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Response of a summertime Antarctic marine bacterial community to glucose and ammonium enrichment

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ABSTRACT: Along the western Antarctic Peninsula, marine bacterioplankton respond to the spring phytoplankton bloom with increases in abundance, production and growth rates, and a seasonal succession in bacterial community composition (BCC). We investigated the response of the bacterial community to experimental additions of glucose and ammonium, alone or in combination, incubated in replicate carboys (each: 50 l) over 10 d in November 2006. Changes in bulk properties (abundance, production rates) in the incubations resembled observations in the nearshore environment over 8 seasons (2001 to 2002 through 2008 to 2009) at Palmer Stn (64.8°S, 64.1°W). Changes in bulk properties and BCC in ammonium-amended carboys were small relative to controls, compared to the glucose-amended treatments. The BCC in Day 0 and Day 10 controls and ammonium treatments were >72% similar when assessed by denaturing-gradient gel electrophoresis (DGGE), length heterogeneity polymerase chain reaction (LH-PCR) and capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) fingerprinting techniques. Bacterial abundance increased 2- to 10-fold and leucine incorporation rates increased 2- to 30-fold in the glucose treatments over 6 d. The BCC in carboys receiving glucose (with or without ammonium) remained >60% similar to that in Day 0 controls at 6 d and evolved to <20% similar to that in Day 0 controls after 10 d incubation. The increases in bacterial production rates, and the changes in BCC, suggest that selection for glucose-utilizing bacteria was slow under the ambient environmental conditions. The results suggest that organic carbon enrichment is a major factor influencing the observed winter-to-summer increase in bacterial abundance and activity. In contrast, the BCC was relatively robust, changing little until after repeated additions of glucose and prolonged (~10 d) incubation.

KEY WORDS: Antarctica · Bacterial community composition · Bioassay · Marine bacterioplankton

INTRODUCTION

Heterotrophic bacteria in oceanic ecosystems (i.e. remote ecosystems, or those isolated from terrestrial inputs of nutrients and organic matter) are predominantly regulated by bottom-up processes emanating from *in situ* primary production of organic matter (Church 2008, Nagata 2008, Robinson 2008). Correlations of bacterial and phytoplankton activity (Cole et al. 1982, Billen et al. 1990) and bioassay studies (Carlson & Ducklow 1996, Church et al. 2000) point toward organic enrichment as the principal factor governing increases in bacterial activity in the marine pelagic environment. Some studies have implicated phosphorus limitation of organic matter utilization (Rivkin & Anderson 1997, Thingstad et al. 1997), but without the

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organic matter supply any increment in bacterial growth would be constrained to that allowed by increased growth efficiency (del Giorgio & Cole 2000). Control of bacterioplankton production is more variable in lakes, but inputs of internal (Cole et al. 1989) or exogenous organic matter (Judd et al. 2006) control the magnitude of bacterial production in many instances.

In polar seas, plankton and microbial communities experience large seasonal changes in environmental conditions which, in turn, lead to large corresponding changes in ecological and biogeochemical properties of the marine ecosystem, including microbial populations (Karl 1993, Karl et al. 1996). A prolonged wintertime period of severely light-limited to nonexistent phototrophic primary production is followed in the spring by large phytoplankton blooms that enrich the upper water column with particulate and dissolved organic matter (Billen & Becquevort 1991, Carlson et al. 2000, Smith et al. 2000). The abundance and activity of heterotrophic bacteria are very low in winter (Murray & Grzymski 2007) but they increase by an order of magnitude, or more, in response to the vernal enrichment process (Kottmeier & Sullivan 1987, Rivkin 1991, Ducklow et al. 2001). Comprehensive and quantitative documentation of all these bulk-system-level processes now exists for many Arctic and Antarctic locations (Pomeroy et al. 1990, Kirchman et al. 2005, Ducklow & Yager 2006, Ducklow et al. 2007, Garneau et al. 2008), showing unequivocal spring–summer maxima and winter minima in bacterial stocks and activity. Yet, in spite of broad agreement about the seasonal patterns, there is little consensus regarding the interplay of biotic and other environmental factors driving the annual pattern of heterotrophic microbial activity in polar seas (Pomeroy & Wiebe 2001, Kirchman et al. 2009).

It is now commonly assumed that the bacterial community composition (BCC) influences seasonal changes in the magnitude of carbon cycling through bacterioplankton (Fuhrman 2009). However, establishing causal relationships is challenging. For example, Alonso-Sáez et al. (2008) concluded that there was no relationship between shifts in the BCC and carbon cycling over the annual cycle at the Blanes Bay Microbial Observatory (BBMO) in the coastal NW Mediterranean Sea. Although still sparse, there is increasing information about the possible community-level shifts in bacterial taxa and their metabolic potentials underlying the bulk changes in bacterial biomass and production in polar waters (Murray & Grzymski 2007). Such information is necessary for understanding the ecological mechanisms responsible for seasonal variations in bacterial carbon cycling. In the seasonally illuminated layer (the upper 50 to 100 m) of Antarctic coastal seas, members of the domain *Bacteria* dominate the assemblage year-round, while members of the *Archaea* vary between 2% (in summer) and 13% (in winter) of the total picoplankton abundance (Murray et al. 1998, Church et al. 2003). Murray et al. (1998) demonstrated a strong, repeatable seasonal cycle of the BCC at Palmer Stn, Antarctica, and later showed that the late winter community comprised — in about equal proportions — *Alphaproteobacteria*, *Gammaproteobacteria* and members of the *Cytophaga-Flavobacteria-Bacteroidetes* (CFB) group (Murray & Grzymski 2007). These same 3 groups also dominated the midsummer bacterial assemblage, each group contributing about one-third of the total standing stock of bacteria (Straza et al. 2010). However, how these changes are related to the parallel variability of bacterial carbon cycling is not known.

The western Antarctic Peninsula region has one of the most rapid rates of climate warming on the planet, with surface air and ocean temperatures having risen by 6°C and 1 to 2°C, respectively, since 1950 (Smith et al. 1996, Vaughan et al. 2003, Meredith & King 2005). The marine ecosystem has responded to the rapid regional climate warming with changes throughout the foodweb from phytoplankton to predators (Ducklow et al. 1995, Montes-Hugo et al. 2009). Microbes in polar seas have also been identified as being potentially vulnerable and sensitive to anticipated climate and ecosystem changes (Morán et al. 2006, Kirchman et al. 2009, Ducklow et al. 2010).

In 2006, we initiated a series of experiments designed to follow how bulk properties (abundance, production, growth rates) and the BCC changed in response to systematic, repeated substrate amendments in large-volume seawater cultures over the course of the annual cycle at Palmer Stn, Antarctica. We amended otherwise untreated seawater and its ambient microbial assemblages with glucose and/or ammonium and followed bulk assemblage properties and the BCC over approximately 10 d. Here we report on the results of a late-spring/early-summer experiment and relate the observed changes in the incubations to observed changes in the ambient waters at the sampling site.

MATERIALS AND METHODS

Study location, sampling and experimental design. Sampling took place in the nearshore waters of Arthur Harbor at Palmer Stn, on Anvers Island, west of the Antarctic Peninsula. Pelagic processes at this location have been well characterized by the Palmer, Antarctica, Long Term Ecological Research Project (PAL LTER) since 1993 (Ross et al. 1996, Ducklow 2008). This region is within the marginal sea ice zone and is characterized by seasonally high biological

productivity despite perennially cold waters (less than 2°C).

Water samples were collected from nearshore LTER Stn B (64.77° S, 64.07° W) on November 3, 2006. Eight carboys (volume of each: 50 l) were acid-cleaned (10% HCl), rinsed 3 times each with Milli-Q water and seawater, and filled with seawater from a depth of 10 m using a submersible Geo-Squirt peristaltic pump (Geotech) and acid-washed silicone tubing. Samples were transferred to a cold room in the laboratory within 1 h of sampling. Following an initial 24 h acclimation period in the laboratory, duplicate carboys were enriched on Days 1, 4 and 8 with organic carbon (+100 µM C as glucose) and nitrogen (+25 µM N as ammonium, NH_4^+), separately and in combination, i.e. +glucose, +NH_4^+, and dual enrichment (+glucose and +NH_4^+) treatments. Two unamended carboys served as experimental controls. All 8 carboys were incubated in the dark at +1°C in a cold room and sampled daily with acid-cleaned, sample-rinsed silicon tubing and a peristaltic pump.

Bulk properties. Samples for bacterial abundance were preserved with 2% formaldehyde and frozen at −80°C until analysis. Bacterial abundance was determined using a Becton-Dickinson FacsCalibur flow cytometer (Gasol & del Giorgio 2000). Samples for bulk dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were stored in 60 ml, acid-washed polyethylene bottles and frozen at −20°C until analysis. DOC and TDN were measured with a Shimadzu TOC-V high-temperature combustion total carbon analyzer and TDN module following Sharp et al. (1993) and Farmer & Hansell (2007). Bacterial production rates were determined immediately after sampling. Bulk ^{3}H-leucine (Leu) incorporation rates and cell-specific ^{3}H-thymidine incorporation rates were used as indices of bacterial production rates and specific growth rates, respectively (Fuhrman & Azam 1980, Kirchman et al. 1985).

Nucleic acid extraction and polymerase chain reaction (PCR) of 16S rRNA genes. Whole-community genomic DNA was collected from the carboys by passing seawater (2 l) through Sterivex filters of pore size 0.22 µm (Millipore). The filter cartridges were stored in 1.8 ml of sucrose lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) at −80°C until further analysis. The DNA sample from 1 carboy of each treatment was used for the analysis of community structure. DNA samples were collected on Days 0 (pooled sample from the 4 carboys), 2, 6 and 10.

Extraction of nucleic acids from Sterivex cartridges was performed according to Murray et al. (1998), with slight modification. Filter cartridges were incubated at 37°C for 45 min with freshly prepared lysozyme (50 mg ml^{-1}) added to the lysis buffer, then at 55°C for 2 h after adding freshly prepared proteinase K (10 mg ml^{-1}) and 2% sodium dodecyl sulfate (SDS). Crude lysates were purified twice by extraction with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, pH 8.0), then twice with an equal volume of chloroform–isoamyl alcohol (24:1). The nucleic acids were precipitated using a one-tenth volume of sodium acetate (3.0 M, pH 5.2) and twice the volume of cold 100% ethanol. Pellets were washed with 70% ethanol before resuspension in 50 to 100 µl of sterile water. DNA concentrations were determined using the quantitative Picogreen assay (Molecular Probes).

The same DNA template was used in independent PCRs for the 3 community fingerprinting methods, as previously described for denaturing-gradient gel electrophoresis (DGGE) (Murray et al. 1996), length heterogeneity PCR (LH-PCR) (Suzuki et al. 1998) and capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) (Ghiglione et al. 2005). About 10 to 100 ng of extracted DNA were used as a template in each PCR reaction. PCR conditions relating to each fingerprinting method, including denaturing, annealing and extension temperatures, number of cycles, and polymerase applied, are summarized in Table 1. All PCR products were checked for proper amplification by agarose gel electrophoresis and again quantified using the Picogreen assay.

Community fingerprints: DGGE electrophoresis was performed with an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.5:1) with a 30 to 70% denaturing gradient. Approximately 500 to 800 ng of PCR product from each sample were obtained by combining several PCR reactions, then loaded into individual lanes in the gel. Gels were run for 1200 V h^{-1} in 1× TAE buffer (40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA). The gels were stained for 12 to 15 min in 1× TAE with SYBRGold nucleic acid stain (Molecular Probes), visualized under ultraviolet illumination, and photographed. Images were analyzed using GelCompare II software (Applied Maths).

LH-PCR electrophoresis was performed on an ABI 3130 4-capillary Genetic Analyzer (Applied Biosystems). For LH-PCR analysis, samples were denatured in an 8:1.5:0.5 mixture of formamide, 5 ng µl^{-1} PCR products, and a GeneScan 400-HD ROX internal size standard. Sample mixtures were heated for 3 min at 94°C, immediately placed on ice for several minutes, and loaded on the sequence analyzer. Electropherograms were analyzed using the PeakScanner software package (Applied Biosystems). LH-PCR profiles were analyzed using local Southern size calling, no peak correction, and a threshold of 25 fluorescence units.

CE-SSCP analysis was performed on an ABI 310 Genetic Analyzer (Applied Biosystems), as previously described (Ghiglione et al. 2005). Briefly, 1 µl (about

Table 1. Primers and PCR conditions applied in this study. *Taq* DNA polymerase and buffers (Fisher-Biotech) were used for denaturing-gradient gel electrophoresis (DGGE) and length heterogeneity polymerase chain reaction (LH-PCR); *Pfu* blunt-ended polymerase and buffers (Promega) were used for capillary electrophoresis single-strand conformation polymorphism (CE-SSCP)

Primer[a]	Sequence (5′–3′)	Annealing temp. (°C) and # PCR cycles[e]	Specificity	Source
DGGE				
GC-358F[b]	CCT ACG GGA GGC AGC AG	Touchdown 65 to 55°C	Eubacteria	Murray et al. (1996)
517R	ATT ACC GCG GCT GCT GG	25 cycles	Universal	Murray et al. (1996)
LH-PCR				
27F[c]	AGA GTT TGA TCM TGG CTC AG	55°C	Eubacteria	Suzuki et al. (1998)
338R	GCT GCC TCC CGT AGG AGT	25 cycles	Eubacteria	Suzuki et al. (1998)
CE-SSCP				
329F	ACG GTC CAG ACT CCT ACG GG	61°C	Eubacteria	Delbès et al. (1998)
533R[d]	TTA CCG CGG CTG CTG GCA C	25 cycles	Universal	Lee et al. (1996)

[a]Primer names incorporate the number corresponding to the16S gene position for *Escherichia coli*
[b]GC clamp sequence: CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC C
[c]Fluorescently labeled at the 5′-end position with hexachlorofluorescein (HEX, Applied Biosystems)
[d]Fluorescently labeled at the 5′-end position with phosphoramidite (TET, Applied Biosystems)
[e]All protocols implemented 3 min initial denaturing at 94°C and 5 min final incubation at the extension temperature (72°C)

10 ng) labeled PCR amplicons was mixed with 18 µl of formamide (Applera) and 1 µl of an internal size standard GeneScan-400 Rox (Applied Biosystems). The mixture was then denatured for 5 min at 94°C and immediately cooled on ice for at least 5 min. Samples were electrokinetically injected (5 s, 12 kV) in a capillary tube (47 cm × 50 µm) filled with a mixture composed of 5.6% GeneScan polymer (Applied Biosystems) and 10% autoclaved glycerol in sterile TBE buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). Electrophoresis was carried out at 15 kV and 30°C for 30 min per sample and phosphoramidite (TET)-labeled amplicons were detected by a laser with a virtual filter C (detection wavelengths 532, 537 and 584 nm). Data were collected with ABI Prism 310 collection software (Applied Biosystems). In order to normalize mobilities from different runs, all electropherograms were calibrated by fixing the positions of peaks produced by the size standard and by using a second-order least-square curve (i.e. linear regression) to provide the best interlane comparison (Genescan analysis software, Applied Biosystems). Peak detection was achieved by computing the first derivative of a polynomial curve fitted to the data within a window that was centered on each data point (Genescan analysis software). The area of each peak was determined by taking into account the full width of the peak measured at half of its height. The relative area of each peak to the total peak area of each pattern was expressed as a percentage to allow inter-sample comparisons (Ghiglione et al. 2005).

***In silico* LH-PCR fragment identification:** Putative taxonomic identities were assigned to LH-PCR fragment lengths using a custom Antarctic 16S rRNA gene sequence data set (Table 2). The custom data set was created by combining 8 clone libraries generated throughout the year from nearshore water collected in the same area near Palmer Stn. Briefly, clone libraries were constructed using 27F and 1391R primers and following the standard operating procedures of the Joint Genome Institute adopted from Ley et al. (2006). A non-redundant library of 1018 Antarctic 16S rRNA gene sequences was formed by clustering 5573 sequences using DOTUR (Schloss & Handelsman 2005) at 99% sequence similarity. Sequences were trimmed *in silico* based on the primers used during LH-PCR analysis (27F & 338R) and binned according to predicted fragment length. Predicted taxonomic assignments for each fragment length bin were made based on phylogenetic relationships to close relatives identified by BLAST (Altschul et al. 1990) and Greengenes (DeSantis et al. 2006), and using sequence-based numerical abundance data of the most representative clusters.

Data analysis. Differences in bacterial abundance and production within and between experimental treatments were determined by analysis of variance (ANOVA). Two-way repeated measures ANOVA was performed using Prism 5 (GraphPad Software) on bacterial abundance and production data from each phase of the experiment with corresponding Bonferroni post hoc tests.

Bacterial biomass was calculated from abundance measurements using 20 fgC cell^{-1}. Bacterial production rates were calculated from leucine incorporation rates assuming 1.5 kgC per mole leucine incorporated (Ducklow et al. 2000). Specific growth rates µ (d^{-1}) for the full bacterial assemblage were calculated by assuming that all cells took up thymidine, and using a

Table 2. Phylogenetic assignment of length heterogeneity polymerase chain reaction (LH-PCR) fragments generated from *in silico* analysis of a custom Antarctic data set (α = *Alpha*-, β = *Beta*-, γ = *Gamma*-, δ = *Deltaproteobacteria*; CFB = *Cytophaga-Flavobacteria-Bacteroidetes*)

LH-PCR fragment length (bp)	Taxonomic assignment	Family/genus
316	α	*Roseobacter* and SAR11
317	α	SAR11 (*Pelagibacter*)
318	α	*Rhodobacter*
319	Plastid and α	Diatom
320	Plastid	
321	Unknown	
322	Unknown	
323	Plastid	
324	Plastid	
325	Plastid and α	
326	α	Uncultivated
327	α	*Magnetospirillum*; uncultivated sulfur-oxidizer
338	γ	SAR93
339	γ	*Pseudomonas*
341	γ	SAR86
	β	*Nitrosospira*
343	δ	SAR324
	γ	Uncultivated *Oceanospirillales*
344	CFB	*Polaribacter*
345	γ	Uncultivated Ant10A4; OM182; others
	CFB	Uncultivated *Flavobacteria*
346	CFB	*Psychroserpens*, *Polaribacter*, uncharacterized uncultivated; uncultivated *Flexibacter*
347	γ	SUP05
	CFB	Uncultivated *Cytophaga*, *Polaribacter*
348	CFB	Uncultivated *Flavobacteria*
349	γ	Uncultivated Ant4D3
350	δ	Nitrospinaceae; *Magnetoglobus*
359	γ	Uncultivated
360	γ	Uncultivated

thymidine conversion factor (CF) of 2×10^{18} cells produced per mole of thymidine incorporated (Fuhrman et al. 1980):

$$\mu = (\text{thymidine incorporation rate} \times CF)/\text{abundance}$$

Data on ambient water column properties, seasonal cycles of bacterial abundance and leucine incorporation can be accessed at: http://oceaninformatics.ucsd.edu/datazoo/data/pallter/datasets.

Comparative analysis of LH-PCR, CE-SSCP and DGGE fingerprints was carried out with the PRIMER 5 software (PRIMER-E). Hierarchical agglomerative clustering of Bray–Curtis similarities among normalized sample profiles was performed using the unweighted-pair-group method with arithmetic averages (UPGMA) from a matrix taking into account the presence or absence of individual peaks or bands and the relative contribution of each peak or band (in percentage) to the total intensity of each pattern.

RESULTS

Environmental context

Fig. 1 shows the average bacterial abundance, leucine incorporation rates and semilabile dissolved organic carbon concentration in surface waters at Palmer Stn B for October 2002 through March 2009, presented as a single seasonal cycle (climatology). On average, mean bacterial abundance is stable for about 6 wk, then increases steadily from December to March. Leucine incorporation rates (a proxy for bacterial production rate) increase to a midsummer peak in January to February, then decline toward the springtime values by late March. Abundance and production levels in the sampling year 2006 to 2007 were mostly typical of the mean values, with a few larger differences. The values on the sampling day for this experiment (3 November) were very close to the mean values (Fig. 1, triangles). In spring 2006 (October to November), both abundance and leucine incorporation rates were stable, varying by 1 to 2×10^8 cells l^{-1}, and by <5 pmol l^{-1} h^{-1}, respectively. In both the long-term average and the period 2006 to 2007, these values started to increase only in early to mid-December. In the period December 2006 to January 2007, abundance and activity exhibited variability of ~3 to 6×10^8 cells l^{-1}, and 10 to 40 pmol l^{-1} h^{-1}, over a time scale of about 10 d. The semilabile DOC (Fig. 1C; i.e. the concentration above the refractory deep water background concentration of 39 µmol C l^{-1}) averaged 13 µmol C l^{-1} over the entire sampling season, and varied by 10 to 50 µmol C l^{-1} from day to day (Fig. 1C, dots). In the period 2006 to 2007 the daily variations in the experimental period were ~5 to 10 µmol C l^{-1} (open triangles).

Incubation results: bulk bacterial properties and dissolved C and N

At the start of the experiment, bacterial abundance, leucine incorporation rates and DOC concentrations were within the observed ranges for ambient levels at the time of sampling (Figs. 2 to 4). Background DOC concentrations remained constant in the control and ammonium-treated carboys over the entire duration of

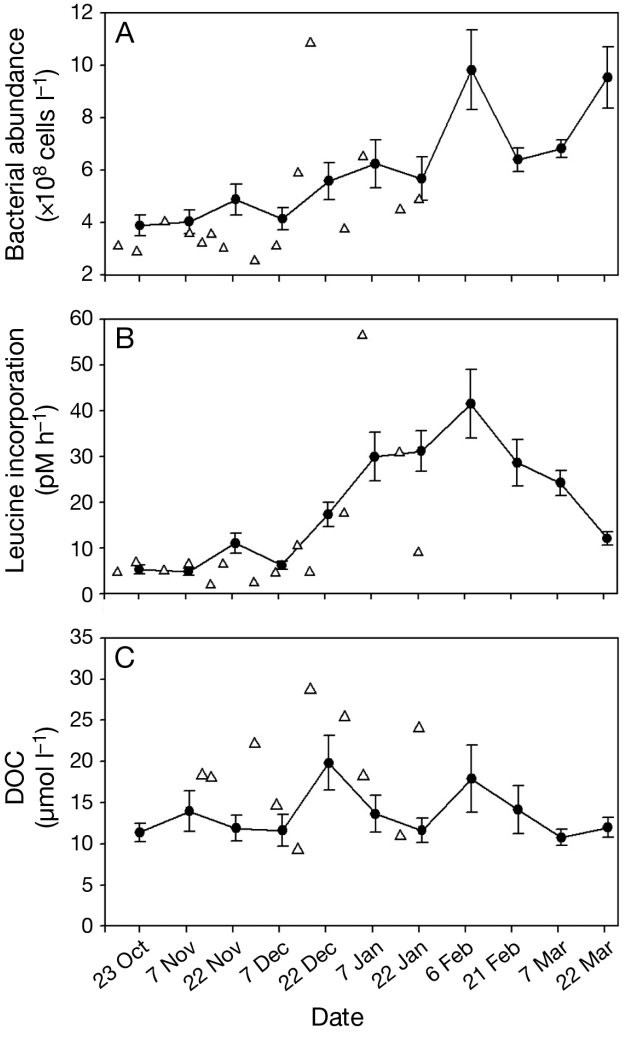

Fig. 1. (A) Bacterial abundance, (B) leucine incorporation rate, and (C) semilabile dissolved organic carbon (DOC) (surface concentration with the deepwater background concentration of 39 µM subtracted). Lines and closed circles refer to (long-term average) bulk surface bacterial properties for Palmer Stn site B, ± standard error of the mean. Open triangles in each plot are the observed data for 2006 to 2007 (i.e. not averaged). Observations were binned and averaged for intervals of 15 d over the October sampling seasons from October 2002 through March 2009. Not every year had full coverage. Dates on the *x*-axis are midpoints of the binned intervals

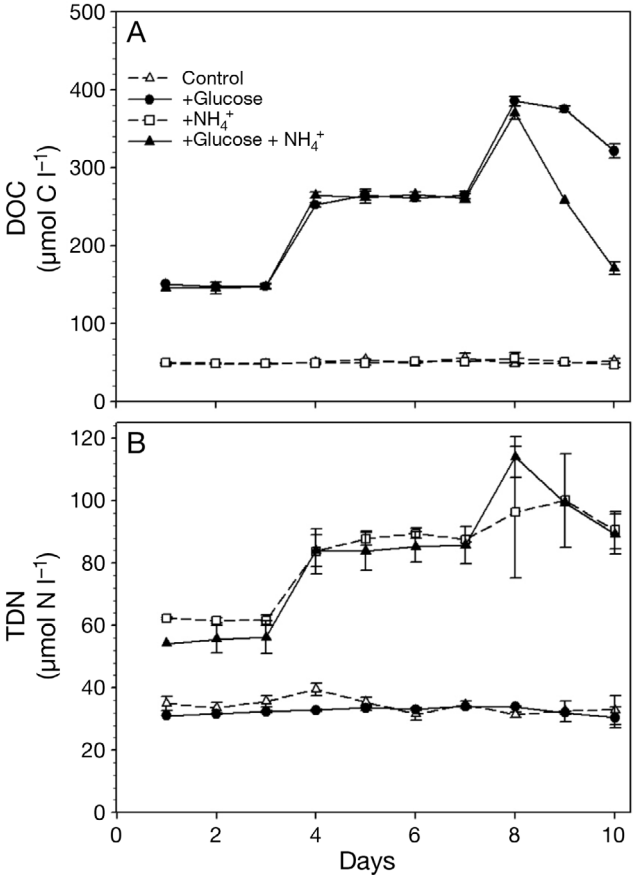

Fig. 2. (A) Dissolved organic carbon (DOC) concentrations and (B) total dissolved nitrogen (TDN) concentrations in experimental carboys. Glucose and/or NH_4^+ were added on Days 1, 4 and 8. Symbols are the means of experiments from 2 carboys. Error bars are standard error of the means

the incubations (Fig. 2A). Background TDN concentrations (nitrate plus DON, see 'Discussion') remained constant in the control and +glucose carboys (Fig. 2B). Following the addition of glucose, the DOC in the +glucose and dual enrichment (+glucose and +NH_4^+) carboys was 145 to 150 µmol C l^{-1}, as expected from the calculated enrichments. The TDN increased by 25 µmol N l^{-1}, also as expected.

Over Days 0 to 4, the initial addition of glucose carbon was not accompanied by a corresponding increase in bacterial biomass (Fig. 3A,B), but it did enhance bacterial production by a factor of about 2 (Fig. 4A, $p < 0.05$). Bacterial abundance increased slightly, from 4 to 7 × 10^8 cells l^{-1} over the first 4 d of incubation in all carboys (Fig. 3A), equivalent to an increase in biomass of about 0.5 µmol C l^{-1} (Fig. 3B). These levels are characteristic of the late-spring, early-summer period (Fig. 1A). Thereafter, abundance remained constant or declined in all carboys until Day 8. Time (rather than treatment) was the main source of variation for both abundance and activity during Days 0 to 4, explaining 80% ($p < 0.0001$) and 81% ($p = 0.0006$) of the total variation, respectively.

After the second additions of substrate on Day 4, the concentrations of DOC and TDN stayed constant until the third addition on Day 8. Bacterial abundance decreased ($p < 0.05$) in all mesocosms after the second additions of glucose; however, the bulk abundance and production also began to diverge in response to the different treatments. The bacterial population in the +NH_4 treatment declined less rapidly and was signifi-

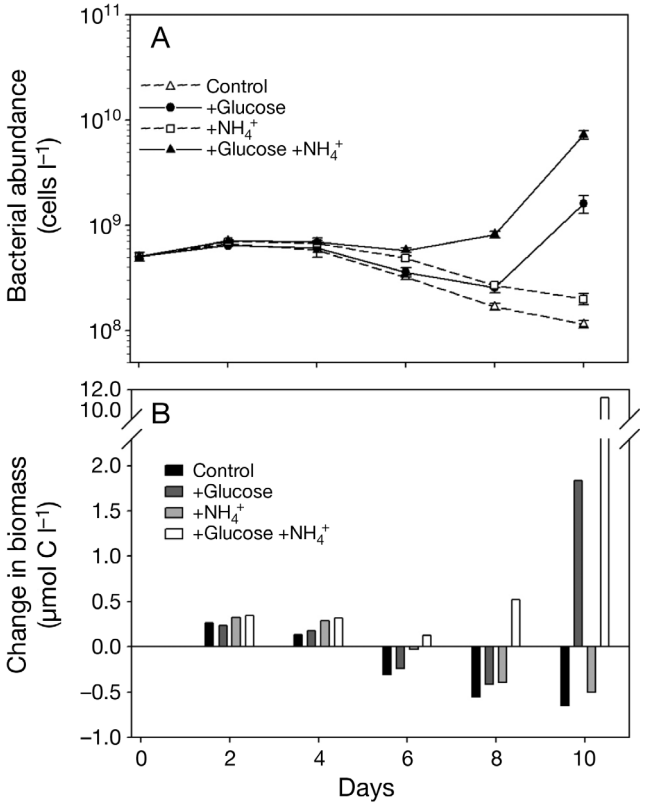

Fig. 3. (A) Bacterial abundance and (B) bacterial carbon biomass change from Day 0 in experimental carboys. Note that panel A is plotted on a log scale to emphasize the changes through Day 8

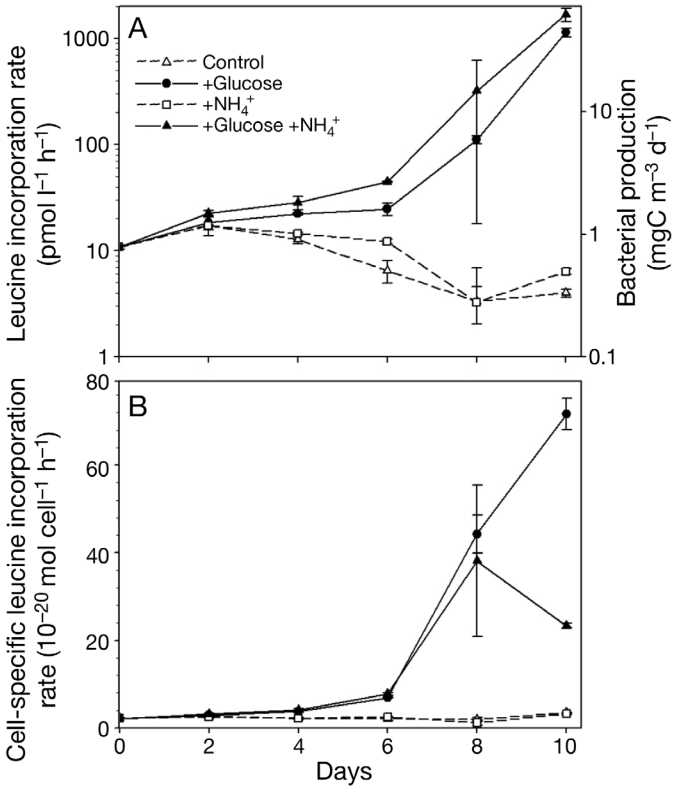

Fig. 4. (A) Rates of leucine incorporation (left axis) and equivalent bacterial production (right axis), and (B) cell-specific leucine incorporation in experimental carboys. Note that panel A is plotted on a log scale to emphasize the changes through Day 6 and the lower degree of change in control and $+NH_4^+$ treatments

cantly elevated compared to control mesocosms on Day 8 ($p < 0.01$). In the dual enrichment (+glucose and $+NH_4^+$) mesocosms, bacterial abundance did not decline below initial levels and was significantly elevated compared to the control mesocosms on Days 6 and 8 ($p < 0.001$). Leucine incorporation rates fell to 12 pM h^{-1} in the $+NH_4^+$ treatment by Day 6, but this activity was significantly elevated ($p < 0.05$) and nearly twice the levels observed in control treatments (Fig. 4A). Despite decreasing abundances, bacterial activity increased in the dual enrichment (+glucose and $+NH_4^+$) treatments ($p < 0.05$), indicating increasing specific growth rates (incorporation per cell). The addition of glucose and NH_4^+ yielded the most pronounced and significant increase, and by Day 6 the rates of leucine incorporation had reached 45 pM h^{-1}. Incorporation rates in the +glucose treatments over Days 5 to 8 were significantly elevated over the control treatments ($p < 0.01$ Day 4, $p < 0.001$ Day 6).

Cell-specific leucine incorporation rates (Fig. 4B) were similar to observed ambient water column values (data not shown) through Day 6 (1 to 8 × 10^{-20} mol $cell^{-1}$ h^{-1}). The glucose-amended treatments diverged from the control and $+NH_4^+$ carboys after Day 4. Mean specific growth rates for the bacterial assemblage in the +glucose and the dual enrichment (+glucose and $+NH_4^+$) carboys also diverged by Day 4 (Fig. 5), while the controls and $+NH_4^+$ carboys remained low and constant until the end of the experiment. The growth rates

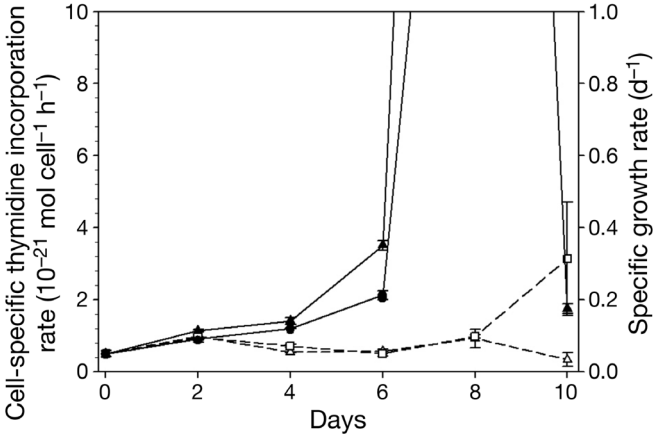

Fig. 5. Cell-specific thymidine incorporation rates and equivalent specific growth rates. Symbols as in Fig. 3. Growth rates were calculated as described in 'Materials and methods'

approximated water column values (0.1 to 0.3 d^{-1}) through Day 6, and then rates in the carboys receiving glucose accelerated to 5 to 6 d^{-1}.

Bacterial responses increased following the third addition of glucose on Day 8 (Figs. 3 to 5). After Day 8, abundance increased to over 10^9 cells l^{-1} in the +glucose and the dual enrichment (+glucose and +NH_4^+) carboys, higher than all but a very few instances observed over 8 yr of seasonal observations (Fig. 1A). Leucine incorporation rates were more than 100 times the ambient values. DOC declined by 50 and 100 µmol l^{-1} in the +glucose and in the dual enrichment (+glucose and +NH_4^+) carboys, respectively, in the 2 d after the third addition of glucose. Bacterial biomass increased by 2 and 12 µmol C l^{-1}, respectively, suggesting conversion efficiencies of about 5 to 10% at this time. TDN also declined in the dual enrichment (+glucose and +NH_4^+) carboy, but not in the +glucose carboy after the final addition on Day 8. The rapid decline in DOC, and the preceding increases in growth rate (Fig. 5), indicate selection and growth of glucose-utilizing bacteria, but only after 6 to 8 d of incubation and 3 additions of glucose. We discount the large increases in abundance, activity and BCC after Day 8 as being highly uncharacteristic of the natural environmental variability in this region, and focus our attention on the preceding period (Days 0 to 6). In general, the variability of bacterial abundance and leucine incorporation rates over Days 0 to 6 was similar to that observed in the seasonal succession between October and January.

Incubation results: bacterial community composition

Like the bulk properties, changes in BCC were most pronounced in the carboys receiving glucose, but only after Day 6. Changes in the control and +NH_4^+ carboys were small and broadly similar. All 3 community fingerprinting methods gave similar results (Table 3, Figs. 6 to 8). At the outset, there were 20 to 30 bacterial operational taxonomic units (OTUs), each contributing >1% of the relative community abundance (Table 3). The apparent species richness did not change greatly over 6 d of incubation in any treatment, even after receiving 2 additions of glucose, and with a doubling of leucine incorporation rates. After Day 6, there were large reductions of OTU richness in the +glucose and in the dual enrichment (+glucose and +NH_4^+) carboys, and small decreases in the other (non-glucose) carboys.

Changes in BCC are presented in more detail in Figs. 6 & 7. Here, we use the control (Fig. 6) and the dual enrichment (+glucose and +NH_4^+) carboys (Fig. 7) to illustrate the nature of changes in BCC over the incubation period. Changes in the control carboy were minor and were limited to the later part of the incubation, after Day 6. The dominant OTUs in the control carboys were putatively assigned as members of the *Alphaproteobacteria*, including *Roseobacter* (316 bp) SAR11 clade (i.e. *Pelagibacter*; 317 bp), and *Rhodobacter* (318 bp)-related organisms, members of the *Cytophaga-Flavobacteria-Bacteroidetes* group (346 bp) and diatom-associated plastids (319 bp) (Table 2). These 5 most abundant fragment lengths contributed 49 to 64% of the total abundance (total peak height) in the control carboys throughout the experiment. No individual OTU that was not present initially became dominant. Changes in relative abundance were mostly due to small changes across the spectrum, rather than large absolute changes in any particular OTU, except for the 319 bp OTU, which fell below the detection threshold in the community by Day 6.

In the dual enrichment (+glucose and +NH_4^+) carboy, the overall spectrum of relative OTU abundance remained similar through Day 6, but with the appearance of 2 fragments affiliated with *Gammaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroidetes* groups (347, 348 bp; cf. Fig. 7, Table 2) as co-dominants by Day 6. These were not present (i.e. <1% of the community) at the start of the experiment. These 2 OTUs then grew rapidly to completely dominate the BCC by Day 10.

When compared, the results of the 3 fingerprinting methods (Fig. 8) all indicated minor changes in the control and +NH_4^+ treatment BCC (more than 72% similarity), even after 6 to 10 d of incubation (Fig. 7). Control and +NH_4^+ BCC were >85% similar on Day 10,

Table 3. Number of peaks as a function of incubation time and experimental treatment (DGGE = denaturing-gradient gel electrophoresis; LH-PCR = length heterogeneity polymerase chain reaction; CE-SSCP = capillary electrophoresis single-strand conformation polymorphism)

Treatment	Day			
	0	2	6	10
DGGE				
Control	19	21	19	15
+NH_4^+	19	14	17	11
+Glucose	19	17	21	5
+Glucose and +NH_4^+	19	18	19	6
LH-PCR				
Control	20	22	19	18
+NH_4^+	20	21	17	17
+Glucose	20	22	22	2
+Glucose and +NH_4^+	20	21	17	2
CE-SSCP				
Control	30	31	30	27
+NH_4^+	30	30	28	27
+Glucose	30	30	26	10
+Glucose and +NH_4^+	30	31	29	4

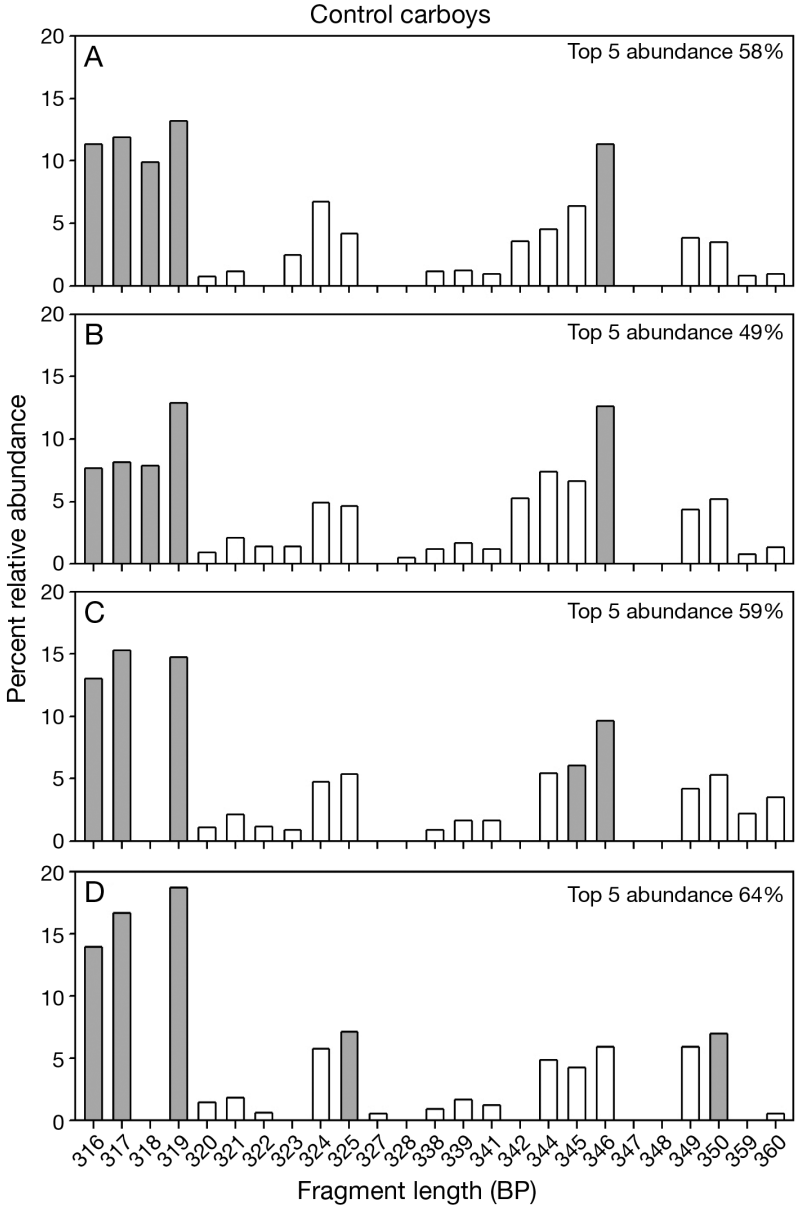

Fig. 6. Relative peak abundance in control treatment carboys revealed by length heterogeneity polymerase chain reaction (LH-PCR) community fingerprinting. (A–D) Days 0, 2, 6 and10, respectively. The gray (shaded) bars are the 5 most abundant operational taxonomic units (OTUs) in the carboy on each succeeding day

as indicated by LH-PCR and CE-SSCP (but they were less similar by DGGE). On Day 6, the BCC in the carboys receiving glucose was still 60 to 70% similar to that in the control and the $+NH_4^+$ samples. At 10 d, the glucose samples diverged greatly from the other samples, and from their initial values. The +glucose and the dual enrichment (+glucose and $+NH_4^+$) BCC were always more than 72% similar during the course of the experiment, with more than 85% similarity at the end of the experiment.

DISCUSSION

Primary production rates frequently exceed 150 µM C m^{-2} d^{-1} (2 gC m^{-2} d^{-1}) during the spring phytoplankton bloom along the western Antarctic Peninsula in November to December (Moline & Prezelin 1996, Smith et al. 2008, Vernet et al. 2008), suggesting a potentially large input of organic matter for bacterial utilization (Morán et al. 2001). Macronutrient concentrations (NO_3^- and PO_4^+) remain high throughout the year but may decrease in December and January in response to the phytoplankton bloom (Ducklow et al. 2007). Nitrate drawdown averages 15 µmol l^{-1}, i.e. from ~30 to 15 µmol l^{-1}. Inorganic nitrogen is usually not limiting. The NO_3:PO_4 ratio in surface waters averages 12 to 13, suggesting a slight excess of P relative to N in these waters. The high primary production rates in spring–summer indicate that iron is not limiting at this time. Dissolved organic carbon concentrations may increase in summer to 45–60 µM, above a deepwater background of 39 µM, and this seasonal enrichment may contribute to the growth of the bacterioplankton community by an order of magnitude (to ~10^9 cells l^{-1}) in summer. When we sampled (3 November 2006), the surface nitrate and phosphate concentrations were 28 and 2.2 mmol l^{-1}, respectively, and primary production was 50 µM C m^{-2} d^{-1} (details at http://oceaninformatics.ucsd.edu/datazoo/data/pallter/datasets).

We sought to investigate the capability of bacterial communities to respond to enrichment with organic carbon and inorganic nitrogen with an experimental approach similar to microcosm approaches employed in other lake and seawater studies (Lebaron et al. 1999, Fisher et al. 2000, Flaten et al. 2003, Kent et al. 2006, Murray et al. 2007, Chrost et al. 2009). Among these studies, the composition of the bacterial community in lakes often responded to inorganic nutrients, whereas the marine assemblages usually responded with increased growth and changes in BCC to additions of organic, but not inorganic, nutrients. Glucose was chosen as a test substrate because it is a simple, precisely defined substrate, and has been used often in other studies (e.g. Fisher et al. 2000, Flaten et al. 2003, San-

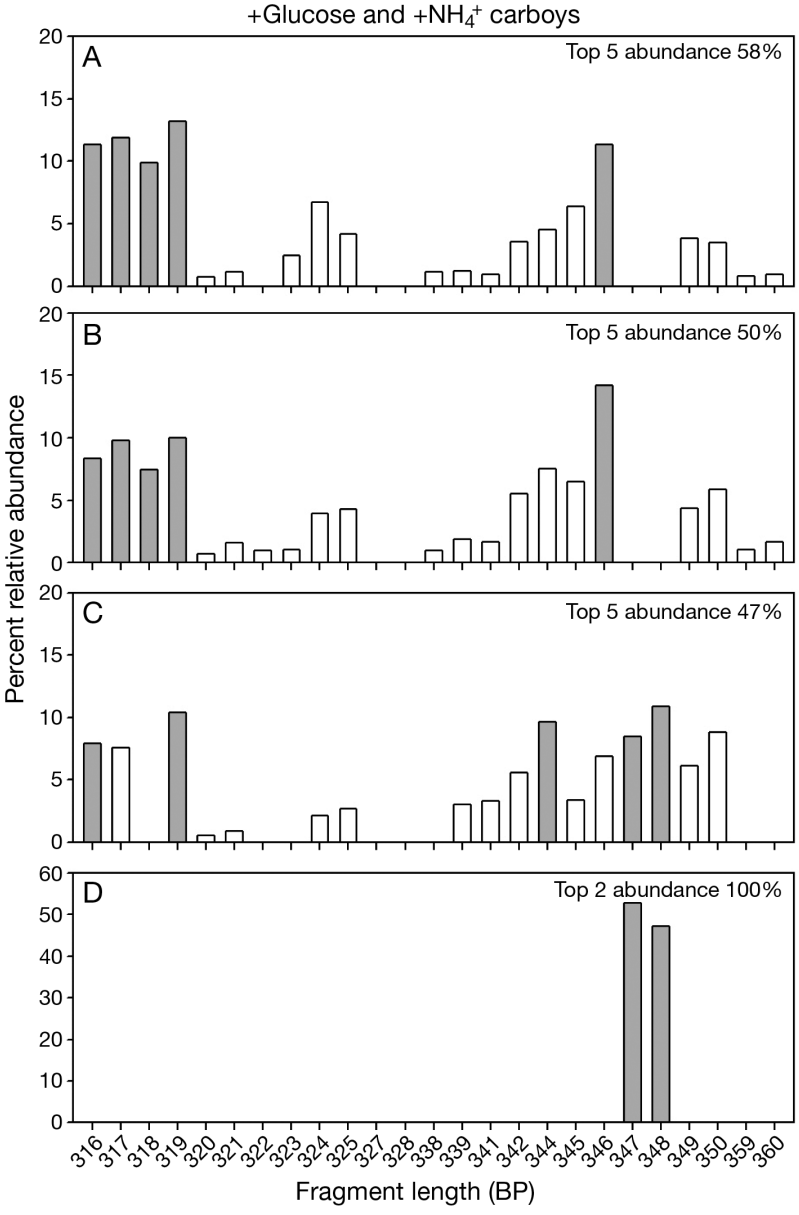

Fig. 7. Relative peak abundance in dual enrichment (+glucose and +NH_4^+) treatment carboys. (A–D) Days 0, 2, 6 and 10, respectively. See Fig. 6 legend for further details

daa et al. 2009). As the treatment level, 100 µmol l^{-1} carbon was selected because it is similar to the level added in previous studies, and to ambient DOC variability observed in subtropical, temperate and polar waters (Carlson & Ducklow 1996, Carlson 2001, Fandino et al. 2001, Church et al. 2002, Manganelli et al. 2009). Ammonium was added at a C:N ratio of 4:1, approximating the bacterial biomass composition (Goldman et al. 1987, Goldman & Dennett 1991). It was chosen as an inorganic nitrogen supplement, even though there was high nitrate, because it is preferred over nitrate for bacterial growth and we wanted to test its effect on carbon utilization. Our incubations were conducted in the dark, which may have influenced possible competition between bacteria and phytoplankton for inorganic nutrients. Here, we discuss the nature of the community response, in the context of observations about the bulk bacterial assemblage in the Palmer Stn region, and in relation to other studies of marine and freshwater bacterial communities, including other incubation studies.

The bacterial community composition and its size and rate of production were typical of spring/summertime conditions in the region and in Antarctic and Arctic coastal waters generally. The dominant members of the community were *Alphaproteobacteria*, *Gammaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroidetes* (Table 2, Figs. 6 & 7), as previously described (Bano & Hollibaugh 2002, Kirchman 2002, Abell & Bowman 2005, Murray & Grzymski 2007, Straza et al. 2010). Murray & Grzymski (2007) reported that the BCC at Palmer Stn in November 1995 and November 2001 was ~50% similar to the BCC in the preceding August (midwinter), and that the October through February BCC samples differed less than 10% from each other, with respect to August samples. The springtime bacterial community we studied appeared surprisingly resistant to experimental perturbation, both from the effects of handling and containment alone, and even from relatively large additions of labile organic carbon and ammonium over a week of incubation, approximating the bulk average generation time of the assemblage.

Bacterial abundance and leucine incorporation in Antarctic coastal waters are very low in winter and early spring, averaging $<2 \times 10^8$ cells l^{-1} and ~2 pmol l^{-1} h^{-1} (H. Ducklow unpubl. obs.). Bacterial production rates in early October and early April average ~1 mmol C m^{-2} d^{-1} in the upper 50 m (euphotic zone), approximating the midwinter values. However, even this very low value may equal or exceed the local primary production rate under severely light-limited conditions (Ducklow et al. 2011). The source and identity of organic matter supporting this low background hetero-

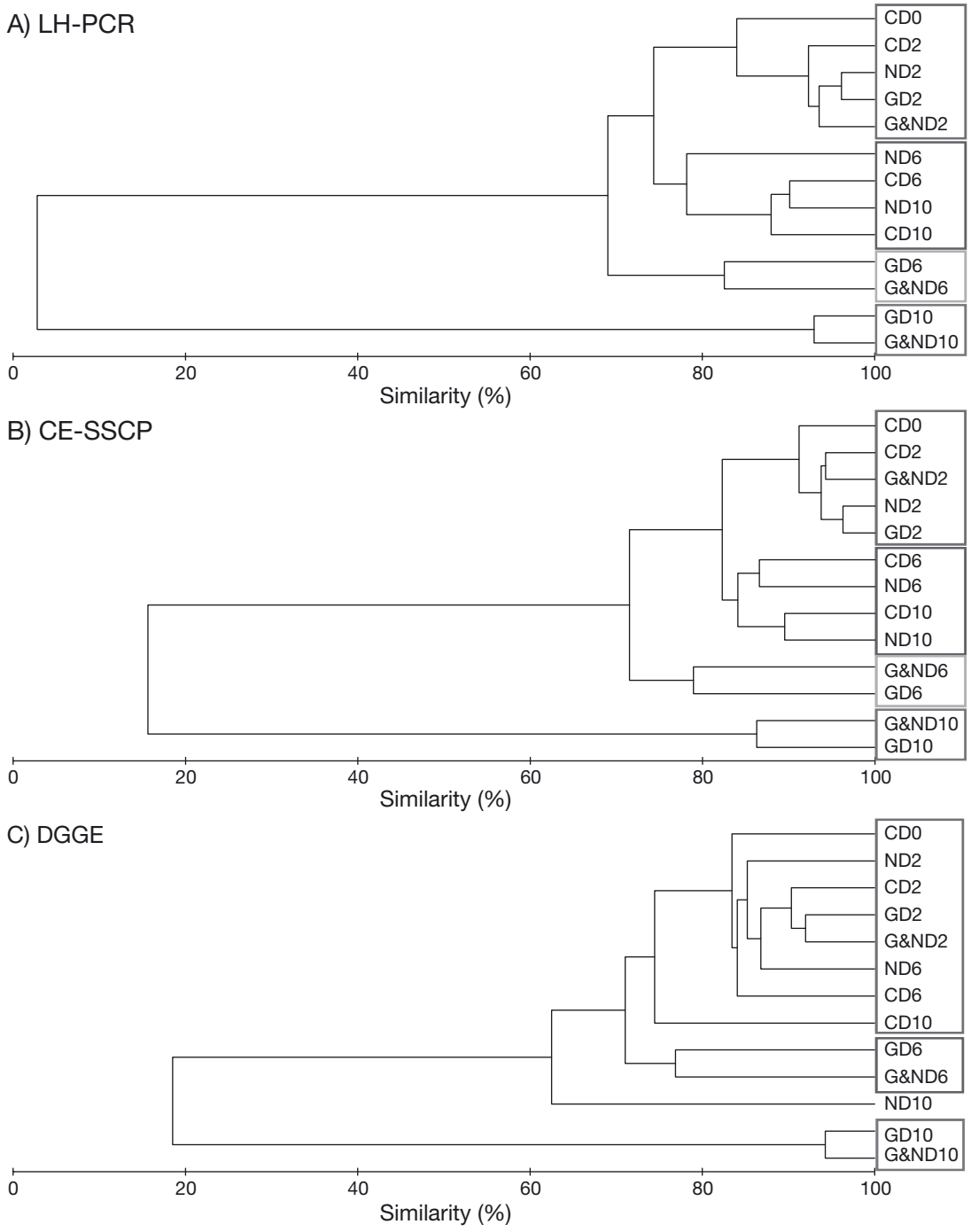

Fig. 8. Similarity of bacterial communities from 4 treatments analyzed by (A) length heterogeneity polymerase chain reaction (LH-PCR), (B) capillary electrophoresis single-strand conformation polymorphism (CE-SSCP), and (C) denaturing-gradient gel electrophoresis (DGGE). Relative intensities of bands/peaks were used for the cluster analysis based on the unweighted pair-group method with arithmetic averages (UPGMA) and Bray–Curtis distance. Samples are labeled by treatment (C: control; G: +glucose; N: NH_4^+) with the time (D, in days) included. Groups with >70% similarity are enclosed in boxes

trophic production are unknown. The early-spring bacterial community response to the spring bloom typically lags behind the vernal increase in primary production and the resulting increase in the organic matter content of the upper ocean by several weeks (Billen & Becquevort 1991, Leakey et al. 1996). Bacterial abundance and production are low and relatively constant until early to mid-December (Fig. 1), whereas pri-

mary production rates typically exceed 50 mM C m^{-2} d^{-1} by mid-November (Moline & Prézelin 1996). This lag may be because labile and semilabile DOC remains mostly low until early December, after which time DOC concentrations are more variable. An alternative explanation is that the community is inherently slow to respond to increased primary production without an extended period of exposure to favorable conditions. Competition with phytoplankton for inorganic nutrients is also possible, but persistently high nitrate and phosphate concentrations make this less likely than other scenarios.

Carbon or nitrogen limitation?

We were not set up to measure glucose or ammonium directly in our experimental samples, so we used DOC and TDN as indices of these nutrient additions. The DOC assay quantitatively revealed the glucose addition of 100 µmol C l^{-1} (Fig. 2A). TDN in seawater potentially includes nitrate, ammonium and bulk dissolved organic nitrogen. Surface nitrate was 28 µmol l^{-1} at the start of our experiment, and the corresponding TDN was 34 µmol l^{-1}, suggesting that [DON + NH_4^+] was ~6 µmol l^{-1}. There are few reliable DON data for this region, but oceanic surface DON concentrations in the vicinity typically average 5 µmol l^{-1} (http://cdiac.ornl.gov/oceans/RepeatSections/), suggesting low NH_4^+ concentrations (≤1 µmol l^{-1}). After the addition of 25 µmol l^{-1} NH_4^+, the TDN averaged 59 µM, exactly 25 µM greater than the initial TDN (Fig. 2B).

Variations between replicates on some days prevented a general conclusion about nitrogen or carbon limitation over the full course of the experiment. Changes in bulk bacterial properties and BCC in the +NH_4^+ treatment generally paralleled the controls, whereas the carboys receiving glucose responded with increased growth and larger changes in BCC, suggesting that the bacterial community was not nitrogen-limited. There was no measurable utilization of NH_4^+ until after Day 8, even in glucose-amended carboys. As noted above, however, there were some slight, but significant, differences between the +NH_4^+ and control treatments, and between the +glucose and the dual enrichment (+glucose and +NH_4^+) treatments on some days, indicating possible ammonium limitation or an effect of ammonium addition on glucose utilization.

Bacterial community composition

In our study, the BCC began to change only after 6 d of incubation and 2 additions of 100 µmol C l^{-1} as glucose on Day 0 and Day 4. A third addition of glucose on Day 8 resulted in a rapid and drastic reduction in OTU numbers within 2 d, as described by the 3 fingerprinting techniques (Fig. 7). Phylogenetic assignment of LH-PCR fragments showed that communities changed from *Alphaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroidetes*, as the dominant groups, to *Gammaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroidetes* after Day 6 in the glucose or the dual enrichment (+glucose and +NH_4^+) treatments. Only 2 OTUs were found on Day 10 in the glucose treatments, with LH-PCR fragments that could be assigned to common groups found in Antarctic ecosystems: *Flavobacteria* and *Flexibacter*-related genera (i.e. *Polaribacter* (Abell & Bowman 2005), and the SUP05 cluster of *Gammaproteobacteria* (Sunamura et al. 2004) which appears to contain chemolithotrophic members and has widespread distribution (Walsh et al. 2009).

Fuchs et al. (2000) observed preferential growth of *Gammaproteobacteria* in dilution cultures taken from English coastal waters (with no substrate additions) over 5 d. The *Gammaproteobacteria* increased from ~8% to between 40 and 60% of the total eubacterial community after 4 d of growth. They concluded that the use of dilution cultures to calibrate bacterial production measurements was compromised by such large changes in the BCC during the incubations. They suggested that sample filtration caused enrichment with organic matter as a consequence of cell breakage. We did not see similar changes in the (unfiltered and undiluted) control samples; however, one might object that the timescale for significant change was longer in our cold samples, compared to those studied by Fuchs et al. (2000). They observed community growth rates of ~1 d^{-1} at 14 and 24°C (judged from changes in abundance in their Fig. 2), although some groups clearly grew faster or slower. We observed similar bulk growth rates at +1°C (Fig. 4), suggesting that our incubation times were not too short to observe changes in the BCC. Teira et al. (2009) conducted 11 dilution experiments over 1 yr in the Ría de Vigo (NW Iberian Peninsula) upwelling system and did not observe such large, systematic changes in the BCC as did Fuchs et al. (2000). Their results were thus similar to ours, suggesting that the BCC was relatively stable in both untreated and amended incubations over short intervals (a few generation times).

The stability of bacterial abundance and production in the experimental carboys reflected the ambient environment: bulk bacterial properties in the ocean were relatively stable over 6 wk (23 October to 7 December in Fig. 1). During this period, bacterial properties did not change appreciably even though primary productivity was high and variable. Another reason for the relative stability of the BCC in the microcosms in

response to experimental addition of glucose over the first 6 d might be the unsuitability of this carbohydrate as a bacterial substrate in Antarctic seawater, even with high dissolved inorganic nitrogen. Glucose amendment did result in significant changes in abundance and activity over 6 d, but these responses did not appear to have a large selective effect on the BCC. While glucose was the only detectable free neutral sugar in the Ross Sea, its concentration was very low (<20 nM, i.e. <120 nM C) and it contributed less than 10% to ambient bacterial production in the period October to November (Kirchman et al. 2001). In comparison, free glucose concentrations were 30 to 70 nM (180 to 420 nM C) in the Central Arctic Ocean, and these concentrations supported from 10 to 97% of the bacterial production (Rich et al. 1997). Straza et al. (2010) observed that, in midsummer 2007, less than 5% of the cells detectable by fluorescence *in situ* hybridization (FISH) took up glucose in samples from the continental shelf and slope waters offshore from our study region. In the same samples, 2 to 30% of the total community (that was visible when stained by 4,6-diamidino-2-phenylindole, DAPI) took up an amino acid mixture (mean 12%) and 5 to 45% took up protein (mean 22%). These observations suggest that bacteria in these waters prefer organic nitrogen-containing compounds to simple carbohydrates, even when inorganic nitrogen is available, as it nearly always is in Antarctic waters. The same preference was observed in Antarctic subpolar waters (Church et al. 2000).

We caution that these results from 1 experiment in 1 season might not hold for other times, locations or treatments. For example, the same experiment repeated during the peak in activity (late January), or in winter, could yield different results, as would the addition of a different carbon source such as protein or amino acids, or incubation in ambient irradiance, which was not possible for our experiment. Finally, our results from a shallow, nearshore location might be different from the results obtained if the same treatments were applied to offshore or deep water samples.

CONCLUSIONS

The response of a late-springtime bacterial assemblage, taken from coastal Antarctic waters, to experimental additions of organic carbon, with or without added inorganic nitrogen, generally resembled the observed variability in the ambient surface ocean bacterial community in response to the spring phytoplankton bloom. Bacterial abundance, production (^{3}H-leucine incorporation rates) and specific growth rates (cell-specific ^{3}H-thymidine incorporation rates) increased by a factor of 2 (abundance) to 10 (production and growth rates) over 8 d in response to the addition of glucose. The lag in the response may have been affected by senescence or death of the enclosed phytoplankton community held in darkness as part of the experimental design, although this did not measurably change the DOC or TDN concentrations. The response was greater when glucose was supplemented with ammonium. The bacterial community was not sensitive to enclosure by itself without the addition of organic matter, nor to the addition of ammonium alone. The results indicate that large-volume (50 l) bioassays conducted over 1 wk to 10 d are reliable tools for exploring the factors causing seasonal changes in Antarctic bacterial communities. However, the full combination of factors influencing changes in the BCC remain to be specified.

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