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Viral and Flagellate Control of Prokaryotic Production and Community Structure in Offshore Mediterranean Waters[▽]

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A dilution and size fractionation approach was used to study the separate and combined effects of viruses and flagellates on prokaryotic production ([^{3}H]leucine incorporation) and community composition (16S rRNA gene PCR and denaturing gradient gel electrophoresis [DGGE]) in the upper mixed layer and the deep chlorophyll maximum in the offshore Mediterranean Sea. Four experiments were established using differential filtration: a resource control without predators (C treatment), treatment in the presence of viruses (V treatment), treatment in the presence of flagellates (F treatment), and treatment in the presence of both predators (VF treatment). The V and VF treatments increased prokaryotic abundance (1.4- to 2.3-fold) and the number of DGGE bands (by up to 43%) and decreased prokaryotic production compared to the level for the C treatment (by 22 to 99%). For the F treatment, significant differences compared to the level for the C treatment were found as well, but trends were not consistent across experiments. The relative abundances of the high-nucleic-acid subgroups of prokaryotes with high scatter (HNAhs) in flow cytometer settings were lower in the V and VF treatments than in the C and F treatments. These differences were probably due to lysis of very active HNA prokaryotes in the V and VF treatments. Our results indicate that the presence of viruses or viruses plus flagellates sustains prokaryotic diversity and controls prokaryotic production by regulating the proportion of the highly active members of the community. Our data also suggest that lysis and grazing control influences the relationship between bacterial community composition and prokaryotic production.

During the last 3 decades, a wealth of information on the mechanisms controlling prokaryotic production in the ocean has been accumulated (26). It is commonly accepted that inorganic nutrients, composition and bioavailability of dissolved organic matter (DOM), and predation are the major factors shaping the spatiotemporal variability of prokaryotic production. Protistan grazing might result in the formation of grazing-resistant prokaryotic cell types (16, 23) and in a stimulation of specific growth rates (39) by recycling nutrients (45, 48). Viruses, considered here to be another type of predator, cause a mortality of prokaryotes that is highly variable but, on average, as important as protistan grazing (summarized in references 56 and 63).

A modeling approach suggested that viral lysis reduces the carbon flow to higher trophic levels and stimulates prokaryotic production (10). Support for this "stimulation of production" hypothesis comes from experimental data showing that viral lysis increases bacterial production and respiration (34) at the food web level. However, in experimental studies with the viral community either present or absent, a negative effect of viruses on bacterial production was typically found (6, 35, 65). It has also been argued that grazing should have a negative effect on viral production, since grazers consume infected cells (5, 58). However, in a set of experiments performed with freshwater systems, viral infection was stimulated in the presence of grazers, suggesting synergistic interactions (41, 54, 55).

Prokaryotic community composition can vary during phytoplankton blooms (4, 40), probably due to changing DOM composition and bioavailability, as suggested by the covariation in the types of ß-glucosidases (4). It has also been suggested that phages control competitive dominants for resource acquisition, thus sustaining diversity (10). This idea has been mathematically described in the "killing the winner" hypothesis (53) and is also applicable to flagellates (52). In a general model, diversity shows a hump-shaped distribution along gradients of increasing resource as well as consumer control (64). However, this relationship can vary for different taxonomic groups of bacteria. In an in situ study conducted in the North Sea, the number of bacterial phylotypes was negatively related to viral abundances and prokaryotic production and respiration (42, 62). Others have shown for the open ocean a positive relationship between the number of phylotypes and productivity (18) and predictability of bacterial community structure over annual cycles (11). Differences in such relationships between environments were also detected (19). Moreover, a latitudinal diversity gradient has been demonstrated for bacterioplankton (12). In a study with coastal water, in the presence of viruses, flagellates, or both types of predators, bacterial production decreased and the number of phylotypes detected by a genetic community fingerprint increased compared to the levels for predator-free controls (65). Overall, there is evidence that protistan grazing (e.g., references 24, 48, and 49) and viral lysis (e.g., references 21, 44, and 61) can affect prokaryotic community composition.

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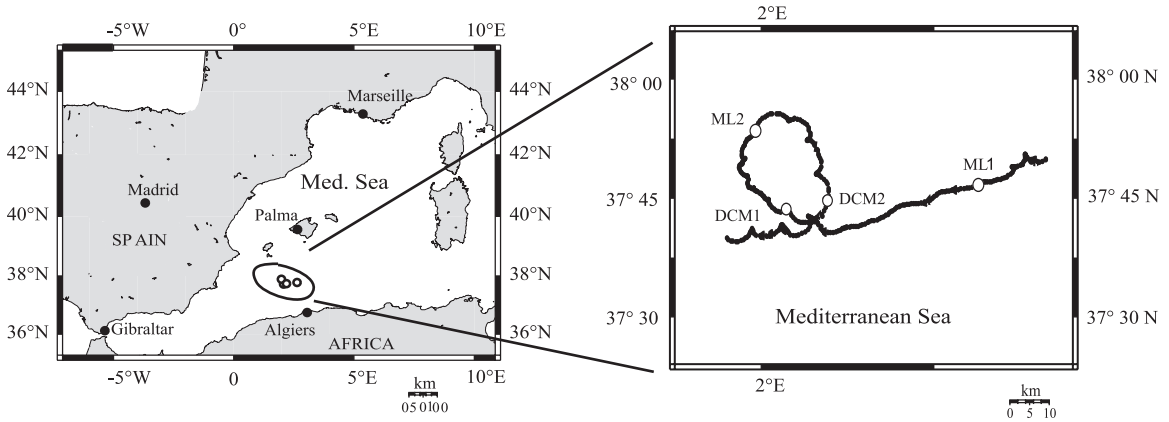

FIG. 1. Study area in the Algerian basin (Western Mediterranean Sea) (left) and track of the buoy deployed near the center of an anticyclonic eddy, with the sampling stations indicated by open circles. Mixed-layer (ML1 and ML2) and DCM (DCM1 and DCM2) regions are shown. Map generated via the Online Map Creation server (http://www.aquarius.geomar.de/omc_intro.html).

Experimental tests of the effect of grazing versus that of viral lysis on prokaryotic production are scarce for the open ocean. Moreover, the links between bacterial production and respiration have mostly been studied for freshwater and coastal marine waters (e.g., references 1, 22, 28, 42, and 62). During an offshore experimental study, in the frame of a mesoscale iron fertilization experiment, changes were detectable in bacterial production, bulk ectoenzymatic activity, and types of β-glucosidase expressed, whereas the number of detectable bacterial phylotypes was remarkably stable (3). This suggests that bacterial production parameters and bacterial community composition were only weakly related. Such observations have also been made in experimental freshwater studies (28).

In the present study, we investigated the relative and combined effects of viral lysis and protistan grazing on prokaryotic production and community composition in offshore Mediterranean waters and compared the findings to those for predator-free incubations (resource control). The types of predators had distinct effects on community composition and production and altered the resource control of prokaryotic diversity. When both types of predators were present, prokaryotic production was consistently reduced and diversity likely sustained, probably by controlling the more-active members of the community.

MATERIALS AND METHODS

Study site and sampling. During the BADE-1 cruise in the Algerian Basin of the western Mediterranean Sea, a stable anticyclonic eddy was followed for 17 days by deploying a drifting buoy in the center of the eddy (Fig. 1). Water samples were collected on 24 September and 2 October 2003 from the mixed layer (20 m; ML1 and ML2) and on 28 September and 5 October 2003 in the deep chlorophyll maximum (DCM) layer (50 m; DCM1 and DCM2) with 12-l NOEX bottles mounted on a conductivity-temperature-depth rosette sampler.

Experimental setup. The general approach was performed as previously described (65), with the following modifications (Fig. 2). Forty liters of seawater collected with the NOEX bottles was filtered through a 0.2-μm polycarbonate cartridge (Durapore; 0.69-m^2 nominal filter area; Millipore) at low pressure (maximum, 10,000 Pa; operated by a 20-liter pressure vessel and a vacuum pump) to obtain cell-free virus-replete fractions of seawater. Half of the filtrate was passed through a 100-kDa-cutoff polysulfone tangential-flow filtration cartridge (Prep-Scale/TFF; 0.23-m^2 nominal filter area; operated by a peristaltic pump at 150,000 Pa; Millipore) to obtain virus-free water. Additionally, 4 liters of seawater was gravity filtered through a 3-μm-pore-size polycarbonate filter (diameter, 47 mm [Millipore]; acid-cleaned polycarbonate filtration units [Sartorius]) to remove ciliates and larger eukaryotes. Subsequently, half of the filtrate was gently filtered (by just turning on the pump but not exerting pressure) through a 0.8-μm-pore-size polycarbonate filter (diameter, 47 mm; Millipore) to remove flagellates. These two fractions of seawater containing cells (<3 μm and <0.8 μm) were diluted to 10% with virus-replete or virus-free seawater. The eukaryote-free fraction (<0.8 μm) was dispensed into virus-free water to obtain the control treatment with no predators (the C treatment) or into the virus-replete fraction to establish the treatment in which viruses but no eukaryotic predators were present (the V treatment). In the same way, water with flagellates (<3 μm) was mixed with virus-free water (with flagellates present) (the F treatment) and, additionally, with a virus-replete treatment (with viruses and flagellates present) (the VF treatment). Three liters of seawater of each treatment was distributed in triplicate 1-liter polyethylene bottles and incubated in the dark at in situ temperature for 59 to 76 h. Samples for viral and prokaryotic counts were taken at ca. 12-h intervals; however, some samples were lost due to technical problems with the −80°C freezer. Samples for prokaryotic production were taken at the beginning, in the middle, and at the end of each experiment. Prokaryotic and viral abundance data are available for these time points. Samples for flagellate abundance and prokaryotic community composition were taken at the beginning (time zero [t_0]) and end (t_{final}; 63 h in ML1, 59 h in ML2, 86 h in DCM1, and 60 h in DCM2) of each experiment. All seawater fractions were prepared in less than 1 hour after collection of samples.

Enumeration of cells and viruses. Samples for counting prokaryotic and viral abundance (1 ml each) were fixed with glutaraldehyde (0.5% final concentration), kept in the dark at 4°C for 30 min, flash frozen in liquid nitrogen, and stored at −80°C until analysis as described by Brussaard (9). Abundances were determined by flow cytometry (FACSCalibur; Becton Dickinson). After thawing, the prokaryotes were stained with SYBR green I solution (Molecular Probes) (at a 1,000-fold dilution of the stock solution) at room temperature in the dark for

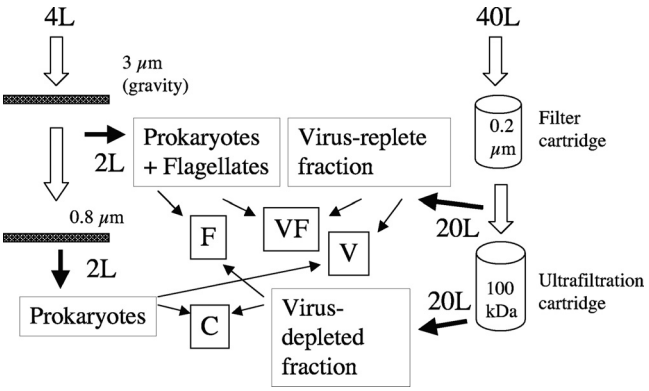

FIG. 2. Flow chart of the experimental design. For details, see the main text. C, control treatment; V, virus treatment; F, flagellate treatment; VF, virus-plus-flagellate treatment.

TABLE 1. Physicochemical and biological characteristics of the water masses used for the experiments

Parameter[a]	Value for:			
	ML1	ML2	DCM1	DCM2
Date	24 September 2003	2 October 2003	28 September 2003	5 October 2003
Longitude	2°38.1′ E	1°59.2′ E	2°05.3′ E	2°11.9′ E
Latitude	37°46.5′ N	37°52.9′ N	37°43.6′ N	37°45.0′ N
Depth (m)	20	20	50	50
Temp (°C)	24.1	24.7	15.1	15.3
Salinity (‰)	37.40	37.35	37.39	37.25
Fluorescence (AU)	0.05	0.06	0.35	0.29
PO_4 concn (μM)	0.015	0.015	0.053	0.025
NH_4 concn (μM)	0.103	0.112	0.153	0.187
PA (10^5 cells ml^{-1})	4.6	7.1	10.2	9.9
% LNA	51	62	65	45
% HNAls	49	37	32	53
VA (10^6 cells ml^{-1})	8.3	7.4	11.3	12.5
HNF (10^3 cells ml^{-1})	3.0	1.5	4.3	5.2

[a] PA, prokaryotic abundance; LNA, low-nucleic-acid prokaryotic population; HNAls, high-nucleic-acid/low-side-scatter prokaryotic population; VA, viral abundance; HNF, heterotrophic nanoflagellate abundance; AU, arbitrary units. Note that HNAhs data are not shown (because they were only ≤3% of the total).

15 min (13). Fluorescent 1-μm latex beads (10^5 beads ml^{-1}) were added to the prokaryotic samples as an internal standard (Polyscience, Inc., Europe). For viral counts, samples were prepared as previously described (9). Briefly, samples were diluted 20-fold in autoclaved and 0.2-μm-prefiltered TE buffer (10 mmol $liter^{-1}$ Tris, 1 mmol $liter^{-1}$ EDTA, pH 8.0) and stained with SYBR green I (Molecular Probes) (at a 20,000-fold dilution of the stock solution) in an 80°C water bath for 10 min before counting. Prokaryotes and viruses were detected by their signatures in a side-scatter-versus-green-fluorescence plot, as described elsewhere (9, 13). Three different prokaryotic subgroups were discriminated in the cytograms on the basis of their nucleic acid fluorescence and side scatter: those with low nucleic acid content (LNA), high nucleic acid content and low side scatter (HNAls), and high nucleic acid content and high side scatter (HNAhs). Cell abundance was determined for each subgroup.

Enumeration of flagellates was carried out with 1% formaldehyde-fixed samples stained with 4′,6′-diamino-2-phenylindole (DAPI) at a final concentration of 0.25 μg ml^{-1} and filtered at low pressure (10,000 Pa) onto 0.2-μm-pore-size black polycarbonate filters. Cells were counted and identified on board on the basis of the size and presence of the nucleus and flagella as described by Sherr et al. (46). These filters were also used to check whether autotrophic flagellates and cyanobacteria were present at the end of each experiment. Detection of autofluorescence was done by switching optical filters to green light excitation.

Determination of prokaryotic production. Prokaryotic production was measured by the incorporation of [^{3}H]leucine (10 nM final concentration; specific activity, 157 Ci $mmol^{-1}$; Amersham) (27) in duplicate samples and one formaldehyde-killed control (2% final concentration). After incubation in the dark at an in situ temperature for 1 h, samples were fixed with formaldehyde, filtered onto 0.2-μm-pore-size filters (cellulose nitrate; Millipore [25-mm diameter]) and rinsed three times with 10 ml of 5% ice-cold trichloroacetic acid. Subsequently, the filters were placed in scintillation vials and stored at −20°C until counting. Back in the laboratory, filters were dissolved in 1 ml ethyl acetate, and after 10 min, 8 ml of scintillation cocktail (Insta-Gel Plus; Canberra Packard) was added. The radioactivity incorporated into prokaryotic cells was measured with a liquid scintillation counter (LKB Wallac, model 1212). The number of disintegrations per minute (dpm) in the formaldehyde-fixed blank was subtracted from the number of dpm in the samples, and the resulting number of dpm was converted into the leucine incorporation rate.

Inorganic nutrients. Samples for inorganic nutrients (NH_4^+, NO_3^-, NO_2^-, and PO_4^{3-}) were analyzed on board the ship within 2 h after the samples were collected and filtered through 0.2-μm filters (type Acrodisc; Gelman Science) with a TRAACS autoanalyzer by using standard methods (38).

DNA extraction, PCR amplification, and denaturing gradient gel electrophoresis (DGGE). Prokaryotic cells from 0.5- or 1-liter samples were collected by vacuum filtration (10,000 Pa) on a 0.2-μm-pore-size polycarbonate filter (47 mm diameter; Millipore) using acid-cleaned polycarbonate filtration units and kept frozen at −80°C until analysis. Nucleic acids were extracted from filters by using several freeze-thaw cycles and enzymatic treatment (proteinase K and lysozyme) as described in more detail elsewhere (61). Instead of the phenol-chloroform extraction step of the original protocol, nucleic acids were extracted with 4.5 M NaCl and chloroform. This slightly modified procedure avoids a toxic chemical and yields genetic fingerprints identical to those obtained with the original method (data not shown). DNA was resuspended in 60 μl of 0.5× TE buffer (10 mmol $liter^{-1}$ Tris, 1 mmol $liter^{-1}$ EDTA, HCl, pH 8.0). One to four microliters of the nucleic acid extracts, quantified with an agarose gel by using a DNA mass ladder (EasyLadder I, no. BIO-33045; Bioline), was used in 50-μl PCR mixtures (1.5 mM $MgCl_2$, 0.25 μM of each primer, and 2.5 U *Taq* polymerase; no. D 5930; Sigma), together with a positive and a negative control. A fragment of the 16S rRNA gene was amplified using the primer pairs 341F-GC/907R and ARC344F-GC/ARC915R for *Bacteria* and *Archaea*, respectively (43).

Five hundred nanograms of PCR products, quantified as described above, was loaded onto acrylamide–bis-acrylamide (8%) gels by using a 30% to 70% (urea and formamide) denaturing gradient. DGGE (DCode universal mutation detection system; Bio-Rad) was run for 18 h and otherwise performed as described previously (43). DGGE gels were stained with a 10× SYBR gold solution (no. S11494; Molecular Probes) distributed over the gel and photographed with a gel documentation system (GelDoc EQ; Bio-Rad). The analysis of band patterns was performed with Quantity One software (Bio-Rad). Only clearly detectable bands were selected for analysis; these bands corresponded to a relative band intensity per lane of 3% of the total band intensity.

Additional bacterial DGGE (INGENYphorU DNA mutation detection system; Ingeny International) was performed with samples from ML1 and DCM1. Selected DGGE bands were excised from the DGGE gels, and the DNA was eluted overnight in autoclaved Milli-Q water at 4°C, checked for purity by rerunning it on a gel, and sequenced (MWG-Biotech, Ebersberg, Germany). Files generated during DNA sequencing were edited using the freeware program 4Peaks 1.6 (A. Griekspoor and Tom Groothuis, mekentosj.com). Up to about 500 bp of the 16S rRNA gene was used in a BLAST search.

Description of community composition. The number of bands was used as a parameter to describe the community composition. All samples from a given experiment were loaded onto the same gel. The use of three standard samples per gel (obtained from a community collected in the Bay of Villefranche, western Mediterranean Sea) allowed for comparison of bands between lanes and thus treatments.

Statistical analysis. Differences between treatments per experiment per time point were tested for significance using parametric analysis of variance (ANOVA) including post hoc test analysis (Fisher's protected least significant difference test). Differences between various predator (V, F, or VF) and control treatments across experiments were tested using paired *t* tests. Testing for normality and homogeneity of variance was performed, and data transformation was done when required (for all data compared per test). Differences were considered significant at a P value of <0.05. The relationship between variables was determined by regression analysis.

RESULTS

Characterization of study sites. The physicochemical and biological characteristics of the sampling sites are summarized in Table 1. PO_4 and NH_4 concentrations were higher in the

TABLE 2. Prokaryotic, viral, and flagellate abundances at the beginning (t_0) and the end (t_{final}) of each incubation in the different experiments[a]

Expt and treatment	Abundance of:					
	Prokaryotes (10^4 ml^{-1})		Viruses (10^6 ml^{-1})		Flagellates (10^3 ml^{-1})	
	t_0	t_{final}	t_0	t_{final}	t_0	t_{final}
ML1						
Control	7.4 ± 2.3	22.1 ± 2.8	0.7 ± 0.1	1.0 ± 0.4	ND	ND
Viruses	7.7 ± 0.5	37.9 ± 3.2	8.1 ± 0.4	4.2 ± 0.2	ND	ND
Flagellates	6.3 ± 0.3	19.9 ± 0.4	1.0 ± 0.2	1.1 ± 0.2	0.22 ± NA	5.4 ± 2.1
VF	7.8 ± 1.1	38.6 ± 2.8	8.1 ± 0.2	4.4 ± 0.4	0.25 ± NA	5.1 ± 1.9
ML2						
Control	12.8 ± 0.8	43.0 ± 8.0	2.2 ± 0.2	3.1 ± 0.6	ND	ND
Viruses	16.4 ± 4.9	61.0 ± 2.2	7.4 ± 0.1	5.7 ± 0.2	ND	ND
Flagellates	14.3 ± 1.9	29.8 ± 4.7	2.2 ± 1.0	1.1 ± 0.2	0.11 ± NA	3.4 ± 0.9
VF	15.9 ± 3.1	63.4 ± 3.9	7.2 ± 0.7	5.5 ± 0.1	0.12 ± NA	2.7 ± 1.2
DCM1						
Control	13.9 ± 0.4	41.5 ± 7.7	1.8 ± 0.3	2.7 ± 0.5	ND	ND
Viruses	13.2 ± 0.4	55.4 ± 12.1	11.3 ± 2.0	5.5 ± 1.3	ND	ND
Flagellates	13.3 ± 3.9	42.7 ± 1.7	1.8 ± 0.1	2.0 ± 0.5	0.37 ± NA	8.5 ± 3.3
VF	15.3 ± 1.7	51.3 ± 3.5	8.3 ± 0.2	4.1 ± 0.5	0.31 ± NA	5.7 ± 2.4
DCM2						
Control	10.6 ± 1.4	56.2 ± 9.9	1.5 ± 0.1	1.2 ± 0.1	ND	ND
Viruses	12.9 ± 1.3	62.9 ± 1.1	10.3 ± 0.8	7.5 ± 1.2	ND	ND
Flagellates	12.6 ± 1.2	74.7 ± 9.2	1.5 ± 0.1	2.8 ± 0.4	0.42 ± NA	9.1 ± 2.2
VF	14.9 ± 1.4	70.3 ± 1.7	9.4 ± 0.5	8.8 ± 0.3	0.41 ± NA	8.5 ± 2.7

[a] Values are means ± standard deviations of results from triplicate incubations. VF, viruses plus flagellates; ND, not detectable; NA, not applicable. For t_0, standard deviations are not given for flagellate abundance, since samples were taken before the distribution of the treatment into the triplicate bottles. The t_{final} values were 63 h in ML1, 59 h in ML2, 86 h in DCM1, and 60 h in DCM2.

DCM layer than in the mixed layer. As expected, chlorophyll fluorescence was five to seven times higher in the DCM layer than in the mixed layer. Prokaryotic and viral abundances were also higher in the DCM layer (Table 1). The LNA bacteria dominated the in situ community in all stations (51 to 65%) except for DCM2 (41%); HNAhs were rare (≤3%). Note that the number of samples is too small for testing of potential differences between the water layers. Overall, data are typical for oligotrophic offshore conditions.

Effect of filtration: dilution efficiency at t_0. Filtration through a 0.8-μm filter resulted in a moderate loss of prokaryotic abundance (14 to 30%), whereas no losses were detected by 3.0-μm filtration. At the beginning of the incubations, prokaryotic abundances in the four treatments of all the experiments varied between 0.7×10^5 and 1.6×10^5 cells ml^{-1}, corresponding to abundances of 17 to 20% of in situ (Tables 1 and 2). Prokaryotic production values at t_0 ranged from 0.95 to 6.0 pmol Leu liter^{-1} h^{-1} and were 20 to 50% higher in the V and VF treatments than in the C and F treatments. This difference was significant ($P < 0.05$; ANOVA). At the beginning of the experiments, the relative proportion of the three prokaryotic subgroups distinguished in the cytograms (LNA, HNAls, and HNAhs) did not differ between the treatments (data not shown) ($P > 0.1$; ANOVA).

Archaea were detected only in the DCM layer, and the number of archaeal DGGE bands was 10 at t_0 in all treatments of both experiments and in all treatments. No bacterial PCR products could be obtained for ML2 and DCM2. At t_0, 27 or 28 bacterial DGGE bands were detected in ML1, compared to 24 in DCM1. One more bacterial DGGE band was detected in the V and F treatments than in the C and VF treatments in ML1.

Initial viral abundances in the V and VF treatments ranged from 0.7×10^7 to 1.1×10^7 particles ml^{-1} and, hence, were similar to the in situ viral abundance (73 to 100% relative to the in situ abundance) (Tables 1 and 2). In the C and F treatments, viral abundance was reduced by 70 to 90% compared to the levels for the V and VF treatments, suggesting that virus reduction was successful. The 3-μm prefiltration removed a relatively small fraction of the flagellates (<30%), whereas 0.8-μm filtration removed virtually all of them. Flagellate abundances at the start of the experiments in the F and VF treatments ranged from 0.1×10^3 to 0.4×10^3 cells ml^{-1} (Table 2). Overall, the experimental approach successfully managed to achieve at t_0 (i) major changes in the abundance ratio between the prey and the two groups of predators in the different treatments and (ii) very similar levels of prokaryotic abundance, production, and diversity (where detectable) among the different treatments.

Effect of predators on microbial abundances and prokaryotic populations. During the initial phase of each incubation, viral abundance decreased in the V and VF treatments (data not shown), suggesting that the reduced host abundance resulted in a viral production rate lower than the decay rate. Later in the experiment (ca. 24 to 36 h), viral abundance increased again or remained constant. At the end of the experiment, viral abundance ranged between 49% and 77% relative to the initial values in the V treatment and between 50% and 94% in the VF treatment (Table 2). At the end of each experiment, viral abundances in the V and VF treatments were

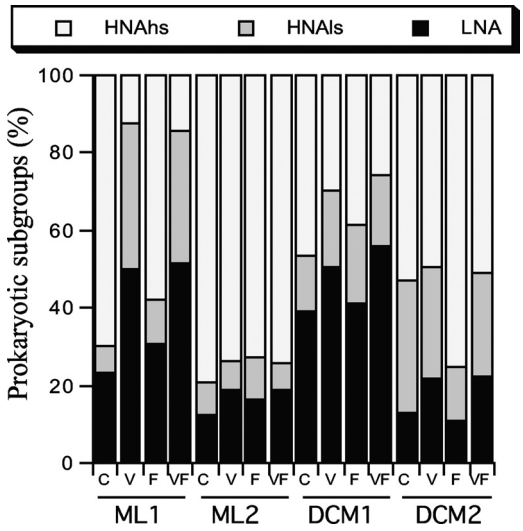

FIG. 3. Proportions (as percentages) of prokaryotic nucleic acid subpopulations to total prokaryotic abundances in the different treatments of the experiments. Shown are values from data points per experiment where prokaryotic abundance was highest (same time point for single experiments). Subgroups: LNA, low nucleic acid; HNAls, high nucleic acid and low scatter; HNAhs, high nucleic acid and high scatter. C, control; V, viruses; F, flagellates; VF, viruses plus flagellates.

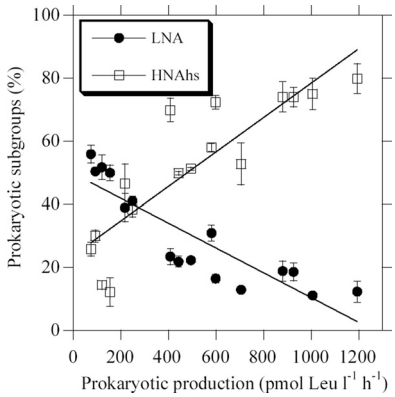

FIG. 4. Relationship between two prokaryotic nucleic acid subpopulations (LNA and HNAhs) and prokaryotic production during the course of the experiments. Values represent means ± standard deviations of results from triplicate incubations. The less significant regression equation for HNAls is shown in Results.

between 3.3- and 11.6-fold (average, 6.3-fold) higher than in the C or F treatment; the differences were significant in all experiments ($P < 0.05$; ANOVA). After ca. 30 to 40 h, a significantly ($P < 0.05$; ANOVA) higher viral abundance was found in the F treatment than in the C treatment in three of the experiments (all except ML2; data not shown), compared to only one experiment at the end of the experiment (Table 2). Viral abundance in the C treatment remained relatively constant and showed an increase only toward the end of the incubation in the ML2 experiment (data not shown). At the end of the experiment in the F and VF treatments, flagellate abundance was between 18- and 30-fold higher than at the beginning and, on average, 2-fold (range, 1.3- to 3.4-fold) higher than the in situ abundance (Table 2). In all experiments, flagellate abundance was higher in the F treatment than in the VF treatment. Across experiments, this difference was significant ($P < 0.05$; paired t test).

Prokaryotic abundances increased during the experiments in all treatments (Table 2). At t_{final}, prokaryotic abundance was significantly ($P < 0.05$; ANOVA) higher in the V and VF treatments than in the C treatment (1.5 to 2.1 times higher in the V treatment and 1.4 to 2.3 times higher in the VF treatment in all experiments). In the F treatment, we found a significant stimulation (1.5-fold) in DCM2, a significant reduction (37%) ($P < 0.05$; ANOVA) in ML2, and no statistically significant effect ($P > 0.1$; ANOVA) in the other experiments in comparison to the level for the C treatment.

For single experiments, the percentages of LNA cells (averaged from the incubations) was significantly higher ($P < 0.05$; ANOVA) in the virus-rich V and VF treatments than in the virus-free C and F treatments (Fig. 3). For time points, when prokaryotic abundance was highest, the percentage of LNA cells ranged from 19 to 59% in the virus-rich V and VF treatments and from 11 to 41% in the virus-free C and F treatments. Differences in the contributions of the HNAhs subgroup among the different treatments were less clear, although in all experiments, the highest proportion was found in either the C or the F treatment and the lowest in the V or VF treatment. HNAhs prokaryotes ranged from 38 to 79% of the total counts in the C and F treatments, compared to 12 to 74% in the V and VF treatments. The HNAls subgroup did not show a characteristic trend between the different treatments, ranging from 7 to 38% in the V and VF treatments and from 7 to 35% in the C and F treatments. On the basis of side scatter, the LNA and HNAls subgroups were also smaller in cell size than the HNAhs subgroup (data not shown). Across all experiments and treatments, the percentages of the LNA (Fig. 4) and HNAls groups were negatively correlated with prokaryotic production ($r^2 = 0.78$ and $P < 0.001$ and $r^2 = 0.26$ and $P < 0.05$, respectively [$n = 16$]). In contrast, the percent HNAhs was positively correlated with prokaryotic production ($r^2 = 0.76$; $n = 16$, $P < 0.001$).

Detection of photoautotrophs at t_{final}. Autotrophic flagellates and cyanobacteria were present at t_{final}, but at abundances much lower than at t_0 and too low for counting. In addition, sequencing of bacterial bands was done for all treatments at t_{final} in two experiments. No plastid or cyanobacterial sequences could be detected in bands excised from DGGE gels.

Effect of predators on prokaryotic production. The development of prokaryotic production in the different treatments over the course of the incubation is shown in Fig. 5. Below, prokaryotic production data are discussed for the time points circa the middle of the experiment and at the end of the experiment. Across treatments and experiments, prokaryotic production ranged from 104 to 1,007 pmol Leu liter^{-1} h^{-1}, except for the VF treatment at t_{final} of the ML1 experiment, where production dropped to less than 10 pmol Leu liter^{-1} h^{-1}. In all experiments, prokaryotic production was significantly lower ($P < 0.05$; ANOVA) in the V treatment than in the C treatment, except for t_{middle} of the DCM2 experiment, where the inverse trend was found. The VF treatment showed the same trend ($P < 0.05$; ANOVA). In the F treatment in the

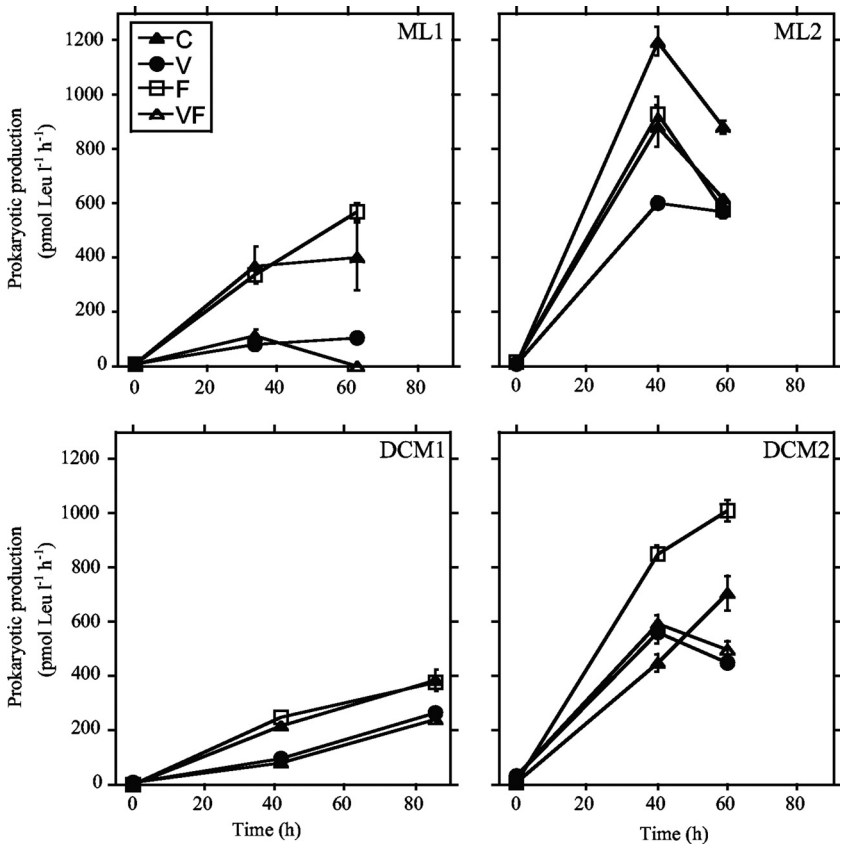

FIG. 5. Prokaryotic production in the different treatments of the four experiments (ML1, ML2, DCM1, and DCM2). Values are means ± standard deviations of results from triplicate incubations; when not visible, standard deviations fall within symbols. C, control; V, viruses; F, flagellates; VF, viruses plus flagellates.

ML1 and DCM1 experiments, the prokaryotic production level was significantly different ($P < 0.05$; ANOVA) from that in the C treatment at only one time point (t_{final} of ML1), whereas it was significantly higher in DCM2 but significantly lower in ML2 at both time points.

Below, stimulation or repression in comparison to the level for the C treatment is discussed for t_{middle} or t_{final} (Fig. 6). In all experiments, prokaryotic production was significantly lower (by up to 22 to 62%) ($P < 0.05$; ANOVA) in the V treatment than in the C treatment (Fig. 6). In the F treatment, the prokaryotic production was significantly higher (by up to 43%) ($P < 0.05$; ANOVA) in DCM2 and lower (by up to 50%) in ML2 than in the C treatment. In all other F treatments obtained from the different sites, no statistically significant effect on prokaryotic production was detected in comparison to the level for the C treatment. In all experiments, prokaryotic production was significantly lower (by up to 26 to 29%) ($P < 0.05$; ANOVA) in the VF treatment than in the C treatment.

Cell-specific prokaryotic production exhibited the same trends as total production (data not shown). Below, only stimulation and repression data from t_{middle} or t_{final} are presented. In all experiments, cell-specific production was significantly lower ($P < 0.05$; ANOVA) in the presence of viruses (V and VF treatments) than in the C and F treatments. Compared to the level for the C treatment, the levels of cell-specific production in the V and VF treatments were reduced by up to 41 to 81% and up to 42 to 99%, respectively. The presence of flagellates only (F treatment) did not induce a consistent effect on cell-specific production in any of the treatments. The cell-specific production levels in the F treatments were up to 60% and 24% higher in two experiments (ML1 and DCM2, respectively; $P < 0.05$; ANOVA) and ca. 20% lower in ML2 ($P < 0.05$; ANOVA) than the levels in the corresponding C treatments and were not significantly different from the levels in the C treatments in DCM1 ($P > 0.05$; ANOVA).

Effect of predators on prokaryotic community structure. For *Archaea*, confinement resulted either in no changes in the number of bands or in a decrease (maximum, 30%) in the number of detected bands. For *Bacteria*, a decrease was always found (when t_0 data were available, i.e., in ML1 and DCM1). The decrease was lower in ML1 (11 to 25%) than in DCM1 (17 to 42%) across the different treatments. However, as described below, strong effects of virus and flagellate manipulation on the number of archaeal and bacterial bands were also observed.

At the end of each experiment, the number of archaeal DGGE bands across the treatments ranged from 7 to 10 (Table 3). Below, predator treatment effects are compared to what was found for the control treatment at the end of the experiment. In the DCM1 experiment, there were 9 bands in the C treatment and 10 in the other treatment; this difference was significant ($P < 0.05$; ANOVA). In the DCM2 experiment, the number of bands was significantly lower (by 10%) ($P < 0.05$;

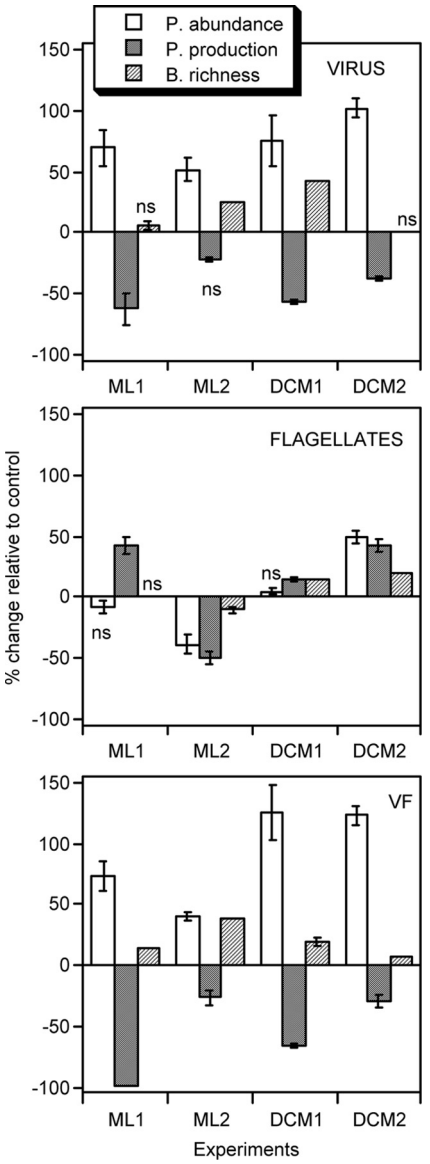

FIG. 6. Effects of the three predator treatments (viruses, flagellates, and viruses plus flagellates) on prokaryotic abundance and production and number of bacterial bands relative to the levels for the control. Values are given as percent stimulation ± standard deviations for triplicate incubations. When no error bars are visible, the standard deviation for triplicates was zero. Data for prokaryotic abundance and number of bacterial bands were calculated as stimulation or repression compared to the level for the C treatment for t_{final}. For prokaryotic production, the strongest difference (t_{middle} or t_{final}) compared to the level for the C treatment was used for calculation. Except for the bars labeled "ns" (not significant), all the other stimulations were significantly different from the control level at P values of <0.05.

ANOVA) in the V treatment than in the C treatment. The decrease in the number of bands was even stronger (ca. 30%) in the VF treatment ($P < 0.05$; ANOVA). Flagellates alone did not have a significant effect ($P > 0.05$; ANOVA) on the number of archaeal bands relative to what was found in the absence of predators (C treatment).

At the end of each experiment, the numbers of bacterial bands across the treatments ranged from 20 to 32 in the mixed layer and from 14 to 30 in the DCM (Table 3). At least one treatment per experiment with predators present (V, F, or VF) had a significant effect ($P < 0.05$; ANOVA) on the number of bacterial bands in all experiments in comparison to what was found for the control, where the predator pressure was relieved. The number of bacterial bands was higher (by 26 to 43%) in the V treatment than in the C treatment in three experiments (Fig. 6). This difference was significant ($P < 0.05$; ANOVA) in two experiments (ML2 and DCM1). When both predators were present, the number of bacterial bands was always significantly higher (from 8 to 39%) ($P < 0.05$; ANOVA) than those in the controls of the four experiments. In the F treatment, the number of bacterial bands decreased by 7 to 14% compared to the level for the C treatment in the mixed layer. However, this effect was significant ($P > 0.1$; ANOVA) only for ML2. The number of bands in the F treatment increased significantly (by 5 to 20%) ($P < 0.05$; ANOVA) compared to the level for the C treatment in the two DCM experiments.

Effects of predators on specific bacterial phylotypes. Considering the identity of bands (i.e., same position on a gel), between 39% and 50% of all bacterial DGGE bands detected per experiment were found in all treatments. Thus, between 50 and 61% of the bands were specific to treatments or combinations of treatments. Note that single experiments were run with single gels and that comparison was made only for single gels. Bands were further classified as treatment specific or sensitive according to their exclusive presence or absence in a certain treatment or combination of treatments. Thus, data are expressed per experiment. Across experiments, a small fraction of the bands, up to 4% (representing one band), was specifi-

TABLE 3. Numbers of archaeal and bacterial bands in the different treatments at the end points of the four experiments[a]

Expt and treatment	No. of bands	
	Archaea	*Bacteria*
ML1		
Control	ND	21 ± 0.0
Viruses	ND	22 ± 0.6
Flagellates	ND	21 ± 0.0
VF	ND	24 ± 0.0
ML2		
Control	ND	23 ± 0.0
Viruses	ND	29 ± 0.0
Flagellates	ND	20 ± 0.6
VF	ND	32 ± 0.0
DCM1		
Control	9 ± 0.0	14 ± 0.0
Viruses	10 ± 0.0	20 ± 0.0
Flagellates	10 ± 0.0	16 ± 0.0
VF	10 ± 0.0	17 ± 0.6
DCM2		
Control	10 ± 0.0	25 ± 0.0
Viruses	9 ± 0.0	25 ± 0.0
Flagellates	10 ± 0.0	30 ± 0.0
VF	7 ± 0.0	27 ± 0.0

[a] Values are means ± standard deviations of results from triplicate incubations. VF, virus plus flagellates; ND, not detected (because no PCR products were obtained).

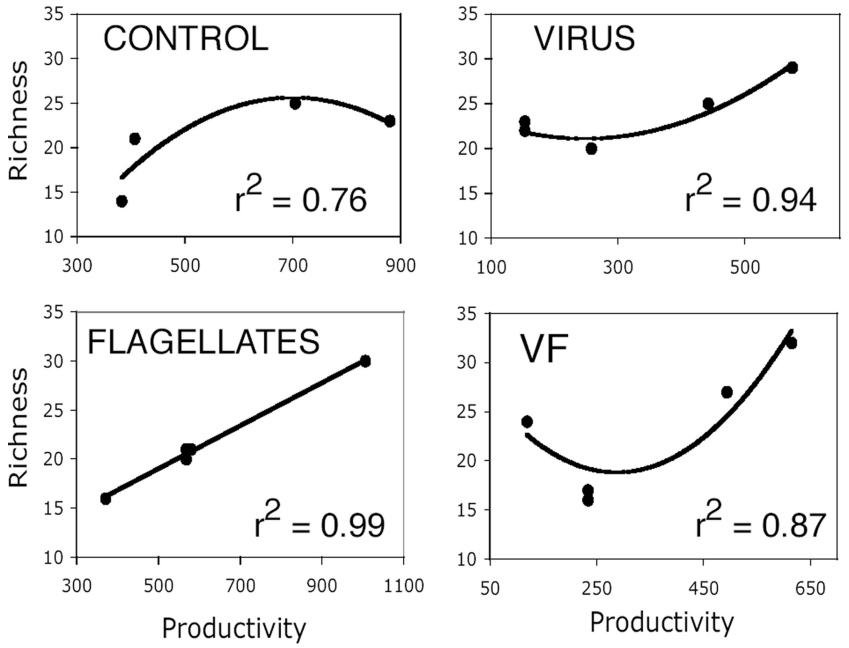

FIG. 7. Relationship between richness and system productivity in the different treatments. Richness is given as numbers of bands and system productivity as prokaryotic production in pmol leucine liter^{-1} h^{-1}. Linear and nonlinear models were tested, and the one with the highest correlation coefficient was chosen ($n = 12$ and $P < 0.001$ for all panels). For the control, $y = 0.00009x^2 + 0.1249x - 18.105$; for viruses, $y = 0.00008x^2 - 0.038x + 25.863$; for flagellates, $y = 0.0219x + 8.1118$; and for viruses plus flagellates, $y = 0.0001x^2 - 0.076x + 29.987$.

cally detected in the predator-free C treatment, indicating that a specific phylotype was strongly controlled by the presence of predators. Between 0 and 17% (i.e., up to four) of the bands were V specific, and up to 8% (i.e., two bands) were F specific, whereas up to 7% and 11% (i.e., two and four bands) were V sensitive and F sensitive, respectively. This suggests that there are phylotypes that either benefit from specific predators or are negatively influenced by their presence. Up to 8% (i.e., up to three) of the bands were found only in the presence of both predators (VF specific), whereas 3 to 6% (i.e., one or two bands) were VF sensitive. In addition, between 3 and 29% (i.e., one to eight) of the bands were V specific (found only in the presence of viruses [in the V and VF treatments]), between 0 and ca. 4% were F specific (present in the F and VF treatments), and between 0 and 15% (i.e., up to five bands) were predator-specific (present in the V, F, and VF treatments).

Link between prokaryotic production and bacterial community composition. By use of all data, the number of bacterial bands was positively correlated to prokaryotic production, although the significance level of this relationship was low ($r^2 = 0.25$; $n = 48$; $P < 0.001$). On the treatment level, linear and nonlinear regression models were used and the model with the best fit was selected (Fig. 7). The number of bands was related to prokaryotic production in the C treatment in a hump-shaped curve ($r^2 = 0.76$; $P < 0.001$; $n = 12$), whereas for the F treatment, this relationship was linear and positive ($r^2 = 0.99$; $P < 0.001$; $n = 12$). For the V and VF treatments, the relationship was U shaped ($r^2 = 0.94$ and $r^2 = 0.87$, respectively; $P < 0.001$; $n = 12$), with a flatter curve for the V treatment.

DISCUSSION

Confinement effects and experimental approach: methodological considerations. Effects of confinement on productivity and on the number of detectable phylotypes have previously been documented (15, 32). Usually, confinement for several days results in a reduction of the number of detected phylotypes, also observed in the present study. However, the effects were moderate (11 to 42%), particularly for the mixed layer (11 to 25%) and for *Archaea* in general (0 to 30%), compared to previously reported values ranging from 50 to 90% (61). With the use of the same experimental approach as that in the present study, confinement effects on the number of bands were even lower for coastal marine bacterial communities (65). As in the offshore study by Winter et al. (61), the effect of confinement was also smaller for *Archaea* than for *Bacteria* in our experiments. Despite the confinement effects, we were able to find treatment effects on the number of bacterial and archaeal bands in all the experiments.

The effect of flagellates might have been underestimated in this study compared to that of viruses, since flagellates were diluted whereas viral abundances were kept roughly at in situ concentrations. However, the flagellate abundance at the end of the experiment was higher than the in situ level (Table 1), indicating that they grazed intensively on the prokaryotic community. Viruses were nondiluted in order to ensure a high rate of encounter with their host cells. This was necessary, since a strong reduction of viral abundance can result in an almost complete reduction of new viral infection (59). For example, this phenomenon is used for estimating viral production by the virus reduction approach (60). Since the viral abundance at the

end of each experiment was between 50 and 90% relative to the in situ abundance in the V and VF treatments, viral impact might have been slightly underestimated in the present study.

Cyanobacterial or plastid DNA could have had an influence on bacterial community structure at the start of each experiment. However, since (i) samples were diluted to 10%, (ii) samples were kept in the dark, (iii) autotrophs decreased in abundance during the experiment, (iv) prokaryotic abundance increased on average fourfold, and (v) we did not detect plastid or cyanobacterial sequences at t_{final}, it is unlikely that cyanobacterial or plastid amplicons contributed to the detected diversity at the end of each experiment. One rationale for diluting the sample for the experiments was that this produces a fraction which contains (besides prokaryotes) the flagellates but is virus replete. Alternative approaches for concentrating prokaryotes and flagellates and reconstituting the F treatment with virus-depleted water have been used (54). However, this concentration procedure can kill or inactivate many flagellates (this study and unpublished data), and the production of cellular debris by the concentration procedure can be problematic in oligotrophic systems depleted in bioavailable DOM.

Ultrafiltration was used to produce virus-depleted water from virus-replete 0.2-μm-filtered water. This could not only remove viruses but also change the organic matter field and thus the bioavailability of DOM. For example, high-molecular-weight (HMW) DOM, which is typically highly bioavailable (2, 37), could be depleted in the virus-free seawater fractions. Thus, comparisons of treatments with 0.2-μm and 100-kDa fractions could be compromised by invalid controls. Indeed, it has been shown that some phylotypes can be favored by HMW DOM (30 kDa and 0.2 μm); however, a consistent effect on the number of bacterial bands was not detected (61). Moreover, the V and VF treatments potentially containing more bioavailable HMW DOM showed slightly higher prokaryotic production levels at t_0. However, prokaryotic production was clearly reduced at t_{middle} and t_{final} (Fig. 5). This has also been shown in studies where the inactivated virus size fraction and thus HMW DOM were added back to controls (6). Further support comes from the finding that the strongest negative effect was found when viruses and flagellates were both present. This indicates that V and VF treatment effects were stronger than the role of DOM changes during preparation of treatments. Thus, the chosen approach seems to be robust in showing effects of virus, flagellate, or combined predators. Nevertheless, it should be kept in mind that while the detection of trends seems to be robust, quantitative inference should be regarded with caution.

Effect of viral lysis and grazing on prokaryotic abundance and production. The proportion of HNAhs prokaryotes increased with prokaryotic production (Fig. 4), suggesting that they were responsible for increases in production. Similar relationships between HNA prokaryotes and prokaryotic production have been reported before for coastal waters of the Mediterranean Sea (14, 29).

Lower prokaryotic production (total and cell specific) in the presence of viruses, as found in all our experiments, was also reported in other studies (6, 35), although an opposite effect was occasionally detected as well (30, 54, 65). The finding that the proportion and abundance of the probably highly active HNAhs prokaryotes were low in the V treatments but that those of LNA were high suggests that viruses preferentially lysed active cells. A link between infection and host production has been reported previously (summarized in reference 56). Since the proportion of LNA increased in the presence of viruses (with the same tendency shown less clearly for HNAls), LNA was probably less susceptible to viral infection. This is consistent with the finding that resistance to viral infection involves metabolic costs as well as the idea that a lower activity reduces infection rates (56). In addition, removing HNAhs could have benefited LNA and HNAls populations by reducing competition. A more efficient use of lysis products by LNA prokaryotes is also possible but less likely, since LNA bacteria were negatively related to prokaryotic production (Fig. 4) and thus probably not growing rapidly.

The finding that prokaryotic abundance was higher in the V and VF treatments than in the predator-free C treatment while prokaryotic production was lower is puzzling. An explanation could be that viral lysis has shifted the prokaryotic biomass to less active and smaller cells, i.e., the LNA cells, hence decreasing production and concurrently increasing abundance. A shift toward smaller cells in the presence of viruses has been shown before (57).

The F treatment did not result in consistent trends in prokaryotic abundance and production. Significant effects were found only in the two experiments with the highest prokaryotic production levels (ML2 and DCM2), but the trends were contrasting in the two layers. We did not find consistent differences in proportion of HNAhs between the F and C treatments, whereas other studies have reported a significant stimulation (8) as well as a reduction in the presence of grazers (14). It has previously been found that grazing can result in reduced total prokaryotic abundance and production but that flagellates also release significant amounts of organic compounds and nutrients, which can stimulate bacterial growth (e.g., reference 49). Variable relative levels of importance of mortality versus nutrient release by flagellates in different environments could explain the lack of consistent trends. Factors related to differences in prokaryotic community composition, such as those found in the present study, could play a role as well.

Our data show that the effects of viruses and flagellates were not simply additive. Nonadditive effects, such as stimulation of viral infection and increase in viral numbers in the presence of flagellates, were also found in other studies (41, 49, 51, 54, 55, 65). In this context, it must be noted that viral abundance was occasionally significantly higher in the F treatment than in the C treatment (Table 2). Stimulation of new infection by grazing is one explanation. However, considering the reduced background of viruses and thus the reduced rates of encounter with hosts in these treatments, provirus induction from prokaryotes or even heterotrophic flagellates is more likely.

Effect of viral lysis and grazing on prokaryotic community composition. The finding that no *Archaea* could be found in the mixed layer is surprising, since *Archaea* are usually detectable by PCR in surface waters of the Mediterranean Sea (31, 36). The rather low abundance of *Archaea* in the upper ocean layers compared to that of *Bacteria* (25), which seems to be due to low archaeal growth rates (17), might have contributed to the fact that we were not able to detect them by PCR. In the presence of viruses in the V and VF treatments, the numbers

of archaeal bands were influenced in one of the two experiments with water of the DCM, whereas flagellates did not have a detectable influence on archaeal fingerprint diversity. An influence of viruses on the presence or absence of archaeal phylotypes could be observed in surface waters of the equatorial Atlantic; however, the number of detected phylotypes was not systematically affected (61). An influence of viruses on archaeal community composition was also found in the Mediterranean Sea (30). Our data suggest a selective removal of archaeal phylotypes rather than stimulation of richness by viruses. The lack of a response of *Archaea* in the F treatment would suggest that they were either not grazed or that grazing was roughly similar for all phylotypes. To our best knowledge, this is the first study of the potential effect of grazing on archaeal community composition.

In the F treatments, the numbers of bacterial bands tended to be lower than the control levels in the mixed layer but were significantly higher than the control levels in the DCM. This suggests that the role of grazing for bacterial diversity can differ even between closely related environments. Interestingly, the repression/stimulation patterns induced by the presence of flagellates were similar for prokaryotic production level and number of bands. A complex pattern between grazing, diversity, and resources has also been reported in previous studies. For example, Matz and Jürgens (33) found that grazing had no significant effect on bacterial community composition, and Simek et al. (47, 49) observed that grazing had a significant effect on bacterial community composition only if bacteria were resource-limited.

The numbers of bacterial bands were generally higher in the V and VF treatments than in the control. Assuming that a higher number of bands means less dominance and more evenness of phylotypes, this suggests that the presence of viruses can sustain richness, hence confirming one prediction of the "killing the winner" hypothesis, i.e., that viral lysis sustains prokaryotic diversity by keeping in check competitive dominants (53). Other studies have, however, shown a decrease in richness (21, 44, 61), thus contradicting some of the predictions of the "kill the winner" hypothesis. Also, in a series of experiments performed in coastal marine systems, viruses stimulated the number of detectable bands (65), and such a stimulation in the presence of viruses was also detected in a freshwater system (54). Hewson and Fuhrman (20) found in various marine environments (including offshore habitats) that viruses have a significant but highly variable impact on bacterial community structure. Our data provide experimental evidence from an offshore marine environment that prokaryotic richness can be stimulated in the presence of viruses. While the above-mentioned studies did not report consistent trends, which could be due to differences in experimental approaches or methods used to assess diversity, they all found significant effects on the prokaryotic community structure. Effects of viruses on bacterial community structure were also detected using fluorescence in situ hybridization (7).

While the "killing the winner" hypothesis is usually linked to viruses, its original formulation in a model also includes grazers (53). Here, we show that the combined effect of viruses and flagellates on the number of bands and fingerprint diversity was more consistent than the effect of one type of predator alone. This was also found in a coastal marine system (65). Moreover, in all experiments, we found bands which were not detected in the VF treatment but were present in all other treatments, and in two experiments, we found bands specific for VF. This suggests that the presence of both types of predators had a specific influence on prokaryotic diversity. Removal of specific bands could be due to phylotypes which were attacked by both types of predators, whereas the appearance of bands in the predator-containing treatments could be due to phylotypes benefiting from the presence of predators, e.g., via the production of DOM or by the removal of competitors. It has been demonstrated for a freshwater reservoir that *Flectobacillus* growth was fastest in the presence of both types of predators (50).

Link between diversity, productivity, and predator pressure. In our experiments, biocomplexity was reduced by removing larger eukaryotes and by exclusion of photosynthetic activity due to dark incubations. In such a system, prokaryotic production serves as a proxy for productivity or resource availability and the control corresponds to the resource-only treatment. The number of bacterial bands was related in a hump-shaped pattern to prokaryotic production in the predator-free treatment (Fig. 7). Such a relationship is often found for general productivity-diversity relationships (64). One of the explanations for such a pattern is that in extreme, i.e., very low- or very high-productivity environments, only a few and very specialized species (or phylotypes) dominate. However, a high number of bands on a genetic fingerprint can also mean more evenness and less dominance of phylotypes. The addition of predators changed this to a relationship which was roughly U shaped in the presence of viruses and both predators but linear when only flagellate predators were present. Thus, viral lysis and flagellate grazing could influence bacterial community structure or dominance patterns differently, depending on the trophic status of the system. Although these conclusions are based on relatively few data points, the relationships were significant and are to our knowledge the first evidence showing that, in offshore microbial communities, viruses and grazers can strongly modify resource control of diversity and the relationship between diversity and ecosystem functions.

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