplankton was dominated by *Teleaulax acuta* (Cryptophyta) in November and by several Chlorophyta (*Chlorohormidium* sp., *Chodatella* sp., *Crucigenia* sp.) and cyanobacteria (*Planktolyngbya contorta, Nodularia spumigena*) in June (Table S3 in Supplement 2). Long Point included a diverse diatom and Cryptophyta assemblage in November, whereas it was dominated by *Hemiselmis* sp. (Cryptophyta) and to a lesser extent by *Chaetoceros* sp. (diatom) in June. The other sampling sites were dominated by diatoms, with several species (*Amphora* sp., *Cocconeis* spp., *Cyclotella* sp., *Cylindrotheca closterium*, *Fragilaria* sp., *Navicula* spp. and *Nitzschia* spp.) observed in November and *Chaetoceros* spp. and *C. closterium* blooming in June (Table S3 in Supplement 2).

Ion Torrent PGM data: α-diversity and taxonomic composition

We amplified the V4 region of the 18S rRNA gene from 10 µm pre-filtered water samples and constructed genetic libraries using Ion Torrent sequencing. Since only a tiny proportion of our reads covered the entire V4 length of about 400 bp, we retained all sequences ≥230 bp for a total of 477 303 reads. After removal of chimeras and singletons, and subsampling at an equal number of reads for all samples, our dataset consisted in 168 550 good quality

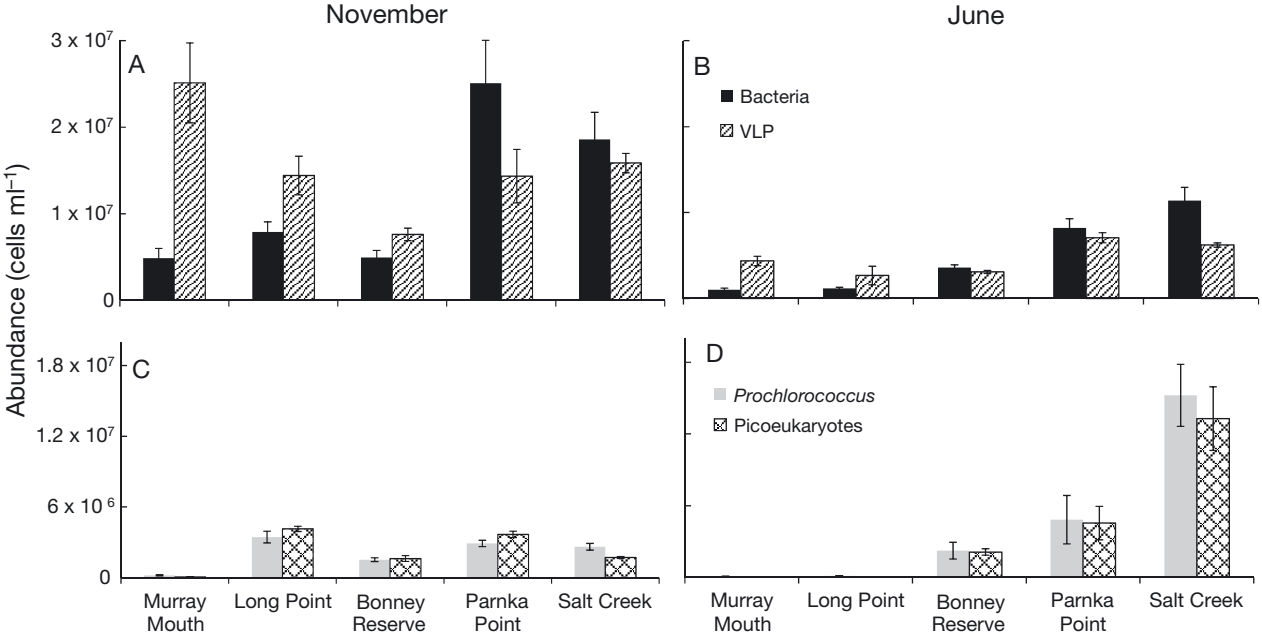

Fig. 2. Abundance of (A,B) bacteria and virus-like particles (VLP), as well as (C,D) *Prochlorococcus* and photosynthetic picoeukaryotes along the Coorong Lagoon in (A,C) November 2012 and (B,D) June 2013. Cells were enumerated using flow cytometry. Each sample was collected and analysed in triplicate, and error bars are standard deviation

reads which clustered into 1204 OTUs based on 97% sequence similarity (Table 2).

Rarefaction curves of OTU richness, Shannon and Simpson-Gini indices as well as phylogenetic diversity were saturated for all the samples (see Fig. S1 in Supplement 1). The June community at Murray Mouth was found to have the highest Shannon and Simpson-Gini indices, whereas the November Salt Creek sample was the most phylogenetically diverse (Table 2). We inferred the taxonomic composition of our reads by comparison with the Protist Ribosomal Database (Guillou et al. 2013) and found a great seasonal and spatial variability among our samples (Fig. 4). Reads affiliated to dinoflagellates, Chlorophyta, Heterokontophyta and Haptophyta were most frequently recovered among the libraries (Fig. 4). The taxonomic affiliation of the OTUs found here as well as their distribution among our samples are available in Table S4 in Supplement 2. Dinoflagellate reads were abundant in most samples, whereas Heterokontophyta sequences, mostly associated with diatoms, were frequently recovered from June libraries from the Murray Mouth and Long Point. Chlorophyta reads were abundant in November libraries from Long Point, as well as November and June libraries from Bonney Reserve, Parnka Point and Salt Creek. High proportions of Haptophyta reads were recovered from the 3 southernmost sampling sites in both seasons (Fig. 5).

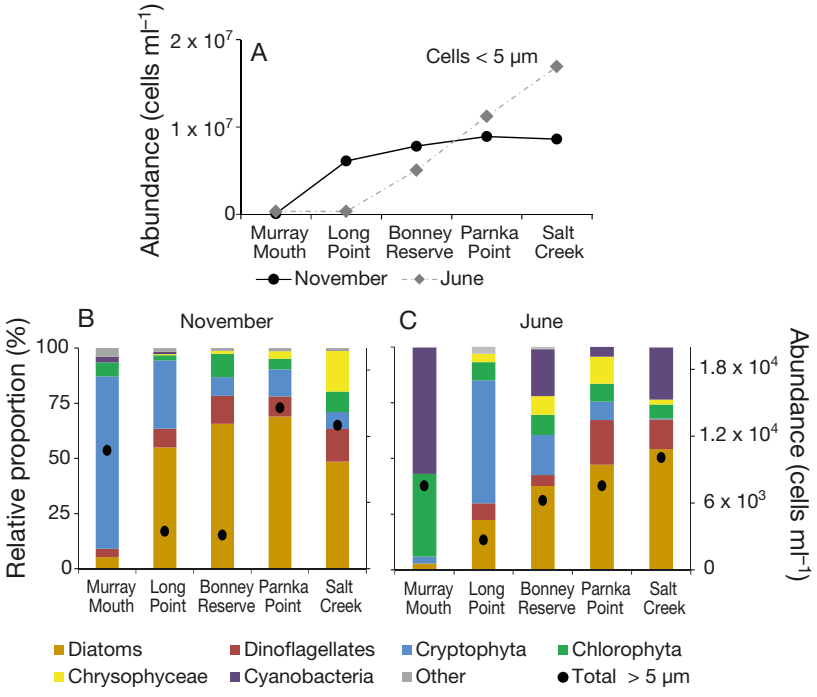

Fig. 3. Microscopy counts of plankton cells in the Coorong Lagoon. (A) Total abundance of cells <5 µm in November 2012 and June 2013, and total abundance and relative proportions of large (>5 µm) phytoplankton taxa observed along the Coorong Lagoon in (B) November 2012 and (C) June 2013

Common OTUs and lineages

Overall, few OTUs were common to more than 2 samples, and 277 OTUs (corresponding to 22% of the dataset), were shared between the 2 seasons (i.e. found in at least in one November and one June sample; Table 3). Only 1 OTU, associated with Pycnococcaceae, was found within all the sam-

Table 2. Sequencing results and analysis of microbial diversity. OTUs: operational taxonomic units; *H*: Shannon index; 1–*D*: Simpson-Gini index; PD: phylogenetic diversity

Date	Sampling site No.	Name	No of reads Raw	Filtered	Subsampled	OTUs	Diversity indices *H*	1–*D*	PD
27 Nov 2012	1	Murray Mouth	21 739	16 855	16 855	181	6.1	0.97	24
	2	Long Point	24 201	21 049	16 855	178	2.2	0.49	24
	3	Bonney Reserve	27 675	23 203	16 855	217	3.5	0.73	31
	4	Parnka Point	35 206	28 317	16 855	264	5.1	0.94	35
	5	Salt Creek	33 277	28 986	16 855	195	4.8	0.92	53
24 Jun 2013	1	Murray Mouth	73 253	47 137	16 855	463	5.1	0.87	40
	2	Long Point	46 623	36 020	16 855	115	2.1	0.56	21
	3	Bonney Reserve	45 694	28 746	16 855	301	5.1	0.94	34
	4	Parnka Point	104 321	67 761	16 855	295	5.1	0.94	35
	5	Salt Creek	65 314	45 237	16 855	157	3.5	0.80	23
Total			477 303	353 311	168 550	1 204			

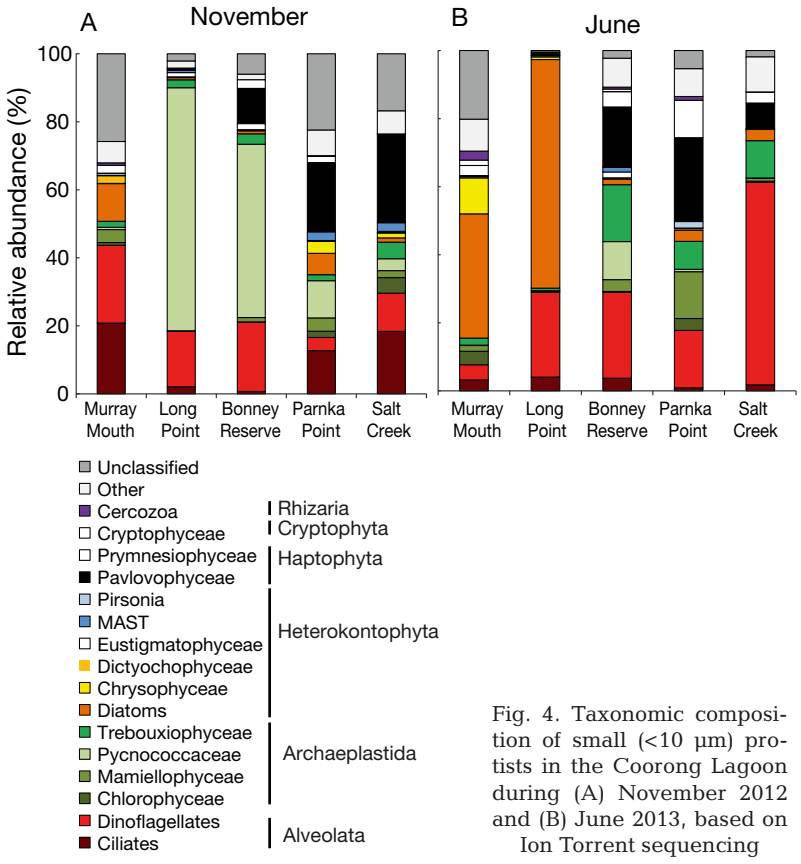

Fig. 4. Taxonomic composition of small (<10 µm) protists in the Coorong Lagoon during (A) November 2012 and (B) June 2013, based on Ion Torrent sequencing

ples, and a very low proportion of OTUs (<1%) were shared among all samples from the same season. The proportion of OTUs found during both seasons was higher for the southern stations of the Lagoon. For example, over 20% of the OTUs were shared between November and June samples from the same station for Bonney Reserve, Parnka Point and Salt Creek, whereas this proportion decreased by half for Murray Mouth and Long Point (Table 3).

While very few OTUs were shared among multiple samples, we found 15 phylogenetic lineages, at the lowest taxonomic level available in the Protist Ribosomal Database (L8), that included OTUs present in ≥8 samples (Table 4). In particular, reads affiliated to Suessiales (dinoflagellates), Chlorellales, and Pycnococcaceae (Chlorophyta) were frequently recovered (>99 reads) from at least 7 samples (Table 4). Surprisingly, the halophilic ciliate *Fabrea* was detected in 8 libraries and frequently recovered from 5 of them (Fig. 6), including a brackish sample. However, it should be noted that our data reflect the presence but not the activity of microorganisms. Pycnococcaceae accounted for >50% of the reads in November libraries from Long Point and Bonney Reserve, in spite of the great differences in salinity measured at these sites (20.3 and 51.3 PSU, respectively). Other lineages were more restricted to a fraction of our sampling sites. In particular, reads from *Chaetoceros* sp. were highly abundant (>50%) in Murray Mouth and Long Point in June, and were not found in any other samples.

β-diversity

Similarities among the different Ion Torrent libraries were investigated by UPGMA based on both Bray-Curtis dissimilarities and weighted UniFrac distances. November and June samples from the same site, or samples from nearby stations collected during the same season tended to group together (Fig. 5). All but one node on the UPGMA tree had high (≥90%) jackknife support values for both weighted UniFrac and Bray-Curtis dissimilarities. Two major clusters were identified, one comprising Murray Mouth samples from both seasons and the Long Point June sample, and another cluster including all the other samples. The repartition of the libraries in these 2 major clusters was also confirmed by NMDS analyses based on both weighted UniFrac and Bray-Curtis dissimilarities (Fig. 6).

Accordingly, some common features in the taxonomic composition can be observed within each of these 2 clusters, and more similarities occurred between libraries from the same or nearby sampling sites (Fig. 5). Several ciliate classes of freshwater origin (Syndiniales group II, Sphaeropleales, *Chrysochromulina*, *Chaetoceros* sp., and Chrysophyceae-clade C) were abundant in the first group of samples. On the other hand, reads affiliated to *Fabrea*, Suessiales, Chlorellales, *Diacronema, Isochrysis*, choanoflagellates, Chrysophyceae-clade F, and marine stramenopiles (MAST) were mostly recovered from the 3 southernmost stations during both seasons as well as Long Point in November.

Influence of environmental conditions

The influence of environmental conditions and geographic distance on the small eukaryote community distribution was analysed with a Mantel test. Geo-

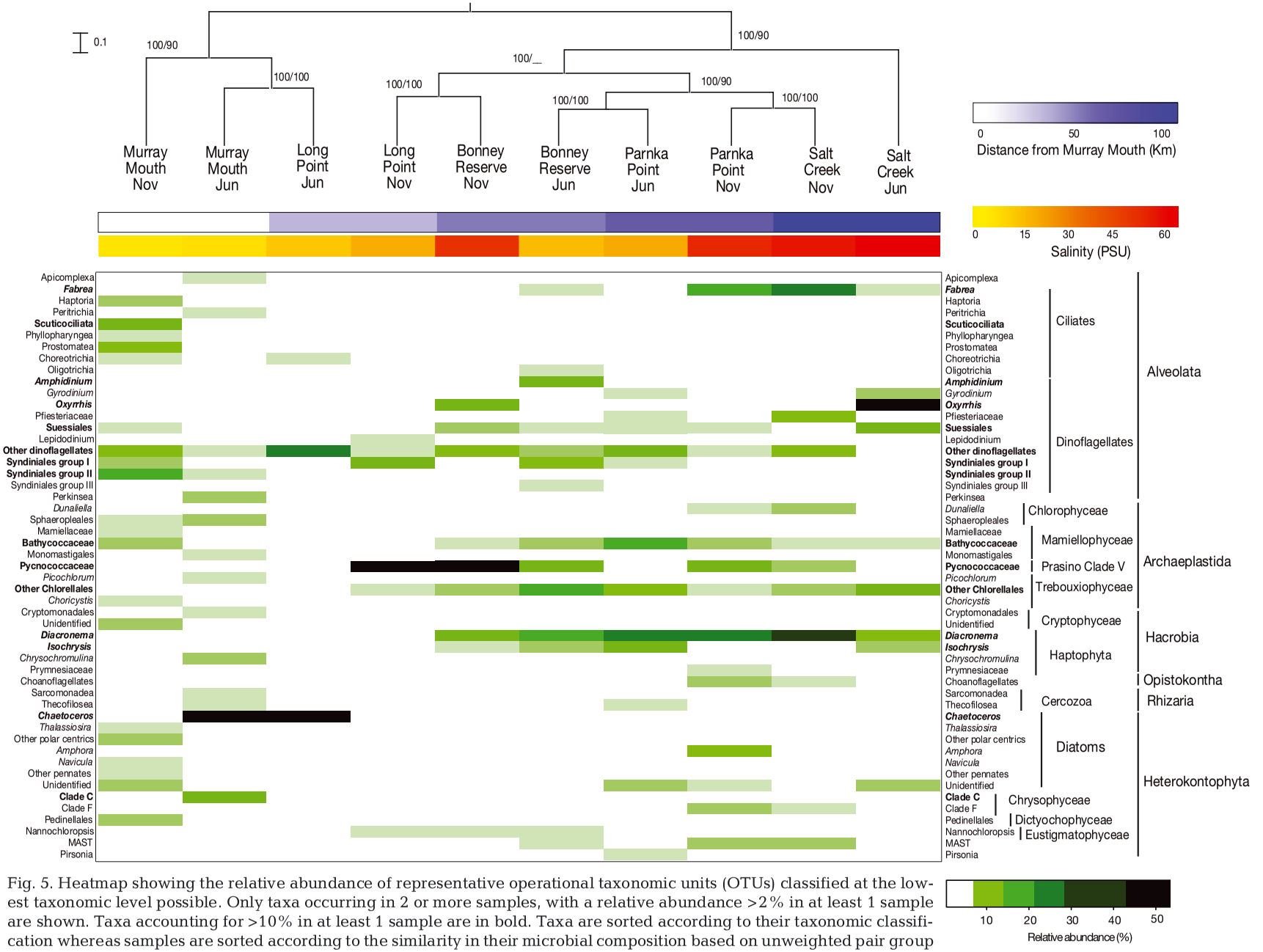

Fig. 5. Heatmap showing the relative abundance of representative operational taxonomic units (OTUs) classified at the lowest taxonomic level possible. Only taxa occurring in 2 or more samples, with a relative abundance >2% in at least 1 sample are shown. Taxa accounting for >10% in at least 1 sample are in bold. Taxa are sorted according to their taxonomic classification whereas samples are sorted according to the similarity in their microbial composition based on unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering analyses. The UPGMA tree based on weighted UniFrac distances is drawn next to the sample names, and numbers at the branch indicate the percentage of jackknife support for weighted UniFrac and Bray-Curtis similarity, respectively. The salinity and the distance from Murray Mouth are indicated next to the sample names in yellow-red and blue shades, respectively

graphic distance between sites significantly affected the small eukaryote community, whereas the examined environmental variables did not seem to have a substantial influence (Table 5). This was confirmed by the fact that both Bray-Curtis and weighted UniFrac dissimilarities between the different protist communities significantly increased as function of their geographic distance (n = 50, R = 0.32 and 0.42, respectively; Fig. 6) and the highest dissimilarity values often occurred between the 2 farthest sampling sites (i.e. Murray Mouth and Salt Creek). In contrast, no relationship was found between small eukaryote dissimilarities and temperature or salinity (Fig. 6).

Table 3. Proportions of operational taxonomic units (OTUs) shared among different groups of samples. Samples were grouped either by location, season or salinity

Cluster	Cluster size	Shared OTUs No.	Shared OTUs %
November–June	2	277	23
Murray Mouth	2	56	9
Long Point	2	27	9
Bonney Reserve	2	106	20
Parnka Point	2	120	21
Salt Creek	2	72	20
North lagoon–November	3	15	1
South lagoon–November	2	115	25
North lagoon–June	3	31	4
South lagoon–June	2	128	28
17-21 PSU	4	11	1
50-65 PSU	4	46	5
50-65 PSU–November	3	64	10
17-21 PSU–June	3	19	3
North lagoon	6	4	0
South lagoon	4	58	6
June	5	2	0
November	5	10	0
All	10	1	0

Table 4. List of phylotypes at the lowest taxonomic level (L8), present in at least 8 samples

Group	Taxon	No of samples present: At least 1 read	>9 reads	>99 reads
Ciliates	*Fabrea*	8	6	5
	Oligotrichia	9	6	2
Dinoflagellates	Pfiesteriaceae	9	7	3
	Suessiales	10	9	7
	Syndiniales group II clade 14	8	7	2
	Syndiniales group II clade 39	8	4	0
Chlorophyta	Chlorellales	9	8	8
	Pycnococcaceae	10	9	7
Heterokontophyta	Chrysophyceae-Synurophyceae clade F	8	6	2
	Nannochloropsis	8	8	3
	Raphid pennates	10	8	3
Cryptophyta	Cryptomonadales	8	4	1
Katablepharidophyta	*Leucocryptos*	8	5	2
Haptophyta	*Diacronema*	9	8	6
	Isochrisis	8	7	4

DISCUSSION

We assessed protist diversity through a coastal lagoon affected by a broad (7.1 to 65.4 PSU) salinity gradient and applied high-throughput amplicon sequencing techniques to an estuarine environment for the first time. The hypervariable V4 region of the 18S rRNA gene provides a detailed overview of protist diversity (Stoeck et al. 2010, Logares et al. 2012) although it can fail to identify some classes in deeper taxonomic details. Similar to the results of a previous study (Mangot et al. 2013) a good portion of dinoflagellate reads could not be identified further because of the poor resolution of the 18S rRNA gene for this class (Saldarriaga et al. 2004, Logares et al. 2007). Our data do not provide evidence for live microorganisms only, but rather for the total community, including dead and senescent cells.

Although the V4 region of the 18S rRNA gene is 416 bp long, reads as short as 200 bp can still be suitable for downstream analyses (Behnke et al. 2011). In the present study, the taxonomic composition of 343 311 reads >230 bp was similar to that obtained from a lower proportion (40 953) of longer (>350 bp) reads (see Fig. S2 in Supplement 1 at www.int-res.com/articles/suppl/a074p263_supp.pdf), although it included more unassigned reads. The high proportion of unassigned sequences (Fig. 4) is thus related to the read length rather than sequencing artefacts. We used all reads >230 bp for downstream analyses because they provided fully saturated rarefaction curves (Fig. S1).

Large phytoplankton

The taxonomic composition of large phytoplankton found here agrees with a previous study based on a much larger dataset

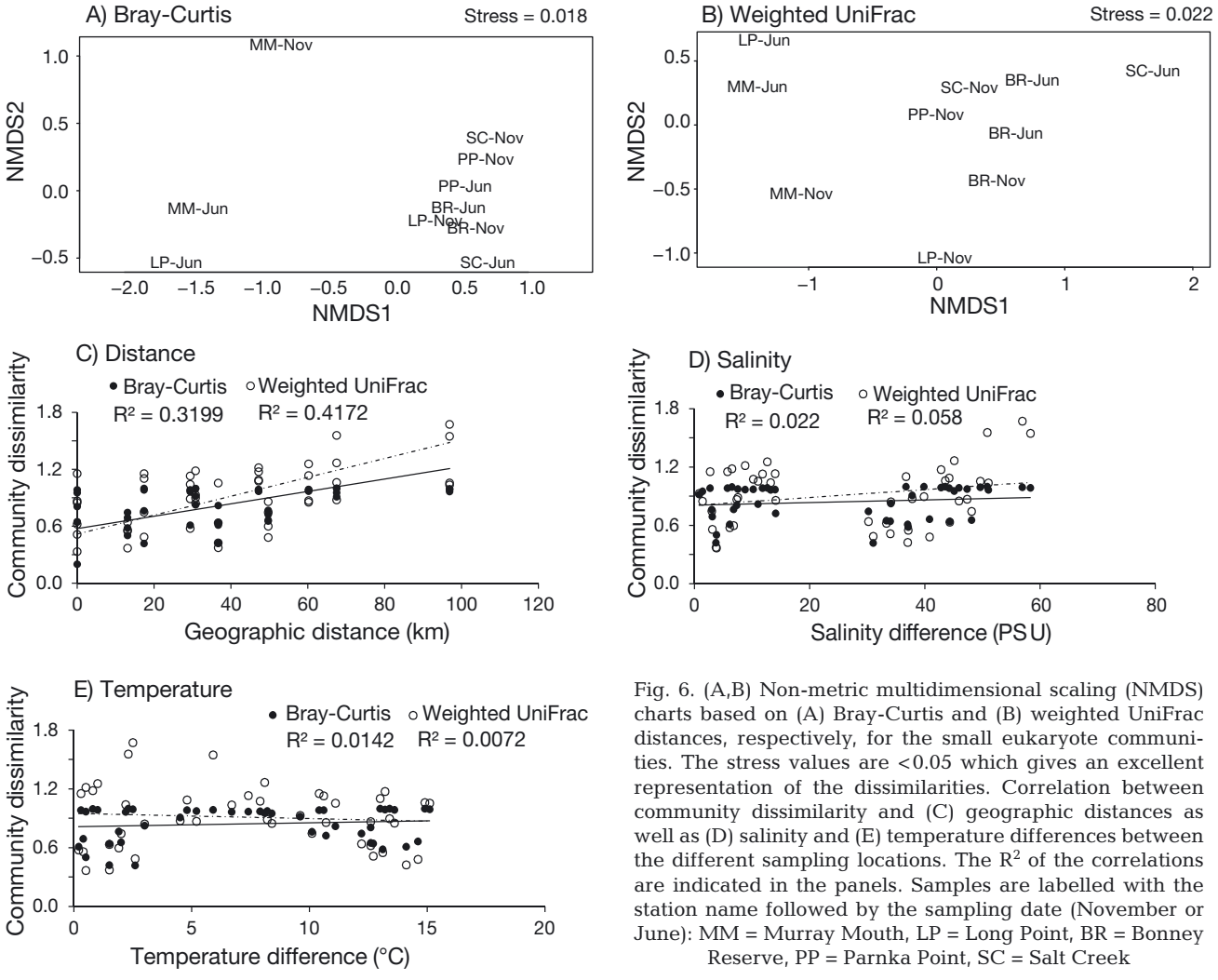

Fig. 6. (A,B) Non-metric multidimensional scaling (NMDS) charts based on (A) Bray-Curtis and (B) weighted UniFrac distances, respectively, for the small eukaryote communities. The stress values are <0.05 which gives an excellent representation of the dissimilarities. Correlation between community dissimilarity and (C) geographic distances as well as (D) salinity and (E) temperature differences between the different sampling locations. The R^2 of the correlations are indicated in the panels. Samples are labelled with the station name followed by the sampling date (November or June): MM = Murray Mouth, LP = Long Point, BR = Bonney Reserve, PP = Parnka Point, SC = Salt Creek

Table 5. Mantel test comparison between community variability (measured as Bray-Curtis dissimilarity as well as weighted UniFrac distances) and temperature, salinity, environmental conditions, and the geographic distance between sampling sites. When the correlation is significant both ρ- and p-values are underlined ($p < 0.05$) or in **bold** ($p < 0.01$). ENV = environmental data

Factor	Community distance			
	Bray-Curtis		Weighted UniFrac	
	ρ	p	ρ	p
Temperature	0.12	0.17	−0.09	0.73
Salinity	0.15	0.14	0.24	0.05
Distance	0.42	0.02	**0.54**	**0.006**
ENV	0.26	0.11	0.33	0.08

(Jendyk et al. 2014) and reflects the general phytoplankton distribution in estuarine waters. Chlorophyta and Cryptophyta are typically found in brackish environments (Pinckney et al. 1998, Murrel & Lores 2004, Marshall et al. 2005) and are likely to be more adapted to salinity shifts from freshwater to brackish conditions, such as those that occur in the vicinity of the Murray Mouth. In spite of the comparable salinities measured during the different seasons at Murray Mouth (Table 1), the November community was dominated by marine species typically occurring in South Australian coastal waters (Leterme et al. 2014, S. Balzano et al. unpubl. data), whereas the June community was mainly comprised of freshwater Chlorophyta and cyanobacteria which can occur in the River Murray (Codd et al. 1994) or other freshwater bodies (Guiry & Guiry 2014).

The phytoplankton community composition at the other stations of the Coorong Lagoon (Fig. 3) suggests better adaptation among diatoms to salinity increases that can exceed seawater values. Consistent with this, some of the diatom species abundant here were previously observed in other hypersaline

environments (Clavero et al. 2000, Gilabert 2001) and successfully cultured in the laboratory under similar high salinities (Clavero et al. 2000, Leterme et al. 2010).

Small eukaryotes: community clustering and shared lineages

The high abundances of bacteria, viruses, *Prochlorococcus* and picoeukaryotes found here (Fig. 2), and the southward increase measured for most populations is consistent with previous studies (Schapira et al. 2009, 2010, Pollet et al. 2010). The high values are likely due to the shallowness of the lagoon, which allows light penetration throughout the water column, and wind stress, which promotes nutrient exchanges. The higher abundances measured at the southern stations might be associated with the lower water turnover rates occurring there, resulting in limited microbial exchanges between the lagoon and both seawater and freshwater.

Our data (Figs. 4 & 5) confirm that estuarine protists are highly diverse, can include representatives from most eukaryotic supergroups (Vigil et al. 2009, Bazin et al. 2014), and similar to other aquatic environments (Marie et al. 2010, Mangot et al. 2013), eukaryotic genetic libraries can be dominated by Alveolata.

The high proportion of reads assigned to *Chaetoceros* sp., recovered from Murray Mouth and Long Point in June (Fig. 4) are likely associated with a phytoplankton bloom. In June, *Chaetoceros* spp. bloomed in Long Point and Bonney Reserve (see Table S3 in Supplement 2 at www.int-res.com/articles/suppl/a074p263_supp.xls) and spores or small cells (i.e. unidentifiable in light microscopy) were also likely present in Murray Mouth resulting in high proportions of *Chaetoceros* spp. reads recovered from that sample (Fig. 6). *Chaetoceros* species can bloom in coastal and estuarine environments (Trigueros et al. 2002, Ake-Castillo et al. 2004) and dominate nanoplankton and picoplankton libraries (Balzano et al. 2012b).

Results from both UPGMA and NMDS indicate that November and June Murray Mouth communities showed some similarities with June Long Point community and differed significantly from all other communities sampled from the Coorong Lagoon (Figs. 5 & 6). Microbial communities from these 3 samples include higher proportions of freshwater taxa (Haptoria, Scuticociliata, Prostomatea, Choreotrichia, Sphaeropleales, Chrysophyceae-clade C; Fig. 5), whereas summer and winter libraries from the 3 southernmost stations, as well as Long Point November library are mostly represented by typically marine (Bathycoccaceae, Pycnococcaceae, Chrysophyceae clade F, MAST) and estuarine (*Oxyrrhis, Diacronema* and *Isochrysis*) taxa as well as known halophilic genera (*Fabrea*, *Dunaliella*) (Pandey & Yeragi 2004, Seoane et al. 2009, Bendif et al. 2011, Not et al. 2012, Guiry & Guiry 2014). These differences are also evident from a trophic point of view, whereby diatoms are the most representative photosynthetic eukaryotes at Murray Mouth during both seasons and at Long Point in June, whereas key marine picoplankters from the families Bathycoccaceae and Pycnococcaceae dominated reads from photosynthetic taxa at the other stations. Similarly, the heterotrophic taxa mostly represented within our libraries were freshwater ciliates and Syndiniales group II for Murray Mouth, and *Fabrea*, *Oxyrrhis* and MAST for the other stations.

The composition of microbial eukaryotes may thus change significantly for the different stations, although halophilic taxa occurred throughout the Coorong Lagoon. Beside the known halophilic genera *Fabrea* and *Dunaliella*, our hypersaline samples were highly represented by reads from the diatom *Amphora* and the heterotrophic dinoflagellate *Oxyrrhis,* which were occasionally observed in hypersaline waters (Buskey et al. 1998, Caric et al. 2011, Keresztes et al. 2012). Moreover, the brackish libraries were dominated by some taxa such as Scuticociliata, Sphaeropleales and Chrysophyceae-clade C, which have a likely freshwater origin but can include halotolerant species previously found in inland and coastal hypersaline lakes (Elloumi et al. 2006, Triadó-Margarit & Casamayor 2013).

In spite of the wide variability in taxonomic composition, the occurrence of 15 phylotypes in most of the samples (Table 4) suggests the presence of temporally and spatially persistent phylogenetic lineages (Shade & Handelsman 2012) in the Coorong Lagoon. Some of these lineages (Suessiales, Chlorellales, Raphid Pennates, Cryptomonadales) are present in most samples because they include a vast number of different taxa which could not be discriminated by the current techniques. Beside these groups, the presence of other persistent lineages at the genus (*Fabrea*, *Nannochloropsis*, *Leucocryptos*, *Diacronema*, *Isochrysis*) or genus-equivalent (Syndiniales group II clade 14, Syndiniales group II clade 39) level might reflect either the ability to survive or grow across a wide salinity range, or a heterogeneous population structure for these taxa. Populations adapted to different ecological niches can occur within the

same genus as reported for *Micromonas* (Foulon et al. 2008) and *Ostreococcus* (Demir-Hilton et al. 2011), and species from the same genus can also differ in their salinity tolerance (Maier Brown et al. 2006, Balzano et al. 2011).

The presence of the hypersaline ciliate *Fabrea* (Pandey & Yeragi 2004) in most of our libraries, including a brackish sample (Table 4) is surprising. Our data only reflect microorganism abundance and not their activity; therefore, we do not know whether the sequences found here reflect active *Fabrea* cells. However, it is remarkable that reads from freshwater, marine and hypersaline taxa were frequently recovered from most of the samples, which span across a broad salinity range.

Syndiniales, which include 2 persistent lineages found here (Table 4) can contribute to the decay of phytoplankton blooms in estuaries (Chambouvet et al. 2008) as well as coastal lagoons (Chambouvet et al. 2011), and their presence among the genetic libraries suggests a similar role in the Coorong Lagoon. Parasitic eukaryotes are an important component of aquatic ecosystems and their ecological role in controlling phytoplankton populations is increasingly evident (Guillou et al. 2008, Christaki et al. 2014).

Microbial community structure

In spite of the broad gradients occurring spatially and seasonally, results from the Mantel test, which allows estimation of the influence of different conditions on the microbial community structure, indicates that salinity does not shape the distribution of small eukaryotes in the Coorong Lagoon (Table 5). This was also confirmed by the fact that both the Bray-Curtis and the UniFrac dissimilarities between the different samples did not significantly increase with increasing salinities (Fig. 6) and that UPGMA dendrograms (Fig. 5) and NMDS analyses based on both Bray-Curtis and weighted UniFrac (Fig. 6) indicated that samples from both brackish and hypersaline environments can cluster together (Fig. 6). Geographic distance is thus the main driver controlling our communities of smaller eukaryotes (Table 5, Fig. 6). This suggests that the exchanges of microbes between the different sampled stations are slow enough to allow the formation of distinct communities with similarities decreasing with distance. On the other hand, such exchanges are likely to be fast enough to prevent species within each community that are adapted to the local salinity to grow and outnumber the others, resulting in a specific salinity-related signature for the local community. Autochthonous communities adapted to local environmental conditions can be formed in estuaries if microbial growth rates exceed water residence time, as found for bacteria (Crump et al. 2004). Information about microbial activities and doubling time is not available in the present study but our data suggest that at least for some species, growth rates were not significantly higher than water turnover, preventing the formation of stable communities adapted to local salinity conditions.

The Coorong Lagoon consists of a long, narrow channel with restricted water exchanges connected to the River Murray and the Southern Ocean only at the Murray Mouth. As a consequence, the Murray Mouth is highly affected by both freshwater and seawater inputs, whereas water exchanges at the other stations are limited and thus turnover rates decrease southward (Webster 2010). Horizontal water mixing at the southern stations of the Coorong Lagoon is mostly driven by wind stress (Webster 2010) and is likely to be lower than at the Murray Mouth. The Coorong Lagoon is therefore likely to exchange microbes with both freshwater and seawater at rates that decrease southward. As a consequence, microbial communities appear to be more influenced by nearby stations than by local environmental conditions. For example, Long Point station in November (although brackish) was more influenced by the nearby hypersaline station Bonney Reserve (Fig. 1) than by the brackish Murray Mouth (Figs. 5 & 6).

The biogeography of planktonic protists is currently under debate, although there is increasing evidence of dispersal limitation as recently demonstrated for microbial eukaryotes inhabiting soils (Bates et al. 2013) and seawater sediments (Bik et al. 2012) across broad geographic distances. At a smaller scale, a primary influence of the geographic distance to the distribution of freshwater protists has been recently demonstrated by comparing microbial communities in 6 lakes in France (Lepère et al. 2013). In contrast with the Coorong Lagoon, where all the sampling sites were connected by water and the greatest distance between them was 96 km, the lakes investigates by Lepère et al. (2013) were further apart (up to 200 km) and were separated by physical barriers. In spite of the shorter distances and the lack of physical barriers which would facilitate the dispersal of microbes throughout the Coorong Lagoon, community dissimilarities between different samples increased with increasing distance (Fig. 6). Prokaryotic communities can experience dispersal limitations at geo-

graphical scales similar or even lower than those studied here, as found for ammonia-oxidising bacteria associated with salt marshes (Martiny et al. 2011) as well as lake bacterioplankton (Wang et al. 2013).

CONCLUSIONS

Microbial communities in inverse estuaries require adaptation to broad salinity gradients. Protists in the Coorong Lagoon are mainly derived from seawater with a minor contribution of freshwater lineages, and include a number of potentially halotolerant taxa. Although the microbial community composition differed significantly between the different stations (Fig. 4), a number of persistent lineages occurred in most stations (Table 4), and are likely to possess the ability to thrive in both brackish and hypersaline conditions.

Geographic distance rather than salinity seems to be the main factor controlling the diversity of smaller microbes in the Coorong Lagoon, and likewise in similar enclosed lagoons. Microbial growth and water turnover are suggested to be the main factors explaining a distance-controlled community structure. Specifically, if the taxa optimally adapted to local salinity conditions do not grow at rates significantly higher than water turnover rates, the community structure is likely to be controlled by other factors, such as geographic distance.

Acknowledgements. Funding was supported under Australian Research Council's Discovery Project funding scheme (DP110101679). Phytoplankton samples were analysed by Microalgal Services, and flow cytometry analyses were carried out by K. Newton. We thank the Department of Environment, Water and National Resources (DEWNR) for allowing us access to the Coorong National Park, Jan Jendyk and Deevesh A. Hemraj for help during the sampling, Jarrad Piper and Ivan Brawley for help in the bioinformatics analyses and 3 anonymous reviewers for the useful comments which significantly improved the manuscript.

LITERATURE CITED

Ake-Castillo JA, Guerra-Martinez SL, Zamudio-Resendiz ME (2004) Observations on some species of *Chaetoceros* (Bacillariophyceae) with reduced number of setae from a Tropical Coastal Lagoon. Hydrobiologia 524:203–213

Balzano S, Sarno D, Kooistra W (2011) Effects of salinity on the growth rate and morphology of ten *Skeletonema* strains. J Plankton Res 33:937–945

Balzano S, Gourvil P, Siano R, Chanoine M and others (2012a) Diversity of cultured photosynthetic flagellates in the northeast Pacific and Arctic Oceans in summer. Biogeosciences 9:4553–4571

Balzano S, Marie D, Gourvil P, Vaulot D (2012b) Composition of the summer photosynthetic pico and nanoplankton communities in the Beaufort Sea assessed by T-RFLP and sequences of the 18S rRNA gene from flow cytometry sorted samples. ISME J 6:1480–1498

Bates ST, Clemente JC, Flores GE, Walters WA, Parfrey LW, Knight R, Fierer N (2013) Global biogeography of highly diverse protistan communities in soil. ISME J 7:652–659

Bazin P, Jouenne F, Friedl T, Deton-Cabanillas AF, Le Roy B, Veron B (2014) Phytoplankton diversity and community composition along the estuarine gradient of a temperate macrotidal ecosystem: combined morphological and molecular approaches. PLoS ONE 9:e94110

Behnke A, Engel M, Christen R, Nebel M, Klein RR, Stoeck T (2011) Depicting more accurate pictures of protistan community complexity using pyrosequencing of hypervariable SSU rRNA gene regions. Environ Microbiol 13: 340–349

Bendif EM, Probert I, Herve A, Billard C and others (2011) Integrative taxonomy of the Pavlovophyceae (Haptophyta): a reassessment. Protist 162:738–761

Bik HM, Sung W, De Ley P, Baldwin JG, Sharma J, Rocha-Olivares A, Thomas WK (2012) Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. Mol Ecol 21:1048–1059

Bragg LM, Stone G, Butler MK, Hugenholtz P, Tyson GW (2013) Shining a light on dark sequencing: characterising errors in ion torrent PGM data. PLoS Comput Biol 9: e1003031

Buskey EJ, Wysor B, Hyatt C (1998) The role of hypersalinity in the persistence of the Texas 'brown tide' in the Laguna Madre. J Plankton Res 20:1553–1565

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K and others (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336

Caric M, Jasprica N, Calic M, Batistic M (2011) Phytoplankton response to high salinity and nutrient limitation in the eastern Adriatic marine lakes. Sci Mar 75:493–505

Casamayor EO, Triadó-Margarit X, Castaneda C (2013) Microbial biodiversity in saline shallow lakes of the Monegros Desert, Spain. FEMS Microbiol Ecol 85:503–518

Chambouvet A, Morin P, Marie D, Guillou L (2008) Control of toxic marine dinoflagellate blooms by serial parasitic killers. Science 322:1254–1257

Chambouvet A, Laabir M, Sengco M, Vaquer A, Guillou L (2011) Genetic diversity of Amoebophryidae (Syndiniales) during *Alexandrium catenella/tamarense* (Dinophyceae) blooms in the Thau lagoon (Mediterranean Sea, France). Res Microbiol 162:959–968

Cheung MK, Au CH, Chu KH, Kwan HS, Wong CK (2010) Composition and genetic diversity of picoeukaryotes in subtropical coastal waters as revealed by 454 pyrosequencing. ISME J 4:1053–1059

Christaki U, Kormas KA, Genitsaris S, Georges C, Sime-Ngando T, Viscogliosi E, Monchy S (2014) Winter-summer succession of unicellular eukaryotes in a meso-eutrophic coastal system. Microb Ecol 67:13–23

Clavero E, Hernandez-Marine M, Grimalt JO, Garcia-Pichel F (2000) Salinity tolerance of diatoms from thalassic hypersaline environments. J Phycol 36:1021–1034

Codd GA, Steffensen DA, Burch MD, Baker PD (1994) Toxic blooms of cyanobacteria in lake Alexandrina, South Australia. Learning from history. Aust J Mar Freshw Res 45: 731–736

Crump BC, Hopkinson CS, Sogin ML, Hobbie JE (2004) Microbial biogeography along an estuarine salinity gradient: combined influences of bacterial growth and residence time. Appl Environ Microbiol 70:1494–1505

Demir-Hilton E, Sudek S, Cuvelier ML, Gentemann CL, Zehr JP, Worden AZ (2011) Global distribution patterns of distinct clades of the photosynthetic picoeukaryote *Ostreococcus*. ISME J 5:1095–1107

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200

Elloumi J, Carrias JF, Ayadi H, Sime-Ngando T, Boukhris M, Bouain A (2006) Composition and distribution of planktonic ciliates from ponds of different salinity in the solar saltwork of Sfax, Tunisia. Estuar Coast Shelf Sci 67:21–29

Faith DP (1992) Conservation, evaluation and phylogenetic diversity. Biol Conserv 61:1–10

Falkowski PG, Raven JA (2007) Aquatic photosynthesis. Princeton University Press, Princeton, NJ

Foulon E, Not F, Jalabert F, Cariou T, Massana R, Simon N (2008) Ecological niche partitioning in the picoplanktonic green alga *Micromonas pusilla*: evidence from environmental surveys using phylogenetic probes. Environ Microbiol 10:2433–2443

Frank-Fahle BA, Yergeau E, Greer CW, Lantuit H, Wagner D (2014) Microbial functional potential and community composition in permafrost-affected soils of the NW Canadian Arctic. PLoS ONE 9:e84761

Gilabert J (2001) Seasonal plankton dynamics in a Mediterranean hypersaline coastal lagoon: the Mar Menor. J Plankton Res 23:207–217

Guillou L, Viprey M, Chambouvet A, Welsh RM and others (2008) Widespread occurrence and genetic diversity of marine parasitoids belonging to Syndiniales (Alveolata). Environ Microbiol 10:3349–3365

Guillou L, Bachar D, Audic S, Bass D and others (2013) The Protist Ribosomal Reference database (PR2): a catalog of unicellular eukaryote small sub-unit rRNA sequences with curated taxonomy. Nucleic Acids Res 41:D597–D604

Guiry MD, Guiry GM (2014) AlgaeBase. National University of Ireland, Galway. www.algaebase.org

Hallegraef GM, Bolch CJS, Hill DRA, Jameson I and others (2010) Algae of Australia: phytoplankton of temperate coastal waters, Vol 432. CSIRO Publishing, Melbourne

Hansen H, Koroleff F (2007) Determination of nutrients. In: Grasshoff K, Kremling K, Ehrhardt M (eds) Methods of seawater analysis. Wiley-VCH, Weinheim, p 159–228

Hurlbert SH (1971) Non-concept of species diversity: a critique and alternative parameters. Ecology 52:577–586

Jeffries TC, Seymour JR, Gilbert JA, Dinsdale EA and others (2011) Substrate type determines metagenomic profiles from diverse chemical habitats. PLoS ONE 6:e25173

Jendyk JG, Hemraj D, Brown MH, Ellis AV, Leterme SC (2014) Environmental variability and phytoplankton dynamics in a South Australian inverse estuary. Cont Shelf Res 91:134–144

Keresztes ZG, Felfoeldi T, Somogyi B, Szekely G and others (2012) First record of picophytoplankton diversity in Central European hypersaline lakes. Extremophiles 16: 759–769

Lefranc M, Thenot A, Lepère C, Debroas D (2005) Genetic diversity of small eukaryotes in lakes differing by their trophic status. Appl Environ Microbiol 71:5935–5942

Lepère C, Domaizon I, Taib N, Mangot JF, Bronner G, Boucher D, Debroas D (2013) Geographic distance and ecosystem size determine the distribution of smallest protists in lacustrine ecosystems. FEMS Microbiol Ecol 85:85–94

Leterme SC, Ellis AV, Mitchell JG, Buscot MJ, Pollet T, Schapira M, Seuront L (2010) Morphological flexibility of *Cocconeis placentula* (Bacillariophyceae) nanostructure to changing salinity levels. J Phycol 46:715–719

Leterme SC, Prime E, Mitchell J, Brown MH, Ellis AV (2013) Diatom adaptability to environmental change: a case study of two *Cocconeis* species from high-salinity areas. Diatom Res 28:29–35

Leterme SC, Jendyk JG, Ellis AV, Brown MH, Kildea T (2014) Annual phytoplankton dynamics in the Gulf Saint Vincent, South Australia, in 2011. Oceanologia 56:757–778

Logares R, Shalchian-Tabrizi K, Boltovskoy A, Rengefors K (2007) Extensive dinoflagellate phylogenies indicate infrequent marine-freshwater transitions. Mol Phylogenet Evol 45:887–903

Logares R, Audic S, Santini S, Pernice MC, de Vargas C, Massana R (2012) Diversity patterns and activity of uncultured marine heterotrophic flagellates unveiled with pyrosequencing. ISME J 6:1823–1833

Logares R, Audic S, Bass D, Bittner L and others (2014) Patterns of rare and abundant marine microbial eukaryotes. Curr Biol 24:813–821

Lozupone C, Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71:8228–8235

Maier Brown AF, Dortch Q, Van Dolah FM, Leighfield TA and others (2006) Effect of salinity on the distribution, growth, and toxicity of *Karenia* spp. Harmful Algae 5: 199–212

Mangot JF, Domaizon I, Taib N, Marouni N, Duffaud E, Bronner G, Debroas D (2013) Short-term dynamics of diversity patterns: evidence of continual reassembly within lacustrine small eukaryotes. Environ Microbiol 15: 1745–1758

Marie D, Partensky F, Jacquet S, Vaulot D (1997) Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. Appl Environ Microbiol 63: 186–193

Marie D, Shi XL, Rigaut-Jalabert F, Vaulot D (2010) Use of flow cytometric sorting to better assess the diversity of small photosynthetic eukaryotes in the English Channel. FEMS Microbiol Ecol 72:165–178

Marshall HG, Burchardt L, Lacouture R (2005) A review of phytoplankton composition within Chesapeake Bay and its tidal estuaries. J Plankton Res 27:1083–1102

Martiny JBH, Eisen JA, Penn K, Allison SD, Horner-Devine MC (2011) Drivers of bacterial β-diversity depend on spatial scale. Proc Natl Acad Sci USA 108:7850–7854

Murrel MC, Lores EM (2004) Phytoplankton and zooplankton seasonal dynamics in a subtropical estuary: importance of cyanobacteria. J Plankton Res 26:371–382

Not F, Siano R, Kooistra WHCF, Simon N, Vaulot D, Probert I (2012) Diversity and ecology of eukaryotic marine phytoplankton. In: Piganeau G (ed) Genomic insights into the biology of algae, Vol 64. Academic Press, London, p 1–54

Pagaling E, Wang H, Venables M, Wallace A and others (2009) Microbial biogeography of six salt lakes in Inner Mongolia, China, and a salt lake in Argentina. Appl Environ Microbiol 75:5750–5760

Pandey BD, Yeragi SG (2004) Preliminary and mass culture experiments on a heterotrichous ciliate, *Fabrea salina*. Aquaculture 232:241–254

Piganeau G, Desdevises Y, Derelle E, Moreau H (2008) Picoeukaryotic sequences in the Sargasso Sea metagenome. Genome Biol 9:R5

Pinckney JL, Paerl HW, Harrington MB, Howe KE (1998) Annual cycles of phytoplankton community-structure and bloom dynamics in the Neuse River Estuary, North Carolina. Mar Biol 131:371–381

Pollet T, Schapira M, Buscot MJ, Leterme SC, Mitchell JG, Seuront L (2010) Prokaryotic aminopeptidase activity along a continuous salinity gradient in a hypersaline coastal lagoon (the Coorong, South Australia). Saline Syst 6:5

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 — approximately maximum-likelihood trees for large alignments. PLoS ONE 5:e9490

Quail MA, Smith M, Coupland P, Otto TD and others (2012) A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 13:341

Saldarriaga JF, Taylor F, Cavalier-Smith T, Menden-Deuer S, Keeling PJ (2004) Molecular data and the evolutionary history of dinoflagellates. Eur J Protistol 40:85–111

Schapira M, Buscot MJ, Leterme SC, Pollet T, Chapperon C, Seuront L (2009) Distribution of heterotrophic bacteria and virus-like particles along a salinity gradient in a hypersaline coastal lagoon. Aquat Microb Ecol 54: 171–183

Schapira M, Buscot MJ, Pollet T, Leterme SC, Seuront L (2010) Distribution of picophytoplankton communities from brackish to hypersaline waters in a South Australian coastal lagoon. Saline Syst 6:2

Seoane S, Eikrem W, Arluzea J, Orive E (2009) Haptophytes of the Nervion River estuary, northern Spain. Bot Mar 52: 47–59

Shade A, Handelsman J (2012) Beyond the Venn diagram: the hunt for a core microbiome. Environ Microbiol 14: 4–12

Shannon CE (1948) A mathematical theory of communication. Bell Syst Tech J 27:379–423

Sherr EB, Sherr BF (2002) Significance of predation by protists in aquatic microbial food webs. Ant Leeuwenhoek 81:293–308

Stoeck T, Bass D, Nebel M, Christen R, Jones MDM, Breiner HW, Richards TA (2010) Multiple marker parallel tag environmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol Ecol 19:21–31

Tomas CR (ed) (1997) Identifying marine phytoplankton. Academic Press, San Diego, CA

Triadó-Margarit X, Casamayor EO (2012) Genetic diversity of planktonic eukaryotes in high mountain lakes (Central Pyrenees, Spain). Environ Microbiol 14:2445–2456

Triadó-Margarit X, Casamayor EO (2013) High genetic diversity and novelty in planktonic protists inhabiting inland and coastal high salinity water bodies. FEMS Microbiol Ecol 85:27–36

Trigueros JM, Orive E, Arriluzea J (2002) Observations on *Chaetoceros salsugineus* (Chaetocerotales, Bacillariophyceae): first record of this bloom-forming diatom in a European estuary. Eur J Phycol 37:571–578

Vaulot D, Eikrem W, Viprey M, Moreau H (2008) The diversity of small eukaryotic phytoplankton (≤3 µm) in marine ecosystems. FEMS Microbiol Rev 32:795–820

Vaulot D, Lepère C, Toulza E, De la Iglesia R and others (2012) Metagenomes of the Picoalga *Bathycoccus* from the Chile coastal upwelling. PLoS ONE 7:e39648

Vigil P, Countway PD, Rose J, Lonsdale DJ, Gobler CJ, Caron DA (2009) Rapid shifts in dominant taxa among microbial eukaryotes in estuarine ecosystems. Aquat Microb Ecol 54:83–100

Wang J, Shen J, Wu Y, Tu C and others (2013) Phylogenetic beta diversity in bacterial assemblages across ecosystems: deterministic versus stochastic processes. ISME J 7:1310–1321

Webster IT (2010) The hydrodynamics and salinity regime of a coastal lagoon — the Coorong, Australia — seasonal to multi-decadal timescales. Estuar Coast Shelf Sci 90: 264–274

Wu QL, Chatzinotas A, Wang J, Boenigk J (2009) Genetic diversity of eukaryotic plankton assemblages in eastern Tibetan lakes differing by their salinity and altitude. Microb Ecol 58:569–581

Zinger L, Gobet A, Pommier T (2012) Two decades of describing the unseen majority of aquatic microbial diversity. Mol Ecol 21:1878–1896

Editorial responsibility: Urania Christaki, Wimereux, France

Submitted: July 29, 2014; Accepted: January 15, 2015
Proofs received from author(s): March 16, 2015