Journal of
Plankton Research

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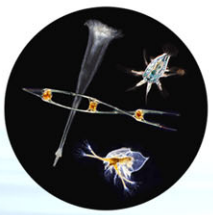

*J. Plankton Res.* (2018) 00(00): 1–13. doi:10.1093/plankt/fbx071

# Growth and cytometric diversity of bacterial assemblages under different top–down control regimes by using a size-fractionation approach

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Received March 26, 2017; editorial decision December 12, 2017; accepted December 19, 2017

Corresponding editor: John Dolan

Zooplankton communities in tropical inland waters are generally characterized by small bodied individuals and the absence of large daphnids. However, the effects of this peculiar food web configuration on microbial compartments have not been tested experimentally. To establish which predator could be responsible for most bacterial loss in a tropical shallow lake, we performed a predation experiment manipulating consumer size fractions. We found that protists had an effect more than four times greater (−86%) than the one exerted by microcrustaceans (−20%), whereas rotifers and nauplii had a minimum effect (−8%). Thus, our results indicate that predation was a crucial factor controlling bacterial abundance and that protists (mainly ciliates) were responsible for most of this loss. Moreover, bacterial community structure was also affected by predation, with a change in the relative proportion of cytometric subpopulations (high-nucleic acid and low-nucleic acid) as a function of different degrees of predation pressure and a decrease in community evenness (assessed by cytometric diversity) with the removal of predators. Therefore, protists play an important role in controlling the abundance and maintaining prokaryotic diversity in warm regions, where zooplankton is present and controlled by juvenile fish throughout the year.

KEYWORDS: clearance rates; flow cytometry; microbial food web; tropical lake food web; selectivity

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## INTRODUCTION

One of the most distinctive features of tropical lakes is the absence of large bodied zooplankton (e.g. Fernando, 1994; Lewis, 1996). As fish reproduction occurs throughout the year, the presence of juvenile fish exerts a nearly permanent predation pressure on zooplankton communities (Lazzaro, 1997), affecting food web configuration. Tropical lakes are usually dominated by small bodied copepods and cladocerans and large daphinids (typically dominant in temperate lakes) are nearly absent in low latitudes (Dumont, 1994). The effects of this food web structure on microbial compartments are still largely unexplored (Sarmento, 2012), but relatively low-bacterial abundance has been frequently reported in tropical freshwater ecosystems (Barros *et al.*, 2010; Roland *et al.*, 2010; Segovia *et al.*, 2016) compared with temperate ones.

The knowledge we have today about the mechanisms controlling bacterial communities comes mainly from studies performed in temperate systems. In those regions, protists are traditionally considered as being mainly responsible for the grazing losses of bacteria, especially heterotrophic nanoflagellates (HNF) (Fenchel, 1982; Sanders *et al.*, 1989, 1992; Berninger *et al.*, 1991). Ciliates are found to be important in structuring bacterial communities when HNF abundance is low (Kisand and Zingel, 2000; Zingel *et al.*, 2007). In turn, several experiments approaching the influence of microcrustaceans suggested a negligible top–down effect on bacterial abundance (Pace and Funke, 1991; Pace and Vaqué, 1994; Adrian *et al.*, 2001; Riccardi, 2002; Agasild and Nõges, 2005; Zingel *et al.*, 2007), including some studies showing relatively high consumption of bacterial biomass by mesozooplankton, but not an effective regulation (Pedrós-Alió and Brock, 1983; Kim *et al.*, 2000). A recent study proposed that in tropical lakes, the major bacterial grazers would be ciliates and small cladocerans (e.g. Bosminids), instead of HNF (Segovia *et al.*, 2016).

It is widely accepted that when in great numbers, cladocerans may exert a significant impact on pelagic bacteria (Pace *et al.*, 1990; Vaqué and Pace, 1992; Gasol *et al.*, 1995; Jeppesen *et al.*, 1996; Cottingham *et al.*, 1997; Wickham, 1998; Hwang and Heath, 1999; Langenheder and Jürgens, 2001). On the other hand, there is consistent evidence that copepods have minimal effects on bacterial communities (Burns and Schallenberg, 1996; Hwang and Heath, 1999; Kim *et al.*, 2000). Rotifers may also feed on bacteria, but generally at very low rates and, therefore, should not be able to affect bacterial abundance (Sanders *et al.*, 1989; Pace *et al.*, 1990; Arndt, 1993; Vadstein *et al.*, 1993) even when they dominate the zooplankton community (Sommaruga, 1995).

It is worth noting that besides direct predation, bacterial community is also subject to indirect cascading predation effects of zooplankton (Jürgens *et al.*, 1994; Kalinowska *et al.*, 2015), which can have a positive influence on bacterial numbers, since their grazing on protists releases bacteria from predation by those organisms. For instance, Fermani *et al.* (2013) observed an indirect effect of rotifers on bacterial abundance through efficient predation on HNF. Another positive effect would be the compensatory growth, in which bacterial abundance would increase in response of the release of nutrients by zooplankton (i.e. excretion and defecation) or the release of carbon from zooplankton grazing on algae (Güde, 1988; Peduzzi and Herndl, 1992; Reche *et al.*, 1997). Virus lysis may also be a major source of bacterial loss (Fuhrman and Noble, 1995), although studies performed in the tropics show a low percentage of visibly infected bacterial cells in natural lakes in the Amazon shallow floodplain lakes (Barros *et al.*, 2010; Almeida *et al.*, 2015) and in contrasting shallow African lakes (Bettarel *et al.*, 2006).

Modifications in bacterial size–structure are also often described as a feedback of increased predation pressure (Pernthaler, 2005), because larger and actively growing bacterial cells are usually preferred by grazers (Andersson *et al.*, 1986; Gonzalez *et al.*, 1990; Langenheder and Jürgens, 2001; Corno *et al.*, 2008). For this reason, bacterial abundance may also remain unchanged in face of predation, because bacterial cells that are less predated may be able to grow and compensate for losses of edible bacteria, simply reallocating their biomass (Jürgens and Güde, 1994; Pernthaler *et al.*, 1996), in the same way, as phytoplankton defence strategies (Sommer, 2008).

Since the first ecological studies using flow cytometry for rapid bacterial enumeration, two cytometric groups of bacteria were identified (Li *et al.*, 1995; Marie *et al.*, 1997), high-nucleic acid (HNA) and low-nucleic acid (LNA), which continued to be used regardless of the environment studied (Bouvier *et al.*, 2007). HNA cells were commonly considered as the most dynamic and active bacterial group, while LNA cells were initially thought to represent inactive or dead cells (Gasol and Morán, 1999; Lebaron *et al.*, 2001, 2002). However, several studies challenge this view, showing that LNA cells are also able to grow (Zubkov *et al.*, 2001; Jochem *et al.*, 2004; Longnecker *et al.*, 2005; Williams *et al.*, 2008; Huete-Stauffer and Morán, 2012) and even be cultivable (Wang *et al.*, 2009). Recently, Vila-Costa *et al.* (2012) sequenced the *16 S rRNA* gene amplicons of both fractions and found that most taxa are in fact related to only one of the cytometric groups, with a small degree of overlapping, suggesting that those fractions have different bacterial compositions.

HNA bacteria are also usually correlated with chlorophyll-*a* and phytoplankton-derived carbon (Li *et al.*, 1995; Bouvier *et al.*, 2007; Morán *et al.*, 2007; Sarmento *et al.*, 2008; Moràn *et al.*, 2011), demonstrating that this group is under a strong bottom–up control. Concomitantly, because HNA cells can be considered as larger in size and fast-growing comparing to LNA cells, predators usually exhibit a preference towards grazing on those components (Gasol and Morán, 1999; Vaqué *et al.*, 2001; Tadonléké *et al.*, 2005; Garzio *et al.*, 2013; Sintes and del Giorgio, 2014; Baltar *et al.*, 2016). Thus, the relative importance of bottom–up and top–down mechanisms mainly influences this fraction of the bacterial community.

We aimed to determine which size fraction of the zooplankton community is responsible for most of the bacterial losses by grazing in a tropical shallow lake. We expected that size fractions containing both ciliates and small cladocerans would contribute more to total bacterial grazing. We also expected that LNA and HNA bacteria would be differentially influenced by grazing, the HNA subpopulation being more affected due to its larger size.

## METHOD

The study was conducted in Garças Lake (22°43′27.18″S; 53°13′4.56″W), located in the Upper Paraná River floodplain. This lake is shallow (mean depth of 2 m) with a 14.1 ha area and permanently connected to the river by a narrow channel. The littoral zone harbours several species of aquatic macrophytes, such as *Eichhornia azurea*, *Nymphaea amazonum*, *Polygonum ferrugineum*, *Polygonum stelligerum* and *Salvinia auriculata* (Thomaz *et al.*, 2009). Transparency is usually lower than 1 m, with total phosphorus varying between 30 and 90 µg/L and total nitrogen between 150 and 300 µg/L (De Araujo Rocha and Thomaz, 2004).

On the sampling day, the water temperature was 26.3°C, pH 6.32, turbidity 25.4 (NTU) and dissolved oxygen 6.32 mg/L. Total depth at the sampling point was 1.2 m and Secchi disk depth was 0.55 m. The water was collected from the sub-surface in 20 L plastic dark carboys, transported to the laboratory in dark conditions and kept at *in situ* temperature.

### Laboratory experiments

Grazing experiments were conducted in the laboratory for 24 h at *in situ* temperature (26°C). We ran the experiment under low-light conditions to avoid excessive growth and competition by phytoplankton (Calbet and Landry, 1999). To test for differences in the total bacterivory attributable to zooplankton size fractions, some of the water was not filtered while other water samples were pre-screened and size fractionated through nylon meshes of different pore sizes. We designated three different predation treatments with (i) unfiltered water containing all bacterivores (adult microcrustaceans + nauplii + rotifers + ciliates + flagellates); (ii) water filtered through a 100 µm mesh, excluding the adult microcrustaceans (nauplii + rotifers + ciliates + flagellates) and (iii) water filtered through a 45 µm composed mainly by protists (ciliates + flagellates). Those fractions will be designated throughout the text as, All, <100 µm and <45 µm (Fig. 1). For the control treatment, we filtered water samples through GF/C glass fibre filters (Whatman) which retain particles larger than 1.2 µm, and only bacteria were able to grow without the interference of predators (GF/C filters are known to have a poor retention efficiency of bacteria; Gasol and Morán, 1999).

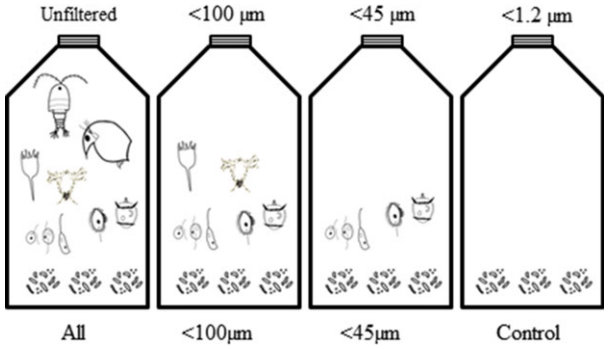


**Fig. 1.** Experiment setup scheme showing the different size-fraction treatments and the control. In the All treatment, adult microcrustaceans, nauplii, rotifers, protists and bacteria were present. In the <100 µm treatment, adult microcrustaceans were removed and only nauplii, rotifers, protists and bacteria were present. The <45 µm treatment was composed mainly by protists and bacteria. The Control treatment had no predators. Headings above indicate manipulation in each treatment and headings below indicate the terminology used throughout the text.

One-litre polyethylene bottles were filled with 800 mL of water with a total of 12 replicates for each treatment and the control. We gently mixed all bottles every 2 h to minimize settling. We sampled water from each treatment for bacterial analysis at the beginning (0 h) and at the end of the experiment (24 h). Additional water samples taken at 12 h were done to track the bacterial abundance during the experiment. Samples were immediately fixed with formalin buffered with borax (1% final concentration) and stored in liquid nitrogen until counting. Water samples for predator abundance estimates were taken at the beginning and at the end of the experiment. Samples fixed with formalin buffered with borax (1% final concentration), Lugol and thiosulphate were used to count all zooplankton and the ciliates. Water samples for flagellate counting by epifluorescence microscopy were fixed with glutaraldehyde (1% final concentration).

### Bacterial and predator counting

We estimated bacterial abundance with a FACSCalibur flow cytometer, staining 200 µL with SYTO-13 (Molecular Probes; 2.5 µmol $L^{-1}$ final concentration) in the dark and run in the flow cytometer. We detected bacteria by plotting the side scatter (SSC) versus FL1 (green fluorescence) and identified two subpopulations of bacteria, LNA and HNA, following Gasol and Del Giorgio (2000). Data were processed using FlowJo V.10 software. Water samples for flagellate counting were filtered through a 0.8-µm polycarbonate black filter and stained with 4',6-diamidino-2-phenylindole (Porter and Feig, 1980), and the abundance estimated by epifluorescence microscopy (Olympus BX51) at 1000 × magnification. Ciliates were counted under an inverted microscope (Olympus CK40) using Utermöhl chambers at 400 × magnification and identified at the lowest taxonomic level possible (Foissner and Berger, 1996; Foissner *et al.*, 1999). Zooplankton was counted under a light microscope (Olympus CX31) using Sedgewick-Rafter counting chambers at 100 × magnification and identified at the species level (Koste, 1978; Reid, 1985; Elmoor-Loureiro, 1997).

### Data analysis

Bacterial net growth rates (NGRs) were calculated for total heterotrophic prokaryotes, HNA and LNA subpopulations assuming exponential growth, $\mu = (\ln N_t - \ln N_0)/t$, where $t$ is the incubation time, $N_t$ is the bacterial abundance after 24 h, $N_0$ is the bacterial abundance at the beginning of the experiment (0 h). We used one-way analysis of variance (ANOVA) to test for differences in bacterial NGR in the predation and the control treatments and used a Tukey test for comparison of means. Predator NGRs were also calculated using the same rationale and the same procedure was used to test for differences in predator abundances and NGR, however only protists, rotifers and nauplii were tested, considering that microcrustaceans were only present at the All treatment.

The effects of predation on bacterial NGR were calculated considering the successive removal of predators in the treatments, as follows:

$$\text{Microcrustacean}_{\text{effect}} = (\text{NGR}_{\text{All}} - \text{NGR}_{<100\mu\text{m}})/\text{NGR}_{\text{control}} \times 100$$

$$\text{Rotifer + Nauplii}_{\text{effect}} = (\text{NGR}_{<100\mu\text{m}} - \text{NGR}_{<45\mu\text{m}})/\text{NGR}_{\text{control}} \times 100$$

$$\text{Protist}_{\text{effect}} = (\text{NGR}_{<45\mu\text{m}} - \text{NGR}_{\text{control}})/\text{NGR}_{\text{control}} \times 100.$$

Thus, predation effects were expressed as the percentage of maximum potential bacterial growth in each treatment relative to the control treatment (absence of predators). We also calculated the HNA/LNA ratio of bacterial abundance in the initial conditions (0 h) and in each treatment at the end of the experiment (24 h). One-way ANOVA was run to test for differences in the treatment effects on the LNA/HNA ratio for each treatment.

Cytometric diversity was analysed using flowDiv package (Wanderley *et al.*, 2015), which calculates ecological diversity indices in binned workspaces for gated populations based on 2D cytograms. Gating was carried out in SYTO-13 stained bacteria cytograms (SSC against FL1) in FlowJo V10, and a 64 bin cytometric fingerprint was obtained based on the number of cells in each bin for each sample (see Quiroga *et al.*, 2017 for more details). A Bray–Curtis dissimilarity matrix was constructed based on the cytometric fingerprint. PERMANOVA was used to test for differences in bacterial composition among treatments. Pielou Evenness ($\mathcal{J}'$) was also calculated and differences were tested using one-way ANOVA.

All analyses were performed using the libraries "multcomp" (Hothorn *et al.*, 2016), "vegan" (Oksanen *et al.*, 2016) and "flowDiv" (Wanderley *et al.*, 2015) in software R 3.1.3 (R Core Team, 2013).

## RESULTS

### Abundance and NGRs

Total bacteria showed higher abundance in control treatments over time, where all predators were excluded, while in all predation treatments, abundances were almost one order of magnitude lower (Fig. 2). Moreover, bacterial abundance in the All treatment decreased over time, whereas in the <45 µm treatment, there was an increase in bacterial abundance at 12 h and then a decrease at 24 h, which coincided with an increase in ciliate abundance at the end of the experiment (Fig. S2).

Accordingly, the calculated bacterial NGRs were significantly higher in the control than in all the other predation treatments. The pairwise comparison of predation treatments showed that All vs <100 µm and <100 µm vs <45 µm were similar between them, while All vs <45 µm were significantly different (Table S1; Fig. 3). The observed bacterial NGR in the predation treatment containing all predators (All) was the only one with negative values, while in the pre-screened treatments (<100 and <45 µm) NGR remained positive but showed very low values. Control treatments showed much higher positive values of NGR, eight times higher than in the predator treatments (Fig. 3).

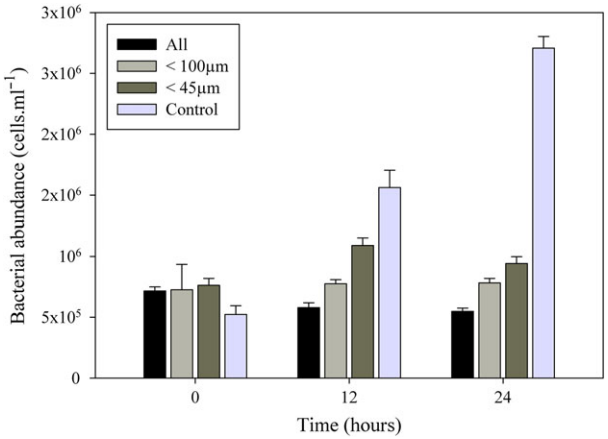


**Fig. 2.** Abundance of total bacteria in the three predator and control treatments over time. Points represent the mean values and bars represent the standard errors.

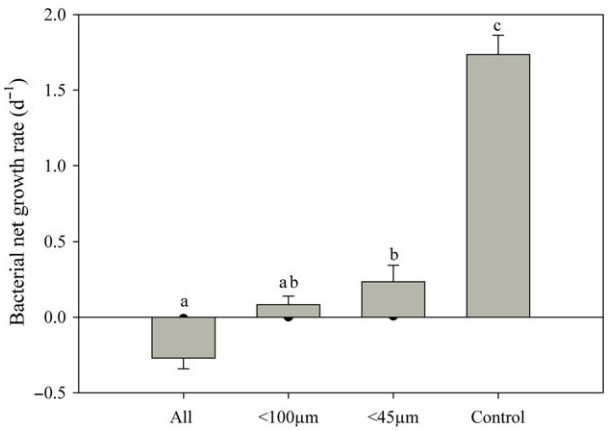


**Fig. 3.** Mean values of bacterial NGRs in the three predator and control treatments. Bars represent the standard errors. Letters in columns indicate statistical significance—treatments not sharing a letter differ significantly at $P < 0.05$ (Tukey's HSD).

HNF abundance was significantly different among treatments at the end of the experiment, with higher values found in the All treatment (see Fig. S1 and Table S1 in Supporting Information). Ciliate abundance was significantly higher in the treatments without microcrustaceans (<100 and <45 µm; Fig. S1; Table S1). The abundance of the three zooplankton groups varied greatly among treatments. Because we intentionally removed microcrustaceans in the pre-screened treatments, they were only present in the All treatments. Rotifers and nauplii were present only at the All and <100 µm treatments, but their abundances were significantly higher in the <100 µm treatments (Fig. S1; Table S1).

NGRs of HNF were not significantly different among treatments at the end of the experiment, and always showed negative values (Fig. S2; Table S2). Ciliates exhibited positive NGR values, which were significantly lower in the All treatment (Fig. S2; Table S2). Rotifers and nauplii were present only at the All and <100 µm treatments, and showed significantly lower and negative values in the All treatment (Fig. S2; Table S2). NGRs of microcrustaceans are not shown, since they were only present in All treatments.

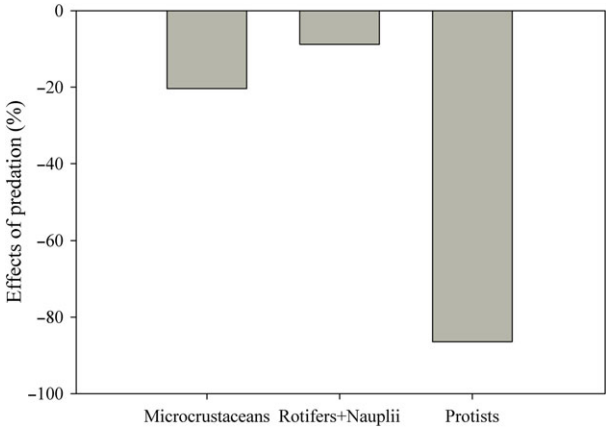


**Fig. 4.** Effects of predation on bacterial NGRs (in terms of percentage of maximum potential bacterial growth in each treatment relative to the control treatment) exerted by three groups of predators.

Effects of predation showed that protists were responsible for most of the predation impact on bacterial communities, with an effect more than four times greater than the one exerted by microcrustaceans. Rotifers and nauplii had a minimal effect on bacterial NGR (Fig. 4).

## HNA and LNA groups

Both HNA and LNA bacteria showed the same patterns as that observed for total bacteria, however the LNA bacterial abundance was not as high as the abundance of HNA bacteria in the control treatments over time (Fig. 5a,b).

We also observed in the cytograms that bacteria at the beginning of the experiment (Fig. 6a and d) and in the predation treatments (Fig. 6b and e) had a higher proportion of the LNA population. An increase in the HNA population, which became more abundant than the LNA, was evident in the control treatment but not in treatments with predators at the end of the experiment (Fig. 6c and f).

The NGRs of both HNA and LNA were significantly higher in control treatments (Fig. 7a). For HNA, NGRs in the All treatments were negative and significantly lower than the other predation treatments, whereas the size-fractioned treatments did not differ between them.

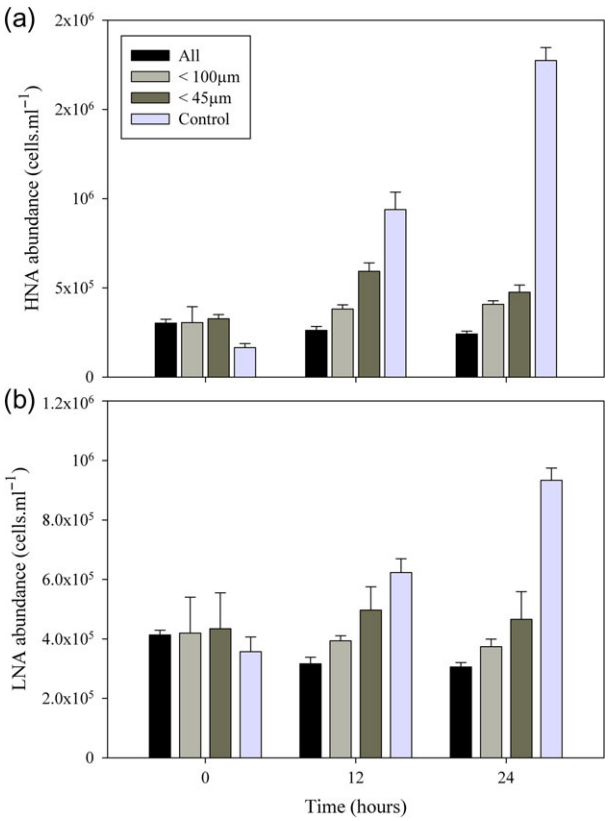


**Fig. 5.** Abundance of HNA (**a**) and LNA (**b**) bacteria in the three predator and control treatments over time. Points represent the mean values and bars represent the standard errors.

For LNA, negative values of NGR were found both in the All and <100 µm treatments, which were similar between them, while NGR in the <45 µm was positive but still no different than the <100 µm treatments (Fig. 7a; Table S4).

Indeed, we found significant differences in the HNA/LNA ratio between the treatments at the end of the experiment. Tukey tests revealed that the treatment containing all predators at 24 h remained similar to the initial conditions. Also, their HNA/LNA ratio was lower than that of the <100 and <45 µm treatments, which were similar between them. The HNA/LNA ratio was significantly higher in the control treatment (Table S5; Fig. 7b).

### Cytometric diversity

PERMANOVA results revealed that the only treatments not significantly different were <100 and <45 µm (Pseudo-$F = 1.76$; $P = 0.14$), whereas all the other treatments were significantly different between them ($P < 0.001$). Pielou´s evenness index ($J'$) results showed that evenness tended to decrease with the removal of predators in the treatments <100 and <45 µm (Fig. 8). Results of the control treatment could not be considered, since they were already lower at the beginning of the experiment, probably due to the filtration to remove predators, which likely removed part of the community.

## DISCUSSION

All 12 replicates for each treatment were extremely robust, which provides consistency to the results found in this experiment. We found that bacterial NGR was significantly higher in the treatment without predators, much lower in the <100 and <45 µm treatments and negative only in the treatment containing all predators. Following the rationale used by Langenheder and Jurgens (2001), considering that the control treatment may represent the carrying capacity of the system, then grazing in all treatments kept bacterial abundances below the maximum abundance they could potentially reach. Especially in the treatment containing all predators, which was closest to the natural conditions of the lake, bacterial abundance seemed to suffer a strong top–down control (i.e. negative growth rates, Fig. 3).

A study comparing bacterial abundance in temperate and tropical regions found that lower numbers in tropical freshwaters, and that bacterial loss by predation was likely to be the cause for the pattern, observed in this study (Segovia *et al.*, 2016). Indeed, the higher bacterial production found in the tropics when compared to temperate systems (Amado *et al.*, 2013), which does not seem to translate into a higher abundance (Roland *et al.*, 2010; Sarmento, 2012), suggests that bacterial losses are likely constraining their growth. In this way, our results add evidence to the idea that predation is a major factor keeping bacteria at low abundance. Additionally, these results indicate that bacterial biomass may be an effective carbon source, as postulated by Azam *et al.* (1983), also in tropical food webs.

Our results on the effect of predation indicated that the treatment containing only protists was responsible for the greatest impact on the bacterial community. Among protists, HNF are usually recognized as the main bacterial grazers in temperate waters (Fenchel, 1982; Sanders *et al.*, 1989, 1992; Berninger *et al.*, 1991). Nevertheless, we found a very low abundance of those organisms in all predation treatments (maximum ~200 cells HNF $mL^{-1}$). Indeed, significantly lower abundances of HNF were found in tropical regions, whereas

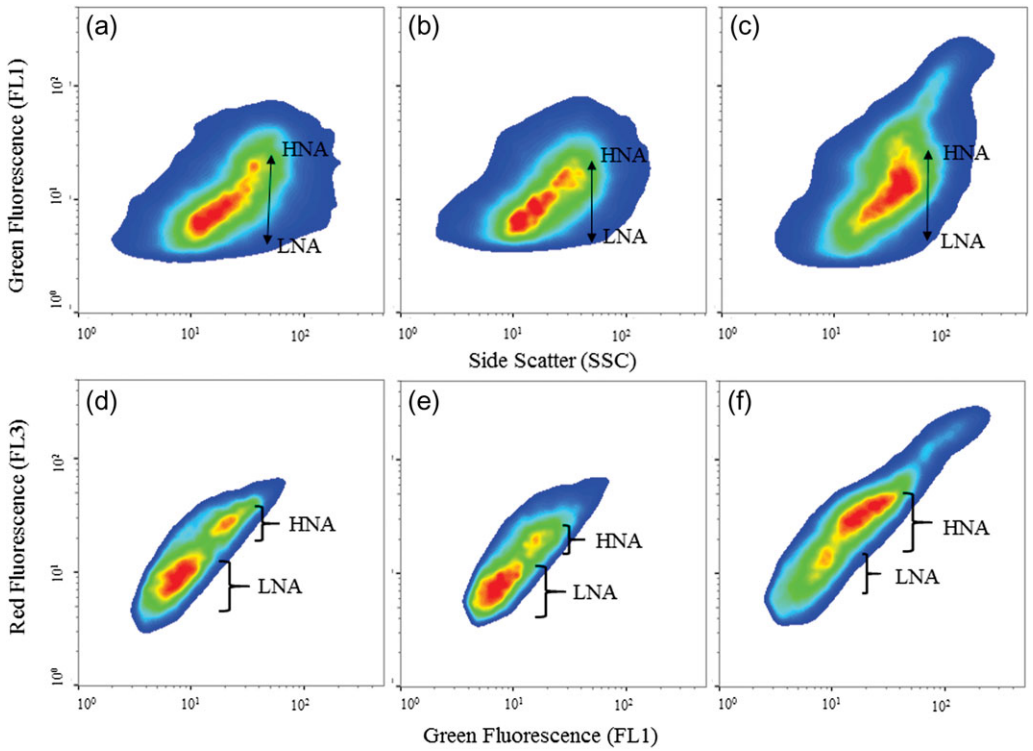


**Fig. 6.** Examples of Syto-13-stained bacteria cytograms of water samples from the beginning of the experiment (**a**,**d**), a predation (**b**,**e**) and a control treatment at the end of the experiment (**c**,**f**), obtained by flow cytometry. Identification of LNA and HNA populations.

in temperate environments, the HNF abundance could be one or two orders of magnitude higher (Segovia *et al.*, 2016). Nevertheless, Tarbe *et al.* (2011) found that HNF were the main grazers of prokaryotic communities in Lake Tanganyika. It is known that ciliates may exert the greatest impacts on bacterial abundance when HNF density is relatively low (Kisand and Zingel, 2000; Zingel *et al.*, 2007). Experiments performed in a temperate reservoir pointed to a higher contribution of ciliates smaller than 20 μm to total bacterivory (e.g. *Cyclidium glaucoma*; Tadonléké *et al.*, 2005). Oligotrichous ciliates, that dominated the ciliate community in our experiment (~97% of total abundance; mainly Tintinnids and *Rimostrombidium lacustris*), are recognized as the greatest bacterivores within the ciliate community (Stabell, 1996; Šimek *et al.*, 2000). Comparing the reported clearance rates of bacteria by HNF (5–31 bact $HNF^{-1}$ $h^{-1}$ in Šimek *et al.*, 2000 and 4–15.4 bact $HNF^{-1}$ $h^{-1}$ in Unrein *et al.*, 2007) with those of oligotrichous ciliates (62 bact $cili.^{-1}$ $h^{-1}$ in Kisand and Zingel, 2000 up to 1782-3220 bact $cili.^{-1}$ $h^{-1}$ in Šimek *et al.*, 2000), it is noteworthy that ciliates may greatly exceed the predation impact on bacteria when compared to HNF. In our study, the mean HNF/ciliates ratio in the treatments was 21.3. Therefore, even considering that flagellates were ~21 times more abundant than ciliates, their low-individual clearance rates (mean reported value of 13.85 bact $HNF^{-1}$ $h^{-1}$, thus 294.7 bact $HNF^{-1}$ $h^{-1}$ for 21 HNF compared to a mean reported value of 1688 bact $cili.^{-1}$ $h^{-1}$ per ciliate) would only exceed the importance of ciliates if this ratio was ~125. Thus, considering the extremely high-consumption rates exhibited by ciliates, together with the very low-HNF abundance registered, we suggest that ciliates were likely the group mainly responsible for the effect of predation on bacteria in this tropical shallow lake.

The effect of predation exerted by microcrustaceans is probably a reflection of the consumption by cladocerans on bacteria. This is because copepods are not efficient predators of picoplankton, showing a minimum impact on bacterial communities (Burns and Schallenberg, 1996; Hwang and Heath, 1999; Kim *et al.*, 2000). On the other hand, cladocerans are capable of feeding on a broad range of particle sizes, including bacteria (Geller and Müller, 1981; Knoechel and Holtby, 1986). A study performed in two temperate lakes actually found that the high densities of small cladocerans, namely *Bosmina longirostris*, in one of those lakes resulted in grazing pressures on bacteria comparable to those found in the other lake, which was dominated by large Daphnids (Vaqué and Pace, 1992).

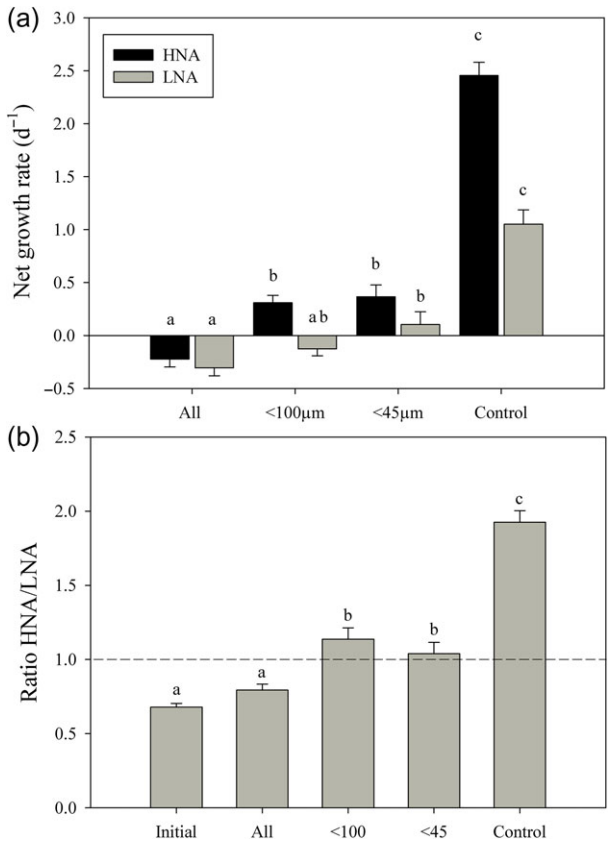


**Fig. 7.** Mean values of HNA and LNA NGRs in the three predator and control treatments (**a**) and mean ratio of HNA/LNA bacterial abundance at initial conditions (beginning of the experiment: 0 h) and at the end of the experiment (24 h) at all the predation and the control treatments (**b**). Bars represent the standard errors. Letters in columns indicate statistical significance—treatments not sharing a letter differ significantly at $P < 0.05$ (Tukey's HSD).

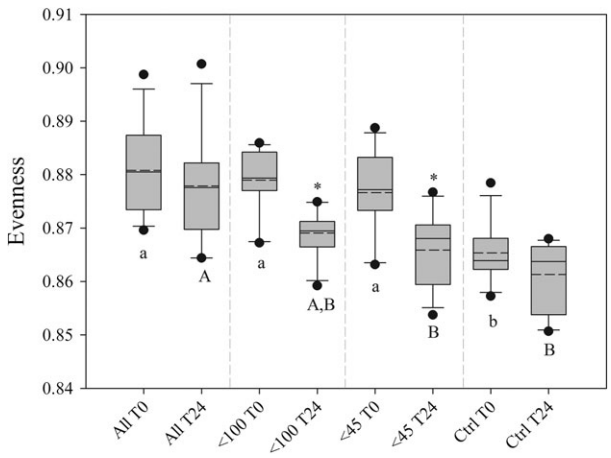


**Fig. 8.** Pielou´s evenness index ($J'$) of each treatment at the beginning (T0) and at the end (T24) of the experiment. Lowercase letters indicate significant differences in T0 among treatments, uppercase letters indicate significant differences in T24 among treatments and asterisks indicate significant differences within the same treatment at T0 and T24.

Indeed, the dominant species found in our experiment, *Bosmina hagmann*i and *Bosminopsis deitersi*, are usually very small (~0.2–0.3 mm; Maia-Barbosa and Bozelli, 2005). Thus, even considering that cladocerans were not as effective as protists in controlling bacterial abundance, they may also be considered important bacterial predators in this tropical environment. Moreover, direct predation on bacteria produces short pathways for the flow of energy and matter, avoiding the losses by ingestion and respiration associated with the passage through the intermediate trophic levels (Lindeman, 1942) of the microbial loop (Ducklow *et al.*, 1986), enhancing the efficiency of carbon transfer through planktonic food webs.

Besides the direct impact on bacteria, microcrustaceans also seem to exert a direct impact on ciliates and rotifers, considering that higher abundances and NGRs of both groups were found after the removal of microcrustaceans from the filtered treatments (Figs S1b and S2). This impact may be the result of a top–down control, which is usually exerted mainly by copepods on rotifers (Brandl, 2005; Miracle *et al.*, 2007) and ciliates (Wickham, 1998; Adrian and Schneider-Olt, 1999; Burns and Schallenberg, 2001). However, it did not result in a trophic cascade in our experiments, because bacterial abundance was actually lower in the unfiltered treatments than in the treatments without microcrustaceans. In contrast, Jürgens *et al.* (1994) found that the removal of microcrustaceans released protists from predation, resulting in higher abundance of protists and, in turn, decreasing bacterial abundance through a cascading effect. However, that experiment lasted 120 h, whereas ours was a short-term experiment (24 h) so that ciliates and rotifers were not able to sufficiently increase their abundances to exert such an impact on bacteria. Short-term incubations are, in general, more realistic, such that the direct predation and trophic cascades observed in our study could be estimated in an additive manner, with less impact of the "bottle effect". Alternatively, microcrustaceans can also indirectly affect protist communities by competing for food resources, since their feeding niches usually overlap (Fenchel, 1980). For instance, competition for bacteria between cladocerans and ciliates likely occurs in freshwater environments, although it does not seem to be as important as the effects of direct predation (Jack & Gilbert, 1994).

We found very little impact exerted by rotifers and nauplii in the predation treatments. Predation rates of rotifers are very low when compared to bacterial growth rates, so that rotifers are usually not able to control bacterial abundance (Arndt, 1993), even when they dominate the zooplankton community (Sommaruga, 1995). Thus, we can infer that rotifers are probably low efficiency feeders of bacterial production (Sanders *et al.*, 1989;

Pace *et al.*, 1990; Vadstein *et al.*, 1993) also in this tropical lake. In fact, secondary production by rotifers was highly correlated to chlorophyll-*a* in lakes of this same floodplain (Dias *et al.*, 2014), which may indicate a preference for herbivory. Nauplii generally prefer larger prey (Finlay and Roff, 2004; Saiz *et al.*, 2014) and may also exhibit a preference towards algae as a food source (Bogdan and Gilbert, 1987; Turner and Tester, 1992).

The positive values of NGR found in the grazing treatments (Fig. 3) show that the predation pressure was not sufficient to control bacterial abundance. This is likely because HNA cells were able to grow fast enough to compensate for the grazing losses (Fig. 7a), thus changing the proportion of HNA/LNA cells (Fig. 7b). Our results differ from those of Pernthaler *et al.* (1996), in which the compensatory growth only counterbalanced the effects of grazing, maintaining similar levels of bacterial abundance and biomass. Sommer (2008) postulated that top–down effects would only affect the size–structure and not the abundance of the organisms if their carrying capacity remains unchanged. Instead, our findings indicate that HNA cells were able to grow and outweigh the grazing losses in the treatments containing predators. Indeed, HNA cells are usually considered as the most actively growing fraction of the community (Gasol *et al.*, 1999; Lebaron *et al.*, 2001, 2002). Our results corroborate this view, since we found higher growth rates for HNA than for LNA bacteria. Moreover, our findings suggest that LNA are also able to grow (Fig. 7a), as reported by other studies (Zubkov *et al.*, 2001; Jochem *et al.*, 2004; Longnecker *et al.*, 2005; Williams *et al.*, 2008; Huete-Stauffer and Morán, 2012), however only in <45 μm and Control treatments, showing that the lower growth rates of this fraction only overcome their loss rates in treatments with fewer or no predators.

The grazer preference of HNA cells by protists has already been observed in several studies (Gasol *et al.*, 1999; Vaqué *et al.*, 2001; Tadonléké *et al.*, 2005; Garzio *et al.*, 2013; Sintes and del Giorgio, 2014; Baltar *et al.*, 2016). The explanation for this selective feeding is that HNA are usually larger (Gasol and Morán, 1999; Baltar *et al.*, 2016) and also more active (Servais *et al.*, 2003; Baltar *et al.*, 2016) than the LNA cells, features long known to be important in the selective grazing by protists (Gonzalez *et al.*, 1990). In a passive manner, cladocerans also exert a higher grazing impact on larger bacterial cells, since they have higher retention efficiency on this fraction of the bacterial community (Güde, 1988; Brendelberger, 1991; Burns and Schallenberg, 1996). Although LNA cells were likely not selected, their much lower growth rates probably hampered their development, consequently preventing a compensatory growth, as proposed by Jürgens and Güde (1994) . Thus, although suffering a greater predation pressure than LNA cells, higher mean values of HNA growth rates were observed in all predation treatments (Fig. 7a), thus HNA cells were responsible for the largest fraction of total bacterial growth (Fig. 3). Nevertheless, the largest proportion of LNA cells in the treatment containing all predators (Fig. 7b) indicate that their small size prevented a heavy predation on this fraction, and contributed to their persistence and dominance, despite their slower growth rates, in situations of strong predation pressure.

Therefore, we found a gradual change in the relative percentage of LNA and HNA as predators were removed, with a shift in the dominance by HNA in the control treatment, under no predation pressure (Fig. 7b). Such a reverse in the HNA/LNA ratio was also found for Gasol *et al.* (1999), who reported the results of two experiments with and without predators. Thus, our results confirm previous findings that the relative proportion of HNA/LNA is a function of the different degrees of predation pressure present in the environments (Tadonléké *et al.*, 2005). In this way, those and our results suggest a modification in the bacterial size–structure in the face of predation (Andersson *et al.*, 1986; Gonzalez *et al.*, 1990; Langenheder and Jürgens, 2001; Corno *et al.*, 2008).

It has been demonstrated recently that the cytometric diversity is highly correlated to bacterial diversity accessed by 16 S amplicon sequencing (García *et al.*, 2015; Props *et al.*, 2016). Considering that HNA and LNA fractions are composed by communities with distinct OTUs (*Operational Taxonomic Unit*; Vila-Costa *et al.*, 2012), it is likely that bacterial composition is also affected by predation in this tropical lake. Indeed, PERMANOVA results showed that composition based on cytometric diversity was distinct between treatments (except between 100 μm and 45 μm). Changes in bacterial community structure were also found in other experimental studies manipulating predators, and have been attributed to differences in predator feeding modes and prey vulnerability (Langenheder & Jürgens, 2001; Zöllner *et al.*, 2003; Corno *et al.*, 2008). Predation exerted mainly by cladocerans results in the dominance of bacteria belonging to the lower end of size classes, with a mean cell volume of 0.08 $\mu m^3$ (Jürgens & Matz, 2002). However, when subject to strong predation pressure exerted by protists, some bacterial taxa are favoured due to their ability to develop grazing-resistant filamentous morphotypes and elongated forms (Pernthaler, 2005). For example, Jürgens *et al.* (1999) found that the removal of metazooplankton led to the dominance of large bacterial rods of the alpha subdivision of *Proteobacteria* and filamentous bacteria belonging to the *Cytophaga-Flavobacterium* cluster. Therefore, changes in the structure of bacterial predators in food webs lead to morphological shifts, which are accompanied by taxonomic changes (Degans *et al.*, 2002).

Furthermore, we found that bacterial community evenness tended to decrease with the removal of predators from the treatments. This agrees with the traditional ecological theories that have shown the important role of predation in maintaining diversity. Paine (1966) stated that predation prevents the dominance of one prey species by restricting their development and hindering resource monopolization. Considering that the treatment with all predators exerted the higher predation pressure (as seen by the lowest bacterial growth rates registered), the successive removal of these predators led to lower predation pressure, and therefore, a decrease in bacterial community evenness. Moreover, the higher bacterial dominance in the treatments containing fewer predators could also be the result of the higher grazing selectivity of protists, which leads to the development of fewer, predator resistant bacterial groups (Pernthaler, 2005).

## CONCLUSION

Altogether, our findings indicate that bacterial loss by predation was a crucial factor controlling bacterial abundance in this tropical shallow lake. Moreover, ciliates were likely the main group responsible for most bacterial loss, thus, bacterial biomass might be an important carbon source for microbial food webs in tropical lakes. Although cladocerans did not exert such an effective control on bacterial abundance, compared to the one exerted by protists, their predation impact suggests a more efficient carbon route, without passing through intermediate trophic levels. Additionally, we found that not only bacterial abundance but also their size–structure and community composition were affected by grazing, resulting in changes in the relative proportion of HNA and LNA cells as a function of different degrees of predation pressure. Community evenness was also affected demonstrating that predation has an important role in maintaining species diversity.

## SUPPLEMENTARY DATA

Supplementary data can be found online at *Journal of Plankton Research* online.

## FUNDING

This study was funded by FAPESP (Processes 2014/14139-3 and 2016/50494-8). B.T.S. and F.E.A. are grateful for the funding and doctoral scholarship provided by the Coordination for the Improvement of Higher Education Personnel (CAPES). We would also like to thank the Brazilian National Council of Technological and Scientific Development (CNPq) for providing doctoral scholarships (B.R.M. and F.M.L.T.) and research productivity scholarships and grants (L.F.M.V.), the Cesumar Institute of Science, Technology and Innovation (ICETI) for support (L.F.M.V.) and the Argentinean Council of Science and Technology (CONICET) for providing the fellowship for young researchers to F.U.

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