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FEATURE ARTICLE

Prokaryotic and diazotrophic population dynamics within a large oligotrophic inverse estuary

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ABSTRACT: The ecology of microbial assemblages inhabiting classical (positive) estuaries has been well documented. However, we know relatively little about the microbial ecology of inverse (negative) estuaries, which exhibit different physical and hydrodynamic properties, including oligotrophy and hypersalinity. We investigated the dynamics of bacterioplankton communities in Spencer Gulf, an inverse estuary in temperate South Australia. We characterised patterns in the overall diversity and composition of the resident microbial assemblage, and tested the hypothesis that pelagic nitrogen-fixing bacteria (diazotrophs) could be an important functional group in the nutrient limited waters of the region. Prokaryotic and diazotrophic communities were evaluated using 16S ribosomal DNA and *nifH* amplicon tag pyrosequencing, respectively. Significant heterogeneity in microbial community composition and diazotrophic population structure was observed, which was driven by shifts in the relative importance of temperate vs. subtropical and oceanic vs. coastal ecotypes of *Cyanobacteria* throughout the inverse estuary. The globally significant unicellular cyanobacterium, UCYN-A '*Candidatus* Atelocyanobacterium thalassa', was the dominant diazotrophic phylotype. Temperature, chlorophyll *a* and nitrogen availability were all significant drivers of bacterioplankton dynamics within the gulf. These results demonstrate the heterogeneous microbiology of inverse estuaries, indicating that specific abiotic and biotic characteristics select for discrete microbial communities, and that pelagic nitrogen fixation may be important in this temperate oligotrophic system.

KEY WORDS: Inverse estuary · Marine bacteria · Community composition · Diazotroph

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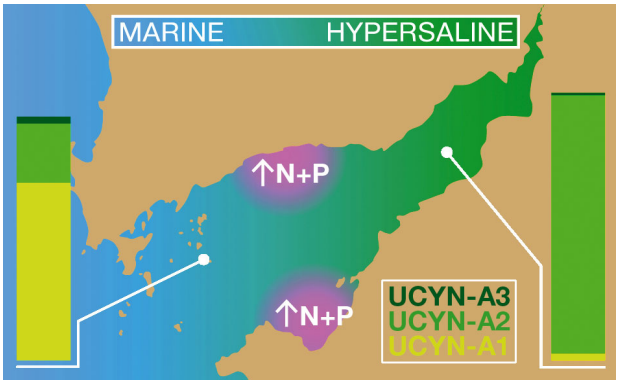

Inverse estuaries, such as Spencer Gulf (South Australia), are dynamic environments. Hypersalinity and heterogeneity in nutrient concentrations drive shifts in bacterioplankton populations, including between ecotypes of the nitrogen fixing '*Candidatus* Atelocyanobacterium thalassa' (UCYN-A).

Image: Greg Love

INTRODUCTION

Estuaries provide a dynamic interface between freshwater and marine microbial communities where terrestrial inputs of allochthonous nutrients play an important role in sustaining biological productivity (Hobbie 1988). In classical or positive estuaries, inflowing fresh water carrying dissolved and suspended materials from terrestrial sources mixes with marine waters (Pritchard 1952), creating environmental gradients that drive heterogeneity in the structure and function of planktonic microbial communities. For instance, gradients in salinity and organic material have been shown to underpin substantial shifts in bacterial community composition and biogeochemical activity (Bouvier & del Giorgio 2002, Poretsky et al. 2010).

Microbial patterns have been well documented in classical estuaries (del Giorgio & Bouvier 2002, Crump et al. 2004, Kirchman et al. 2005, Kan et al. 2007). In these systems, longitudinal salinity gradients can result in a corresponding transition in abundant taxa, for example, from marine type *Alphaproteobacteria* and *Cyanobacteria* through to freshwater associated *Betaproteobacteria* (Bouvier & del Giorgio 2002, Bernhard et al. 2005, Kirchman et al. 2005). Furthermore, unique estuarine communities may evolve when water residence times are sufficient (Crump et al. 2004, Herlemann et al. 2011). Conversely, to the best of our knowledge, there is no information concerning the taxonomic identity or the spatial distribution of microbial communities in inverse (or negative) estuaries.

In inverse estuaries, freshwater inputs at the head of the estuary are low or absent (Eyre 1998), and an excess of evaporation over precipitation increases the salinity of the inflowing seawater (Pritchard 1952) giving rise to strong salinity gradients and hypersaline regions at the enclosed head of the system (Nunes Vaz et al. 1990). Compared with classical estuaries, inverse estuaries do not receive significant inputs of allochthonous nutrients from rivers at the head (Smith & Veeh 1989), and can become seasonally closed to oceanic inflow at the mouth (Petrusevics 1993), which can result in the formation of relatively oligotrophic waters (Middleton et al. 2013).

Inverse estuaries are typically found within arid climates (Eyre 1998) and one such region is the South Australian Gulf system (Nunes Vaz et al. 1990) where the largest of 2 adjacent gulfs, Spencer Gulf, encompasses 21 700 km^2 and extends over 300 km between 32° S to 35° S (Smith & Veeh 1989). Spencer Gulf receives no riverine input of fresh water (Bowers & Lennon 1987), experiences fluctuations in temperature from approximately 12°C in the winter to 24°C in summer (Nunes Vaz et al. 1990) and has a well-established internal clockwise circulation pattern of oceanic inward flow along the western side of the basin and an outflow along the eastern side of the gulf (Bye & Whitehead 1975). The upper, hypersaline region of Spencer Gulf is significantly more productive than the lower region (Smith & Veeh 1989). As there is little terrestrial nutrient input here, seagrass-based nitrogen fixation has previously been suggested to be an important source of biologically available nitrogen for supporting primary production (Smith & Veeh 1989). However, blooms of the diazotrophic cyanobacterium *Trichodesmium* have been reported in Spencer Gulf (Paxinos 2007), suggesting that pelagic microbial nitrogen fixation could play a previously unrecognised role in nitrogen cycling in these waters.

The sedimentology and the physical and hydrographical properties of Spencer Gulf have been extensively studied (Lennon et al. 1987, Tiller et al. 1989, Nunes Vaz et al. 1990, Petrusevics 1993, Fernandes et al. 2006, Petrusevics et al. 2011), as has the chemical state of the gulf and the interactions between nutrient availability, productivity and salinity (Smith & Veeh 1989). It has been demonstrated that this system exhibits distinct spatial patterns in phytoplankton biomass (Bierman et al. 2008) that are potentially associated with limited nitrogen availability (Middleton et al. 2013), but relatively little is known about the microbiology of the system. Due to a distinct lack of inflowing fresh water and allochthonous nutrients in this estuary, we predicted that the microbial assemblage will be dominated by marine taxa adapted to life in the coastal zone. However, similar to classical estuaries, we expected local differences in water body parameters to result in species sorting mechanisms that ultimately influence bacterioplankton population dynamics. In addition, we suspected that nitrogen-fixing bacteria could be an important functional group within this system. To test our hypotheses, we determined both the overall bacterioplankton community composition, and the structure of the pelagic diazotrophic microbial assemblage throughout this temperate oligotrophic gulf.

MATERIALS AND METHODS

Sampling

Samples were collected from 5 locations within Spencer Gulf between 17–20 April 2011, onboard the FV '*Atlas*'. Water samples were collected during the austral autumn when dense gulf water from the northern region of the gulf is transported out into the adjacent shelf waters via the formation of a gravity current along the eastern basin, triggering the influx of oceanic water in the west. Samples were collected from surface and bottom waters (approximately 1 m below and above the surface and bottom respectively) as the gulf was expected to be stratified if the flushing had not already occurred. Site locations were based on previously defined spatial regions and were chosen in an attempt to sample spatial heterogeneity in environmental conditions, whereby 3 sites were sampled in the southern basin and 2 in the middle basin of the gulf (Fig. 1). Vertical profiles of temperature, salinity, oxygen and fluorescence were

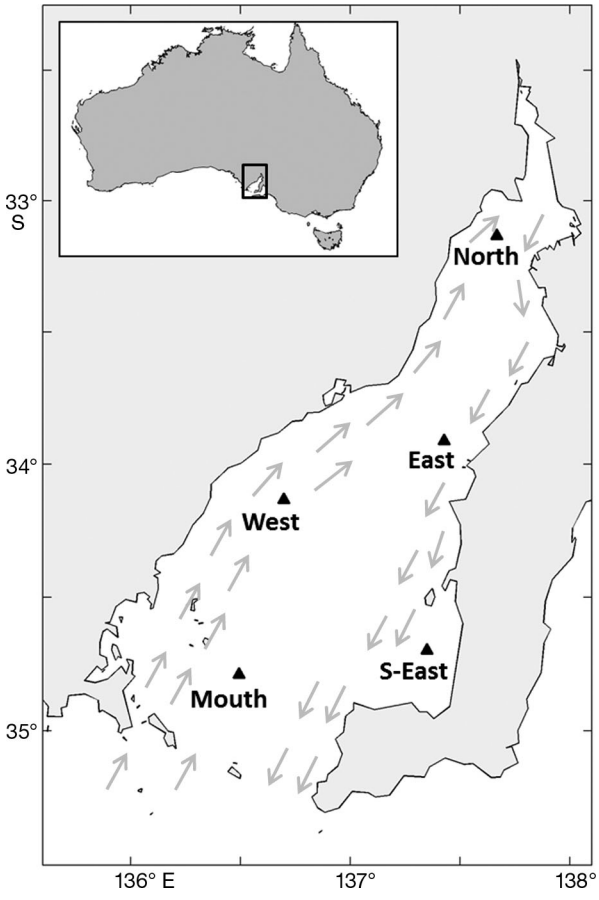

Fig. 1. Sampling locations within Spencer Gulf, in temperate South Australia, including a simplified view of the clockwise circulation pattern within the gulf (grey arrows)

made at each site using a Seabird 19 Plus CTD equipped with a WetLabs fluorometer and SBE43 dissolved oxygen sensor. Water samples were collected using Niskin bottles attached to the CTD assembly.

Biotic and abiotic characteristics

For each water sample collected, 100 ml was filtered through a 0.45 µm filter for macro-nutrient analysis, then frozen and stored at −22°C prior to laboratory analysis. Dissolved ammonium (NH_3, APHA-AWWA-WCPF 1998a, detection limit 0.071 µM), oxides of nitrogen (NO_X, APHA-AWWA-WCPF 1998b, detection limit 0.071 µM), phosphate (PO_4, APHA-AWWA-WCPF 1998c, detection limit 0.032 µM) and silicate (SiO_2, APHA-AWWA-WCPF 1998d, detection limit 0.333 µM) were determined by flow injection analysis with a QuickChem 8500 Automated Ion Analyser.

The pigment composition of water samples was measured using high pressure liquid chromatography (HPLC). Two-litre water samples were filtered through 0.4 µm glass fibre filters (GF/F, Whatman), which were snap-frozen in liquid nitrogen and stored at −80°C prior to analysis via the gradient elution procedure of Van Heukelem & Thomas (2001) on an Agilent 1200 series HPLC system (South Australian Research and Development Institute, Aquatic Sciences).

Bacterioplankton community composition

Water samples (4 l) collected at each site were filtered onto 0.2 µm polycarbonate membrane filters (Millipore), immediately quick frozen in liquid nitrogen and stored at −80°C prior to analysis. Microbial genomic DNA was extracted using the Power Water DNA extraction kit (MoBio) following the manufacturer's protocols. Partial 16S rRNA gene fragments spanning the V1-V3 variable regions were amplified using the primers 27F (5′-AGR GTT TGA TCM TGG CTC AG-3′) and 519R (5′-GWA TTA CCG CGG CKG CTG-3′, Lane 1991) and the following reaction conditions: a single-step 30 cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen) comprising 94°C initial denaturation (3 min), 28 cycles of 94°C (30 s) 53°C (40 s) and 72°C (1 min), with a final elongation step at 72°C (5 min). The resultant 16S rRNA gene amplicon products were pooled in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience); samples were sequenced using Roche 454 FLX Titanium (Molecular Research LP) instruments and reagents following the manufacturer's guidelines (Dowd et al. 2008). 16S rRNA gene sequences were analysed and processed using the Quantitative Insights into Microbial Ecology software (QIIME; Caporaso et al. 2010). Briefly, samples were rarefied to 1337 sequences to ensure even sampling effort across samples, sequences were clustered at 97% sequence identity using UCLUST (Edgar 2010) and taxonomy was assigned according to the latest version of the SILVA database (111; Quast et al. 2013).

Nitrogenase gene presence and diversity

In order to explore the composition of the diazotrophic community within Spencer Gulf, the gene encoding the enzyme nitrogenase (*nifH*, Zehr et al. 1997, 2003) was amplified and sequenced. A nested PCR protocol was used to amplify an approximately 359 bp region of the *nifH* gene using 2 sets of degen-

erate primers (Zehr & McReynolds 1989, Zehr & Turner 2001), nifH3 reverse primer (5′-ATR TTR TTN GCN GCR TA-3′) and nifH4 forward primer (5′-TTY TAY GGN AAR GGN GG-3′), nifH1 forward primer (5′-TGY GAY CCN AAR GCN GA-3′) and nifH2 reverse primer (5′-ADN GCC ATC ATY TCN CC-3′) respectively. Microbial community template DNA (1 µl) was added to 25 µl GoTaq Colourless Mastermix (Promega), 2 µl of both forward and reverse primer (10 µM working stock) and 20 µl molecular grade water. The first stage of the PCR used nifH4 and nifH3 primers and the following reaction conditions: 95°C initial denaturation (GoTaq activation), and 30 cycles of 95°C denaturation (1 min), 48°C annealing (1 min) and 72°C extension (1 min), followed by a final extension at 72°C (10 min). The second stage used the nifH1 and nifH2 primer pair and 1 µl of PCR product from the first stage as template, following the same reaction conditions. PCR products were purified using the Ultra Clean PCR Clean-up Kit (MoBio Laboratories) following the manufacturer's instructions.

The *nifH* amplicons were sequenced by 454 pyrosequencing (Roche, FLX Titanium; Molecular Research LP) after an additional 10 PCR cycles with custom barcoded nifH1 and nifH2 primers under the same reaction conditions (Dowd et al. 2008, Farnelid et al. 2011, 2013). Between 902 and 2517 *nifH* sequences were recovered from each sample. Raw sequences were quality filtered, clustered at 95% sequence identity (Penton et al. 2013) using UCLUST (Edgar 2010) and rarefied to 902 sequences per sample in QIIME (Caporaso et al. 2010). This resulted in 285 *nifH* operational taxonomic units (OTUs), and putative taxonomy was assigned using BLASTn (Altschul et al. 1990) against the NCBI Nucleotide collection database. Representative sequences of 14 *nifH* OTUs that contributed to the majority of sequences across all samples (~60%) were aligned to best match *nifH* nucleotide sequences identified from the BLASTn using ClustalW (Thompson et al. 1994). In addition, taxonomy was inferred using FrameBot, to compare translated *nifH* sequences to the Ribosomal Database Project's *nifH* protein database (Fish et al. 2013, Wang et al. 2013). A maximum likelihood phylogenetic tree of aligned *nifH* nucleotide sequences was constructed in MEGA6 (Tamura et al. 2013).

Statistical analyses

Rarefied sequence data were square root transformed and a resemblance matrix was generated using Bray-Curtis similarity; environmental parameters were normalised and a resemblance matrix was generated using Euclidean distance (Clarke & Warwick 2001). Analysis of similarities (ANOSIM) was used to test the hypothesis that different regions of Spencer Gulf harboured distinct microbial communities (Clarke 1993). Distance-based linear modelling (DistLM) and distance-based redundancy analysis (dbRDA) were used to identify the relationship between the multivariate biological data and the available predictor variables (Legendre & Anderson 1999, McArdle & Anderson 2001). The contribution of discrete OTUs to the observed differences in community assemblage as a function of sampling location was determined using similarity percentage analysis (SIMPER; Clarke 1993). All statistical analyses were performed in the PRIMER + PERMANOVA software package (v6; Clarke & Warwick 2001).

RESULTS

Biotic and abiotic characteristics of Spencer Gulf

Samples were collected during the austral autumn, when the outflow of dense gulf water along the bottom of the eastern edge of the system into the adjacent shelf waters was expected to occur. Water temperatures at this time were consistent with seasonal oscillations and the inverse nature of the gulf, ranging from 18.08°C to 19.21°C (Table 1), with higher temperatures associated with the most northern site (19.21°C), compared with the mouth (18.62°C) and the adjacent shelf waters (~17.5°C, IMOS 2014 Ocean Current http://oceancurrent.imos.org.au/SAgulfs/2011/2011041819.html [accessed 14/03/2014]). Salinity increased along the western edge of Spencer Gulf reaching a maximum in the north and decreasing again along the eastern side of the basin, ranging from 36.78 to 39.66 (Table 1). Phosphate concentrations were below the limit of detection at each site (<0.032 µM; Table 1) and NO_x concentrations (limit of detection 0.0714 µM) could only be determined in 2 samples corresponding to the surface and bottom samples taken from the western sampling site (Table 1, Fig. 1). This was also the site at which total nitrogen and total phosphorus peaked (Table 1). However, chlorophyll *a* (chl *a*) concentrations were greatest along the eastern edge of the basin with the majority attributable to the <5 µm planktonic fraction (Table 1). Measured biotic and abiotic characteristics of Spencer Gulf demonstrated greater partitioning due to horizontal rather than vertical spatial scales (Fig. S1 in

Table 1. Abiotic and biotic characteristics of sampling locations within Spencer Gulf. Samples were obtained at the surface (S) and 1 m above bottom (B). nd = no data available, Bd = below detection limit, TN = total nitrogen, TP = total phosphorus

Sample	Mouth		West		North		East		S-East	
	S	B	S	B	S	B	S	B	S	B
Bottom depth (m)	45	45	25	25	15	15	25	25	15	15
Temperature (°C)	18.62	18.69	19.15	19.03	19.21	19.19	18.58	18.90	18.45	18.08
Salinity (PSU)	36.78	36.81	36.86	36.88	39.61	39.66	37.99	38.38	36.84	36.87
Fluorescence (mg m^{-3})	0.23	0.23	0.16	0.16	0.31	0.30	0.26	0.29	0.08	0.22
Oxygen (mg l^{-1})	7.15	7.60	7.07	7.65	7.30	nd	7.50	nd	7.67	nd
TN (µM)	3.82	4.16	15.49	16.49	9.92	10.92	9.28	9.42	10.85	8.28
TP (µM)	Bd	Bd	0.90	1.00	0.25	0.26	0.28	0.28	0.33	0.26
NH_3 (µM)	0.20	0.20	0.36	0.47	0.27	0.33	0.23	0.15	0.48	0.43
NO_x (µM)	Bd	Bd	0.42	0.33	Bd	Bd	Bd	Bd	Bd	Bd
PO_4 (µM)	Bd	Bd	Bd	Bd	Bd	Bd	Bd	Bd	Bd	Bd
SiO_2 (µM)	0.44	0.69	0.64	0.63	1.04	1.46	0.46	0.74	0.55	0.80
Chl *a* (>5 µm; mg m^{-3})	0.05	0.05	0.04	0.04	0.07	0.12	0.07	0.06	0.05	0.05
Chl *a* (<5 µm; mg m^{-3})	0.35	0.37	0.22	0.21	0.27	0.38	0.39	0.49	0.22	0.35
Total 16S Sequences	6722	10292	1337	1524	7876	8596	8191	6359	5717	11406

the Supplement at www.int-res.com/articles/suppl/a074p001_supp.pdf), suggesting that the gulf was not significantly stratified at the time of sampling.

Bacterioplankton community composition

At the phylum level, the major bacterial groups across Spencer Gulf were the *Cyanobacteria*, *Proteobacteria* and *Bacteroidetes* (Fig. 2a). Minor shifts in the relative abundance of each phylum were observed across the different sampling locations and depths. Notably, *Proteobacteria* contributed ~43% of sequences in the western edge of the gulf whilst *Cyanobacteria* were most abundant, comprising ~70% of sequences, at the northern site (Fig. 2a).

At the genus level, *Synechococcus* was the dominant group of *Cyanobacteria* (approximately 90% of cyanobacterial sequences), with 2 distinct OTUs abundant across all samples (Fig. 2b). Shifts in the relative proportion of *Synechococcus* reflect a similar pattern to the overall Cyanobacterial relative abundance (Fig. 2a). The *Synechococcus* OTUs were representatives of 2 distinct marine clades, the predominantly temperate, coastal Clade I (100% identical to CC9311, Toledo & Palenik 1997) and the predominantly warm, open ocean Clade II (99% identical to WH8109, Waterbury et al. 1986). Both strains were most abundant in the northern samples, towards the head of the gulf, totalling 53% and 58% of the bacterioplankton community in the surface and bottom waters, respectively (Fig. 2b), with the Clade I OTU more abundant than the Clade II OTU in all samples (Fig. 2b). *Prochlorococcus* was also detected at all sites, but was substantially less abundant than *Synechococcus*. Near the mouth of the gulf, where conditions were most 'oceanic', *Prochlorococcus* sequences contributed up to 8% of total sequences, but only represented 1% of sequences in the surface samples of the eastern gulf waters (Fig. 2b).

Genus level representatives from the most abundant *Gamma-* and *Alphaproteobacteria* included *Pseudoalteromonas*, *Psychrobacter*, *Alcanivorax*, *Alteromonas*, *Halomonas*, '*Candidatus* Pelagibacter' and a *Rhodobacteraceae* OTU that could be not identified to genus level (Fig. 2b). *Pseudoalteromonas* abundance was particularly high in the western basin samples, totalling 19% of sequences in the bottom water samples. This was complementary with a peak in *Psychrobacter*, which comprised 22% of the community composition in the surface sample at this site, where it was 2 orders of magnitude more abundant than in the surface waters of the mouth, north and south-eastern samples (Fig. 2b). Notably, '*Candidatus* Pelagibacter', a ubiquitous marine bacterium which typically comprises 30% of marine bacterioplankton communities (Morris et al. 2002), comprised only 0.22% to 3.3% of the overall microbial community (and was not detected at all in samples from the south-eastern region of the basin, Fig. 2b).

Microbial communities across the sampling sites were statistically different (ANOSIM, $p < 0.05$, Global $R = 0.83$), with the greatest differences in community composition observed between the west and east samples of the gulf (SIMPER; 48.37% dissimilarity). However, there were no significant differences between surface and bottom water communities (ANOSIM, $p > 0.05$, Global $R = 0.136$). DistLM

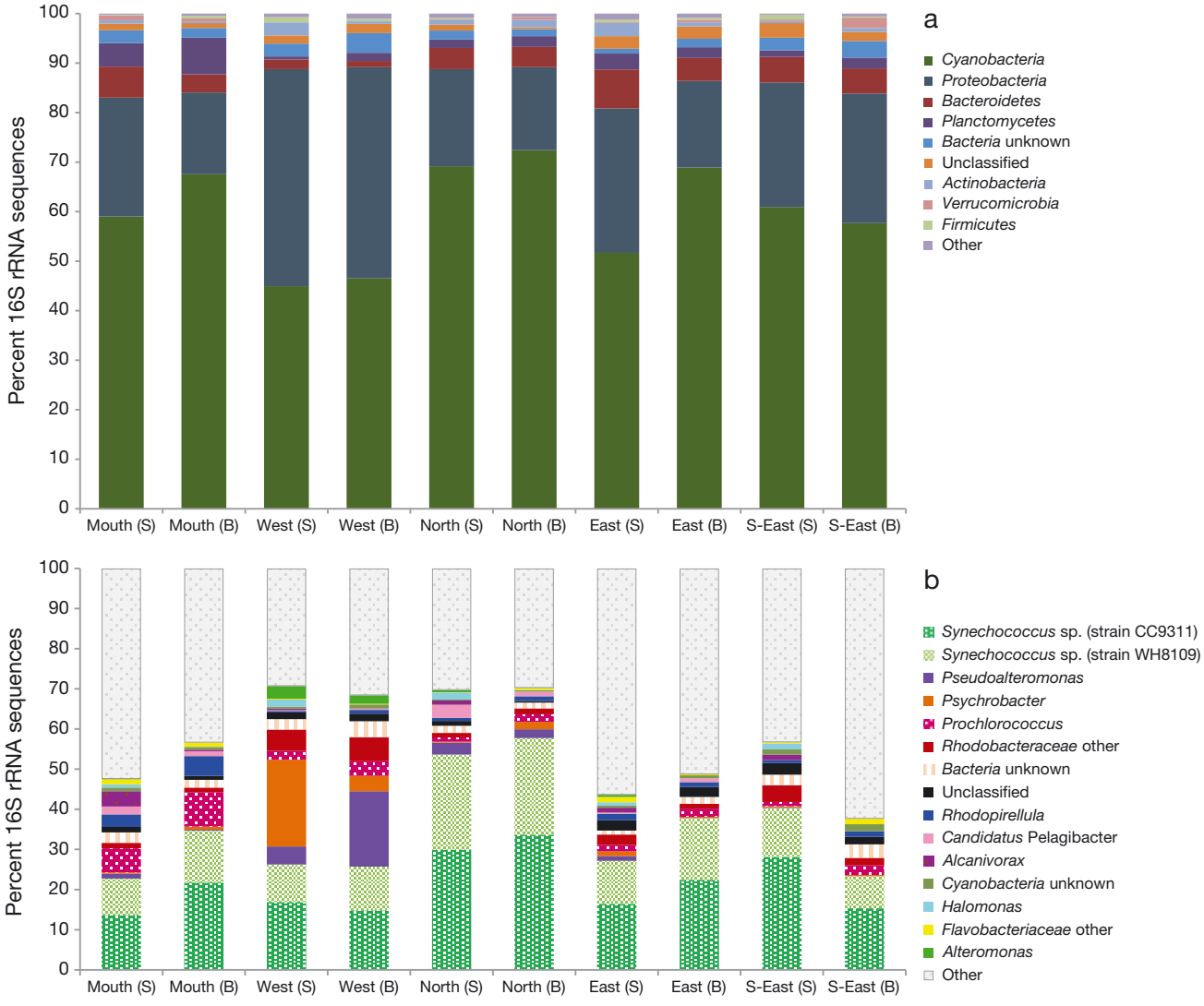

Fig. 2. Bacterioplankton community composition across Spencer Gulf for surface (S) and bottom (B) samples at the (a) phylum and (b) genus and OTU levels (where available)

identified temperature, salinity, total nitrogen, total phosphorus and chl *a* (<5 µm) as variables that explained a significant ($p < 0.05$, Table S1 in the Supplement) proportion of the differences in microbial community composition. Distance-based redundancy analysis, constrained with the significant variables, revealed that the microbial assemblage inhabiting the western edge of the gulf and those in the north were clearly distinct from those at the other sampling locations (Fig. 3). Communities in the western basin, which displayed a higher proportion of *Gammaproteobacteria* (Fig. 2), correlated with total nitrogen and total phosphorus (Fig. 3). Communities in the north, which displayed the highest proportion of *Cyanobacteria* (Fig. 2), correlated with higher values of temperature and salinity (Fig. 3).

Detection of nitrogen-fixing cyanobacteria

Due to the low number of sequence reads within the western basin samples (1337), and the loss of a minimum of 4380 reads from the remaining samples, we re-analysed the 16S rRNA sequence data to identify any rare or unique taxa that were lost from the overall community composition during the initial rarefaction. By excluding the western basin samples, we were able to rarefy the data to 5717 sequences. After doing this, the overall patterns observed within the microbial community composition at the phyla and genus level did not change, but a greater access to the rarer organisms in the samples was provided. Within these rarer taxa, we identified 4 OTUs that were 97% similar to the nitrogen-fixing bacterium, unicellular

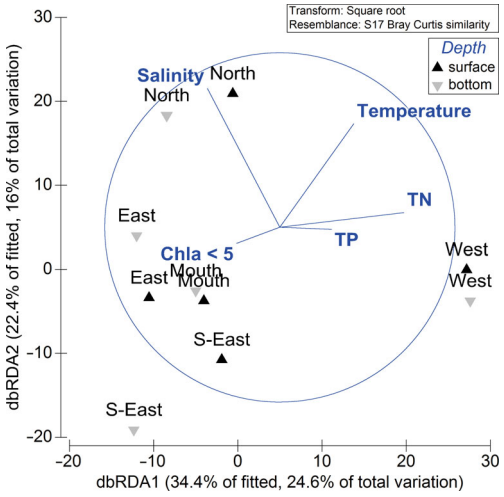

Fig. 3. Distance-based redundancy analysis constrained by environmental variables that correlated significantly (distLM, $p < 0.05$) with the pelagic microbial community composition. TN = total nitrogen, TP = total phosphorus, chl *a* < 5 = chl *a* < 5 µm size fraction

cyanobacteria group A (UCYN-A, *'Candidatus* Atelocyanobacterium thalassa', Fig. S2 in the Supplement). The majority of the UCYN-A 16S rRNA gene sequences belonged to one OTU, which was 99% similar to 16S rRNA genes isolated from the coccolithophore *Braarudosphaera bigelowii* found in the coastal seas of Japan (Hagino et al. 2013), which are believed to be associated with UCYN-A (Thompson et al. 2012). In total, we observed 131 sequences assigned to the UCYN-A OTUs, which contributed between 0.03 and 0.86% to the total number of sequences per sample. The majority of UCYN-A sequences occurred in the samples obtained from the northern region of Spencer Gulf (Table S2 in the Supplement). To our knowledge, this is the first report of UCYN-A sequences observed in an untargeted 16S rRNA gene profile obtained from a pelagic microbial community; the abundance of UCYN-A, and other nitrogen-fixing bacteria, is typically too low in the environment to be detected without approaches that either target them using specific 16S primers or target the *nifH* gene. Indeed, when we looked for the presence of UCYN-A 16S rRNA gene sequences across a range of marine microbial communities within other regions of the Australian marine environment (totalling 101 samples, 577 417 16S rRNA gene sequences and 38 188 OTUs based on ≥ 97% similarity, J. Seymour, M. Brown et al. unpubl. data), including regions where high levels of diazotrophic activity are known to occur and where UCYN-A has been shown to be a dominant diazotroph (Montoya et al. 2004, Moisander et al. 2010), UCYN-A sequences were present in only 1 additional sample (133 sequences total; Table S2).

Diversity of pelagic diazotrophs

In order to further investigate the diversity of potential diazotrophs within Spencer Gulf, we amplified and sequenced the *nifH* gene (Zehr & Turner 2001, Zehr et al. 2003). Clustering of 902 *nifH* sequences per sample resulted in a total of 285 *nifH* OTUs (Edgar 2010) at 95% nucleotide sequence similarity (Penton et al. 2013). Among these, there were 14 OTUs that contributed to ~60% of sequences across all samples (Fig. 4). A number of *nifH* OTUs were observed at every site, but the relative abundance of diazotroph

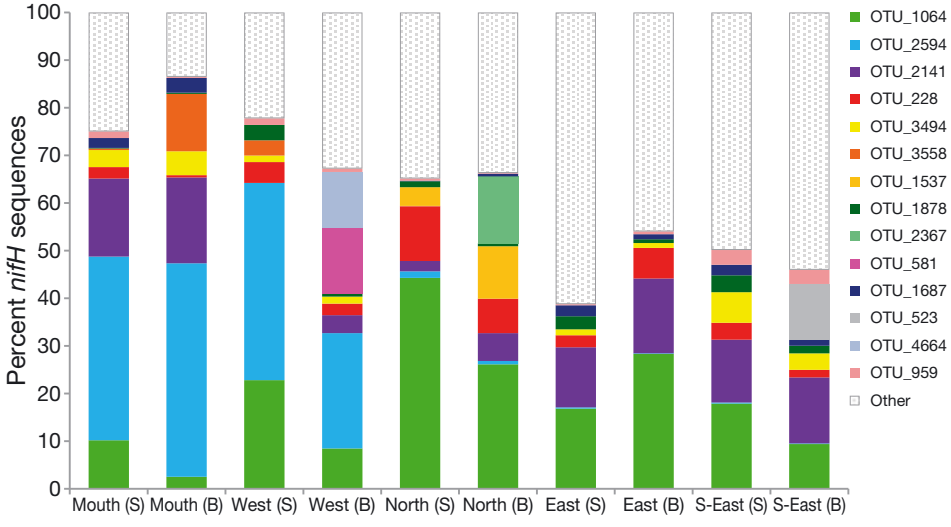

Fig. 4. Diazotrophic community composition across sites sampled within Spencer Gulf for surface (S) and bottom (B) samples. Only *nifH* OTUs that comprised > 60% of total *nifH* sequences are shown

OTUs varied depending on sampling location (Fig. 4). For example, OTU_1064 dominated the diazotrophic community in the surface waters of the northern sampling site, where it represented 44% of *nifH* sequences, but was less abundant in the surface and bottom waters of the mouth where it represented 10 and 2.5% of sequences, respectively. The representative sequence from this OTU was 99% similar to a clone isolated from the eastern Mediterranean (JUL_HO1_RNA_7, accession number EF568484, Man-Aharonovich et al. 2007), which clusters with UCYN-A *nifH* sequences recently identified as ecotype UCYN-A2 (Fig. 5, Thompson et al. 2014). Conversely, in the samples collected from the mouth of the gulf and the western edge of the basin, OTU_2594 was the most abundant OTU and contributed up to 45% of sequences (Fig. 4). This organism, which was negligible (<1% of sequences) at the remaining sampling sites, is 100% similar to an uncultured clone (accession number KC013147) isolated from the Hawaii Ocean Time-series in the North Pacific Subtropical Gyre (Bombar et al. 2013) and clusters with sequences that are representative of the UCYN-A1 ecotype (Fig. 5, Thompson et al. 2014). At the protein level, these 2 OTUs shared 100% identity with UCYN-A (ACJ53724, Zehr et al. 2008). None of the most abundant diazotrophs were identified as *Trichodesmium* strains at the nucleotide or protein level.

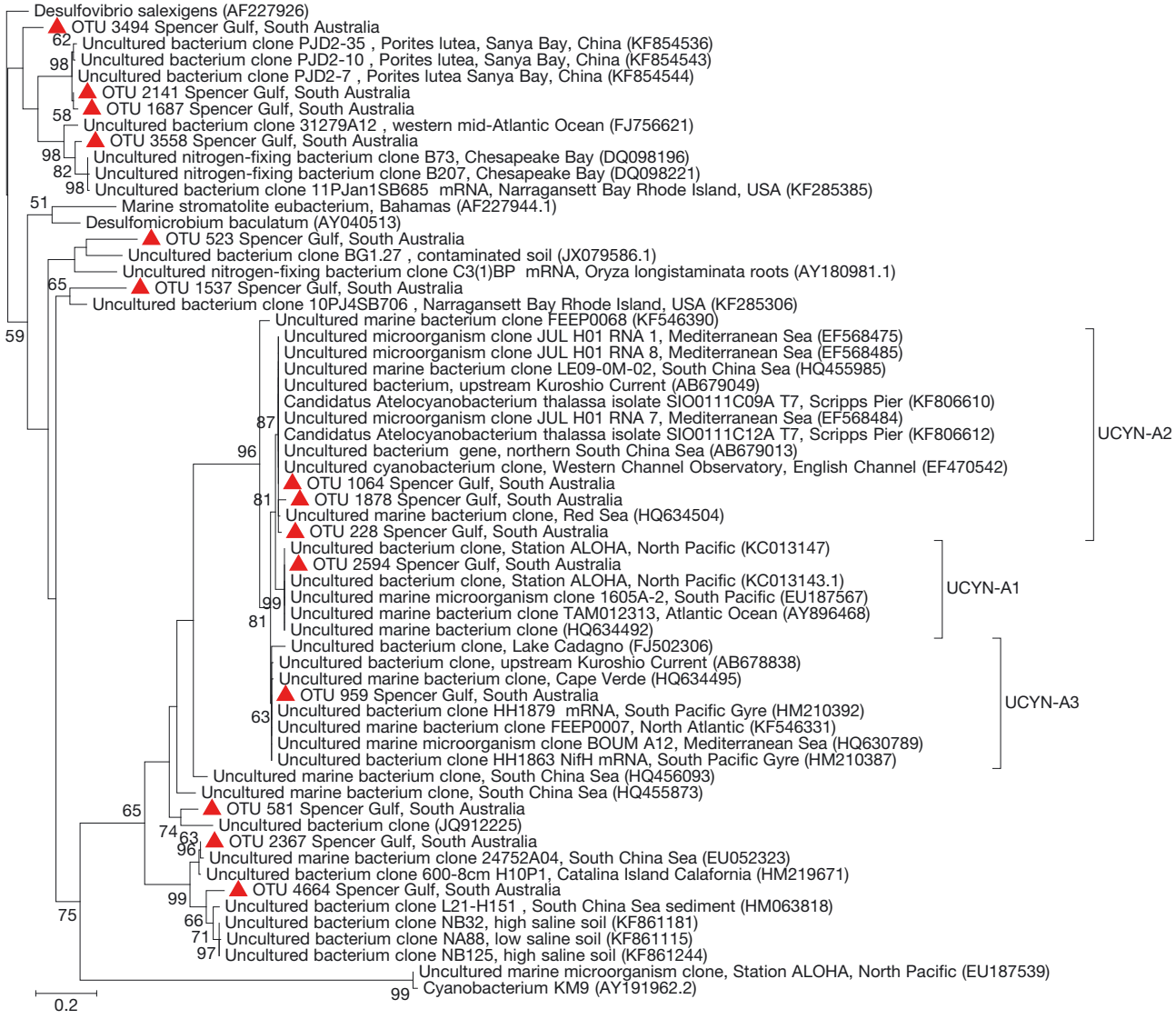

Fig. 5. Maximum likelihood phylogenetic tree, using the Jukes-Cantor correction, of partial *nifH* sequences recovered from this study (indicated by red triangles) and previously isolated *nifH* genes including accession numbers and source or geographical origin where available. Bootstraps over 50% for 1000 permutations, and a scale bar of 0.2 base pair substitutions are shown

Overall, we observed a shift in the putative pelagic diazotrophic community from the mouth towards the head and between the west and east sides of the gulf (Fig. 4). The observed differences in *nifH* OTU abundance and composition between sites were statistically significant (ANOSIM, $p < 0.001$, Global R = 0.83), with the greatest difference observed between the mouth and northern gulf sites (SIMPER, 78.42% dissimilarity) and the greatest similarity between eastern edge and south-eastern basin samples (SIMPER, 65.89% dissimilarity). No significant differences between surface and bottom water communities were observed (ANOSIM, $p > 0.05$, Global R = –0.164). DistLM identified temperature, salinity and silicate as significant ($p < 0.05$, Table S1) predictor variables to explain the differences in diazotroph community composition. Distance-based redundancy analysis, constrained by the significant environmental variables, revealed that northern diazotroph communities, which contained a higher proportion of UCYN-A2, clustered together. Samples from the mouth, west and south-east separated along a gradient defined by temperature, which corresponds with a decrease in the relative abundance of UCYN-A1 (Figs. 4 & 6).

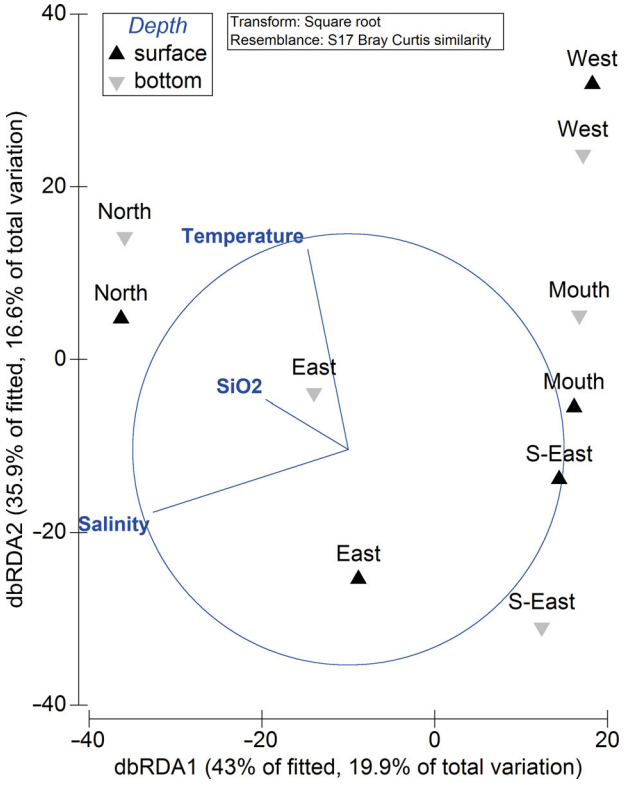

Fig. 6. Distance-based redundancy analysis constrained by environmental variables that correlated significantly (distLM, $p < 0.05$) with the diazotrophic community composition

DISCUSSION

Previous research has shown Spencer Gulf to be a nutrient limited system (Smith & Veeh 1989, Middleton et al. 2013), even though nutrient enrichment associated with highly productive aquaculture industries within the gulf is believed to be high in some locations (Fernandes et al. 2007, Lauer et al. 2009). Despite the oligotrophic conditions of the gulf, satellite derived phytoplankton estimates combined with *in situ* chl *a* measurements suggest that phytoplankton abundances are relatively high (Petrusevics et al. 2011), including in the northern region of the gulf (Bierman et al. 2008) despite no riverine source of nutrients. In the northern region, effluent discharge from terrestrial industry, including a lead smelter and steelworks, results in the local enrichment of trace elements and heavy metals (Ward & Young 1981, Ferguson et al. 1983, Tiller et al. 1989). The impact of these local allochthonous nutrients on the bacterio- and phytoplankton are unknown. In addition, the identity and diversity of microorganisms, which are ultimately involved in the biogeochemical cycling of the system, have not yet been explored. Which leads to the question: What are the key microorganisms underpinning the biogeochemical cycling of this system?

Spencer Gulf is characterised by complex temporal circulation patterns and physical dynamics that are unique to the region (Nunes Vaz et al. 1990). During the summer months, the whole gulf becomes isolated from the adjacent shelf waters due to temperature and salinity fronts (Petrusevics 1993, Petrusevics et al. 2011). During autumn and winter months, the release of a high density gravity current along the eastern side of the gulf triggers the inflow of oceanic water from the Southern Ocean along the west (Bye & Whitehead 1975, Lennon et al. 1987). A relatively long residence time of 9 mo (Smith & Veeh 1989) and these complex temporal dynamics result in different water body characteristics within different areas of the gulf, with discrete physico-chemical properties (Fig. S1). Although we did not investigate temporal dynamics in the present study, our data indicate that there are spatially distinct ecological niches within Spencer Gulf at any given point in time, which correlate with differences in environmental characteristics such as temperature and salinity (Figs. 3 & 6).

In classical estuaries, salinity is a major structuring factor of microbial communities, for example driving a transition from *Alphaprotoebacteria* and *Gammaproteobacteria* to *Betaproteobacteria* and *Actinobacteria* from high to low salinities respectively (Bouvier & del Giorgio 2002, Crump et al. 2004, Herlemann et

al. 2011). Compared with classical estuaries, we observed no taxa that are associated with riverine inputs, such as freshwater clades of *Betaproteobacteria* and *Actinobacteria*. Instead, we observed primarily marine associated taxa such as *Synechococcus*, *Prochlorococcus* and *Gammaproteobacteria* including *Psychrobacter* and *Pseudoalteromonas*. However, one substantial difference between the data presented here and typical marine microbial communities is the relatively low abundance of SAR11 sequences. Whereas SAR11 typically comprises near to 30% of marine microbial communities (Morris et al. 2002, Schattenhofer et al. 2009), the Spencer Gulf system was dominated by *Synechococcus* sequences. Previous work in the adjacent South Australian Shelf Waters demonstrated the seasonal dominance of *Synechococcus* within picophytoplankton communities in this region (van Dongen-Vogels et al. 2011, 2012), but the relationship between picophytoplankton abundance and heterotrophic bacterial abundance has not previously been explored within this system.

Synechococcus ecotypes from Clade I and Clade II (Fuller et al. 2003) dominated the microbial assemblage in the mouth, north and eastern waters of the gulf. The CC9311 *Synechococcus* ecotype (Toledo & Palenik 1997) from Clade I (Fuller et al. 2003) possesses unique adaptations to life in the coastal zone, with genome content reflecting the importance of trace element availability such as iron and copper for enzymatic functions, and ammonia as an important nitrogen source (Palenik et al. 2006). Ecotypes of *Synechococcus* from Clade I are typically most abundant in temperate waters (Tai & Palenik 2009, Choi et al. 2013); therefore, the overall dominance of the Clade I *Synechococcus* ecotype throughout the temperate, coastal environment of Spencer Gulf is not surprising (Partensky et al. 1999, Zwirglmaier et al. 2008).

On the other hand, the second most abundant OTU in our samples belonged to the *Synechococcus* Clade II, and closely matched a strain (WH8109) originally isolated from the Sargasso Sea (Waterbury et al. 1986). Members of Clade II are generally abundant in subtropical waters (Zwirglmaier et al. 2008, Choi et al. 2013). In this study, the OTU matching WH8109 was most abundant in the northern region of the gulf, where the partitioning of the northern microbial community was significantly correlated with temperature and salinity (Fig. 3). Conditions in the northern region of Spencer Gulf can seasonally become subtropical in nature, with water temperatures reaching 20 to 24°C during summer months (Nunes Vaz et al. 1990). The shifts in the dominant *Synechococcus* ecotypes in particular (Huang et al. 2012) could reflect a shift from temperate to subtropical water properties (Choi et al. 2013).

Our data also revealed that the western edge microbial community was distinct compared with assemblages associated with the remaining sampling locations. This change in overall microbial community composition correlated with higher total nitrogen and phosphorus concentrations in the western region of the gulf (Fig. 3). A substantial increase in OTUs belonging to the genus *Psychrobacter* occurred in the surface waters, while an increase in *Pseudoalteromonas* occurred in the bottom waters of the western edge, indicating a shift towards a more copiotrophic bacterial community. *Pseudoalteromonas* are classic marine copiotrophs (Lauro et al. 2009); this increase in copiotrophic taxa in the western edge of the basin is potentially related to the organic and inorganic nutrient inputs from nearby aquaculture industries (Fernandes et al. 2007, Lauer et al. 2009), which are believed to be the most significant source of allochthonous nutrients in Spencer Gulf (Middleton et al. 2013).

The western edge samples were not only considerably different to the other regions within the gulf; they also contained the lowest number of sequence reads per sample. Since 16S rRNA amplicon pyrosequencing is particularly effective at discriminating the whole of the prokaryotic assemblage including the organisms in low abundance (Sogin et al. 2006, Brown et al. 2009), we re-analysed the community profile data to remove the western edge samples and probe more deeply into the composition of the remaining sites. This resulted in an additional 4380 sequence reads per sample, and the detection of a globally significant, yet typically rare at the community level, nitrogen-fixing cyanobacterium UCYN-A. Diazotrophs are typically only detected using specific primer sets (Church et al. 2005, Short & Zehr 2007, Goebel et al. 2010) and are not often detected within whole community surveys such as this, suggesting that within this system they represent a relatively higher proportion of the bacterioplankton community.

The most abundant UCYN-A 16S rRNA gene sequences in the present study were 99% similar to 16S rRNA genes isolated from its symbiont, the coccolithophore *Braarudosphaera bigelowii* (Hagino et al. 2013), and these sequences were predominantly found in the northern sampling region within Spencer Gulf (Table S2). The presence of a pelagic diazotrophic community was confirmed using a *nifH* gene targeted approach, and UCYN-A sequences comprised the most abundant *nifH* OTUs across Spencer Gulf. Sequences closely related to a coastal UCYN-A

strain from the Mediterranean Sea (Man-Aharonovich et al. 2007), which cluster within the UCYN-A2 ecotype (Fig. 5), were present throughout the gulf but most abundant in the northern region. Meanwhile, at the mouth and western edge of Spencer Gulf, sequences most similar to that of UCYN-A1 and an open-ocean UCYN-A strain detected in the North Pacific Subtropical Gyre (Bombar et al. 2013) dominated.

While generally considered an open ocean cyanobacterium (Farnelid et al. 2011, Thompson et al. 2014), UCYN-A has been shown to be present and actively expressing *nifH* in estuarine waters, for example at the mouth of the Chesapeake Bay estuary in salinities as low as 16.9 (Short & Zehr 2007), in the Amazon River Plume (Foster et al. 2007, Goebel et al. 2010) and in semi-enclosed seas such as the Mediterranean (Man-Aharonovich et al. 2007). Moreover, most recently, UCYN-A has been detected in 2 temperate classical estuaries (salinities ranging from 11 to 24) in an area between the North Sea and Baltic Sea, where substantial rates of nitrogen fixation were observed, with a significant proportion attributed to the unicellular (<10 µm) size fraction (Bentzon-Tilia et al. 2014). However, only 16% of all diazotroph sequences within their study were identified as UCYN-A, with a greater proportion of diazotrophs belonging to the *Gammaproteobacteria* (Bentzon-Tilia et al. 2014). UCYN-A is evidently widely distributed throughout a range of aquatic environments.

Indeed, recently Thompson et al. (2014) identified 3 ecotypes of UCYN-A; the UCYN-A1 ecotype appears to have a broad distribution throughout the major ocean basins and, based on their assessment of publically available *nifH* sequences, UCYN-A2 appears to have a more limited coastal ocean distribution. Within the context of the present study, the distinct geographical ranges of these 2 UCYN-A clades, and the observed differences in their relative abundance, reflects a transition from more oceanic to coastal ecotypes. While the genomic and physiological differences between UCYN-A clades are yet to be determined, the partitioning of the northern diazotroph assemblages correlating with an increase in silicate and temperature (Fig. 6) suggests that UCYN-A ecotypes (and/or indeed UCYN-A's prymnesiophyte host) may occupy distinct niches, as has been previously observed in other globally significant cyanobacteria (Partensky et al. 1999, Palenik et al. 2006, Huang et al. 2012). However, within a single environment, these ecotypes co-occur (Figs. 4 & 5), suggesting that UCYN-A clades can also occupy the same ecological niche at a given point in time (Thompson et al. 2014).

Targeting genes encoding the nitrogenase enzyme complex (such as *nifH*) has proved successful in determining putative diazotroph phylogeny and diversity (Zehr et al. 1997, 2003). Many previous studies have used *nifH* clone libraries and/or qPCR with both DNA and RNA from marine environments (Church et al. 2005, Goebel et al. 2010, Moisander et al. 2010, Turk-Kubo et al. 2012). These studies have revealed that UCYN-A has a broader distribution and higher abundance in cooler waters, relative to other diazotrophic taxa (Moisander et al. 2010). In addition, a database compiled of oceanic qPCR-based *nifH* abundances revealed that UCYN-A are globally the most abundant diazotrophic group, which contributes significantly to global nitrogen fixation (Luo et al. 2012). Few studies have used amplicon sequencing to target the *nifH* gene of pelagic microbial assemblages (Farnelid et al. 2011, 2013, Bentzon-Tilia et al. 2014), making direct comparisons with the published literature difficult. Regardless, the data we present here furthers our understanding of the distribution of UCYN-A in marine systems, and provides further evidence for the differential distribution of UCYN-A ecotypes.

Rates of nitrogen fixation were not measured during the present study but the detection of UCYN-A in both the whole community (16S rRNA) survey and the *nifH* targeted survey suggests that pelagic N_2 fixation by marine cyanobacteria could play an important role in supporting production in this region. Previously, nitrogen fixation associated with seagrass meadows was proposed to be a significant source of fixed nitrogen inputs to Spencer Gulf (Smith & Veeh 1989). To the best of our knowledge, this is the first assessment of the potential for nitrogen fixation within the water column of Spencer Gulf, and within an inverse estuary in general. However, the presence of biochemically important phylotypes does not necessarily translate to active bacteria or biochemical functions, and direct measurements of pelagic biological nitrogen fixation throughout Spencer Gulf are required to better understand the N cycling dynamics within the inverse estuary.

The known influx of oceanic water along the western edge of Spencer Gulf suggests that the origin of UCYN-A in the gulf is the temperate waters of southern Australia, but the potential for diazotrophy in this region has not been investigated. Within the gulf itself, diazotroph community composition correlated significantly with temperature, salinity and silicate concentration, which all increased towards the northern sampling site, where the coastal UCYN-A ecotype was most prevalent. Previous observations and measurements have also shown high concentrations

of iron in the upper Spencer Gulf region (Harbison 1984, Dupavillon & Gillanders 2009). Water column iron concentrations were not determined in the present study, but we hypothesise that iron concentrations will be higher in northern gulf waters compared with other regions of the basin and the adjacent shelf waters, since oceanic iron concentrations tend to be limiting (Brand 1991, Moore et al. 2013). Iron is a limiting co-factor of the nitrogenase enzyme and consequently diazotroph activity (Moore et al. 2009, 2013, Turk-Kubo et al. 2012, Ward et al. 2013). If there are consistently high concentrations of iron in this system, the environmental properties of Spencer Gulf (e.g. oligotrophic and suspected iron enriched) could provide highly favourable conditions for diazotrophy. The low abundance of SAR11 type sequences in our 16S rRNA dataset suggests that considerable environmental filtering of taxa has occurred, and that the conditions prevailing in the gulf at the time of sampling represent reasonable habitat parameters for those organisms observed.

CONCLUSIONS

Our study demonstrates the existence of substantial heterogeneity in the composition of the marine bacterial community inhabiting an inverse estuary, which incorporates shifts in the relative proportion of ecologically significant phylotypes. The complex physical and hydrodynamic properties of inverse estuaries, such as Spencer Gulf, have the potential to underpin spatial patterns in bacterioplankton communities. With little to no terrestrial nutrient input and long residence times, inverse estuaries are relatively oligotrophic when compared to classical estuaries and coastal zones, potentially providing a niche within coastal regions for microbial groups that are competitive under low nutrient regimes, such as the nitrogen-fixing bacteria observed in this study. As a consequence, inverse estuaries, such as Spencer Gulf, represent ideal habitats for expanding our understanding of the drivers of marine microbial ecology and biogeography and are regions of potentially unique biogeochemistry.

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