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Bacterial biodiversity in deep-sea sediments from two regions of contrasting surface water productivity near the Crozet Islands, Southern Ocean.

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Abstract

The relationship between surface-derived particulate organic matter (POM) and deep-sea sediment bacterial abundance, community structure and composition was investigated in two different sediment layers from two zones of contrasting surface water productivity in the southern Indian Ocean. Bacterial sediment communities from high chlorophyll (HC) and low chlorophyll (LC) sites were characterized and compared using direct counts, clone library construction, denaturing gradient gel electrophoresis (DGGE) and fluorescence in situ hybridization (FISH). Of the 1566 bacterial clones generated from the sediment communities, 1010 matched published 16S rDNA sequences at ≥ 97% identity. A comparison of surface sediment clone libraries showed that at least one third of all identified operational taxonomic units (OTUs) were common to both HC and LC sites. DGGE community profiles were consistent (82% similar) and evenness of the major phylogenetic groups was 96% similar between surface sediment communities, where *gamma*- and *alpha*-Proteobacteria were dominant. Sediment communities shared similarly high biodiversity, while species richness was marginally higher at the LC site. Intra-site shifts in bacterial abundance and composition were observed with increasing sediment depth. Despite the differences in organic matter input between sites, the consistency observed between HC and LC sediment communities pointed to 1) the extent of remineralisation by mega and meio-fauna was a factor affecting the quantity and quality of POM available to sediment bacteria, 2) sampling during the early 'nutrient assimilation phase' of the bacterial response to freshly deposited POM or 3) the action of bacteria in the water column could affect the quantity and quality of POM available to sediment bacteria. Although factors other than these may explain the observed similarities, this first comparison of deep-sea sediment communities in relation to surface-derived productivity may be useful in further elucidating the role of sediment bacteria in carbon remineralisation in the deep-sea environment.

1. Introduction

Deep-sea sediments represent largest carbon sink on Earth. The functionally diverse bacteria harboured in deep-sea sediments comprise up to 90% of the benthic biomass (Dixon and Turley 2000) and provide a fundamental contribution to global carbon recycling through the sequestration and remineralization of organic matter (Houghton 2007). The characterisation of bacteria that inhabit deep-sea sediments, and the determination of their diversity, community structure and abundance, are the first steps towards a fuller understanding of the key role they play, yet owing the challenges of sampling such an extreme and complex biosphere, the diversity of deep-sea sediment bacteria and the environmental factors that control their diversity and distribution are poorly understood (Jørgensen and Boetius 2007).

Resource availability in the form of particulate organic matter (POM) from primary production is thought to be a key determinant of benthic diversity in marine systems (Levin *et al.* 2001; Rex *et al.* 2006; Ruhl *et al.* 2008). The intimate link between diversity and the nutritional and biochemical attributes of the surrounding marine environment can be exploited through the comparison of diversity/distribution patterns to indicate environmental variation resulting from global change (Jørgensen and Boetius 2007; Ruhl *et al.* 2008). Evidence for a clear link between bacterioplankton diversity and surface water productivity has been widely reported (Schäfer *et al.* 2001; Horner-Devine *et al.* 2003; West *et al.* 2008). Shallow marine benthic and mesocosm studies have both demonstrated that temporal changes in sediment bacterial abundance and distribution are not independent of surface-derived labile organic matter (Polymenakou *et al.* 2005, Rosselló-Mora *et al.* 1999). A shallow-water study in the North Sea found that differences in bacterial community composition coincided with overlying productivity although no differences in diversity were observed (Franco *et al.* 2007). In the deep sea, studies have reported rapid increases in sediment bacterial activity in response to POM input from the euphotic zone (Lochte 1992; Witte *et al.* 2003; Moodley *et al.* 2005). However, information concerning the relationship between the input of POM and bacterial community structure, diversity and abundance in deep-sea sediments is lacking.

In this study we compared the bacterial community structure, diversity and abundance of deep-sea sediments from two contrasting regions of productivity in the Southern Ocean, in order to examine the relationship between surface-derived POM and deep-sea bacterial diversity and distribution. In high nutrient, low chlorophyll

(HNLC) regions such as the Southern Ocean, the seasonal flux of particulate organic carbon from eutrophic surface waters constitutes an important nutrient resource for the deep-sea communities (Gooday 2002). In the Indian Ocean sector of the Southern Ocean, a perennial diatom bloom, driven by natural iron enrichment of seawater from island sediment sources (Planquette *et al.* 2007), occurs to the northeast of the Crozet Islands. The bloom enhances the exportation of particulate organic carbon to the seafloor in an otherwise oligotrophic HNLC environment (Pollard *et al.* 2009; Venables *et al.* 2007). Organic carbon fluxes of up to 440 mg Cm^{-2} d^{-1} are deposited on the seafloor to the east of the Crozet Islands, compared to 28-46 mg Cm^{-2} d^{-1} at an oligotrophic site south of the islands (Salter *et al.* 2007).

Bacterial communities from sediments within an HNLC region to the south of the Crozet Islands were compared with those from a eutrophic region to the northeast of the islands where particulate organic matter was present on the deep seafloor from an overlying surface-water bloom. The abundance, richness, structure and evenness of each community were analysed and compared using four culture-independent methods: 4',6'-diamidino-2-phenylindole (DAPI)-staining, the construction of clone libraries, denaturing gradient gel electrophoresis (DGGE) and fluorescence *in situ* hybridization (FISH).

This study represents the first comparison of deep-sea sediment communities from contrasting regions of surface water productivity. The work was undertaken as a component of the multi-disciplinary benthic Crozet program, designed to investigate the influence of quantity and quality of surface-derived organic matter on biological diversity and geochemistry in the deep-sea benthos for carbon cycling models, flux budgets, ecology, and palaeoceanographic interpretations.

2. Materials and Methods

2.1. Study sites and sampling

Samples were collected between 14/12/05 and 05/01/06 during a research cruise (RRS *Discovery*; D300) to the Indian Ocean sector of the Southern Ocean. The sampling stations are represented in Figure 1. Sediment cores were collected using a Bowers-Connelly megacorer (Gage and Bett 2005) from a eutrophic site (site HC; high chlorophyll) within the diatom bloom area to the east of the Crozet Islands (46 °S, 56 °E) and from an oligotrophic site, (site LC; low chlorophyll) to the south of the islands (49 °S, 51 °E). Bottom water depths were between 4191 and 4320 m. Five replicate cores from each site were sectioned through a vertical profile at 1 cm intervals to a final depth between 15 and 30 cm depending on core length. Sediment subsamples (1g) were taken from each depth layer into sterile 2.2 ml Eppendorfs, for analysis of bacterial abundance, for molecular analysis by DGGE and FISH and for the construction of clone libraries. Individual core sections were stored at -20 °C in sterile, vented Petri dishes sealed with parafilm. Sample handling was performed at 2-4°C.

Samples were collected following the peak of the diatom bloom in surface waters in November. Surface chlorophyll-a levels at the HC site ranged between 0.55-0.74 mgm^{-3} and between 0.42-0.47 mgm^{-3} at the LC site. At the time of sampling particulate organic matter was visibly present on the seafloor at the HC site and chlorophyll-a concentration was 3-fold higher in HC surface sediments (Smith C.R. *et al*. unpublished data). Total organic carbon and total nitrogen concentrations were similar in both sediments (Holtvoeth J. *et al.* unpublished data).

2.2 Direct counts

Bacterial abundance was analyzed at the following sediment depths: 0-1, 4-5, 9-10, 14-15 and 19-20 cm. Sediment samples (n= 5) were diluted 1:10 in sterile, 0.2 μm filtered seawater and homogenized briefly using a vortex mixer. Cells were harvested by vacuum-filtration (<30 kPa) of the suspension through a 25 mm, 0.22 μm, black, polycarbonate filter membrane (Porectics, Osmotics Inc., MA). Cell fixation was performed directly on the filter membrane using 2 ml paraformaldehyde [4% wt/vol] (Sigma-Aldrich, Poole) for 30 minutes according to the method of Schallenberg *et al.*

(1989) followed by three washes (5 ml) with sterile seawater to remove excess fixative. Filters were stained with DAPI (5 µg ml^{-1}) for 5 min, rinsed twice with sterile seawater (5 ml) and phosphate buffered saline (PBS; 10 mM sodium phosphate [pH 7.2], 130 mM NaCl [pH 7.4]), air dried, and mounted in CitiFluor (CitiFluor Ltd., Canterbury). Enumeration was conducted using a Leitz Labalux epifluorescence microscope fitted with a 50W mercury, high-pressure lamp, UV excitation and a filter set for DAPI (340 to 380 nm excitation filter, 400 nm beam splitter, and 430 nm barrier). A minimum of 1000 cells was counted per sample. Bacterial cell counts were expressed as mean number of cells per gram (dry weight) of $sediment^{-1}$ (± 1 standard deviation; SD).

2.3 DNA isolation from environmental samples

DNA was extracted from subsamples (0.5 g) of thoroughly mixed sediment from surface (0-1 cm depth) and deep (91-20 cm depth) layers from three replicate cores per site using the FastDNA SPIN kit for soil (Qbiogene, Cambridge) according to the manufacturer's instructions, with bead beating for 5 min. Two additional washes of the extracted, matrix bound DNA were performed using the salt/ethanol wash component of the kit to enhance removal of humic acids. DNA was eluted into 50 µl sterile water (pH 8.0). The five extractions from each core were combined in a Microcon YM100 column (Millipore UK Ltd., Hertfordshire) and concentrated by centrifugation according to manufacturer's instructions. Concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, MA).

2.4. Clone library construction

DNA extractions from each site were pooled for PCR in order to construct two clone libraries per site for surface and deep sediments: HC surface (0-1 cm), LC surface (0-1 cm), HC deep (19-20 cm), LC deep (19-20 cm). 16S rRNA genes were amplified by PCR using the primers 8F (5'AGA GTT TGA TCC TGG CTC AG 3') and 1492R (5' TAC GG(C/T) TAC CTT GTT ACG ACTT 3') from VH Bio Ltd. (Gateshead). The PCR reaction mixture contained 5 ng DNA, 1 µl each primer (10 mM), 10 µl 10x NH_4-based reaction buffer, 2.5 µl $MgCl_2$ (50 mM), dNTPs (20 nmol each), 0.3 µl BIOTAQ polymerase 0.05 U $µl^{-1}$ Biotaq™ DNA polymerase (Bioline Ltd., London) and 0.8 µl bovine serum albumin [10 $mgµl^{-1}$] (Sigma-Aldrich, Poole) and was adjusted to a final volume of 30 µl using sterile water. PCR amplification was performed on a DNA Engine Tetrad 2 Peltier Thermocycler (Bio-Rad Life Sciences, Hertfordshire) under

the following conditions: initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min 15 s, 72 °C for 1 min and a final elongation step at 72 °C for 10 min. Pure *E.coli* DNA and marine water bacterial DNA were incorporated into each PCR run as positive controls. PCR products were cleaned using the Illustra GFX PCR DNA purification kit (GE Healthcare UK Ltd., Chalfont St Giles).

PCR products were ligated into a vector according to the pGEM-T Easy Vector protocol (PromegaUK Ltd., Southampton). Ligated products were transformed into ultra efficient XL-2 blue MRF competent cells (Stratagene, CA, USA) with addition of 200mM IPTG (isopropyl-beta-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside, Bioline Ltd) and plated onto Luria agar containing ampicillin [100 $\mu g ml^{-1}$]. Resultant colonies were amplified in a PCR reaction mix containing 25 µl ReddyMix (Abgene Ltd., Epsom), 0.5 µl M13F primer (5'-CGC CAG GGT TTTCCC AGT CAC GAC-3') and M13R (5'-GGC AGG AAA CAG CTA TGA CC-3') under the following conditions: 95°C for 5 min followed by 29 cycles of 95°C for 30 s, 58.5°C for 30 s and 72°C for 1 min 10 s. M13 PCR products were cleaned and eluted into 50 µl sterile distilled water using purification columns.

Sequencing reactions were performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cheshire) in a forward direction using the M13F primer and products were run on a MegaBace500 automated DNA capillary sequencing system (Molecular Dynamics, CA). Additional sequencing was performed under contract by Macrogen Inc. (Korea).

2.5. Denaturing gradient gel electrophoresis

HC and LC surface (0-1 cm) sediment DNA was extracted from three replicate sediment cores per site and amplified by PCR using the primer combination 341F-GC (Muyzer *et al.* 1993) and 907R (Muyzer and Smalla 1998). Each reaction mix contained 5 ng DNA with 1 µl each primer (10 mM), 5 µl 10X PCR reaction buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCL [pH 8.8], 0.1% Tween-20, 15 mM $MgCl_2$), 200 µM of each deoxyribonucleotide triphosphate (Bioline Ltd., London) 1.25 µl bovine serum albumin [BSA; 10 $mg\mu l^{-1}$] (Sigma-Aldrich, Poole) and 0.05 U μl^{-1} Taq polymerase (Bioline Ltd., London). The volume was adjusted to 25 µl with DNase and RNase-free 0.1 µm-filtered sterilized water (Sigma-Aldrich, Poole). PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 5 min, then 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min followed by a final

elongation step at 72 °C for 15 min. PCR reactions were conducted in duplicate.PCR products (ca. 550 bp long) were evaluated by electrophoresis on 1.5% (wt/vol) agarose gels (30 min, 100 V). Duplicate PCR reactions were combined and concentrated into 20µl using MinElute columns (Qiagen Ltd., Crawley) for loading, with a reference lane, onto the gel. Vertical gel casting and running were undertaken according to Helms (1990) using the INGENYphorU-2 System (Ingeny International BV, The Netherlands). A 50-70% denaturing gel containing 6.5% [v/v] acrylamide/bis-acrylamide was cast using 50 µl ammonium persulphate [20%] and 5 µl TEMED (Sigma-Aldrich, Poole) to initiate polymerisation. The gel was run overnight at [120 V, 18 h] and post-stained with SYBR Gold nucleic acid stain (Invitrogen Ltd, Paisley).

Banding patterns for each DGGE profile were processed, normalized and statistically analyzed using Gel Compar II software v3.5 (Applied Maths, Belgium). A similarity matrix based on band positions and contribution of the optical intensity of each band to the total intensity of each DGGE profile (densitometric curve) was generated and these data were used for cluster analysis (Pearson Correlation; UPGMA).

DGGE bands that appeared at the same position in the profile from different sites were excized. Bands were excized into clean 0.5 ml tubes containing 30 µl, 0.1X TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at 4ºC overnight to allow elution of DNA. 2 µl eluted DNA was used in a PCR with 341F and 907R as previously described. PCR products were purified and eluted into 30 µl, 0.1 µm-filtered, nuclease-free water for cloning and sequencing.

2.6. Fluorescence *in situ* hybridization

Bacterial group evenness was determined in surface (0-1 cm) and deep (19-20 cm) sediment samples (n=5) from HC and LC sites using group-specific, 16S rRNA-targeted oligonucleotide probes. Bacterial cells were harvested by vacuum-filtration and fixed on 0.2 µm polycarbonate filters as described for direct counts. The following 5' CY-3-labelled, oligonucleotide probes (VH Bio Ltd, Gateshead) were used in this study: EUB338 (Eubacteria; Amann *et al.* 1990), NON338 (negative control), ARCH915 (Archaea) (Stahl and Amann 1991), ALF968 (most *α*-Proteobacteria), GAM42a (*γ*-Proteobacteria; Glöckner *et al.* 1999), CF319a (Cytophaga-Flavobacteroides cluster; Manz *et al.* 1996) and PLA46 (Planctomycetales; Neef *et al.* 1998).

Each probe was added at a final concentration of 50 ng ml^{-1} to 250µl of hybridisation solution [0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS] containing 10% [v/v] formamide (for EUB338, NON338), 20% (for ALF968) and 35% [v/v] formamide (for GAM42a, PLA42, CF319, ARCH915). A volume of 20 µl hybridization solution containing probe was added directly to cells retained on the filter paper. Cells were incubated in a humid atmosphere [46°C, 90 min] and rinsed twice with 5 ml prewarmed [37 °C] wash buffer [20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS] containing NaCl in concentrations of 450 mM NaCl (for EUB338 and NON338), 225 mM NaCl (for ALF968) and 80 mM (GAM42a, CF319a, PLA46 and ARCH915). Cells that hybridized with EUB338 were counterstained with DAPI (5 µg ml^{-1}). Air-dried cells were mounted in Vectashield (Vector Labs, CA) and enumeration was undertaken by epifluorescence with a CY-3 filter unit (550-570 nm excitation emission wavelength).

A minimum of 1000 DAPI-stained bacterial cells within 10-20 randomly selected fields of view, or 250 random fields of view were enumerated using an Olympus BX51 epifluorescence microscope fitted with a tetramethyl rhodamine filter set (WU filter set; BP520-550/DHC70/BA580IF) for CY3 (550-570 excitation emission wavelength). The mean number of hybridized cells g^{-1} dry sediment was calculated for each probe.

2.7. Clone library and data analyses

Sequence files were checked for base calling accuracy. Cloned sequences were submitted as BLASTN searches against the EMBL-EBI nucleotide sequence database to identify the closest matching relative. A local BLAST search was conducted using BioEdit software v7.0.9 (Hall 1999), to determine the similarity of sequences within and between Crozet HC and LC libraries. Sequences that showed ≥ 97% compositional similarity to each other were considered the same operational taxonomic unit (OTU). A value of 97% similarity is commonly set as the threshold to encompass the sequence microheterogeneity that may occur between closely related taxa (McCaig *et al.* 1999). Clone libraries were compared statistically based upon the total number of OTUs, the abundance of each OTU and the number of OTUs shared between sites. Compositional similarity between libraries was estimated using Sørensen's coefficient according to the equation: $S = S_{12} / [0.5(S_1 + S_2)]$, where S_{12} is the number of sequences common to both libraries, and S_1 and S_2 are the total

number of sequences in the first and second libraries respectively (Martin 2002). Sequences were deposited in the EMBL database under accession numbers FM213482 - FM215048.

Taxonomic classification of 16S rRNA gene sequences from clone libraries was performed using the ribosomal database project classifier (Wang *et al.*, 2007) and by phylogenetic analysis using arb (Ludwig *et al.*, 2004). 16S rRNA gene clone library sequences were aligned in arb using the SINA aligner (Pruesse *et al.*, 2012) and aligned sequences were added using parsimony into the all species living tree project release 108 phylogenetic reconstruction (Yarza *et al.*, 2008). Species and phylogenetic groups that were unrelated to the clone library sequences were removed from the final tree.

Direct count and FISH data were compared between site and between sediment depths using one-way analyses of variance tests. Significant differences (at $P < 0.05$) were tested using post hoc Tukey's tests to identify which pairs of means were significantly different. The Primer 5 program (Plymouth Marine Laboratory, Plymouth; Clarke and Warwick 2001) was used to conduct multivariate statistical analysis to investigate the sources of differences and compare FISH data at phylogenetic group level between HC and LC. Non-transformed data for probes GAM42a, ALF968, CF319a, and PLA46 for both sites and depths was clustered based on group averages following generation of a Bray Curtis similarity matrix. Visualization of maximum likelihood distance matrices was achieved by multidimensional scaling (MDS) plots based on the similarity matrix. A SIMPER analysis was conducted to identify the bacterial group that contributed most to observed differences. Multivariate statistical analysis was conducted to examine differences in sequence composition between HC and LC clone libraries and maximum likelihood distance matrices were visualized by MDS ordination.

3 Results

3.1. Direct counts

The number of cells observed at the selected sites in the surface sediments (0-1 cm depth) were 3.65 (±0.6) $x10^9$ (HC site) and 2.98 (±0.5) $x10^9$ (LC site) cells g (dry weight) $sediment^{-1}$. There were no significant differences in cell counts between HC and LC sediments down to 20 cm depth ($P = 0.14$) or between depths within a site (P

= 0.07). There was no significant correlation between cell counts and sediment depth; however, a slight trend towards decreasing cell number with increasing depth was observed in both sediments.

3.2. Clone library analysis

In total, 1566 useable clones were generated across four clone libraries (two per sample site, at 0-1 cm and 19-20 cm sediment depths). Local BLAST analysis of nucleotide sequences showed that each clone library comprised between 114 and 315 OTUs (sequences with ≥ 97% compositional similarity to each other; Table 1). BLASTN analysis of all sequences against the EMBL-EBI database resulted in 1010 matches (at ≥ 97% identity) to 380 different 16S rDNA published sequences. Clones matching the following five published phylotypes only were identified in all four libraries; uncultured bacterium clone 062d24 (EU734968), uncultured bacterium clone A20 (AY373407), uncultured bacterium clones b78-22 (EU286986), s26-120 (EU287420) and s26-96 (EU287396).

The number of clones screened within HC and LC surface sediment libraries (594 and 636 clones respectively) was sufficient to permit an unbiased assessment of richness using the non-parametric Chao index (Chao 1984). The richness curves derived for both libraries approached an asymptote. OTU richness, (i.e. the number of OTUs present) was 30% greater in the LC surface library compared to the HC library. Diversity estimates (Table 1; (D)) were similarly high in sediments from both sites. Using Good's coverage calculation (Good 1953), a maximum of 425 sequences was estimated as sufficient to cover approximately half of the diversity in Crozet surface sediments. According to Sørensen's similarity coefficient, more than a third (36%) of surface sediment-derived clones were common to both HC and LC surface libraries, when approximately half of the total estimated diversity of the sediments was covered in the analysis. However, a decrease in compositional similarity between sites was observed with increasing sediment depth. HC and LC deep sediment libraries shared greatest compositional similarity with their surface counterpart (9% and 12% respectively).

Most of the clones (between 70 and 88%) in each library represented uncultured bacteria with less than 1% of all the clones matching cultivated bacterial sequences (at ≥ 97% identity). Only 2 clones were closely related to named species; *Sphingomonas* sp. and *Ulvibacter antarcticus* strain imcc310. The largest proportion

of clone library sequences in surface sediments were *gamma*-proteobacteria (25 to 34 %) compared to *alpha*-proteobacterial sequences in deep sediments (29 to 37 %; figure 2 a,b). The proportion of delta and *epsilon*-proteobacteria ranged from 10 to 12 % in surface sediments to 6 to 13 % in the deeper, 19-20 cm sediment depth. Actinobacteria were more abundant in surface sediments (12 and 15 %) whereas planctomycetes were more abundant in deeper sediments (12 and 13 %). Sequences related to Bacteriodetes were over twice as abundant in surface sediments (7 to 9 %) compared to the deeper sediment (3 to 4 %). Gemmatidonomadetes were found in low abundance (1 %) in the HC surface and deep sediments but were absent from the LC sediments.

Of the surface sediment clones that gave EMBL-EBI database matches at (≥ 97% identity (822), more than 60% were originally isolated from deep-sea environments. The distribution of clones according to original isolation source was similar for HC and LC surface sediment libraries (Figure 3). Almost one third of the clones in each surface sediment library were Arctic and Antarctic marine isolates. In the deep sediment libraries, clones matching Arctic and Pacific nodule province phylotypes were dominant.

3.3. DGGE analysis

The degree of similarity between HC and LC sediment bacterial communities was assessed by comparison of surface sediment DGGE profiles. Overall, replicate banding patterns (from three cores per site) were highly consistent between HC and LC sites (Figure 4 [A]). The consistency in banding patterns was observed across eight independent replicate gels, which suggests that DGGE was highly reproducible. Between 20 and 25 banding positions were observed in the surface sediment DGGE profiles. Comparison of the densitometric curves for each profile showed similar distributions of peaks and relative peak densities between sites (Figure 4 [B]). The most prominent bands (labelled A-J; Figure 4 [A]) were excised for cloning and sequencing from equivalent positions in the HC and LC profiles. Analysis of the cloned bands showed that the same microorganisms were dominant in the sediments of both sites. Seven of the ten bands sequenced comprised more than one OTU. An OTU was defined as any sequence that shared ≤ 97% sequence identity with another sequence in the HC and LC clone libraries. Table 2 shows the EMBL-EBI BLASTN matches for the most dominant OTU identified in each band. The dominant OTU

represented in DGGE bands A, B, C and I (Table 2) were also present in all four clone libraries.

Similarity analysis (Pearson Correlation, UPGMA clustering) showed that HC and LC surface sediment DGGE banding patterns were 82% similar and thus both communities shared most of the same dominant members. The mean intrasite similarity value (n= 3) for the LC site was lower than the mean intersite value (82%). This indicated a clear overlap in composition of the sediment bacterial communities between HC and LC sites.

3.4. FISH analysis

The six phylogenetic groups detected by FISH were the Eubacteria, *gamma*-Proteobacteria, *alpha*-Proteobacteria, CFB cluster, the Planctomycetales and the Archaea. No significant differences in the relative abundance (evenness) of these groups was found with site at either sediment depth, although evenness was found to differ significantly with increasing sediment depth (Table 3). A SIMPER analysis of the data showed that the CFB cluster was the principle contributor (47%) to the observed dissimilarity in evenness between communities with sediment depth, followed by the Planctomycetes (24%). The consistency in phylogenetic group evenness observed between sites is shown by the close clustering (based on Bray Curtis maximum likelihood distances) of HC and LC surface and deep replicate samples (Figure 5). Surface and deep sediment replicates (n=3 per site) shared on average 96% and 92% similarity in phylogenetic group evenness respectively, regardless of site.

The contribution of each phylogenetic group to the total number of DAPI-stained cells was lower in the deep compared to surface sediments, resulting in a high percentage of undetected prokaryotes (~40%) at both deeper within sediment sites (Figure 6). The *gamma*- and *alpha*-Proteobacteria were the only groups to show relative consistency in their contribution to the DAPI-stained cell count between sites and depths, comprising up to 27 and 21% respectively, depending on the sample. For HC and LC sites, eubacterial abundances were 2.9 (±0.08) $x10^9$ and 2.7 (±0.2) $x10^9$ cells g (dry wt) $sediment^{-1}$ in surface sediments respectively and 1.7 (±0.09) $x10^9$ and 1.8 (±0.3) $x10^9$ cells g (dry wt) $sediment^{-1}$ in the deep sediments. A significant decrease in eubacterial abundance was observed with vertical depth ($P < 0.001$) within each site but not between sites. On average 68 ± 9% (± SD) of DAPI-stained cells in both

HC and LC surface sediment samples were detected using the universal probe for Eubacteria; EUB338. In contrast, eubacterial cells accounted for only 48 ± 6% of the DAPI-stained count in the deep sediment samples. Between 96 and 117% of the eubacterial cell count was identified with probes for the major phylogenetic groups. The detection of up to 17% more cells using phylogenetic group probes in some cases, indicated that cells might have bound to more than one group-specific probe or alternatively, the eubacterial probe was unable to detect certain groups (Llobet-Brossa *et al.* 2002).

In concurrence with the clone library data, members of the *gamma*-Proteobacteria were predominant in the Crozet sediments. Abundance of *gamma*-Proteobacteria ranged between 0.94–1.13 and 0.98-1.1 x 10^9 cells g (dry wt) sediment^{-1} for HC and LC sites respectively. Numbers of *alpha*-Proteobacteria were never greater than 75% of the *gamma*-Proteobacteria followed by the CFB cluster and the Planctomycetes. In the deeper sediments, Planctomycete abundance was higher than the CFB cluster (by approximately 20%) at both sites. On average, hybridizations to all group-specific probes were greater than hybridizations to the negative control probe NON338. The highest numbers of Archaea were observed in the surface sediment communities at a maximum of 4.6 x10^8 cells g (dry wt) sediment^{-1}.

4. Discussion

This study compared the bacterial communities of deep-sea sediments from two contrasting regions of high and low productivity in the Southern Ocean. Results showed that there was a high similarity between surface sediment bacterial communities from regions with very different overlying water productivities, suggesting that 1) the extent of remineralisation by mega and meio-fauna was a factor affecting the quantity and quality of POM available to sediment bacteria, 2) sampling occurred during the early 'nutrient assimilation phase' of the bacterial response to freshly deposited POM or 3) activity of bacteria in the water column could affect the quantity and quality of POM available to sediment bacteria. Although factors other than these may explain the observed similarities, this is the first comparison of deep-sea sediment communities in relation to surface-derived productivity in the Southern Ocean and may be useful in further elucidating the role of sediment bacteria in carbon remineralisation in the deep-sea environment.

Bacterial cell counts observed in this study were similar to those described for sediments in the Crozet region previously (De Wit *et al.* 1997) and consistent with values for marine sediments from other sub-polar and polar locations (Sahm and Berninger 1998; Ravenschlag *et al.* 2001; Bowman *et al.* 2003; Quéric and Soltwedel 2007).

Direct count analysis of the Crozet sediments indicated no difference in bacterial standing stocks between HC and LC sites to a sediment depth of 20 cm, despite the three-fold higher concentration of chlorophyll-a and the presence of overlying organic matter at the HC site. In addition, all three community analysis techniques (clone libraries, DGGE, FISH) provided corroborative evidence to demonstrate that despite the differences in POM input, HC and LC surface sediment bacterial communities shared strong similarities in composition (36%), structure (82%) and evenness (96%). The consistency in bacterial standing stocks observed in a comparison of oligotrophic and eutrophic sediments from the Porcupine Abyssal Plain (Eardly *et al.* 2001) were attributed to interannual variation in the quality and quantity of deposited organic matter, the rapid remineralization of recently deposited organic matter by benthic bacterial communities and the time of sampling. It is feasible that the across-site consistency in standing stocks and community composition observed in Crozet sediments, whilst probably linked to similar TOC and CN concentrations within both sediments (Wolff G.A. *et al*. unpublished data), might also be partially attributed to the quality of the organic matter rather than the quantity. Although HC sediments had three-fold higher chlorophyll-a content, analysis of organic matter freshness within the sediments (chlorophyll-a to pheaphorbide ratio) showed little difference in quality between HC and LC sites (Smith C.R. *et al*. unpublished data; Wolff G.A. *et al*. unpublished data).

In this study, the potential for higher abundances of mega-fauna at the HC site (Billett D.S.M. *et al.* unpublished data), previously reported generally elevated formaniferal populations (Hughes *et al*. 2007) and also the observation that macrofauna are responsible for most of the initial processing of labile organic matter (Witte *et al.* 2003) should not be discounted as a regulators of bacterial standing stock or community composition at the HC site. Evidence for major differences in the structure of water column bacterial communities between the HC and LC sites suggested rapid remineralization of POM by water column bacteria in this region. The rapid micro, meio and macrofaunal metabolism of fresh POM in food-limited environments has the potential to delay or reduce the incorporation of POM into the sediment, thus

limiting its availability to sediment-dwelling microorganisms (Bett *et al* 2001). Recent evidence suggests that in regions of high surface water productivity most of the total primary production is consumed within the water column, where it supports elevated biomasses of organisms compared to oligotrophic regions (Steinberg *et al.* 2008). This suggests that for the most part, bacterial communities within the sediments would potentially remain relatively unaffected by the presence of overlying matter whilst abundance of water-column and seabed-dwelling organisms were elevated.

At the time of sampling organic matter present at the HC site was relatively recent, only days or weeks old (Salter 2007). In response to fresh organic matter, the growth of the standing stock at the HC site may have been limited as a result of rapid bacterial assimilation of nutrients and the potential for energy expenditure to be driven towards metabolic processes rather than replication. In a usually food-limited environment, bacteria in immediate contact with organic matter within upper sediment layers often show low growth efficiency and a retarded replication response to POM input (Turley 2000, Witte *et al.* 2003). Evidence for two-fold higher respiration rates at the HC compared to the LC site (Homoky *et al.* 2009) indicated a bacterial response in activity to POM input in HC sediments. This suggested a higher turnover of microbial cells at the HC site, which was not correlated to cell numbers or diversity. In Crozet surface waters, Zubkov *et al.* (2007) detected 10-fold and 1.5-fold differences in microbial turnover of POM and biomass respectively between HC and LC sites, yet, bacterial standing stocks were directly comparable. While responses in bacterial activity and biomass to POM input have been reported (Boetius and Lochte 1996; Ducklow *et al.* 2001; Obernosterer *et al.* 2008; West *et al.* 2008) these responses are not always correlated to the abundance or standing stock of bacteria (Rex *et al.* 2006).

As shown by clone library and DGGE analyses, the high bacterial diversities in HC and LC sediments were comparable to each other and to the bacterial diversities of other marine sediments (Li *et al.* 1999a; Li *et al.* 1999b; Lopez-Garcia *et al.* 2003). More than 70% of the sequences obtained gave BLASTN matches to uncultured bacteria and < 30% of these were affiliated to a phylogenetic group or species. This illustrated the vast extent of the bacterial biodiversity present within the Crozet sediments still to be characterized.

The lower species richness observed in HC compared to LC sediments (740 versus 899 OTUs), could be attributed to the higher input of organic matter to the HC site or

regional variability in sediment biogeochemistry and chlorophyll-a content (Polymenakou *et al.* 2005). It is important to note that Chao-1 richness estimates are sensitive to sampling effort (Bissett *et al.* 2006) and a greater sampling effort at the LC site may also account for some of the additional richness at this site. The negative correlation between organic matter input and richness observed in the present study supports the postulation of Levin *et al.* (2001) that species richness is lower in nutrient-rich areas as the enhanced resources support larger populations where the dominance of a few species occurs, often leading to a decrease in habitat heterogeneity. However, the productivity-diversity relationships exhibited by metazoans might not apply to bacteria. Indeed, one of the most likely explanations for the decrease in diversity in highly productive areas is that the high organic loading leads to hypoxia, which is certainly not the case around the Crozet islands. Further contrasting evidence from a study of shallow North Sea sediments found higher bacterial species richness at the site with greater food availability (Franco *et al.* 2007). However, the processes that define species richness in sediments are largely unknown and it has been suggested that deep-sea processes are likely to differ from those occurring in shallow waters (Moodley *et al.* 2005).

Bacterial OTUs common to HC and LC sediments were identified within clone libraries and by DGGE. It is likely that these dominant, widespread bacteria represented key species of the Crozet region while the rarer OTUs might correspond to species with a greater sensitivity to variations in organic matter input and thus thrive in a more specialised niche. The dominance of *gamma*-Proteobacteria in deep-sea sediments followed by the *alpha*-Proteobacteria has been reported elsewhere (Nercessian *et al.* 2005, Li *et al.* 1999a, Li *et al.* 2009b). These bacterial groups comprise the keystone microorganisms of deep, marine sediments. A number of *Gamma*-proteobacterial sequences identified in surface sediments were related to bacterial clones JTB255 and AT-s2-59, members of the recently acknowledged GMS clade (Gillan and Pernet 2007). This clade has been linked to sulphur oxidation and cycling in marine sediments. The presence of members of the CFB cluster in Crozet surface sediments provided evidence for other diagenetic and remineralization processes owing to the recognized involvement of this group in the degradation of deposited organic matter and complex macromolecules (Cottrell and Kirchman 2000, Fandino *et al.* 2005). Although shifts in abundance and diversity of the CFB cluster with organic input have been described (Rosselló-Mora *et al.* 1999; Rink *et al.* 2007; Bissett *et al.* 2008), their abundance in surface sediments was consistent between HC and LC sites. Unusually, Planctomycete-related sequences were only identified in

surface sediments in this study, although elsewhere Planctomycetes have also been isolated from the deep layers of shelf (Hunter *et al.* 2006), deep-sea (Li *et al.* 1999a; Reed *et al.* 2002) and subseafloor sediments (Inagaki *et al.* 2006; Webster *et al.* 2006). Planctomycete abundance levels observed in this study (comprising 3-9% of the library), were similar to those in Wadden Sea sediments where rates of aerobic remineralization of overlying algal biomass were high (Musat *et al.* 2006). The presence of one planctomycete clone AGG27 identified in HC surface sediments was potentially an indicator of fresh POM at this site as it was previously isolated from phytodetrital marine aggregates off the Californian coast (Delong *et al.* 1993). Although the precise ecological role of the Planctomycetes remains undefined, recent studies of marine Planctomycete genomes have revealed a wealth of sulphatase and C1 metabolism genes indicating a specialist role for these bacteria in the initial breakdown of sulphatated heteropolysaccharides (Woebken *et al.* 2007). Abundance levels of *beta*-Proteobacteria-, Acidobacteria-, Gemmatimonadetes- (previously candidate division KS-B) and Chloroflexibacter-related sequences were consistent with those described for Antarctic, Eastern Mediterranean and Baltic sediments (Bowman *et al.* 2003; Polymenakou *et al.* 2005; Edlund *et al.* 2008). *Beta*-Proteobacteria tend to predominate in freshwater ecosystems and show lower incidence in sediments (Glöckner *et al.* 1999). Sequences related to this group were present in the Crozet HC and LC surface sediment libraries but absent from both deep sediment communities. All *beta*-proteobacterial sequences in surface sediments corresponded to the same phylotype, an uncultured Nitrosospira bjs81-042 isolated from cold seep sediment in the Japan Sea (Arakawa *et al.* 2006). This organism is most likely an ammonium-oxidizer like other members of the Nitrosospira group. A lower redox potential in the deep Crozet sediments compared to the surface might explain the increased presence of acidobacterial sequences in deeper sediments (Edlund *et al.* 2008).

Differences in community composition between the HC and LC sites were more pronounced in the deeper sediments (20.9% similar) than in surface sediments (36.0% similar). Although the lower sampling effort devoted to deeper sediment layers might partly account for the lower level of similarity between deep sediment communities, the greater biogeochemical heterogeneity observed with increasing sediment depth (Marsh *et al.* 2007) might also contribute to the dissimilarities. The presence of carbonate-rich turbidite inputs in deep HC sediments compared to basalt-based turbidite inputs in the LC deep sediments were deposited as a result of significantly different circulatory regimes (Marsh *et al.* 2007). In addition, the absence

of the CFB cluster from the LC deep site, implied a less effective diffusion of nutrients from overlying phytodetritus at the LC site, perhaps because of higher variability in the annual supply of nutrients from the sediment surface (West *et al.* 2008).

HC and LC deep sediment communities at both sites shared few similarities with their surface sediment counterparts (8.8% and 12.3% respectively). Intra-site community differences between sediment depths were greater than community differences between sites. Evidence for an intra-site trend in decreasing bacterial abundance with increasing sediment depth in addition to significant shifts in community composition, richness and evenness was attributed to a decrease in the quality and availability of organic carbon with depth (Danovaro *et al.* 2000; Parkes *et al.* 2000). The biogeochemical depth-related gradient that occurs in sediments, results in localized habitats for metabolically diverse bacteria (Koizumi *et al.* 2003). Depth-related changes in Crozet sediment biogeochemistry were reflected by lower richness and diversity of deep sediment communities compared to the surface. In addition, an increase in number of Gemmatimonadetes, *delta*-Proteobacteria, and Acidobacteria was observed in the deep sediment communities compared to the surface sediments and members of both the Planctomycetes and the CFB cluster were absent from the LC deep site. It was likely that many of the organisms present in the deeper communities favoured hypoxic or reduced conditions (Mussmann *et al.* 2005; Heijs *et al.* 2008). Of the major phylogenetic groups examined, the CFB cluster was primarily responsible for the large structural shift in evenness of the major groups observed with increasing sediment depth. A similar trend was described for Antarctic shelf sediments down to 21 cm sediment depth (Bowman *et al.* 2003). The depth-related decrease in abundance of this group might have resulted from a decline in the availability of labile organic matter. However, as reported elsewhere (Urakawa *et al.* 2000), the majority of depth-related changes in the Crozet sediment communities occurred as a result of changes in the relative abundance of the minor bacterial groups (*beta*-Proteobacteria, Acidobacteria, Gemmatimonadetes and Chloroflexibacter), rather than the overall assemblage composition. In Antarctic sediments, an abundance of Acidobacteria in deeper sediment layers suggested sulphidogenic activity (Bowman *et al.* 2003).

Productivity and substrate availability are documented as strong determinants of bacterial community composition in marine systems (Van Hannen *et al.* 1999; Polymenakou *et al.* 2005; West *et al.* 2008). Indeed, Bienhold *et al.* (2012) analyzed the link between phytodetritus input, diversity and activity of bacterial communities of

the Siberian continental margin (37–3427m water depth), they found that dominant bacterial taxa showed strong positive or negative relationships with phytodetritus input. However, in this study surface sediment bacterial communities from high chlorophyll and low chlorophyll sites were not significantly different to infer a direct correlation of bacterial abundance, community structure and composition with productivity. Rather, the high similarity between surface sediment bacterial communities from different productivity regimes reflected the stability of the deep-sea environment. Our results suggest that the extent to which POM regulates the abundance, structure and composition of deep-sea sediment bacterial communities may depend upon the quality of POM, in addition to the availability, which may be reduced by the enhanced presence of sea-bed macro and meio-fauna and the potentially slower response by bacteria to fresh POM during the early stages of POM deposition. This study is the first comparison of deep-sea sediment bacterial communities in relation to differences in overlying surface water productivity. However, further work is required in order to better understand the role of deep-sea bacterial communities in organic matter recycling and the processes that regulate their structure and composition.

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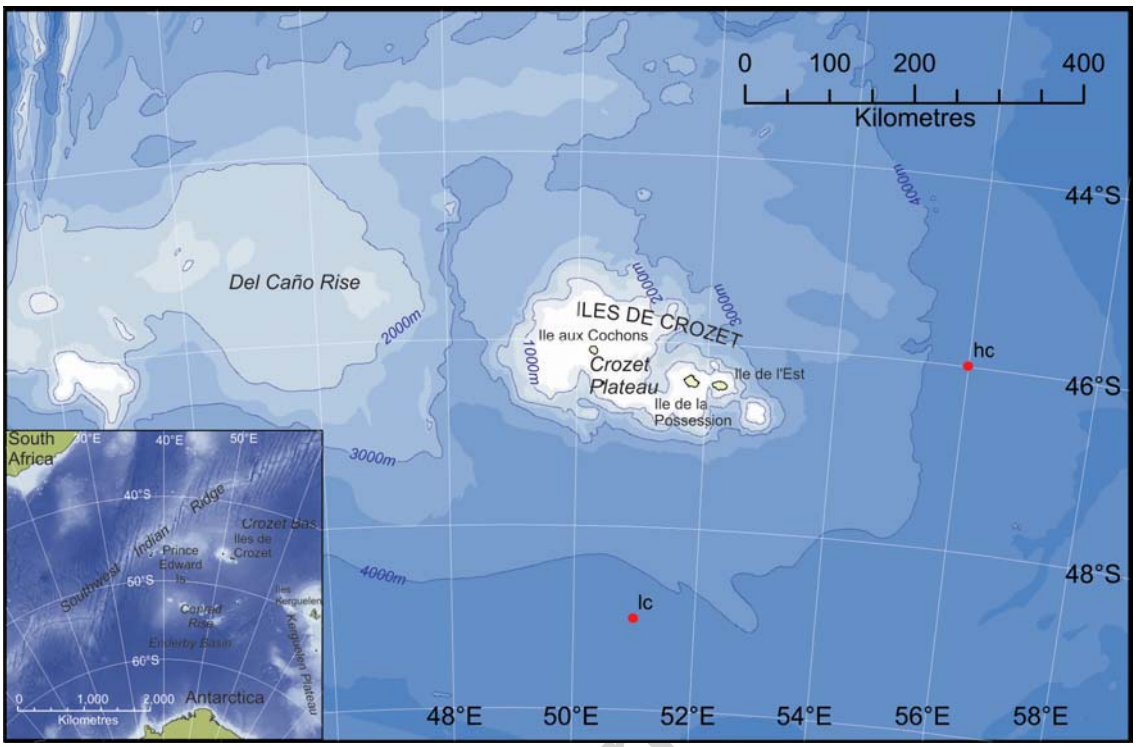


Figure 1. Study sites around the Crozet Archipelago. hc – high chlorophyll M5 site east of the islands in the region of the phytoplankton bloom. Lc – low chlorophyll M6 site south of the islands.

Highlights

- Bacterial sediment communities from high and low chlorophyll sites were compared.
- Bacterial population densities were consistent.
- 96% similarity was found across major phylogenetic groups.
- DGGE community profiles were consistent.
- One third of all clones identified were common both HC and LC sites.

Table

Clone library	HC surface [0-1 cm]	LC surface [0-1 cm]	HC deep [19-20 cm]	LC deep [19-20 cm]
Number of clones generated	594	636	167	169
Number of OTUs[a]	295	315	114	125
EMBL-BLASTN matches ≥ 97% identity	397	425	93	95
Chao1 species richness estimate	740	899	360	425
Simpson diversity index (D)[b]	0.99	0.99	0.99	0.99
Coverage of diversity (%)[c]	50.3	50.4	31.7	26.0
Compositional similarity (%) [d]	36.0		20.9	

[a] No. of unique sequences showing ≤ 97% identity with recovered sequences from this study
[b] 0 = homogenous; 1 = diverse
[c] Method of Good (1953)
[d] Sørensen's coefficient (Martin 2002)

Table 1. Comparison of total screened clones, number of OTUs, estimated total sequence richness, diversity and coverage for 16S rDNA clone libraries for sediment bacteria at two sediment depths from high surface chlorophyll (HC) and low surface chlorophyll (LC) regions near the Crozet Islands, Southern Ocean.

Band	Closest EMBL-EBI BLASTN matches [Accession #]	Isolation source	Sequence Identity (%)
A	Uncultured *Cytophaga* sp. clone BD7-10 [AB015585.1]	Deep-sea sediment	99
B	Uncultured *Cytophaga* sp. clone BD7-10 [AB015585.1]	Deep-sea sediment	99
C	Uncultured *gamma*-Proteobacterium clone JTB148	Japan Trench sediment	99
D	Uncultured *gamma*-Proteobacterium clone VHS-B1-32	Harbour sediment	98
E	Uncultured bacterium clone B78-47 [EU287011.1]	Arctic sediment	100
F	Uncultured bacterium clone P13-57 [EU287150.1]	Arctic sediment	99
G	Uncultured bacterium clone EPR3965-I2-Bc30	Sea-floor lava	99
H	Uncultured *Cytophaga* sp. clone JdFBGBact_79	Sea-floor basalts	97
I	Uncultured bacterium clone A20 [AY373407.1]	Deep-sea sediment	99
J	Uncultured Gram-positive bacterium clone BNT33-12	Cold-seep sediment	96

Table 2. Closest EMBL-EBI BLASTN matches to DGGE band sequences isolated from equivalent positions in the banding patterns of the HC and LC sediment profiles.

Probe	Specificity	Between sites	Between depths within sites
EUB338	Eubacteria	NS	SD (F= 111.3, DF 1, P < 0.0001)
GAM42a	*Gamma*-Proteobacteria	NS	SD (F= 16.9, DF 1, P = 0.003)
ALF968	*Alpha*-Proteobacteria	NS	SD (F= 80.4, DF 1, P < 0.0001)
CY319a	CFB cluster	NS	SD (F= 299.7, DF 1, P < 0.0001
PLA46	Planctomycetes	NS	SD (F= 183.2, DF 1, P < 0.0001)
ARCH915	Archaea	NS	SD (F= 32.3, DF 1, P < 0.0001)

Table 3. Results of ANOVA tests comparing abundance data for group-specific probes to identify significant differences between HC and LC sites and surface (0-1 cm) and deep (19-20 cm) samples. Where significant differences were detected, F-ratio (F), degrees of freedom (DF) and probability value (P) are given. NS = not significant, SD = significant difference

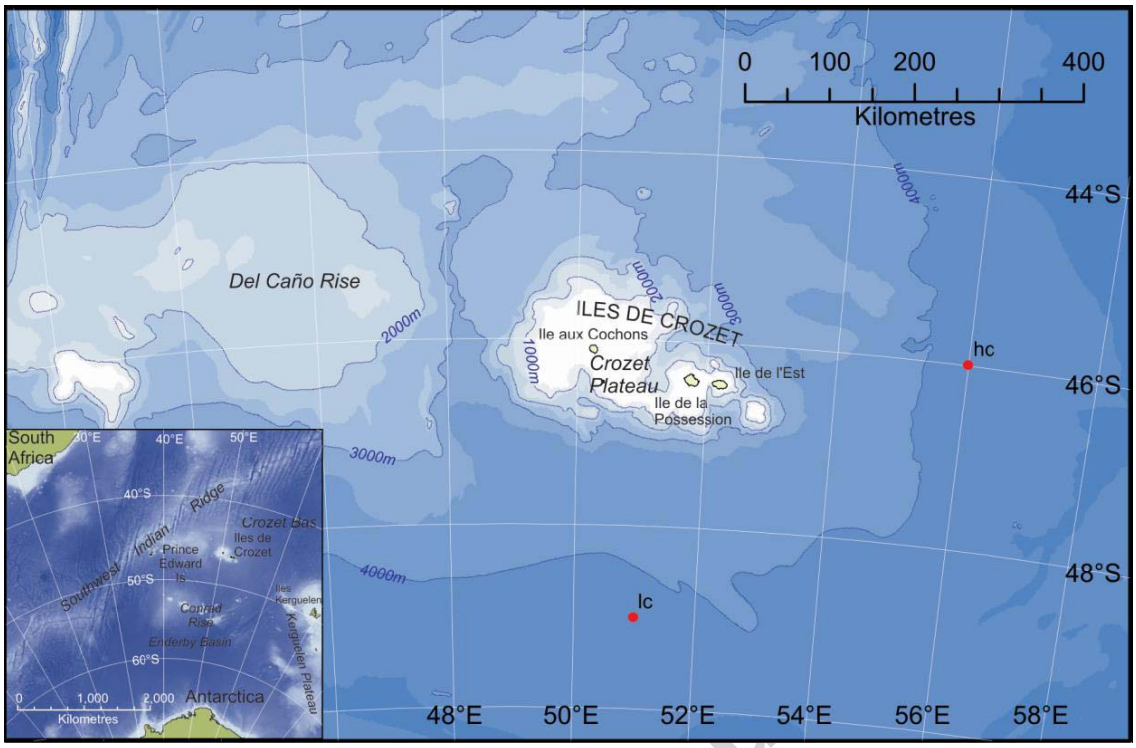


Figure 1. Study sites around the Crozet Archipelago. hc – high chlorophyll M5 site east of the islands in the region of the phytoplankton bloom. Lc – low chlorophyll M6 site south of the islands.

Figure 3

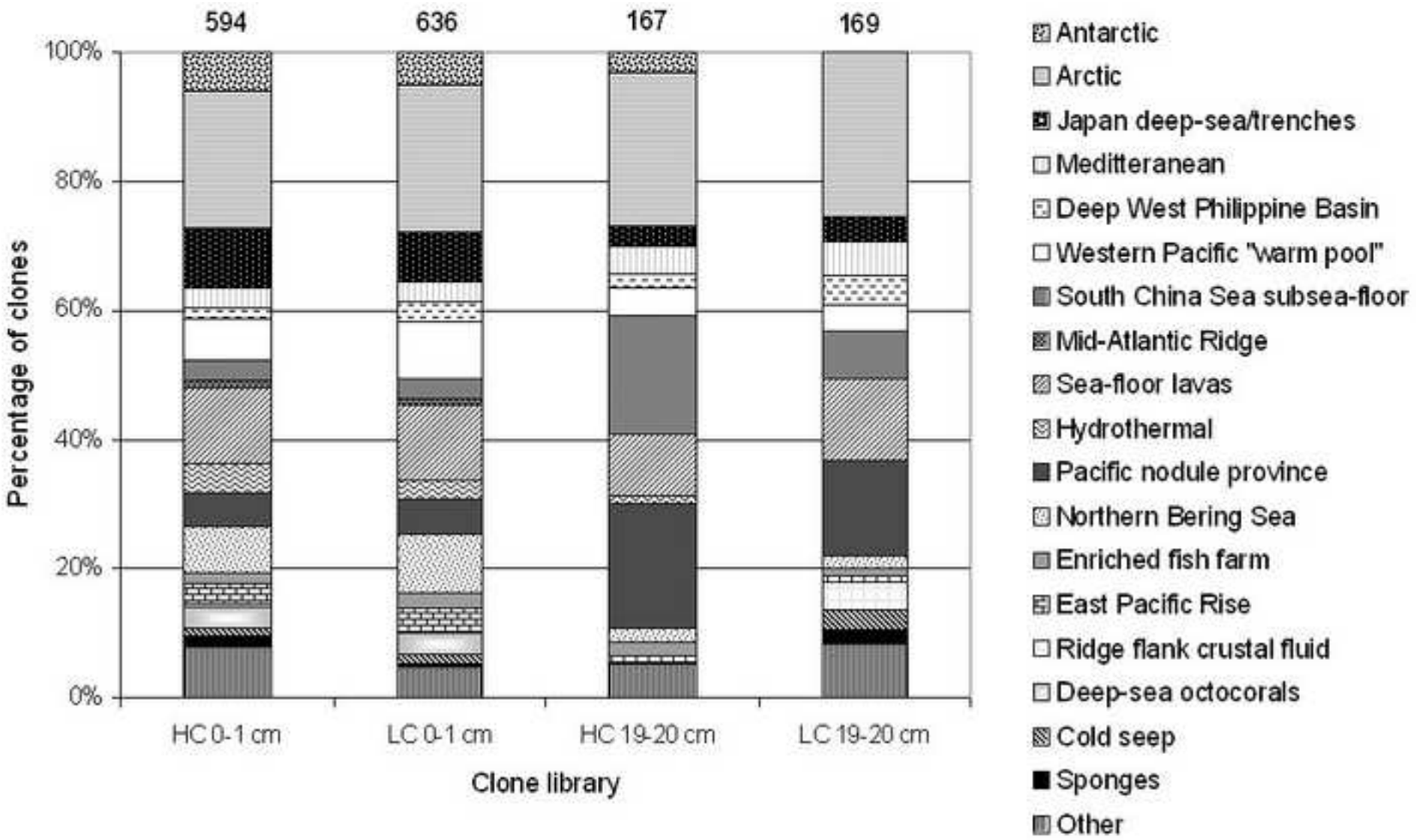


Figure 4a

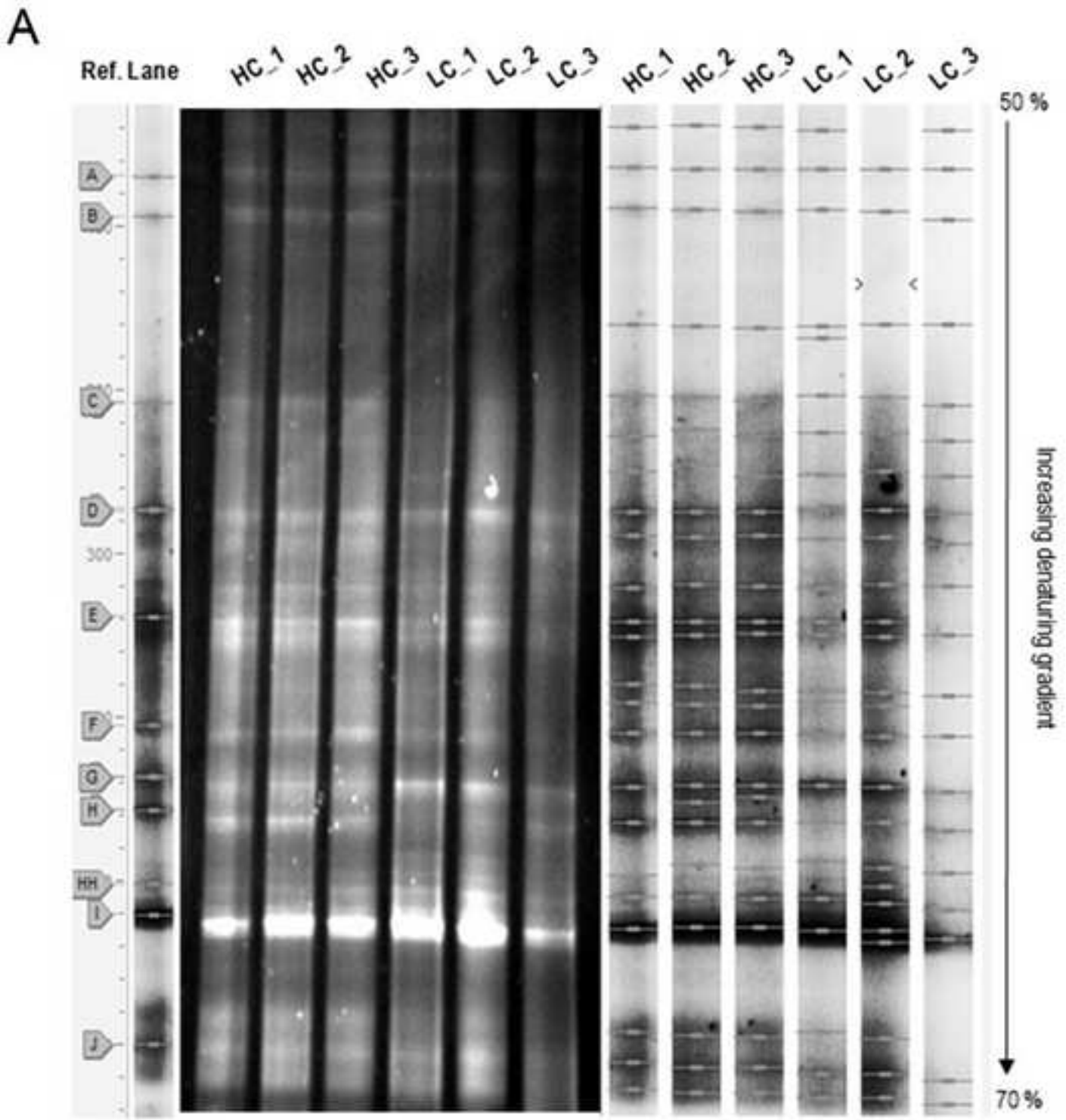
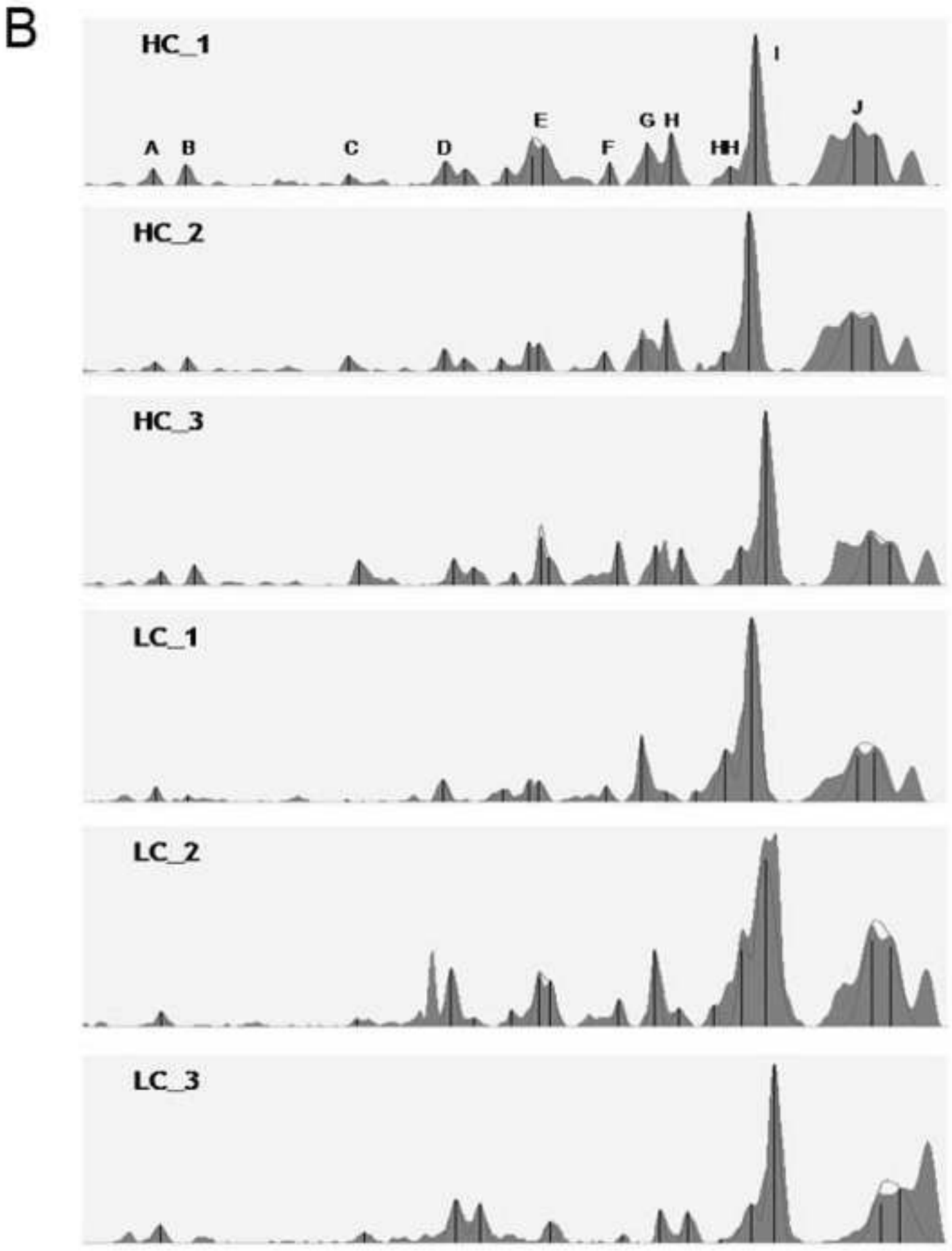


Figure 4b



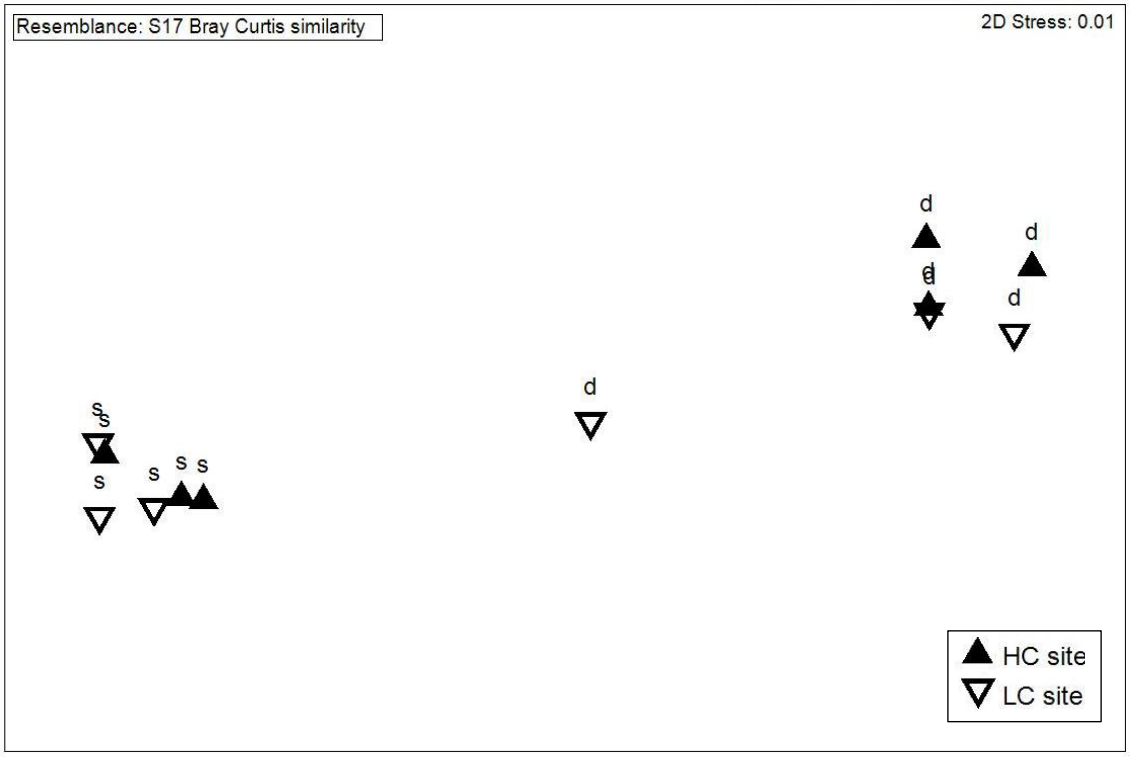


Figure 5. MDS ordination showing differences in the evenness of four bacterial phylogenetic groups (the *gamma*- and *alpha*-Proteobacteria, the CFB cluster and the Planctomycetes) between replicate samples (n=3) from HC and LC surface (s) and deep (d) sediments.

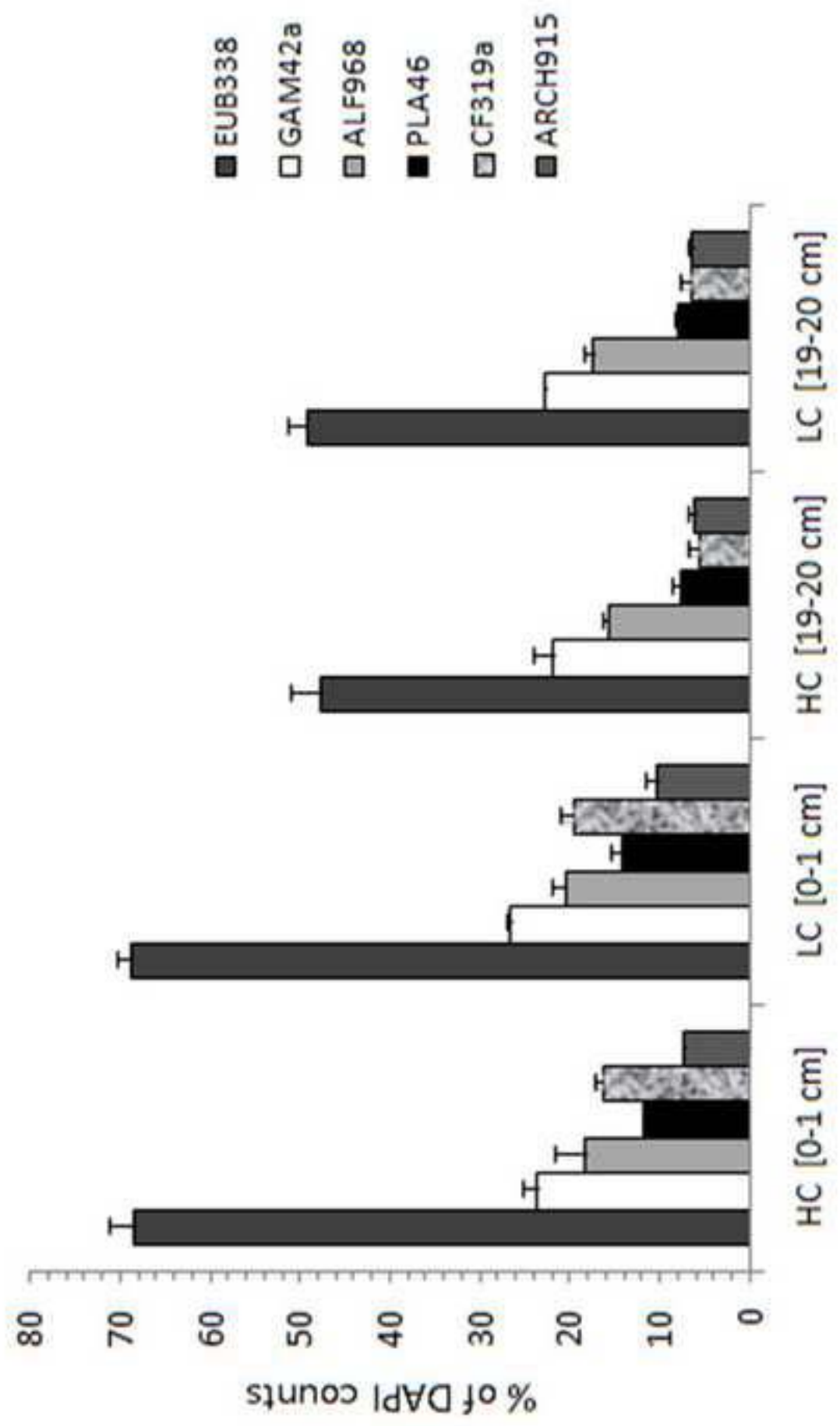
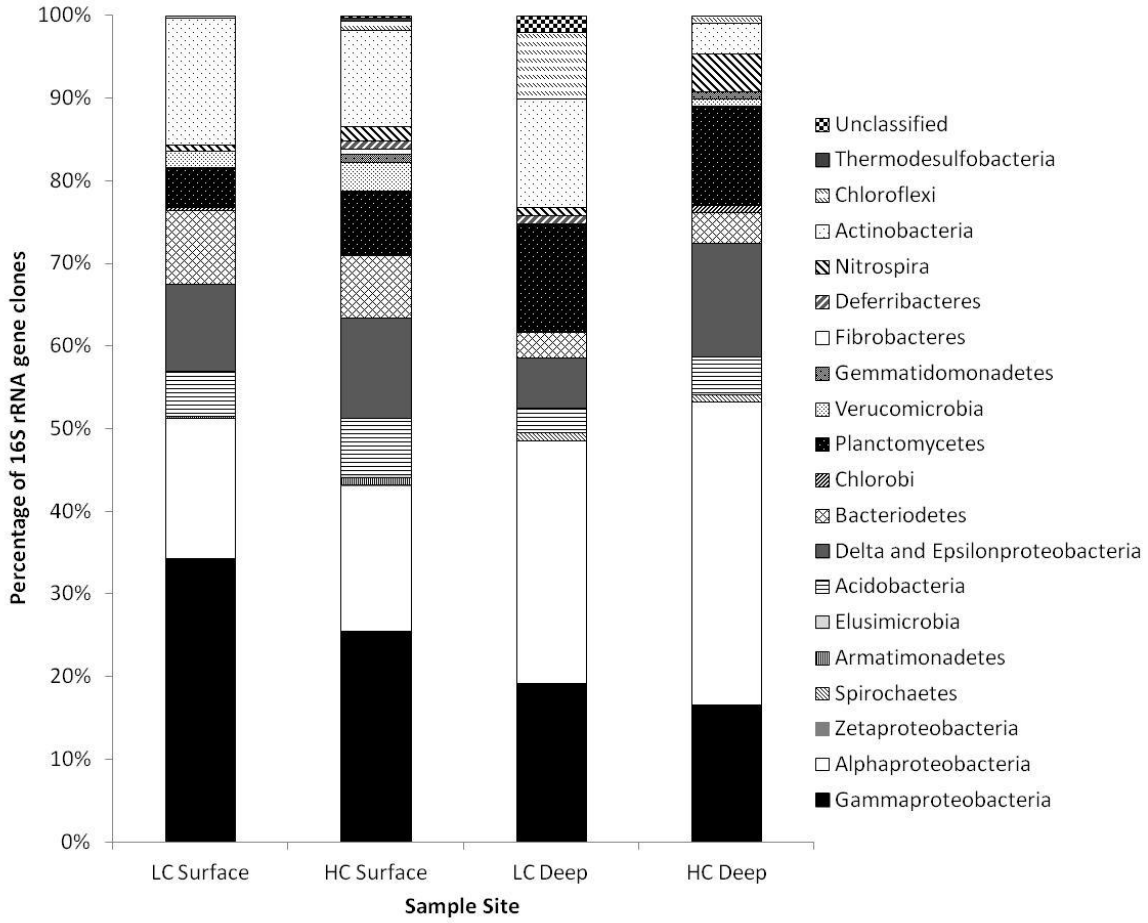

EUB338
GAM42a
ALF968
PLA46
CF319a
ARCH915
% of DAPI counts
0
10
20
30
40
50
60
70
80
HC [0-1 cm]
LC [0-1 cm]
HC [19-20 cm]
LC [19-20 cm]

Figure 6

Figure 2



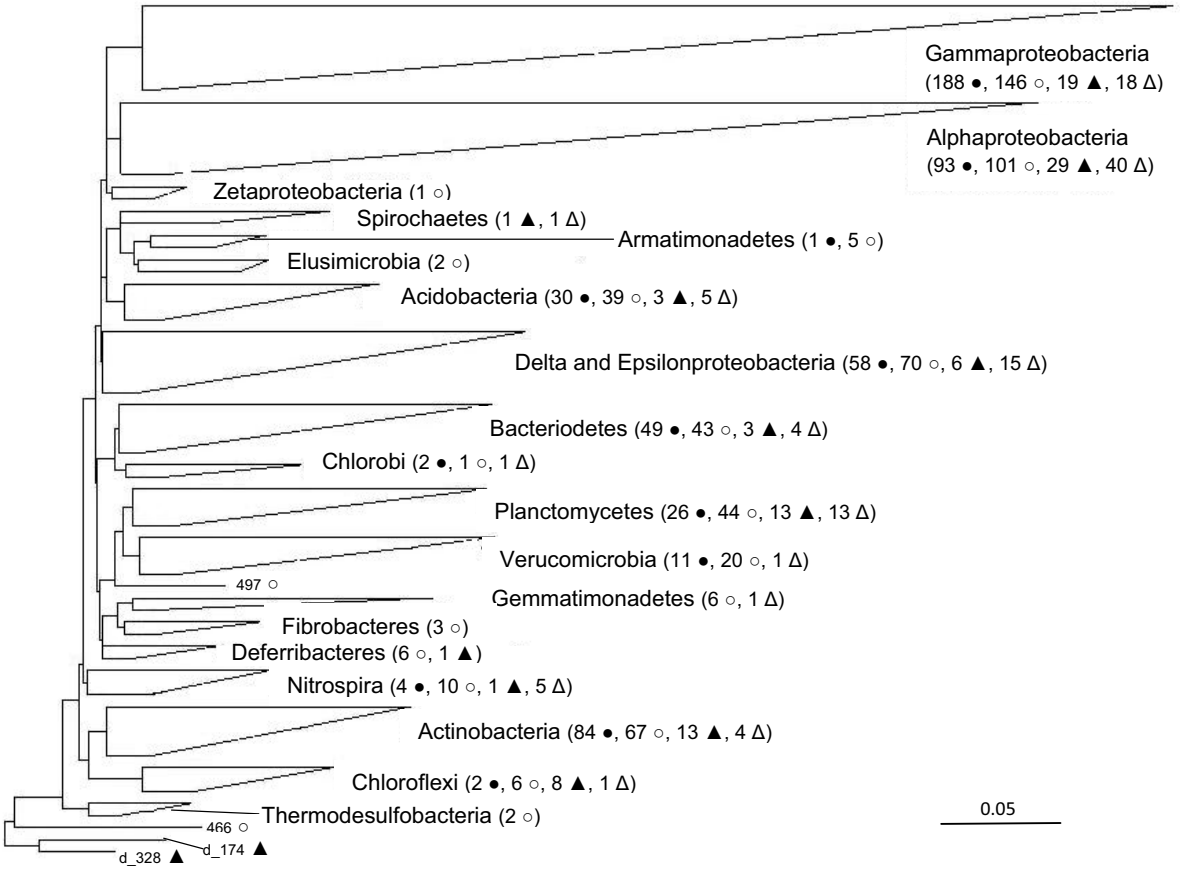


Figure Legends

Figure 1. Study sites around the Crozet Archipelago. HC – high chlorophyll M5 site east of the islands in the region of the phytoplankton bloom. LC – low chlorophyll M6 site south of the islands.

Figure 2a. Percentage distribution of uncultured group-affiliated bacterial sequences within sediment libraries: HC surface, LC surface, HC deep and LC deep.

Figure 2b. Phylogenetic tree summary of 16S rRNA gene sequences obtained from clone libraries showing the number of clones from each site in each phylogenetic group (○ LC surface; ● HC surface; Δ LC deep; ▲ HC deep). Scale bar represents units of sequence divergence.

Figure 3. Breakdown of clones in each library by original isolation source. 'Other' includes surface water, glacier, mud volcanoes, microbial mats, submerged steel, Baltic Sea and Gulf of Mexico sediments. Data compiled from EMBL-EBI sequence database. Number of clones in each library is shown above each bar.

Potentially move into supplementary information:

Figure 4A. Unprocessed and processed DGGE gels showing banding patterns for three HC and LC surface sediment (0-1 cm) replicate samples and reference lane. 50-70% denaturing gradient. Negative image shows automatically assigned dominant bands.

Figure 4B. Densitometric curves for the DGGE profiles of three replicate High Chlorophyll (HC) and Low Chlorophyll (LC) surface sediment samples. Vertical black lines within a peak indicate the presence of a band in the gel. Peak height corresponds to density.

Figure 5. MDS ordination showing differences in the evenness of four bacterial phylogenetic groups (the *gamma*- and *alpha*-Proteobacteria, the CFB cluster and the Planctomycetes) between replicate samples (n=3) from HC and LC surface (s) and deep (d) sediments.

Figure 6. Relative cell counts for each phylogenetic group as a percentage of the total number of DAPI-stained cells within surface (0-1 cm) and deep (19-20 cm) sediment layers from the HC (high chlorophyll) and LC (low chlorophyll) sites.