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Research Article

RESEARCH ARTICLE

Picocyanobacteria aggregation as a response to predation pressure: direct contact is not necessary

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One sentence summary: The aggregation of single-cell picocyanobacteria into colonies can be induced either by direct or indirect contact with different grazers like flagellates, rotifers and cladocerans.

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ABSTRACT

Picocyanobacteria (cells <2 μm) can be found either as single-cells (Pcy) or embedded in a mucilaginous sheath as microcolonies or colonies (CPcy). It has been demonstrated that phenotypic plasticity in picocyanobacteria (i.e. the capability of single-cells to aggregate into colonies) can be induced as a response to grazing pressure. The effect of the presence of different predators (cladocerans and rotifers) on the morphological composition of picocyanobacteria was studied in a natural community, and it was observed that the abundance of CPcy significantly increased in all treatments with zooplankton compared with the control without zooplankton. The aggregation capability was also evaluated in a single-cell strain by adding a conditioned medium of flagellates, rotifers and cladocerans. The proportion of cells forming colonies was significantly higher in all treatments with conditioned medium regardless of the predator. These results suggest that the aggregation of Pcy can be induced as a response to the predation pressure exerted by protists and different zooplankters, and also that Pcy has the capability to aggregate into CPcy even without direct contact with any predator, most probably due to the presence of an infochemical dissolved in the water that does not come from disrupted Pcy cells.

Keywords: picocyanobacteria; grazing resistance; aggregation; eutrophic shallow lakes

INTRODUCTION

Picocyanobacteria (i.e. cyanobacteria smaller than 2 μm) are an important component within the pelagic communities in both freshwater (Stockner and Shortreed 1991; Weisse 1993; Stockner, Callieri and Cronberg 2000; Sánchez-Baracaldo, Handley and Hayest 2008; Camacho *et al.* 2009; Callieri 2017) and marine environments (Li 1995; Partensky *et al.* 1996; Agawin, Duarte and Agustí 2000).

In natural communities, picocyanobacteria exist as single-cells (Pcy) or embedded within a mucilaginous sheath as microcolonies or colonies (CPcy). Some evidence suggests that certain genera of CPcy (e.g. *Aphanothece*, *Aphanocapsa*, *Cyanodictyon*) could have unicellular stages in their life cycle (Komárková and Šimek 2003), and that microcolonies could be transitional forms between single-cells and colonial morphotypes (Callieri, Cronberg and Stockner 2012). It is well documented that the phenotypic plasticity in picocyanobacteria, i.e. the capability of single-cells to aggregate into colonies, is tied to environmental conditions (e.g. Crosbie, Pöckl and Weisse 2003; Jezberová and Komárková 2007; Komárek *et al.* 2014; Huber *et al.* 2017).

Among the environmental variables that would induce picocyanobacteria to change from one morphotype to another, light

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conditions in the water and grazing have been identified as the most important factors. For example, it has been shown that some picocyanobacterial strains were stimulated to form colonies when they were exposed to different conditions of UV radiation to prevent photodamage (Callieri, Lami and Bertoni 2011). Jezberová and Komárková (2007) and Callieri *et al.* (2016) demonstrated that the development of colonies could be stimulated by the presence of a mixotrophic flagellates as an adaptation to escape from predation.

Grazing pressure by protists (i.e. flagellates, ciliates) induces morphological changes in heterotrophic bacteria as well (Hahn and Höfle 2001; Matz, Deines and Jürgens 2002; Corno 2006). The development of grazing-resistant morphotypes, such as filamentous, spiral-shaped or aggregated, seems to be a common phenomenon in more productive environments (Güde 1989; Jürgens and Güde 1994) and can be found in the major phylogenetic groups such as alpha- and beta-Proteobacteria, and the Cytophaga-Flavobacterium phylum (Jürgens *et al.* 1999; Šimek et al. 1999, 2001; Corno and Jürgens 2006).

Most studies dealing with phenotypic changes as a response to grazing pressure by zooplankton have been focused on large cladocerans like *Daphnia* spp. (Jürgens and Matz 2002, and references therein). However, it is known that rotifers and small cladocerans, that usually dominate in eutrophic systems, might also graze upon bacterial-size particles (Hwang 1999; Work and Havens 2003). Consequently, they could also theoretically induce morphological changes in picocyanobacteria.

In the Pampa region of Argentina (South America), there are thousands of eutrophic shallow lakes where *Daphnia* spp. is virtually absent due to the high density of zooplanktivorous fish (Colautti *et al.* 2015). Therefore, zooplankton is dominated by rotifers and, in certain periods, by small cladocerans belonging to the genera *Bosmina* and *Moina* (Diovisalvi, Rennella and Zagarese 2015a; Diovisalvi *et al.* 2015b). Picocyanobacteria dominate phytoplankton biomass in many lakes from this region and the proportion of cells forming colonies (CPcy) ranges from 0 to 80% (Izaguirre *et al.* 2014; Fermani *et al.* 2015; Huber *et al.* 2017). Interestingly, higher values were associated with higher *Bosmina* spp. abundance. Moreover, Huber *et al.* (2017) experimentally demonstrated that the size of picocyanobacteria colonies increases in the presence of *Bosmina* spp. However, there is no evidence regarding the role of rotifers in the phenotypic structure of the picocyanobacterial community.

Another information gap to fill is whether the formation of aggregates in picocyanobacteria could be induced without direct contact between prey and predators (i.e. mediated by infochemicals). Some evidence suggests that heterotrophic bacteria belonging to Bacterioidetes and alpha-Proteobacteria can sense diffusible chemical cues secreted by protozoan predators and respond by forming inedible filaments or microcolonies, respectively (Corno and Jürgens 2006; Blom *et al.* 2010). Therefore, the same response could be expected for picocyanobacteria.

The aim of the present work was to compare the effect of different predators (flagellates, rotifers and cladocerans) on the morphological composition of picocyanobacterial natural communities and to evaluate the effect of infochemicals produced by these predators on the ability of a Pcy strain to form colonies. We hypothesized that (H1) all the potential grazers can stimulate colony formation in single-cell picocyanobacteria, although *Bosmina* would induce the strongest response (i.e. a higher proportion of aggregated cells than other grazers), and (H2) prey–predator direct contact is not necessary to induce the aggregation of single-cell picocyanobacteria into colonies.

MATERIALS AND METHODS

In order to test our hypothesis, we conducted two experiments exposing both (i) a natural planktonic community to the direct contact of different grazers, and (ii) a strain of Pcy to prey–predator indirect contact by adding a conditioned medium of different grazers.

The natural planktonic community and the strain of Pcy came from Chascomús Lake (35° 36'S, 58° 02'W), located in the Pampa region of Argentina. This lake is relatively large (30.1 km^2), shallow (~2 m depth), hypertrophic (mean chlorophyll-a 250 $\mu g\ L^{-1}$) and turbid (mean Secchi depth 10 cm) (Torremorell *et al.* 2007; Diovisalvi *et al.* 2010).

Phytoplankton is composed of cyanobacteria, chlorophytes and diatoms, although colonial and single-cell picocyanobacteria dominate (Huber *et al.* 2017). The abundance of heterotrophic flagellates (mean: 3.5×10^4 individuals (ind.) mL^{-1}) and ciliates (427 ind. mL^{-1}) does not show a clear seasonal pattern (Fermani *et al.* 2013).

Strains isolation and culture conditions

The strain of picocyanobacteria (CH-040) was isolated from Chascomús Lake and corresponds phylogenetically to Clade PV of the genus *Synechococcus* based on 16S RNA information (Huber 2017). It was maintained in BG-11 (blue-green) medium (Rippka 1988), which is specific for freshwater cyanobacteria, at 20°C, 11 $\mu mol\ m^{-2}\ s^{-1}$ and a 12:12 h light–dark cycle photoperiod.

The zooplankton used in the experiments was also isolated from this lake. Several individuals of *Keratella tropica* (rotifer), *Brachionus havanaensis* (rotifer) and *Bosmina huaroniensis* (cladoceran) were manually selected and transferred to a glass container moderately hard synthetic freshwater medium (see Table 6 in Weber 1993, hereafter mentioned as EPA medium from the acronym of Environmental Protection Agency of United States). All cultures were fed with *Chlamydomonas reinhardtii* Dangeard, 1888, cultured in MWC (Modified Wright's Cryptophyte) medium (Guillard and Lorenzen 1972).

The culture of the mixotrophic flagellate *Ochromonas tuberculata* (CCAP 933/27) was obtained from the Algae and Protozoa Culture Collection (www.ccap.ac.uk). This strain was proved to be phagotrophic (Parry *et al.* 2001). It was maintained in MWC medium at 20°C, 18 $\mu mol\ m^{-2}\ s^{-1}$ and 12:12 h light–dark cycle photoperiod. The culture was not axenic and the abundance of bacteria fluctuated between 2 and 5×10^6 cells mL^{-1} which was sufficient food for the flagellates throughout the experiment. Considering that many mixotrophic chrysophytes do not survive under complete darkness (e.g. Caron *et al.* 1993; Keller *et al.* 1994), we opted to grow the culture under low light intensity. Under these conditions, and using FLB (fluorescently labeled bacteria), we estimated a cell-specific grazing rate of around 1 bacterium $flagellate^{-1}\ h^{-1}$ (Costa, pers. comm.) that should be enough to allow the potential production of infochemicals.

Experimental setup

Experiment with natural planktonic community and direct prey–predator contact

The experiment consisted of four treatments: Control, containing only prefiltered water (i.e. with all the planktonic communities except zooplankton); WZ, with addition of a mix of zooplankton representative of the natural conditions at the sampling date; Bos, with addition of *Bosmina huaroniensis;* and Kt, with addition of *Keratella tropica*.

The water was collected from Chascomús Lake on 31 October 2017, and it was prefiltered through a 45 μm mesh to remove the zooplankton. Sixteen 10 mL glass containers were filled up to the top with prefiltered lake water. Five of them corresponded to the Control, five to the WZ treatment, three to Bos and three to Kt treatment. Each glass container for Bos treatment was filled with 10 individuals of *B. huaroniensis* to reach an abundance of 1000 ind. L^{-1}, while for Kt treatment 50 individuals of *K. tropica* were added to reach an abundance of 5000 ind. L^{-1}. These values correspond to the maximum recorded abundance of cladocerans and rotifers in the lake (Fermani *et al.* 2013; Diovisalvi *et al.* 2015b; Huber *et al.* 2017). These individuals were manually isolated from the initial culture and kept without any type of food for 24 h prior to the experiment. Experimental containers of WZ were filled with a zooplankton concentrate from Chascomús Lake. On average, each experimental unit contained 14 cladocerans (*Bosmina longirostris* and *B. huaroniensis*), 47 rotifers (*K. tropica, Keratella americana* and *Filinia* sp.) and 7 copepods (calanoids and ciclopoids). Cladocerans concentration was a bit higher than that reported for Bos treatment, while the final concentration of rotifers was very similar to the abundance in Kt treatment.

All glass containers were incubated in a plankton wheel for 6 days at 20°C, 18 μmol m^2 s^{-1} and a 12:12 h light–dark cycle photoperiod. Samples for counting picocyanobacteria, heterotrophic flagellate and ciliate abundance were collected at the beginning (T_0) and at the end (T_f) of the experiment. The final time was selected based on previous experiments where a clear response was observed after this incubation time without mortality of zooplankters (Huber *et al.* 2017). Only the initial and final times were sampled since sampling at intermediate times could have removed the zooplankters, modifying the number of grazers during the experiment and, consequently, altering the results.

Two types of samples were collected at each time: samples preserved with P+G (1% paraformaldehyde + 0.05% glutaraldehyde final concentrations) and samples fixed with 2% acidified Lugol's iodine solution.

Picocyanobacteria abundance was estimated by epifluorescence microscopy. Samples preserved with P+G were filtered through a 0.22 μm pore-size black polycarbonate filter following the standard 4.6 diamidino-2-phenylindole (DAPI) staining procedure (Porter and Feig 1980). All samples were diluted (1:30) with 0.22 μm prefiltered lake water due to the high amount of organisms and suspended particulate matter. Details of sample processing for epifluorescence are described in Fermani *et al.* (2013). For the dilution step, a final volume of 40 μL from the original water sample was filtered. The picocyanobacteria were observed under green light excitation at a magnification of 1000× in a Nikon Eclipse 80i epifluorescence microscope as described in Fermani *et al.* (2013). Different cyanobacterial morphotypes were defined based on the number, shape and size of the cells following the classification by Huber *et al.* (2017) and summarized in Supplementary Table S1, see online supplementary material. Epifluorescence counting was performed to determine the abundance (cells mL^{-1}) of Pcy, microcolonies and short trichomes. Also, the number of cells per colony of *Aphanocapsa*-like, *Cyanodictyon* sp. and *Eucapsis* sp. was estimated on these samples.

The sizes of cells were estimated by image analysis from epifluorescence following the technique of Massana *et al.* (1997) and using Image-Pro Plus 4.5 software (Media Cybernetics).

The number of colonies per milliliter (i.e. *Aphanocapsa*-like, *Cyanodictyon* sp. and *Eucapsis* sp.) was estimated by inverted microscope on samples fixed with acidified Lugol's iodine solution. In this case, samples were diluted 1:50 and chambers of 10 mL were allowed to sediment for 24 h. A counting error of <20% was accepted in estimating the abundance (Venrick 1978). Details of the protocol are described in Iachetti and Llames (2015). The number of cells per mL of colonial morphotypes was estimated by multiplying the colonies per mL (estimated with inverted microscope) by the number of cells per colony (estimated with the epifluorescence microscope).

Heterotrophic flagellates were enumerated by epifluorescence microscopy. Samples preserved with P+G were diluted 1:30 and 3 mL were stained with DAPI and filtered through a 0.8 μm pore-size black polycarbonate filter, following the same procedure as described above for autotrophic picocyanobacteria. For the dilution step, a final volume of 100 μL from the original water sampled was filtered. Heterotrophic flagellates were observed under UV and blue light excitation at a magnification of 1000×. A minimum of 100 flagellates per sample were counted.

Ciliates were enumerated on samples fixed with acidified Lugol's iodine solution. The high concentration of particulate matter present in lake water made it difficult to count under the inverted microscope, therefore 6 mL of subsamples were bleached with a few drops of thiosulfate and subsequently fixed with 2% formalin (Macek, Pestová and Martínez Pérez 2008). Afterwards, between 0.5 and 1 mL was stained with DAPI, gently filtered through a 2 μm pore-size black polycarbonate filter (MSI) (Sherr and Sherr 1993) and mounted following the same procedure as described above. Filters were examined at 1000× magnification using an epifluorescence microscope under blue light and UV excitation (Fermani *et al.* 2013). A minimum of 400 fields were counted.

The *in situ* zooplankton abundance of WZ treatment at T_0 was determined by pouring 50 L of lake water through a 45-μm mesh and this filtered water was preserved in 4% formalin. Rotifers were counted under a compound microscope on a 1-mL Sedgwick–Rafter counting cell. Cladocerans were identified under a dissecting microscope in a 5-mL Bogorov counting chamber. The zooplankton of all treatments was counted at T_f to evaluate the survival rate.

Experiment with a strain of Pcy and indirect prey–predator contact

For this experiment the picocyanobacteria strain was maintained with BG-11 medium (Stanier *et al.* 1971; Rippka 1988) in a semi-continuous culture at exponential growth phase in order to minimize the number of clumped cells. The experiment was run in triplicate using glass containers of 10 mL of capacity. A total of 18 glass containers were filled with 8 mL of picocyanobacteria culture and 2 mL of conditioned medium (for the different treatments) or culture medium (for Controls), setting the initial abundance of picocyanobacteria to $\sim 2 \times 10^6$ cells mL^{-1}.

Four treatments with conditioned medium were established. For this purpose, the culture in which each predator was grown was filtered using a 0.22 μm membrane filter (Biofil™). The cultures used contained *B. huaroniensis* (Bos) with an initial abundance of 1.2×10^3 individuals L^{-1}, *K. tropica* (Kt) with 3×10^3 ind. L^{-1}, *B. havanaensis* (Bh) with 3×10^3 ind. L^{-1} and *O. tuberculata* (Och) with 3.8×10^7 cells L^{-1}. Additionally, two different Controls were included. One with the addition of BG-11 medium, the medium used to grow the picocyanobacteria (Control-BG11), and another with the addition of EPA modified medium, the medium used for the cultivation of zooplankters (Control-EPA).

Besides *Bosmina*, which have an impact on the Pcy–CPcy dynamic (Huber *et al.* 2017), and rotifers, which dominate zooplankton in the lake (Diovisalvi *et al.* 2015b), we also included a phagotrophic protist since they are usually considered the main bacterivores in lakes. Therefore, we decided to use *Ochromonas*

as they feed on Pcy and induce a change in their morphology when they are grown together (Jezberová and Komárková 2007).

Samples (1 mL) were taken at the beginning of the experiment (T_0) and after 24, 48 and 96 h of incubation for determining the abundance of picocyanobacteria. On each occasion, the volume removed was replaced with conditioned medium or culture medium depending on the treatment/Control. Glass containers were placed in a plankton wheel using the same conditions of light and temperature as for the first experiment. Picocyanobacteria (Pcy and CPcy) were enumerated using an epifluorescence microscope. The procedure was similar to the previously described experiment; the only difference was that the dilution used was different depending on changes in picocyanobacterial abundance along the time course of the experiment.

Data analysis

Statistical analysis was performed with the Infostat statistics analysis program (Di Rienzo *et al.* 2019), which connects to the R environment through an integrated interpreter. In order to compare all treatments against the Control, a one-way ANOVA or a Kruskal–Wallis test was carried out for the first experiment, depending on whether the data for analysis were parametric or non-parametric. An unbalanced design was planned for this experiment because we increased the number of replicates in WZ, and therefore also in the Control, in order to control the variance. When the ANOVA or Kruskal–Wallis results showed that the variations were significant ($P < 0.05$), we tested for significant differences among treatments by *post hoc* comparisons using a Tukey's test. For the second experiment we ran a generalized linear mixed model (GLMM) analyzing two types of effects, i.e. the effect of the treatments on picocyanobacteria and the effect of time on the treatments.

RESULTS

Experiment with natural planktonic community and direct prey–predator contact

At the beginning of the experiment (T_0) the abundance of picocyanobacteria was 1.3×10^7 cells mL^{-1}, of which 34% were single-cell forms (Pcy) and the rest appeared to form different types of aggregates (CPcy) (Fig. 1A, Supplementary Table S1): 62% corresponded to different types of colonies and microcolonies and 4% to short trichomes. Cell morphology of Pcy and CPcy looked very similar under the microscope, all of them were phycocyanin-rich, without aerotops (gas vesicles), and with almost identical cell-sizes of 1.07 ± 0.3 μm (Supplementary Table S1). Among the CPcy, three main different morphotypes were identified: *Cyanodictyon* sp. with an initial abundance of 6×10^6 cells mL^{-1}, *Aphanocapsa*-like with 4.8×10^5 cells mL^{-1} and *Eucapsis* sp. with 1.3×10^5 cells mL^{-1}. Microcolonies, without a defined colonial morphology, were observed as well (3.6×10^5 cells mL^{-1}). Additionally, other aggregates were also identified with fairly similar cell morphology, such as *Merismopedia* sp. (1.2×10^5 cells mL^{-1}) and *Rhabdoderma* sp. (1.4×10^5 cells mL^{-1}).

After 6 days of incubation (T_f) the total number of picocyanobacterial cells (Pcy+CPcy) did not decrease with respect to the initial abundance (9.2×10^6 cells mL^{-1}). While the abundance of Pcy was significantly ($P < 0.05$) lower in all treatments (~1.5×10^6 cells mL^{-1}) compared to the Control (3.5×10^6 cells mL^{-1}), the abundance of CPcy cells was significantly higher

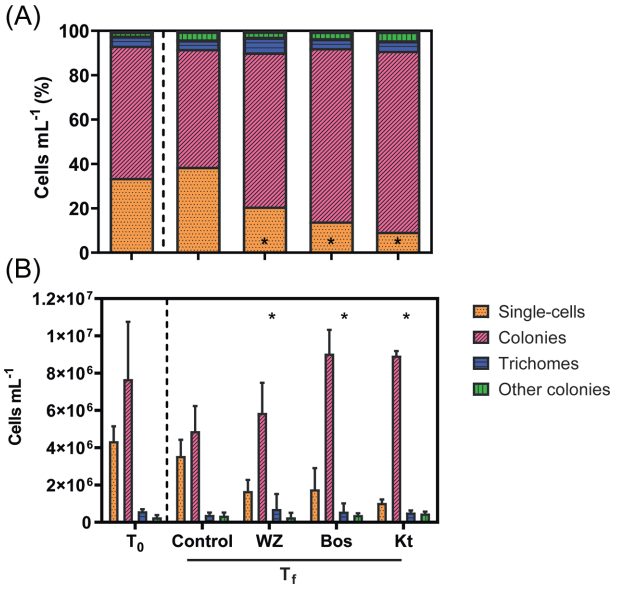

Figure 1. Relative contribution **(A)** and absolute abundance **(B)** of picocyanobacteria morphotypes at the beginning (T_0) and after 6 days of incubation (T_f) in the experiment with the natural planktonic community. Colonies include microcolonies, *Aphanocapsa*-like, *Eucapsis* sp. and *Cyanodictyon* sp. Error bars indicate standard deviation. Asterisks indicate statistically significant differences ($P < 0.05$).

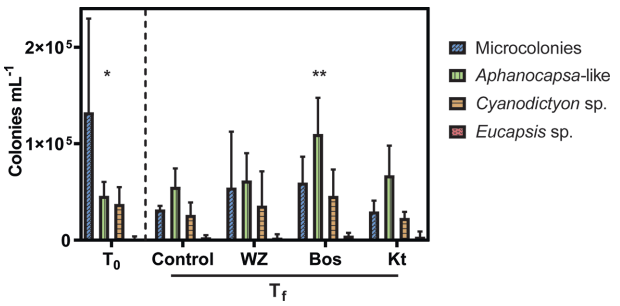

Figure 2. Abundance of the main morphotypes of CPcy at the beginning (T_0) and after 6 days of incubation (T_f) in the experiment with the natural planktonic community. Error bars indicate standard deviation. Asterisks indicate statistically significant differences ($P < 0.05$).

(Fig. 1B; Supplementary Fig. S1, see online supplementary material). No significant differences were observed among the treatments with different predators (i.e. WZ, Bos, Kt). The relative abundance of Pcy and CPcy cells showed the same trend. Pcy increased to 39% in Control, whereas in treatments containing zooplankton it decreased significantly ($P < 0.05$), reaching 21, 14 and 9% in WZ, Bos and Kt treatments, respectively (Fig. 1A). Regarding cells forming colonies or aggregates (CPcy), there was a subtle (not significant) decline in Control from 59% at T_0 to 53% at T_f. Contrarily, a significant increase ($P < 0.05$) in abundance of CPcy was observed in all treatments with zooplankton: 69% in WZ, 78% in Bos and 82% in Kt.

Differences in CPcy morphotypes among treatments were mainly observed in *Aphanocapsa*-like (Fig. 2). In Bos treatment, the abundance increased significantly by doubling the number of colonies per mL ($P < 0.05$). On the other hand, microcolonies tended to decrease in all treatments compared with T_0.

The number of cells per colony did not vary significantly among treatments in most CPcy morphotypes ($P > 0.05$), microcolonies (average 4.5 cells colony^{-1}), short trichomes (average 4.3

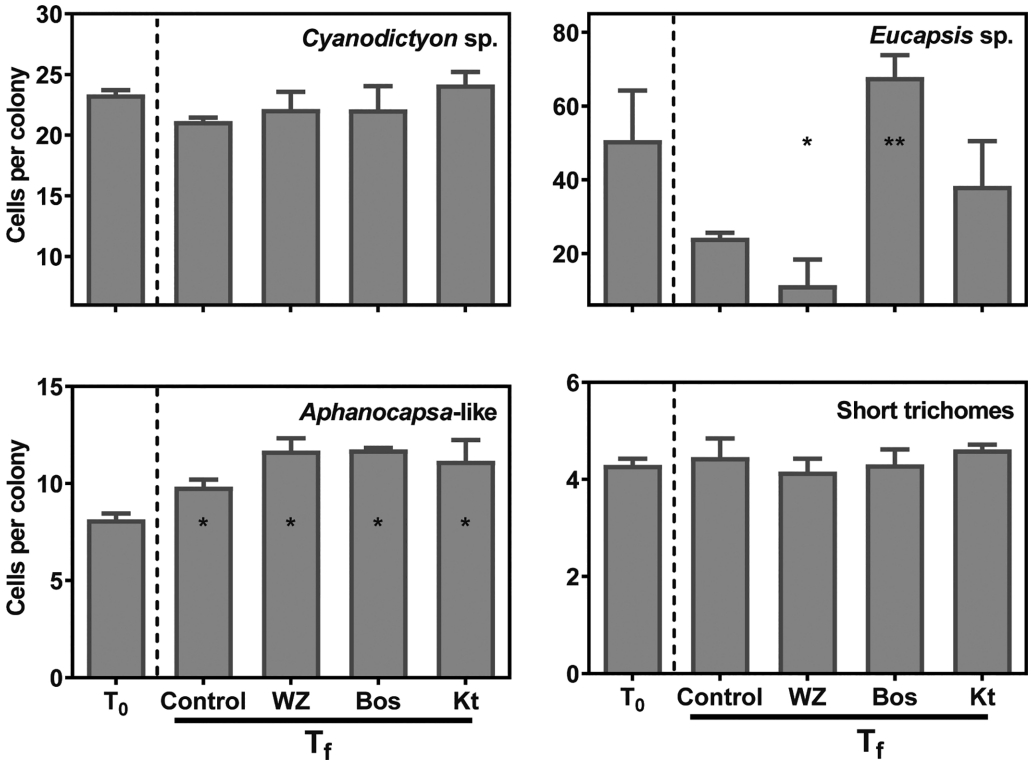

Figure 3. Number of cells per colony of the main CPcy morphotypes at the beginning (T_0) and after 6 days of incubation (T_f) in the experiment with the natural planktonic community. Error bars indicate standard deviation. Asterisks indicate statistically significant differences ($P < 0.05$); * and ** (in *Eucapsis* sp.) indicate significant differences between WZ and Bos treatments.

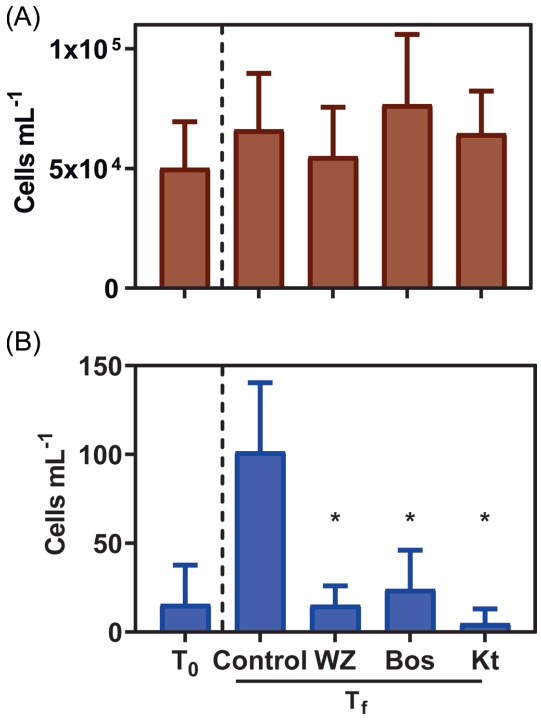

Figure 4. Abundance of flagellates **(A)** and ciliates **(B)** at the beginning (T_0) and after 6 days of incubation (T_f) in the experiment with the natural planktonic community. Error bars indicate standard deviation. Asterisks indicate statistically significant differences ($P < 0.05$).

cells colony^{-1}) and *Cyanodictyon* sp. (average 22.5 cells colony^{-1}). *Aphanocapsa*-like increased significantly ($P < 0.05$) from 8.1 cells colony^{-1} at T_0 to more than 11 cells colony^{-1} in Kt, WZ and Bos at T_f (Fig. 3). Significant differences in the average colonial size of *Eucapsis* sp. were observed in WZ ($P < 0.05$) which decreased to 11.4 cells colony^{-1} and in Bos which reached 67.8 cells colony^{-1}.

The *in situ* abundance of heterotrophic flagellates was 5.2 × 10^4 flagellates mL^{-1}. No significant differences ($P > 0.05$) were observed among treatments and Control at T_f (Fig. 4A). Ciliate initial abundance was 12 ciliates mL^{-1}. At the end of the experiment, the abundance was significantly higher in the Control (mean: 102 ciliates mL^{-1}) compared with the other treatments ($P < 0.05$) (Fig. 4B). The zooplankton community in the lake was mostly represented by small cladocerans (*B. longirostris* and *B. huaroniensis*) and rotifers (*K. tropica*, *K. Americana*, *B. havanaensis* and *Brachionus caudatus*), while copepods were the least abundant group (Supplementary Table S2, see online supplementary material). At the end of the experiment, zooplankton survival was ~75% for Bos and ~100% in Kt treatments.

Experiment with a strain of Pcy and indirect prey–predator contact

The total number of cells (Pcy+CPcy) at the beginning of the experiment was 1.7 × 10^8 cells mL^{-1}. The abundance of picocyanobacteria increased 2–3-fold in all treatments containing conditioned medium and remained fairly constant in controls. At the beginning of the experiment, ~20% of the cells appeared aggregated. However, this percentage decreased to 11 and 14% in Control-BG11 and Control-EPA, respectively, after 4 days of incubation (Figs. 5 and 6). Differences between Controls were

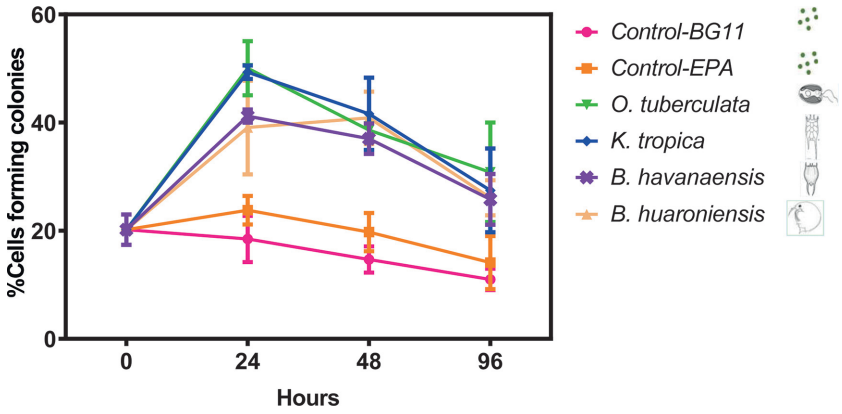

Figure 5. Percentage of picocyanobacteria cells forming aggregates in each treatment and Controls during the 4 days of incubation in the experiment with conditioned medium. Error bars indicate standard deviation.

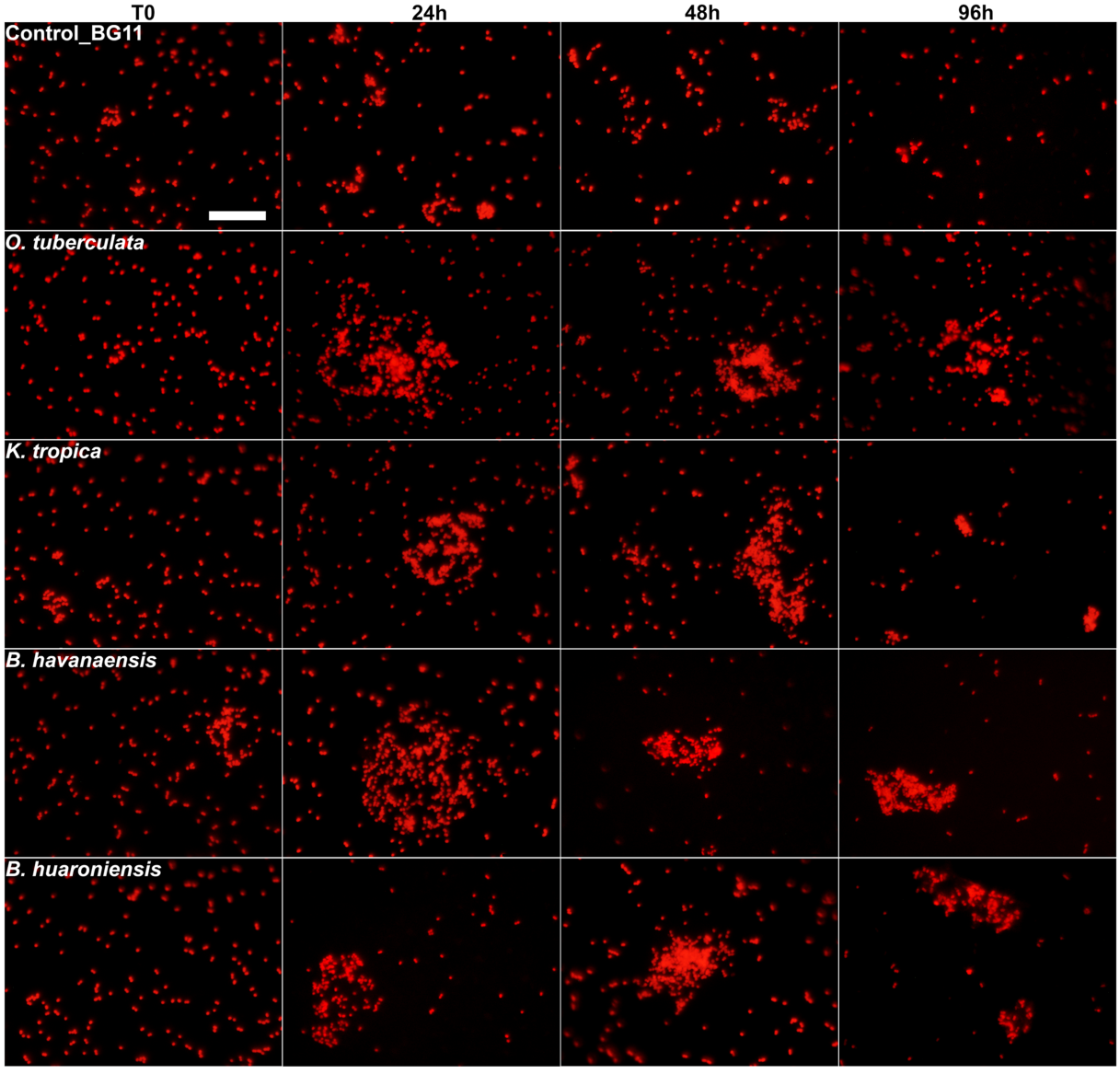

Figure 6. Epifluorescence micrographs under green light excitation of the Pcy strain in each treatment and at different incubation times (T_0, 24, 48 and 96 h) in the experiment with conditioned medium. Scale bar, 20 μm.

not significant at any time ($P > 0.05$). Contrarily, the four treatments containing conditioned medium increased significantly the percentage of aggregated cells (Fig. 5), as well as the size of the colonies (Supplementary Fig. S2, see online supplementary material). After a day of incubation, cells forming aggregates surpassed 40% in all cases. At 48 h of incubation, there was a slight decrease in the majority of treatments with conditioned medium, although differences compared with the Controls ($P < 0.05$) were found throughout the course of the experiment (24, 48 and 96 h). No significant differences ($P > 0.05$) were observed among treatments with conditioned medium. In addition, no significant differences were found among sampling times ($P > 0.05$).

DISCUSSION

It is well established that the aggregation of single-cell microorganisms offers refuge against grazers (Pernthaler 2005; Stal 2017). For example, some eukaryotic phytoplankton like the green algae *Scenedesmus* (Verschoor *et al.* 2004), as well as different groups of heterotrophic bacteria, form microcolonies (Matz *et al.* 2004) or aggregates (Blom *et al.* 2010; Corno, Villiger and Pernthaler 2013) as a defense strategy to avoid predation. In picocyanobacteria, Jezberová and Komárková (2007) and Callieri *et al.* (2016) demonstrated that the development of aggregates could be stimulated by the presence of a mixotrophic flagellated chrysophyte. However, in these latter experiments, prey and predators were placed in the same experimental container. In the present work, we experimentally confirm the aggregation of single-cell picocyanobacteria into colonies as a strategy of defence against grazers and also demonstrate that direct contact between prey and predator is not necessary to stimulate the aggregation of Pcy into CPcy cells, confirming hypothesis H2. Additionally, our results suggest that different predators such as zooplankters (i.e. rotifers, small cladocerans) and nanoflagellates could trigger the same response.

The planktonic colonial cyanobacteria *Microcystis aeruginosa* generally grow as single-cells under culture conditions. However, it has been shown that their cells aggregate when treated with the microcystin toxin that the same species produces (Sedmak and Eleršek 2006), with disrupted *Microcystis* cells (Becker 2010) or with spent *Daphnia* medium (Becker 2010). Contrarily, other experiments with the green algae *Scendesmus obliquus* and the rotifer *Brachionus calyciflorus* demonstrated that induction of colony formation in *S. obliquus* occurs through chemicals that are released by grazing, and not through chemicals produced by a predator or prey alone (Verschoor, Zadereev and Mooij 2007). In our case, the response was observed using conditioned medium from different zooplankters (feeding on *Chlamydomonas* sp.) and from *O. tuberculata* (maintained in a non-axenic culture medium feeding on bacteria), which suggests that infochemicals do not come from disrupted Pcy cells. In accordance with our results, Corno and Jürgens (2006) used dialysis bags to demonstrate that a *Flectobacillus* (Bacteroidetes) strain can sense diffusible chemical cues secreted by protozoan predators and respond by forming inedible filaments. Interestingly, this phenotypic change response was induced when the flagellate grazed on *Flectobacillus* or on *Pseudomonas putida* (Proteobacteria).

Some studies addressing the role of *Daphnia* in the formation of colonies of different algae suggested that the evolution of infochemicals in this phenotypic response could be a non-volatile organic cue with a low molecular weight (Von Elert and Franck 1999; Van Holthoon *et al.* 2003). Similarly, Yasumoto et al. (2005, 2008) studied, by chromatography, the infochemical released by *Daphnia* in the presence of *Scenedesmus* and found eight different types of aliphatic sulfates. However, to date there is no proof that these chemicals are released by live daphnids and by other grazers (e.g. rotifers, flagellates). More studies are needed to unravel the exact composition of the chemical cues responsible for defense induction in each particular species.

Most experiments performed to evaluate the phenotypic plasticity of bacteria and picocyanobacteria in response to grazing have been focused on protists, in particular flagellates. This is because heterotrophic and mixotrophic flagellates are commonly considered to be the main picoplankton grazers in pelagic waters (Sherr and Sherr 2002; Pernthaler 2005), whereas the effect of larger zooplankters is usually underestimated or considered to be of secondary importance (Callieri, Cronberg and Stockner 2012), despite picoplankton being within the prey size preferred by small-sized zooplankters. Previous evidence showed that *Bosmina* feed on prey <19 μm (Burns 1968, Gliwicz 1969). In particular, some estimations indicated that these small cladocerans prefer prey between 1.5 and ~5 μm (Ross and Munawar 1981; Geller and Müller 1981). Also, different species of the rotifer *Keratella* seem to prefer particles of ~0.5–2 μm (Ooms-Wilms 1997; Ronneberger 1998). Therefore, single-cell Pcy and small microcolonies of few cells are within the size-range of prey preferred by *Bosmina* and *Keratella*.

Here we evaluated the structuring effect of a rotifer (*K. tropica*) and a small cladoceran (*B. huaroniensis*) on a natural community of Pcy–CPcy. Despite the fact that the volume used in the first experiment might have limited the normal behavior of *Bosmina*, the effect on the aggregation of Pcy was indeed observed, since the abundance of single-cell Pcy decreased and the abundance of CPcy cells increased with the presence of different zooplankters (Fig. 1). These results are in agreement with previous work by Huber *et al.* (2017) adding that rotifers can also trigger the same response. Even though this could be explained by a faster removal of small Pcy, thus favouring CPcy dominance, we did not find differences in the total number of picocyanobacteria cells (Pcy+CPcy) between the initial and the final time. Moreover, Huber *et al.* (2017) demonstrated that several genotypes, i.e. ITS (internal transcribed spacer)-16S rDNA sequences, of picocyanobacteria can be found *in situ* either as single cells or as colonies. An indirect effect, mediated through a trophic cascade, could also be a plausible explanation for the aggregation. However, the results of the second experiment showed that the conditioned medium that came from the culture of *Bosmina* induced the aggregation in a Pcy strain as well. These results indicate that grazing by zooplankton could induce *in situ* aggregation of single-cell picocyanobacteria.

In eutrophic shallow lakes, the abundance of rotifers and small cladocerans can be remarkably high (Sommaruga 1995; Jürgens and Jeppesen 2000). In Chascomús Lake more than 5000 rotifers L^{-1} and 1000 cladocerans L^{-1} can be found during certain periods (Fermani *et al.* 2013; Diovisalvi *et al.* 2015b; Huber *et al.* 2017). Particularly, Huber *et al.* (2017) recorded a significant increase in the number of cells per colony of *Cyanodictyon* sp. (from 20 to 70 cells colony^{-1}) after a peak of cladocerans (>1000 ind. L^{-1}). In line with this, a flow cytometric 10-year study of picocyanobacteria dynamics performed in the same lake indicated an increase of red fluorescence (i.e. chlorophyll) and light scatter (i.e. size) during periods of high abundance of small cladocerans, which can be associated with higher abundance, and larger, CPcy (Quiroga, pers. comm.). Based on these previous results, *Bosmina* was expected to induce a stronger response than others grazers (i.e. rotifers). However, contrary to our hypothesis H1,

we found that the presence of all grazers had a similar effect on the proportion of CPcy in a natural community.

It was demonstrated that colony size of *S. obliquus* increased with *B. calyciflorus* infochemical concentration (Verschoor *et al.* 2004). Following this idea, we speculate that the lack of differences among zooplankters in our study could be due to a high infochemical concentration in all the treatments, probably higher than what is commonly found in the lake. Unfortunately, the nature of these chemical signals, how they are released and sensed by the picocyanobacteria, and the doses necessary to trigger a response, is still unclear.

Besides flagellates and zooplankton, small-sized ciliates (<30 μm) are also important picoplankton grazers in more productive waters (e.g. Beaver and Crisman 1989; Šimek et al. 2000, 2019). In Chascomús Lake, ciliate abundance fluctuates from nearly undetectable to >1000 ind. mL^{-1} (Fermani *et al.* 2013) and is usually dominated by species of relatively small size (10–20 μm) mainly represented by *Halteria* spp., *Urothichia* sp. and Tintinnids (Fermani, pers. comm.). In our experiment, only a few taxa of *Halteria* spp. were observed. This genus is able to graze a mean of 40 Pcy $ciliate^{-1}$ mL^{-1} (Zingel *et al.* 2007) and 1–3 × 10^3 bacteria $ciliate^{-1}$ h^{-1} (Šimek *et al.* 2000) in highly productive environments. Even though no experiments were performed to confirm the effect of ciliates on the aggregation of picocyanobacteria, they should proably induce the same response as observed here for other grazers.

In the experiment using the whole natural planktonic community, the abundance of flagellates did not differ between T_0 and T_f in any of the treatments, while ciliates increased in the Control from 12 to 102 ciliates mL^{-1} at the end of the experiment. The absence of zooplankton in the Control would explain this increment. Despite this, the proportion of CPcy only slightly changed between T_0 and T_f, thus flagellates and ciliates were able to maintain a fairly constant proportion of CPcy. This would suggest either that the ciliates do not have the same effect on the aggregation of Pcy like the other grazers, or that the increase in the abundance of ciliates in the Control probably was not high enough to impact significantly on the structure of Pcy–CPcy assemblage.

Complex trophic interactions might have occurred among zooplankters, ciliates and flagellates. All of them are able to feed on picoplankton, but also on other grazers (e.g. rotifers can ingest flagellates). At this point of the discussion it is important to mention that despite us being unable to disentangle all this complexity, the effect of the zooplankton in the structure of picocyanobacteria is evident: high abundance of *Bosmina* and *Keratella* resulted in a higher proportion of CPcy. We cannot rule out that this response could be the consequence of multiple indirect trophic interactions, and probably the aggregation of Pcy could be stimulated indirectly through a cascading effect. Nevertheless, the results of the experiment with conditioned medium suggest that the zooplankton can also induce the aggregation of picocyanobacteria mediated by infochemicals. Therefore, both mechanisms are probably operating in natural systems.

The response of the different CPcy morphotypes varied among treatments. The abundance of colonies of *Aphanocapsa*-like increased when *Bosmina* was added, while *Eucapsis* sp. increased their number of cells per colony. These results seem to contrast with those previously obtained by Huber *et al.* (2017), who observed the most evident response in *Cyanodictyon* sp. However, during their experiment the abundance of *Aphanocapsa*-like and *Eucapsis* sp. was very low (<20% of the CPcy), which limits the detection of a clear response for these morphotypes. Most probably, this apparent discrepancy among experiments could be explained by differences in the initial abundance of the main CPcy morphotypes that changes along the annual cycle, and at the time of this experiment *Aphanocapsa*-like was dominant.

Finally, another point to be considered is that our experiments were carried out with non-axenic Pcy strains. Yang *et al.* (2006) tested the colony-inducing effect in an axenic culture of *M. aeruginosa* when grazed by the flagellate *Ochromonas*, and observed a relatively weak response. Cruz and Neuer (2019) compared the growth of two strains of marine picocyanobacteria (*Synechococcus* and *Phrochlorococcus*) in axenic and non-axenic conditions, and demonstrated that heterotrophic bacteria enhance aggregation in both strains. This evidence suggest that heterotrophic bacteria play a key role in the aggregation of picocyanobacteria. Therefore, future studies should contemplate not only the trophic prey–predator interaction but also the interactions involving the associated prokaryote communities.

CONCLUDING REMARKS

The results obtained in the present work contribute new evidence about the aggregation capability of single-cell picocyanobacteria. We demonstrated for the first time that grazers from different evolutionary lineages could induce the aggregation of single-cell picocyanobacteria, and that direct contact between prey and predator is not necessary. From our experimental design, we could answer key questions to provide further understanding of the phenotypic plasticity of picocyanobacteria, such as those devised by Stal (2017): What causes picocyanobacteria to form aggregates? Are there infochemicals involved in the aggregation of Pcy? Who produces them? The results obtained in this work also open new questions about whether different grazers produce the same infochemical molecules or if during their evolutionary history picocyanobacteria have developed different capabilities to sense them.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of Interest. None declared.

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