Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in abundances, activity and composition

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Abstract

Quantitative and qualitative changes in bacterial communities from the Mediterranean Sea were compared in duplicate batch mesocosms with or without addition of inorganic nutrients. Methods including traditional microbial ecology techniques, molecular biology and flow cytometry were combined to determine abundances, production, cell size, activity, culturability and taxonomic diversity of bacterial cells. Addition of nutrients and confinement resulted in an increase of bacterial densities which were rapidly controlled by protozoan grazing. Changes in bacterial activity and morphology were observed during the growth phase of bacteria and under grazing pressure. The proportion of medium-size and culturable cells increased during the growth phase. These cells were preferentially consumed by grazers resulting in a strong limitation of bacterial production. As a consequence of the grazing pressure, large cells were produced and contributed to the remaining bacterial productivity after grazing. Grazing had an effect on the taxonomic composition of bacterial communities by preferentially eliminating γ-Proteobacteria, α-Proteobacteria were preserved. It seems that some species from the genera Ruegeria and Cytophaga may have developed defence strategies to escape predation. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Knowledge on the role and control of bacterioplankton in biogeochemical cycles and energy fluxes is in permanent progress. A current subject of discussion is whether the abundance and productivity of pelagic bacteria are preferentially controlled by grazing or nutrients [1–4]. An important issue in aquatic microbial ecology is to elucidate the relative importance of resource limitation, grazing, and viral mortality on the functional and taxonomic diversity of bacterioplankton communities [5,6].

In coastal eutrophic areas, the nature and availability of inorganic and organic nutrients may play an important role in the energetic activation and inactivation of bacterial species. Therefore, nutrients may affect the taxonomic structure of natural communities both temporally and spatially.

Heterotrophic nanoflagellates and ciliates are usually...
the major consumers of bacteria in aquatic systems [7]. It has been suggested that very small and large bacteria are partly or totally protected from nanoprotozoan grazing whereas active cells within the medium size-class are preferentially consumed [5,8]. Whether large and very small bacteria exhibit an ecologically significant defence strategy against protozoan grazing, is still a matter of debate. Furthermore, it is unknown to what extent selection due to grazing pressure affects the taxonomic structure and activity of natural communities. A key question is to identify and to understand the ecological role and behaviour of the bacterial cells which have developed adaptation strategies to escape grazing pressure in natural aquatic ecosystems.

The introduction of flow cytometry and molecular biological techniques to microbial ecology has provided new approaches for investigating the structure of microbial communities [9,10]. In a previous paper [11], we have shown that small and large cells were produced as a consequence of grazing pressure and that grazing influences the genetic diversity of bacterial communities by eliminating some populations and by selecting other species [6]. In this study we used a unique combination of methods and provide strong evidence that sequential growth and grazing result in important changes in morphology, composition and activity of natural bacterial communities. Changes in the structure and composition of the bacterial community were monitored by denaturing gradient gel electrophoresis (DGGE) and by sequencing of 16S rRNA gene fragments. These results are reported in the accompanying paper by Schäfer et al. [12] whereas results on the fluctuation of demographic parameters, activity, cell size distribution are presented in this paper and discussed in regard to DGGE results.

2. Materials and methods

2.1. Experimental mesocosms

The mesocosms consisted of four rectangular tanks of 400-l capacity, 60 cm in height, 60 cm width and 110 cm length [11]. Each mesocosm was filled with 300 l of natural seawater collected in June 1997 from 1 m depth at a station (42°31′ N – 03°11′ E) located 2 miles off Banyuls-sur-Mer, France (North-western Mediterranean coast). Natural seawater was filtered through a 200-μm nylon mesh. This filtration removed large metazoans and detritus. Each mesocosm was temperature-controlled to match the temperature of Mediterranean coastal water at the time of the experiment, by placing the mesocosms into larger tanks (30 cm height, 80 cm width, 140 cm length) continuously alimented by natural seawater pumped from the shore. The temperature of the water was 18.2–19.5°C during the experiment. Mesocosms were illuminated by a fluorescent ramp consisting of five tubes (36 W, 120 cm) providing illumination on a 12:12 h light:dark period. Photosynthetically active irradiance (PAR) was measured with a Li-cor quantum sensor. The PAR at the upper part of mesocosms was 200 μE m⁻² s⁻¹. The mesocosms were mixed by means of an immersed pump taking water from two opposite bottom corners and driving the water back below the surface. Two mesocosms were not amended and used as duplicate controls to follow changes in the initial microbial community due to confinement (hereafter referred to as ‘control’ replicate 1 or replicate 2). Two mesocosms were used as duplicate tanks (hereafter referred to as ‘experimental’ mesocosms replicate 1 and replicate 2) to follow the effect of inorganic nutrients by supplementing them with NaNO₃ (5.1 μM), NH₄Cl (1.8 μM) and KH₂PO₄ (0.6 μM).

2.2. Sampling strategy

Five litres of each mesocosm were taken daily to analyse nutrients, phyto- and bacterioplankton counts and bacterial production. The temporal evolution of total bacterial counts was used to determine the sampling times at which all other parameters including the bacterial genetic diversity were also analysed. For these points, 10 l was taken and mixed. These six (we had 6 points for molecular biology) sampling times were chosen corresponding to the starting time of each mesocosm, to the first significant increase in bacterial counts, to the peak, and then, after a significant decrease of total counts and at the end of experiments defined by the stabilisation of total counts.

2.3. Nitrogen and phosphorus analysis

Subsamples for determination of nitrate and phosphate concentration were collected in polyethylene bottles and frozen (−20°C) until analysis. Nitrate and phosphate concentrations were determined with a Skalar autoanalyser according to Treguer and Le Corre [13]. For ammonium analysis, subsamples were collected in 100-ml glass bottles and processed according to Koroleff’s manual method [14]. The reagents were added immediately after collection and measurements were performed within 4–24 h using a spectrophotometre (Spectronic 401).

2.4. Counts of culturable bacteria

Culturable bacteria were enumerated by plating 100 μl of serial dilutions of each water sample on marine agar plates (MA, Difco, Detroit, MI, USA). In preliminary assays R2A medium prepared with a seawater base was also used, however, marine agar was chosen for the assays since it yielded higher counts. After 7 days of incubation at room temperature (20 ± 3°C) colony forming units (CFU) were enumerated. Counts did not increase after prolonged incubation.
2.5. Flow cytometric analysis of bacteria

Total counts of SYTO 13-stained bacteria were performed with a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an air-cooled argon laser (488 nm, 15 mW). Living samples were stained with SYTO 13 (Molecular probes, Eugene, OR, USA) at 5 μM for 30 min. Fluorescent beads (1.0 μm; Polysciences, Warrington, PA, USA) were systematically added to samples to normalise cell fluorescence emissions and scatters. According to their green fluorescence (FL1) and right angle light scatter (RALS, related to cell size) signatures, different bacterial populations may be discriminated and enumerated. Bacterial enumeration by flow cytometry performed as described above was found to be in close concordance with bacterial enumeration by epifluorescence microscopy after DAPI staining [15,16].

To calibrate the flow cytometric scatter measurements, bacterial cell lengths and biovolumes were estimated from epifluorescence examination of 4,6-diamidino-2-phenylindole (DAPI)-stained cells of fixed (2% formaldehyde) samples (n = 17) [16]. The relationship between RALS and cell volumes showed high and significant correlation (r = 0.869; P ≤ 0.0001) [16]. The positive correlation was also obtained when using cell lengths rather than biovolumes [16]. Several bacterial cell populations were defined via their RALS and green fluorescence (FL1) cytogram obtained for each sample. Cells with high RALS values corresponded to long rods observed by epifluorescence microscopy. From one sample to another, these populations were not exactly located at the same place on cytograms. Thus, to objectively group the different populations observed in all samples analysed, a clustering method (Ward’s algorithm, [17] was applied on all the identified populations. Each population was characterised by its RALS and FL1 mean values. Clustering was carried out with the ‘R package’ [18]. Three main clusters with an increasing estimated mean size (cell length) (small ≤ 1.0 μm; 1.0 μm < medium ≤ 2.5 μm, and large > 2.5 μm) were discriminated in each sample.

2.6. Cell size measurement

Size measurement of DAPI-stained cells was carried out from microphotographs of at least 10 randomly selected microscopic fields. The lengths and widths (μm) of at least 100 single cells were measured from slide projection and calibrated with a micrometric slide. Bacterial cell volume estimations were based on the assumption that all bacteria are spheres or cylinders with two hemispherical caps.

2.7. Microscopic counts of protozoa

The abundance of protozoa was determined by epifluorescence microscopy (Leitz, Laborlux D) after DAPI staining. Twenty millilitres of glutaraldehyde-preserved (0.5% final concentration) water samples were stained with DAPI (10 μg ml⁻¹, final concentration) for 15 min. Filters were mounted on microscopic slides and stored at +4°C until examination. Stained protists were collected by filtration on a 0.8-μm pore size black filter (Nuclepore). Picocells (0.2–2 μm in diameter) and nano-sized (2–20 μm in diameter) micro-organisms were identified, counted and measured at a magnification of 1250× and 625× while micro-sized (20–200 μm) micro-organisms were analysed at a magnification of 125×. A minimum of 100 organisms per filter was counted. Autotrophic species were distinguished from heterotrophs by the red auto-fluorescence of chlorophyll a observed under blue light excitation. Dinoflagellates, flagellates and ciliates were enumerated distinctly. Data presented in this paper concern only the heterotrophic protozoa.

2.8. Bacterial activity

Incorporation of ³H-thymidine. Four 10-ml subsamples were incubated in the presence of the four different concentrations (6.5, 13, 26 and 39 nM) of tritiated thymidine (Amersham 84 Ci mmol⁻¹) for 1-2 h in the dark at the temperature of the mesocosms. After incubation, cold trichloroacetic acid was added (final concentration 5%) and the samples were filtered through 0.2-μm pore size cellulose acetate membrane. Radioactivity associated with the filters was estimated by liquid scintillation. Incorporation rates expressed in pmol l⁻¹ h⁻¹ at the different thymidine concentrations were calculated and plotted against the added thymidine concentrations. The maximum incorporation rates were estimated by best fitting a hyperbolic function to the experimental data using software based on the least squares criterion [19]. Data presented in the paper are maximum incorporation rates. Cellular productions were calculated by multiplying the maximum thymidine incorporation rates by a theoretical conversion factor of 0.5×10¹⁸ cells produced per mol of thymidine incorporated [20]. Specific thymidine incorporation rates were calculated by dividing cellular productions by total bacterial counts.

Incorporation of ³H-leucine. Four subsamples of 10 ml were incubated in the presence of the four different concentrations of leucine (2 nM of tritiated leucine – Amersham 151 Ci mmol⁻¹ with 0, 25, 50, 75 nM non-radioactive leucine) for 1–2 h in the dark at the temperature of the mesocosms [19]. After incubation, cold trichloroacetic acid was added (final concentration 5%) and subsamples were heated at 85°C for 30 min after acidification following the procedure proposed by Kirchman et al. [21] to specifically measure the incorporation into proteins. After cooling, the samples were filtered through 0.2-μm pore size cellulose acetate membrane. Maximum incorporation rates into proteins were calculated as for thymidine incorporation. Data presented in the paper are maximum incorporation rates into proteins.
2.9. Fluorescent in situ hybridisation (FISH)

Bacterial cells were filtered on black 0.2-µm pore-size polycarbonate membrane filters (47 mm diameter, Nuclepore) to obtain 50–100 bacterial cells per microscopic field. The volume of water to be hybridised was determined from flow cytometric counts. After filtration, cells were fixed by overlaying the filters with 4% cold paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 30 min at room temperature. The fixative was removed by applying gentle vacuum, and the filters were rinsed with filter-sterilised PBS. Next, the filters were air dried and stored at −20°C until further processing.

Probes EUB, ALF, BET, GAM and CFB used for hybridisation at the community level are reported in Table 1. These probes were labelled with the indocarbocyanine fluorescent dye CY3 (Biological Detection Systems, Pittsburgh, PA, USA) and stored at −20°C.

Prior to in situ hybridisation, filters were cut into small sections (0.5–1 cm²). These sections were placed on cover-slips, covered with 16 µl of hybridisation buffer containing 2 µl (50 ng µl⁻¹) of the respective fluorescent probe, and hybridised in petri-slides (Millipore) inside an incubator at 46°C for 90 min. Hybridisation buffers and washing procedures were those described by Alfreider et al. [28]. To determine total bacterial abundance, the filter sections were stained for 10 min with DAPI (final concentration 2.5 µg ml⁻¹) at room temperature prior to microscopic examination. Preparations were put on glass slides, mounted in epifluorescence-specific oil (Olympus) and inspected by epifluorescence microscopy (BX70, Olympus, France). Filter sets for DAPI were BP 330–385 nm and BA-420 nm and for CY3 were BP-520–530 nm and BA-580-IF nm (Olympus).

Between 400 and 600 DAPI-stained bacteria were counted on each hybridised filter. Unhybridised filters were also examined as controls for the presence of orange fluorescent bacterium-sized particles (Cyanobacteria, chlorophyll-containing detritus and other organic detritus). When detected, these counts were deduced from hybridised counts.

2.10. Hybridisation of bacterial isolates

A total of 664 isolates was randomly selected from the different times and mesocosms using marine agar (Difco) for isolation. Preparation of genomic DNA from the pure cultures of selected isolates and amplification of 16S rDNA by PCR were done as described by Rainey et al. [29]. 16S rDNA amplicons of strains were immobilised on a nylon membrane and hybridised against taxon specific oligonucleotide probes as described by Pukall et al. [26]. Probes used in this study are given in Table 1. These probes were selected based on preliminary identification of the most dominant isolates in seawater from the same location [26].

2.11. Phylogenetic analysis

The nearly complete 16S rDNA sequence of strain CH611 was analysed according to the cycle sequencing protocol described by Rainey et al. [29]. The sequence was manually aligned and analysed using the DSMZ-internal database consisting of more than 5000 16S rDNA sequence entries, including also those from the Ribosomal Database Project [30] and EMBL. The nucleotide sequence of strain CH611 was deposited in the EMBL nucleotide sequence databases under the accession number AJ295988.

Table 1
Specific 16S RNA and 23S RNA probes used for FISH and microscopic examination and for oligonucleotide hybridisation of isolates

| Probe  | Target position | Sequence (5’-3’) | FA (%)/TA<sub>m</sub> | Specificity          | Reference |
|--------|----------------|-----------------|-----------------------|----------------------|-----------|
| FISH analyses | | | | | |
| EUB    | 16S 338–355 | GCT GCC TCC COT AGG AGT | 0 | Eubacteria | [22] |
| ALF    | 16S 19–35  | GCT TCG YTC TGA GCC AG | 20 | α subclass of Proteobacteria | [23] |
| BET    | 23S 1027–1043 | GCC TCG CCA CTT COT TT | 35 | β subclass of Proteobacteria | [23] |
| GAM    | 23S 1027–1043 | GCC TCG CCA CAT COT TT | 35 | γ subclass of Proteobacteria | [23] |
| CFB    | 16S 319–336 | TGG TCG GTG TCT CAG TAC | 15 | Cytophaga-Flavobacterium cluster of CFB | [24] |
| Hybridisation of isolates | | | | | |
| ALF    | 16S 19–35  | GCT TCG YTC TGA GCC AG | 58 | α subclass of Proteobacteria | [23] |
| Ruergeria | 16S 155–175 | GTA TTA TCT ACC GTT TCC AGT G | 68 | R. algicola | [25] |
| Sulfitobacter | 16S 81–108 | CCG CCG CTC ACC CGA AGG | 64 | S. pontiacus | This study |
| GP4    | 16S 73–97  | CAT CTT CTA GCA AGC TAG AAA TG | 62 | Alteromonas macleodii | [26] |
| GP1    | 16S 73–97  | CAT CAT CTA GCA AGC TAG ACA TG | 63.5 | A. macleodii | [26] |
| GP3    | 16S 73–97  | CAG CCA AGT GCT ACT TCC TG | 68 | A. macleodii | [26] |
| CFB    | 16S 319–336 | TGG TCG GTG TCT CAG TAC | 56 | Cytophaga-Flavobacterium cluster of CFB | [24] |
| Gram-positive | 16S 1199–1215 | TCA TCA TGC CCG TTA TG | 50 | Gram-positives | [27] |

*FA (%): final concentration of formamide used in the hybridisation buffer for FISH analyses according to Alfreider et al. [28] or TA<sub>m</sub>: melting temperature (°C) of oligonucleotide probes used for membrane hybridisation of isolates.
3. Results

The results presented are those obtained from replicate 1 of both experimental and control mesocosms. Similar trends were obtained for the two replicates of each mesocosm. Total counts between the two replicates of control and experimental mesocosms correlated well ($r^2 = 0.76$, $P < 0.0001$, $n = 14$) and $r^2 = 0.84$, $P < 0.0001$, $n = 14$, respectively) suggesting the good reproducibility of results. Furthermore, the structure of bacterial communities at different sampling times was also reproducible [12].

3.1. Nutrients

The initial seawater was characterised by low concentrations of phosphate, nitrate and ammonium, being close to the detection limit (Fig. 1). In the experimental mesocosm, added phosphorus, nitrate and ammonium were rapidly consumed during the first 3 days and then stabilised (Fig. 1B). The first growth phase was supported by dissolved organic carbon (DOC) present in the original seawater but also from organic carbon released by protozoa during the grazing phase. The interesting observation in all mesocosms was that the exhaustion of nutrients from the third day of incubation was compatible with the significant emergence of bacterioplankton densities and production, especially in the enriched mesocosms but also to a lesser extent in the control (Figs. 2 and 3). The substrates for bacterial growth during the second growth phase in experimental mesocosms were expected to become available through the excretion by protozoa.

3.2. Bacteria and protozoa abundances

Initial bacterial numbers were about $5 \times 10^5$ cells ml$^{-1}$ in both mesocosms (Fig. 2). Although bacterial abundances increased during the first days in both mesocosms, this increase was more important in the experimental mesocosm ($6.8 \times 10^6$ cells ml$^{-1}$ after 96 h) as compared to the control ($1.5 \times 10^6$ cells). The peaks of abundance were observed after 48 h in the control and 96 and 216 h in the enriched mesocosm. In the experimental mesocosm, the first increase of total bacterial counts was followed by a rapid increase in counts of nanoprotozoa, which was delayed by approximately 24 h. Nanoprotozoa were themselves rapidly controlled by microprotozoa. In experimental mesocosms, the control of nanoprotozoa by microprotozoa allowed a second increase of bacterial density.

![Fig. 1. Temporal changes of ammonium, nitrate and phosphorus concentrations in replicate 1 of control and experimental mesocosms. Similar trends were observed in duplicate mesocosms. The grey area corresponds to the intense grazing phase.](image1.png)

![Fig. 2. Temporal changes in total bacterial, nanoprotozoan and microprotozoan counts in replicate 1 of control and experimental mesocosms. The grey area corresponds to the intense grazing phase.](image2.png)
counts until stabilisation. The phase of grazing corresponding to an important decrease of total bacterial counts is identified in grey on all figures.

3.3. Bacterial activity

The rate of $^3$H-thymidine incorporation into DNA was used to estimate bacterial cell replication [31] and that of $^3$H-leucine into bacterial proteins to estimate the rate of protein synthesis [21]. Although both methods are usually well correlated [32], they give complementary information on the bacterial metabolism as one them is a measure of cell multiplication and the other is a measure of biomass synthesis [33]. Maximum incorporation rates of thymidine and leucine presented the same trends although slight differences were observed in some situations (Fig. 3). In the initial seawater, the rates of leucine incorporation into protein and thymidine incorporation into DNA were about 500 and 15 pmol l$^{-1}$ h$^{-1}$, respectively. The incorporation rates increased slightly during the first 2–3 days in the control mesocosm and then fluctuated. In the experimental mesocosm, both incorporation rates increased exponentially until day 2 and 3, showing a biphasic fluctuation inversely related to nanoprotozoa densities. The high values of bacterioplankton activities in the experimental mesocosm remained higher than those measured at the beginning of the experiment. An interesting aspect is that leucine and thymidine incorporation values showed an unusual divergence in the experimental mesocosm after the peak of nanoprotozoa biomass and when bacterial counts increased for a second time after 144 h. Such divergence is due to the fact that thymidine incorporation increased whereas leucine incorporation rates roughly stabilised. This clear and strong change in the incorporation values of thymidine and leucine suggest that the bacteria had reduced their protein synthesis activity but still had increased DNA duplication rates. A similar but less pronounced divergence between thymidine and leucine incorporation rates was observed in the control.

The specific thymidine incorporation rates reported in Table 2 are estimates of the mean activity per bacterial cells. This cellular activity increased strongly at the end of the grazing phase in experimental mesocosms and then remained high until the end of incubation. In the control mesocosms, specific cellular activity also increased after 144–168 h and was then maintained at high values.

3.4. Culturable bacterial cells

The fraction of culturable cells was rather high in the initial seawater (about 12–14%) and increased during the growing phase in both mesocosms (Fig. 4), reaching a maximum of 26% in the control between 48 and 96 h. In the control, the culturability was even higher than in the experimental mesocosm and decreased after 96 h. This is probably due to intense grazing on culturable cells in the experimental mesocosm since the percentage of culturable cells increased strongly during the first 24 h but was rapidly controlled.

3.5. Bacterial cell size

In both, control and experimental mesocosms an in-

### Table 2

| Time (h) | Control 1 | Control 2 | Experimental 1 | Experimental 2 |
|----------|-----------|-----------|----------------|----------------|
| 0        | 0.028     | 0.031     | 0.025          | 0.021          |
| 24       | 0.043     | 0.036     | 0.024          | 0.044          |
| 48       | 0.036     | 0.038     | 0.032          | 0.058          |
| 72       | 0.036     | 0.043     | 0.026          | 0.030          |
| 96       | 0.036     | 0.033     | 0.014          | 0.027          |
| 120      | 0.033     | 0.039     | 0.031          | 0.028          |
| 144      | 0.047     | 0.035     | 0.061          | 0.067          |
| 168      | 0.030     | 0.049     | 0.070          | 0.071          |
| 192      | 0.046     | 0.049     | 0.047          | 0.059          |
| 216      | 0.054     | 0.075     | 0.058          | 0.072          |
| 240      | 0.049     | 0.074     | 0.062          | 0.071          |
| 264      | 0.051     | 0.058     | 0.066          | 0.081          |
| 288      | 0.074     | 0.113     | 0.072          | 0.093          |
| 312      | 0.050     | 0.102     | 0.051          | 0.070          |
crease in the bacterial size was clearly observed after the peak of nanoprotozoa density, strongly suggesting that bigger bacteria were not efficiently grazed (Fig. 5). The relative contributions of cells to small, medium and large sizes as determined from flow cytometric scatter measurements are presented in Fig. 6. The fraction of small cells decreased slowly during the incubation period in both mesocosms. The proportion of medium-sized cells decreased rapidly up to the peak of grazers at time 144 h while larger cells increased at the same time in both mesocosms. Then, large cells were submitted to a small decrease after 172 h in all mesocosms, corresponding to the peak of microprotozoa. These results are congruent with those obtained by microscopic observation and also suggest that a fraction of very small cells was not responsive to environmental changes. Small cells remained below 10% of total counts and decreased slightly during the incubation in both mesocosms. During the last sampling times in the experimental mesocosm the fraction of large cells decreased whereas that of medium-sized cells increased.

3.6. FISH

The fraction of cells hybridising with probe EUB was higher than 90% of total cells in all samples (Table 3). No cells hybridised with probe BET suggesting the absence of β-Proteobacteria in our samples. Probe ALF hybridised to less than 20% of total bacteria in the initial seawater but this fraction increased to a maximum of 33.6 and 54.8% at 144 h in control and experimental mesocosms, respectively. Inversely, the proportion of cells hybridising to probe GAM decreased slightly up to time 144 h. It suggests that α-Proteobacteria are those which preferentially escape from grazing pressure. Although the accurate number of cells hybridising to probe CFB could not be determined in most cases due to the low frequency of these cells (less than 0.2%), some long cells (>20 μm) observed at time 144 h hybridised with this probe and represented 0.8% of total cells in the experimental mesocosm. This low detection limit for microscopic counts corresponds to the detection of one cell within 500 DAPI-stained cells examined in a total of 20–30 microscopic fields.
3.7. Identification of bacteria and group specific affiliation of isolates

Isolates were randomly selected from the different sampling points and hybridised with group-specific probes (Table 4). The proportion of \( \alpha \)-Proteobacteria increased during the phase of increasing protozoa counts suggesting that some of these strains were able to escape from the grazing pressure or that they were growing faster than they are consumed. Especially the fraction of Ruegeria algicola isolates increased following the grazing pressure and represented 25% of all isolates at time 144 h. Among the different strains isolated at time 144 h, one strain, strain CH611, was found to form long cells (mean length 15.8 \( \pm \) 7.2 \( \mu \)m, \( n = 60 \) cells) and was identified by sequencing of the 16S rRNA encoding gene as a close relative of Ruegeria gelatinovorans, showing a sequence similarity value of 95.9%. This strain also hybridised with the \( R. \) algicola probe due to probe-specificity limitation. The proportion of isolates hybridising with the different Alteromonas probes decreased with increasing incubation time. A large proportion of isolates could not be affiliated to the phyla against the different probes were directed.

4. Discussion

Bacterial growth was rapidly stimulated by addition of nutrients in experimental mesocosms whereas growth was more limited in control mesocosms and more likely due to be due to confinement and sample processing. Previous studies in the Mediterranean sea have indicated N- and P-limitation of phytoplankton and bacteria [34]. In all mesocosms, the increase of total bacterial counts was followed by a rapid control of bacterial abundances by grazing. Although viruses were not studied and may also contribute to this decrease, important changes in the activity, taxonomic diversity and cell size distribution of bacterial communities were observed concomitant with the evolution of both bacterial and Protozoa counts. The two control mesocosms were analysed to ensure that changes observed in the experimental mesocosms were not only due to confinement and to the environmental conditions during the time of incubation.

4.1. Dynamics of bacterial populations

The percentage of culturable cells in the initial seawater

| Time (h) | 0 | 48 | 96 | 144 | 216 | 312 |
|---------|---|----|----|-----|-----|-----|
| ALF     | 15(27) | 45(43) | 41(41) | 59(55) | 37(51) | 68(56) |
| Ruegeria| 5(15) | 6(8) | 8(10) | 25(23) | 11(15) | 34(22) |
| Sulfitobacter | 7(15) | 6(8) | 24(16) | 11(12) | 5(7) | 7(4) |
| GP4     | 15(12) | 2(3) | 13(3) | 2(11) | 0(7) | 0(4) |
| GP1     | 3(12) | 0(2) | 3(2) | 2(0) | 5(0) | 0(2) |
| GP2     | 8(7) | 21(6) | 20(8) | 2(4) | 0(0) | 0(5) |
| CFB     | 1(13) | 0(11) | 0(14) | 4(11) | 5(12) | 5(4) |
| Gram-positive | 10(5) | 15(6) | 12(8) | 9(14) | 37(15) | 0(7) |
| Not identified | 47(23) | 17(29) | 10(24) | 23(5) | 16(15) | 27(22) |

Percentages were determined from strains randomly isolated in experimental mesocosms. Values in brackets are the percentages obtained in control mesocosms.
was surprisingly high since CFU are generally below 1% of total cells in marine waters. High percentages of CFU in seawater were also reported by Fukami et al. [35] and in that study the fraction of culturable bacteria was submitted to important changes in both, numbers and composition during the degradation of diatoms. The high percentage of CFU found in this study suggests that a significant fraction of viable and cultured species was present within the initial natural community. Some of the isolates collected at the onset of incubation hybridised with different probes. Isolates hybridising with *Alteromonas* and *Sulfitobacter* probes may represent a significant fraction of CFU counts since these genera were highly represented in both DNA and RNA DGGE profiles [12]. However, an important fraction of isolates did not hybridise with any of the probes tested in this study and may also belong to some other species such as those identified by DGGE [12].

The low representativity of *ß*-Proteobacteria within isolates from seawater has been reported in other studies [9,35]. Inversely, *ß*-Proteobacteria were preferentially selected during the growth phase and this increase was also observed on DGGE profiles [12] in both control and experimental mesocosms. Riemann et al. [36] found that bacteria related to the *ß*-Proteobacteria and to *Cytophagaales* are fast-growing bacteria preferentially selected during the decomposition of diatoms. These bacteria are probably able to rapidly adjust their growth rate to new growth conditions inducing rapid changes in the composition of the bacterial community.

In the experimental mesocosm, the rapid decrease of the percentage of culturable cells was due to a more rapid increase of total counts since absolute culturable counts continued to increase until 120 h, when their numbers started to decrease (data not shown). In fact, this decrease in the percentage of culturable counts probably results from the selective feeding of nanoprotozoa on culturable bacterial cells and/or from the growth of non-cultured cells (i.e., viable and active cells unable to grow on marine agar media) [37]. Although species and genera were not quantified by FISH at the community level, the high proportion of isolates hybridising with the *Sulfitobacter* probe and the high occurrence of these genera in both DNA and RNA-derived DGGE profiles [12] suggest that, at least, cells belonging to these genera were actively growing at the time of sampling and were highly represented in the culturable fraction during the initial growth phase.

A few isolates hybridising with the CFB probe were found in most mesocosms. Again, this was congruent with DGGE results [12] and suggest that the abundance of these cells was maintained relatively constant during all phases of the experiment. The occurrence of CFB cells was lower when determined at the community level but cells hybridising with this probe and detected at 144 h were very large suggesting that they may be able to escape grazing (see below).

These changes in demographic parameters were concomitant with the increasing mean cell size of the community and with an increasing grazing pressure. Different mechanisms may explain these changes in both morphological and structural parameters.

The proportion of cells within the fraction of large cells (>2.5 μm) increased throughout the experiment and at least the largest cells within this fraction may escape to grazers. Similar results have been reported by Jürgens et al [6] in freshwater ponds and by Pernthaler et al. [38] in an experimental system using protists with contrasting feeding modes. Different reports have suggested morphological changes into larger cells as a possible defence mechanism to escape grazers [38,39]. Nevertheless, these large cells can only result from the growth of nutrient-responsive cells and their resistance to grazing may be also independent of any defence mechanism since grazing edibility probably changes to grazing resistance with small changes in cell elongation [6]. It suggests that *ß*-Proteobacteria may be responsive to the added nutrients and become large enough so that phagotrophic protists preferentially crop these active cells whereas *ß*-Proteobacteria are less responsive and/or become much larger and inedible as already observed by Jürgens et al [6]. The occurrence of large *ß*-Proteobacteria isolated at the peak of grazing supports this hypothesis. *Ruegeria* species may belong to this group of species since the number of isolates hybridising with the corresponding probe increased after the grazing peak. This was also supported by DGGE analyses reported by Schäfer et al. [12], which showed that DGGE-bands corresponding to *Ruegeria* were especially dominant in the patterns corresponding to the post-grazing phase of the incubation. Alternatively, the increase in the proportion of *ß*-Proteobacteria can also be explained for some species by fast growing cells which can divide faster to eventually outgrow the predation pressure, or more likely, because predation creates conditions allowing for higher specific growth rates. The second hypothesis seems to be confirmed by the fact that higher specific thymidine incorporation rates occurred only after the strong decrease in total bacterial counts. In this experiment, species able to rapidly adapt their growth rate may belong to *ß*-Proteobacteria as already observed by others [36]. It should explain why they were dominant after the grazing peak and also why the thymidine vs. leucine ratio increased, suggesting that cells divided more quickly. *Sulfitobacter pontiacus* may belong to this category of species since the number of isolates hybridising with the specific probe increased rapidly suggesting that this species was very responsive to the added nutrients but decreased rapidly with increasing grazing pressure. These observations were confirmed by molecular analyses [12].

Similarly, cells hybridising with the CFB probe were present at very low densities but their proportion increased at time 144 h and some very large bacteria hybridised with this probe. It suggests that these species also develop filamentous forms, although they did not represent a signifi-
cant part of the whole community. It should be interesting to further understand to what extent these species contribute to the bacterial production. Similar large bacteria belonging to the CFB group have already been reported [6]. Hahn et al. [40] suggested that for some members of the CFB group, the formation of large cells is growth rate controlled and that this mechanism is a phylogenetically widely distributed defence strategy against grazing. Although the deterministic or stochastic nature of this mechanism remains unknown, it results in a strategy to preserve the diversity of bacterial species in natural marine systems.

The demographic pattern classification as defined through r and K strategies for higher organisms [41] have only rarely been applied to bacteria [42] but our results suggest that such strategies may exist within the bacterial world. An r strategist microbe should display rapid growth and dominate situations where resources are temporarily abundant (species which develop defence mechanisms such as an increasing growth rate). Whereas K strategists should be species with lower growth rates and less variations in densities and which play a key role in the maintenance of ecosystems. Thus, oligotrophic species are possible r strategists. Although further investigations are needed to confirm these hypotheses, results from this study suggest that S. pontiacus may develop an r strategy. In contrast, R. gelatinovorans displays a permanent large morphotype whereas R. algicola forms large cells by cellular elongation only under grazing pressure. Both species would represent K strategists.

### 4.2. Dynamics of bacterial activity

Gasol et al. [37] suggested that protozoa preferentially crop the active fraction of the bacterial community if they select their prey according to size, considering that activity is positively related to cell size. The decrease of bacterial production under increasing grazing pressure may sustain this hypothesis. However, alternative mechanisms have been proposed and Pernthaler et al. [4,43] suggested that: ‘bacteria can adopt at least two distinct strategies as a reaction to intense flagellate predation: to outgrow predation pressure or to develop inedible, inactive filaments’. The grazing-resistant species R. gelatinovorans isolated in this study displays a large morphotype (mean cell length 15.8 ± 7.2 μm) independently of the growing conditions since filaments were observed under a large range of growth rates (data not shown). Thus, this species does not develop a grazing-resistance mechanism, such as that suggested for R. algicola and the cells became detectable when edible bacteria were removed at high grazing rates. These cells probably grow slowly and contribute to the bacterial production. There is no clear evidence today that large bacteria are active or inactive cells. Further studies are needed to better understand the ecological role of these species. Ubiquitous marine species of the genera Ruegeria and Roseobacter have already been found in many marine coastal waters and along eutrophication gradients in the north Adriatic Sea [37,44,45].

It was already shown from our mesocosms that activity increases with cell size [46]. If we accept that large cells have a higher contribution to the bacterial production, the selection of large bacteria may result in the selection of active and productive cells which may explain the stability of bacterial production that was observed in our mesocosms after grazing. However, it is not known if the large bacteria grow permanently with these, with respect to grazing, advantageous morphological properties or if they express these characteristic features only under strong grazing pressure. If most of these large bacteria express these features only under grazing pressure, their selection may have little effect on the taxonomic structure and thus, on the functioning of natural communities.

### 4.3. Conclusions

Our results suggest that nutrients and grazing play a key role in shaping the morphologic, genotypic and phenotypic composition as well as the activity of bacterial communities by regulating abundances. Although the role of viruses, which also contributes to this regulation, was not considered in this study, it was shown that grazing plays a key role in the control of growing cells and thus, in the regulation of bacterial production. Although our data are insufficient to demonstrate a causal link between the shifts in community composition and activity, we hypothesise that substrate and grazers may drive bacterial population successions and consequently the variations in bacterial production. Paradoxically and inversely to higher organisms, the less active cells are more likely to survive than growing and productive cells which do not develop grazing-resistance mechanisms. As grazing may affect the production of cells with the highest growth rates, it may eliminate preferentially the most active cells within natural communities and regulate bacterial productivity, if we assume that size and activity are positively correlated. Therefore, grazing probably contributes to limit the dominance of species developing an r-strategy. Consequently, regulation processes have a significant effect on the metabolic activities of the whole communities but probably have a lower effect on the metabolic potential of these communities because the effect of grazing on bacterial genetic diversity is more limited. This limitation is due to the fact that grazing is density-dependent and also because some marine species are able to develop defence mechanisms such as rapid growth and/or induction of a size polymorphism. We hypothesise that species belonging to the genera Ruegeria, Cytophaga, Sulfitobacter and Flavobacterium may play a key ecological role along eutrophication gradients since they are ubiquitous species probably able to escape to the grazing pressure as it often occurs in such highly productive areas.
Studies that simultaneously measure changes in the phylogenetic composition and in the bulk activity and biochemistry of the bacterial community are just beginning to be done. New techniques such as cell sorting and flow cytometry to physically isolate the cells sharing similar activities or properties will be of great interest to further investigate the relationships between community composition and activity.

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