



PERGAMON

Deep-Sea Research II 46 (1999) 1791–1811

DEEP-SEA RESEARCH
PART II

Bacterial community composition during two consecutive NE Monsoon periods in the Arabian Sea studied by denaturing gradient gel electrophoresis (DGGE) of rRNA genes

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Received 9 September 1998; received in revised form 18 December 1998; accepted 8 January 1999

Abstract

Horizontal and vertical variations in bacterial community composition were examined in samples collected during two Joint Global Ocean Flux Study (JGOFS) Arabian Sea cruises in 1995. The cruises, 11 months apart, took place during two consecutive NE Monsoon periods (January and December). Bacteria were harvested by filtration from samples collected in the mixed layer, mid-water, and deep sea at stations across the study area. Total bacterial community genomic DNA was analyzed by PCR amplification of 16S rRNA gene fragments, followed by denaturing gradient gel electrophoresis (DGGE). In total, 20 DGGE bands reflecting unique or varying phylotypes were excised, cloned and sequenced. Amplicons were dominated by bacterial groups commonly found in oceanic waters (e.g., the SAR11 cluster of α -Proteobacteria and cyanobacteria), but surprisingly none of the sequenced amplicons were related to γ -Proteobacteria or to members of the Cytophaga-Flavobacter-Bacteroides phylum. Amplicons related to magnetotactic bacteria were found for the first time in pelagic oceanic waters. The DGGE banding patterns revealed a dominance of ≈ 15 distinguishable amplicons

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in all samples. In the mixed layer the bacterial community was dominated by the same ≈ 15 phylotypes at all stations, but unique phylotypes were found with increasing depth. Except for cyanobacteria, comparison of the bacterial community composition in surface waters from January and December 1995 showed only minor differences, despite significant differences in environmental parameters. These data suggest a horizontal homogeneity and some degree of seasonal predictability of bacterial community composition in the Arabian Sea. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

Bacteria play a major role in carbon dynamics of marine ecosystems (Pomeroy, 1974; Azam et al., 1983; Fuhrman, 1992), consuming a large, but highly variable, fraction of primary production (Cole et al., 1988). In addition, their biomass is often comparable to that of the phytoplankton, even in the euphotic zone (Fuhrman et al., 1989; Cho and Azam, 1990; Simon et al., 1992). Despite their well-documented importance in marine biogeochemistry, heterotrophic prokaryotes generally have been pooled in a “black box” because of the difficulty in distinguishing the species that comprise a given community. This difficulty stemmed from an inability to distinguish bacterial species in complex communities based on morphology and from uncertainties about the biases introduced in culture-based investigations. A greater appreciation of the complexity of microbial communities came with introduction of rRNA gene sequencing as a tool in bacterial systematics (Woese, 1987) and its application in marine environments (e.g., Giovannoni et al., 1990; DeLong et al., 1993; Fuhrman et al., 1993; Mullins et al., 1995; Pinhassi et al., 1997; Rappé et al., 1997; Rath et al., 1998).

The biogeochemical implications of the phylogenetic diversity observed in marine microbial communities are not yet well understood, but there is indirect evidence that bacterial species composition is an important variable controlling the rates and patterns of organic matter hydrolysis. Martinez et al. (1996) found that ectohydrolytic enzyme activities and profiles were highly variable among different bacterial isolates from the Southern Californian Bight. In addition, shifts in bacterial species dominance have been observed both on a short time scale of days to weeks (Rehnstam et al., 1993; Fandino et al., 1998) and on a seasonal basis (Höfle and Brettar, 1995; Pinhassi et al., 1997; Murray et al., 1998). Together these results suggest that enzymatic processing of organic matter can dramatically change due to shifts in bacterial species dominance. Thus, knowledge of the composition of bacterial communities, and how that composition varies over space and time, is likely to be of major importance for understanding the role of bacteria in marine biogeochemistry. However, our knowledge of spatial and temporal variability in marine bacterial communities is still quite limited.

Dramatic seasonal variations in physical forcing and a pronounced mid-water oxygen minimum zone make the Arabian Sea a particularly interesting region for

investigating spatial and temporal variability in microbial communities. As part of the US JGOFS program in the Arabian Sea, we obtained samples during two consecutive NE Monsoon periods from environments ranging from coastal to open ocean and surface to deep sea. In this first study of the phylogenetic composition of bacteria in the Arabian Sea, we sought to determine whether variations in environmental conditions (including the extensive oxygen minimum zone) are reflected in changes in the bacterial community composition.

The approach we used is based on community-level analysis of bacterial 16S rRNA gene fragments using denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993). In this technique, mixtures of PCR-amplified fragments are electrophoretically carried through a gradient of denaturant in a polyacrylamide gel. Sequence variations affect the exact position at which the fragments denature and cease migration through the gel. The resulting banding pattern provides a graphical representation of the composition of the bacterial community. This approach allows a rapid visual comparison of communities among different samples and specific members of the community can subsequently be identified by excising and sequencing bands of interest (e.g., Ferris and Ward, 1997; Kowalchuk et al., 1997; Øvreås et al., 1997).

Using this approach, we compared community composition from different depth zones at five stations during two NE Monsoon periods in the Arabian Sea. We found the bacterial communities to be dominated by ≈ 15 phylotypes, which were generally related to common oceanic groups. Several phylotypes were identified, which were unique to the deep sea samples. In surface waters, we detected only minor differences in the composition of the bacterial community despite major spatial and temporal variations in other biological and physical variables.

2. Methods

2.1. Sample collection

Samples were collected as part of two JGOFS Arabian Sea cruises in 1995 aboard the RV *Thomas G. Thompson*: Cruise TN043 in January and Cruise TN054 in December (Fig. 1). Although collected 11 months apart, samples from both cruises are from different stages of NE Monsoons (Smith et al., 1998). For each cruise, bacterial community genomic DNA was collected from two coastal as well as three offshore stations (up to ca. 1500 km from shore). A total of 14, depth-integrated seawater samples (30–200 l) were collected and analyzed. Seawater was collected with Niskin bottles from three different strata: surface waters (0–80 m), oxygen minimum zone (200–1000 m) and deep-sea samples (2000–4000 m). Samples, anywhere from one to nine discrete depths within a particular stratum, were pooled in 50 l carboys to create the integrated samples. Samples were subsequently filtered through serially connected 47 mm Gelman A/E prefilters and 0.2 μm pore-size capsule filters (Gelman) at a pressure ≈ 1 bar. Capsules were immediately frozen (-80°C) and kept frozen until extraction.

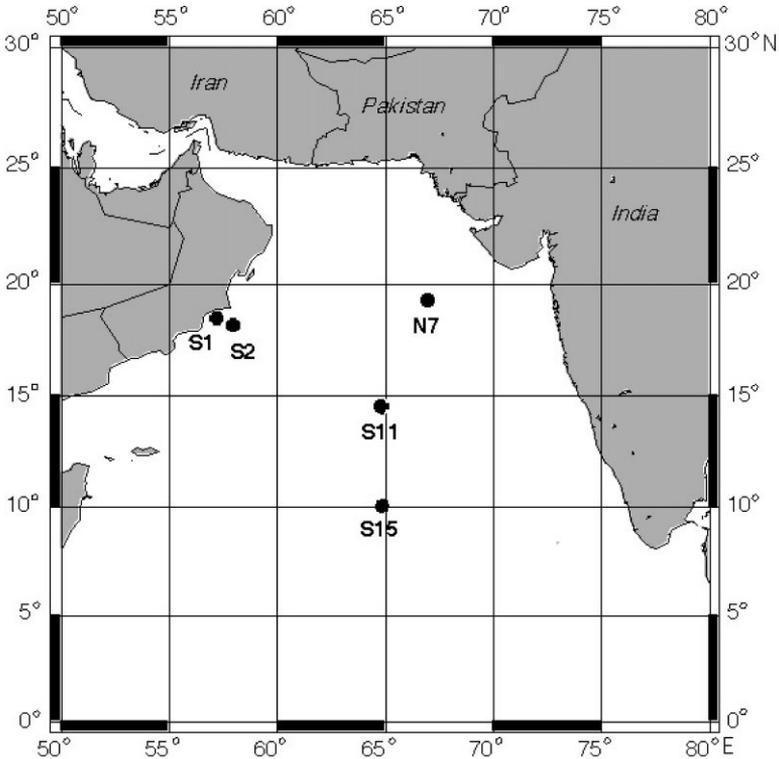


Fig. 1. Map of the Northern Arabian Sea showing station locations.

2.2. DNA extraction

DNA was extracted by the method of Fuhrman et al. (1988) with slight modifications. Twenty ml STE buffer (100 mM NaCl; 10 mM Tris hydrochloride, pH 8.0; 1 mM EDTA, pH 8.0) containing 0.1 vol. of 10% sodium dodecyl sulphate was added to each capsule. The capsule was boiled for 1 min in a microwave oven, left to sit for 2 min, and the lysate subsequently decanted and precipitated with 0.1 vol. of 10.5 M ammonium acetate and 2 vols. of 100% ethanol. The pellet was resuspended in 500 μ l TE buffer (10 mM Tris - 1 mM EDTA; pH 8.0), transferred to a polypropylene centrifuge tube, extracted twice with 500 μ l phenol/chloroform/isoamyl alcohol (25 : 24 : 1), extracted again with 500 μ l chloroform/isoamyl alcohol (24 : 1) and precipitated with ammonium acetate/ethanol. The purified nucleic acids were resuspended in TE and quantified fluorometrically (PicoGreen; Molecular Probes).

2.3. PCR-amplification

Bacterial 16S rRNA genes were PCR-amplified using a universal primer complementary to position 517-534 (5'-ATTACCGCGGCTGCTGG-3') and a

bacterial primer complementary to position 341–358 plus a GC clamp (5'-CGCCCCCGCCGCGCGGGCGGGGCGGGGGCACGGGGGG CCTAC-GGGAGGCAGCAG-3'; Mulyer et al., 1993). The 40 base pair GC clamp (underlined) was attached to the bacterial primer to prevent complete melting of amplicons during DGGE (Sheffield et al., 1989; Mulyer et al., 1993). PCR reactions (100 μ l) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 2.25 mM MgCl₂, 0.8 mM deoxynucleotide triphosphates, 0.5 μ M of each primer, 2 units of polymerase (Amplitaq Gold, Perkin-Elmer) and 10–20 ng of template DNA. Reactions were overlain with mineral oil. Initial denaturation was at 94°C for 5 min followed by a thermal cycling program as follows: *denaturation* for 1 min at 94°C; *primer annealing* for 1 min at an initial 65°C, decreasing 1°C every two cycles to a final of 50°C (Touchdown PCR; Don et al., 1991); *primer extension* for 3 min at 72°C. Thirty cycles were run followed by a final 7 min incubation at annealing temperature (72°C). Negative controls, in which the template was replaced by an equivalent volume of sterile water (MilliQ-purified, autoclaved), were included in each batch of PCR reactions.

2.4. DGGE and cloning

The size of the PCR products was confirmed by agarose gel electrophoresis. Products from triplicate reactions were pooled, precipitated, resuspended in TE buffer, quantified (PicoGreen, Molecular Probes) and 500 ng of PCR product was loaded on 8% polyacrylamide gels (acrylamide:*N,N'*-methylenebisacrylamide 37 : 1) containing denaturant gradients of 25–43% or 25–49%, top to bottom (where 100% is defined as 7 M urea and 40% vol/vol formamide). When analyzed on different gels, PCR products from the same batch were always used. Electrophoresis was performed with a hot bath DGGE unit (CBS Scientific, Del Mar, California) using 0.5 \times TAE running buffer (20 mM Tris, 10 mM acetate, 0.5 mM Na₂EDTA, pH 8.2) at 60°C for 5.5 h at 200 V. Gels were stained for 30 min in SYBR Green I nucleic acid stain (1 : 10,000 dilution; Molecular Probes), destained for 10 min in 0.5 \times TAE, and photographed with UV transillumination.

DGGE bands were excised using a sterilized razor blade and the DNA eluted overnight at 37°C in 400 μ l 1 \times SSC buffer (0.6 M NaCl, 60 mM trisodium citrate, pH 7). The eluate was centrifuged briefly to pellet acrylamide fragments. The supernatant was LiCl₂/ethanol precipitated, resuspended in 10 μ l TE and cloned using the Original TA Cloning Kit or the TOPO TA Cloning kit (Invitrogen). The presence of inserts was checked by restriction digest with EcoRI and agarose gel electrophoresis.

DGGE profiles of reamplified, cloned DNA were used to check for heteroduplexes and to confirm the position of cloned bands relative to the original sample. Heteroduplexes form during mixed template PCR's when annealing occurs between similar but nonidentical products (Fernandez et al., 1993). Reamplified heteroduplexes were seen as two dominant bands at the lower end of the DGGE-gel, presumably representing homoduplex molecules, and one or two fainter bands higher up on the gel representing the relatively unstable heteroduplex molecules. Note that to ensure detection of heteroduplexes, reamplification was always performed using a single

colony from the original plating of transformed cells. During the bi-directional replication of a cloned heteroduplex, the non-homologous strands will resolve into separate plasmids. Therefore, restreaking of an original colony containing a mixture of the two plasmids could result in separation of the component sequences into different colonies, which would make a heteroduplex undetectable.

2.5. Sequencing and phylogenetic analysis

Bi-directional sequencing was performed with the ABI PRISM sequencing kit (Perkin Elmer) using an automated ABI DNA sequencer. Sequences were aligned to known sequences using RDP (Ribosomal Database Project; Maidak et al., 1997) or BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990). All sequences were analyzed by the RDP program CHECK_CHIMERA. Phylogenetic relationships were inferred by the neighbor-joining method using the Phylogeny Inference Package (PHYLIP) version 3.572 (Felsenstein, 1993). Phylogenetic trees were edited using Treeview (Page, 1996).

2.6. Comparison of DGGE and cell counts

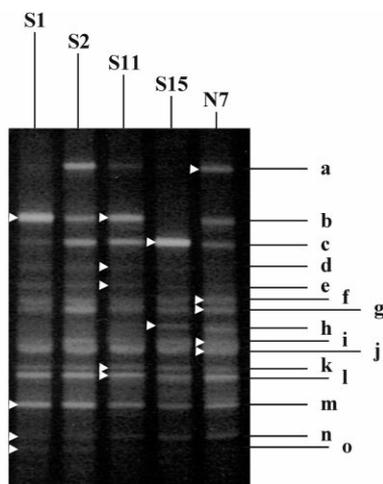
Relative brightness of DGGE bands was calculated by scanning (DuoScan, Agfa) and image analysis (RFLPScan, CSP, Inc.) of a gel photo representing integrated surface samples collected during the December cruise. For each gel lane, the intensity of the individual bands was expressed as percentage of the integrated intensity for the entire lane.

Relative abundances of *Synechococcus* and *Prochlorococcus* spp. in the integrated surface samples used for DGGE were estimated from flow cytometric data collected as previously described (Campbell et al., 1998). Counts of *Synechococcus*, *Prochlorococcus*, and heterotrophic bacteria at the 4–9 depths that comprised each integrated surface sample were used to estimate final cell concentrations in the pooled samples. Relative abundance of each group was then expressed as a percentage of the sum of the concentrations of all three groups. Since samples collected for DGGE analysis and for flow cytometry were from different casts, the same depths were not always available (7 out of 31 cases). In these instances, counts were taken from the next nearest depth or estimated by linear interpolation between the next deepest and next shallowest depths.

3. Results

3.1. Spatial variation

A DGGE profile of bacterial communities in surface samples from two coastal and three mid-oceanic stations in December is shown in Fig. 2. Bands numbered 1–15 were cloned and 12 of those clones were sequenced. Bands 5, 6 and 15 were not sequenced because they were found to be heteroduplexes. The profile shows bacterial



Clone	Taxon	Closest relative	Similarity	
a	NAS1	Chloroplast	OSC182	98%
b	NAS2	Cyanobacteria	SAR7 (<i>Synechococcus spp.</i>)	99%
c	NAS3	Prochlorophyte	<i>P. marinus</i>	99%
d	NAS4	-	-	-
e	NAS5	Heteroduplex	-	-
f	NAS6	Heteroduplex	-	-
g	NAS7	α -Proteobacteria	CS308 ("Magnetococcus")	95%
h	NAS8	α -Proteobacteria	<i>B. bacilliformis</i>	92%
i	NAS9	α -Proteobacteria	SAR464	100%
j	NAS10	α -Proteobacteria	CS308 ("Magnetococcus")	95%
k	NAS11	α -Proteobacteria	PLY43/SAR95	99%
l	NAS12	δ -Proteobacteria	SAR324/SAR276	98%
m	NAS13	α -Proteobacteria	FL1	100%
n	NAS14	δ -Proteobacteria	SAR276	99%
o	NAS15	Heteroduplex	-	-

Fig. 2. DGGE profiles of surface samples collected in December 1995 with gradient of 25–43% denaturant. Excised, cloned and sequenced bands are indicated by arrows and are sequentially numbered according to distance from the top of the gel with the prefix NAS standing for North Arabian Sea. For explanation of clone NAS4 see discussion. The depth range covered by each sample is S1 (1–85 m); S2 (0–60 m); S11 (0–80 m); S15 (0–70 m); N7 (0–60 m).

communities consisting of ≈ 15 distinguishable phylotypes in each sample. The only obvious spatial variation was in relative band intensity of clones related to chloroplasts and cyanobacteria (NAS1, NAS2 and NAS3). The relative intensity of the cyanobacterial bands suggested an inverse relationship between *Synechococcus* (most abundant near shore) and *Prochlorococcus* (most abundant offshore), which correlated with the relative cell abundances determined by flow cytometry (Fig. 3). The correlation was very strong for *Prochlorococcus* ($r = 0.98$, $p = 0.001$), while *Synechococcus* showed a higher degree of variability ($r = 0.81$, $p = 0.108$). Most other dominant bands appear at all stations with indistinguishable relative intensities. One

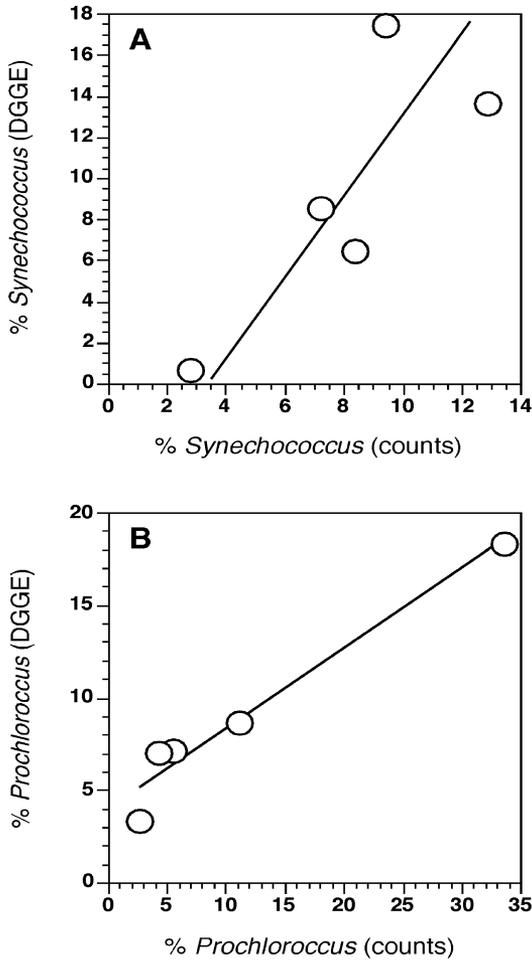


Fig. 3. Comparison of relative DGGE band intensity vs relative cell abundance for (A) *Synechococcus* and (B) *Prochlorococcus* in integrated surface samples collected in December 1995. Curves fit by Model II linear regression are (A) $y = 2.00x - 6.82$ and (B) $y = 0.44x + 3.96$.

exception is NAS8, which appears to be dominant only at the stations further offshore (S15 and N7).

3.2. Variation between cruises

DGGE patterns for surface samples from two coastal stations and one offshore station were compared for the January and December cruises (Fig. 4). The only major changes observed were in relative brightness of the bands representing cyanobacteria and chloroplast phylotypes (NAS1, NAS2 and NAS3). Only minor differences could

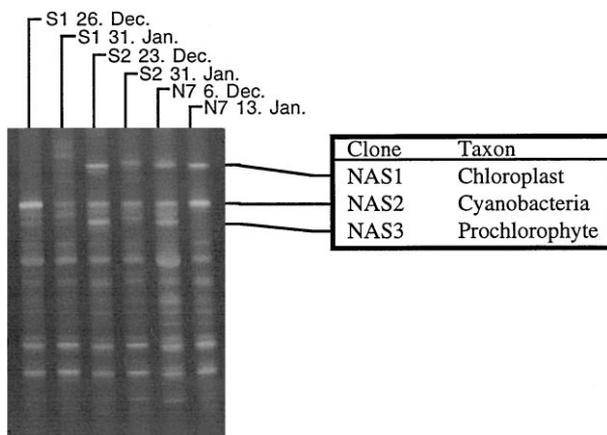


Fig. 4. DGGE profiles of surface samples for the January and December 1995 cruises with gradient of 25–43% denaturant. Labeling of cloned bands is as described in Fig. 2 legend. The depth ranges covered by each sample are (from right): S1 (1–85 m; 0–80 m); S2 (0–60 m; 20–60 m); N7 (0–60 m; 20 m). Labels on lanes indicate station number followed by date of collection.

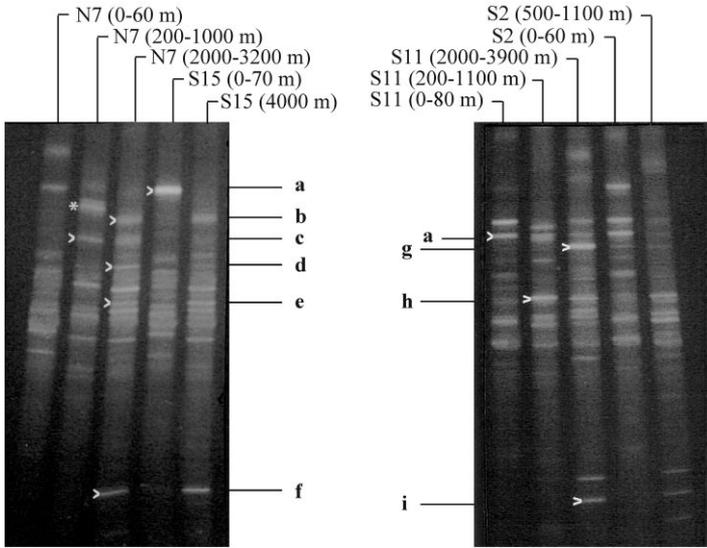
be resolved in the presence or intensity of bands representing heterotrophic bacterio-plankton.

3.3. Variation with depth

DGGE patterns for mixed layer, mid-water and deep-sea samples in December are presented in Fig. 5. Two bands were unique to samples from depths below 2000 m (NAS17, NAS20). Another band (NAS19) was detected below 2000 m at station N7, but is also seen as a faint band from 200 to 1000 m. Two other bands were detected only below 500 m (NAS21, NAS24). Of these, NAS21 was detected in the deep-sea samples from stations N7, S15 and S11, but was also seen in the mid-water zone at station S2. NAS23 was seen as a bright band below 200 m. Several potentially unique bands did appear in samples from the oxygen minimum zone (200–1000 m). However, due to the limited resolution of the gel we only excised one dominant band (Fig. 5) that was with certainty specific for the suboxic zone. Unfortunately, we were unsuccessful in cloning and sequencing this latter band. Eight bands from deep-sea samples which were not dominant in surface samples were cloned and sequenced.

3.4. Phylogenetic analysis of cloned bands

A phylogenetic tree was constructed to visualize the affiliation of the cloned phylotypes to database sequences (Fig. 6). Phylogenetic analyses show that the 18 different bands fall into six phylogenetic bacterial groups. Several sequences branched within the radiation of oxygenic phototrophs which includes cyanobacteria, Prochlorophytes and chloroplasts (NAS1, NAS2 and NAS3). This was expected considering



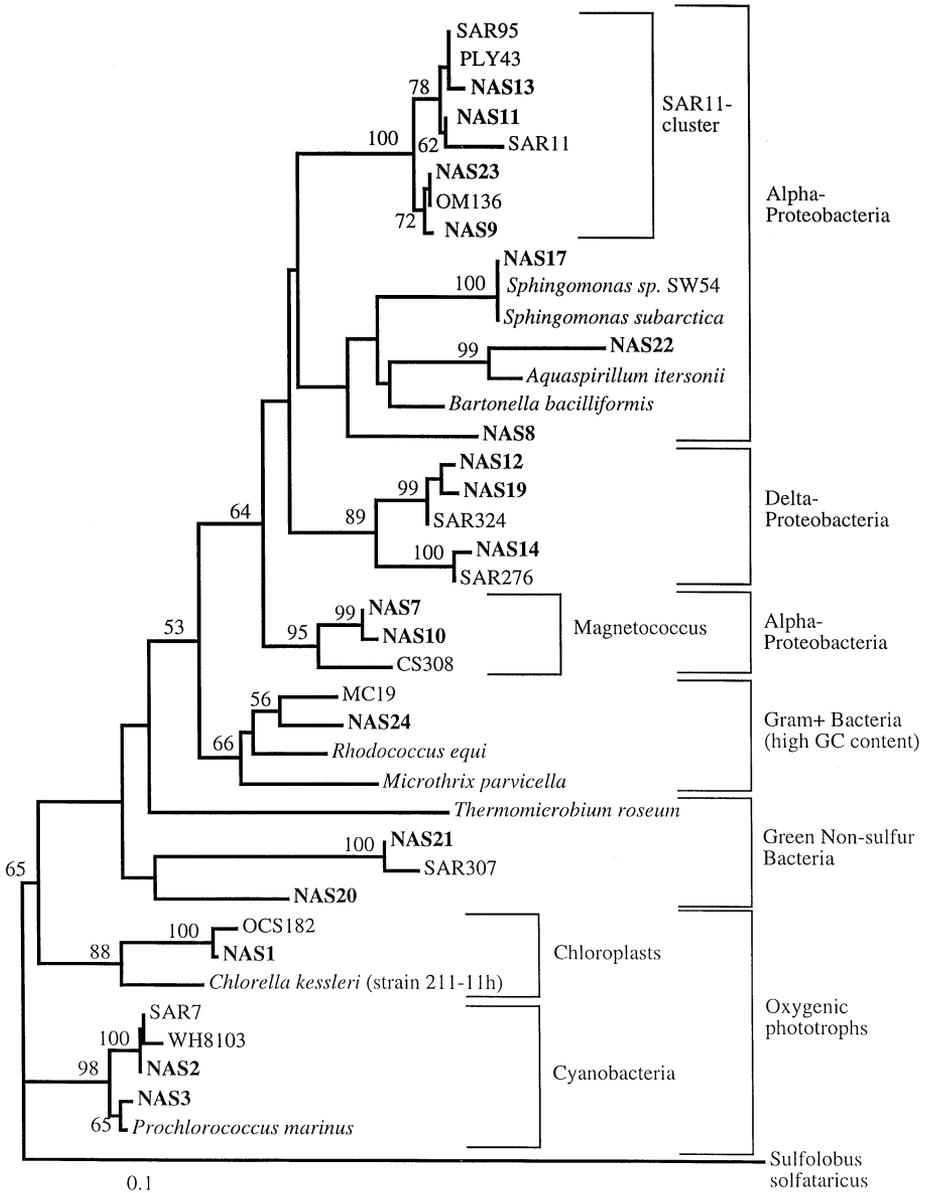
Clone	Taxon	Closest relative	Similarity	
a	NAS16	Prochlorophyte	<i>P. marinus</i>	99%
b	NAS17	α -Proteobacteria	<i>Sphingomonas sp.</i>	100%
c	NAS18	Eubacterium	OCS307	88%
d	NAS19	δ -Proteobacteria	SAR324	98%
e	NAS20	Gr. Non-Sulfur bact.	<i>D. ethenogenes</i>	89%
f	NAS21	Gr. Non-Sulfur bact.	SAR307	98%
g	NAS22	α -Proteobacteria	<i>A. itersonii</i>	94%
h	NAS23	α -Proteobacteria	OM136	100%
i	NAS24	Gram +, high GC	MC19	94%

Fig. 5. DGGE profiles of different depth strata at four stations in December 1995 with gradient of 25–49% denaturant. Two gels were used. Labeling of cloned bands is as described in Fig. 2 legend. Asterisk denotes band which is specific for the oxygen minimum zone. Labels on lanes indicate station and depth range of sample.

Fig. 6. Phylogenetic tree showing relationships of the sequences found in our samples to representative bacterial 16S rRNA genes. The cultured bacteria and the sequences of the closest relatives to our sequences were retrieved from GenBank. The tree was inferred by the neighbor joining method using 173 aligned bases beginning at the equivalent to base 341 and towards the 3'-end of the 16S rRNA molecule (*E. coli* numbers). The number of bootstrap replicates supporting the branching order, from a total of 100 replicates, is shown at the segments. Only values > 50% are shown. An Archaea (*Sulfolobus solfataricus*) was used as an outgroup. Identification of environmental clones are as follows: SAR, Sargasso Sea (Giovannoni et al., 1990; Britschgi and Giovannoni, 1991; Giovannoni et al., 1996; Field et al., 1997; Wright et al., 1997); MC, Mount Coot-tha region (soil), Australia (Stackebrandt et al., 1993); SW, Seawater, Antarctic (Bowman et al., 1997); CS, Lake Chiemsee Sediment, Germany (Spring et al., 1992); OCS, Oregon Coast Study, USA (Rappé et al., 1998); PLY, marine environments (Rochelle et al., unpublished); OM, Ocean Margins, Cape Hatteras, USA (Field et al., 1997). The scale bar indicates substitutions per nucleotide position.

the abundance and global distribution of these groups in ocean waters. Clone NAS2 was found to have chimera characteristics (but see discussion).

Most amplicons were related to α -Proteobacteria. Members of the SAR11-cluster (Giovannoni et al., 1990; Field et al., 1997) were found in both surface (NAS9, NAS11 and NAS13) and deep waters (NAS23). This extends the reported range of these groups from the Mediterranean Sea and the Atlantic and Pacific Oceans (Fuhrman



et al., 1993; Mullins et al., 1995; Benlloch et al., 1995) to include the Arabian Sea. Several clones (NAS8, Fig. 2; NAS17 and NAS22, Fig. 5) were affiliated with the genus *Sphingomonas*. Amplicon NAS8, which also falls in the α -Proteobacteria, is not closely related to any known sequence in the RDP or BLAST databases. Its nearest relative is *B. bacilliformis* with a similarity of 92%. This clone is seen as a bright band only in surface samples from station S15 and N7, but is absent at other stations. NAS17, seen only below 2000 m, was identical to seven closely related species of α -Proteobacteria (not shown), including *Sphingomonas sp.* SW54 isolated from Antarctic waters (Bowman et al., 1997). NAS22 is a very bright band at station S11 (2000–3900 m), but it is also seen as a very faint band at station S2 both in surface waters and in the oxygen minimum zone. This clone is related (94% similarity) to *Aquaspirillum itersonii* which is affiliated with the genus *Azospirillum* (Gram-negative, nitrogen-fixing bacteria).

Two clones (NAS7 and NAS10, Fig. 2) are closely related to a group of uncultured, magnetotactic cocci within the α -subgroup of Proteobacteria found in freshwater sediments (Spring et al., 1992). This group forms a distinct branch separate from other α -Proteobacteria, but the branching order is not well supported by bootstrap values.

Three clones (NAS12 and NAS14, Fig. 2; NAS19, Fig. 5) are related to a recently discovered gene lineage of the δ -subdivision of Proteobacteria. This gene lineage forms a monophyletic cluster within δ -Proteobacteria but shows no specific relationship to any known δ -Proteobacterial 16S genes in databases (Wright et al., 1997). This lineage was found to be most abundant in the lower surface layer of both the Atlantic and the Pacific Oceans, which is consistent with our finding of NAS12 and NAS14 in surface waters in the Arabian Sea. However, NAS19 was found only below 2000 m at station N7.

NAS18, which is seen as a relatively bright band below 200 m at station N7 (Fig. 5), was not closely related to any known species (88% similarity to an environmental clone, OCS307; Giovannoni et al., 1996). However, NAS18 was found to have chimera characteristics with one portion of the sequence identical to an environmental clone (MC13) and the other to *Campylobacter sp.* (ϵ -Proteobacterium).

Clones related to the high GC content subgroup of Gram-positive bacteria (NAS24) and to Green Non-Sulfur Bacteria (NAS20 and NAS21) were found in deeper waters (Fig. 5). Consistent with its high GC content (59%), NAS24 is seen as the lowest band on all the profiles from below 2000 m. NAS20 was only distantly related to Green Non-Sulfur Bacteria (similarity = 89%). NAS21, a distinct band near the bottom of the gel, is closely related to a gene lineage of Green Non-Sulfur Bacteria. This lineage was recently discovered at 250 m depth in the Sargasso Sea by Giovannoni et al. (1996) who found it to be most abundant at the lower end of the deep chlorophyll maximum. We only detected NAS21 at depths below 500 m.

4. Discussion

4.1. Methodology

A major advantage of DGGE over other current methods for community analysis is the relative ease with which qualitative comparisons among samples can be made.

The visual representation provided by the banding pattern and the accessibility of the bands for sequence analysis allows one to quickly focus sequencing efforts only on bands of interest. Because of these advantages, DGGE has found application in a variety of marine and freshwater environments (Murray et al., 1996; Teske et al., 1996; Øvreås et al., 1997). Like other PCR-based approaches, however, this method is subject to possible biases introduced by the amplification reaction such as chimera formation (Kopczynski et al., 1994; Wang and Wang, 1997), heteroduplex formation (Ferris and Ward, 1997), template annealing (Suzuki and Giovannoni, 1996), and preferential amplification of some DNA templates (Reysenbach et al., 1992). In addition, differences in genome size and number of 16S rRNA genes among bacteria (Farrelly et al., 1995) make strictly quantitative interpretations questionable. The unknown extent of these possible biases calls for caution in the use and interpretation of results based on PCR amplification. Nevertheless, several lines of evidence suggest that DGGE can provide a reasonably accurate representation of gross community composition. In this section we discuss some observations on various aspects of the methodology including potential problems and its general reliability.

We assessed the extent to which heteroduplexes and potential chimeras influenced the results by analyzing bands excised from the DGGE gels. In the course of these analyses we found that one must be wary of potential contamination of individual bands with traces of other amplicons. This can be observed when excised bands are directly reamplified by PCR and run again on DGGE. Under such conditions, a single band may yield a dominant band, but also many additional bands matching others from the original community pattern (results not shown). Such contamination is not surprising since some smearing in gel separations is to be expected. Therefore, any given DGGE band cannot be assumed pure, but only a significant enrichment for sequences with a given melting point. Therefore, to obtain cleaner sequencing and more easily interpreted heteroduplex results, eluted bands were cloned prior to further analysis.

To ensure that a selected clone contained the intended sequence, each was checked by reamplification and DGGE to verify realignment with the originally excised band. In one instance, a picked clone (NAS4) was found to realign to a different band (NAS13), suggesting that we had inadvertently cloned a contaminating amplicon (Fig. 2). Sequencing confirmed that the NAS4 contaminant clone was identical to NAS13. To get a rough idea of the frequency with which “contaminants” might be cloned, we reamplified seven clones prepared with DNA eluted from a single band (NAS23, Fig. 5). In this case we found that six out of seven clones aligned with the original band (data not shown). This indicates that while the target sequence may predominate, contaminant clones can occur at a significant frequency. Fortunately, it is relatively easy to use reamplification and DGGE to verify clone identities as well as identify and eliminate heteroduplexes prior to investing in sequence analysis. Since only one clone was sequenced from each band, this protocol would not have discriminated among any co-migrating bands having different sequences, but the same melting point. The rRNA sequence diversity in the samples may therefore be underestimated.

Identification of chimeras has some degree of uncertainty and requires careful consideration of the resulting probability. For example, one of our two candidates, clone NAS2, is a possible chimera composed of *Synechococcus* (SAR7) and *Prochlorococcus* (*P. marinus*). Each part composing this purported chimera has 100% match to the sequences of the component species, which indicates a very high probability of being a chimera (Robison-Cox et al., 1995). However, for several reasons we speculate that this sequence is a natural chimera, as has been proposed for some other sequences (Sneath, 1993). First, the 100% match to *Prochlorococcus* and not to *Synechococcus* in one half of the sequence is the result of a single base-pair change. Second, this clone was a very bright band and no other major band was identified which could have contributed to the formation of this chimera. Third, the spatial distribution of this amplicon was in accordance with that determined by flow cytometric counts of *Synechococcus* spp. (discussed below).

One current limitation of DGGE is the shortness of the amplified fragments which can be separated. However, it has previously been shown that phylogenetic analyses based on such partial sequences are largely congruent with those calculated using most of the 16S gene (Schmidt et al., 1991; Rath et al., 1998). Even so, some loss of resolution is to be expected when using shorter sequences (e.g., NAS17 could not be resolved from six other closely related species). In addition, because we have focused only on the variable region, deeper phylogenetic relationships were not well resolved (e.g., note the low bootstrap values for the *Magnetococcus* branch). Nevertheless, by using 16S rRNA gene fragments of ca. 170 base-pairs in the variable V3 region (Neefs et al., 1990), we were able to distinguish most phylotypes from their relatives and determine phylogenetic relationships among our clones. Most clones were closely related to sequences in the databases (89–100% similarity; average 97%), while three clones were only distantly related (NAS8, NAS18 and NAS20). Although NAS18 may be a chimera, NAS8 and NAS20 appear to be phylogenetically undescribed species. One of our clones (NAS17) was 100% identical to a cultured bacterium.

Positive correlations between relative band brightness and cell counts for *Prochlorococcus* and *Synechococcus* spp. suggest that DGGE can, at least in some cases, provide semi-quantitative information about community composition. The correlation was remarkably strong for *Prochlorococcus*, but the higher variability seen with *Synechococcus* is not surprising. Although some of the variability in the relationship between cell counts and band brightness may represent artifacts of PCR amplification, there are other potential sources of error for which we did not control. One source may be diel variations in DNA content per cell (i.e., genome copy number) resulting from synchronization of cell cycle in the populations. Slopes of the regression lines in Fig. 3 suggest that DGGE tended to overestimate *Synechococcus*, and underestimate *Prochlorococcus* abundances, perhaps reflecting differences in growth rates or ribosomal gene copy number. There are also likely to have been substantial and variable losses resulting from pre-filtration through glass fiber filters (done for DGGE samples, but not for flow cytometry). Despite all these potential sources of error, DGGE was able to discriminate at least the major variations in the relative cyanobacterial abundances.

Overall, we found DGGE to perform consistently and reliably with replicate PCR reactions and DGGE gels yielding identical patterns. Bands from two different samples, but at the same vertical position in a gel, were found to have identical sequences (NAS3). Likewise, bands with the same relative position on two separate gels yielded identical sequences (NAS3, Fig. 2 and NAS16, Fig. 5). The expected power of DGGE to resolve closely related sequences also was confirmed by finding two distinct bands that differed by only a single base pair (NAS7 and NAS10). We observed some gel-to-gel variation in exact band positions, but relative positions remained stable. This means that any amplification bias for or against a 16S fragment is constant. Together with the cyanobacterial data discussed above, this suggests that variations in bacterial community composition are reflected by variations in DGGE banding patterns.

4.2. Horizontal and seasonal predictability

The most striking result from the spatial and temporal DGGE profiles was that the dominant amplicons, which were related to heterotrophic bacterial phylotypes, were very similar among surface samples from coastal and off shore stations, even when sampled 11 months apart. Since sample collections were at similar points in the seasonal cycle, our results do not exclude the possibility of seasonal variations, but do at least suggest a predictability in the community composition for a particular season. We note that the similarities in DGGE banding patterns were observed despite significant variability in some other biological parameters between cruises, and/or across the study area. To illustrate, data from the US JGOFS Data System (<http://www1.whoi.edu/arabianobjects.html>) were used to calculate average parameter values for surface water at the same stations and over the same depth ranges (0 to ≈ 100 m) that were sampled for DNA extractions on the two cruises. Average primary productivity ranged over 3-fold ($\approx 7\text{--}21 \mu\text{g C l}^{-1} \text{ h}^{-1}$), while average leucine and thymidine incorporation in the surface layer ranged over 7-fold ($\approx 17\text{--}129 \text{ pmol l}^{-1} \text{ h}^{-1}$) and 15-fold ($\approx 0.4\text{--}6.4 \text{ pmol l}^{-1} \text{ h}^{-1}$), respectively. Chlorophyll a and bacterial abundance ranged over 2.5 to 3-fold ($\approx 0.2\text{--}0.6 \mu\text{g l}^{-1}$ and $0.6\text{--}1.5 \times 10^6 \text{ ml}^{-1}$, respectively) while 7 to 9-fold ranges were observed in average abundances of *Prochlorococcus spp.* and *Synechococcus spp.* ($\approx 4\text{--}30$ and $2.5\text{--}23 \times 10^4 \text{ cells ml}^{-1}$, respectively).

Bacterial communities have been shown to change both on a seasonal basis (Lee and Fuhrman, 1991; Gordon and Giovannoni, 1996; Pinhassi et al., 1997; Murray et al., 1998) and on a short-term basis (days to weeks, Rehnstam et al., 1993; Fandino et al., 1998; Murray et al., 1998), indicating that biotic and abiotic factors regulate the success of bacterial populations within the community. In this context, it is surprising to find little difference in community composition of heterotrophic bacterioplankton obtained from stations up to 1500 km apart representing coastal and offshore environments of the Northern Arabian Sea. These findings suggest that individual bacterial populations can to some extent adapt to, and proliferate in, a changing environment. Thus, even some pronounced environmental changes would not necessarily result in population successions.

4.3. Vertical distributions

In contrast to the apparently uniform bacterial community composition along horizontal gradients, most amplicons appeared to have a depth-dependent distribution. This is consistent with observations for a few specific phylotypes from the Atlantic and Pacific Oceans (Giovannoni et al., 1996; Gordon and Giovannoni, 1996; Field et al., 1997; Wright et al., 1997). An interesting example is clone NAS21, which is closely related (98% similarity) to SAR307, an environmental clone related to Green Non-Sulfur bacteria found by Giovannoni et al. (1996). They used probes to investigate the distribution of the SAR202 gene cluster (containing SAR307) and found a distinct maximum at the lower boundary of the chlorophyll maximum in both the Pacific and Atlantic Oceans. Based on this distribution, Giovannoni et al. speculated that members of this cluster are aerobic photo-heterotrophs. We found the NAS21 band to be brightest in the deepest samples (below 2000 m) and undetectable above 500 m. This suggests that, while phototrophy may occur within the SAR202 cluster, it is unlikely to be a universal feature of the group.

Two bands aligning with the cyanobacterial bands were found below 2000 m at station S11. The detection of these phototrophs in the deep ocean may be the result of transport via fecal pellets and/or marine snow (Silver and Alldredge, 1981; Silver and Gowing, 1986). We note that since these bands from the deep samples were not sequenced, it is theoretically possible that they simply represent some unrelated bacterial groups having DNA with similar melting points (Kowalchuk et al., 1997). However, this seems unlikely given the exact alignment of both bands between surface and deep samples.

We expected to find a pronounced effect of the oxygen minimum zone on bacterial community composition. For example, this zone (200–1000 m) is known to be a site of extensive denitrification (see Naqvi 1994 for review), suggesting that denitrifiers would be abundant. Several potentially unique bands did appear in samples from the oxygen minimum zone. However, due to the limited resolution of the gel we can not be certain that most were actually specific to those samples. An attempt to sequence one band that was with certainty specific for the suboxic zone (Fig. 5) was unsuccessful. Thus, although we have no evidence for denitrifiers in our samples, they could well have been represented among the amplicons and may have turned up with the sequencing of more bands.

Overall, our comparison of DGGE profiles from different depths revealed that, although the number of apparently dominant phylotypes was similar for each depth stratum, pronounced differences in composition are seen with depth. Similar differences have been observed in the Antarctic (Murray et al., 1998), the Baltic Sea (Höfle and Brettar, 1995), and in a stratified lake in Norway (Øvreås et al., 1997); however, in the latter two studies the number of dominant phylotypes was lowest in deeper waters. It should be noted that our use of depth-integrated samples complicates interpretations of vertical variations in diversity and will mask finer scale vertical variations in bacterial community composition. Despite these limitations, the depth profiles suggest that the environmental conditions associated with increased depth are highly selective.

4.4. Diversity

Overall, the phylogenetic groups of bacteria found in the Arabian Sea are similar to common groups found in other oceans, e.g., cyanobacteria, α - and δ -Proteobacteria, Gram-Positive bacteria, and Green Non-Sulfur bacteria. Notable exceptions are the presence of clones related to magnetotactic bacteria and the lack of γ -Proteobacteria (GP) and members of the Cytophaga–Flavobacter–Bacteroides phylum (CFB). It is surprising that no GP's and CFB's were found, since these have routinely been found in other oceans (Britschgi and Giovannoni, 1991; Fuhrman et al., 1993; DeLong et al., 1993; Benlloch et al., 1995; Pinhassi et al., 1997; Suzuki et al., 1997). This is unlikely to be caused by discrimination against these groups by the DGGE analysis, since investigations of other communities using identical primers and procedures in the same laboratory have turned up dominant members from these groups (Fandino et al., 1998; L. Riemann, unpublished results).

Somewhat different results were found in a deep (2500 m) Arabian Sea sample analyzed by cloning and sequencing of PCR-amplified rDNA (J. A. Fuhrman and A. Davis, unpublished results). They analyzed 18 bacterial clones of PCR-amplified DNA sampled from station S15 in January 1995, and found one archaeal clone with the remaining 17 clones in the α -Proteobacterial clade containing *Roseobacter denitrificans*. This observation of one dominant phylogenetic cluster differs from our findings of diverse phylogenetic groups using DGGE and sequencing. Differences in both sampling (pooled depths vs. a discrete depth) as well as methodology (cloning vs. DGGE, different primer sets and PCR conditions) may well contribute to this apparent discrepancy.

In surface waters we found two clones (NAS7 and NAS10) related to a group of uncultured, magnetotactic cocci found in freshwater sediments (Spring et al., 1992). Magnetotactic bacteria are not restricted to sediments but have also been found in a highly stratified estuary near the oxic/anoxic transition zone (Bazyliński et al., 1995). However, to our knowledge this is the first report of clones related to magnetotactic bacteria in oceanic waters. Since magnetotactic bacteria are adapted to exploit oxic/anoxic transitions and some have been shown capable of denitrification (Bazyliński and Blakemore, 1983) it is tempting to speculate that they are important in denitrification in the upper oxygen minimum zone. However, any such speculation has to be reconciled with the presence of these clones in the surface samples which were collected above the oxycline.

The low number of distinguishable bands (≈ 15) on the DGGE gels argues for a comparably low number of dominant bacterial phylotypes, although it does not exclude the possibility of other phylotypes occurring in smaller numbers, being non-amplifiable or being masked by co-migration. Similar results have been found by DGGE for estuaries (Murray et al., 1996; Teske et al., 1996) and a lake (Øvreås et al., 1997), but higher numbers of amplicons (≈ 20 – 30) were observed in Antarctic waters (Murray et al., 1998). The number of dominant phylotypes detected by PCR-DGGE seems low compared to analysis of clone libraries composed of cloned PCR-amplified community DNA (e.g., Fuhrman et al., 1993; Benlloch et al., 1995; Fuhrman and Davis, 1997). These authors find very few identical clones within the same sample

indicating a higher bacterial species diversity than estimated here. To our knowledge, a comparison of direct cloning vs. DGGE of PCR products using identical samples has not been reported. Therefore, it is not known whether, or to what extent, differences in methodology contribute to the apparent differences in estimated diversity.

Many studies to date which have undertaken a molecular characterization of bacterial communities have naturally tended to focus on differences and novelties. This has been invaluable in extending our knowledge about the extent of microbial diversity. However, it also can foster the perception that natural marine bacterial communities are overwhelmingly complex and variable. In contrast, we found that DGGE fingerprints of bacterial community composition in the surface waters of the Arabian Sea were nearly identical, both across the ocean basin and for two different samplings separated by nearly a year. This suggests that complete characterization and ecological study of the numerically dominant bacterial phylotypes in the ocean could be both a fruitful and manageable endeavor.

Acknowledgements

We thank H. Ducklow for logistical support, D.C. Smith for assistance in sampling, J.A. Fuhrman and A. Davis for advice on sample collection and processing and for sharing unpublished sequence data, G. Toledo for assistance in generating the phylogenetic tree, and R.T. Barber and J. Marra for sharing their data on chlorophyll a and primary production. This work was funded by NSF grants to F.A. (OCE 93-12695), L.C. (OCE 93-11246, OCE 96-12509), M.R.L. (OCE 93-11246), and H. Ducklow (OCE 95-00601). This is U.S. JGOFS contribution number 492.

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