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ORIGINAL PAPER

Changes in salinity and temperature drive marine bacterial communities' structure at Potter Cove, Antarctica

Edgardo A. Hernández[1,4] · José L. Lopez[3] · Anouk M.-T. Piquet[2] · Walter P. Mac Cormack[1,4] · Anita G. J. Buma[2]

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Abstract

Coastal areas of the West Antarctic Peninsula (WAP) constitute a rich and biodiverse marine zone. Despite these ecosystems being supported by the microorganism's activity, the structure of microbial communities is insufficiently studied. As WAP is the area most affected by global warming worldwide, the increased glacier melting caused by the global warming and the consequent increase of the water runoff could be deeply affecting these microbial communities. To advance knowledge about the structure of microbial communities and its response to the environmental factors, a full-year study of marine bacterioplankton was conducted at Potter Cove, Antarctica. Multivariate analysis based on denaturing gradient gel electrophoresis (DGGE) and environmental data revealed a seasonal pattern in the structure of the bacterioplankton community, with spring–summer clustering separately from autumn–winter samples. Salinity, temperature and particulated matter were the main environmental driving forces. Based on the seasonal patterns, five bacterial clone libraries were performed from three sampling sites (E1, inner cove; E2, outer cove; and E3, mouth of a creek). Phylogenetic analysis of libraries generated 301 operational taxonomic units (OTUs), revealing the enormous richness and high diversity of these communities. Proteobacteria (68%), Bacteroidetes (20%) and Actinobacteria (8%) were the most represented phyla. During summer, bacterial community from E1 resembled that observed in E3, whereas during winter it resembled the E2 community. Results evidenced the influence of glacial meltwater input and showed the high variability of the bacterioplankton from inner cove. This study contributes to the better understanding of the structure of the Potter Cove marine ecosystems and could be reflecting the behavior of other similar ecosystems from WAP.

Keywords Bacterioplankton · Salinity · Temperature · Clone libraries · Potter Cove · Antarctica

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✉ José L. Lopez
jlopez@ffyb.uba.ar

[1] Departamento de Microbiología Ambiental, Instituto Antártico Argentino, 25 de Mayo 1143, B1650HMK San Martín, Provincia de Buenos Aires, Argentina

[2] Department of Ocean Ecosystems, Energy and Sustainability Research Institute, University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

[3] Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 4to piso, C1113AAD Buenos Aires, Argentina

[4] Facultad de Farmacia y Bioquímica, Instituto NANOBIOTEC UBA-CONICET, Universidad de Buenos Aires, Junín 956 6to piso, Buenos Aires, Argentina

Introduction

Despite the crucial role of the bacterioplankton communities in the Southern Ocean, their abundance, diversity and activity remain insufficiently studied, in particular with regard to changes along environmental gradients (Wilkins et al. 2013a, b; Luria et al. 2014).

Bacterioplankton growth is controlled by several factors, such as temperature (Ducklow 2000; Kirchman et al. 2009) and nutrients availability (Simon and Rosenstock 2007; Newton and McMahon 2011). Ultimately, the composition of the bacterioplankton community determines its growth rates, but studies about microbial ecology focused on Southern Ocean bacterioplankton diversity are just at a very early stage. However, high rates of bacterioplankton growth have been reported in Antarctic and sub-Antarctic waters, with average values similar to those of lower latitude marine areas (Kelley et al. 1999). Also, molecular-based studies using

PCR denaturing gradient gel electrophoresis (PCR-DGGE) (Abell and Bowman 2005; Murray and Grzymski 2007) and clone libraries (Brinkmeyer et al. 2003; Gentile et al. 2006) suggested that Antarctic bacterioplankton diversity is relatively high and comparable with other non-polar marine ecosystems.

Antarctic seas undergo the most extreme seasonal variations worldwide, due to changes in sea-ice cover, day length and solar irradiation. However, seasonal changes of bacterioplankton diversity in Southern Ocean have been scarcely analyzed to date. In this sense, Murray and Grzymski (2007) compared PCR-DGGE profiles of the 16S rRNA gene obtained from Anvers Island (West Antarctic Peninsula) during two annual cycles and observed drastic seasonal changes in community structure (expressed as Sorenson's index, C_S) which proved to be highly reproducible. In contrast, studies using pyrosequencing of the V6 region of the 16S rRNA gene to describe bacterial assemblages in the Arctic Ocean showed no significant difference between summer and winter communities (Kirchman et al. 2010). This remarkably stable bacterial community structure found in the Arctic using this molecular approach was not reported for the bacterioplankton of the Southern Ocean.

As the studies on temporal and spatial changes covering a full annual cycle are scarce for the coastal Antarctic marine bacterioplankton (Ghiglione and Murray 2012), in the present study we conducted the analysis of the bacterial community structure in the surface water from Potter Cove, West Antarctic Peninsula (WAP). To achieve this objective, culture-independent studies were essential, since only a small fraction of naturally occurring bacterial assemblages can be cultured using currently available methods (Eilers et al. 2000).

Our hypothesis was that bacterial communities from Potter Cove would be highly affected by the seasonal environmental changes such as the summer freshwater runoff, glacier melting and calving, photic conditions and biogeochemistry.

To test our hypothesis, temporal and spatial variability of bacterial community was investigated using DGGE obtained from three sampling sites located in a salinity gradient. Diversity, dominance and richness indexes were calculated and related to relevant environmental parameters using multivariate analysis tools. Also, these indexes were calculated from the OTUs obtained in five clone libraries and beta diversity analyzed using Venn diagrams. Results confirmed our hypothesis about dependence of the bacterial community structure on the seasonal environmental changes present in Potter Cove.

Materials and methods

Site description and sampling

Sampling was carried out in Potter Cove (62°14′S, 58°40′W), 25 de Mayo/King George Island, Antarctica. The surface waters of this cove exhibit marked seasonal changes, with cyclic processes of freezing and thawing of sea ice. Also, a huge input of glacial melt water during spring and summer significantly modifies its physicochemical characteristics. Potter Cove is divided into an outer and an inner basin, separated by a 30-m-depth transversal sill. Due to these topological characteristics, the main creek drains freshwater into the inner part of the cove, where significant surface currents circulate clockwise (Curtosi et al. 2007). The outer part of the cove is less affected by these factors and exhibits similar characteristics to those of the open waters of the adjacent Admiralty Bay. Based on these features, three sampling sites were selected: station 1 (E1; 62°13.935′S, 58°39.990′W) located in the inner zone, station 2 (E2, 62°14.011′S, 58°41.443′W) in the outer zone and station 3 (E3; 62°14.064′S, 58° 39.402′W) at the mouth of the Potter Creek (Fig. 1). From spring to summer, the Potter creek is the main source of freshwater coming from precipitations and glacier-ice melting. The sampling effort process covered a 15-month period, from December 2007 to February 2009. During spring and summer, when the cove was free of ice, samples were taken every 15 days at each sampling station. During autumn and winter, sampling was performed monthly at E1 and E2. Station E3 was not sampled during this period because the flow of freshwater from Potter creek ceased and the shallow marine waters close to the mouth of the creek were completely frozen. A total of 46 samples were obtained during the annual sampling period. Surface seawater samples were taken using Niskin bottles (10 L) manipulated from Zodiac boats. Niskin bottles had been previously washed with 1 N HCl and further gently rinsed with sterile distilled water. From each water sample, 8 L was successively filtered onto 3- and 0.22-µm cellulose acetate membranes (Millipore, USA). Filters were kept at − 80 °C for further DNA extraction in the laboratory. One L was used for pigment analysis and 1 L for estimating suspended particulate matter (SPM), both fractions previously filtered on Whatman GF/F filters (Whatman International Ltd., Maidstone, UK).

Measurement of environmental parameters

Temperature and salinity profiles were obtained in the sampling sites using a MicroCTD (Falmouth Scientific

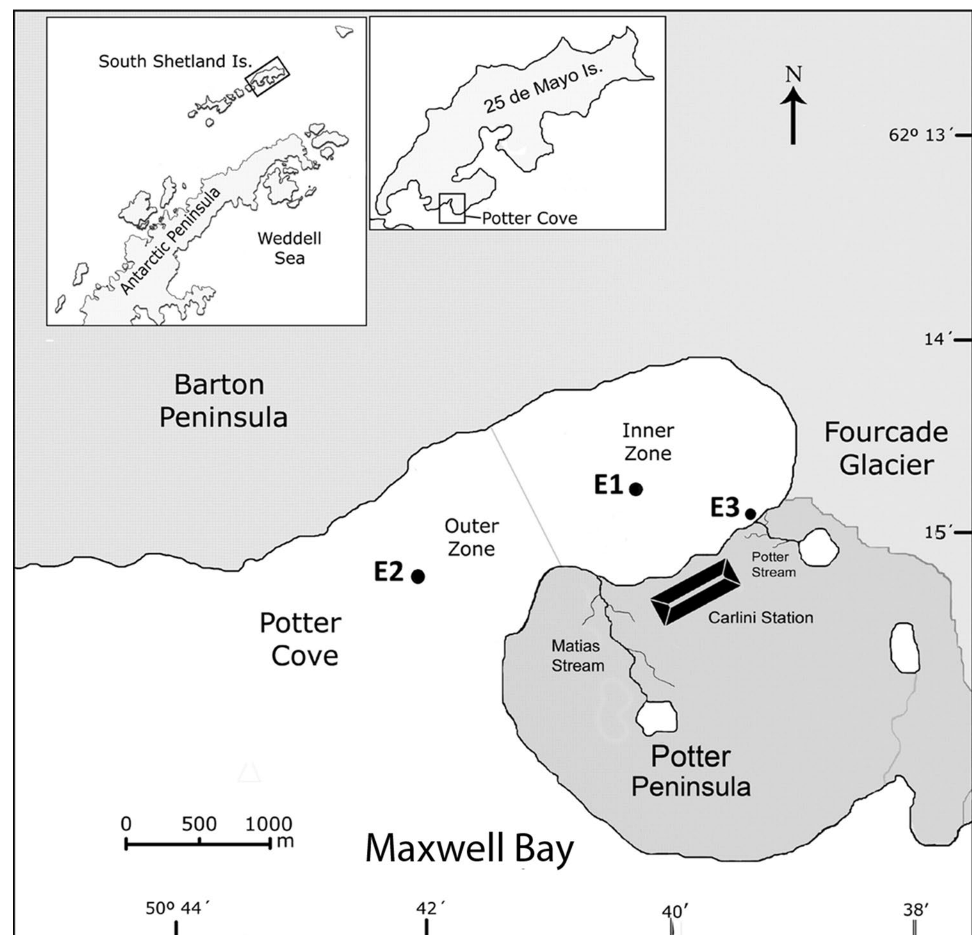

Fig. 1 Sampling sites within the Potter Cove, 25 de Mayo (King George) Island and location of the cove in the Antarctic Peninsula

Inc. FSI MCTD-3). Total suspended particulate matter (SPM) was gravimetrically estimated from 1 L of water filtered on precombusted Whatman GF/F filters. After filtration, filters were rinsed twice with distilled water to remove salts, then dried for 24 h at 60 °C and weighed (W_{SPM}). Total SPM was obtained by subtracting the weight of precombusted filter from the weight of the dried SPM filter. Finally, the difference between weights of the SPM filter before and after combustion for 5 h at 500 °C provided the organic matter (OM) value, while the remainder material represented the inorganic matter (IM). Chlorophyll-a (Chl-a) concentration was spectrophotometrically determined according to Strickland and Parsons (1972) using a UV–VIS 139 spectrophotometer (Hitachi, Japan). Detailed information on environmental sampling as well as analytical procedures is given in Hernández et al. (2015).

Fingerprint analysis of bacterioplankton community

Genomic DNA (gDNA) was extracted from a fraction of approximately 2 cm^2 of the 0.22-µm membrane filters using the Ultra Clean soil DNA isolation kit (MoBIO, USA). For clone libraries, a fragment of the 16S rRNA gene was amplified using the bacterial primer set 27f (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1492r (5′- TACGGYTAC CTTGTTACGACTT -3′) where Y = C or T. The reaction mixture (50 µL total volume) consisted of 1x PCR buffer, 2.5 mM $MgCl_2$, 2% DMSO, 0.2 mM dNTPs, 0.2 µM of each primer and 2 U of *Taq* DNA polymerase (Gold Taq, Eurogentec, Belgium).

Although we know that the DGGE analysis provides only a partial picture of the microbial assemblage present in an environment (Schiaffino et al. 2016), we applied this technique to make an initial screening of the 46 samples obtained from the annual cycle and to select samples for further clone library analysis. For DGGE, a PCR was performed from the genomic DNA (20 to 30 ng per reaction) to amplify a fragment of approximately 433 bp of the bacterial 16S rRNA gene using the 968-GCf and 1401r primer set. Primer sequences as well as reaction and cycling conditions can be found in Piquet et al. (2010a, b). The quality and concentration of the PCR products were checked by electrophoresis on 1% agarose gels in 1x TAE buffer and subsequently stained with ethidium bromide. Size and yield of PCR products were estimated by comparison with a DNA smart ladder (Eurogentec, Belgium).

Molecular fingerprints of the bacterioplanktonic community were generated by DGGE using the DGGE PhorU system (Ingeny, Goes, the Netherlands). Optimal band separation was obtained on a 6% polyacrylamide gel using a 40% to 60% urea–formamide DNA denaturing gradient. A 100% urea-formamide was defined according to Muyzer and Smalla (1998) as 7 M urea (Bio-Rad, Veenendaal, the Netherlands) and 40% deionized formamide (Sigma-Aldrich Chemie B.V.). For each sample, we loaded 200 ng PCR product supplemented with 1x loading buffer (0.05% w/v bromophenol blue, 40% sucrose, 0.1 M EDTA pH 8.0, 0.5% sodium lauryl sulfate). The DGGE was run for 16 h at 60 °C and 100 V in 0.5x TAE buffer. [TAE buffer is composed of 0.04 M Tris base, 0.02 M sodium acetate and 10 mM EDTA (pH 7.4).] A marker sample was added to each gel as a reference for subsequent band pattern analysis. The DGGE band profiles were digitalized and normalized using flanking marker bands with BioNumerics version 3.5 (Applied Maths NV, Belgium) as described in Piquet et al. (2008). Band patterns were transformed into both a presence/absence matrix and a relative abundance matrix, using BioNumerics. The relative abundance matrix was used to estimate the Shannon diversity (H), richness (R) and dominance (D) (Online resource 2). These indices were coupled to the environmental data (Chl-a, SPM, OM, IM, salinity and temperature) for multivariate analysis [principal components analysis (*PCA*), discriminant analysis (*DA*), multivariate analysis of variance (*MANOVA*)]. *MANOVA* was tested using *Wilks, Pillai, Lawley–Hotelling and Roy statistics*. Samples for different seasons were compared using a *Hotelling test* with *Bonferoni's corrected level* (InfoStat program, Di Rienzo et al. 2011). Detailed information about the mentioned environmental parameters can be observed in Hernández et al. (2015).

Construction of clone libraries

Five 16S rDNA clone libraries were constructed as described previously (Piquet et al. 2010a, b).

Based on the results from the cluster analysis of the DGGE pattern (obtained from presence/absence matrixes), we selected five samples as follows: 3 summer samples on January 12, 2009, corresponding to stations E1, E2 and E3, and 2 winter samples on July 31, 2008, corresponding to stations E1 and E2. The selected samples from winter and summer represented clearly different similarity clades obtained by Pearson's correlation from DGGE pattern analysis (see Fig. 2). During summer, the salinity gradient between sampling sites was the highest on January 12, 2009, with E3 showing the lowest reported value of 29.62 psu.

PCR products were cloned in the pGEM-T Easy vector system (Promega Benelux B.V.) and transformed into *Escherichia coli* strain TOP10 following the manufacturer's protocol. Two hundred colonies with positive insert were randomly selected and grown on LB broth. The plasmid was isolated using a HP GenElute Plasmid Miniprep kit (Sigma-Aldrich). Rarefaction curves indicating bacterial 16S rRNA richness were made by using Analytic Rarefaction (Holland 2003).

Clone libraries and phylogenetic analysis

For each sample, 200 white colonies were selected and amplified from the vector's T7-SP6 sites. Thirty ng of PCR product was cleaned using ExoSAP-IT (Affymetrix, Cleveland, USA) and used as template in the sequencing reaction performed with BigDye® 3.1 Terminator buffers (Applied Biosystems) and 0.2-mM T7 primer. Sequence products were cleaned using standard isopropanol precipitation and analyzed on an automated Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned using the online Clustal Omega-Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/services/web_clustalo/). Alignment was manually edited using BioEdit. Identity matrix was calculated from this alignment by using the accessory to BioEdit.

The 16S rDNA phylogenetic trees were constructed from the set of all the sequences obtained. These sequences were purified to remove chimeras using the online Bellerophon program (Huber et al. 2004). Subsequently, all the sequences (881) were grouped in OTUS with 97% identity using Mothur v.1.37.0 (Schloss et al. 2009). For each OTU, the representative sequence was obtained using the same program. The search for the first 5 nearest neighbors of each representative sequence of the OTUS was conducted with SILVA database release 128 (Quast et al. 2013). Redundant sequences were removed. The sequences were aligned using RDP aligner (Cole et al. 2009). Finally, the phylogenetic analysis was performed with one set of 1192 sequences (our sequences and those from SILVA) by means of the RAxML program available on the online site CYPRESS Science Gateway 3.3 V. (Miller et al. 2010) using default parameters. Venn diagrams were obtained using the online version of Bioinformatics.psb.ugent.be/webtools/venn.

Nucleotide sequence accession numbers

The sequences obtained in this study are available in the GenBank database under accession numbers: MK782596 a MK782750 (BacE1sum); MK796249 a MK796404 (BacE-1win); MK820176 a MK820365 (BacE2win); MK774826 a MK775011 (BacE2sum); MK820414 a MK820603 (BacE-3sum). Data correspond to unassembled sequences.

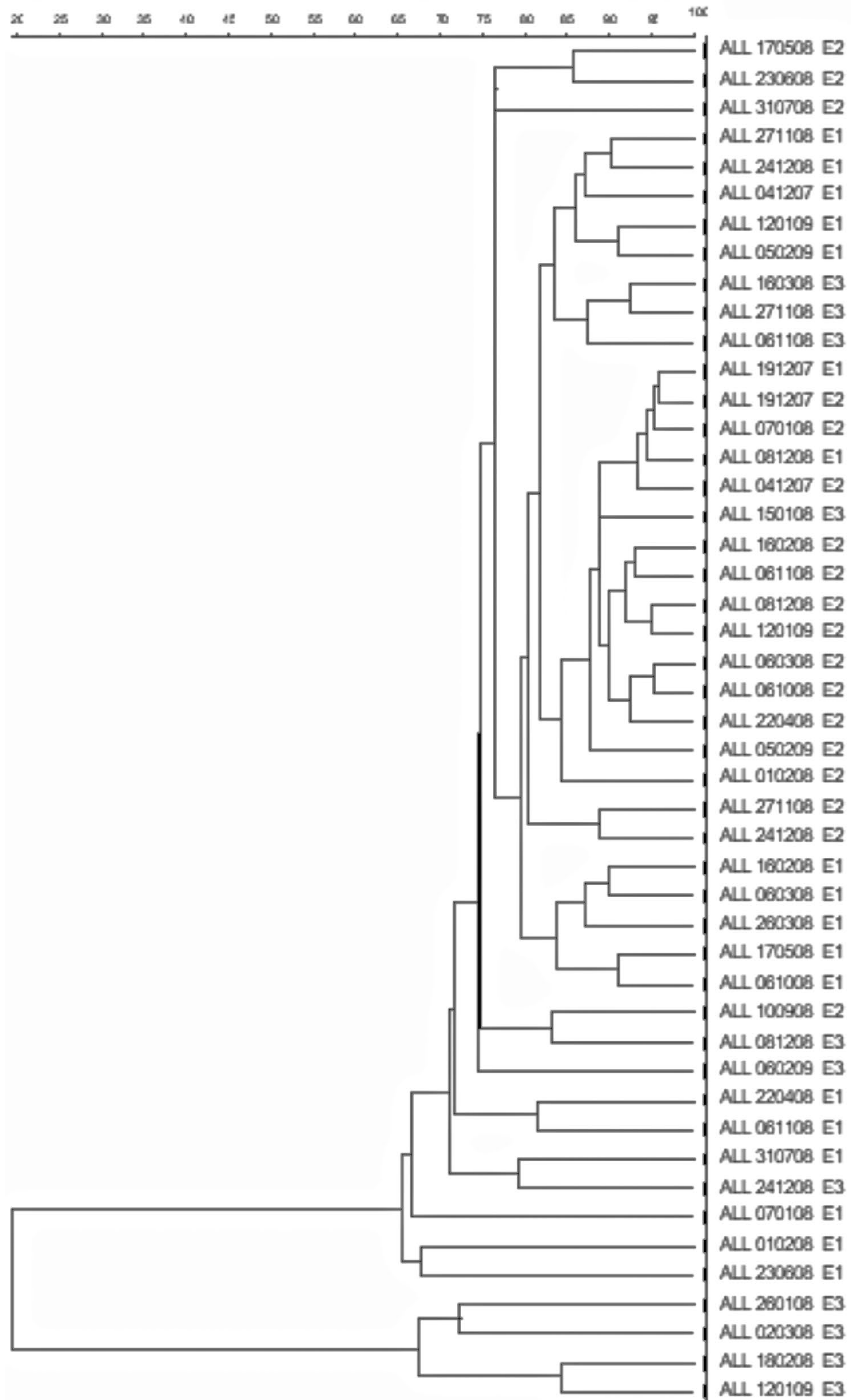

Fig. 2 Similarity dendrogram based on Pearson's correlations calculated from denaturing gradient gel electrophoresis (DGGE) analysis of samples from Potter Cove surface seawater. Samples which were selected for the clone libraries are indicated by the boxes

Results

Environmental parameters

Temporal changes in water temperature showed similar trends for the three sampling locations. Minimum values were − 1.69 °C, − 1.40 °C and − 0.29 °C for E1, E2, and E3, respectively. E3 temperatures tended to be higher than those of the other two stations during the periods when this station could be monitored. The highest temperatures were recorded in the inner cove, with 2.89 °C and 3.94 °C for E1 and E3, respectively, whereas the highest temperature at E2 (outer cove) was 1.98 °C.

Salinity showed very different temporal trends at the three sampling stations. Salinities measured at the inner cove (E1) and near the meltwater stream (E3) were variable and showed a clear decrease during summer, when E3 exhibited values as low as 29.62 psu (21/01/2009). In contrast, salinity values in E2 proved to be more stable, maintaining relatively high values even during summer (E2 summer average = 33.58 ± 0.63 psu). Chl-a showed similar patterns at E1 and E2, increasing during spring (when they reached maximum values close to 2.3 µg L^{-1}) and evidencing no major phytoplankton biomass increase during summer.

Finally, SPM showed higher values during summer, when freshwater runoff was maximal. Analysis of the SPM showed that it was mostly composed of inorganic matter, with annual average values of 81.4% for E1, 76.4% for E2 and 91.2% for E3 (results not shown). The inorganic fraction of the SPM was the main factor responsible for the observed seasonal changes. Additional figures showing the evolution of the environmental parameters during the whole sampling period can be found in Hernández et al. (2015).

Fingerprint analysis of bacterial communities

The DGGE analysis (performed with samples taken all along the year as described in the Materials and Methods section) revealed a heterogeneous community with spatial and temporal variability (Online resource 1). The number of bands per sample ranged from 17 (in some spring samples obtained from E2 and E3) to 42 (in a sample of E1 during winter) and showed an average of 28.95 (SD 6.35). Differences between sampling stations were evident. A clear decrease in richness was observed in the spring and summer samples at E1 and E2 compared with those observed in the autumn and winter samples (Online resource 2). The winter samples of E1 showed the highest richness (39.00, SD 4.24). Similar trends to those observed for richness were observed in diversity values. Diversity, estimated by the Shannon index, showed high values in all stations, with the lowest ones observed in a sample from E3 during spring (2.61) and a maximum (3.51) observed during winter at E1.

Multivariate analysis

The *PCA* evidenced that samples ordinated in the spaces, determining two different clusters, with the two main components explaining 85.6% of the variance (Online resource 3). The first component explained 61.7% of the variance and separated the autumn and winter samples (left) from the spring and summer ones (right). In the *PCA*, autumn and winter samples (E1 and E2) were explained by the variables salinity, diversity and richness, and winter samples at station E2 were closely related to high salinity values. In contrast, spring and summer samples were related to temperature, SPM, OM, Chl-a and bacterial dominance. E1 summer and E3 (summer and spring) samples were closely grouped and related with high temperatures, OM and IM. When the same variables considered for *PCA* were used for a *DA* (considering only seasons as grouping criteria), results based on the confidence ellipses confirmed the grouping spring and summer. Also, autumn samples showed only a low overlapping with summer spring groups. However, winter samples showed to be the most distinct group of samples (Online resource 3). These results support our criterion for the selection of samples to be cloned for the libraries analyses.

Multivariate analysis of variance (*MANOVA*) applied to compare samples obtained in the different seasons evidenced a high level of significance ($p < 0.001$). *MANOVA* showed no significant differences either between winter and autumn samples, or between summer and spring samples. However, samples from winter differed significantly from the spring–summer ($p < 0.05$) and samples from summer differed significantly from the autumn–winter samples, confirming the statistical significance of the clustering observed with the *PCA* and *DA*. With respect to spatial variability, only E2 showed significant differences with E3 ($p < 0.05$).

Finally, the robustness of the multivariate analyses, demonstrating significant differences between summer and winter communities, also strengthens our sample selection for the clone libraries (3 libraries from summer and two libraries from winter, as described in the Materials and Methods section).

Clone libraries analysis

General description of the bacterial community structure from Potter Cove

The 881 bacterial 16S RNAr nucleotide sequences obtained from the five microbial communities (E1 winter

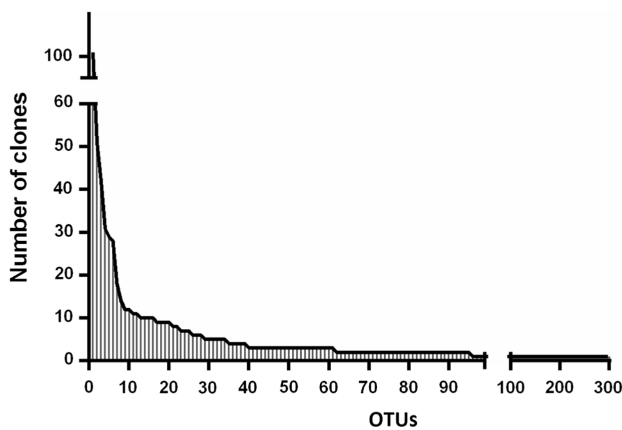

Fig. 3 Histogram showing number of sequences corresponding to each operational taxonomic unit (OTU). A high fraction of the OTUs (261 out of 301) were represented by only three or less sequences

Table 1 Diversity indices for each clone library from Potter Cove seawaters

Library	ACE (richness)	Shannon (diversity)	Simpson (diversity)	Berger–Parker (abundance)
E1-SUM	68.63	3.05	0.104	0.30
E1-WIN	498.63	3.84	0.033	0.12
E2-SUM	382.97	3.87	0.034	0.11
E2-WIN	590.26	4.31	0.012	0.07
E3	431.77	4.09	0.025	0.13

E1-SUM station 1 in summer, *E1-WIN* station 1 in winter, *E2-SUM* station 2 in summer, *E2-WIN* station 2 in winter, *E3* station 3 in summer, *ACE* abundance-based coverage estimator

and summer; E2 winter and summer and E3 summer) were analyzed using Mothur GUI software. (Average neighbor appeared to form the most robust OTUs.) When 0.2 and 0.1 genetic distances values were considered, 32 and 138 OTUs were obtained, respectively. Finally, we based our classification on a genetic distance of 0.03 that determined 301 OTUs, 68.4% of which (206/301) were represented by singletons, 11.3% by doubletons (34/301) and 7.3% (22/301) by three sequences. The presence of 87.0% (261/301) of the OTUs containing only 1–3 sequences evidenced the high richness and low dominance of the studied microbial communities. Only 34 out of 301 OTUs (11.3%) contained 5 or more sequences. Figure 3 shows the number of sequences corresponding to each OTU of the libraries. The rarefaction curve calculated from all the libraries showed that the sampling correctly represented the structure of the bacterial community (Online resource 4).

Representative sequences of each OTUS were used for further phylogenetic studies (see molecular phylogeny subsection).

As indicated by ACE, Shannon, Simpson and Berger–Parker indices, Potter Cove's bacterial communities are characterized by a high richness, high diversity and low abundance (Table 1).

Temporal dynamics of bacterial populations

When only temporal variations of bacterial communities were considered, it was observed that more than 50% of the multiple sequences OTUS were present in winter and summer. Also, the OTUs having the higher number of sequences were detected in both seasons, whereas those exclusive of one season were represented by 2–10 sequences. This result shows that a high fraction of the community ribotypes are stable members, present all along the year, whereas winter- or summer-exclusive OTUs are detected as minoritary members (Online resource 5). In this sense, when only singletons were analyzed, it was observed that 95 out of 206 were isolated in winter, whereas 111 were isolated in summer, showing no significant differences among them and evidencing that the high richness exhibited by the bacterial community of each season is mainly supported by the OTUs of lesser abundance. In this sense, the decrease in diversity observed during summer (see Shannon index in Table 1) could be related to the growth of a few OTUs that increase their abundance during this season. For example, OTU1 increased by almost 2.5 times the number of sequences in summer (71 sequences) compared with those observed in winter (30 sequences). While OTU1 is the most evident example, 9 out of 11 of the most represented OTUs (which comprised 309 sequences) increased their presence during summer, as OTU3 (from 15 to 27 sequences) or OTU 5 (from 4 to 25 sequences) among others (Online resource 5).

Spatial dynamics of bacterial populations

Spatial distribution of OTUs containing 2 or more sequences is shown in the Venn diagram of Online resource 6. The number of OTUs exclusive to one sampling site was 17 (exclusively from E1), 23 (exclusively from E2) and 12 (Exclusively from E3). In addition, 20 OTUs were shared by E1–E2, whereas 7 OTUS were shared by E1–E3 and by E2–E3. Finally, 9 OTUs were detected in the three sampling sites. It is important to emphasize that the four most abundant OTUs (comprising a total of 225 sequences) only contained clones isolated from Stations E1 and E2 (Online resource 6), providing evidence regarding the marine origin of these ribotypes that would enter from open sea and would be established in the interior of the cove. The OTU 5 only contained clones isolated from E3 and E2, with a major proportion of such clones coming from E3. This fact could be reflecting a terrigenous origin of OTU 5 (Online resource 6).

See phylogenetic analysis section for taxonomic information on OTUs 1–5.

The three studied stations presented a high proportion of singletons. In E2 singletons represented 51.4% (106/206), while in E1 and E3 20.0% (41/206) and 28.6% (59/206) of the all singletons were observed. Statistical analysis (*Chi square*) showed that the differences E1–E2 and E2–E3 were significant ($p < 0.05$), while the E1–E3 difference was not significant.

Phylogenetic analysis of bacterial populations and spatial–temporal dynamics

The phylogenetic analysis of the bacterial communities of Potter Cove was performed based on the OTUs grouping (3% level). For this purpose, the five nearest neighbors of each representative sequence were obtained using SILVA (after the elimination of the redundant sequences). The phylogenetic tree (Online resource 7) evidenced a high resolution of the main taxonomic branches (high values of bootstrap) and only a minor fraction of the sequences included in this analysis (28/301) did not group with sequences previously described. Twenty-five of such sequences represented singletons, and the remaining three represented OTUs with two or three sequences. Details of the taxonomic affiliation of the 301 OTUs from all libraries as a whole and discriminated by station and seasons are shown in Figs. 4 and 5 and Online resource 8.

When the sequences from the five libraries were grouped, we observed that phylum Proteobacteria was the dominant one, with Bacteroidetes and Actinobacteria comprising the major part of the remaining sequences (Fig. 4a). Alphaproteobacteria (57.0%) and Gammaproteobacteria (41.5%) represented 98.5% of the sequences ascribed to the phylum Proteobacteria (Fig. 4b).

In E1, Proteobacteria represented almost 90% of the sequences in summer and 84% in winter. These percentages were smaller at station E2 (70% in summer, 65% in winter and even lower at station E3 (37% in summer, Fig. 5a). Gammaproteobacteria was the most abundant class of Proteobacteria, mainly during summer at station E1 (78%, Fig. 5b). In winter at station E1 and in both seasons at station E2, proportions of this class were smaller and similar (49% in E1 winter, 56% and 50% in E2 winter and summer,

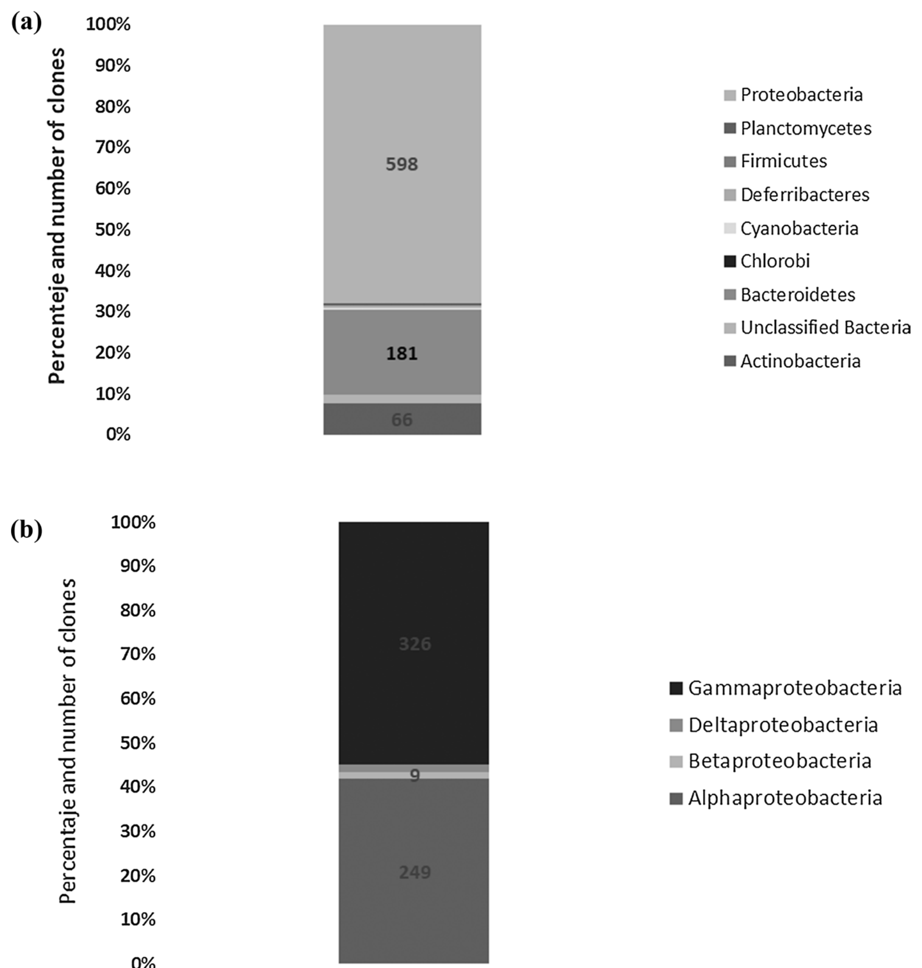

Fig. 4 Percentage of clones ascribed to the most representative operational taxonomic units (OTUs) of the five libraries taken as a whole. **a** Clones grouped by phyla. **b** Clones ascribed to the phylum Proteobacteria separated by classes

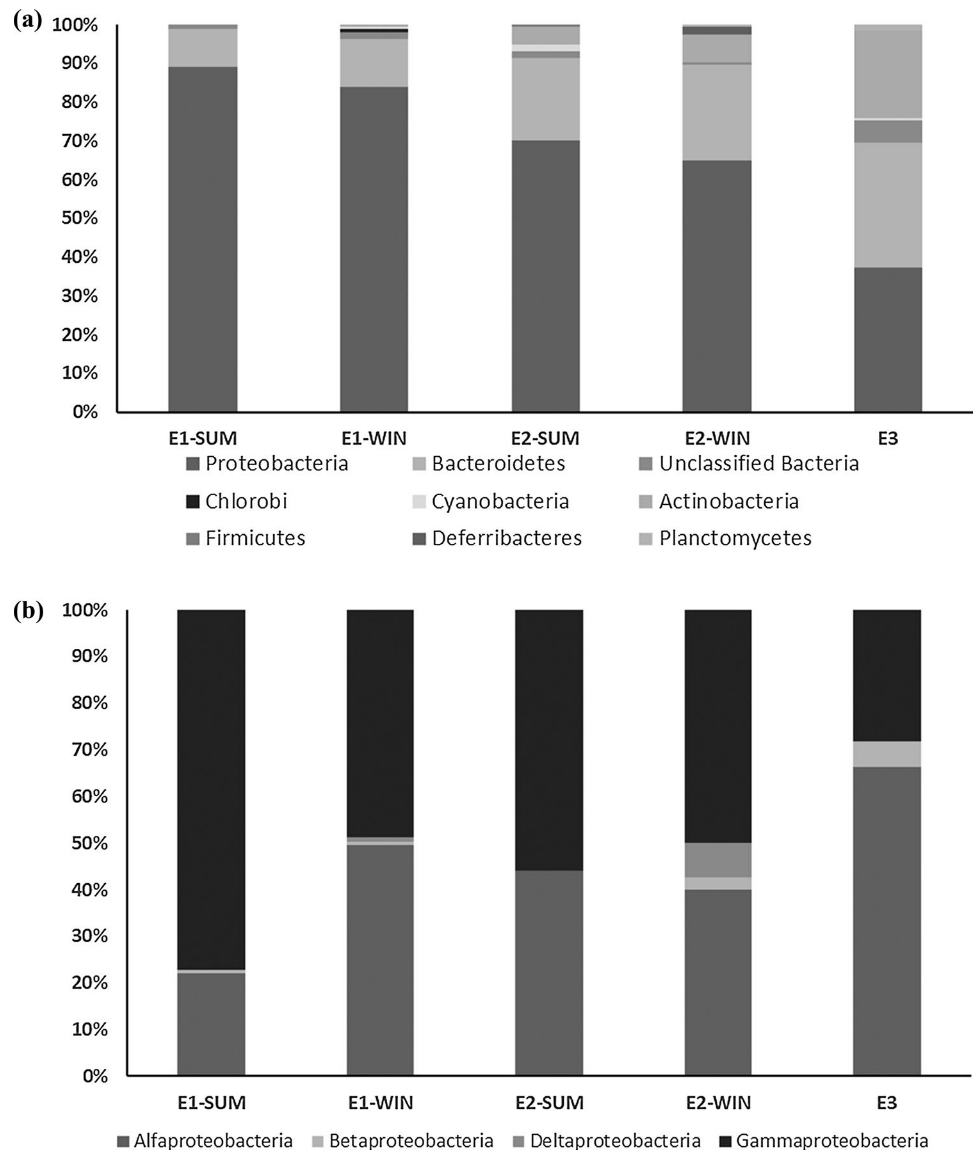

Fig. 5 Percentage of clones ascribed to the most representative operational taxonomic units (OTUs) for each of the five libraries. **a** Clones grouped by phyla. **b** Clones ascribed to the phylum Proteobacteria separated by classes. *E1-SUN* station 1 in summer; *E1-WIN* station 1 in winter; *E2-SUM* station 2 in summer, *E2-WIN* station 2 in winter, *E3* station 3 in summer

respectively). In the summer library of station E3, the class Alphaproteobacteria showed the highest percentage among Proteobacteria sequences (66%), whereas Gammaproteobacteria covered only 27.8% of the sequences, thus highlighting the dramatic effect of the freshwater runoff on the coastal marine bacterial community at Potter Cove.

Comparison of the clone libraries showed that from winter to summer at station E1, Gammaproteobacteria increased from 64 to 108 sequences, whereas Alphaproteobacteria (order Rhodobacterales) decreased from 65 to 31 sequences. In both cases, these differences were highly significant ($p < 0.001$). At station E2, by contrast, Gamma- and Alphaproteobacterias showed no significant differences between winter and summer. However, within the class of Alphaproteobacteria, the order Rhodobacterales increased from 20 (winter) to 37 (summer) sequences ($p < 0.001$) and Sphingomonadales from 3 (summer) to 13 (winter) sequences ($p < 0.001$). The taxa found at station E3 showed significant differences when compared with stations E1 and E2. Here, 23% of the sequences (43 sequences) were assigned to Actinobacteria. Finally, at station E3 neither sequences ascribed to the genera *Pelagibacter* or *Polaribacter*, both considered typical marine bacteria, were detected (Online resource 9)

The phylum Bacteroidetes was present in all clone's libraries and showed similar percentages when we compared summer with winter periods for E1 and E2, but the number of sequences present in E2 was nearly twice over those observed in E1. This phylum reached the highest percentage (32%) in summer at station E3 (Fig. 5a).

The spatial and temporal comparative analysis of the OTUs with two or more sequences was made using the Venn diagram. Although station 1 showed a significant number of exclusive OTUs (17 OTUs), most of them (11) were present all throughout the year (Fig. 6). Linking these results with

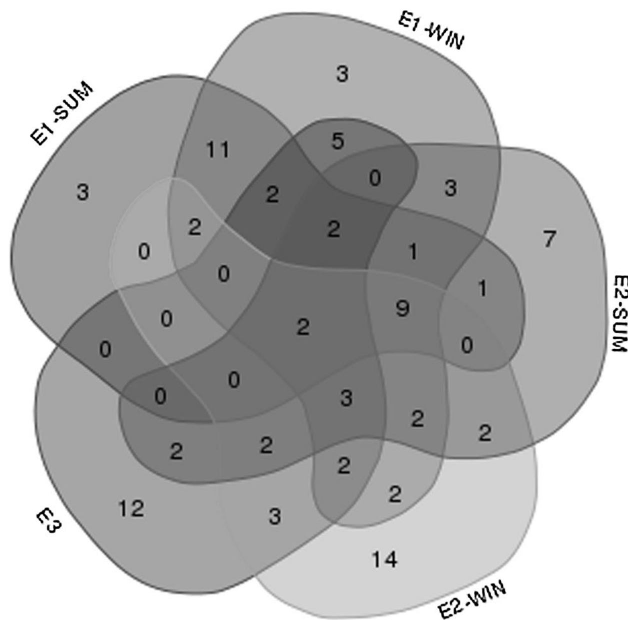

Fig. 6 Venn diagram of operational taxonomic units (OTUs) with 2 or more sequences obtained from the five clone libraries. *E1-SUN* station 1 in summer, *E1-WIN* station 1 in winter, *E2-SUM* station 2 in summer, *E2-WIN* station 2 in winter, *E3* station 3 in summer

those from the taxonomic study, it was made evident that in E1 the only 3 summer-exclusive OTUs were Polaribacter and 2 unclassified Gammaproteobacteria, whereas *Thalassospira*, *Alkanivorax* and Flavobacteriaceae were the three winter-exclusive OTUs (see Online resource 9). Both, E2 in both seasons, and E3 in summer, showed a high proportion of exclusive OTUs. In the case of the more oceanic station E2, only two OTUs [an Actinobacteria (*Euzebya*) and a Gammaproteobacteria (Alteromonadaceae)] resulted common to winter and summer, the remaining OTUs being exclusive of one of the seasons. In this station, exclusive OTUS were much more abundant in winter than in summer (14 in winter and 7 in summer). This observation reveals the high degree of seasonal variability of this bacterial community.

The 19 OTUs winter-exclusive and containing multiple sequences (Online resources 5 and 9) were assigned at least to class level. On the other hand, 24 out of 25 summer-exclusive OTUs also were assigned to class level. Complete information on the exclusive and shared OTUs by stations, the name of each OTU and its taxonomic assignment as well as the number of sequences included in each OTU is presented as Online resources 8 and 9.

In relation to the community members shared by both marine stations (E1 and E2) during winter and summer, nine multiple-sequence OTUs were detected (Fig. 6). It is interesting to highlight that this group contained nine OTUs with 8 or more sequences and included the four more abundant ones (OTUs 1–4, Online resource 9). These OTUs were affiliated to: Oceanospirillales (1 OTU, 101 sequences, *Balneatrix*); SAR 11 (1 OTU, 51 sequences, clade Surface 1 and 1 OTU, 8 sequences, clade surface); Rhodobactereaceae (1 OTU, 42 sequences, Candidatus *Planktomarina* DC5 80 3 lineage); Oceanospirillales (1 OTU, 31 sequences, ZD0405 uncultured, 2 OTUs, 18 + 12 sequences, SAR86); Alteromonadales (2 OTU, 12 + 10 sequences, SAR92). These nine OTUs comprised 285 out of 881 sequences.

The two OTUs that were shared by the 3 stations in the two seasons (comprising a total of 18 clones) were ascribed to *Halomonas* and *Marinobacter*, two genera included in the class Gammaproteobacteria.

Discussion

It is known that our planet is undergoing a dramatic climate change, and the WAP is perhaps the site where this phenomenon is occurring at its maximum expression (Meredith and King 2005; Gille 2008). In this region, where the diversity of macroorganisms is low, microorganisms play a crucial role on both the energy flux and biomass production, thus being decisive for the functioning and stability of the entire Antarctic ecosystem. This is especially true of the coastal ecosystems of the Antarctic Peninsula, whereby knowledge of the structure and dominant groups of microorganisms is vital for a better understanding of the changes caused by global warming and the concomitant input of freshwater derived from the increase in the average temperature of the environments.

With our hypothesis in mind (that bacterial communities from Potter Cove would be affected by the seasonal environmental changes), in this work, an annual sampling was carried out in three marine stations having different degrees of melting waters influence, in order to determine the main groups of bacteria presents and how they vary according to location and seasons.

Also, in the analysis of the results, we included our own previously reported (Hernández et al. 2015) physical and chemical variables (salinity, temperature, SPM) corresponding to the same sampling effort, in order to relate it with the observed changes in bacterial community structure.

The analysis of the environmental parameters from our study and its relationship with the bacterial community structure evidenced that during spring and summer Potter Cove undergoes a significant increase in the SPM. As was previously reported (Klöser et al. 1994), the main fraction of those SPM corresponds to inorganic matter. This increase is mainly originated from freshwater runoff, although contribution from direct melting and calving glacier front cannot be discarded. This freshwater input leads to a decrease in

salinity in the inner part of the cove, which mainly affected E3 and to a much lesser extent E1.

DGGE data evidenced that environmental changes occurring during spring and summer were accompanied by a decrease in bacterial diversity, caused by lower richness values and an increase in dominance of some bacterial groups. It is important to note that these observations are based on a technique that only allows for the retrieval of the more abundant or dominant OTUs. These seasonal changes in bacterial community structure based on DGGE analysis were also observed by Murray and Grzymski (2007) and Grzymski et al. (2012) who reported that Sorenson's index calculated from DGGE profiles suggested that bacterial community of marine Antarctic surface coastal waters is drastically different in winter and summer. A similar phenomenon had been previously mentioned by Murray et al. (1998).

As was stated in the results section, it is possible that the decrease in diversity evidenced by DGGE during spring–summer at Potter Cove could be determined by a faster growth of some bacterial groups taking advantage of the higher organic substrate levels provided by the phytoplankton, as well as groups able to adapt to the lower salinity values caused by the estival freshwater input (Luria et al. 2017). In this sense, the phylogenetic analysis of the clone libraries evidenced that groups increasing during summer were diverse, comprising members of Oceanospirillaceae, Rhodobacteraceae, Nocardiaceae, SAR11 clade (Alphaproteobacteria) and Flavobacteriaceae.

The clear grouping of the spring–summer samples from E1 with the spring–summer samples of E3 showed by the *PCA* analyses (and mostly explained by the high SPM and temperature values, as well as the low salinities) suggests that, during summer, bacterial communities from stations E3 (located at the mouth of the creek) and E1 (located at the inner part of the cove) are influenced by the freshwater input and that during summer both stations share several bacterial groups. However, during autumn–winter, when meltwater impact is absent, samples from E1 grouped with E2, suggesting that during autumn–winter environmental conditions and associated bacterial community in Potter Cove are mostly oceanic.

The most represented OTU (OTU1) belonged to Oceanospirillaceae family and was identified as *Balneatrix*. Although formally the genus *Balneatrix* includes only one non-marine species, the pathogenic *Balneatrix alpica*, the closeness of this species to members of the genus *Oceanospirillum* was noted since its first description (Dauga et al. 1993). Also, several works have mentioned *Balneatrix* as a major component of marine bacterial communities from several marine habitats, as Bongue Sound, USA (D'Ambrosio et al. 2014), Gulf of Mexico (Liu et al. 2017) and the Pacific sector of the Arctic (Han et al. 2014). It is interesting to note that Han et al. (2014) found significant proportion of *Balneatrix* only in seawater samples, but this group was absent or scarce in surface mixed layers having lower-salinity areas. In accordance with this observation, we found sequences ascribed to *Balneatrix* in both E1 and E2, but no sequences were detected in the relatively low-salinity station E3. Another publication (Moreno-Pino et al. 2016) also reported not only a clear dominance of Gammaproteobacteria but also of *Balneatrix* assigned sequences in Fildes Bay area of 25 de Mayo (King George) Island. Although the exact taxonomic location of these *Balneatrix*-like sequences within the Oceanospirillaceae family remains an open question, their abundance in both E1 and E2 suggests that members of this genus (or of other genera closely related to *Balneatrix*) are dominant in several coastal Antarctic marine environments. Another question that remains unexplored is the ecological role and metabolic activity of this abundant exclusively marine group of bacteria.

Another point to highlight is the fact that the four most represented OTUS (1–4) were obtained from marine waters and were absent in the relatively low-salinity waters from E3 (see Online resource 6). These OTUs were identified as *Balneatrix*, a member of the SAR 11 clade, a Rhodobacteraceae and an uncultured member of the Oceanospirillales. These OTUs could be representing groups originating in the open sea that would become established in the inner part of the cove, increasing their presence during summer. It is also relevant to note that those bacterial groups that were dominant in E3 samples were detected either only in E2 or, when present in E1, their proportion resulted, in general, smaller in number than those observed in E2. This is the case with the five most represented sequences from E3. One of the reasons to explain these observations could be the reported circulation regime of the surface waters at Potter Cove. As was previously reported (Klöser et al. 1994), a cyclonic circulation pattern with water masses entering the cove through the northern area and leaving the cove through the southern sector is the dominant feature. In this context, microbes being transported by the freshwater from Potter and Matias streams would be flowing near the southern coast, affecting E2 (at the mouth of the cove) in a stronger way than the E1 area. Under this condition, grouping between E1 summer and E3 would not be caused by the few dominant groups of E3, but mainly by the numerous low represented OTUs, many of which are shared by the two sites. In the same way, during winter, when E3 area is frozen and does not have influence on E1, this area is only affected by E2, determining the grouping of winter samples of E1 and E2 evidenced in the *PCA* analysis. In summary, the richness of the bacterial communities of Potter Cove would be influenced by ribotypes provided by both freshwater and open seawaters. General results of the phylogenetic analyses, showing the dominance of Proteobacteria and, to a lesser extent, Bacteroidetes and Actinobacteria phyla, are consistent with previously reported

studies in coastal waters of the Antarctic Peninsula (Straza et al. 2010; Grzymski et al. 2012). Published results obtained from metagenomes of both Arctic and Antarctic surface coastal sediments (Matos et al. 2016) showed a similar bacterial community structure, at phyla level, compared to water samples obtained from Potter Cove. Moreover, our findings, which are based on clone libraries, are in good agreement with results based on small subunit (SSU) rRNA gene hypervariable amplicon sequencing obtained from coastal waters off Palmer station (Luria et al. 2014). These authors also reported a dominance of Gamma- and Alphaproteobacteria, with a high prevalence of Rhodobacterales in surface samples (accounting for 43% of the Alphaproteobacteria). In addition, a higher bacterial diversity was found during winter when compared with summer samples. In our work, the previously reported broad distribution and high abundance of the order Rhodobacterales (Giebel et al. 2009; Fu et al. 2013) were also observed and were accompanied by a great diversity, with 19 OTUs (96 sequences) assigned to members of the order. It is interesting to note that the most abundant OTU of Rhodobacterales (OTU3, 42 sequences) seems to have a marked seasonal variation in Potter Cove, with a greater dominance in summer compared to winter (see Online resource 5b). This temporal change was caused mainly by changes occurring in E2, where OTU3 was abundant and almost exclusively present during summer. The marked seasonal variability of Rhodobacterales was highlighted by Gilbert et al. (2012) who found, working with samples from a temperate marine coastal site off Plymouth (UK), that Rhodobacterales (which were dominated by the Roseobacter clade) exhibited a peak in spring, when nutrient concentrations were low and primary productivity was high. These authors also found an opposite peak of SAR11 clade, which peaked during winter, a phenomenon that was not observed by us in Potter cove, where similar number of sequences of the abundant OTU2 (SAR11 clade) were detected in both winter and summer seasons. As was mentioned above, these Rhodobacterales OTUs were exclusively marine and were not detected at all in E3. The genera *Pelagibacter* and *Polaribacter* were not present in E3, which would suggest that they also represent marine sequences.

In Potter Cove waters, we observed the almost total absence of Cyanobacteria, which represent the main group of primary producing prokaryotes in many regions of the planet, including Antarctic freshwaters and soils (Zakhia et al. 2008). This scarce presence of Cyanobacteria is a common feature in Antarctic and Arctic marine waters (Koh et al. 2012) including Potter Cove (Landone Vescovo et al. 2014) and rules out the question about the existence or not of other prokaryotic group assuming the ecological role, as predominant phototrophs, that cyanobacteria perform in other marine systems. Many Rhodobacterales, mainly grouped under the marine Roseobacter clade, are aerobic anoxygenic phototrophs (AAP). However, it was reported that these AAP bacteria are primarily heterotrophic organisms, able to obtain energy from light using bacteriochlorophyll a-depending photosynthetic reaction centers, but with no photoautotrophic CO_2 fixation capacity. In this way, the significant presence of these microorganisms in Antarctic marine waters would not be related to their role as primary producers; thus, their physiological role in coastal polar marine environments that enables them to dominate there remains unclear. Despite lacking O_2 production and CO_2 reduction ability, it was reported that the AAP are especially abundant in oligotrophic environments (as Potter Cove waters are) and are able to regulate their metabolism in accordance with light intensity (Hauruseu and Koblížek 2012). They can also assimilate organic carbon at a higher efficiency rate compared to their strict heterotrophic neighbors (Yurkov and Csotonyi 2009). So, all these properties, added to their ability to absorb light even when only wavelengths in the near infrared are available (Sato-Takabe et al. 2012), suggest a relevant role of the AAP in the marine carbon cycle. In addition to Rhodobacterales, also a significant number of sequences (21), located in 7 different OTUS, were assigned to genus *Erythrobacter*, another group of AAP (Koblížek 2015). We consider that elucidation of the ecological role of the AAP in Potter Cove should represent a major objective in the near future in order to achieve a better understanding of the energy flux and carbon cycle in this cove and other coastal Antarctic marine environments where high latitude and the annual freezing–thawing cycles of surface waters give to the light regime unique features.

The above-mentioned effect of salinity became evident when the structure of communities was observed at phyla and class levels. E3 in summer exhibited a highly different pattern, with a higher proportion of Betaproteobacteria (6%), that was poorly represented in E2 and almost absent in E1 (see Fig. 5). This small proportion of Betaproteobacteria in marine waters is in agreement with those reported in other studies in coastal Antarctic marine waters (Grzymski et al. 2012). It is possible that Betaproteobacteria are being transported to the basin by Potter and Matias creeks and represent microorganisms of terrestrial origin. In this sense, a recent clone libraries-based study on the composition of bacterial communities of soils and sediments from Potter Cove and Peninsula showed that, although scarcely represented in marine sediments, members of class Betaproteobacteria were abundant in soils, representing 20–25% of the clones for the most anthropized soils (Vazquez et al. 2017). Also, in the same work, Actinobacteria represented one of the most abundant taxa detected in control and low impacted soils, which is in accordance with the high proportion of members of this phylum detected in E3 (23% of the clones). These recently published results support the hypothesis

that the main differences exhibited by E3 compared with E2 and E1 during summer are due to the bacterial groups transported from soil to the marine basin by the water runoff caused by the glacier melting. These results of the bacterial community at phyla and classes levels also support the hypothesis that the structure of the surface bacterioplankton community at Potter Cove is strongly conditioned by salinity, as was reported for phytoplankton community of the cove by Hernando et al. (2015). However, some mechanical/physical stress caused by calving events (Pasotti et al. 2015) as well as releasing nutrients and other microelements by glacier melting (Alderkamp et al. 2012) cannot be discarded as factors influencing bacterial community in this marine ecosystem

Another key, albeit poorly studied, point for the understanding of the homeostasis of the Potter Cove ecosystem is the dynamics of the Nitrogen cycle. We detected a scarce number of *Nitrosomonas* and *Nitrobacter* sequences, which suggests a low level of nitrification activity mediated by bacteria. This observation could be related to the dominance, in this marine environment, of the ammonia oxidizing archaea (AOA, phylum Thaumarchaeota) that was reported in a previous work of our own at Potter Cove performed during the same period (Hernández et al. 2015). However, deeper analysis of the proportion and activity of the Potter Cove Archaea based on high-throughput sequencing will be required to infer the relevance of these prokaryotes within the nitrogen cycle of this environment.

In conclusion, the present work has shown that the microbiota in the inner part of Potter Cove is far from having a stable structure. On the contrary, it showed clear changes between summer and winter, mainly dependent on the salinity changes associated with the freshwater runoff as well as on the microbiota transported by the creek waters and spilled on the marine basin. Thus, while in summer the structure of the bacterial community resembles that observed in coastal areas near the mouth of these creeks, during winter its structure is closer to that shown by the more oceanic waters, typical of the areas outside of the cove. Our results revealed the enormous richness and high diversity of the bacterial communities of this Antarctic marine ecosystem, as well as its high seasonal and spatial variability. Also, the numerous not classifiable ribotypes (Cole et al. 2007) represent an original contribution to databases, with sequences that could either be endemic to the study site, or simply not have been described elsewhere in the world.

In addition, it helps to understand how a combination of environmental factors seems to affect the bacterial community composition and set a baseline for upcoming studies evaluating the response of Potter Cove ecosystem to environmental changes. This work showed that the study area should be considered, in future studies, as a variable biological system compared to the open seawaters, with large seasonal and spatial changes of its microbiota dictated by environmental changes, especially in salinity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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