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Changes in marine prokaryotic community induced by varying types of dissolved organic matter and subsequent grazing pressure

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We analysed changes in the abundance, biomass, activity and composition of coastal marine prokaryotic communities after the addition of organic substrates, such as glucose, leucine and yeast extract, and the effect of grazing pressure exerted by nanoflagellates. The addition of a carbon source (i.e. glucose) promoted the growth of Gammaproteobacteria, while a combined source of C and N (i.e. leucine) favoured the development of Alphaproteobacteria. The addition of yeast extract, a complex substrate rich in N and growth factors, promoted the proliferation of Alphaproteobacteria and Gammaproteobacteria. Grazing pressure exerted by nanoflagellates produced marked differences on the size structure of the prokaryotic biomass. A pronounced tendency to filamentation and aggregation was observed in the glucose treatment, while in the case of yeast extract, small and mainly freely dispersed prokaryotes were maintained throughout the incubations. Thus, the final community in the yeast extract treatment showed a high percentage of edible biomass, while an important fraction of potentially grazing-resistant prokaryotes (more than 50% of total prokaryotic biomass) was detected in the microcosms enriched with glucose. These results suggest a marked effect of DOM sources on the development of grazing-resistant prokaryotes.

INTRODUCTION

Substrate availability, bacterivorous protists and viruses are the main factors controlling the abundance, activity and composition of prokaryotes in marine systems (Fuhrman, 1999; Pernthaler, 2005). Heterotrophic prokaryotes use organic compounds as a source of carbon and energy, and different bacterioplankton groups show distinct dissolved organic matter (DOM) uptake patterns (Cottrell and Kirchman, 2000; Alonso-Sáez and Gasol, 2007). For example, *Alphaproteobacteria* are very active in the uptake of low-molecular-weight DOM, such as glucose and amino acids, whereas members of the *Bacteroidetes* group actively take up high-molecular-weight DOM (Cottrell and Kirchman, 2000; Elifantz *et al.*, 2005). These uptake patterns also depend upon the concentration of the available organic compounds (Alonso and Pernthaler, 2006). Thus, sporadic episodes of seawater enrichment with organic compounds can stimulate the growth of specific components of the bacterioplankton assemblage. This is the case with *Gammaproteobacteria*, which have been associated with nutrient pulses (Eilers *et al.*, 2000), and with *Bacteroidetes*, which has been associated with upwelling events (Suzuki *et al.*, 2001).

Grazing pressure, exerted mostly by heterotrophic nanoflagellates (HNF), constitutes another important force shaping the phenotypic and genotypic composition of bacterioplankton (Jürgens and Matz, 2002; Sherr and Sherr, 2002). Bacterivorous protists graze upon different types of prokaryotes at different rates

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(Ayo *et al.*, 2001), and their selectivity has been linked to many prey characteristics, such as size, surface properties, motility, aggregation capability, division state, biochemical cell composition and cell surface properties (Jürgens and Matz, 2002). However, prokaryotes have also developed different mechanisms to resist capture, ingestion and digestion by bacterivorous protists (Jürgens and Güde, 1994), including the development of complex morphologies, filamentation, miniaturization and aggregation. The appearance of grazing resistance in a sizeable fraction of the prokaryotic biomass has important ecological implications, because it sequesters particulate carbon and results in decreased efficiency of carbon and energy transfer through the trophic web (Pernthaler, 2005).

Although several studies have assessed grazing-resistant prokaryotes in freshwater ecosystems (Jürgens *et al.*, 1999; Hahn and Höfle, 2001; Šimek *et al.*, 2007), less is known regarding their relevance in marine systems (Jürgens *et al.*, 2000; Massana and Jürgens, 2003). The purpose of the present study was to explore the potential development of grazing-resistant prokaryotes in coastal marine waters after stimulating bacterial growth by adding different organic substrates. Despite the limitations of laboratory experiments in imitating *in situ* conditions, the addition of organic compounds to seawater samples would simulate the input of autochthonous or allochthonous biodegradable organic matter in natural systems. Such inputs of organic matter can lead to rapid growth of prokaryotes and, subsequently, to growth of bacterivorous nanoflagellates, leading to the eventual establishment of a grazing-resistant fraction of prokaryotes. We analysed changes in abundance, biomass, activity and prokaryotic community composition in laboratory microcosms enriched with glucose as a carbon source, leucine as a combined source of carbon and nitrogen and yeast extract as a complex source of nutrients and growth factors.

METHOD

Sampling and strategy

Coastal seawater was sampled from the Bay of Biscay (43°25′3.5″N, 2°56′56.6″W) at ~0.5 m depth during high tide, 10 m from the shore. Two experiments were carried out on 31 July 2001 (Experiment I) and 4 September 2001 (Experiment II). Samples were taken in 25 L polypropylene tanks pre-cleaned with dilute acid and processed in the laboratory within 2 h of sampling. Samples were filtered through 8 μm polycarbonate filters in order to remove ciliates and zooplankton and, thus, avoid interactions due to the grazing of ciliates upon HNF. Gravity filtration was applied to the samples in order to minimize the potential enrichment of samples with DOC generated by cell disruption during the filtration procedure. The filtered seawater was distributed to four 2 L flasks, each of which was filled to 1.5 L. We did not add any organic substrates to one of the flasks; this flask was used as a control. The other three flasks were enriched with glucose (56 μM), leucine (74 μM) or yeast extract (1.5 mg L^{-1}). These concentrations were chosen with the aim of stimulating bacterial activity but producing only a moderate increase in bacterial abundance, within the range of values that can be found in natural samples. For this purpose, prior to the experiment, we ran a test to analyse the effect of the addition of different substrate concentrations on bacterial growth in seawater from the same location (results not shown). As a reference, total DOM concentration in seawater from the same marine area is in the range of 0.2–0.4 mM C (Unanue, 1992). Solutions of glucose, leucine and yeast extract were prepared with Milli Q water and filtered through 0.2 μm polycarbonate filters immediately before beginning the experiments. Microcosms were maintained in the dark at 20°C with 120 rpm agitation for 206 h in the first experiment and for 313 h in the second experiment. *In situ* temperature during sampling was 19°C in Experiment I and 20°C in Experiment II.

The temporal evolution of the microbial communities was followed by measuring the following parameters: freely dispersed and aggregated prokaryotic abundance, HNF abundance, biomass and size distribution of the prokaryotic community, thymidine incorporation as an indicator of prokaryotic growth, β-*N*-acetylglucosaminidase activity as an indicator of grazing activity and phylogenetic composition of the freely dispersed prokaryotic community by fluorescence *in situ* hybridization (FISH).

Microbial counts, size distribution and biomass of prokaryotes

Freely dispersed prokaryotes were counted by epifluorescence microscopy after staining triplicate 10 mL formalin-fixed samples with DAPI (4′,6′-diamidino-2-phenylindole, final concentration 2 μg mL^{-1}) (Porter and Feig, 1980). Within 1 day of preservation at 4°C, the stained samples were filtered through 0.2 μm pore-size black Millipore polycarbonate filters. The filters were examined under a Nikon epifluorescence microscope, and between 400 and 600 prokaryotes were counted in at least 20 randomly selected fields. Total prokaryotic abundance was measured as described

previously (Velji and Albright, 1986). Immediately before staining, 100 μL of sterile 100 mM tetrasodium pyrophosphate was added to 1 mL subsamples, shaken for 90 min and cooled at 4°C for 30 min. Then, the subsamples were placed in ice and sonicated (100 W, 3 s pulses for 3 min) to disperse the particles. Aggregated prokaryotic abundance was estimated by subtracting the freely dispersed prokaryotic abundance from the total prokaryotic abundance. For HNF counts, 10 mL subsamples were fixed with paraformaldehyde (final concentration 2%) and stained with DAPI (final concentration 2 $\mu g\,mL^{-1}$). HNF in 50–100 randomly selected fields were counted in triplicate samples.

The biovolume of the freely dispersed prokaryotes was determined using a semiautomatic image analysis system (Massana *et al.*, 1997). Images of freely dispersed prokaryotes stained with DAPI were recorded with a Hamamatsu C2400 camera, digitalized with video card LG-3 (Scion Corporation) and processed with National Institutes of Health Scion Image 1.62 software. For each sample, at least 200 prokaryotes were measured. The biovolume of freely dispersed prokaryotes was transformed into biomass using an allometric model (Norland, 1993).

Heterotrophic prokaryotic production

Thymidine incorporation rate was measured as indicator of heterotrophic prokaryotic production. Samples were processed following the method of Fuhrman and Azam (Fuhrman and Azam, 1980). Triplicate 5 mL subsamples were incubated with 50 nM [methyl-^{3}H]thymidine (80–90 Ci $mmol^{-1}$, Radiochemical Centre) and incubated with 120 rpm agitation for 1 h at 20°C in the dark. The saturating thymidine concentration and the appropriate incubation time were determined prior to the experiment. After incubation, the subsamples were chilled on ice and 5 mL of ice-cold 10% (w/v) trichloroacetic acid (TCA) was added to each subsample. The mixtures were kept on ice for 5 min and then filtered through 0.2 μm membrane filters. The filters were rinsed five times with 5 mL of ice-cold 5% (w/v) TCA, dried and placed in scintillation vials with 1 mL of ethyl acetate. After 20 min, 10 mL of scintillation liquid (BCS, Amersham) was added to the vials and they were radioassayed by liquid scintillation (Tri-Carb 2000CA Packard Instruments). Formaldehyde-killed controls (2% final concentration) were processed in a similar manner.

Phylogenetic composition of the prokaryotic community

FISH with rRNA-targeted oligonucleotide probes was used to analyse changes in the composition of prokaryotes in Experiment I (Amann *et al.*, 1995). Fifteen millilitre subsamples were withdrawn for each time point and fixed with 45 mL of paraformaldehyde (4%) at 4°C for 3 h in the dark. Subsamples were filtered through 0.2 μm polycarbonate filters (45 mm diameter Millipore) and rinsed twice with 20 mL of 10 mM PBS buffer. Filters were air-dried and stored in Petri dishes at −20°C to await further processing. Sections of the filters were hybridized with the following oligonucleotide probes synthesized with CY3 fluorochrome at the 5′ end (Innogenetics): EUB338 for most *Bacteria*, ARCH915 for most *Archaea*, ALF968 for most *Alphaproteobacteria*, BET42a for most *Betaproteobacteria*, GAM42a for most *Gammaproteobacteria* and CF319a for many groups belonging to the *Bacteroidetes* group. Hybridizations were performed following the procedure of Glöckner *et al.* (Glöckner *et al.*, 1999). After hybridization, the filter sections were stained with DAPI (final concentration 1 $\mu g\,mL^{-1}$) and mounted on microscope slides with Citifluor AF1. Between 10 and 15 fields per filter were analysed with a Nikon epifluorescence microscope, and between 6 and 600 hybridized cells were counted from a total of 300–1000 DAPI counts.

N-acetylglucosaminidase activity

N-acetylglucosaminidase activity was measured as an indicator of grazing activity (Vrba *et al.*, 1996). The fluorogenic substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (MUF-NAG) was added to 3 mL triplicate subsamples at a final concentration of 3 μM to measure grazing activity. The saturating concentration had been determined in previous experiments. Fluorescence at 342 nm excitation and 453 nm emission was measured with a Perkin Elmer LS 50B spectrofluorometer immediately after the addition of the substrate and after 3–5 h of incubation at 20°C in the dark. Fluorescence was converted into MUF concentrations by using 10 nM MUF standards.

RESULTS

Temporal dynamics of microbial communities

In all of the experimental microcosms, the microbial communities of grazers and prey went through the characteristic succession pattern with three defined stages: a first stage characterized by prokaryotic growth; a second stage characterized by HNF growth, intense grazing and a consequent decrease in prokaryotic biomass; and a third stage characterized by a decrease

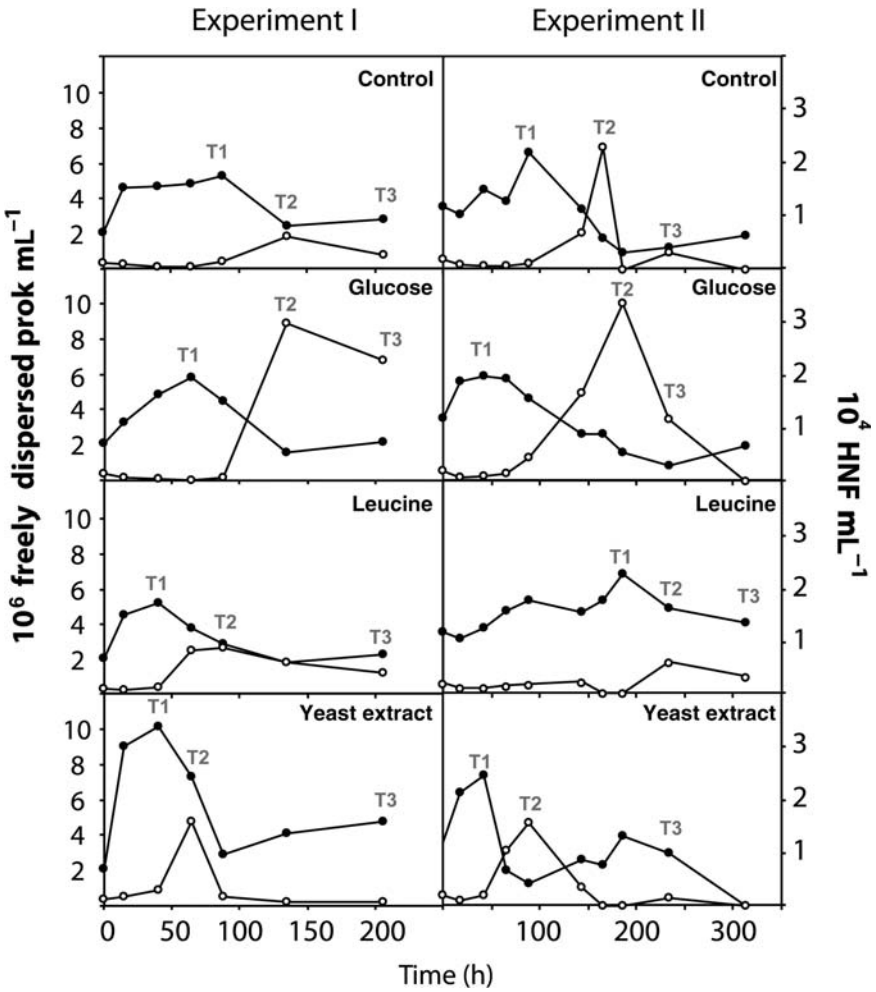

Fig. 1. Evolution of freely dispersed prokaryotic abundance (filled circles) and HNF abundance (empty circles) during Experiments I and II. T1, time of maximum prokaryotic abundance; T2, time of maximum nanoflagellate abundance; T3, final situation. The variation coefficient was lower than 10% for prokaryotic counts and lower than 45% for HNF counts.

in protist abundance and the development of potentially grazing-resistant prokaryotes (Jürgens and Güde, 1994). Four key points in time were analysed: the initial situation (T0); the peak of prokaryotic abundance (T1); the peak of HNF abundance (T2); and the final situation (T3), in which a grazing-resistant prokaryotic community was established (Fig. 1).

The number of freely dispersed prokaryotes at the beginning of the incubations was 2.1×10^6 and 3.6×10^6 prokaryotes mL^{-1} in Experiments I and II, respectively (Fig. 1). Their abundance increased during the first hours, with peaks at 25–100 h, depending on the type of enrichment. The dynamics of freely dispersed prokaryotes were similar in both experiments, the only exception being the microcosm enriched with leucine in Experiment II, which had a peak at 190 h. The earliest peak and the maximum increase in abundance at the initial stage corresponded to the yeast extract treatment. Decreases in the number of prokaryotes, associated with increases in the number of HNF, were also more pronounced in the yeast extract microcosms. HNF abundance at the beginning of Experiments I and II was 0.12×10^4 and 0.21×10^4 HNF mL^{-1}, respectively (Fig. 1), and the highest numbers of HNF were observed in the microcosms to which glucose was added.

Changes in size structure and aggregation of the prokaryotic community

Prokaryotes at the beginning of the experiments were characterized by their small size, with an average length of 0.81 and 0.60 μm in Experiments I and II, respectively. The length of most prokaryotes was in the range of 0.50–0.75 μm, and filaments were not detected. In contrast, there was a tendency towards increases in cellular size and elongation throughout the incubations

(Fig. 2). This tendency was less marked in Experiment II than in Experiment I, in which filaments were detected in all microcosms (Fig. 2), and reached maximal values of length (i.e. 21, 25, 18 and 15 μm for control, glucose, leucine and yeast extract treatments, respectively). Thus, despite the fact that bacterial abundances were approximately in the same range in both experiments (Fig. 1), the freely dispersed bacterial biomass was generally higher in Experiment I than in Experiment II. At the end of the experiments (T3),

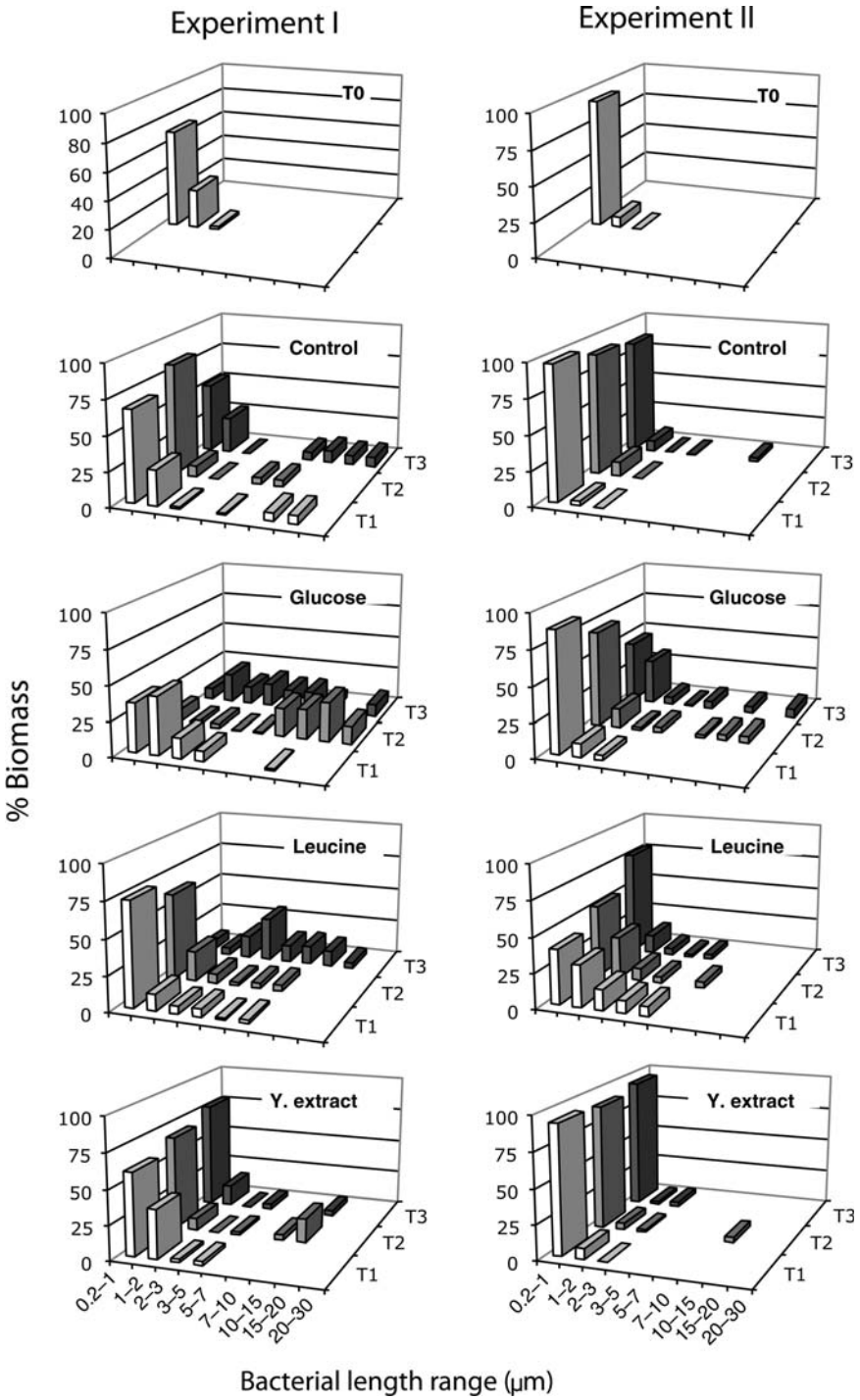

Fig. 2. Size distribution of freely dispersed prokaryotic biomass at the initial situation (T0), at the time of maximum prokaryotic abundance (T1), at the maximum nanoflagellate abundance (T2) and at the final situation (T3) for the different treatments.

freely dispersed biomass was 119, 189, 224 and 143 μgC L^{-1} in Experiment I and 26, 25, 81 and 50 μgC L^{-1} in Experiment II, for control, glucose, leucine and yeast extract treatments, respectively.

Despite these differences in total biomass values, changes in size structure and tendency towards elongation were always more marked for glucose and leucine additions, when compared with the addition of yeast extract (Fig. 2). When pooling data from both experiments, the percentage of freely dispersed biomass in the form of filamentous cells (i.e. cells longer than 3 μm) was significantly higher after the addition of glucose than in the control and yeast extract treatment (Wilcoxon signed-rank test, $n = 6$, $P = 0.04$). Even if the results were not statistically significant for the addition of leucine, it is remarkable that at the end of Experiment I, 71% of the freely dispersed biomass belonged to cells larger than 3 μm in this microcosm.

Similar to filamentous prokaryotes, at the beginning of the experiments, we did not detect aggregated prokaryotes. However, they appeared after the prokaryotic growth phase and were present in sizeable numbers by the end of the experiments (Fig. 3). The percentage of aggregated prokaryotes after the addition of glucose was significantly higher than that of the control, leucine and yeast extract treatments (Wilcoxon signed-rank test, $n = 6$, $P = 0.027$, 0.027 and 0.028, respectively). At the end of the incubations, the percentages of aggregated prokaryotes after addition of glucose (82% and 62%) and leucine (72% and 38%) were substantially higher than after addition of yeast extract, with <20% of aggregated prokaryotes (Fig. 3).

Microbial activities

Enriching the microcosms with organic substrates caused an increase in thymidine incorporation compared with the control microcosms, mostly in the first stage of prokaryotic growth (Fig. 4A). The maximum thymidine incorporation rate was observed in those microcosms enriched with yeast extract, with values eight to nine times higher than in the control microcosms, followed by the leucine and glucose microcosms (Fig. 4A). The specific activity of HNF ranged from <1 to 7 fmol MUF HNF^{-1} h^{-1}, with maximum values in the microcosms enriched with yeast extract and minimum values in the microcosms enriched with leucine (Fig. 4B).

Phylogenetic composition of the prokaryotic community

At the beginning of the experiment, we detected 64% of the prokaryotes using FISH (Fig. 5). Percentages of *Archaea* were low throughout the experiments (from 1% to 6% of DAPI counts). *Gammaproteobacteria* and *Bacteroidetes* were the most highly represented (13% and 18% of DAPI counts, respectively), and together with *Alphaproteobacteria*, comprised 40% of the total counts. The percentage of non-hybridized prokaryotes was 44% on average, but reached unusually high values at T1 in the leucine-treated microcosms (67%) and at the end of the incubation period in the yeast extract microcosms (73%). After the prokaryotic growth phase, *Gammaproteobacteria* increased their abundance in the microcosm enriched with glucose, *Alphaproteobacteria* were more abundant in the microcosm enriched with leucine, and both *Gammaproteobacteria* and *Alphaproteobacteria* increased in the microcosm enriched with yeast extract. The composition of the prokaryotic communities was strongly affected thereafter, as a consequence of grazing pressure (Fig. 5). The abundance of *Gammaproteobacteria* was drastically reduced in the microcosms enriched with glucose and yeast extract, and *Bacteroidetes* abundance increased in the microcosm enriched with leucine.

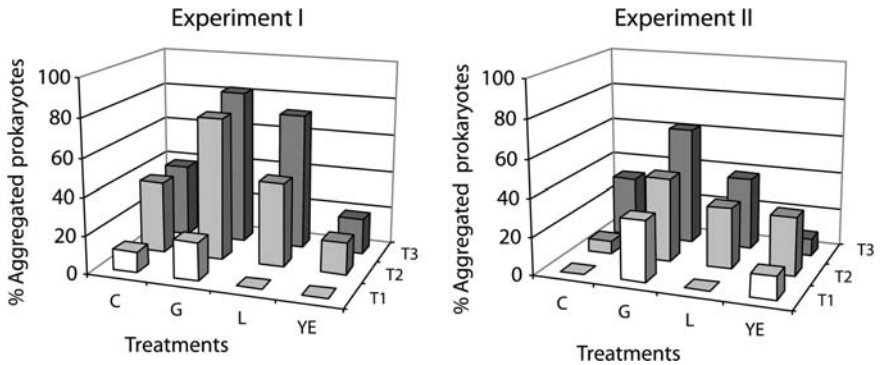

Fig. 3. Evolution of the percentages of aggregated prokaryotes at the time of maximum prokaryotic abundance (T1), at the time of maximum nanoflagellate abundance (T2) and at the final situation (T3) in control (C), glucose (G), leucine (L) and yeast extract (YE) treatments.

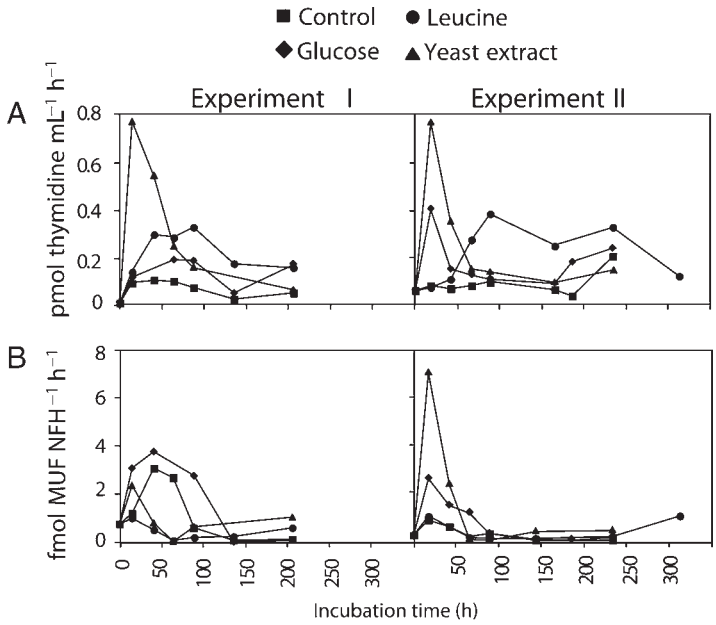

Fig. 4. (**A**) Evolution of thymidine incorporation and (**B**) evolution of specific β-*N*-acetylglucosaminidase activity through the incubation time in both experiments. Values are the average of three replicates. The variation coefficient was lower than 10% for both measurements.

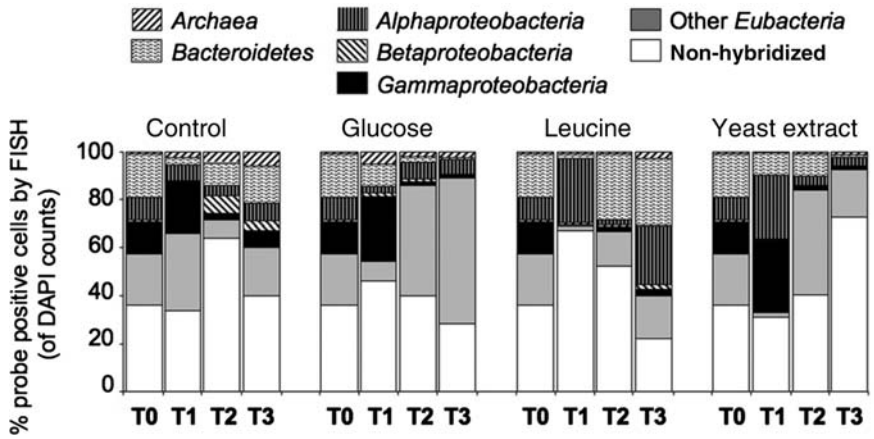

Fig. 5. Composition of the prokaryotic community in Experiment I as measured by FISH at the initial situation (T0), at the time of maximum prokaryotic abundance (T1), at the time of maximum HNF abundance (T2) and at the final situation (T3).

DISCUSSION

Naturally occurring seawater enrichment, due to exudation of organic carbon by phytoplankton during blooms or to allochthonous organic matter input, usually results in substantially enhanced prokaryotic growth. Such bursts of prokaryotic growth are, in general, tightly coupled to their consumption by bacterivorous protists, which transfers part of the carbon to higher trophic levels through the microbial loop. However, a variable fraction of prokaryotic biomass can be inedible to bacterivores, which results in decreases in the efficiency of carbon transfer. Because prokaryotes consume up to 50% of the carbon produced by phytoplankton (Cole *et al.*, 1988; Ducklow and Carlson, 1992), the appearance and development of grazing-resistant prokaryotes can have important implications with regard to the amount of carbon reaching higher trophic levels.

The composition of allochtonous or autochtonous organic matter inputs to marine waters can vary substantially, depending on the origin and diagenetic state. For example, the composition of photosynthetic organic carbon exudates differs depending on the dominant algal species and on environmental factors such as the

nutrient status (Nagata, 2000). Changes in the abundance, biomass and composition of prokaryotic communities in response to different sources of DOM have been widely analysed in experimental studies (Kirchman and Rich, 1997; Eilers *et al.*, 2000; Pinhassi and Berman, 2003). However, the effect of the composition of DOM sources on the development of grazing-resistant prokaryotes has been almost entirely overlooked in marine systems, and constitutes the objective of the present study.

The experimental design (i.e. substrate additions in bottle incubations with 8 μm pore-size pre-filtered seawater samples) introduced different artefacts, one being the removal of grazers such as ciliates or zooplankton, which can also act as natural sources of DOC and regenerated nutrients. This precludes the extrapolation of the results to the functioning of natural communities, and thus, our findings can only be regarded as insights into the potential of marine bacterial communities to develop grazing resistance mechanisms. The data presented are also quite restricted (only two experimental replicates), but despite these limitations, our results clearly support the view that marine bacterial communities can develop important grazing-resistant mechanisms such as filamentation and aggregation after episodes of DOC enrichment. Furthermore, filamentation and aggregation were significantly higher after the addition of a single C-source (i.e. glucose), than after the addition of rich source of carbon and nutrients such as yeast extract.

In one of the few previous studies carried out in marine waters (Jürgens *et al.*, 2000), filaments did not appear, even in the glucose- and amino acid-enriched microcosms, but these experiments were performed with open ocean water samples. Differences in the bacterioplankton composition between coastal and open oligotrophic ocean may explain these differing results, because filamentous prokaryotes have been observed previously in microcosms with coastal marine waters (Caron *et al.*, 1988; Shiah and Ducklow, 1995).

At the beginning of the experiments, the dominant phylogenetic groups were *Bacteroidetes*, *Gammaproteobacteria* and *Alphaproteobacteria*, which are the predominant groups in marine coastal systems (Cottrell and Kirchman, 2000). As a consequence of the growth of those prokaryotes best adapted to substrates that we added, different prokaryotic groups were selected: *Gammaproteobacteria* and *Alphaproteobacteria* predominated in the microcosms enriched with glucose and leucine, respectively, and both groups were found in high abundance in the microcosms enriched with yeast extract. Accordingly, studies assessing the single-cell activity of these prokaryotic groups by microautoradiography combined with FISH have shown that *Alphaproteobacteria* are among the most active groups in the uptake of amino acids (Cottrell and Kirchman, 2000). Stimulation of *Gammaproteobacteria* by high concentrations of glucose has also been reported (Pinhassi and Berman, 2003; Harvey *et al.*, 2006), and indeed, this group is known to include members with high growth rates that respond quickly to most increases in labile DOM (Eilers *et al.*, 2000; Pinhassi and Berman, 2003; Yokokawa *et al.*, 2004).

The grazing pressure exerted by the nanoflagellates produced further changes in the phylogenetic composition of the prokaryotic communities, such as decreases in the abundance of *Gammaproteobacteria* (in glucose and yeast extract treatments) or *Alphaproteobacteria* (in leucine and yeast extract treatments). A high percentage of non-hybridized prokaryotes were found in the microcosms enriched with leucine or yeast extract, which could be due to limitations of the FISH method. Also, a large fraction of detected cells in the glucose or yeast extract microcosms could not be identified by the general probes. The coverage of the general probes used (such as Alf968, Beta42a, Gam42a and CF315) is fairly good for marine communities (Amann and Fuchs, 2008). However, these probes are not exhaustive and it is plausible that some members of these groups growing in the incubations could not be detected by the general probes. Another possibility is that the non-identified cells were affiliated to other groups not tested like Actinobacteria, which are poorly detected with the FISH technique (Sekar *et al.*, 2003) and are favoured under conditions of high bacterivorous pressure (Šimek *et al.*, 2005) because of their generally small size (Hahn *et al.*, 2003). Although Actinobacteria is typically a freshwater group, some actino-bacterial-type sequences have been found in marine systems (Rappé *et al.*, 1999).

The changes that we observed during the phase of intense grazing support the idea that some populations can dominate the bacterioplankton community when carbon and nutrient limitation is the only main selection factor, but different populations of prokaryotes can successfully compete when there are other major selection factors, such as grazing. Interestingly, although marked changes in prokaryotic assemblage composition in response to bacterivorous grazing have been shown in freshwater studies (Jürgens *et al.*, 1999; Šimek *et al.*, 2001), no changes were found in a study that was carried out with chemostats with marine samples (Massana and Jürgens, 2003). The differences between their results and ours underline the importance of the DOM supply with regard to the appearance of grazing-resistant morphotypes because in the chemostat study, no additional DOM was supplied.

In this work, we examined the two main types of resistance mechanisms, aggregation and morphological changes after the addition of different DOC sources. The complex substrate, i.e. yeast extract, was a rich source of nutrients and growth factors required by prokaryotes; hence, the addition of this substrate produced the highest peaks in prokaryotic production. However, the prokaryotes growing in the yeast extract-enriched samples were mostly small and freely dispersed, and therefore were potentially edible (Figs 2 and 3). In contrast, after the addition of glucose and leucine, there was a marked tendency towards aggregation and filamentation (Figs 2 and 3). The addition of carbon in the form of glucose or leucine probably forced the communities to a situation of starvation by the limitation of other essential nutrients. In accordance with our results, some studies have shown that N- and P-limited cells showed increasing biovolume with the time of starvation, and can form filaments or swollen rods (Holmquist and Kjelleberg, 1993). Even if the addition of leucine also provided N, this substrate is more difficult to metabolize than other amino acids, and thus, the N could have been not so readily available for the synthesis of cell compounds. Starvation of bacterial communities could also explain the low hybridization efficiencies found with FISH technique as has been reported before (Oda *et al.*, 2000).

In accordance with studies carried out in the Central Atlantic Ocean (Jürgens *et al.*, 2000), in our experiments, aggregates appeared during prokaryotic growth, before the growth of HNF and increased in quantitative importance during the grazing process. This suggests that aggregation is an effective mechanism of grazing resistance. Although there are protists that specialize in grazing upon aggregated prokaryotes (Caron, 1987; Artolozaga *et al.*, 2000), it has been shown that protists graze upon them far more slowly than upon freely dispersed prokaryotes; up to seven times more slowly in the case of specialized nanoflagellates (Artolozaga *et al.*, 2002). Remarkably, aggregation was strongly influenced by the DOM supply; it was significantly higher in the microcosms enriched with glucose (over 60% of prokaryotes were aggregated) than in the microscosm enriched with yeast extract (<20% of prokaryotes, Fig. 3). Aggregation appears to be the result of the synthesis of exopolymeric compounds (Decho, 1990), which could have been promoted after the addition of a carbon source as glucose (Spiglazov *et al.*, 2004).

Miniaturization did not appear to be a grazing-resistance mechanism in these experiments, because in most cases, the smallest prokaryotes (range 0.2–0.5 μm) diminished in importance during the grazing phase. At the other extreme, it has been demonstrated that prokaryotes in the range of 3–5 μm have a reduced vulnerability to flagellate grazing (Šimek *et al.*, 1997; Jürgens *et al.*, 1999). To estimate the grazing-resistant prokaryotic biomass, we made the assumption that the prokaryotes accessible to HNF were freely dispersed prokaryotes smaller than 3 μm in length, including the smallest cells, and 30% of the aggregated biomass. This percentage, although speculative, was chosen taking into account the fact that the capacity of specialized protists to graze on the aggregates depends on the location of the prokaryotes, and the prey located within the aggregate are less edible but more abundant than those located on the aggregate surface. Interestingly, the β-*N*-acetylglucosaminidase activity of HNF did not correlate with the total prokaryotic biomass, but we did find a significant relationship with the biomass of edible prokaryotes calculated as explained above (Spearman correlation, $P < 0.01$). Considering these criteria, the percentage of grazing-resistant biomass strongly depended upon the added substrate; the addition of yeast extract only yielded 8–16% grazing-resistant biomass, whereas glucose and leucine led to higher percentages of 46–64% and 27–70%, respectively. For this comparison, a conversion factor of 20 fg C cell^{-1} was applied to aggregated bacteria (Simon *et al.*, 1990; Turley *et al.*, 1996), which was in the lower range of the values estimated for freely dispersed bacteria in the incubations. Therefore, these estimations are probably conservative because aggregated bacteria have been shown to be larger than freely dispersed bacteria (Simon *et al.*, 2002), and thus, the amount of aggregated biomass could have been even larger.

It should be taken into account, however, that the resistance strategies are only partial and temporal. For example, aggregates can be colonized by bacterivores that specialize in grazing upon aggregated prokaryotes (Artolozaga *et al.*, 2000), and aggregates can disintegrate (Biddanda and Pomeroy, 1988). Moreover, other grazers, such as ciliates and zooplankton (which were removed from our experimental microcosms by prefiltration), can take up both large and aggregated prokaryotes, with the consequent transfer of energy and matter directly to these larger organisms. Despite this, our results indicate that the immediate transfer of carbon through the microbial loop after an episode of seawater enrichment can be markedly different depending upon the composition of the organic matter source. The input of a complex mixture of organic compounds, covering a wide range of requirements for prokaryotes, promoted their growth and division, as well as their consumption by HNF and the consequent transfer of carbon. However, the input of non-balanced organic

matter, with an excess of carbon, such as glucose, promoted the development of grazing-resistance mechanisms such as elongation and aggregation, which, in turn, limited the transfer of carbon to higher trophic levels. Although more data are needed, the composition of DOM and nutrient limitation seem to be important factors influencing the establishment of grazing-resistant prokaryotes, and consequently should be taken into account in future studies.

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