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Changes in marine bacterioplankton phylogenetic composition during incubations designed to measure biogeochemically significant parameters

Abstract—Bottle incubations, during which the activity and growth of prokaryotes is monitored during several days, are frequently carried out to study functional aspects of marine prokaryotic assemblages. These experiments will relate directly to in situ activities if all populations grow harmonically during the incubation. We tested whether this was the case by analyzing the composition of bacterial assemblages at the beginning and at the end of the incubation by denaturing gradient gel electrophoresis. Five experiments were done in different Antarctic regions. Bacterial assemblages north and south of the Polar Front were very different. In all cases, the final assemblages were very different from the initial ones, and these changes were often accompanied by a significant decrease of diversity indices. Our experiments included treatments with different temperature and organic matter amendments. Whereas the increase in temperature tested had a minor effect on prokaryotic growth rate and specific composition, the addition of organic matter strongly stimulated growth rate and selected a particular bacterial assemblage in some experiments but not in others. A significant component of bacterial assemblages from waters south of the Polar Front appeared to be *Polaribacter franzmannii,* a gas vacuolated bacterium of the Cytophaga-Flavobacterium-Bacteroides group that was originally isolated from Antarctic sea ice. This phylotype was enriched and dominated in almost all final assemblages. Our results indicate that long-term bottle incubations mostly measure the activity of a few opportunistic bacteria and not that of the original assemblage. This should be taken into account if data obtained in these experiments are used for balancing whole ecosystem carbon budgets and to derive biogeochemical conclusions.

Controlled incubations are often used to determine the activities of marine prokaryotic assemblages. In some cases, incubations last only a few hours, during which the composition of the assemblage is probably stable. Examples of such short-term incubations are experiments to estimate prokaryotic production (Kirchman et al. 1985) or the proportion of actively respiring cells (Sherr et al. 1999*a*). Other experiments, however, require much longer incubation times. In such experiments, grazing by protists is minimized by dilution or filtration, and the response of the prokaryotic assemblage is measured by cell growth. Examples of this approach are incubations used to determine empirical conversion factors of leucine or thymidine incorporation to bacterial production (Kirchman and Ducklow 1993; Ducklow et al. 1999) or to estimate prokaryotic growth efficiency or rates of dissolved organic carbon degradation (Carlson and Ducklow 1996; del Giorgio and Cole 1998). Other examples are bioassays to study potential limiting factors, such as temperature or organic and inorganic nutrients (Kirchman

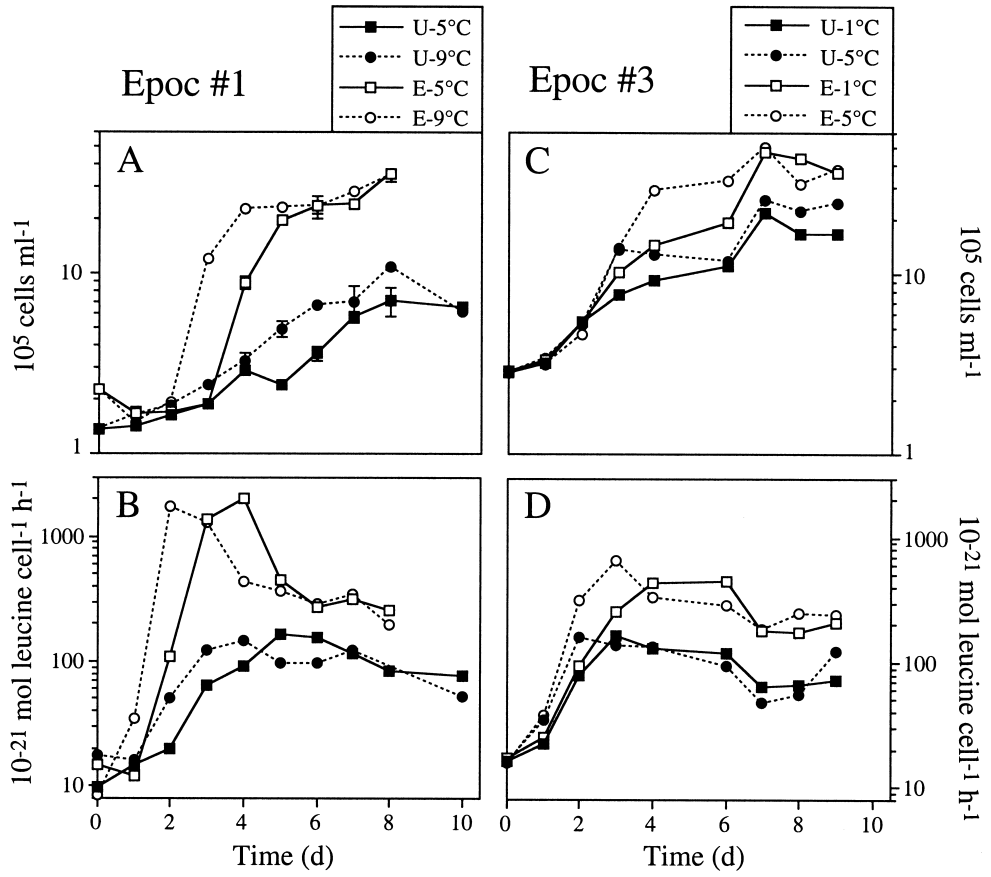

Fig. 1. Changes in prokaryote cell counts in (A) Epoc 1 and (C) Epoc 3. Changes in cell-specific leucine incorporation in (B) Epoc 1 and (D) Epoc 3. Results for unenriched (U) and DOM-enriched (E) treatments incubated at two temperatures. Error bars for cell counts are standard errors between replicates (not visible when smaller than the plot symbol).

1990). It is important to differentiate these experiments from those designed to investigate how prokaryotic assemblage composition responds to factors such as algal blooms (Riemann et al. 2000) or different types of grazers (Simek et al. 1999). In the latter cases, the experimental variable is phylogenetic composition, whereas in the former cases the variable is metabolic activity.

Bottle incubations that last >24 h (often >1 week) are much longer than prokaryotic division times. A crucial assumption of these long-term experiments is that the growth of the prokaryotic assemblage is harmonic, i.e., all natural populations grow proportionally and species composition does not change during the incubation. Previous studies have shown a dramatic increase in culturability (ZoBell 1943; Ferguson et al. 1984) and average metabolic activity (Sherr et al. 1999*b*) of marine bacteria during bottle incubations, although these effects seemed minimized in large volume incubations (ZoBell 1943; Lee and Fuhrman 1991). Recent data indicate that predator removal can affect bacterial composition (Suzuki 1999), especially if the assemblage was previously under a strong grazing pressure (Simek et al. 1999). In any case, the assumption that the composition of prokaryotic assemblages remains stable during bottle incubations must be verified.

In the present article, we present data from several bottle incubations performed in different Antarctic regions. Some bottles were incubated at in situ conditions, to determine empirical conversion factors for leucine incorporation, whereas others were incubated at higher temperatures and/or were amended with organic matter. Instead of following the bacterial dynamics in detail in a single incubation, we chose to compare the composition of the initial and final bacterial assemblages after many different treatments using the molecular fingerprinting technique denaturing gradient gel electrophoresis (DGGE). Although DGGE is not the most complete technique for describing microbial diversity, it offers the best compromise between the need to process a significant number of samples and the information generated (Muyzer et al. 1997).

Five experiments were done (Table 1): two in the Scotia Sea, North (Epoc 1) and South (Epoc 2) of the Polar Front; two in the Weddell Sea, one near the ice-edge (Epoc 3), and the other slightly North (Epoc 4); the last station was located in the Southwestern end of Gerlache Strait (Epoc 5). Seawater from a depth of 10 m (except 25 m in Epoc 5) was collected with Niskin bottles attached to a rosette with a conductivity, temperature, depth probe. Chlorophyll *a* concentration was determined by fluorometry. GF/A (1.6 μm

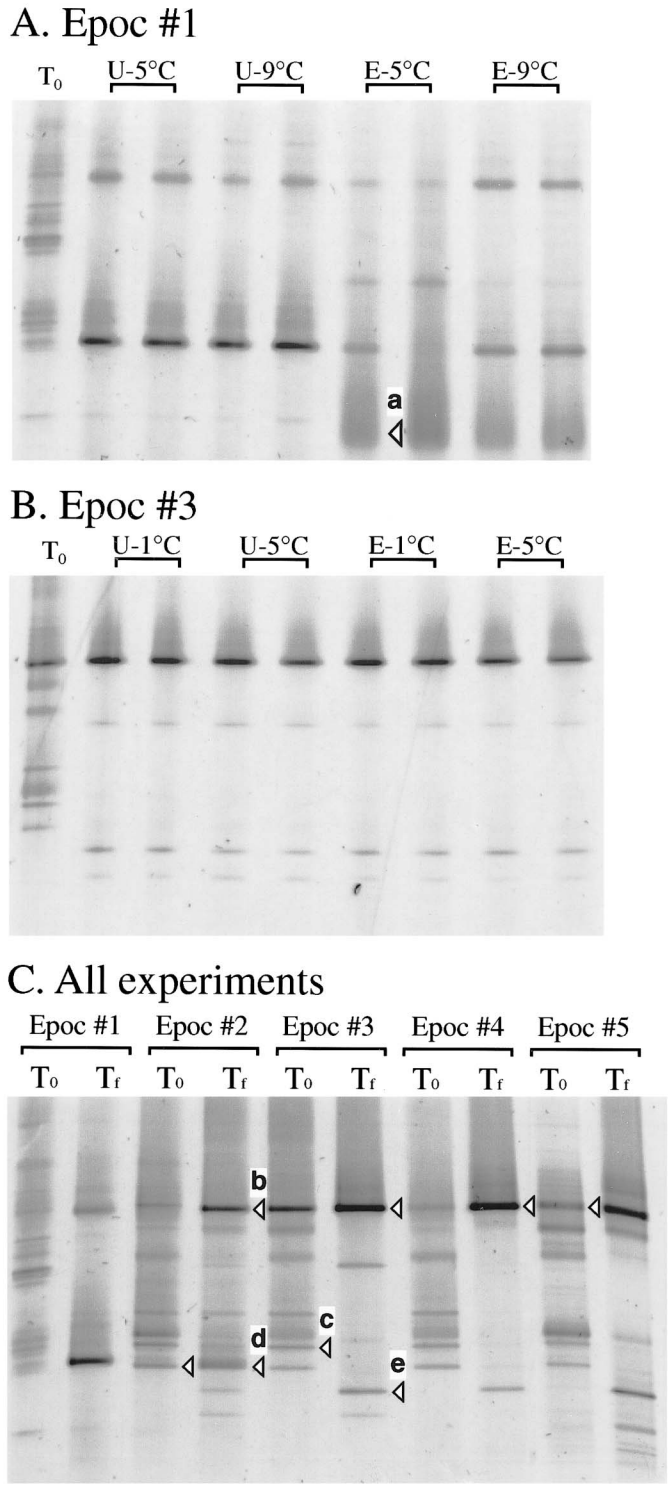

Fig. 2. DGGE gel comparing initial and final bacterial assemblages in (A) Epoc 1, (B) Epoc 3, and (C) the initial and U in situ final samples for the five experiments. (A) and (B) show results for U and DOM-enriched (E) treatments incubated at two temperatures. Bands excised from the gels and sequenced are indicated by arrowheads. Their closest matches in the database are (a) *Vibrio;* (b) *Polaribacter;* (c) *Roseobacter;* (d) *Octadecabacter;* and (e) *Roseobacter*.

nominal pore size) and GF/F (0.7 μm nominal pore size) filtered seawater was generated by use of a peristaltic pump, mixed in equal parts, and dispensed into 2-liter polycarbonate bottles. Bottles were incubated in the dark for 8–10 d at nearly in situ temperature or 4°C and 8°C above this temperature (Table 1). Some bottles were amended with dissolved organic matter (DOM; 5 μM acetate plus 5 μM glucose). Most treatments were carried out in duplicate. To avoid contamination by DOM or microorganisms, glass-fiber filters were precombusted before use (450°C for 4 h) and tubing for filtration and bottles for incubations were rinsed with bleach, acid-milliQ water, milliQ water, and sample water in succession.

Samples for prokaryotic counts were fixed daily with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and allowed to sit in the dark for 10 min prior to transfer to liquid nitrogen. Prokaryotic counts were performed with a FACSCalibur flow cytometer after staining with Syto13 (Gasol and del Giorgio 2000). Cytometry data were also used to calculate the average cell volume and the percentage of high DNA bacteria (%HDNA). Leucine incorporation rates (Kirchman et al. 1985) were measured every other day in alternate replicates by the protocol explained in Smith and Azam (1992), with use of 40 nM of leucine as tracer. Microbial biomass was collected on 0.2 μm Durapore filters (Millipore) by vacuum filtration at the beginning (between 2 and 4 liters) and at the end of each incubation (between 0.4 and 1.5 liters). Filters were covered with lysis buffer and stored at −70°C. Nucleic acids were extracted by digesting preparations with lysozyme, proteinase K, and sodium dodecyl sulfate, extracting the nucleic acids with phenol-chloroform-isoamyl alcohol, and then desalting and concentrating the nucleic acids with a Centricon-100 concentrator (Schauer et al. 2000). The recovered DNA was quantified by a Hoechst dye fluorescence assay. Extractions yields averaged 0.22, 1.43, and 5.63 μg of DNA per liter of sample at time 0, final time of unenriched treatments, and final time of enriched treatments, respectively.

One nanogram of DNA was used as template for polymerase chain reaction (PCR) amplification of a fragment of bacterial 16S rDNA. The reactions contained 200 μM of each of the deoxynucleoside triphosphates, 0.3 μM of each of the primers (the bacterial specific 358f, with a 40-bp GC-clamp, and the universal specific 907r; Muyzer et al. 1997), 1.5 mM $MgCl_2$, and 0.02 U μl^{-1} of *Taq* DNA polymerase. The PCR was performed with the following program: initial denaturation at 94°C for 1 min; 10 touchdown cycles of denaturation (at 94°C for 1 min), annealing (at 65°C–55°C for 1 min, decreasing 1°C each cycle), and extension (at 72°C for 3 min); 20 cycles of denaturation (at 94°C for 1 min), annealing (at 55°C for 1 min), and extension (at 72°C for 3 min); and a final extension at 72°C for 5 min. The PCR product was quantified by agarose gel electrophoresis by use of the Low DNA MASS Ladder (GIBCO BRL). DGGE was performed as described in Muyzer et al. (1997). A 6% polyacrylamide gel with an 80%–40% gradient of DNA-denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide) was casted. Around 800 ng of PCR product were loaded for each sample, and the gels were run at 100 V and 60°C for 16 h in 1× TAE buffer. The gels

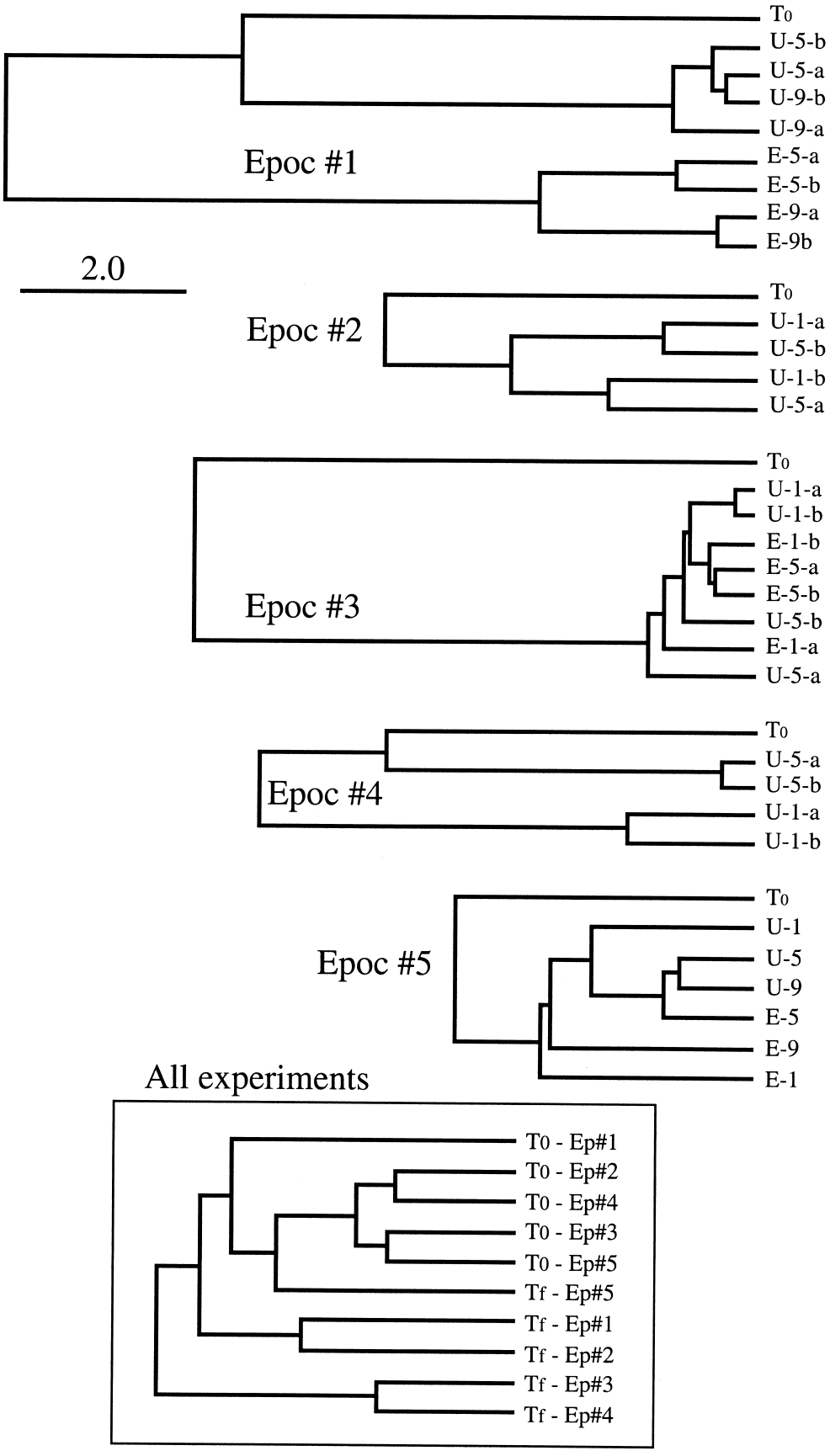
T0
U-5-b
U-5-a
U-9-b
U-9-a
Epoc #1
E-5-a
E-5-b
E-9-a
E-9b
2.0
T0
U-1-a
U-5-b
U-1-b
U-5-a
Epoc #2
T0
U-1-a
U-1-b
E-1-b
E-5-a
E-5-b
U-5-b
E-1-a
U-5-a
Epoc #3
T0
U-5-a
U-5-b
U-1-a
U-1-b
Epoc #4
T0
U-1
U-5
U-9
E-5
E-9
E-1
Epoc #5
All experiments
T0 - Ep#1
T0 - Ep#2
T0 - Ep#4
T0 - Ep#3
T0 - Ep#5
Tf - Ep#5
Tf - Ep#1
Tf - Ep#2
Tf - Ep#3
Tf - Ep#4

Table 1. Summary of the bottle experiments performed, including the characteristics of the sampling site and the treatments: incubation temperatures (separated by slashes), enrichment with dissolved organic matter (DOM), and number of replicates.

Experiment	Date (1998)	Coordinates	Temp. (°C)	Chl *a* (μg L^{-1})	Incubation temp. (°C)	Enrichment	Replicates
Epoc 1	15 Dec	56°19′S, 57°39′W	4.7	0.5	5/9	none/DOM	2
Epoc 2	16 Dec	59°21′S, 55°45′W	0.6	0.6	1/5	none	2
Epoc 3	19 Dec	63°19′S, 52°18′W	−0.9	2.7	1/5	none/DOM	2
Epoc 4	21 Dec	62°18′S, 52°20′W	−0.6	1.3	1/5	none	2
Epoc 5	27 Dec	64°58′S, 63°23′W	−0.4	4.9	1/5/9	none/DOM	1

were stained with the nucleic acid stain GelStar (FMC BioProducts) for 30 min and visualized with UV radiation in a Fluor-S MultiImager (Bio-Rad). Some bands were excised from the gel and transferred to tubes containing sterile water and glass beads. The polyacrylamide was disrupted in a Mini Bead beater, and tubes were incubated overnight at 4°C. After a short centrifugation pulse, the supernatant was used for a PCR reamplification with use of the same primers. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) and were partially sequenced with the Thermo Sequenase Dye Terminator Cycle Sequencing Kit (Amersham). Sequences were submitted to BLAST search (http://www.ncbi.nlm.nih.gov), to find the closest relatives in the database.

Images of DGGE gels were analyzed with the Diversity Database software (Bio-Rad), as described by Schauer et al. (2000). The software recorded an intensity profile through each lane, detected the bands, and measured its intensity after application of a rolling disk as background subtraction. Next, bands occupying the same position in the different lanes were identified. A matrix was constructed, taking into account the presence or absence of individual bands in each lane and the relative contribution of the band to the total intensity of the lane. This matrix was used to calculate a distance matrix by use of normalized Euclidean distances (root-mean-squared differences). A dendrogram showing the relationships among samples was obtained by UPGMA (unweighted pair-group method with arithmetic averages) in cluster analysis. The number of bands and their relative intensity were also used to calculate the Shannon diversity index (H') for each bacterial assemblage, according the following formula:

$$\mathrm{H}' = -\sum_{i=1}^{n} p_i \ln p_i,$$

where n is the number of bands in the sample and p_i the relative intensity of the ith band.

Filtration of seawater through GF/F and GF/A filters resulted, as expected, in the removal of essentially all predators and a reduction in prokaryote counts (on average, 47% of unfiltered seawater). Filtration also reduced the %HDNA on average from 62% to 50%. In all treatments (Table 1) there was an increase of cell counts during incubation (data for two experiments are shown in Fig. 1A,C), accompanied by an increase of the mean cell volume and the %HDNA (data not shown). Replicate bottles had very small standard errors for cell counts. The other three experiments behaved similarly. Overall, prokaryote numbers (initial values of 2–4 $\times$ 10^5 cells ml^{-1}) increased more dramatically in the DOM-enriched treatments (average ratio of highest to initial abundance of 17.1) than in the unenriched treatments (average ratio of 5.6). Incubation temperature had no effect on maximal prokaryote concentration. Temperature did, however, reduce the lag time before growth started (for example, in Epoc 1, the lag time was 1 d shorter at 9°C than at 5°C). Prokaryote numbers remained high up to the end of the incubation, likely because heterotrophic flagellates never developed significantly. Community leucine incorporation increased during the first days and remained high thereafter (data not shown). This resulted in a peak of cell-specific leucine incorporation in the first days, between day 2 and day 6, depending on the treatment (Fig. 1B,D), which was responsible for the increase in prokaryote biomass. Integrated leucine incorporation evolved similarly in the first four experiments, with a moderate effect of temperature and a very significant effect of DOM enrichment: average values were 128 and 20 nmol leucine L^{-1} for enriched and unenriched incubations, respectively. In Epoc 5, on the other hand, the effect of DOM enrichment was less marked, and the effect of temperature was more apparent.

Changes in the phylogenetic composition of the bacterial assemblages from the beginning to the end of each incubation were estimated by DGGE (Fig. 2A,B as examples). Gels revealed changes in composition during the incubation and among treatments. DGGE fingerprints were processed by image analysis and simple statistics. First, dendrograms with samples in each experiment were constructed (Fig. 3) and, second, the Shannon diversity index (H') for each sample was calculated. On average, H' values were 2.13, 1.51, and 1.15 for the initial, final unenriched, and final enriched assemblages. Changes in diversity indices were analyzed with two ANOVA analyses (Table 2), the first comparing H' val-

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Fig. 3. Dendrograms comparing all samples from each Epoc experiment, obtained by analysis of the DGGE fingerprints by Euclidean distances and UPGMA. T_0 are the initial samples, and final samples are coded as U (unenriched) or E (enriched), the temperature of incubation (1°C, 5°C, or 9°C), and the replicate bottle (a or b). The dendrogram in the lower box compares initial and final samples from all Epoc experiments. Scale bar indicates normalized Euclidean distances.

Table 2. Significance of differences in Shannon diversity indices (H′) in Epoc experiments according to ANOVA analyses (Epoc 5 was not included because it lacked replicates). The first ANOVA compares H′ values in the initial and final assemblages in each treatment; the second ANOVA compares the effect of treatments on H′ values of final assemblages.

	Change of H′ values during incubation				Effect of treatment on H′ values of final assemblage		
Experiment	U in situ	U + 4°C	E in situ	E + 4°C	Temperature	Enrichment	Interaction
Epoc 1	***	***	***	***	*	***	**
Epoc 2	NS	NS	†	†	NS	†	†
Epoc 3	***	***	***	***	NS	NS	NS
Epoc 4	*	NS	†	†	*	†	†

* $0.05 > P > 0.01$; ** $0.01 > P > 0.005$; *** $P < 0.005$.
† Not applicable.

ues between the initial and the final samples in each treatment and the second comparing H' values in final samples from different treatments. Dendrograms clearly indicated that final assemblages were always different from the initial ones, although not all experiments behaved in the same way. In Epoc 1, a large reduction in H' values resulted in highly significant differences between initial and final values in all treatments. In fact, most initial bands disappeared during the incubation (Fig. 2A). The dendrogram showed the selection of a very different final assemblage in DOM treatments (with significantly different H' values), whereas temperature had only a marked effect in the enriched treatments. In Epoc 2, the final assemblages were different from the initial, but there was neither a change in H' values during the incubation nor an effect of temperature on final H' values. In Epoc 3, there was again a large and significant decrease in H' values, but in this case the same final assemblage was selected in all treatments with similar H' values. In Epoc 4, assemblages incubated at higher temperature were relatively similar to the initial one, and H' values only changed in assemblages incubated at in situ temperature. Finally, in Epoc 5, final assemblages were rather similar to the initial one, and opposite trends were observed in both treatments: H' values decreased with temperature in unenriched treatments and increased with temperature in enriched treatments.

In order to unequivocally compare the bacterial assemblages from the different experiments, two samples from each experiment (the initial and ''U in situ'' final sample) were run in the same DGGE gel (Fig. 2C). The sample taken north of the Polar Front (Epoc 1) was very different from the other four samples, which were collected in the Southern Ocean (see dendrogram inside box in Fig. 3). The four latter samples had most of their bands in common, and they did not group by geographic proximity; instead, the sample south of the Polar Front (Epoc 2) grouped with one sample in the Weddell Sea (Epoc 4), whereas the sample in Weddell Sea near the ice-edge (Epoc 3) grouped with the sample in the Gerlache Strait (Epoc 5), rather full of ice at the time of sampling. As was indicated before, the smallest changes during incubations occurred in Epoc 5 and, thus, the final assemblage from this experiment was closest to the initial one. The final assemblages of the four remaining experiments grouped differently than the initial ones: final assemblages from Scotia Sea (Epoc 1 and 2) grouped together, whereas final assemblages from Weddell Sea (Epoc 3 and 4) grouped together as well.

Some of the most prominent DGGE bands were sequenced (marked in Fig. 2). Band **b** was excised from four different samples, and its sequence was very similar (98%) to the gas vacuolated bacterium *Polaribacter franzmannii,* of the Cytophaga-Flavobacterium-Bacteroides group. This phylotype was always present in samples taken south of the Polar Front and dominated the respective final assemblages. In addition, although it had not been detected in the initial assemblage of Epoc 1, it appeared as the second dominant band at the end of this experiment. Despite the large number of indeterminations in sequences of bands **c** and **d,** both unequivocally affiliated with marine α-Proteobacteria: band **c** with *Roseobacter* sp. and band **d** with *Octadecabacter antarcticus.* Both phylotypes were important components of the initial assemblages south of the Polar Front, and both disappeared during the incubation, with the exception of band **d** in Epoc 2. Band **d** also dominated the final assemblage of Epoc 1. The sequence of band **e** was closest to *Roseobacter* sp. Shippagan (97% similarity). This phylotype was absent from initial assemblages but appeared in all final assemblages south of the Polar Front. We also sequenced the band appearing in enriched treatments of Epoc 1 (Fig. 2A). Its sequence was highly similar (97%) to the γ-Proteobacterium *Vibrio marinus.*

We measured significant prokaryotic growth in all bottle incubations. Prokaryotic growth under near in situ conditions seems to be stimulated by a variety of factors: reduction of grazing pressure, reduction of competition by diluting populations, and bottle effects (ZoBell 1943; Ferguson et al. 1984; Sherr et al. 1999*b*). In addition, the filtration step might enrich the filtrate with nutrients, although we used the least problematic of all possible filters (Gasol and Morán 1999). Our replicates always evolved similarly, in contrast with the recent report of different bacterial populations developing in replicate marine enrichments (González et al. 1999). The latter were explained by stochastic differences in the composition of the inoculum, which was much smaller in their experiment than in ours. We estimated that between 80% and 95% of the prokaryote cells at the end of experiments had been produced during the incubation. The obvious question to ask is what is the identity of these newly produced prokaryotes, and this was addressed by DGGE. Al-

though DGGE has several potential limitations, such as chimeras and heteroduplex formation or PCR biases (Muyzer et al. 1997), it is very useful for comparison of assemblages and for giving a rough estimate of bacterial diversity (Schauer et al. 2000). Marine archaea are known to be present and abundant in Antarctic waters (Massana et al. 1998), but we only followed the dynamics of bacteria. It is doubtful whether archaea developed in our bottles, because all attempts to enrich or isolate them have failed. In order to process many treatments, we only compared the initial with the final sample. In some cases (unenriched bottles in Fig. 1A), prokaryotic growth occurred throughout the incubation, and thus the analysis of initial and final assemblages was justified. When growth stopped some days before collection of the final sample, the maintenance of prokaryote numbers thereafter, together with the decrease of cell-specific leucine incorporation, suggest absence of growth and mortality and, therefore, maintenance of the bacterial composition.

The experiments started with different bacterial assemblages. The assemblage north of the Polar Front (Epoc 1, Subantarctic Region, temperature >4°C) was very different from that in the other four samples, collected in properly Antarctic waters and at temperatures ~0°C. These data, together with additional data from the same cruise (R. Massana, unpublished results), confirm the role of the Polar Front as an important biological barrier for many planktonic populations (Karl 1993). The four Antarctic samples did not group by geographic proximity but by water column properties: the two more ''oceanic'' samples from the Scotia and Weddell Seas clustered together, as did the two samples from ice-edge zones in the Weddell Sea and Gerlache Strait.

In all experiments, incubation at near in situ conditions selected an assemblage that was different from the initial one. In most cases there was also a dramatic reduction of diversity indices: many DGGE bands disappeared, and only a few bands dominated final assemblages. A recent study found that removal of bacterivores caused a shift in bacterial composition of marine coastal assemblages after two days of incubation (Suzuki 1999). By using a technique also involving a PCR step (LH-PCR), the author demonstrated that in the absence of bacterivores some phylotypes that were rare in the original samples ended up dominating the final assemblages. Another study demonstrated that the removal of bacterivores only affected bacterial composition (i.e., biomass share of different phylogenetic groups as estimated by FISH) if the assemblage was previously under a strong grazing pressure (Simek et al. 1999). Our data confirm and expand these previous observations with Antarctic bacterial assemblages.

The effect of different treatments on the composition of the final assemblage was less dramatic than had been expected. The increase in temperature did not stimulate prokaryotic growth or affect the composition of the final assemblages. Temperature apparently did not limit prokaryotic growth or the increase was too small to force a change. Ducklow et al. (1999) also found that temperature increases of up to 4°C did not stimulate prokaryotic growth in experiments performed in the Ross Sea polynya. Additions of glucose and acetate resulted in a very large stimulation of prokaryotic growth and activity, but effects on bacterial composition were variable: in Epoc 1 an assemblage dominated by *V. marinus* developed, whereas in Epoc 3 and 5 the assemblage was similar to that of unenriched treatments. These two later experiments were carried out in richer waters (higher chlorophyll values) than the first, and their assemblages might have contained phylotypes already adapted to low-molecular-weight carbon sources. Thus, the same phylotypes could proliferate in unenriched and enriched bottles, although growth could be strongly enhanced in the later case. Smalla et al. (1998) also found that the populations selected in BIOLOG wells were important in the initial assemblages only when this assemblage was adapted to utilize food sources similar to those in BIOLOG wells.

The dominant phylotypes at the end of incubations were very similar to *Vibrio* (Epoc 1 enriched), *Octadecabacter* (Epoc 1 unenriched), and *Polaribacter* (the other cases). The fact that enriched phylotypes are most similar to cultivated species is not surprising, because isolated bacteria have encountered a similar enrichment process to our incubation conditions. *Octadecabacter* and *Polaribacter* are recently described bacterial species isolated from Antarctic sea ice and water and share the trait of having gas vesicles. They were not only enriched during the incubations, but they were also abundant in the environment. Gas vesicles, not usually found among the heterotrophic bacteria of temperate waters, seem to be an adaptation to regulate buoyancy of bacterial cells in the stratified interfaces between sea ice and water (Gosink et al. 1997). It is surprising to find these bacteria dominant in the plankton and enrichments, which lack such strong vertical gradients. The question of how well the original assemblage might be represented by culturable phylotypes appears to be related to the trophic status of the community. Thus, in relatively rich systems, such as marine coastal communities (Pinhassi et al. 1997), phylotypes retrieved in pure culture seem to be also important in the original assemblage, whereas in oligotrophic oceanic systems most phylotypes present in the sample belong to non cultured organisms (Mullins et al. 1995). Our results agree with this scenario: in Southern Ocean waters, and especially in the richer sites (Epoc 3 and 5), the cultured phylotype *Polaribacter* was abundant in the natural assemblage.

Our results show that in long-term bottle incubations designed to estimate functional properties of prokaryotic assemblages, there is commonly a very significant change in species composition when incubated without predators at in situ temperature. These changes often involve a large reduction in bacterial diversity. Similar experiments should be performed in other temperate and tropical systems to determine the generality of this result. Assemblages in bottles do not grow harmonically. Rather, some populations grow faster than others and end up dominating the final assemblage. These populations may or may not be those that are abundant at the start of the experiment. Activities measured during bottle incubations correspond to these fast-growing populations. Whether these values are extrapolable to the whole natural assemblage is unclear. In this context, Suzuki (1999) postulated that the populations selected during bottle incubations were the ones actively growing in situ but maintained at low abundances by grazing pressure. A thorough investigation of whether variability in, e.g., leucine-to-car-

bon conversion factors is ''ecological'' (i.e., all phylotypes respond equally to the same ecological situation) or ''phylogenetic'' (different phylotypes respond differently) is clearly needed, and until this is done, results obtained from bottle incubations should be interpreted with caution. Our results emphasize the need for developing alternative methods not involving long incubations to investigate the activity of natural prokaryotic assemblages.

Ramon Massana,[1] *Carlos Pedrós-Alió, Emilio O. Casamayor, and Josep M. Gasol*

Departament de Biologia Marina i d'Oceanografia
Institut de Ciències del Mar, CSIC
Passeig Joan de Borbó s/n
08039 Barcelona, Catalunya, Spain

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[1] Corresponding author (ramonm@icm.csic.es).

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