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Response of bacterial and viral communities to nutrient manipulations in seawater mesocosms

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ABSTRACT: Changes in natural bacterial and viral assemblages were studied in seawater mesocosms manipulated with inorganic (nitrate + phosphate) and inorganic + organic (glucose) nutrient additions. As inferred from the gel band patterns obtained by DGGE, only moderate changes within the bacterial community took place when mineral nutrients were added alone. Supplementing the mineral nutrients with glucose in excess of what the bacteria could consume led, however, to major changes in band patterns. Based on fluorescence *in situ* hybridisation (FISH), the major bacterial response was identified as an increase in the population of γ-*Proteobacteria* with a smaller response in α-*Proteobacteria*. Sequencing of bands from the DGGE gels indicated that glucose + mineral nutrients led to a *Vibrio*-dominated bacterial community. A specific FISH probe was designed from a band sequence affiliated to *Vibrio splendidus*, and linked a large-celled bacterial morphotype to the DGGE-gel bands dominating in glucose-amended mesocosms. A similar difference in the response of the viral populations among treatments was demonstrated using pulsed field gel electrophoresis (PFGE). The number of bands on DGGE gels and PFGE gels were similar (mean ratio 0.98). We suggest an interpretation of these results where coexistence of nutrient-competing bacterial hosts is controlled by viral lysis. We also suggest that the success of large bacteria in glucose-replete treatments was not based on superior glucose-utilisation abilities, but rather on an advantage in competition for limiting mineral nutrients derived from the combination of a large cell surface with a low cellular content of the limiting element, possible for cells with large C-rich inclusion bodies.

KEY WORDS: Bacteria · Virus · Community composition · DGGE · PFGE · FISH · *Vibrio*

INTRODUCTION

Introduction of methods such as fluorescence microscopy (Zimmermann & Meyer-Reil 1975) and thymidine incorporation (Fuhrman & Azam 1980) gave quantitative data required to support the idea that heterotrophic bacteria play a key role in the biogeochemical cycling, not only of carbon, but also of the elements limiting phytoplankton growth (Williams 1981, Azam et al. 1983). Many aspects of the functioning of the microbial part of the pelagic food web could then be studied from a 'black box' perspective without resolving the bacterial community into its sub-populations. One could thus circumvent the massive methodological problems involved in trying to understand bacterial processes of natural systems at the level of species and sub-populations. With the introduction of new molecular techniques, however, one now has tools allowing the return to questions at this level of resolution. With the insights gained in the meantime from 'black box'

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studies of the microbial food web, a major present challenge is to merge the 2 approaches into a coherent description that links diversity to control mechanisms for biogeochemical cycles, and that links food web interactions to the control of diversity within the bacterial community.

It is reasonable to expect that changes in bacterial community composition are driven by environmental factors that affect bacterial abundance and activity (Riemann & Middelboe 2002). Important factors expected to control bacterioplankton communities include nutrient supply, temperature, grazers and viruses (see e.g. Upton et al. 1990, Pernthaler et al. 1996, Šimek et al. 1999, Wommack et al. 1999, Riemann et al. 2000, Castberg et al. 2001). To understand how growth, productivity and diversity of bacterioplankton is regulated, it is necessary to understand the coupling between genetic and functional aspects of the microbial community. Molecular tools can be used, not only to characterise the genetic diversity within a microbial community, but also to differentiate bacterial species and their dynamics in natural environments without cultivation. Profiling of microbial communities using fingerprinting methods have mainly been based on analysis of PCR products amplified from community DNA with a subsequent separation on DGGE/TGGE (Muyzer et al 1993, Wintzingerode et al. 1997). DGGE bands depict the predominant community members, and variations in the signal intensity are raw estimates of *in situ* changes of population abundance (e.g. Øvreås et al. 1997, Casamayor et al. 2000a). Sequencing of 16S rDNA molecules from clone libraries or DGGE has been especially informative, and fluorescent oligonucleotide probes are therefore important molecular tools to enumerate particular bacterial populations and determine their spatial distribution (Amann et al. 1995, Casamayor et al. 2002). Application of these molecular techniques to mesocosm experiments is one possible approach towards the goal of linking identity (genotype) and function (phenotype) within the bacterial community. Simple Lotka-Volterra-based models illustrate that microbial biodiversity, population dynamics, and biogeochemical cycling may be seen as intimately coupled aspects of the pelagic food web (Thingstad 2000). Although the past decade has produced significant progress in the understanding of diversity and dynamics of bacterial species (Riemann & Middelboe 2002), our ability to understand and eventually predict the ecological role of microorganisms still seems crucially dependent upon a much increased understanding of how identity and function are linked.

We here report changes in biodiversity within bacterial and viral communities induced by nutrient manipulation of mesocosms in Isefjorden, Denmark. Phytoplankton responses and responses in bulk properties of this experiment have been reported elsewhere (Joint et al. 2002).

MATERIALS AND METHODS

Experimental set-up. The mesocosm experiment was performed at Søminestasjonen, a field station located at Isefjorden, a shallow Danish fjord on the west coast of Zealand, Denmark, during the period 20 to 31 May 2000. The mesocosms consisted of polyethylene bags, and each mesocosm was fixed to a pontoon bridge. The mesocosms were filled with 1.7 m^3 surface water from the fjord, and thereafter continuously mixed using an airlift system moving water from the bottom of the bags to the top, through a tube into which air was injected in the middle of the tube. Sampling was performed using a Plexiglas tube, collecting an integrated water sample of the entire water column in the bags. Inorganic nutrients were added as nitrate ($NaNO_3$), phosphate (NaH_2PO_4), silicate (Na_2SiO_4) and glucose as carbon source. For a detailed description of the mesocosm set-up, see Joint et al. (2002).

In this study we focus on 5 mesocosms: a control (#5) receiving no additions, 2 mesocosms that received phosphate and nitrate as a low (#1) and high (#2) daily dose in the Redfield ratio, and 2 mesocosms (#8 and #9) that received the same dose of mineral nutrient amended with an excess of glucose-C relative to a Redfield C:N:P ratio of 106:16:1 (Table 1). The experiment was terminated after 6 d by a storm destroying the plastic enclosures, leading to leakage between the fjord and the mesocosms.

DNA extraction. For DNA extraction, water samples (1 to 5 l) taken from the mesocosms were prefiltered through a 48 µm mesh membrane, and subsequently

Table 1. Nutrient additions as daily doses. Nitrogen (N) was added as nitrate, phosphorus (P) as orthophosphate, and organic-C (C) as glucose

#5 Control	#1 Balanced low	#2 Balanced high
No additions	800 nM N 50 nM P 0 nM C C:N:P = 0:16:1	1600 nM N 100 nM P 0 nM C C:N:P = 0:16:1
	#8 Balanced low + C	#9 Balanced high + C
	800 nM N 50 nM P 63600 nM C C:N:P = 1272:16:1	N: 1600 nM P: 100 nM C: 21200 × 6 nM C:N:P = 1272:16:1

filtered through a 2 µm filter before the bacterioplankton community were collected on a 0.22 µm Sterivex filter column. In total, 1.8 ml of lysis buffer (40 mM EDTA; 50 mM Tris, pH 8.3; 0.75 M sucrose) was added to the filters and the samples were kept at –20°C until DNA was extracted. DNA was extracted as described in detail by Schauer et al. (2000).

DGGE. These analyses were performed with samples from the different mesocosms at different times. Extracted genomic DNA (fraction size 2 to 0.2 µm) was used as target DNA in the polymerase chain reaction (PCR) to amplify fragments suitable for subsequent DGGE analysis using primer combinations PRBA 8f (5′-AGAGTTTGATCCTGGCTCAG-3′) and PRUN 518r (5′-ATTACCGCGGCTGCTGG-3′). A 40 bp rich GC clamp was attached to the 5′ end of the PRBA 8f primer as described by Muyzer et al. (1993). Amplification was performed as previously described (Øvreås et al. 1997). The PCR amplification produced 500 bp long products, which were subsequently separated by DGGE using a linear gradient of urea and formamide. DGGE was performed with a Hoefer Scientific SE600 vertical dual-cooler system. PCR samples were loaded onto 8% (wt/vol) polyacrylamide gels in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2 EDTA, pH 7.4). The linear gradient of urea and formamide ranged from 30 to 60% denaturant. The electrophoresis was run at 60°C, first for 10 min at 20 V and subsequently for 20 h at 60 V. After electrophoresis, the gels were stained for 45 min with SYBR Gold nucleic acid stain (MolecularProbes) in TAE buffer. The gels were rinsed in distilled water and photographed. DNA fragments to be nucleotide sequenced were punched from the gel and processed as previously described (Øvreås et al. 1997). The gels were analysed using a software program developed by Svein Norland (Dept. of Microbiology, University of Bergen), where presence/absence of bands was recorded. Clustering is based on the simple matching algorithm, while the dendrogram is drawn applying the group average method.

Pulse field gel electrophoresis (PFGE). Two litres of sample were concentrated using the Vivaflow 200 tangential flow module with a cut-off at 100 000 (Vivascience) following the manufacturer's procedure. To this sample 20 µl of a 10% Tween 80 solution was added. The sample was shaken vigorously to disperse viruses attached to bacteria or particles in the sample, followed by centrifugation in a swing-out centrifuge (Beckmann J2-HS) at 7500 × *g* for 10 min at 4°C. The virus particles were subsequently concentrated by ultracentrifugation (Beckman L8-M with SW-28 rotor) for 2 h at ~100 000 × *g* at 10°C. The virus pellet was resuspended and incubated overnight in 200 µl SM buffer (0.1 M NaCl; 8 mM $MgSO_4 \cdot 7H_2O$; 50 mM Tris-HCl; 0.005% [w/v] glycerine) (Wommack et al. 1999) at 4°C.

Equal volumes of virus concentrate and molten 1.5% InCert agarose (FMC) were mixed and dispensed into plug moulds. After the gel had solidified, plugs were punched out from the moulds into a small volume of buffer (250 mM EDTA; 1% SDS) containing 1 mg ml^{-1} Proteinase K. The plugs were incubated in the dark at 30°C overnight. The Proteinase K digestion buffer was decanted and the plugs were washed 3 times, 30 min each in TE (10 mM Tris-Base; 1 mM EDTA, pH 8.0). The virioplankton agarose plugs were stored at 4°C in TE 20:50 (20 mM Tris; 50 mM EDTA, pH 8.0).

Viral plugs, and plugs containing phage lambda concatamers (Bio-Rad) serving as molecular weight markers, were placed into wells of a 1% SeaKem GTG agarose (FMC) gel in 1 × TBE gel buffer (90 mM Tris-borate; 1 mM EDTA, pH 8.0) with an overlay of molten 1% agarose. A total of 0.1 µg of *Hin*dIII-digested lambda fragments (Promega) were used as a DNA standard for quantification. Samples were run using a Bio-Rad DR-II CHEF Cell (Bio-Rad) electrophoresis unit operating at 200 V, with pulse ramps from 20 to 45 s at 14°C for 23 h in 0.5 × TBE tank buffer (45 mM Tris-borate, and 1 mM EDTA, pH 8.0). After electrophoresis, the gels were stained for 30 min in SYBR Green I (MolecularProbes) according to manufacturer's instructions and digitally scanned for fluorescence using a laser fluoroimager (FujiFilm, FLA2000). The gels were analysed using a software program designed by Svein Norland, Dept. of Microbiology, University of Bergen, where presence/absence of bands were recorded.

Transmission electron microscopy (TEM) and X-ray element analysis of individual cells. Cells were harvested directly onto grids by centrifugation in a Beckman model L8-70M preparative ultracentrifuge, using a SW41 swing-out rotor for 10 min, at 7000 × *g* at 20°C. We used aluminum grids (100 mesh, Agar Scientific) supported with a carbon-coated formvar film, and after centrifugation the grids were air-dried. The unfixed and unstained cells were viewed and analysed for elements in a Philips CM 200 electron microscope. Sample grids were mounted between beryllium rings, and the microscope was operated in scanning mode at a tilt angle of 38°, 80 kV acceleration voltage, magnification between 5000 and 10 000×, spot size of 14 nm (spot size 3), and an accumulation time (live time) of 30 s. X-ray spectra were recorded from areas that circumscribes the specimen (Norland et al. 1995). The light element detection system consisted of EDAX detector DX-4 supported with SIS Soft Imaging Software. In this system we identify cells by the imaging system, the cell area is marked out, and an identical area is located in a particle-free area.

Total cell counts and fluorescent *in situ* hybridisation (FISH). Water samples measuring 1 to 5 ml from

each mesocosm bag were concentrated onto white polycarbonate filters (25 mm diameter; pore size 0.2 µm; Osmonics) by applying a low vacuum. The filters were subsequently fixed by the methods of Glöckner et al. (1999) and stored at –20°C until use.

All oligonucleotide probes used in this study were synthesised with a CY3 fluorochrome at the 5′ end (Interactiva Biotechnologie). Each filter was cut into 4 sections prior to hybridisation with labeled oligonucleotides. All hybridisations were performed at 46°C for 2 h in a hybridisation solution containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 35% formamide, 0.01% sodium dodecyl sulfate and 50 ng of CY3-labeled oligonucleotide. Hybridisations, washings and DAPI staining of filters were performed as outlined by Glöckner et al. (1999). Labeled oligonucleotides used in this study included the domain-specific probes EUB338 for *Bacteria* (Amann et al. 1990), and ARCH915 for *Archaea* (Stahl & Amann 1991). The group-specific probes ALF968 (Glöckner et al. 1999), BET42a (Manz et al. 1992), and GAM42a (Manz et al. 1992) were used to detect the α, β and γ subclasses of *Proteobacteria*. Probe CF319a detected members of the cytophaga-flavobacterium cluster of CFB phylum (Manz et al. 1996). Two probes were also designed in this study to detect *Vibrio*-related organisms, Vib-197: and Vib-620. The complimentary NonEUB338 served as the negative control for non-specific binding (Wallner et al. 1993). Probes BET42a and GAM 42a were used with competitor oligonucleotides as described previously (Manz et al. 1992).

Filter sections were visualised with a Zeiss Axioplan epifluorescence microscope fitted with a 50 W high pressure mercury bulb and specific filter sets (DAPI [Zeiss 01]; CY3 [Chroma HQ 41007; Chroma Tech]). Bleaching was avoided by viewing each microscope field first with the CY3 filter set before switching to the DAPI filter set. For each sample and probe, replicate filters and more than 500 cells were counted. For DAPI examination, more than 2000 cells were counted per sample. The mean abundances and standard deviations were calculated from the counts of 20 randomly chosen fields on each filter. All counts were corrected by subtracting the counts obtained with the negative control NonEUB338.

For determining total bacterial counts only, 1 to 5 ml of water was filtered onto black polycarbonate filters (dimensions as before), stained with DAPI as outlined previously (Porter & Feig 1980), and examined by epifluorescence microscopy as described above.

Design of *Vibrio*-specific probes. Two probes were designed in this study for detection of *Vibrio*-related organisms, Vib-197 (AGGTCCGAAGATCCCCCT) and Vib-620 (ACAGCACTCTAGTTCACCAG). Probes were designed using the PROBE_Design service of the ARB software package. Vib-197 probe design was based on retrieved DNA sequence of DGGE bands from the mesocosm bags (see Fig. 5, Band 20). Vib-620 probe was based on the theoretical predicted sequence to the closest related organism, *Vibrio splendidus*. Probes were checked for positive specificity against *V. splendidus* (NCIMB 1). The closely related species *V. ordalii* DF1K (NCIMB 2168), *V. ordalii* DF3K (NCIMB 2167), *V. natriegens* (NCIMB 1901) and *Methylococcus capsulatus* (Bath) failed to hybridise the probe with conditions used. Control whole-cell hybridisations were performed as outlined previously (Amman et al. 1995) using the conditions of 46°C for 2 h in hybridisation solution containing 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 35% formamide, 0.01% sodium dodecyl sulfate and 50 ng of CY3-labeled oligonucleotide.

RESULTS

Bacterial total counts and morphology changes

Changes in bacterial abundance in this experiment are reported in detail in Joint et al. (2002). Briefly, there was a moderate net increase from ca. 1.6×10^6 cells ml^{-1} at the start of the experiment to 3.4 and 4.5×10^6 cells ml^{-1} in Bags 1 and 2, respectively, on Day 5. Adding glucose together with mineral nutrients gave a more marked net increase, with a maximum abundance of 7.4×10^6 cells ml^{-1} reached in Bag #9 on Day 4 and final (Day 5) values of $6.3 \times$ and 6.8×10^6 cells ml^{-1} in Bags #8 and #9, respectively. Bacterial production estimated from leucine or thymidine incorporation showed a similar pattern, with a slight tendency towards increased bacterial production in Mesocosms #1 and #2 receiving inorganic nutrients. A much larger stimulation was seen in Mesocosms #8 and #9 where glucose was added together with inorganic nutrients (Joint et al. 2002). Examination by fluorescence microscopy and TEM revealed a new population of large rod-shaped bacteria emerging in the mesocosms where glucose had been added (Fig. 1). Because of the large cell size of this population, the effect of glucose on bacterial biomass was probably much larger than the effect on bacterial abundance. These cells had a molar C:N:P ratio of 260:37:1 and large electron-thin intracellular areas, indicating internal stores of C-rich material (Fig. 1E,F).

FISH

The fraction of microorganisms hybridising with the *Bacteria*-specific EUB338 probe was between 35 and

75% of DAPI total counts in the control mesocosms (#5) and from 35% at Day 0 to approximately 95% at Day 4 for the glucose-treated mesocosms (Fig. 2).

Group-specific probes for some groups within the subdomain *Proteobacteria* (α, β and γ) were used on all mesocosms (Fig. 3A–C) to ascertain the composition of the bacterial community. The γ-*Proteobacteria* had the largest response to the addition of glucose, with the population increasing from around 5% at Day 0 to 35% for Mesocosm #8 (Day 5) and 42% for Mesocosm #9 (Day 4). The control mesocosm (#5) had no such large increase of γ-*Proteobacteria*. The α-*Proteobacteria* also increased in the glucose treated bags, though the response was not as pronounced (from ~2 to ~10% of total population). The presence of β-*Proteobacteria* in all mesocosms throughout the experiment was low, and these results are in agreement with other studies in marine environments (Glöckner et al. 1999, Giovannoni

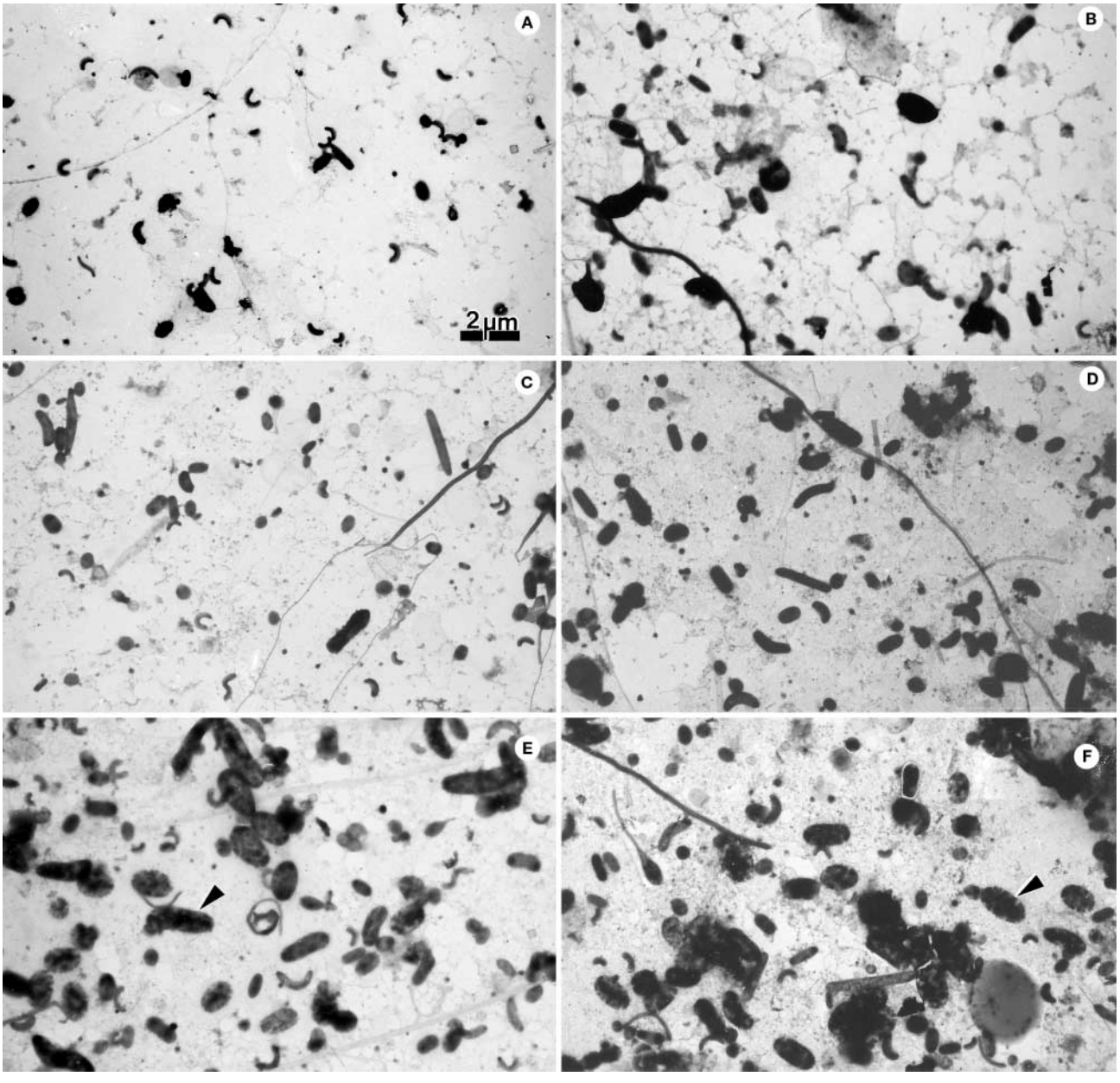

Fig. 1. Transmission electron micrograph of positively stained bacteria from the mesocosm experiment. (A) Bag #1, Day 0; this reflects the start of the experiment. The bacterial community is characterised by the presence of relatively few and small bacteria. (B) The control bag (Bag #5) at Day 5, (C) Bag #1, Day 5, (D) Bag #2, Day 5, (E) Bag #8, Day 5 and (F) Bag #9, Day 5. In Bags #8 and #9, the large rod-shaped bacteria (arrows) are dominating

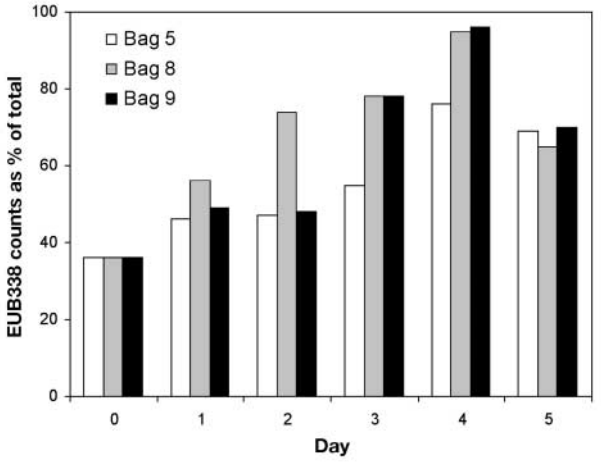

Fig. 2. Fraction of bacterioplankton, as determined by *in situ* hybridisation, with rRNA-targeted fluorescent oligonucleotide probe EUB 338 for *Bacteria*, as percentage of the total bacterial count (DAPI) in control mesocosm (Bag #5). The mesocosms added high amounts of glucose in addition to mineral nutirents (Bags #8 and #9)

& Rappé 2000). The cytophaga-flavobacterium population increased in all the mesocosms, including the control, from ~1 to ~12% at the end of the experiment. Archaeal populations within all the mesocosms were below the detection limits, indicating that they were a minor component of the prokaryotic assemblage (results not shown). Thus, neither confinement nor glucose addition favoured growth of marine Archaea.

The detected α-, β- and γ-*Proteobacteria* and cytophaga-flavobacterium population accounted for less than 10% of the total DAPI counts at the start of the experiment. On Day 4, this had increased to 29% in Mesocosm #5, 51% in Mesocosm #8 and 62% in Mesocosm #9. Increasing glucose concentrations thus led to increased detected levels for these groups of organisms. However, other phylogenetic groups known to be widespread in marine ecosystems, such as *Planctomycetales* (Schlesner 1994), were not probed in this study, and the coverage of the proteobacterial probe set is known to be incomplete. For example, the GAM42a probe has been reported not to detect all deep branching bacteria in the gamma subclass of *Proteobacteria* (Glöckner et al. 1998, 1999). Therefore, we carried out a PCR-based fingerprinting technique (DGGE) using universal bacterial primers in order to expand the view of the bacterial assemblage.

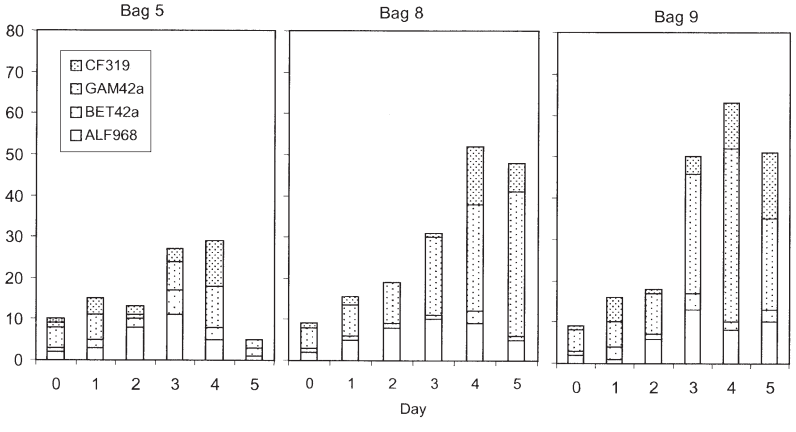

Fig. 3. Community structure of the bacterioplankton as determined by *in situ* hybridisation with rRNA-targeted fluorescent oligonucleotide probes in the mesocosms. Probe data are given as percent of cells detectable after staining with DAPI. Specifications of the probes are as follows: EUB338 for *Bacteria*; CF319 for the cytophaga-flavobacterium cluster; ALF968 for α-subclass *Proteobacteria*; BET42a for β-subclass *Proteobacteria*; and GAM42a for γ-subclass *Proteobacteria*

DGGE

Each microbial assemblage in the size range 2 to 0.2 µm produced reproducible DGGE fingerprints with 10 to 19 bands visible on the gels. As inferred from the DGGE band pattern, the control mesocosm (#5) had a very stable bacterioplankton community. After 2 d, 1 additional population was emerging, but none of the original community members seemed to disappear during the experimental period in the control mesocosm. The addition of mineral nutrients alone (#1 and #2) induced a moderate change in band pattern, with 2 to 3 extra bands emerging. The most marked changes in band pattern were found when glucose was added in addition to mineral nutrients (#8 and #9), suggesting a more profound alteration of the bacterial community structure. The number of bands increased during the experimental period, and the original bands seemed to be preserved during the observational period. We could also detect important changes in the relative intensity of some of the bands, suggesting changes in the relative abundance of the different 16S rDNA-defined populations. Cluster analysis based on the presence of bands and their relative intensity (Fig. 4) gave 3 main clusters. One contained all samples from the control mesocosms, the closest cluster to this contained all samples from mesocosms receiving mineral nutrients only (#1 and #2), and the most distant cluster contained all samples from mesocosms receiving mineral nutrients and glucose (#8 and #9). Shannon index based on number of bands and their intensity was in the range 1.5 to 2.0 for all mesocosms, with an increasing trend throughout

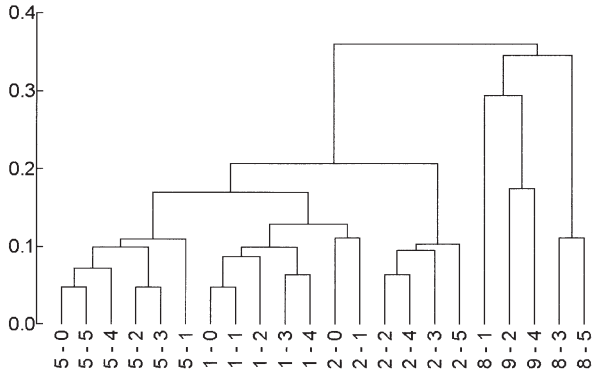

Fig. 4. Dendrogram showing the similarity of DGGE patterns from different mesocosms and sampling time (e.g. Bag #5, Day 2 is marked 5-2). Clustering is based on the simple matching algorithm, while the dendrogram is drawn applying the group average method

the experiment, indicative of an increase in bacterial diversity during the experimental period.

DGGE sequencing, phylogenetic analysis and specific probes

Selected bands from the DGGE were sequenced to better describe the phylogenetic diversity of the amplified rDNA from the mesocosms. At the start of the experiment, the amplicons were related to *Actinobacteriales* (gram-positive) and *Planctomycetales* and several uncultured *Proteobacteria* (Fig. 5, Table 2). At the middle and end of the experimental period, 16S rDNA sequences were retrieved from organisms previously known to be cultivable and targeted by the probes used. DGGE analysis from glucose-amended mesocosms revealed some very dominant bands. Sequencing analysis of these bands showed that they were phylogenetically affiliated to *Vibrio splendidus*. Suspecting these bands to correspond to the microscopically observed population of large rod-shaped cells in the same mesocosms, 2 species-specific FISH probes (Vib-197 and Vib-620) were constructed, based on the sequencing analysis. The large dominating bacterium did indeed hybridise to both these probes (Fig. 6). Several of the other bands taken from these mesocosms were also phylogenetically affiliated to the *Vibrio* genus. This large rod shaped *Vibrio* species constituted a very small percentage of the bacterial population in the control mesocosm (#5) throughout the experiment. In the glucose treated mesocosms however, they reached a peak of ~7 and ~13% of the total DAPI counts at Day 4 in Mesocosms #8 and #9, respectively. This corresponded to approximately $\frac{1}{3}$ the population of γ-*Proteobacteria* detected, *Vibrio* species being a subset of this phylogenetic group.

PFGE

Virioplankton PFGE fingerprints were obtained for viral genomes with molecular sizes between 25 and 630 kb. We found between 5 and 21 bands with an average of 13 bands in the mesocosms. The lowest number of bands were retrieved at the start of the experiment (Day 0), whereas the highest numbers of bands were found at the end of the experiment (Day 5) in the mesocosm where glucose was added at higher concentrations (#9). The ratio between the number of bands on PFGE and DGGE gels from the same sample varied between 0.5 and 1.5 with a mean of 0.98. As was the case for DGGE, samples from Mesocosm #5 clus-

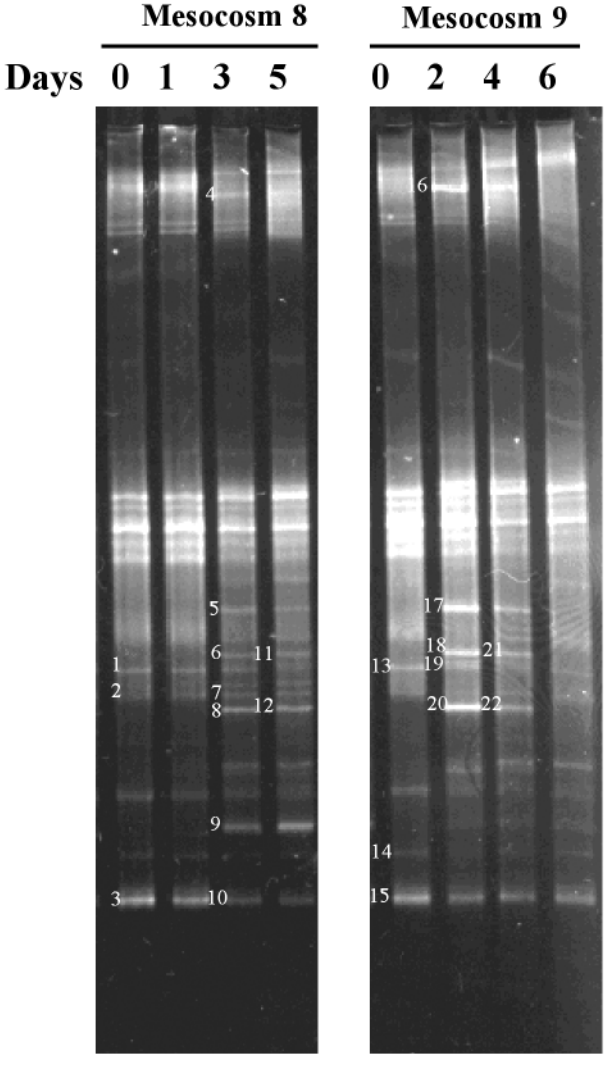

Fig. 5. DGGE profiles of the bacterioplankton community composition over time in Mesocosms #8 and #9. A total of 22 bands were excised from the DGGE gels and amplified by PCR, and the nucleotide sequences were determined according to Table 2

Table 2. Sequence information from dominating bands on DGGE-gels in glucose-amended mesocosms (#8 and #9)

Band	% similarity[a]	Alignment[b]	Mesocosm	Day	Closest relative	Taxonomic description
1	81	321/395	8	0	Unidentified proteobacterium	β-*Proteobacteria*
2	89	286/319	8	0	Uncultured proteobacterium	α-*Proteobacteria*
3	86	373/433	8	0	*Agrococcus jejensis*	Firmicutes
4	94	479/506	8	3	*Vibrio* sp	γ-*Proteobacteria*
5	97	498/513	8	3	*Vibrio* sp. TK327	γ-*Proteobacteria*
6	96	458/475	8	3	*Vibrio* sp. TK327	γ-*Proteobacteria*
7	88	401/454	8	3	Uncultured proteobacterium	α-*Proteobacteria*
8	99	501/502	8	3	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
9	89	397/442	8	3	*Marinomonas protea*	γ-*Proteobacteria*
10	87	375/433	8	0	*Agrococcus jejensis*	Firmicutes
11	97	460/475	8	5	*Vibrio* sp. TK327	γ-*Proteobacteria*
12	99	501/502	8	5	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
13	81	321/395	9	0	Unidentified proteobacterium	β-*Proteobacteria*
14	88	203/230	9	0	Unidentified planctomycetales	Planctomycetales
15	86	373/433	9	0	*Agrococcus jejensis*	Firmicutes
16	95	480/506	9	2	*Vibrio* sp	γ-*Proteobacteria*
17	97	498/513	9	2	*Vibrio* sp. TK327	γ-*Proteobacteria*
18	98	499/508	9	2	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
19	88	421/474	9	2	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
20	99	498/503	9	2	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
21	98	499/508	9	4	*Vibrio* sp. Etwt2	γ-*Proteobacteria*
22	99	501/502	9	4	*Vibrio* sp. Etwt2	γ-*Proteobacteria*

[a]Sequences were aligned to the closest relative using BLAST (Altschul et al. 1997). The similarity was calculated with gaps not taken into account

[b]The part of the total sequence used in alignment

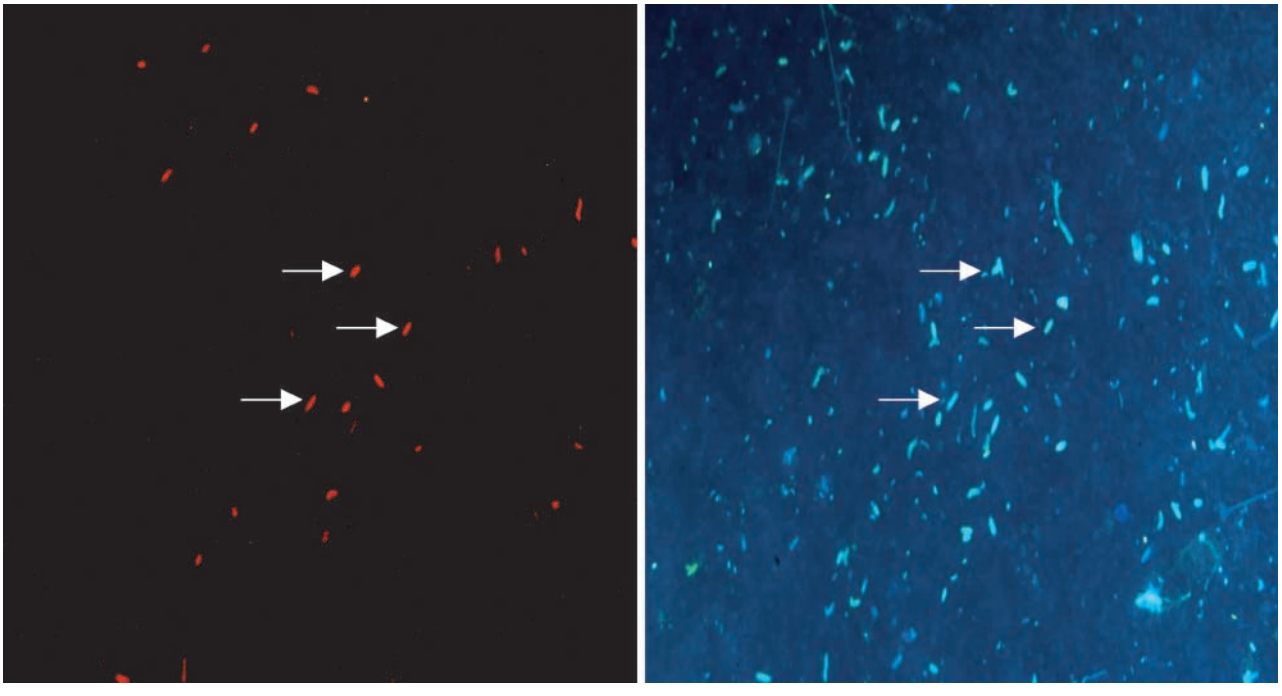

Fig. 6. Epifluorescence photomicrographs of *in situ* hybridisation with CY3-labeled Vib-620 probe (left) and corresponding DAPI-stained cells (right). Photomicrographs represent the same field of view. The sample is from Bag #9 at Day 4 of the experiment. Large rod-shaped *Vibrio* cells are positively probed (see arrows for 3 examples)

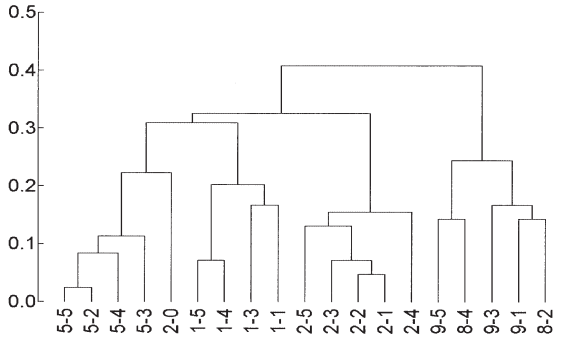

Fig. 7. Dendrogram showing the similarity of pulsed field gel electrophoresis (PFGE) patterns from different mesocosms and sampling time (e.g. Bag #5, Day 2 is marked 5-2). Clustering is based on the simple matching algorithm, while the dendrogram is drawn by applying the group average method

tered together (Fig. 7), suggesting moderate changes in the viral population in the control. Two neighbouring clusters contained samples from Mesocosms #1 and #2, respectively, while the most distant cluster contained samples from Mesocosms #8 and #9. The most profound changes in viral community composition thus appeared to take place in mesocosms receiving mineral nutrients and glucose together. Shannon diversity indices based on number of bands and their relative intensities were in the range of 1.4 to 3.5.

DISCUSSION

The most notable changes in nutrient-manipulated seawater were found in mesocosms receiving glucose and inorganic nutrients together. Here, a significant increase in bacterial cell counts, biomass, and production was accompanied by major changes in community composition of both bacteria and viruses. Addition of mineral nutrients alone had a much smaller effect on abundance, biomass, and community composition. Bioassay measurements suggested that at the start of the experiment there was a mixture of N and C limitation of bacterial growth, whereas in the mesocosms supplemented with N, P and glucose, N was the nutrient most likely to limit bacterial growth (Joint et el. 2002). Phytoplankton biomass and production was reduced in the presence of excess labile DOC, interpreted as a result of increased competition from energy- and carbon-replete bacteria in glucose-amended mesocosms, but pigment analysis suggested little change in the phytoplankton community composition for any of the treatments (Joint et el. 2002). An effect of organic loading on the composition of the bacterioplankton communities has been found also by others (Pernthaler et al. 1998, Bosshard et al. 2000). The response to organic enrichment would, however, be expected to be closely linked to the state of the ecosystem present at the start of the experiment, and should therefore be generalised only with great caution. In systems with mineral limited bacteria (see e.g. Pomeroy et al. 1995, Rivkin & Anderson 1997, Thingstad et al. 1998, Zohary & Robarts 1998) it seems reasonable to expect a different type of response.

In our experiment, the amount of glucose added to the mesocosms was much larger than the amount the bacteria could consume (Joint et al. 2002). In Mesocosms #8 and #9 it is thus difficult to envisage any important competition for glucose between bacteria able to use this as their main source of energy and carbon. Given enough organic C, bacteria will have to compete, not only with each other, but also with phytoplankton for available nitrogen and phosphate substrates. The nitrate and phosphate added was consumed to below detection limit in Mesocosms #1, #2, #8, and #9 (Joint et al. 2002). Given this background, we hypothesise a shift from a bacterial community dominated by species with a high ability to compete for organic carbon sources (in Mesocosms #5, #1 and #2) to a community dominated by species with a high ability to compete for mineral nutrients (in Mesocosms #8 and #9). In this scenario, the success of large morphological forms in Mesocosms #8 and #9 may at first sight seem to be in contradiction to the usual assumption that small size is an advantage for nutrient competition at permanently low concentrations. Big bacterial cells, able to keep storage inclusions like glycogen, have been observed also in the field, and were suggested to be better competitors in fluctuating environments (Casamayor et al. 2000b). However, since diffusion transport increases in proportion to cell size (e.g Jumars et al. 1993), large cell size could actually be an advantage rather than a disadvantage, also at permanently low substrate concentrations. The requirement would be a mechanism allowing an increase in size without a concomitant increase in the cellular requirement for the limiting nutrient. Using excess organic C to 'blow up the balloon' under N- or P-limited conditions would be such a mechanism. This seems to have been the case in Mesocosms #8 and #9 where dominating cells had large electron-thin inclusions, probably containing C-storage material. X-ray analysis revealed a particularly low P content relative to C in these cells (molar C:N:P = 260:37:1). In natural unperturbed populations, X-ray analysis has shown a C:N:P ratio in bacterial biomass of 50:10:1 (Norland et al. 1995, Fagerbakke et al. 1996). A size-increasing strategy such as this would presumably also shift the predation pressure towards larger size-selective predators (Šimek et al. 1999), potentially reducing predatory pressure. If this interpretation is correct, the strategy used by

Vibrio splendidus and other large-celled bacterial species would be analogous to what has previously been suggested for diatoms (Thingstad 1998). In the proposed diatom scenario, silicate is used to build an exosceleton that permits a large, nutrient-free vacuole, with a large cell, a large surface area, and a low cellular N and P content as the result.

The bacterial community structure was analysed using *in situ* hybridisation with fluorescent rRNA-targeted oligonucleotide probes (FISH). This application has been successfully applied to characterisation of bacterial communities in lakes, on lake snow aggregates, coastal waters and marine environments (Glöckner et al. 1996, Pernthaler et al. 1998, Simon et al. 1999). The low fraction registered as *Bacteria* at the start of our experiment (36%) is lower than the values reported from studies in oligo- and mesotrophic lakes (Glöckner et al. 1996, Pernthaler et al. 1998), and much lower than that which has been reported in oceanic environments such as the Southern Ocean (Simon et al. 1999). The proportion of cells visualised with the EUB probe reached, however, 96% in the mesocosms where glucose was added in large amounts (#9).

This increase in probe-detectable cells was probably due to a transformation of bacteria to an actively growing state with high ribosome content, rather than to a shift towards a higher proportion of *Bacteria* in the community. This is consistent with results obtained from the leucine incorporation assay, where a significant increase in bacterial production was detected in Mesocosms #8 and #9 (Joint et al. 2002). The effect was, however, not simply due to the added nutrients alone since the control mesocosm (#5) also has an observable increase, though not as dramatic as in the glucose-treated mesocosms.

In the beginning, the group-specific probes used in this study (ALF968, BET42a, GAM42a and CF319) assigned only 10% to known groups, which is in agreement with the results obtained from sequencing of dominant bands on DGGE. In the mesocosms that received glucose (#8 and #9), the sum of hybridised cells increased to 50–63% of the total DAPI counts after Day 3 of the experiment. The control mesocosms (#5) experienced a more moderate increase to 29% of the total DAPI counts detected with the accumulated probe set at Day 4. The presence of β-*Proteobacteria* was low in all mesocosms throughout the experiment, a result consistent with other studies in marine environments (Glöckner et al. 1999, Giovannoni & Rappé 2000). The cytophaga-flavobacterium population increased from ~1 to ~12% in all mesocosms, including the control, at the end of the experiment. With no pronounced effect of nutrient additions relative to control, a confinement effect has probably been the reason for the increase of this population. Archaeal populations were a minor component of the prokaryotic assemblage within all the mesocosms. *Archaea* were thus either not favoured by any of the experimental conditions of this experiment, or their response was too slow for this 6 d experiment.

Bacterial community composition profiles during the 6 d of the mesocosm study was analysed by DGGE of PCR amplified 16S rDNA. The number of bands on DGGE gels and the diversity as measured by the Shannon index increased over time in the mesocosms. Since our experimental period was relatively short, this effect may be a transient phenomenon where the community still comprised declining, but not yet outcompeted, sub-populations of the initial community, co-existing with emerging populations of species favoured by the new conditions. We can thus not infer from our study whether adding glucose or mineral nutrients would eventually lead to a new steady state with increased or decreased bacterial diversity.

Sequence information from the DGGE gels suggested that some of the dominating bands retrieved from the mesocosms at the start of the experiment showed phylogenetic affiliation to planctomyces and actinomycetes. Glöckner et al. (2000) have found actinobacteria to account for up to 63% of the bacterioplankton biomass in lake water. A major taxon of obligate marine bacteria within the order *Actinomycetales* has recently been discovered in ocean sediments. Populations of these bacteria are found to be persistent and widespread, and the majority of isolates require seawater for growth (Mincer et al. 2002). Actinomycetes have also been detected in several marine environments by DGGE, with subsequent sequencing of the bands (L. Øvreås unpubl.). In a study where the culturability and *in situ* abundance of pelagic bacteria from the North Sea were analysed, Eilers et al. (2000) found that the majority of isolates belonged to the *Pseudoalteromonas*, *Alteromonas* and *Vibrio* genera. These organisms were easily cultured, but they only constituted a minor fraction of the bacterioplankton community, and they were not detected in the 16S rRNA library. FISH data from these samples revealed that these organisms represented less than 1% of the total cell counts, whereas CFB was identified by FISH to comprise 17% of the total cells. These data are consistent with our finding that the addition of glucose enriched the community in easily culturable organisms such as the *Vibrio* genera identified from sequence analysis of DGGE bands.

A morphologically characteristic cell type in glucose-amended mesocosms was identified as being related to *Vibrio splendidus*, and other DGGE bands were also representatives of the *Vibrio* genus. Therefore, these results indicate that we have several coexisting populations with similar morphology (Casamayor et al.

2002), and/or that we detected some artefacts such as multioperons inside the same population, resulting in a considerable degree of microdiversity. The *Vibrio* genus is one of the best-known marine taxa. Vibrio-related populations are abundant in eutrophic coastal environments and are able to form temporal blooms with a patchy distribution (Heidelberg et al. 2002). It used to be claimed as one of the major components of the bacterial flora of marine bacterioplankton communities (Rehnstam et al. 1993). The growth strategy of vibrios has been studied in detail, and the bacteria are easily culturable on agar plates (Nyström et al. 1990a,b). It has been shown that these marine bacteria can survive carbon starvation for extended periods of time, and can grow rapidly at high substrate concentrations with a high cellular rRNA content. If our interpretation of this experiment is correct, this genus also has members able to compete for mineral nutrients under carbon- and energy-replete conditions.

For both DGGE and PFGE, the number of bands detected is a conservative estimate of the total number of bands, since it depends on the detection threshold. By applying ribosomal intergenic spacer analysis (RISA), generally fewer numbers of bands were detected. However the major changes in bacterial community composition were also seen in Mesocosms #8 and #9 (data not shown). The lowest number of PFGE bands were detected at the start of the experiment and the numbers of bands seemed to increase with time and treatment. For DGGE, both the number of bands and the pattern of evolution in different mesocosms were similar to those for PFGE. This is illustrated by the near 1:1 ratio (ranging between 0.5 and 1.5, mean 0.98) between PFGE and DGGE band numbers, and provides circumstantial evidence that theories assuming viral control of bacterial diversity (Thingstad 2000) may capture important aspects of system functioning. By similar principles as for viruses, however, any loss mechanism selective among bacteria such as, e.g., size, shape or taste selectivity in protozoan grazing, could theoretically add to the regulation of bacterial diversity. Species-specific viral lysis thus probably works in combination with effects from predation, as demonstrated experimentally by several investigators (Šimek & Chrzanowsli 1992, Jürgens & Güde 1994, Pernthaler et al. 1996, Šimek et al. 1999, 2001).

PFGE has been used to analyse natural variability of viruses in marine virioplankton. By PFGE, Wommack et al. (1999) found that the Chesapeake Bay virioplankton community composition contained an average of 11 viral subpopulations, whereas Steward et al. (2000) found 14 to 35 bands in 4 marine samples. When the same methods were applied in a seawater enclosure in Raunefjorden (Norway), the number of bands varied between 4 and 16 with an average of 11 bands (Larsen et al. 2001). Following a bloom of *Emiliania huxleyi*, the overall number of viral genome bands was lower, ranging from 6 to 10 bands with an average of 8 (Castberg et al. 2001). The average of 13 bands found in this study is thus consistent with previously published studies.

In the idealised Lotka-Volterra model analysed by Thingstad (2000), total bacterial abundance at steady state is controlled by protozoan grazing, while lytic viruses control the number of co-existing host-populations within this community. In this conceptual framework, the number of niches is thus a kind of top-down-controlled property of the system. Which species will fill these niches is, however, also determined by the relative abilities of the different bacterial species to compete for the substrate limiting bacterial growth rate in the given system, and is thus a feature also controlled by bottom-up mechanisms. In our experiment, the similarity in band numbers on the PFGE and DGGE gels, the co-variation seen in treatment-induced changes in these patterns, and the success of bacterial morphotypes argued to have a strategy suited for mineral nutrient competition in glucose-replete treatments are results that are at least qualitatively consistent with such a conceptual framework. Similar lines of reasoning at the higher, 'black-box' level of functional groups would suggest that glucose addition should stimulate bacterial, relative to phytoplankton, success in the competition for limiting mineral nutrients. Consistent with this, a reduction was indeed found in chlorophyll levels in glucose amended mesocosms (Joint et al. 2002). The results of this experiment may thus seem encouraging in suggesting that relatively simple models combining algal-bacterial predation with protozoan grazing and viral lysis may provide a useful conceptual framework for further exploration of the links between the 'black-box' level of food-web descriptions, and descriptions with a resolution approaching species level.

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