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Antarctic sea-ice microbial communities show distinct patterns of zonation in response to algal-derived substrates

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ABSTRACT: Antarctic sea ice provides a stable environment for the colonisation of diverse and highly specialised microbes that play a central role in the assimilation and regulation of energy through the Antarctic food web. However, little is known about how the bacterial community composition changes within the sea ice, the functional role bacteria play in the sea-ice microbial loop, and the effect of variations in the environment on these patterns and processes. Using terminal restriction fragment length polymorphism (T-RFLP) analysis of the total (16S rRNA gene) and the active (16S rRNA) community, we characterised spatial patterns in Antarctic sea-ice bacterial communities (SIBCO). In addition, bacterial enzymatic activities were identified using synthetic fluorogenic substrates. Both bacterial community structure and enzymatic activity were compared to changing environmental conditions vertically down through the sea ice. The structure of the DNA- and RNA-derived bacterial communities exhibited strong vertical zonation through the ice. There was no direct relationship between changes in chlorophyll *a* (chl *a*) and bacterial numbers; however, there were direct relationships between chl *a,* bacterial community structure and metabolic function, thus providing evidence for a coupling of the microbial loop. The SIBCO were involved in phosphate recycling and selectively assimilated proteins over carbohydrates in response to the N- and P-poor environment. We propose that Antarctic sea ice has an active microbial loop in which the spatial dynamics of bacterial communities are influenced by algal-derived substrates and nutrient availability.

KEY WORDS: Antarctica · Bacteria · Community structure · Ice core · Metabolic function · Phosphate · Protease · Sea ice

INTRODUCTION

Microbes play key roles in biogeochemical processes, ultimately sustaining almost every other form of life on Earth. In Antarctic sea ice, the sea-ice microbial community (SIMCO) provides an indispensable food supply for Antarctic krill (Thomas & Dieckmann 2002, Quetin et al. 2007) and may serve as an inoculum for bloom events at the receding ice edge in the austral summer (Lizotte 2003, Arrigo & Thomas 2004). The SIMCO also produces a large amount of energy in the Southern Ocean's food webs, where most of this biomass is produced as high-molecular-weight dissolved organic matter (DOM) and particulate organic matter (POM) from algal-derived photosynthate (Arrigo & Thomas 2004, Gross 2005). However, the sea-ice bacterial community (SIBCO) has its own role in the assimilation of carbon (C) and nutrients by stimulating productivity through the microbial loop (Azam et al. 1983, 1991).

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Little is known of the changes in SIBCO composition vertically through the sea ice or spatially at any one depth or of how these changes may be influenced by the environment (Martin et al. 2011). As sea ice forms, planktonic microbes are incorporated from the water column into the semi-solid matrix (Mock & Thomas 2005). These planktonic microbes are exposed to a large shift in environmental conditions, from seawater at ~1.8°C to a completely enclosed ice matrix that has steep gradients of salinity, light, temperature and nutrient concentration (Thomas & Dieckmann 2002). There is a shift to more psychrophilic species in the sea ice in comparison to the water column, suggesting acclimation to the sea-ice environment (Brown & Bowman 2001, Maas et al. 2012). However, studies to date have all been based on total genomic DNA from environmental samples, which identify the total community, including active, dormant and dead cells. Since RNA is less stable than DNA, it can be used as an indicator of metabolically active populations (Mills et al. 2004, 2005, Gentile et al. 2006), which may give higher resolution for linking community composition to the environment (Miskin et al. 1999, Rodríguez-Blanco et al. 2010). Comparisons of the 16S rRNA gene (community composition) and the 16S rRNA (activity) are important for gaining a greater understanding of the total community diversity and function (Nogales et al. 2001, Moeseneder et al. 2005, Gentile et al. 2006, West et al. 2008, Rodríguez-Blanco et al. 2010).

An active microbial loop has been inferred by a positive relationship between algal biomass (using chlorophyll *a* [chl *a*] as a proxy) and bacterial numbers (Stewart & Fritsen 2004, Pusceddu et al. 2009) or bacterial productivity (Martin et al. 2009, Ducklow et al. 2012). However, in Antarctic environments, bacterial and algal coupling is highly variable between locations and seasons (Pomeroy & Wiebe 2001, Stewart & Fritsen 2004, Ducklow et al. 2012). Bacteria also show a significant time lag in the response to available growth substrate (Ducklow 1999, Martin et al. 2011), and this lag increases as bacteria shift from utilising low-molecular-weight photosynthates to hydrolysing large extracellular macromolecules (Billen 1990, Billen & Becquevort 1991). Thus, in addition to revealing algal-bacterial relationships, investigations of bacterial enzyme activity may also provide additional insight into microbial loop dynamics and high-molecular-weight DOM composition.

Sea-ice bacteria are highly metabolically active, with active members comprising more than a third of the total bacterial biomass (Junge et al. 2002, Brinkmeyer et al. 2003, Martin et al. 2008). In contrast, only 1 to 10% of the total bacterial community in temperate environments is metabolically active (Gasol et al. 1995, del Giorgio et al. 1996, Sherr et al. 1999). Bacteria release enzymes into the environment to hydrolyse large polymers of DOM and assimilate C, nitrogen (N) and phosphorus (P). Sea-ice bacteria produce enzymes such as chitobiase, aminopeptidase, phosphatases, lipase and β-glucosidase (Helmke & Weyland 1995, Bergamasco et al. 2003, Yu et al. 2009). Different patterns of enzymatic activities can be found depending on the quality and composition of the DOM available in the environment (Sala & Güde 2004) and may be linked to altered bacterial community composition, with particular bacterial phylogenetic groups responding differently to changing DOM and nutrient concentrations (Keinänen et al. 2002, Pinhassi et al. 2004, Nelson et al. 2011). Relationships between these enzymes can indicate C, N and P limitation. The principal sources of C, N and P are enzymatic degradation products of cellulose, proteins, chitins and peptidoglycan (the principle sources of organic N) and mineralize P from nucleic acids, phospholipids and other ester phosphates. Phosphatase activity has been used as a measure of P deficiency (Sala et al. 2001), and hydrolysis of peptides and proteins, a major pool for organic N, indicates N paucity (Keil & Kirchman 1991). Thus, the ratio of phosphatase activity (PH) to protease activity (PR) (PH:PR) may give an indication of P versus N limitation. PH:PR would be high under P-poor and low under N-poor conditions (Sala et al. 2001). The ratio of protease to β-glucosidase activity (PR:β-Glu) indicates the quality of the C pool and C versus N limitation, where a PR:β-Glu ratio > 1 indicates proteolysis and N limitation, and a ratio < 1 indicates glycolyis and C limitation (Christian & Karl 1995).

The aim of this research was to gain better insights into the environmental drivers of bacterial community structure and function in Antarctic sea ice and to identify the role bacteria play in the sea-ice microbial loop. First, we applied T-RFLP to both the total bacterial community (DNA-derived) and the active bacterial community (RNA-derived) to assess spatiotemporal variation in their community composition. Second, we measured bacterial enzyme activity to ascertain the components of DOM utilised by the SIBCO. Finally, we investigated the relationships between community structure and function and how these in turn relate to changes in the environment. We propose that Antarctic sea ice has an active microbial loop in which zonation patterns of bacterial communities are influenced by chl *a* and relative nutrient availability.

MATERIALS AND METHODS

Sample collection

A total of 20 sea-ice cores were collected during the austral summer from Granite Harbour (77° 0′ S, 162° 54′ E) from 18 to 29 November 2009. The samples were taken from land-fast sea ice, collected 1000 m from the nearest land and a distance of 10 km from the ice edge. Four cores were collected using a Kovaks ice coring drill on each of the following days: 18, 21, 24, 27 and 29 November. The top, middle and bottom 10 cm 'zones' from each ice core were removed. Each zone was melted into 3 times the volume of 0.2 µm filtered and autoclaved sea water to prevent osmotic shock (sensu Ryan et al. 2004). The melting time was 1 to 2 h. This process was a compromise between maintaining a realistic RNA-derived population and avoiding physiological shock due to melting. The microbes were collected by filtering through a 0.22 µm polycarbonate filter (Millipore). The filter was stored in RNA*later*™ (Ambion) at −20°C for up to 1 mo until processing.

DNA/RNA extraction

Genomic DNA (gDNA) was extracted from one half of the polycarbonate filters following the DNeasy™ protocol (Qiagen) with the following modifications. The filter was incubated at 37°C in enzymatic lysis buffer (40 mM EDTA, 50 mM Tris-HCl [pH 7.4], 0.75 M sucrose, 15% Tween 80) for 30 min with lysozyme (final concentration 1 mg ml^{-1}; Sigma). Proteinase K (final concentration 100 µg ml^{-1}; Roche Diagnostics) and buffer AL were then added, and the sample was incubated at 55°C overnight. To each sample, 0.5 volume 99% molecular biology grade ethanol (Sigma) was then added, and the DNeasy™ protocol was then followed according to the manufacturer's instructions starting from Step 4. The DNA was quantified using Quant-iT PicoGreen (Molecular Probes).

RNA was extracted from the other half of the polycarbonate filter using the RNAeasy™ protocol (Qiagen) with some modifications. The filter was placed in a RTL buffer with 0.1 mm beads. The samples were homogenised using a Ribolyser (Hybaid) on setting 4.0 for 30 s followed by incubation on ice for 1.5 min, then both steps were repeated. The RNeasy™ kit's protocol was then followed from Step 4 in the product information sheet with subsequent DNase (Sigma) digestion. RNA was quantified using the Quant-iT RiboGreen (Molecular Probes) and converted to cDNA using SuperScript™ III First-Strand Synthesis SuperMix and random hexamers as primers with subsequent DNase treatment (Invitrogen). To rule out DNA contamination, 5 samples without reverse transcriptase were also included. The samples were again quantified before PCR amplification.

PCR amplification of 16S rRNA gene and T-RFLP analysis

The bacterial 16S rRNA gene was amplified from the genomic DNA (gDNA) and transcribed cDNA using the primers 27Fm-6-FAM (5′-AGR GTT TGA TCC TGG CTC AG-3′) and 1391R-6-Hex (5′-GAC GGG CGG TGW GTR CA-3′) (Applied Biosystems). Two 50 µl PCR reactions were performed for each gDNA and cDNA sample separately. Each reaction contained 0.09 mM of each fluorescent primer, 0.01 mM of each primer with no fluorophore, 20 ng of gDNA or 2 ng cDNA, 1 U iProof™ DNA polymerase (Bio-Rad), 200 µM of each dNTP and 1.5 mM $MgCl_2$. PCR cycling conditions were as follows: initial denaturing at 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 45 s, annealing at 50°C for 60 s and extension at 72°C for 1 min, with a final extension of 72°C for 7 min in an Applied Biosystems 2730 PCR Machine (Applied Biosystems). The 16S rRNA gene band was visualized by gel electrophoresis on a 1% agarose gel. Samples with a correctly sized band of ~1600 base pairs were column purified following the spin column protocol (Zymogen) and quantified using Quant-iT PicoGreen kit (Molecular Probes). PCR amplicons (400 ng) were digested using 10 U of *Alu*I according to the manufacturer's instructions (Fermentas). Following restriction enzyme digestion, the digested sample was quantified, and 10 ng $µl^{-1}$ of the digested PCR amplicon was sent for analysis on the ABI prism 3730 capillary sequencer run under GeneScan mode with the LIZ 500 internal standard (Applied Biosystems; Allan Wilson Centre, Palmerston North, NZ).

Bacterial community analyses

Since each T-RFLP peak may be derived from >1 species, the identification of restriction fragment lengths (RFLs) and their abundance was interpreted with caution. Thus, RFLs were tentatively identified via comparison with a clone library. The 16S rRNA

genes from the gDNA and cDNA amplicons used for T-RFLP were cloned using TOPO TA per the manufacturer's instructions (Invitrogen). Individual clones were amplified using M13F and M13R primers (final concentration 0.1 mM) using Platinum® Blue Supermix following instructions in the TOPA TA manual. Clones containing the correct size insert were detected by gel electrophoresis and column purified (Zymogen). A total of 80 M13 amplicons were digested with *Alu*I as described above, and 28 unique RFLP patterns were observed. These unique RFLP patterns were analysed by T-RFLP analysis as described above. The unique M13 PCR products were sequenced using an ABI Prism 3100 genetic analyzer (Macrogen). Chimera sequences were eliminated using Chimera-Check (Cole et al. 2003) and Pintail (Ashelford et al. 2005). The non-chimeric sequences were submitted to the public EMBL-EBI database (www.ebi.ac.uk/) with accession numbers HG941671 to HG941698.

The raw T-RFLP data were filtered and binned using a pipeline method modified from the algorithm described by Abdo et al. (2006). The standard deviation of baseline signals within each of the T-RFLP profiles was determined, and peaks whose heights were <3 standard deviations from the baseline signals were considered noise. Absolute peak areas were transformed into percentage values to normalise the total fluorescence variations among samples. The raw electropherograms of the DNA- and RNA-derived RFL profiles from the top, middle and bottom of the sea-ice core are shown in Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a073p123_supp.pdf and were tentatively identified from the best BLAST hits of the clone libraries (see Table S1 in the Supplement).

We investigated the effect of 2 fixed factors — sea-ice zone (3 levels: top, middle and bottom) and method (2 levels: DNA versus RNA) — on the bacterial community profiles using a permutational multivariate analysis of variance (PERMANOVA) and subsequent pairwise analyses (Anderson 2001, McArdle & Anderson 2001) on the RFL data. Analyses used Bray-Curtis similarity matrices and a $\log(x + 1)$ transformation. Significant patterns identified by the PERMANOVA were visualised using a canonical analyses of principal coordinates (Anderson & Willis 2003). Note that we also investigated the effects of a third factor (day of ice core collection) to account for possible temporal sampling biases and found that there was no change in the DNA- or RNA-derived profiles over the 11 d time frame.

Enzyme assays of bacterial activity

Sea-ice samples were collected directly from the ice core without thawing by scraping into a 25 ml vial and were stored immediately at −20°C. When ready for processing, the samples were thawed and put through a 20 µm mesh to remove any algal material. The activity of enzymes that hydrolyse lipid, chitin, cellulose and proteins were investigated. The following synthetic substrates (final concentration: 39 µM) were used to test for bacterial enzyme activity in each thawed sample: lipase, 4-Methylumbelliferyl oleate; chitinase, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide; β-glucosidase, 4-Methylumbelliferyl β-D-glucopyranoside; phosphatase, 4-Methylumbelliferyl phosphate; and protease, L-Leucine-7-amido-4-methylcoumarin hydrochloride. Each sample was run in triplicate, and the following positive controls were run concurrently with the samples: lipase from wheat germ, chitinase from *Streptomyces griseus*, β-glucosidase from almonds, alkaline phosphatase and Proteinase K (Sigma), following the protocol by Maas et al. (2013). Samples were incubated in a fluorescent plate reader (Turner BioSystems) at 10°C for 6 h, and the fluorescence was measured (excitation 365 nm and emission 410 to 460 nm) every 5 min. The maximum uptake rate was calculated for each sample and for each enzyme using the Michaelis-Menten equation in pmoles fluorescence produced per minute per bacterial cell.

Environmental parameters

For chl *a*, 100 ml of the melted sea-ice sample was filtered onto a 47 mm GF/F filter and extracted in 10 ml of methanol over 12 h in the dark at 4°C. The extracted chl *a* was subsequently measured on a fluorometer (10AU Turner Designs) using the acidification protocol of Evans et al. (1987). Sea ice was collected directly from the sea-ice core in sterile tubes and stored at −20°C until analysis for nutrient concentrations. Nutrient samples were analysed for nitrate, nitrite, phosphate, ammonia and silicate using flow injection analysis on a Skalar San++ Automated Ion Analyser (Skalar). Samples for bacterial abundance were fixed in gluteraldehyde (0.05% v/v of final concentration) and frozen at −20°C until processed. Total bacterial cell counts were measured using Hoescht dye (Sigma-Aldrich) and a Becton Dickinson LSRII flow cytometer (BD biosciences) and were calibrated using Becton Dickinson TruCount beads (sensu Martin et al. 2011). ANOVA using the

general linear model was performed on normalised bacterial numbers between the top, middle and bottom of the ice core.

Community-environment relationships

Mantel-like RELATE tests were used to determine correlations among community composition, enzymatic activity and environmental drivers. Relationships between environmental parameters and enzyme activity were calculated using regression analysis in SigmaPlot v10. To construct an explanatory model for bacterial community structure, we used a distance-based multivariate multiple regression technique (McArdle & Anderson 2001) on those RFLs driving group separation in multivariate space. Environmental predictors were investigated for co-linearity, but none of the relations exceeded a Pearson's correlation of 0.75, and therefore, all of these predictors were included in the model selection stage. All predictors were normalised and fitted conditionally in a step-wise manner, with tests based on 9999 permutations of the residuals under a reduced model (Anderson 2001). To balance model performance with parsimony, model selection was based on Akaike's information criterion (Akaike 1973) with a second-order bias correction applied to account for the relatively low number of response observations relative to the number of predictor variables (Hurvich & Tsai 1989, Burnham & Anderson 2004). All analyses were completed using Primer v6 (Clarke & Gorley 2006) and PERMANOVA+ (Anderson et al. 2008).

RESULTS

Zonation patterns of the SIBCO

The sea ice at Granite Harbour was 3.1 m thick with a 10 cm cover of snow. Despite 2 glaciers flowing into the Harbour, the salinity of the water immediately under the ice was 32. Temperature up through the sea ice decreased from −1.8°C to −4.4°C within the ice core and was −6°C at the top of the core. To test for PCR bias, 1 environmental sample (Granite Harbour bottom ice zone) was analysed 6 times using the T-RFLP technique. No differences were found among the replicates (data not shown).

The PERMANOVA showed that there was a significant effect of sea-ice zone (pseudo-F = 12.73; p-*perm* = 0.0001) and method (pseudo-F = 14.29; p-*perm* = 0.0001) on bacterial community composition. The bacterial DNA- and RNA-derived profiles were distinct from each other within the bottom, middle and top of the ice core (pairwise comparisons, $p < 0.001$). When investigating the DNA- or RNA-derived profiles separately, the bacterial community compositions were distinct between zones (pairwise comparisons, $p < 0.001$; Fig. 1).

The raw RFL profiles showed many RFLs that were present in both the DNA- and RNA-derived communities throughout the sea ice core (Fig. S1 in the Supplement). Several RFLs were identified as driving these differences in community profiles (Fig. 1, Table 1) and were identified from the best BLAST matches in searches of the clone libraries. The bottom of the ice core was characterised by the RFLs 132r and 146r in both the DNA- and RNA-derived communities (Fig. 1). These RFLs were tentatively described as *Arthrobacter* sp., *Erythrobacter* sp., *Psychrobacter glacialis, Paracoccus* sp. and *Planococcus psychrotoleratus*. Additional RFLs characterised only the bottom ice DNA-derived community (Fig. 1a) and were tentatively described as *Brevundimonas mediterranea*, *Flavobacteria degerlachei*, *Polarobacter dokdonensis* and *Williamsia serinedens* (Table 1). RFLs that drove the separation in the bottom ice active community were implied from the clone library as *Octadecabacter antarcticus, Parococcus* sp. and *Rhodococcus* sp. The bacteria that characterized the middle DNA-derived community were *Glaciecola pallidula*, *Micrococcus* sp., *Parococcus* sp. and *P. glacialis,* while the RNA-derived middle ice community was characterised by *P. dokdonensis*. *Erythrobacter* sp., *Octadecabacter antarcticus, Pelagiocola litoralis, P. psychrotoleratus, Psychroflexus torquis, Rhodococcus* sp. and an uncultured *Sphingobacteria* were common to both the DNA- and RNA-derived mid zone communities. The DNA-derived community at the top of the sea-ice core was characterised by *Arthrobacter* sp. and *P. psychrotoleratus*, whereas the RNA-derived community was characterised by *Erythrobacter* sp., *P. dokdonensis*, *Bacillus* sp., *G. pallidula*, *P. glacialis* and *Psychroflexus torquis*.

Nutrient concentration and enzyme activity by zone

Bacterial abundance ranged from 6.5×10^3 to 1.4×10^5 cells ml^{-1} of melted sample. There was no change in bacterial numbers through the sea-ice core (ANOVA $F_{2,59} = 0.37$, $p = 0.692$), nor was there a rela-

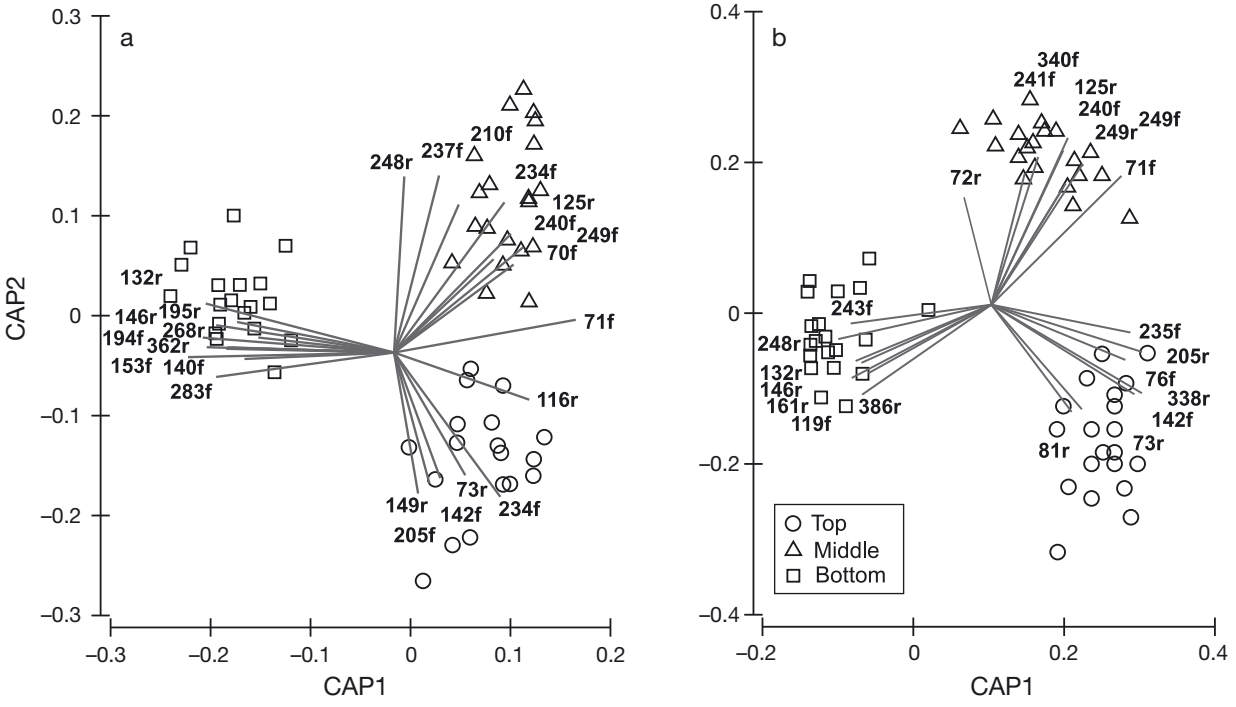

Fig. 1. Canonical analysis of principal coordinates of bacterial communities in 3 different zones (top, middle and bottom) of Antarctic sea ice. Bacterial communities were identified using T-RFLP profiles from (a) the rRNA gene and (b) rRNA. Overlaid onto the canonical analysis are vectors of the subset of restriction fragment lengths (RFLs) identified by Pearson's correlation, indicating the association of RFLs with particular sites. The directions of the vector lines indicate the relationship of each RFL to the site groupings in multivariate space, with the length of each vector proportional to the strength of the variance explained

Table 1. Restriction fragment lengths (RFLs) identified by clone library analysis most responsible for driving separation between ice-core zones at Granite Harbour. RFLs were defined by Pearson's correlation >0.6 in the DNA (■), RNA (■) or both (⊡) communities. White indicate an absence of these bacterial species

Identity and accession number	Bottom	Middle	Top
Arthrobacter sp. (DQ341426)	⊡		■
Bacillus sp. (AF414443)			⊡
Brevundimonas mediterranea (AJ244706)	■		
Erythrobacter sp. (EF512713)	⊡	⊡	■
Flavobacterium degerlachei (AJ557886)	■		
Glaciecola pallidula (FR746107)		■	⊡
Micrococcus sp. (EU394442)		■	
Octadecabacter antarcticus (FJ998358)	■	⊡	
Paracoccus sp. (AM275338)	⊡	■	
Pelagiocola litoralis (AY165584)		⊡	
Paracoccus sp. (AM275338)	■		
Planococcus psychrotoleratus (AY771711)	⊡	⊡	■
Polaribacter dokdonensis (DQ481463)	■	■	■
Psychrobacter glacialis (AJ539102)	⊡	■	⊡
Psychroflexus torquis (AY167320)		⊡	⊡
Rhodococcus sp. (FN397657)	■	⊡	
Uncultured *Sphingobacteria* bacterium (FN433448)		⊡	
Williamsia serinedens (FN673550)	■		

tionship with chl *a* concentration (linear regression $F_{1,59} = 0.15$, p = 0.702). The predominant nutrients in Antarctic sea ice were ammonia (8 to 13 µM) followed by nitrate (1 to 5 µM). Nitrate concentrations were highest at the bottom of the ice core, along with silicate and phosphate, whereas ammonia was highest within the ice core (Fig. 2a). At the bottom of the ice core over the 11 d time frame, nitrate and phosphate increased, whereas all the other nutrients decreased (see Fig. S2 in the Supplement). Chl *a* concentrations were low within the sea-ice core, between 0.0 and 0.4 mg m^{-2}, but increased over the 11 d period at the bottom of the ice core from 4.01 to 49 mg m^{-2} (Fig. S2). The PH:PR ratios were 6.3 ± 0.7 (mean ± SE) in the bottom zone, 7.0 ± 0.9 in the middle and 5.8 ± 0.5 in the top zone. The PR:β-Glu ratio decreased up through the ice core, where the highest nutrient quality of 6.7 ± 0.9 was at the bottom,

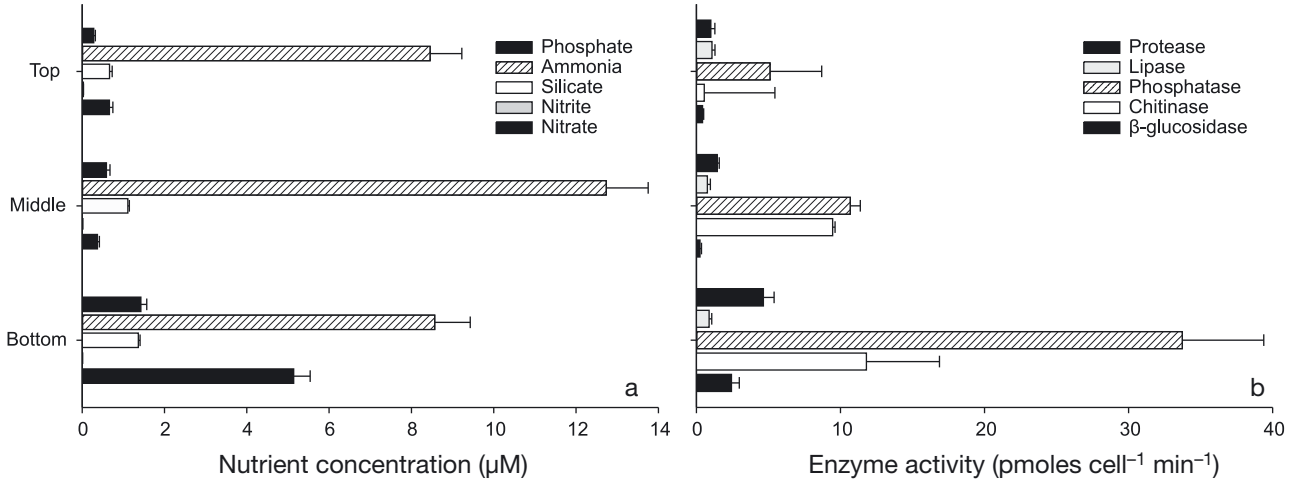

Fig. 2. (a) Nutrient concentrations and (b) enzymatic activity at the top, middle and bottom of the sea-ice core. Data are the mean of all 20 cores ± SE

Table 2. Distance-based permutational linear model relating rRNA-based bacterial communities to nutrient availability and metabolic function. Model selection was based on Akaike's information criterion with a second-order bias correction applied (AICc), with the total variation explained (r^2) by each best-fit model shown (% total). Analyses based on 9999 random permutations of the raw data

Predictor	AICc	Pseudo-*F*	p-value	% variability	% total
[Chlorophyll *a*]	180.21	3.8799	0.0034	14.99	
[Phosphate]	180.16	2.4791	0.0369	8.97	23.96
Protease activity	181.21	4.403	<0.001	11.8	11.8

the ratio was 3.7 ± 0.8 in the middle, and the lowest ratio of 1.0 ± 0.1 occurred at the top of the ice core. The total N (NH_4^+, NO_3^- and NO_2^-) to P (PO_4^{3-}) ratio increased up through the ice core from 9:1 at the bottom, 22:1 in the middle and 32:1 at the top of the ice core. The rate of bacterial enzymatic activity was divided by total bacterial cell counts to estimate the activity per bacterial cell. Bacterial activity generally decreased up through the sea ice core, apart from lipase activity, which was higher at the top of the core (Fig. 2b).

Environmental factors and activity driving zonation patterns

Bacterial enzymatic activities were significantly correlated with environmental factors (r = 0.373, p = 0.030) and bacterial community composition (r = 0.256, p = 0.011). Chl *a* and phosphate formed the best-fit model for explaining spatial variations in RNA-derived bacterial community composition across sea-ice zones, together accounting for 24% of the total variation. In contrast, when modelling spatial variations in RNA-derived community metabolic function, protease activity alone formed the best-fit model, accounting for 11.8% of the variation (Table 2). The chl *a* concentration in the sea ice was positively associated with protease activity (linear regression $F_{1,59} = 7.78$, p = 0.0069).

DISCUSSION

Bacteria play a vital role in global nutrient cycles (Azam & Worden 2004), but understanding what controls their distribution and functional roles is challenging. While previous studies have examined microbial loop dynamics (Martin et al. 2011), bacterial community diversity (Martin et al. 2009, Koh et al. 2010, Maas et al. 2012) and enzymatic function (Bergamasco et al. 2003, Celussi et al. 2010) in Antarctic sea ice, our study is the first to complete a comprehensive simultaneous assessment of both changes in community composition and metabolic function in relation to changing environmental conditions. Bacterial communities exhibited distinct patterns of zonation down through the sea ice, with results depending heavily on the target molecule (i.e. DNA or RNA). Previous research has shown similar findings for variations in bacterial community structure depending on whether rDNA or rRNA was extracted (Nogales et al. 2001, Moeseneder et al. 2005, Gentile et al. 2006). While there are biases inherent to both extraction techniques, we believe

these to be minimal as the raw electropherograms showed similar profiles from both the DNA- and RNA-derived communities, and therefore, a similar bacterial community was extracted using both methods.

The patterns of zonation identified are likely driven, in part, by the parallel changes in nutrient and environmental conditions, where chl *a*, nitrate, phosphate and silicate concentrations decreased toward the top of the ice core, and ammonium was lowest at the bottom of the ice core. Temperature decreases with increased proximity to the ice-air interface, whereas irradiance and salinity increase (Martin et al. 2011). The harsh gradients of the environment up through the sea-ice core may drive changes in community composition through selection and adaptation to the environment. Bacterial communities at the top of the ice core may be better adapted to colder temperatures and lower DOM and nutrient availability. These shifts were seen in both the DNA- and RNA-derived communities, suggesting that a large proportion of the DNA-derived community was active to show these shifts. Blazewicz et al. (2013) mentions several limitations of rRNA as an indicator for microbial activity; however, the obvious shift in community structure shows that these communities are actively responding to their environment. Antarctic sea ice is dominated by psychrotolerant and psychrophilic bacteria, with a shift to more psychrophilic species closer to the surface (Brown & Bowman 2001). The characteristic species at Granite Harbour were predominantly psychrotrophic and psychrophilic bacteria, many of which have been previously described in Antarctic sea ice (Brown & Bowman 2001, Maas et al. 2012). When investigating both the DNA- and RNA-derived profiles, there was a significant shift in the bacterial community up through the sea-ice core. Temperature decreased significantly up through the ice core, which may suggest the selection of psychrophilic bacteria that are more adapted to a colder environment. This was also suggested when identifying the bacteria driving the changes between the different zones. Using the clone library tentatively, the bottom of the ice core was characterised by psychrotolerant bacteria such as *Psychrobacter glacialis*, *Arthrobacter* sp., *Flavobacterium degerlachei* and *Williamsia serinedens*, with a shift to more psychrophilic bacteria within the ice core, such as *Polaribacter dokodonensis* and *Psychroflexus torquis*.

There was no relationship between bacterial numbers and chl *a*, which traditionally has been referred to as an uncoupling of the microbial loop (Stewart & Fritsen 2004, Martin et al. 2009). Bacteria have a strong positive relationship with chl *a* when low-molecular-weight photosynthates are abundant (Ducklow et al. 2012), and newly synthesized exudates from active phytoplankton are incorporated into bacteria within hours (Morán et al. 2001). However, algal-bacterial uncoupling can occur in the presence of grazers, which produce high-molecular-weight algal-derived DOM that provides a substrate for bacteria. Under these conditions, bacteria have a 15 to 30 d lag in their response to available growth substrates (Billen 1990, Billen & Becquevort 1991, Martin et al. 2011), which leads to an apparent weakening of the microbial loop (Billen 1990). There was no shift in the DNA- or RNA-based profiles over the 11 d time period, suggesting that at the time of our sampling, the bacterial community was lagging behind the start of the sea-ice algal spring bloom. Top-down factors such as nanoflagellate grazing (del Giorgio et al. 1996) and bacterivorous protists and viruses (Suttle 2007) regulate bacterial populations, which was out of the scope of this study. However, this lack of a relationship between bacterial numbers and chl *a* may suggest that bacteria are less dependent on photosynthates and more dependent on high-molecular-weight DOM produced by grazing, detritus turnover and/or zooplankton excretion.

Bacteria respond to changing nutrient conditions by regulating the synthesis and activity of their enzymes, therefore indicating the nutritional status of a biological system (Sala et al. 2010). Our results indicate that the SIBCO utilises components of high-molecular-weight DOM by actively synthesizing enzymes in the order phosphatases > chitinases > proteases > β-glucosidase > lipases. In marine systems, these enzymes are usually in the order proteases > phosphatases > β-glucosidases > chitinases > lipases (Hoppe et al. 2002), as similarly found by Celussi et al. (2010) in Antarctic sea water. The relative activities of specific enzymes have been used to infer those compounds that are important to bacterial nutrition (Christian & Karl 1995, Sala et al. 2001). Phosphatase activity serves multiple ecological functions by increasing P pools during cellular P limitation and as a 'secondary' function by regenerating utilisable organic C and N sources (Hoppe & Ullrich 1999). High phosphatase activity indicates a low availability of phosphate, whereas protease activity indicates N deficiency; thus, the PH:PR ratio gives an indication of P versus N limitation (Sala et al. 2001). The PH:PR ratio was > 1 at all sites, suggesting that phosphatase activity was higher than protease activity and that the Antarctic sea ice at the time of

sampling was more P-limited than N-limited. The β-BG:PR ratios were < 1 at all sites, suggesting that protease activity was higher than β-glucosidase activity, an indication of N rather than C limitation. Proteins comprise the most utilisable fraction of the marine organic N pool and may suggest a preference of sea-ice bacteria for amino acids. Hedges et al. (2001) showed that marine plankton is about 50 to 60% protein, 20 to 25% carbohydrates and 20 to 30% lipids by weight; thus, the enzymatic activities of the SIBCO may simply reflect the macromolecule make-up of sea-ice DOM, targeting proteins. The β-BG:PR ratios in our study were highest at the bottom of the ice core, suggesting that sea-ice bacteria are more actively involved in the degradation of proteins over carbohydrates. However, at the top of the ice core, this becomes reversed and carbohydrates are the focus of assimilation.

DOM quality can have a strong effect on bacterial community composition where inorganic nutrient concentrations may also play a role in algal-bacterial coupling (Wheeler & Kirchman 1986, Currie 1990). In Antarctic sea ice, high concentrations of nitrate (300 µM) and phosphate (42 µM) are frequently recorded associated with dense, actively growing algae (Thomas & Dieckmann 2002). However, at Granite Harbour, the concentration of these compounds was 60- and 24-fold lower than those high values, respectively. The low concentrations of these nutrients in our sea-ice samples suggest both N and P limitation and thus a lower biomass of algae in comparison to the previous year (R. O. M. Cowie & K. G. Ryan pers. obs.). In oceans, nitrate and phosphate concentrations occur in a ratio of 16:1. Redfield proposed that this ratio was controlled by phytoplankton, which release N and P at a similar ratio (15:1 to 16:1) during remineralisation (Tyrrell 1999). Nitrate:phosphate (NO_3^-:PO_4^{3-}) ratios were 3.6:1 at the bottom of the ice core and lower through the ice core, deviating from the Redfield ratio. Any deviations from this ratio are thought to result from N fixation (higher ratio) or denitrification (lower ratio; Koeve & Kähler 2010). However, cyanobacteria are not present in Antarctic sea ice (Koh et al. 2012), and therefore, denitrification may have a greater role over N fixation. Klausmeier et al. (2004) predicted that a low N:P ratio could occur during exponential growth in bloom-forming phytoplankton and that a high N:P ratio occurs when resources are scarce and there is low primary production. The low N:P in our samples may suggest high primary production and the beginning of the spring bloom at the bottom of the sea ice. Also, over the 11 d time frame, chl *a*, phosphate and nitrate increased, indicating the beginnings of a bloom event. Nitrate concentration decreased up through the ice core, to be replaced by high ammonia concentrations within the ice core. Over the time period, as nitrate concentrations increased, ammonia concentrations decreased. Ammonia may become the dominant N source when nitrate concentrations are low. The total N (NH_4^+, NO_3^- and NO_2^-) to P (PO_4^{3-}) ratio increased up through the ice core, suggesting that the sea-ice microbial community moves from a high primary production to low primary production environment. Alternatively, the low concentrations of NO_3^- and PO_4^{3-} may suggest that Antarctic sea ice is multi-nutrient co-limited where both nutrients are reduced to levels too low for cellular uptake.

Chl *a* and phosphate concentrations accounted for 24% of the community variation between zones. The variation between bacterial communities explained by phosphate concentrations may suggest that instead of up-regulating enzyme activities to modify their environment, bacteria with phosphatase activities become more dominant in the community in response to P limitation. Similarly, Yu et al. (2009) found that bacteria isolated from Arctic sea ice could only assimilate certain nutrients and found a high number of lipase- and protease-producing bacteria. Phosphatase activity has been shown in many species of marine bacteria, primarily from the genera *Pseudomonas, Chromobacterium, Bacillus* and *Flavobacteria* (Martinez et al. 1996), and has also been found in psychrophilic *Arthrobacter* isolates (De Prada & Brenchley 1997) and *Psychroflexus torquis* (Bowman et al. 1998). While *Arthrobacter* was found in both fractions at the bottom of the ice core, it was found only in the DNA fraction at the top of the ice core, unlike *Bacillus* and *Psychroflexus torquis*, which were active at the top of the ice core, suggesting that these groups may have become more dominant at lower phosphate concentrations due to their phosphatase activity. Protease activity accounted for 11% of the community variation between sea-ice zones. Many bacteria, including *Psychrobacter*, *Arthrobacter* and *Plancococcus* but not *Paracoccus* or *Psychroflexus torquis*, have protease activity (Groudieva et al. 2004, Yu et al. 2009). Phosphatase and protease activity is used to assimilate phosphate and proteins respectively from DOM and were highest at the bottom of the sea-ice core. Here, RFLs that corresponded to *Plancococcus, Psychrobacter* and *Arthrobacter* characterised this community, and these groups may be dominant due to their ability to liberate P and N in response to co-nutrient limitation in sea ice. Furthermore, protease activity increased as chl *a* concentra-

tion increased, indicating that not only does the bacterial community shift in response to chl *a* concentration but also their metabolic activity responds to increased algal biomass.

In summary, our results show that the Antarctic SIBCO can contribute significantly to the microbial loop through hydrolysis of the major organic constituents in sea ice and that the bacterial community structure is influenced, to some degree, by nutrient availability and the composition of algal-derived substrates. Zonation in sea ice not only leads to distinct nutrient characteristics but also results in differences in the bacterial community composition and metabolic activity. Environmental NO_3^- and PO_4^{3-} concentrations were low due to high primary production and the beginnings of the spring bloom at the bottom of the ice core. In response, bacteria actively released phosphatases and selectively assimilated proteins over carbohydrates, increasing the N and P pool. Although there was no relationship between bacterial numbers and chl *a*, suggesting an uncoupling of the microbial loop, our results clearly show the loop is indeed active and that bacteria respond to chl *a* through changes in enzymatic activity and shifts in bacterial community composition.

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