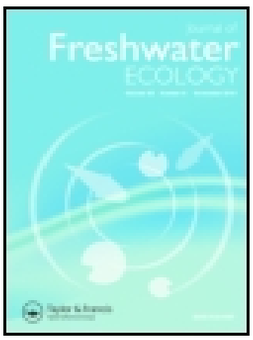

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Competition and protist predation are important regulators of riverine bacterial community composition and size distribution

Giampiero Batani[a], Germán Pérez[b], Gabriela Martínez de la Escalera[b], Claudia Piccini[b] and Stefano Fazi[a]

[a]Water Research Institute, National Research Council of Italy (IRSA-CNR), Rome, Italy; [b]Department of Microbiology, Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Montevideo, Uruguay

ABSTRACT

Among the bacterivorous protists, heterotrophic nanoflagellates (HNFs) are considered to be the main grazers of bacteria in freshwaters due to their size-selective grazing. In this work, we assessed the change of a riverine bacterial community in controlled incubations, where HNFs' predation pressure was initially released through filtration. Filtration did not prevent the passage of cysts, which grew in the enrichments afterwards. Data on the composition of the bacterial community were gathered by Catalyzed Reporter Deposition Fluorescent *In situ* Hybridization (CARD-FISH) using 16S probes targeting phylogenetic groups. Bacterial cell size was also examined using image analysis. Overall, the initial filtration directly (through release of predation pressure) or indirectly (through competition among bacterial groups) affected the bacterial community composition. When nanoflagellate abundance rose, a reduction of bacterial abundance and changes in cell size distribution were observed. *Gamma-Proteobacteria* and *Actinobacteria* were the groups showing the greatest reduction in abundance. *Beta-Proteobacteria* showed a reduction of cell size and were found in aggregates. *Alpha-Proteobacteria* and *Actinobacteria* developed two distinct filamentous morphotypes: short, segmented rods and long chains of rods. Our results showed that the release of the predation pressure and the successive rise of the nanoflagellates changed the bacterial community in terms of composition at large phylogenetic scale. HNF grazing is highly group-specific and seems to reconstruct the community based on cell size, and thus, not only drastically changing the bacterial community composition, but also increasing its functional diversity.

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Bacteria; nanoflagellates; aggregates; filaments; functional diversity; river ecology

Introduction

Planktonic bacteria are important players in biogeochemical cycles. In aquatic ecosystems, the main factors that have been identified as influencing bacterial communities are substrate supply, lysis by viral infections, and protozoan and metazoan predation (Jürgens & Matz 2002). A great deal of laboratory and field evidence shows that protists' grazing is one of the major forces shaping the bacterial community structure (Pernthaler et al. 1996, 1997; Šimek et al. 1997; Jürgens et al. 1999; Pernthaler 2005; Corno & Jürgens 2008; Chen et al. 2011; Chow et al. 2014). Thus, a more comprehensive understanding of the bacteria–protist interaction is one of the central issues in carbon transfer in aquatic systems and, more generally, in freshwater ecology. Among the bacterivorous protists,

CONTACT Stefano Fazi fazi@irsa.cnr.it

heterotrophic nanoflagellates (HNFs) are widely considered to be the main grazers of bacteria in freshwaters (Jürgens et al. 1997; Arndt et al. 2000; Jacquet et al. 2005; Weitere et al. 2005; Bong & Lee 2011). HNFs' grazing can impact both the standing stock and taxonomic structure of bacterial communities, as well as size and morphology of each bacterial phylogenetic group (Šimek et al. 1999; Fazi et al. 2008).

Biovolume is an important characteristic of bacterial cells because it shapes the contribution of microbes to total biomass and biogeochemical cycling. Since biomass depends on cell size as well as cell abundance, the distribution of biovolume within bacterial groups is as important as abundance (Straza et al. 2009). The analysis of the cell biovolume is, therefore, a crucial step towards a better understanding of the contribution of each picoplanktonic population to community dynamics and carbon flux in the water column (Schattenhofer et al. 2009). In particular, as the carbon-to-volume ratio has been shown to vary with cell volume, the community cell volume distribution needs to be carefully considered when modelling the food web and nutrient cycling at the ecosystem level (Simon & Azam 1989; Malfatti et al. 2010). Most of the bacterioplankton cells of both marine and freshwater environments are small in size (volume $< 0.03\ \mu m^3$). However, the numerically predominant fractions in aquatic environments under high grazing pressure are represented by ultramicrobacteria (volume $< 0.001\ \mu m^3$) or large filaments (volume $> 1\ \mu m^3$; Hahn et al. 2003; Corno et al. 2008; Justice et al. 2008; Jousset 2012). HNF grazing on bacteria is size-selective with a preference for medium-sized bacteria (Chrzanowski & Šimek 1990; González et al. 1990; Šimek & Chrzanowski 1992; González 1996; Corno et al. 2008; Glücksman et al. 2010). Large-sized bacteria, such as filamentous bacteria (Hahn et al. 1999) and microcolony-forming bacteria (Hahn et al. 2000), may exceed a species-specific upper ingestion limit of HNFs, thus providing these bacteria with a refuge from grazing. Bacterial resistance against predation depends on morphological plasticity of each bacterial strain (Pernthaler 2005). However, it is still a matter of debate whether some resistance forms, such as filamentous, are due to a change in the morphology of non-filamentous bacteria (Pernthaler et al. 1997), if bacteria evolve to become more filamentous (Corno & Jurgens 2006), or if permanently filamentous bacteria become more abundant under high grazing pressure (Hahn et al. 1999; Justice et al. 2008). Another open question is whether filament formation is triggered by chemical stimuli released by the predator (Pernthaler et al. 1997; Corno & Jurgens 2006; Blom et al. 2010) or if it is growth rate dependent (Hahn et al. 1999; Salcher et al. 2007). On the other hand, it is well known that aquatic bacteria developing in microcolonies embedded in a complex matrix produce a wide range of extracellular polymeric substances such as polysaccharides, proteins, nucleic acids, and lipids (Hahn et al. 2004; Salcher et al. 2007) and are protected from nanoflagellates' predation. The shift to the aggregation cell type may be a passive consequence of selective feeding on single cells (Hahn et al. 2004; Corno & Jürgens 2008), but microcolony formation can also be specifically induced in the presence of predators by cell–cell communication (quorum sensing; Matz et al. 2004; De Kievit 2009).

Most of the experimental studies aimed at following prey–predator dynamics between picoplanktonic and nanoplanktonic components of freshwaters food web have been based on (1) protist inoculum in the bacterial growth media (Pernthaler et al. 1996; Šimek et al. 1997; Posch et al. 1999; Salcher et al. 2005); (2) community studies in short-term experiments, by measuring bacterial edibility for protozoa over a few days using an optical clearance-rate test (Thelaus et al. 2008), (3) by estimating the feeding activity of specific grazers detected by Fluorescent *In situ* Hybridization (FISH) for a few hours with tracer preys (Massana et al. 2009); or (4) the responses to stress conditions that may affect the interplay between protists and bacteria (Fazi et al. 2008). Following the latter approach, here, we assessed the change of a riverine bacterial community in controlled incubations where water was pre-filtered to initially release the HNF predation pressure. It has been demonstrated that physical treatment options, including filtration (Oemcke and van Leeuwen 2005; Worsfold et al. 2009), have greater potential for protist removal over chemical treatments (Doblin & Dobbs 2006). However, the filtration process is not only inevitably accompanied by changes in bacterial cell physiologies and cell destruction, as a result of the physical separation of bacteria and

grazers, including suspended particulates-associated microbiota, but it is also likely to influence nutrient availability and bacterial growth (Takeshi & Jacquet 2008), thus enhancing competition among different bacterial groups (Bohannan & Lenski 2000; Corno et al. 2008) or the effect of prokaryotic viruses (phages) and fungi (Boer et al. 2005; Weinbauer et al. 2007; Chow et al. 2014).

Because phages and flagellates consume the same prey, an antagonistic interaction may be expected; that is, a decrease in the activity of one type of consumer could result in an increase in resources for the other consumer of bacteria (Maki & Yamamura 2005; Chow et al. 2014). As phages typically do not trespass genus boundaries and, as phage infection is density-dependent, phages should limit competitive bacterial dominants and thus allow fewer competitive bacterial types to survive (Fuhrman & Suttle 1993; Thingstad et al. 1993). Thus, viral mortality can be considered as a mechanism that potentially increases species evenness in the bacterial community. In the same way, fungi can also affect bacterial community composition by removing or creating bacterial niches (Boer et al. 2005).

We followed bacteria−protists dynamics for 8 days, starting from a condition in which protists were eliminated by filtration, a process which did not prevent the passage of cysts that grew in the enrichments afterwards. We intended to (1) detect the direct or indirect effects of HNF removal on bacterial community structure; (2) assess the potential morphological plasticity of bacterial clusters when they start to be exposed to the protist predation.

Data on the composition of the bacterial community were gathered by Catalyzed Reporter Deposition Fluorescent *In situ* Hybridization (CARD-FISH), particularly analyzing the dynamics of five phylogenetic clusters: *Proteobacteria* ($\alpha-\beta-\gamma$), *Bacteroidetes*, and *Actinobacteria*. By coupling CARD-FISH with image analysis (Posch et al. 2007; Fazi et al. 2008; Posch et al. 2009; Salcher et al. 2010), we described changes in the distribution of the individual cells in volumetric classes for each of the analyzed phylogenetic clusters.

Methods

Experimental set up

Water was collected at Santa Lucía River (Uruguay) in 5-L, clean acid-washed bottles and transported to the laboratory in an icebox (4 °C). Water samples were filtered through GF/C filters (nominal pore size 1.2 μm, Whatman™) and the experiment was set up. Three independent replicates were conducted in sterile flasks (1 L), which were maintained at the *in situ* temperature (20 °C) in the dark with gentle shaking (IKA KS 130 orbital shaker). Water was sampled after 0, 20, 95 and 192 h from the starting of the experiment. Dissolved inorganic nitrogen (DIN, as the sum of NO_2^--N, NO_3^--N and NH_4^+-N) and soluble reactive phosphorus (SRP) were determined by standard methods (APHA 1995) at the beginning (0 h) and the end (192 h) of the incubations.

Cell numbers of bacteria and protists

The abundance of bacteria (BAB) and HNFs was evaluated by staining with 4′-6-diamidino-2-phenylindole (DAPI) (Porter & Feig 1980). Water samples were fixed with NaOH-buffered formaldehyde solution (FA, 1% v/v final concentration, pH = 7.4) for a maximum of 24 h. Depending on the concentration of organisms, between 0.5 and 5 mL of samples per replicate bottle were filtered onto black polycarbonate membrane filters (pore size 0.2 μm, 25 mm diameter, Nuclepore Corporation, Pleasanton, USA) by gentle vacuum (<0.2 bar), stained with DAPI, and total bacterial and protist abundances were determined in these preparations by epifluorescence microscopy (Leica DC 350 F) at a magnification of 1000× (Jürgens & Montserrat Sala 2000). Additional aliquots of fixed samples were filtered onto polycarbonate membrane filters (pore size 0.2 μm, 47 mm diameter, Nuclepore Corporation, Pleasanton, USA) and stored at −20 °C until further processing (CARD-FISH staining).

Bacterial community composition by CARD-FISH

CARD-FISH was performed following the protocol optimized by Fazi et al. (2005, 2013). The following rRNA-targeting HRP-labelled probes (Biomers, Ulm, Germany) were used: ALF968, targeting sequence types affiliated with *alpha-Proteobacteria*; BET42a for *beta-Proteobacteria*; GAM42a for *gamma-Proteobacteria*; CF319a for *Bacteroidetes* (formerly *Cytophaga-Flavobacterium-Bacteroides*); HGC69a for *Actinobacteria* (Loy et al. 2007). The stained filter sections were inspected on an epifluorescence microscope (Leica DM LB 30, Leica Microsystems, Wetzlar, Germany) at 1000× magnification. At least 300 cells were counted in >10 microscopic fields randomly selected across the filter's sections. The relative abundance of hybridized cells was estimated as the ratio of hybridized cells to total DAPI-stained cells.

Biovolume of the different populations

A Leica DC 350F high-resolution camera (Leica Microsystems, Wetzlar, Germany) was used to capture 1300 × 1030 TIFF gray-scale images of DAPI-stained cells at a color depth of 8 bits and a resolution of 0.1 mm per pixel. Image filtering was performed using the software ImageJ (version 1.37, National Institutes of Health, Bethesda, MD, USA). More than 10 images randomly captured across each of the three replicate filters were processed at UV excitation to detect DAPI-stained cells. The binary images were generated and processed according to Amalfitano et al. (2008). To determine the size of bacterial cells from different populations, image pairs of the same microscopic field were captured at UV and blue light excitation in order to detect cells hybridized by CARD-FISH with specific probes. After spatial filtering and segmentation, a contour mask was created around hybridized cells in each image and applied to the corresponding segmented images from DAPI staining. Size measurements were performed separately for each population from DAPI-stained cells as described above. This procedure was also repeated separately for each of the filamentous cells observed in some bacterial strains. The same image pairs analyzed in order to detect cells hybridized by CARD-FISH were further processed measuring only the biovolume of the filamentous cells without their context. Finally, the biovolume of each bacterial population was divided into 13 cell volumetric classes (μm^3), according to Fazi et al. (2008), for further analysis.

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 5.0 for Mac OS X (GraphPad Software, San Diego, CA, USA). If required, data were log-transformed prior to analysis in order to approximate normality (Kolmogorov−Smirnov test); if this was not obtainable, parametric tests were replaced by their non-parametric alternatives such as Wilcoxon test for paired comparisons.

One-way repeated measures analysis of variance (RM-ANOVA) was utilized to analyze changes of bacterial abundance in time, with bottles as subject factors, time as the repeated factor, and bacterial abundance as the categorical factor; two-way RM-ANOVA was utilized to analyze the abundance of the four bacterial clusters. RM-ANOVA was also performed to analyze changes in time of the biovolume distributions of each bacterial cluster.

Results

Nutrient concentrations did not show any significant change during incubation. At the beginning of the experiment, DIN and SRP concentrations were 29.4 ± 2.9 μM and 17.9 ± 0.4 μM and at the end 29.0 ± 0.1 μM and 18.8 ± 1.2 μM, respectively (paired *t*-test, $p \geq 0.05$). Overall, the total BAB showed values ranging from 4.8×10^6 to 9.9×10^6 cells/mL, while HNFs reached an abundance of 8.2×10^4 cells/mL (Figure 1). At the beginning of the experiment (0 h), BAB showed an average value of $9.2 \times 10^6 \pm 2.0 \times 10^6$ cells/mL, while the presence of nanoflagellates was not observed.

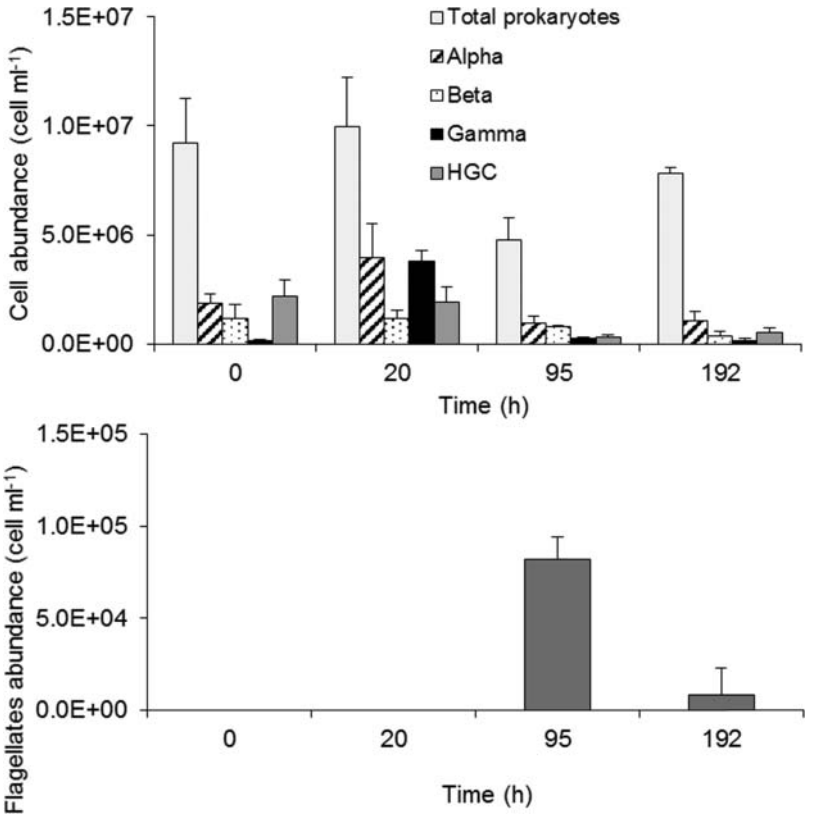

Figure 1. Abundances of bacteria and flagellates (cell/mL) at time 0, 20, 95 and 192 h. At all incubation times, taxonomic composition of the bacterial community as analyzed by CARD-FISH is shown. Error bars indicate standard deviation (alpha = *alpha-Proteobacteria*; beta = *beta-Proteobacteria*; gamma = *gamma-Proteobacteria*; HGC = *Actinobacteria*; total *prokaryotes* = DAPI-stained bacteria).

At 20 h, still in the absence of nanoflagellates, BAB was $9.9 \times 10^6 \pm 2.3 \times 10^6$ cells/mL. At 95 h, the appearance of nanoflagellates ($8.2 \times 10^4 \pm 1.2 \times 10^4$ cells/mL) resulted in a lowering of BAB ($4.8 \times 10^6 \pm 9.9 \times 10^5$ cells/mL). BAB increased again at the end of the experiment (192 h), reaching a mean value of $7.8 \times 10^6 \pm 3.2 \times 10^5$ cells/mL, when nanoflagellates disappeared almost completely ($8.3 \times 10^3 \pm 1.4 \times 10^4$ cells/mL).

One-way RM-ANOVA, with bottles as subject factors, time as the repeated factor, and bacterial abundance as the categorical factor, showed a significant difference in the mean values among time points ($F = 5.46$; $p = 0.038$; df = 11). All pairwise multiple comparisons (Student–Newman–Keuls Method) showed a significant decrease only between time 20 h vs. 95 h ($p = 0.037$) and time 0 h vs. 95 h ($p = 0.042$). The inverse relationship between the abundance of predators (nanoflagellates) and that of prey (bacteria) was statistically significant ($n = 36$; $r^2 = 0.9$; $p < 0.05$).

At the beginning of the experiment, the bacterial community was dominated by *alpha-Proteobacteria* and *Actinobacteria*, with abundance values of $1.9 \times 10^6 \pm 7.0 \times 10^5$ and $2.1 \times 10^6 \pm 2.9 \times 10^5$ cells/mL, respectively. At 20 h, *alpha-Proteobacteria* increased to $3.9 \times 10^6 \pm 6.2 \times 10^5$ cells/mL. Interestingly, *gamma-Proteobacteria* were almost completely absent at 0 h and showed the highest abundance at 20 h ($3.8 \times 10^6 \pm 3.2 \times 10^5$ cells/mL). *Actinobacteria*, instead, did not undergo any significant numerical variations at 20 h compared to the beginning of the experiment. After the appearance of the nanoflagellates in the water (95 h), the abundance of all bacterial groups decreased; *alpha-Proteobacteria* and *Actinobacteria* reached their minimum value of $9.7 \times 10^5 \pm 1.0 \times 10^5$ and $3.2 \times 10^5 \pm 7.0 \times 10^4$ cells/mL, respectively. *Beta-Proteobacteria* decreased

throughout the experiment, with the minimum value of $3.8 \times 10^5 \pm 1.6 \times 10^5$ cells/mL at 192 h. *Cytophaga-Flavobacteria* abundance was always below the detection limit. Two-way RM-ANOVA, with the abundances of the different bacterial groups as categorical factors, showed significant differences among groups ($F = 13.78$; $p = 0.002$; df = 3) and time ($F = 204.71$; $p < 0.001$; df = 3) and a significant interaction between groups and time ($F = 35.09$; $p < 0.001$; df = 9). All pairwise multiple comparisons (Student–Newman–Keuls Method) for *alpha-Proteobacteria* showed significant differences among all time points except between time 95 h vs. 192 h ($p = 0.567$). For *beta-Proteobacteria*, only the 192 h time point was significantly lower than all the other ones ($p < 0.05$). For *gamma-Proteobacteria*, only the 20 h time point was significantly higher than all the other ones ($p < 0.001$). *Actinobacteria* abundance significantly differed only between time 0−20 h vs. 95−192 h ($p < 0.001$).

The percentage of the prokaryotic cells not affiliated with any of the four analyzed groups decreased from 41.7% at the beginning of the experiment (0 h) to 0% at 20 h. Afterwards, when the nanoflagellates appeared, this percentage increased again, up to 71.8% at 192 h (Figure 1).

One-way RM-ANOVA, with bottles as subject factors, time as the repeated factor, and biovolume of the total DAPI-stained cells as the categorical factor, showed a significant change in the mean biovolume through time ($F = 3.57$; $p = 0.023$; df = 11), with all pairwise multiple comparisons showing significant differences between time points 0 and 95 h ($p = 0.023$) and between time points 20 and 95 h ($p = 0.036$).

The size distributions of the four phylogenetic groups are shown in Figure 2. One way RM-ANOVA showed a significant change in the mean biovolume through time for all the four clusters (*alpha-Proteobacteria*, $F = 6.25$; *beta-Proteobacteria*, $F = 5.71$; *gamma-Proteobacteria*, $F = 10.31$; *Actinobacteria*, $F = 4.66$; all $p < 0.01$; df = 11), with all pairwise multiple comparisons showing significant differences between time points (Table 1):

- *Alpha-Proteobacteria*, relatively small at time 0 h, with a peak of abundance corresponding to the volumetric class of 0.032 μm^3, increased in size after 20 h (significant difference between 0 and 20 h; $p = 0.013$). Afterwards, their biovolume first decreased at 95 h and then increased again at 192 h, significantly differing in both times from 20 h ($p = 0.002$ and $p = 0.003$, respectively).
- Large *gamma-Proteobacteria* cells became abundant at 20 h, with a peak corresponding to the size class of 0.128 μm^3. Afterwards, a decrease of their biovolume (significant difference between 20 and 95 h; $p < 0.001$) was observed, along with a drastic reduction in abundance.
- Both *beta-Proteobacteria* and *Actinobacteria* did not significantly differ in biovolume between 0 and 20 h. *Beta-Proteobacteria* cells size decreased at 95 h, but the only significant differences in biovolume were observed between 0–20 and 192 h ($p = 0.003$ and $p = 0.005$, respectively). *Actinobacteria* cell size increased at the end of the experiment (192 h), with significant differences in biovolume between 0 and 95−192 h ($p = 0.020$ and $p = 0.044$, respectively) and between 20 and 95−192 h ($p = 0.029$ and $p = 0.041$, respectively).

Interestingly, the formation of cell aggregates was observed at 95 h, particularly for *alpha-* and *beta-Proteobacteria*. Moreover, the appearance of filamentous morphotypes (volumetric classes range: 0.512–2.4 μm^3) belonging to *alpha-Proteobacteria* (short-segmented rods) and *Actinobacteria* (long chains of rods) was observed at the end of the experiment (192 h) (Figure 3).

Discussion

In this work, we combined a time-series microcosm study with highly sensitive analytical techniques, such as CARD-FISH and image analysis, to trace ecosystem predator–prey dynamics under laboratory conditions. Time-series microcosm studies based on the reconstruction of two- and three-level food chains of bacteria and protists have been proven to be highly descriptive with regard to predator–prey dynamics in nature and their role in shaping the food web and the carbon cycle in

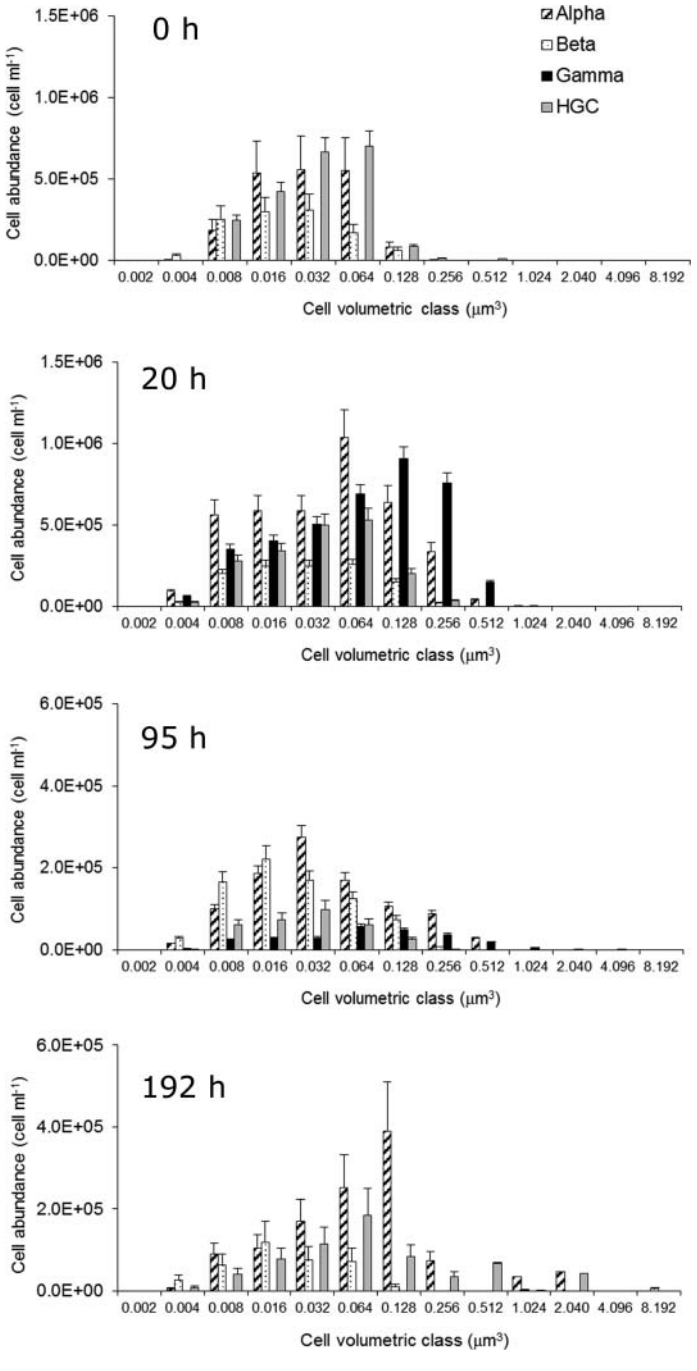

Figure 2. Distribution of bacterial abundances in size classes at time 0, 20, 95 and 192 h as analyzed by image analysis. Data are expressed in cell/mL. The four panels show different scales on the *Y*-axis (alpha = *alpha-Proteobacteria*; beta = *beta-Proteobacteria*; gamma = *gamma-Proteobacteria*; HGC = *Actinobacteria*).

the water column (Balčiūnas & Lawler 1995; Pernthaler et al. 1996; Šimek et al. 1997; Posch et al. 1999; Petchey 2000; Salcher et al. 2005; Thelaus et al. 2008; Massana et al. 2009; Malfatti et al. 2010). However, the majority of these works do not take into account environmental stress conditions that may affect the interplay between protists and bacteria (Fazi et al. 2008). Here, we followed a size-

Table 1. All pairwise multiple comparison (Student–Newman–Keuls Method) among biovolume through time for all the four analyzed clusters (ALF = *alpha-Proteobacteria*; BET = *beta-Proteobacteria*; GAM = *gamma-Proteobacteria*; HGC = *Actinobacteria*). Acronyms are reported only for the significant comparisons ($p < 0.05$).

	20 h	95 h	192 h
0 h	ALF	HGC	BET-HGC
20 h		ALF-GAM-HGC	ALF-BET-HGC
95 h			–

fractionation approach via filtration to study bacterial community abundance and composition over time after an initial release of the HNF predation pressure (Šimek et al. 2001). Filtration did not prevent the passage of HNF cysts that grew in the enrichments afterwards, allowing us to follow the changes in bacterial community abundance, composition and biovolume when nanoflagellates' abundance rose. Indeed, we could not exclude the effect of other factors, such as removal of non-encysting protists and effects due to bacteriophages and fungi. However, in line with Fazi et al. (2008), we intended to capture and depict the changes in community composition and morphology structure when a bacteria consortium starts to be exposed to the protist predation, emerging within the same microbial consortium, without any inoculum of external HNFs.

The total abundance of planktonic bacteria at the beginning of the incubation (before the appearance of the HNFs) resembled the *in situ* abundance and was similar to that reported for other freshwater environments (McManus et al. 2004; Freese et al. 2006). During the first 20 h, the total bacterial abundance did not change and all the identified bacteria were affiliated with the four analyzed clusters. Moreover, an enrichment of *gamma-Proteobacteria* was observed. This bacterial group reached a relative abundance of approximately 40% in accordance with previous observations in microcosm incubations (Puddu et al. 2003). Fuchs et al. (2000) hypothesized that the fast-growing *gamma-Proteobacteria* would fill the niche of typical r-strategists, which rapidly exploit extra nutrients when they become available. Members of this group are adapted to high nutrient concentrations and therefore grow well under culture conditions (Glockner et al. 1999). In addition, Puddu et al. (2003) hypothesized that the lack of grazing in their incubations, with a consortium of bacteria from coastal waters, could result in a disadvantage for other bacteria in competing with the dominant *gamma-Proteobacteria*. This is in line with many other works where an enrichment of fast growing r-strategists bacteria was observed after grazer removal (Eilers et al. 2000; Šimek et al. 2005; Posch et al. 2007; Salcher et al. 2007; Grossart et al. 2008; Hutalle-Schmelzer et al. 2010; Newton and McMahon 2011; Neuenschwander et al. 2015). In this study, the initial filtration removed the protists and the changes observed in the bacterial community composition between 0 and 20 h could be due to changes in competition among different bacterial groups in the absence of predation (Bohannan & Lenski 2000), or the effect of prokaryotic viruses (phages) and fungi (Boer et al. 2005; Weinbauer et al. 2007; Chow et al. 2014). A constant reduction of the community's relative diversity has been observed in several studies where the predator was removed (Bohannan & Lenski 2000; Corno et al. 2008); the absence of the predator promoted greater competition for the resources among different bacterial strains, particularly in low-resource environments. Protist removal was also associated with synergistic and antagonistic effects between HNFs and viruses on bacterial activity and diversity, suggesting group-specific vulnerabilities to the two sources of mortality (Weinbauer et al. 2007). Similarly, fungi can also affect bacterial community composition (Boer et al. 2005). This is particularly evident in fungus-associated bacteria, with a shift towards some bacterial strains as the dominant members of the bacterial community of fungal surfaces (Artursson & Jansson 2003; Boer et al. 2005).

After an initial lag phase, HNFs appeared in the water and their abundance increased afterwards. Owing to the pre-filtration of the water, the finding of HNFs in the incubations can be attributed to the existence of cysts that could pass through the GF/C pores (Foissner 2007; Weisse 2008). It has been claimed, in fact, that the development of cysts in adult flagellates can be affected by nutrient

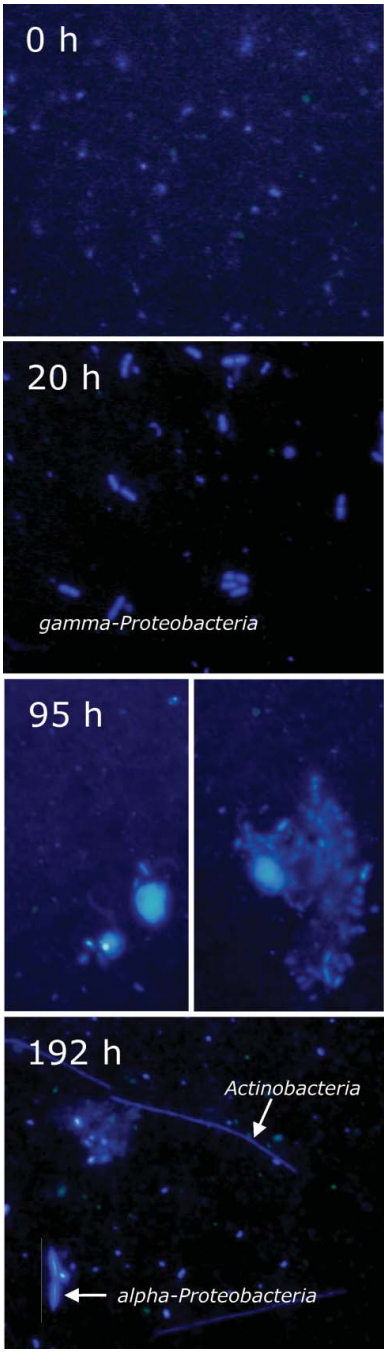

Figure 3. Micrographs of prokaryotes at epifluorescence microscope after DAPI staining. (A) Typical small bacteria at 0 h; (B) large dividing cells at 20 h, mainly *gamma-Proteobacteria*; (C) appearance of flagellates and bacteria aggregation in a microcolony near a flagellate at 95 h; (D) the arrows indicate the appearance of the two filamentous morphotypes: large- and short-segmented rods (*alpha-Proteobacteria*) and long chains of rods (*Actinobacteria*).

availability and by bacterial abundance and size range of edibility (Varnam & Evans 2000). In a previous study, Fazi et al. (2008) found the emergence of flagellates after a few hours of incubation in favorable environmental conditions and after the colonization of the water by edible bacteria.

The abundance of HNFs in different pelagic habitats varies greatly, but typically ranges from 100 to 10,000 cells/mL in lakes, rivers and marine surface waters (Boenigk et al. 2002), according to the values of HNFs' abundance measured in this work. Moreover, in our study, the ratio of HNFs to heterotrophic bacteria was approximately 1:1000, comparable to that found by other authors (Sanders et al. 1992), although this estimation has been disputed (Gasol & Vaque 1993). The drastic numeric reduction of flagellates at the end of the experiment could be explained by the density-dependent mechanism (Abrams & Ginzburg 2000). After the appearance of flagellates, there was a drastic decrease of *alpha-* and *gamma-Proteobacteria* abundances, large cells very attractive to flagellates, and an increase of the total bacteria not affiliated with the four analyzed groups, probably indicating the appearance of phylogenetic clusters resistant to predation (Pernthaler 2005; Corno et al. 2008). Then, it can be argued that, in our study, changes in bacterial community composition could have been driven by both direct (feeding, from 20 h onwards) and indirect (e.g. changes in competition among different bacterial groups, in the first 20 h of incubations) effects, which cannot be separated.

After the appearance of HNFs, a morphological modification of the bacterial groups was also observed. *Beta-Proteobacteria* showed an overall reduction of cell size. Moreover, both *alpha-* and *beta-Proteobacteria* were observed within aggregates, suggesting that the formation of aggregates could give resistance against predation as soon as the nanoflagellates emerge. Some small rods or cocci *beta-Proteobacteria* are strongly affected by predation (Jürgens et al. 1999; Posch et al. 2001; Šimek et al. 2001; Gasol et al. 2002; Salcher et al. 2008), giving rise to a paradox of the aquatic microbial ecology (Salcher et al. 2005), since these *Proteobacteria* are very abundant in freshwater ecosystems (Methé & Zehr 1999; Glöckner et al. 2000; Burkert et al. 2003; Gao et al. 2005; Lemke et al. 2009). In this work, *beta-Proteobacteria* was not the dominant group and their abundance did not change significantly after the appearance of nanoflagellates, probably due to their ability to form small aggregates. This result is partly in line with numerous studies (Jürgens et al. 1999; Šimek et al. 2001; Burkert et al. 2003; Hahn et al. 2004; Alonso-Sáez et al. 2009), in which *beta-Proteobacteria*, when exposed to high predation pressure, formed aggregates and microcolonies. Here, we also detected small *alpha-Proteobacteria* cells forming microcolonies in association with *beta-Proteobacteria*. This result is confirmed by the work of Salcher et al. (2005), in which they found a cluster of *beta-Proteobacteria* (BET3-446) and species of the genus *Caulobacter*, belonging to *alpha-Proteobacteria*, associated in aggregates as a consequence of increased nanoflagellates' abundance. Therefore, after the appearance of HNFs, changes in the interactions between *alpha-* and *beta-Proteobacteria* were observed, suggesting that the aggregation of bacteria in microcolonies could be a defense mechanism against predation (Matz & Jürgens 2003; Jousset 2012).

At the end of the experiment, *alpha-Proteobacteria* and *Actinobacteria* developed into two distinct filamentous morphotypes resistant to predation, consisting of short, segmented rods and long chains of rods, respectively. The a*lpha-Proteobacteria* do not normally represent the highest proportion of bacteria in freshwater systems (Methe & Zehr 1999; Glöckner et al. 2000; Klammer et al. 2002; Lemke et al. 2009). However, in line with our findings, filaments forming *alpha-Proteobacteria* are often found under high grazing pressure (Jürgens et al. 1999; Šimek et al. 1999; Weitere et al. 2005; Thelaus et al. 2008). Regarding the highly diverse group of the *Actinobacteria*, it has been claimed that some of the most abundant freshwater *Actinobacteria* would not suffer size changes induced by protists digestion, because of their cell wall structure (Sekar et al. 2003; Tarao et al. 2009; Šimek et al. 2013) or their constantly small cell size (Hahn et al. 2003; Salcher 2013). However, bacterial community taxonomic and morphological changes induced by predation are very species-specific, particularly when bacterial groups consisting of many different morphological species, such as *Actinobacteria*, are exposed to variable environmental conditions (Jürgens & Matz 2002). It has been seen, for example, that the same flagellate (*Bodo saltans*) activated different bacterial morphological responses in different experimental systems: no filaments appeared in chemostat experiments

(Posch et al. 1999; Šimek et al. 1999), although the development of filaments had been observed in a previous study (Šimek et al. 1997). In this work, the development of filamentous *Actinobacteria* could be explained by taxonomic changes of the bacterial community. However, to fully understand whether the development of filaments is a direct response to enhanced grazing pressure or the result of bacterial community shifts due to other factors, such as competition among different bacterial strains, further investigations are required involving the performance of fitness essays by comparing how well flagellates can consume non-filamentous and filamentous bacterial cells within each bacterial group.

Cytophaga-Flavobacteria were not found in this study, in contrast with many studies where *Bacteroidetes* have been found to be a frequent component of the riverine bacterial community (Crump et al. 2009; Read et al. 2015) or the main group forming filamentous cells under high grazing pressure (Salcher et al. 2005). On the contrary, other studies (Puddu et al. 2003; Crespo et al. 2013) have demonstrated that *Cytophaga-Flavobacteria* are usually enriched on particulate organic detritus, and they could be specialists for particulate organic matter degradation, which was removed by filtration in this experiment.

To conclude, our results showed that the analyzed bacterial clusters differently respond to HNFs' predation pressure by changing in abundance and morphology with important implications on the overall community composition, and, more generally, on the biochemical cycling into the water column. HNFs' grazing is highly group-specific, determining changes of the bacterial community towards a similarity with its composition at the beginning of the experiment. An interesting point is that even though the bacterial community is similar in terms of diversity at the beginning and at the end of the experiment, nanoflagellates seem to reconstruct the bacterial community based on cell size, thus also increasing its functional diversity. These results pinpoint the importance of predators and top-down regulation for prey community composition and abundances. Further investigations, coupling biovolume and taxonomic identification with activity estimation at single-cell level, could certainly expand our knowledge on ecosystem functioning and stability.

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Notes on contributors

Giampiero Batani received his MSc degree from the University *La Sapienza* of Rome and the Water Research Institute, National Research Council of Italy (IRSA-CNR), Rome, Italy in 2013, under the supervision of Dr Stefano Fazi. He is currently a PhD candidate at the University of New South Wales, Sydney, Australia. His research interest focuses on host-micro-organisms interactions and their role in aquatic environments.

Germán Pérez received his MSc degree at the PEDECIBA, Universidad de la República of Uruguay, under the supervision of Dr Claudia Piccini. He is currently working at the Agronomy Faculty and his research focus is on cyanobacterial ecology in rice soils.

Gabriela Martínez de la Escalera is a PhD candidate of PEDECIBA, Universidad de la República of Uruguay, under the supervision of Dr Claudia Piccini. Her thesis deals with molecular ecology and diversity of toxic bloomforming cyanobacteria.

Claudia Piccini obtained her PhD degree in microbiology from PEDECIBA, Universidad de la República of Uruguay. She is a researcher at the Instituto de Investigaciones Biológicas Clemente Estable of Uruguay. Her research focuses on the ecology of heterotrophic and autotrophic freshwater bacteria.

Stefano Fazi received his PhD degree in aquatic ecology from the University of Parma, Italy. He is a researcher in aquatic microbial ecology at the Water Research Institute, National Research Council of Italy (IRSA-CNR), Rome, and an adjunct professor at the University *La Sapienza* of Rome, Italy. His research focuses on the role and the structure of microbial communities in aquatic ecosystems.

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