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# Shifts in cell size and community composition of bacterioplankton due to grazing by heterotrophic flagellates: evidence from a marine system

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**ABSTRACT:** The effect of flagellate grazing on bacterioplankton community composition, e.g. cell size and diversity, has been well studied in fresh waters, but much less is known for marine systems. We conducted experiments with communities from an oligotrophic bay in the NW Mediterranean Sea; size fractionation was used to alter grazing by flagellates, and incubation in dialysis bags was used to retain otherwise *in situ* conditions. Grazing reduced bacterial abundance and growth. In contrast to a current model of the effects of grazing, cell size distribution was unimodal and not bimodal. Significant shifts towards larger cell size classes were observed. This is in accordance with the idea that increasing the cell length is a bacterial strategy to reduce susceptibility to grazing by small flagellates. Only weak evidence was found for the complementary strategy of reducing cell size. In all experiments, bacterial community structure as assessed by PCR-based 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) showed significant differences associated with the presence of grazers. Sequence analyses of DGGE bands were used to select probes for catalyzed reporter deposition-fluorescence *in situ* hybridization analysis for specific taxa. In our experiments, *Polaribacter* showed patterns of defense against grazing, while *Roseobacter* was highly susceptible to grazing. Although the effect of grazing may be stronger in freshwater than in oligotrophic marine systems, our data support the hypotheses that (1) increasing the cell size is a bacterial defense strategy against grazing by small flagellates typical in marine systems and (2) grazing influences bacterial community composition.

**KEY WORDS:** Flagellates · Bacteria · Diversity · Life strategies · Grazing defense

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## 1. INTRODUCTION

General models of bacterioplankton dynamics usually include variable resistance against grazing (and viral lysis) as a mechanism partially explaining the diversity and production of bacterioplankton (Thingstad 2000, Šimek et al. 2002, Winter et al. 2010). These models suggest 2 basic survival strategies for producers such as bacterioplankton, i.e. become a competition specialist to acquire essential substrates or become a defense specialist to reduce mortality

due to grazing or viral lysis. An analysis of studies performed with freshwater and marine communities supports the major assumptions of these models (Winter et al. 2010).

Bacterivory has been previously considered as comparatively nonspecific, i.e. the grazing pressure would be evenly distributed among bacterial types or species. However, there is now ample evidence that protistan grazers (mainly heterotrophic flagellates) strongly influence the morphological as well as taxonomic diversity of bacteria (Šimek et al. 1999, Mas-

sana & Jürgens 2003), i.e. they can affect bacterial species in different ways. The interactions between grazers and their prey are manifold. Some protistan species are generalists in that they can consume a large range of prey sizes, and others are specialists feeding on prey with very small size ranges (Pernthaler 2005). The resistance of bacteria against grazing in freshwater systems has been explained by various mechanisms such as motility, cell surface chemistry, toxicity, cell size or cell aggregation (see also Jürgens & Güde 1994, Jürgens et al. 1999, Hahn & Höfle 2001). A common model suggests that grazing induces a bimodal distribution of bacterial cell sizes in a community with a peak of very small cells and a peak of very large cells, which are both in the size range less vulnerable to grazers, hence called grazing-resistant cell sizes or morphotypes (Pernthaler 2005). It is now also well documented that there is a large variety of mechanisms involved in selective feeding by protists (Montagnes et al. 2008) and conversely that sudden shifts in the bacterial prey community can induce significant changes in a grazer flagellate community (Šimek et al. 2013, Grujić et al. 2015, 2018).

In marine systems, the diversity of flagellates is comparatively well studied (Massana et al. 2009); however, more studies are needed on bacterial mechanisms of resistance against grazing (González et al. 1993, Havskum & Hansen 1997, Engström-Öst et al. 2002, Massana & Jürgens 2003, Baltar et al. 2016). There is evidence that flagellates graze preferentially on medium-sized and more active fractions of bacteria (González et al. 1990, Gasol et al. 1995, del Giorgio et al. 1996). However, there also appears to be a large-sized refuge because with regard to *Synechococcus*, flagellate grazing is minimal when the *Synechococcus* population is dominated by large dividing cells (Dolan & Šimek 1999) in marine environments with generally very small (2–3 µm) flagellate predators (e.g. Christaki et al. 2001, Tanaka et al. 2003). Also, it has been shown that cell size is related to phylogeny in marine waters (Straza et al. 2009, Yooseph et al. 2010). While several studies suggest that bacterial diversity can be influenced by protistan grazing in marine systems (e.g. Suzuki 1999, Yokokawa & Nagata 2005, Zhang et al. 2007, Bonilla-Findji et al. 2009), it seems that the effect of grazing on the size and community structure could be a less prominent phenomenon in marine than in freshwater systems (Massana & Jürgens 2003, Baltar et al. 2016). Overall, the mechanisms of bacterial adaptation to predation are poorly understood in marine systems (Strom 2000).

To study the potential effect of grazing on bacterial cell size and diversity, we used a well-tested combined filtration dialysis bag approach (e.g. Šimek et al. 2001) in an oligotrophic coastal environment in the NW Mediterranean Sea. This approach consists of (1) a <3 µm fraction (enhanced bacterivory) promoting flagellate growth and consequently also grazing of bacteria by removing grazers of flagellates such as ciliates or filter-feeding zooplankton and (2) a <0.8 µm fraction (without grazing). In addition, dialysis bags allow for the exchange of inorganic nutrients and dissolved organic matter, thus minimizing resource limitation. Three experiments were performed to examine grazing effects on bacterial communities differing in community structure and biomass. In our experiments, we found that the presence of grazers (1) changed bacterial community composition (as assessed by PCR-based 16S rRNA gene DGGE analysis), (2) stimulated *Polaribacter* and repressed *Roseobacter* (as detected by catalyzed reporter deposition-fluorescence *in situ* hybridization [CARD-FISH] analysis) and (3) induced an increase in cell size as a potential strategy to reduce the grazing impact of very small heterotrophic nanoflagellates (HNF) known to dominate marine systems (Christaki et al. 2001, Unrein et al. 2007, Massana et al. 2009).

## 2. MATERIALS AND METHODS

### 2.1. Study site and experimental design

The study was conducted in 2011 and 2012 in the Bay of Villefranche located in the NW Mediterranean Sea. The bay is influenced by the Liguro-Provençal Current, a cyclonic current running from east to west along the French Riviera (Bonilla-Findji et al. 2010). Under prevailing conditions, this current enters the bay, which results in continuous water renewal. There is no pronounced continental shelf in this steeply descending bay. Water stratification begins typically in March, is strongest in summer and starts to erode in fall. The Bay of Villefranche is considered an oligotrophic coastal system with maximum chl *a* concentrations typically less than 1 µg l<sup>-1</sup> (Sheldon et al. 1992).

Water samples for experiments were collected from ca. 0.5 m water depth with a Niskin bottle close to the pier (*Posidonia* seagrass meadow; water depth: ca. 2–3 m) and at a permanent coastal station (Point B: 43°51'10"N, 07°19'00"E; bottom depth: >90 m; >1 m off the pier) at the entrance of the bay. A final volume

of 12 l was carried in a 15 l plastic container to the laboratory for further processing and size fractionation. Three experiments were performed: the first from 20 to 24 September 2011 with water only from Point B (Expt PtB-2011) and the second and third from 22 to 26 May 2012 with water collected from both Point B (Expt PtB-2012) and the pier (Expt Pier-2012).

The experimental design and protocols were similar to those described in detail by Šimek et al. (2001) using 2 size fractions of water: with and without flagellate grazers. The fraction with grazers was obtained via filtration through 3.0  $\mu\text{m}$  pore size filters, which removed the predators of bacterivorous HNF such as ciliates and filter-feeding zooplankton, thus supposedly allowing rapid HNF growth (enhanced bacterivory), and the grazer-free fraction was obtained via filtration through 0.8  $\mu\text{m}$  pore size filters, which removed all bacterivorous protists (without grazing). Water samples (ca. 5 l) were filtered by gravity filtration through 3  $\mu\text{m}$  (diameter: 142 mm; Millipore, Isopore<sup>TM</sup> membrane filter TSTP). Half of the filtrate was then filtered through 0.8  $\mu\text{m}$  pore size polycarbonate filters (diameter: 142 mm; Millipore, Isopore<sup>TM</sup> membrane filter ATTP) by applying only low vacuum. The 0.8  $\mu\text{m}$  filtration step was conducted in heat-sterilized steel Millipore filter holders to minimize the possibility of HNF contamination of the grazer-free fraction. All tubes and containers used were acid cleaned (0.1 N HCl) and rinsed with Milli-Q and sample water.

The different water fractions were placed into 700 ml pretreated (rinsed 3 times with 60°C distilled water for 15 min) dialysis tubes (diameter: 76 mm; 12–14 kD molecular weight cutoff; Spectra/Por 2, Spectrum Laboratories) closed with specific clips (Spectra/Por, Spectrum Laboratories), sealing the water enclosed and thus avoiding a possibility of contamination of different fractions by microbes from surrounding water. Notably, while filling the tubes, they were kept partially flat (without any air bubbles); this allowed them to be closed with additional clips into smaller separated compartments of the bag that were released as a subsample without direct contact with a major volume of the water sample incubated in the tubes. The dialysis bags were incubated at the pier in triplicate. Temperature was kept at *in situ* values using a flowing seawater incubator, and the dialysis bags were incubated in the dark to minimize the effects of autotrophic population growth. Approximately 80 ml compartments of the bags were separated by a clip just prior to taking a subsample for cell counts from each dialysis bag at time point (T) 0, T24, T48, T72 and T96 h. Samples for CARD-FISH and

genetic fingerprints were taken from the different size fractions before filling the dialysis bags and at T96 h from all replicates. Thus, less than half of the incubation volume was removed for time course samples during the incubation.

## 2.2. Sizing and enumeration of microorganisms

Samples for counting and cell sizing of bacteria were fixed with formaldehyde (2% final concentration, v/v), stained with DAPI (final concentration 0.1 mg ml<sup>-1</sup>, w/v) and inspected using an epifluorescence microscope (Olympus AX 70). For each sample, images with 300 to 450 DAPI-stained bacterial cells were recorded for sizing and cell dimension processed with semiautomatic image analysis systems (NIS-Elements 3.0, Laboratory Imaging) as described previously (Posch et al. 1997, Šimek et al. 2013). Bacterial biomass was calculated from the allometric relationship between cell volume and carbon content as described by Norland (1993).

For the enumeration of total bacterial abundance by flow cytometry, water samples were preserved with glutaraldehyde (0.5% final concentration) for 30 min at 4°C and then flash frozen in liquid nitrogen and stored at -80°C until analysis. Bacteria were stained with SYBRGreen I and counted by flow cytometry (FACSCalibur, Becton & Dickinson) using the protocol of Gasol & del Giorgio (2000) for bacteria and Brussaard et al. (2004) for viruses. *Synechococcus*, *Prochlorococcus*, and autotrophic picoeukaryotes and nanoeukaryotes were counted as described previously (Jacquet et al. 2006).

For HNF counting, 50 ml samples were fixed (formaldehyde, 2% final concentration, v/v), 10–20 ml subsamples were stained with DAPI and filtered through 0.8  $\mu\text{m}$  pore size black polycarbonate filters (Osmonics), and HNF abundance was determined via epifluorescence microscopy as described in detail previously (Šimek et al. 2001).

## 2.3. CARD-FISH

Group-specific oligonucleotide probes (Thermo-Hybrid) and the CARD-FISH protocol (Pernthaler et al. 2002) were used based on sequence analysis of genetic fingerprints, which showed consistent patterns across experiments (see Section 3). The following oligonucleotide probes were selected to track time course changes of specific groups (genera) of bacteria: *Glaciecola* (probe GPU622), *Roseobacter*

(probe ROSEO536) and *Polaribacter* (probe PB223) (Brinkmeyer et al. 2000). Proportions (% of total bacteria) of FISH-positive bacteria were counted by epifluorescence microscopy and derived from inspection of >1000 DAPI-stained bacterial cells.

#### 2.4. DNA extraction and PCR

Cells from 0.5 l samples were collected on 0.2 µm pore size polycarbonate filters (diameter: 0.47 mm; Whatman), and filters were stored at –80°C until analysis. DNA was extracted from the filters and purified as described elsewhere using the HEATSALT protocol (Hansson et al. 2009). Briefly, the protocol combines a heat and salt lysis with a simultaneous salt extraction step followed by further purification steps.

Conditions of the touchdown PCR and chemicals were as described in Schäfer & Muyzer (2001). Cleaned nucleic acid extract (1–4 µl) was used as a template in a 50 µl PCR reaction (1.5 mM MgCl<sub>2</sub>, 0.25 µM of each primer and 2.5 U *Taq* polymerase; Sigma; no. D5930) together with a positive and a negative control. A fragment of the 16S rRNA gene was amplified using the primer pairs 341F-GC and 907RM for *Bacteria* (Schäfer & Muyzer 2001). The bacterial primers had the modifications suggested to also detect *Gammaproteobacteria* (Sánchez et al. 2007).

#### 2.5. DGGE and sequencing

DGGE procedures followed those described by Schäfer & Muyzer (2001). PCR products (500 ng) were separated into bands by electrophoresis for 18 h at 100 V on acrylamide and bis-acrylamide (6%) gels prepared using a gradient of 30 to 70% (urea and formamide) using an INGENYphorU DNA mutation detection system (Ingeny International). DGGE gels were photographed with a gel documentation system (GelDoc EQ; Bio-Rad) after 15 min staining with a 10X SYBR Gold solution (Molecular Probes; no. S11494). Analysis of band patterns between lanes was performed with Quantity One software (Bio-Rad) using a variety of exposure times. Band intensities were recorded using a rolling circle correction for background staining. All samples from a specific experiment were loaded on the same gel.

Selected bands were excised. The DNA was eluted overnight in autoclaved Milli-Q water at 4°C, checked for purity by re-running it on a gel and sequenced (MWG-Biotech). Sequences were edited using the

freeware program 4Peaks 1.6 (A. Griekspoor & T. Groothuis, mekentosj.com). More than 500 bp of the 16S rRNA gene were used in a BLAST search.

#### 2.6. Statistical analyses

Data comparison between treatments and time points was done using the Student's *t*-test. Across experiments, a paired *t*-test was used. Data were log or arcsine transformed to satisfy the requirements of normality and homogeneity of variance necessary for parametric statistics;  $p < 0.05$  was considered significant. Bacterial (apparent) growth rates ( $\mu$ ) were calculated from the first 3 to 4 time points by applying linear regression after ln transformation of data with the slope as the growth rate. Because of the strong increase of HNF abundance towards the end of the experiments, the growth rate of HNF was calculated from the last 2 time points.

Statistical analysis of gels was carried out using R (R Project for Statistical Computing, R 2.6.1). Statistical analysis of band patterns was performed using the relative band intensity within a lane as well as a binary matrix of presence vs. absence data. Cluster diagrams were created using the UPGMA. The Jaccard similarity coefficient was used for binary data (presence vs. absence of bands), whereas the Bray-Curtis similarity was used when the relative intensity of each band was also taken into account. ANOSIMs were also performed using the R package Vegan. The program returns an R-value between –1 and +1 and the significance level;  $p < 0.05$  was considered significant. The generated R-value indicates the differences between DGGE profiles, and a value of zero represents the null hypothesis  $H_0$  (i.e. there is no difference between groups).

### 3. RESULTS

#### 3.1. *In situ* conditions

Two experiments were performed with water collected at Point B at the entrance of the oligotrophic Bay of Villefranche in September 2011 (hereafter PtB-2011) and May 2012 (hereafter PtB-2012). In May 2012, an additional experiment was performed with water collected at the pier of the institute (hereafter Pier-2012). Conditions were different among the experiments. The water temperature was 22.8°C in 2011 and 16.7°C in 2012 (Table 1). Nutrient data suggested a phosphorus and perhaps also nitrogen

Table 1. Physicochemical and autotrophy related parameters of water from the Bay of Villefranche used in experiments. Chemical data are given in  $\mu\text{M}$ . *T*: temperature; *Synech*: *Synechococcus* ( $10^4 \text{ ml}^{-1}$ ); *Prochl*: *Prochlorococcus* ( $10^4 \text{ ml}^{-1}$ ); *PicoEuk*: autotrophic picoeukaryotes ( $\text{ml}^{-1}$ ); *NanoEuk*: autotrophic nanoeukaryotes ( $\text{ml}^{-1}$ ); PtB: Point B; <DL: below detection limit; ND: not determined

Expt	<i>T</i> (°C)	Salinity	$\text{NH}_4$	$\text{NO}_3$	$\text{NO}_2$	$\text{PO}_4$	Chl <i>a</i>	<i>Synech</i>	<i>Prochl</i>	<i>PicoEuk</i>	<i>NanoEuk</i>
PtB-2011	22.8	38.21	0.02	0.13	0.02	<DL	0.09	1.72	4.7	858	31
PtB-2012	16.7	37.99	0.23	0.18	0.04	0.08	0.17	0.89	<DL	875	144
Pier-2012	16.9	28.12	ND	ND	ND	ND	ND	0.78	<DL	1050	78

co-limitation in September 2011. Chl *a* concentrations were  $0.09 \mu\text{g l}^{-1}$  in 2011 compared to  $0.17 \mu\text{g l}^{-1}$  in 2012. The highest abundances of *Synechococcus*, *Prochlorococcus* and heterotrophic bacteria were found in 2011, whereas the highest abundances of picoeukaryotes, nanoeukaryotes, HNF and viruses were found in 2012. Slight differences were also detected in water taken from the station Point B and the pier in 2012, which were collected on the same day (Table 2).

At all sampling stations, bacteria were characterized by a unimodal distribution of classes of cell lengths (Fig. 1). The dominant size class was  $>0.4\text{--}0.8 \mu\text{m}$  cell lengths ( $>60\%$  of total abundance), followed by the size class  $>0.8\text{--}2 \mu\text{m}$  (ca. 20%). The contribution of bacteria with a cell length  $>2 \mu\text{m}$  (representing a potentially grazing-resistant morphology) ranged from 1.5 to 6.1% of total abundance, corresponding to 7.9–24.5% of total bacterial carbon. The different bacterial genera *Glaciecola*, *Polaribacter* and *Roseobacter* as detected by CARD-FISH ranged from ca. 1 to 5% in the premanipulation communities (Table 2). At PtB-2011 and Pier-2012, 15 DGGE bands were detected, compared to 13 DGGE bands at PtB-2012 (Table 2). Of the potentially 25 different bands (as identified from 2 gels), 20% were common to all 3 sampling occasions, 40% occurred in 2012, 20% occurred at PtB-2012 and 20% occurred at PtB-2011 and Pier-2012. UPGMA dendrograms showed that bacterial community composition differed among 3 communities and that the 2012 communities were

more similar to each other than to the 2011 community (Fig. 2).

### 3.2. Microbial abundance and growth in experiments

Across experiments, filtration through  $0.8 \mu\text{m}$  resulted in a moderate reduction of bacterial abundance compared to  $3 \mu\text{m}$  filtered or unfiltered samples ( $14 \pm 3.5\%$ ); bacterial abundance was not affected by  $3 \mu\text{m}$  filtration. In the  $0.8 \mu\text{m}$  fraction, no protists were detected and no growth of flagellates was observed during the experiments (hence the treatment term ‘without grazing’). Flagellate abundance was reduced by  $13.9 \pm 4.6\%$  due to filtration through  $3 \mu\text{m}$  filters compared to unfiltered water. In the  $3 \mu\text{m}$  treatments, no ciliates were observed at T0 h and during the time course of the experiments. In this treatment, flagellate abundance increased moderately during the first 72 h and showed a strong increase between T72 and T96 h (Fig. 3) (hence the treatment term ‘with enhanced bacterivory’). Flagellate growth was highest in Expt PtB-2011 and lowest in Expt Pier-2012 (Table 3).

Bacterial abundance increased in all treatments (Fig. 3). At the end of all the experiments, the bacteria in the treatment without grazing appeared to be about to enter the stationary growth phase, whereas in the grazing treatment, bacterial abundance was decreasing, with the most dramatic drops observed

Table 2. Heterotrophic parameters of water from the Bay of Villefranche used in the experiments. Bacterial genera are given as percentage of total counts. Bacterial cells  $>2 \mu\text{m}$  are considered grazing resistant. #bands: number of DGGE bands; HNF: heterotrophic nanoflagellates; PtB: Point B

Expt	Bacteria	Bacteria $>2 \mu\text{m}$ (%)	C content bacteria $>2 \mu\text{m}$ (%)	#bands	<i>Glaciecola</i>	<i>Polaribacter</i>	<i>Roseobacter</i>	HNF
PtB-2011	7.4	6.1	24.5	15	2.7	4.7	2.8	0.21
PtB-2012	6.2	1.5	7.9	13	2.2	0.7	4.5	0.32
Pier-2012	5.9	4.8	12.1	15	3.8	1.5	3.6	0.44

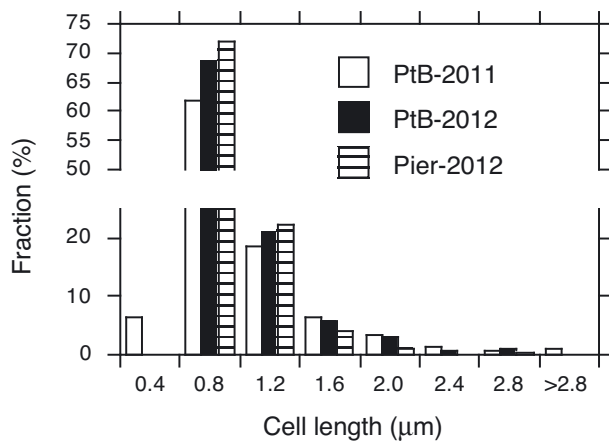


Fig. 1. Size distribution of bacteria in water samples from the Bay of Villefranche used for the experiments: Point B (PtB)-2011, PtB-2012 and Pier-2012

in PtB-2011 and PtB-2012. In all experiments, bacterial abundance was significantly lower in the treatment with enhanced bacterivory than without grazing at T96 h (*t*-test,  $p < 0.05$ ). Bacterial apparent growth rates did not differ in a consistent way between treatments across experiments and were highest in Expt PtB-2012 and lowest in Expt PtB-2011 (Table 3). In Expt Pier-2012, bacterial apparent growth rate was significantly higher in the treatment with enhanced bacterivory than without grazing.

### 3.3. Cell size structure of bacteria in experiments

At the start of the experiments, the average volume and length (and consequently calculated carbon content of cells) did not differ significantly between treatments (across experiments, paired *t*-test,  $p > 0.1$ ; see also Fig. 2). In Expt PtB-2011, the volume, length and carbon content of cells increased with time in both treatments and was significantly higher at T96 h than at T0 h (Table 4). In addition, these parameters were 2.0-, 1.4- and 1.7-fold, respectively, higher in the treatment with enhanced bacterivory than without grazing; the differences were significant (*t*-test,  $p < 0.05$  for all parameters). In the other experiments, there was no significant difference for these parameters between T0 and T96 h or between treatments at T96 h (*t*-test,  $p > 0.05$ ).

To analyze the bacterial cell size structure, we combined cell length data from the triplicates parsed into 8 size classes. In the beginning of all 3 experiments, there were only minor differences between the 2 treatments. The size class 0.4–<0.8  $\mu\text{m}$  was dominant at the beginning of the experiment (data

not shown) and remained dominant at the end of all experiments in all treatments (Fig. 4). However, there were significant treatment effects for specific size classes in each experiment (see below).

In all experiments, there was a shift in the size class distribution due to the presence of flagellates (Fig. 4). Specifically, in Expts PtB-2012 and Pier-2012, the relative abundance of the smallest size class (<0.4  $\mu\text{m}$ ) was significantly higher in the treatment with enhanced bacterivory than without grazing (*t*-test,  $p < 0.05$ ; Fig. 4B,C). In Expts PtB-2011 and Pier-2012, the larger size class 0.8–<1.2  $\mu\text{m}$  was significantly greater in the treatment with enhanced bacterivory (*t*-test,  $p < 0.05$ ; Fig. 4A,C). For larger size classes, between 3 and 4 size classes were significantly greater in the treatment with enhanced bacterivory than without grazing, and none of these size classes exhibited the reverse trend. In contrast, the size class 0.4–<0.8  $\mu\text{m}$  was significantly greater in the treatment without grazing in Expts PtB-2012 and Pier-2012. Despite the tendency of a shift towards very small and large cells induced by the presence of grazers, the cell distribution remained roughly unimodal for bacterial cell length (Fig. 4), biovolume and bacterial carbon content (data not shown).

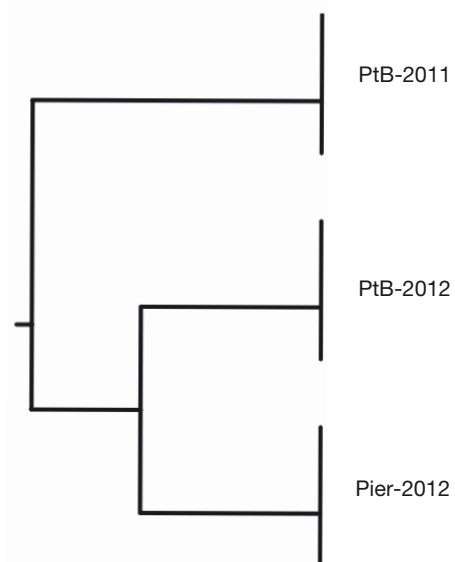


Fig. 2. Bacterial community composition for the 3 experiments: Point B (PtB)-2011, PtB-2012 and Pier-2012. Data from DGGE analysis were used to calculate UPGMA cluster dendrograms (presence vs. absence of bands). Per sampling, a non-prefiltered, a 3  $\mu\text{m}$  prefiltered and a 0.8  $\mu\text{m}$  prefiltered sample were used. These 3 samples showed an identical presence–absence pattern. Maximum dissimilarity was 19%. Note that clustering using relative band intensities yielded the same pattern with slight differences between filter fractions

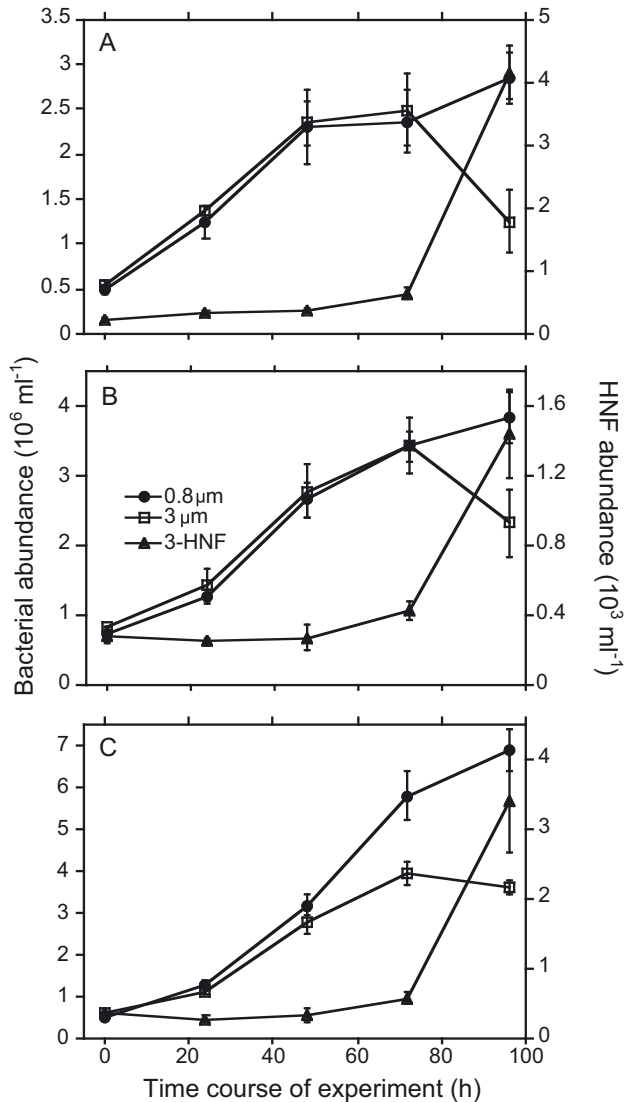


Fig. 3. Time course changes of bacterial and heterotrophic nanoflagellate (HNF) abundance in treatments with enhanced bacterivory (3  $\mu\text{m}$  prefiltration) and without grazing (0.8  $\mu\text{m}$  prefiltration). (A) Expt Point B (PtB)-2011; (B) Expt PtB-2012; (C) Expt Pier-2012. Data are means  $\pm$  SD of triplicate incubations

### 3.4. Bacterial community composition and sequencing in experiments

In initial waters from all the treatments (including unfiltered samples, see also Fig. 1), there was no difference in the presence-absence pattern of DGGE bands. Also, the UPGMA dendrograms based on band intensity showed only small differences between treatments at T0 h (<5% Bray-Curtis similarity).

At T96 h, UPGMA dendrograms, based on band intensity, showed that the 2 treatments grouped into 2 separate clusters in all experiments (Bray-Curtis

dissimilarity, 13% for Expt PtB-2011, 10% for Expt PtB-2012, 19% for Expt Pier-2012) (Fig. 5). ANOSIM showed that this grouping was statistically significant in all experiments ( $p < 0.05$ ). Considering presence vs. absence patterns and relative band intensities ( $t$ -tests,  $p < 0.05$ ), 1 band was stimulated in the treatment with enhanced bacterivory compared to without grazing (uncultured *Alteromonas/Glaciecola*)

Table 3. Apparent growth rates of bacteria and heterotrophic nanoflagellates (HNF) in the experiments. Data are means  $\pm$  SD of triplicate incubations. A  $t$ -test was used to assess differences in bacterial growth rates between treatments. PtB: Point B; NA: not applicable; NS: not significant

Expt/ Treatment	Growth rate ( $\text{d}^{-1}$ )	
	Bacteria	HNF
<b>PtB-2011</b>		
0.8 $\mu\text{m}$	$0.79 \pm 0.084$	NA
3 $\mu\text{m}$	$0.72 \pm 0.049$	$1.90 \pm 0.338$
Significance	NS	NA
<b>PtB-2012</b>		
0.8 $\mu\text{m}$	$0.91 \pm 0.015$	NA
3 $\mu\text{m}$	$0.87 \pm 0.011$	$1.21 \pm 0.314$
Significance	$p < 0.05$	NA
<b>Pier-2012</b>		
0.8 $\mu\text{m}$	$0.91 \pm 0.029$	NA
3 $\mu\text{m}$	$1.21 \pm 0.024$	$1.77 \pm 0.314$
Significance	$p < 0.05$	NA

Table 4. Volume, length and carbon content of cells in the experiments. Data are means  $\pm$  SD of triplicate or 6 incubations. A  $t$ -test was used to assess differences in bacterial growth rates between treatments. PtB: Point B; T: time point; NS: not significant

Expt/ Treatment	Cell volume ( $\mu\text{m}^3$ )	Cell length ( $\mu\text{m}^3$ )	Cell carbon (fg)
<b>PtB-2011</b>			
T0 h	$0.067 \pm 0.0066$	$0.70 \pm 0.023$	$16.1 \pm 0.49$
T96 h: 0.8 $\mu\text{m}$	$0.129 \pm 0.0373$	$0.90 \pm 0.045$	$24.6 \pm 4.19$
T96 h: 3 $\mu\text{m}$	$0.257 \pm 0.0560$	$1.24 \pm 0.084$	$41.1 \pm 1.84$
Significance (0.8 vs. 3 $\mu\text{m}$ )	$p < 0.05$	$p < 0.05$	$p < 0.05$
<b>PtB-2012</b>			
T0 h	$0.064 \pm 0.0103$	$0.74 \pm 0.068$	$15.3 \pm 1.87$
T96 h: 0.8 $\mu\text{m}$	$0.062 \pm 0.0081$	$0.72 \pm 0.024$	$15.4 \pm 1.47$
T96 h: 3 $\mu\text{m}$	$0.064 \pm 0.0048$	$0.72 \pm 0.004$	$16.6 \pm 0.74$
Significance (0.8 vs. 3 $\mu\text{m}$ )	NS	NS	NS
<b>Pier-2012</b>			
T0 h	$0.062 \pm 0.004$	$0.72 \pm 0.035$	$15.2 \pm 0.75$
T96 h: 0.8 $\mu\text{m}$	$0.060 \pm 0.006$	$0.71 \pm 0.048$	$14.9 \pm 0.83$
T96 h: 3 $\mu\text{m}$	$0.053 \pm 0.007$	$0.66 \pm 0.034$	$13.7 \pm 1.47$
Significance (0.8 vs. 3 $\mu\text{m}$ )	NS	NS	NS

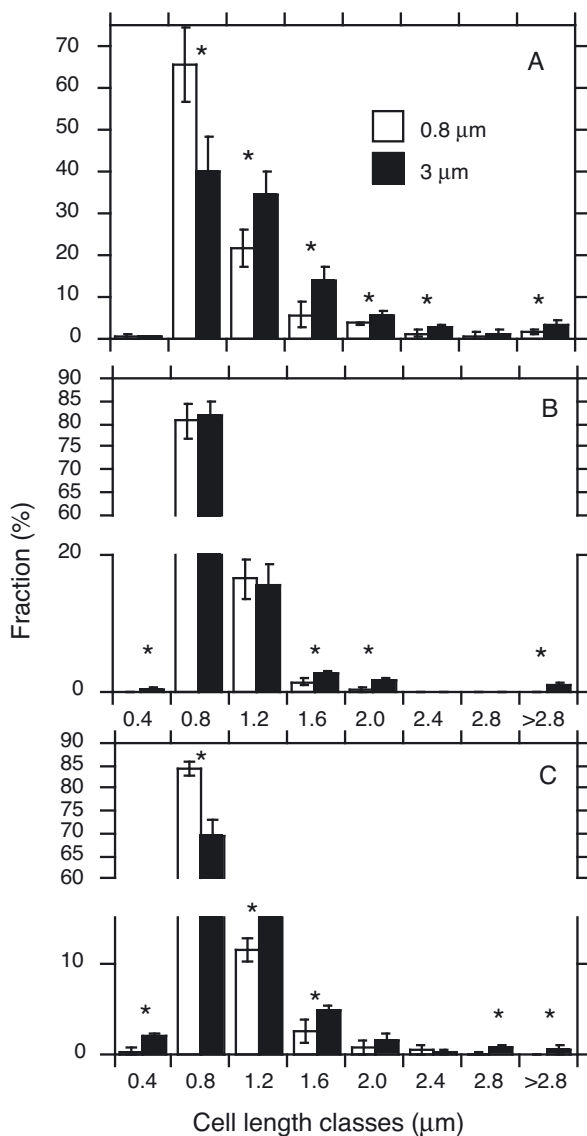


Fig. 4. Size distribution of bacteria at the end of the experiments in treatments with enhanced bacterivory (3  $\mu\text{m}$  pre-filtration) and without grazing (0.8  $\mu\text{m}$  pre-filtration). (A) Expt Point B (PtB)-2011; (B) Expt PtB-2012; (C) Expt Pier-2012. Data are means  $\pm$  SD of triplicate incubations. \* $p < 0.05$  ( $t$ -test)

and 1 band was repressed in the treatment without grazing compared to with enhanced bacterivory (*Flavobacterium* sp. clone) in Expt PtB-2011 (Table 5). In Expt PtB-2012, 3 bands were stimulated in the treatment with enhanced bacterivory compared to without grazing (*Polaribacter*, *Vibrio gigantis*/*V. crassostrea*), and 2 bands were stimulated in the treatment without grazing compared to with enhanced bacterivory (uncultured *Glaciecola*) ( $t$ -tests,  $p < 0.05$ ; Table 5). In Expt Pier-2012, 1 band was stimulated in the treatment with enhanced bacterivory compared to without grazing (*V. gigantis*, *V. crass-*

*ostrea*), and 5 bands were stimulated in the treatment without grazing compared to with enhanced bacterivory (uncultured *Glaciecola*, uncultured *Roseobacter*, uncultured *Alteromonas/Glaciecola*) ( $t$ -tests,  $p < 0.05$ ; Table 5). Overall, 7% of the bands differed between the treatments in Expt PtB-2011 compared to 23% in Expt PtB-2012 and 26% in Expt Pier-2012. The sequence analysis was used in addition to previous work (Malits et al. 2009, Cattaneo et al. 2010) for selecting CARD-FISH probes.

### 3.5. CARD-FISH analysis of communities in experiments

Filtration had only slight and inconsistent effects on the relative abundance of bacterial genera at the start of the experiment (data not shown). Together with an increase in total bacterial abundance, the relative abundance of all the targeted bacterial genera increased in all experiments. The apparent growth rates varied strongly among experiments and ranged from 0.1 to 3.3  $\text{d}^{-1}$  for *Glaciecola*, 0.1 to 7.6  $\text{d}^{-1}$  for *Polaribacter* and 7.4 to 10.3  $\text{d}^{-1}$  for *Roseobacter* in incubations without grazers (Table 6). While the relative abundance of *Glaciecola* and *Polaribacter* remained below 5% at the end of the experiments, the relative abundance of *Roseobacter* was  $>7\%$  (Fig. 6). At the end of the experiment, the relative abundance of *Glaciecola* was either not significantly different (PtB-2011 and PtB-2012) between the 2 treatments ( $t$ -test,  $p > 0.05$ ) or significantly higher in the treatment with enhanced bacterivory (Pier-2012) ( $t$ -test,  $p < 0.05$ ). The relative abundance of *Polaribacter* was in all experiments significantly higher in the treatment with enhanced bacterivory than without grazing ( $t$ -test,  $p < 0.05$ ). The relative abundance of *Roseobacter* was lower in the treatment with enhanced bacterivory than without grazing, and this difference was significant in 2 experiments (PtB-2011 and Pier-2012) ( $t$ -test,  $p < 0.05$ ) as well as across experiments (paired  $t$ -test,  $p < 0.05$ ).

## 4. DISCUSSION

### 4.1. Relevance of *in situ* data for experimental conditions

Different communities were encountered in the 3 experiments in terms of both abundance and community composition (i.e. DGGE data), with the

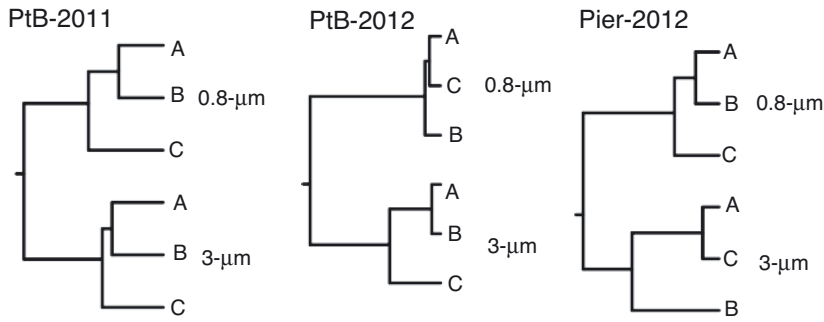


Fig. 5. Bacterial community composition at the end of the experiments in treatments with enhanced bacterivory (3 µm prefiltration) and without grazing (0.8 µm prefiltration). Data from DGGE analysis were used to calculate UPGMA cluster dendrograms (including relative band intensity). Three replicates per treatment (A, B and C) were used for the analysis. The Bray-Curtis dissimilarity between treatments was 13% for Expt Point B (PtB)-2011, 10% for Expt PtB-2012 and 19% for Expt Pier-2012

strongest differences found between 2011 and 2012 (Tables 1 & 2). Thus, an important aim of this study was achieved, i.e. that the 3 experiments were performed with communities differing in initial community structure and biomass. The experimental treatment employed to examine grazing effects did not result in abnormally high HNF abundances. The highest HNF abundances found in the 3 experiments ( $1.4\text{--}4.0 \times 10^3$ ) were well within the range of seasonal variations of abundances found *in situ* in the Bay of Villefranche ( $<10 \times 10^3$  cells ml<sup>-1</sup>) (e.g. Mostajir et al. 1995). Thus, the experimental abundances were realistic for *in situ* conditions and suggest typical grazing pressure.

#### 4.2. Effect of grazing on bacterial biomass and growth

In all experiments, bacterial abundance was significantly lower in the treatment with enhanced bacterivory than without grazing at T96 h, and HNF abundance increased strongly in all experiments in the 3 µm treatment from T72 to T96 h. This could be due to a lack of microzooplankton such as ciliates removed by the 3 µm prefiltration. Together, these data suggest that towards the end of the experiment, grazing by HNF was exerting a significant effect on the bacterioplankton communities.

The decrease in bacterial abundance in the treatment with enhanced bacterivory was strongest in Expt PtB-2011 where HNF abundance and estimated HNF growth rate were highest. Thus, the grazing effect was likely strongest in Expt PtB-2011. The growth rates of HNF exceeded bacterial growth rates by 2.5-fold in Expt PtB-2011 and by 1.9-fold in Expt Pier-2012, corresponding with marked decreases in bacterial abundance in the treatments. In Expt Pier-2012, HNF growth rate was only 1.3-fold higher than bacterial growth rate, and the bacteria reached highest abundances among the 3 experiments. This suggests a lower grazing control. Presumably, a longer incubation would have ended with a more marked

Table 5. Effect of presence vs. absence of flagellates on DGGE bands and phylogenetic affiliation of sequences from DGGE bands with closest uncultured and cultured matches. Data are from the end of the experiment. Band ID also corresponds to the labeling of the experiments: PB11: Point B (PtB)-2011; PB12: PtB-2012; Pier12: Pier-2012). Treatment effects (stimulation): +F: with heterotrophic nanoflagellates (HNF); -F: without HNF; I: based on band intensity; PA: based on presence vs. absence of bands. Number of bases used to calculate sequence similarity is given in parenthesis in the fourth column

Band ID	Treatment effect	Closest match (environmental or culture)	Sequence similarity in % (number of bases)	Taxonomic group
PB11-3	-F (PA)	<i>Bacteroidetes</i> bacterium, <i>Flavobacterium</i> sp. clone	100 (539)	<i>Bacteroidetes</i>
PB11-12	+F (I)	Uncultured <i>Alteromonas</i> , <i>Glaciecola</i> <sup>a</sup>	89 (561)	<i>Gammaproteobacteria</i>
PB12-6	+F (PA)	<i>Polaribacter</i> sp. DOT-118-2	100 (556)	<i>Bacteroidetes</i>
PB12-12	-F (I)	Uncultured <i>Glaciecola</i>	100 (567)	<i>Gammaproteobacteria</i>
PB12-16	-F (I)	Uncultured <i>Glaciecola</i>	100 (567)	<i>Gammaproteobacteria</i>
PB12-20	+F (I)	<i>Vibrio gigantis</i> sp., <i>V. crassostrea</i> sp.	99 (564)	<i>Gammaproteobacteria</i>
PB12-21	+F (I)	<i>Vibrio gigantis</i> sp., <i>V. crassostrea</i> sp.	100 (564)	<i>Gammaproteobacteria</i>
Pier12-13	-F (I)	Uncultured <i>Glaciecola</i>	100 (566)	<i>Gammaproteobacteria</i>
Pier12-17	-F (I)	Uncultured <i>Glaciecola</i>	100 (568)	<i>Gammaproteobacteria</i>
Pier12-18	-F (I)	Uncultured <i>Roseobacter</i> <sup>a</sup>	89 (562)	<i>Alphaproteobacteria</i>
Pier12-19	-F (I)	Uncultured <i>Alteromonas</i> , <i>Glaciecola</i>	100 (566)	<i>Gammaproteobacteria</i>
Pier12-20	+F (PA)	<i>Vibrio crassostrea</i> sp., <i>V. gigantis</i> sp.	100 (557)	<i>Gammaproteobacteria</i>
Pier12-21	-F (I)	Uncultured <i>Alteromonas</i> , <i>Glaciecola</i>	100 (567)	<i>Gammaproteobacteria</i>

<sup>a</sup>Bad sequence

Table 6. Apparent growth rates of catalyzed reporter deposition-fluorescence *in situ* hybridization populations in the experiments. Data are means  $\pm$  SD of triplicate incubations. A *t*-test was used to assess differences in bacterial growth rates between treatments. PtB: Point B; NS: not significant

Expt/ Treatment	Growth rate ( $d^{-1}$ )		
	<i>Glaciecola</i>	<i>Polaribacter</i>	<i>Roseobacter</i>
<b>PtB-2011</b>			
0.8 $\mu$ m	0.1 $\pm$ 0.26	0.1 $\pm$ 0.06	9.4 $\pm$ 0.42
3 $\mu$ m	0.2 $\pm$ 0.08	0.3 $\pm$ 0.07	2.3 $\pm$ 0.22
Significance	NS	$p < 0.05$	$p < 0.05$
<b>PtB-2012</b>			
0.8 $\mu$ m	3.3 $\pm$ 0.56	0.8 $\pm$ 0.14	10.3 $\pm$ 0.94
3 $\mu$ m	1.7 $\pm$ 0.15	3.3 $\pm$ 0.18	5.0 $\pm$ 0.26
Significance	NS	$p < 0.05$	$p < 0.05$
<b>Pier-2012</b>			
0.8 $\mu$ m	1.0 $\pm$ 0.05	7.6 $\pm$ 0.56	7.4 $\pm$ 2.09
3 $\mu$ m	1.8 $\pm$ 0.16	10.2 $\pm$ 0.73	5.5 $\pm$ 0.70
Significance	$p < 0.05$	$p < 0.05$	NS

decline in bacterial abundance in the treatment with enhanced bacterivory. Such patterns of declining bacterial abundance with increasing HNF abundances have been shown in numerous experiments (Šimek et al. 2001, Pernthaler 2005, Pernthaler & Amann 2005). Our data support previous findings that grazing by HNF can control bacterial abundance and biomass in marine waters (Baltar et al. 2016). However, our question was how does HNF grazing qualitatively affect the bacterioplankton in oligotrophic marine waters?

### 4.3. Bacterial cell size as defense strategy

The current model of bacterivory, based mainly on work in freshwater ecosystems, suggests the existence of an edible size range of bacteria with very small and very large cells either being resistant to grazing or showing reduced susceptibility to grazing (Šimek & Chrzanowski 1992, Pernthaler 2005). In our experiments, no clear bimodal size distribution pattern was detected, although there was a tendency that the presence of grazers favored very small and large cells. The existence of very small bacteria with a small genome size is typical for marine systems (Yooseph et al. 2010), and such cells were also found in our study. For example, there are bacteria (ultramicrobacteria) with a diameter of 0.2 to 0.3  $\mu$ m, and these do not increase in size even in nutrient-rich cultures conditions (Velimirov 2001). Such bacteria, while rather adapted to oligotrophic conditions, might also benefit from reduced grazing. The fact that there

was only a weak increase in the relative abundance of very small cells in the treatments with HNF could be due to bacterial growth rates which were too low to permit a detectable response in the time frame of our experiments.

In contrast, we found evidence in all 3 experiments that the presence of grazers induced shifts in the cell size distribution of bacteria towards larger cells. To our knowledge, this is one of the few studies (see

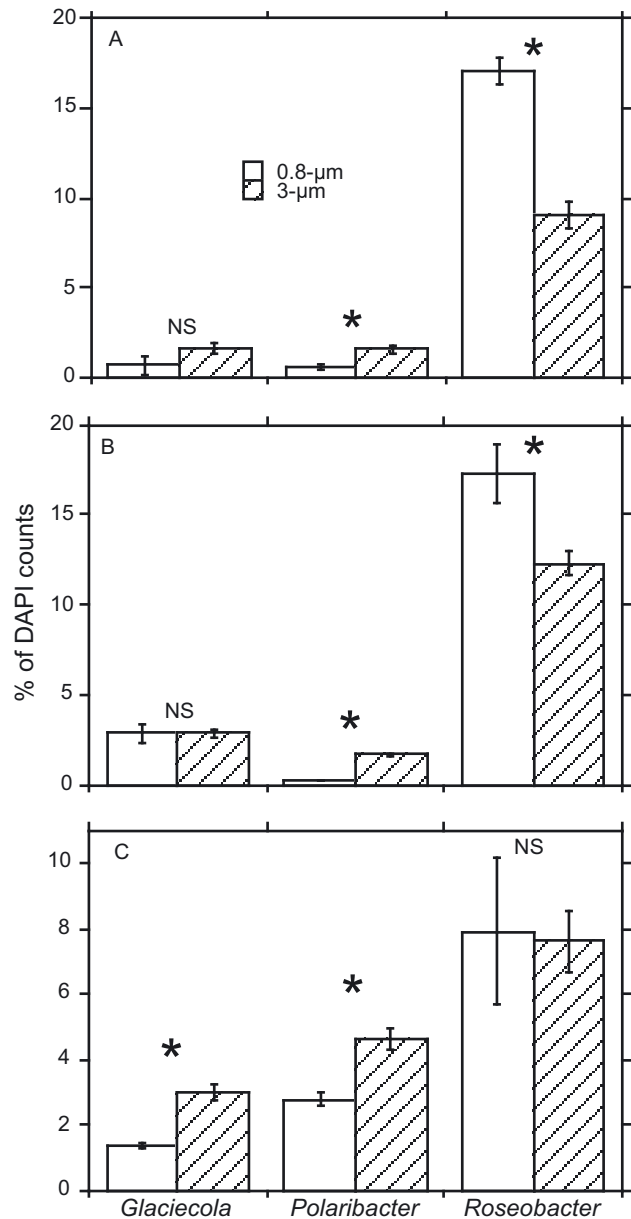


Fig. 6. Relative abundance of fluorescence *in situ* hybridization probe-targeted groups at the end of the experiments in treatments with enhanced bacterivory (3  $\mu$ m prefiltration) and without grazing (0.8  $\mu$ m prefiltration). Data are means  $\pm$  SD of triplicate incubations. (A) Expt Point B (PtB)-2011; (B) Expt PtB-2012; (C) Expt Pier-2012. \* $p < 0.05$  (*t*-test)

Section 1) showing that increasing the size could also be a defense strategy of bacteria against grazing by small flagellates in comparatively oligotrophic marine systems. The arbitrary threshold of 2  $\mu\text{m}$  used for grazing resistance seems valid, since the median cell size (or its equivalent spherical diameter) of flagellates was ca. 2.0 to 2.5  $\mu\text{m}$  (data not shown); thus, grazing on bacterial cells larger than 2  $\mu\text{m}$  should be reduced compared to smaller cells. Notably, HNF communities in the Bay of Villefranche are dominated by cells <2  $\mu\text{m}$  (Tanaka et al. 2003). Our results suggest that the size class of bacteria most vulnerable to grazing by HNF was 0.4 to 0.8  $\mu\text{m}$ . Consequently, even medium-sized cells ranging from 0.8 to 2  $\mu\text{m}$  in size appeared to be relatively grazer resistant. While we confirm that increasing cell size could be a survival strategy in marine waters, the response detected was much weaker than that in some freshwater systems. In freshwater systems, long filaments and aggregation of cells occur frequently in the presence of flagellates and can dominate in terms of biomass (e.g. Šimek et al. 2001). Also, some bacteria within the typical edible size range in fresh waters (e.g. some groups of *Actinobacteria*) are discriminated against by bacterial grazers and, thus, do not support growth of natural HNF communities (Tarao et al. 2009, Šimek et al. 2013). So far, not much attention has been paid to such aspects in natural marine bacterioplankton; however, it is conceivable that more hidden strategies of bacterial defense against grazing by HNF also exist in marine systems (Baltar et al. 2016).

#### 4.4. Effect of grazing on bacterial community structure

In all 3 experiments, bacterial community structure, as indicated by the DGGE patterns, differed between the treatments with enhanced bacterivory and without grazing and, thus, support some previous findings. Bacteria can respond in various ways to grazing (see Section 1); it is also known that flagellates show feeding specificity (Montagnes et al. 2008) and, vice versa, that prevailing prey types can have a strong effect on the composition of the HNF community (Šimek et al. 2013, Grujčić et al. 2015). Such direct interactions could be complemented by indirect interactions such as the release of organic material and inorganic nutrients by flagellates (thus, changing bacterial competition) to shape bacterial diversity. Interestingly, for all 4 major phylogenetic groups detected by DGGE

analysis, we also found sequences that were not affected by the presence of flagellates, suggesting resilience to changes by bacterivory (Baltar et al. 2016). This indicates (at least at the level of the phylogenetic resolution of DGGE analysis) that there could be phylotypes which are neither directly nor indirectly affected by grazing.

The DGGE analysis indicated that bacteria closely related to *Alteromonas/Glaciecola* did increase in treatments without grazing. Thus, these bacterial types might be vulnerable to grazing. It is well known that *Alteromonales* contain strains that grow fast when grazers are removed (Beardsley et al. 2003, Allers et al. 2007). A potentially opportunistic life strategy of *Glaciecola* is supported by previous experimental findings from the Bay of Villefranche, where it was shown by DGGE analysis that *Glaciecola* reacted positively to a disturbance induced by the addition of soot (Cattaneo et al. 2010). While the DGGE data from our experiments support the hypothesis that *Glaciecola* contain phylotypes that are susceptible to grazing, the CARD-FISH data showed no clear trend for susceptibility to grazing, and in 1 experiment, grazing even favored *Glaciecola*.

*Vibrio* phylotypes were positively affected by the presence of HNF as indicated by DGGE analyses, and they are often seen as opportunists (Suttle 2007) or copiotrophs (Lauro et al. 2009). This is also supported by a study in the Bay of Villefranche (Weinbauer et al. 2006). In addition, it has been shown that *Vibrio* is susceptible to grazing (Beardsley et al. 2003). However, it is also known that some *Vibrio* strains can form filaments (Ehara et al. 1997). The closest relative detected by DGGE band sequencing was *V. gigantis*, which can make large cells of 2 to 3  $\mu\text{m}$ , which are potentially too big for efficient ingestion by small flagellate cells of 2 to 3  $\mu\text{m}$  diameter (see Section 4.3). Thus, *Vibrio* seems to have invented versatile life strategies in marine waters.

*Roseobacter* was negatively affected by the presence of grazers in our study as indicated by both DGGE and CARD-FISH analyses. However, a lack of grazing effect was found for *Roseobacter* in another study (Beardsley et al. 2003), whereas a DGGE phylotype of *Roseobacter* was negatively affected by viruses in the Bay of Villefranche (Malits & Weinbauer 2009). The high detected (apparent) growth of *Roseobacter* supports findings indicating that *Rhodobacteriaceae*, likely owning an opportunistic life strategy, benefit from phytoplankton blooms and can dominate communities (Allers et al. 2007). Overall, the mechanisms explaining how *Roseobacter* manages to be an abundant bacterial group in some oceans (Selje et al.

2004) are still unclear. One explanation could be that *Roseobacter* also consist of strains with very different life strategies. Our data indicate that the *Roseobacter* strains in the Bay of Villefranche could be dominated by potentially fast-growing phylotypes vulnerable to grazing, the life strategy known, for example, for *Limnohabitans* bacteria in fresh waters (Jezbera et al. 2006, Šimek et al. 2014). It suggests that this life strategy, i.e. a large growth potential of bacteria allowing for overriding the effect of their enhanced grazing-induced mortality, is omnipresent in aquatic systems.

Both DGGE and FISH analyses showed that *Polaribacter* were positively affected by the presence of HNF. *Polaribacter* can grow into filaments (e.g. the type species *P. filamentus*) (Gosink et al. 1998) which could protect the cells against grazing. Thus, *Polaribacter* strains in the Bay of Villefranche are candidates for a genus with an enhanced, morphology-related grazing resistance in marine waters.

## 5. CONCLUSIONS

The effect of grazing on the control of bacterial community growth and composition has been previously documented for marine systems. Our data support this idea for oligotrophic coastal waters. We also found evidence that increasing the cell size could be a defense mechanism against grazing by small bacterivores in marine systems and that a significant amount of carbon (ca. 8–25%) could be bound in grazing-resistant bacteria. However, the size-related grazing defense strategies, such flock or filament forming (Jürgens & Güde 1994, Pernthaler 2005), seem to be less important in marine than in freshwater systems. Nevertheless, we demonstrated that defense against grazing by changing the cell size is likely also an efficient strategy for marine bacteria, and we identified important genera that are potential candidates for defense strategists against grazing. Future, more detailed studies, e.g. on ingestion of specific genera by flagellates have to be performed to separate the direct, indirect and neutral effects of grazing and advance the field of grazing resistance in marine systems.

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