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Research Article

RESEARCH ARTICLE

Rotifers weaken the efficiency of the cyanobacterium defence against ciliate grazers

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One sentence summary: The presence of rotifers able to feed on cyanobacterial mucilage led to decreased effectiveness of inducible defence in two ways, by increasing dispersion of the cyanobacterial trichomes, thus loosening cyanobacterial mats, and by ingestion of the exopolysaccharide material covering trichomes.

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ABSTRACT

Cyanobacteria can protect themselves through limited dispersion and by increasing the compactness of the mucilage-covered cyanobacterial mat as well as by producing sheaths covering their trichomes. These features have been used in research to measure their degree of inducible defence. The influence of the presence of the rotifers *Lecane inermis* on the effectiveness of *Phormidium* sp. (Ph2) cyanobacterium defence was investigated. Experiments were conducted on the ciliates *Pseudomicrothorax dubius* and *Furgasonia blochmanni*, specialised in the ingestion of filamentous cyanobacteria. The most compact were cyanobacterial mats that were subjected exclusively to ciliates and the most dispersed were mats in the presence of rotifers alone. The presence of rotifers feeding on cyanobacterial mucilage led to the decreased effectiveness of the defence in two ways, by increasing the dispersion of cyanobacterial trichomes, thus loosening the cyanobacterial mat, and through the ingestion of the exopolysaccharide material covering the trichomes. As a result, in the presence of rotifers and the high density of ciliates, almost all the trichomes were removed. Moreover, in comparison with other treatments, a higher number of ciliates and rotifers remained active until the end of the experiments. This is the first report to show how rotifers can weaken the defence of cyanobacteria.

Keywords: inducible defence; trophic interactions; microbial ecology; *Lecane inermis*; *Phormidium* sp.

INTRODUCTION

Cyanobacteria are one of the oldest organisms living on earth. Owing to their extreme phenotypic plasticity, they can dominate various environments. Gaining a superior position over other organisms, cyanobacteria often form blooms, which are frequently harmful because of their ability to produce toxins (Carmichael 1997; Pearl *et al.* 2001; Huisman, Matthijs and Visser 2005 and literature within). Even though cyanobacteria have many enemies, the top-down effect (predation) on cyanobacteria in water bodies is considered to be weak (Kâ *et al.* 2012; Liu *et al.* 2018; Ger *et al.* 2019 and the literature within). Moreover, due to their very low palatability, these organisms are often treated as a dead end in trophic webs (Gerphagnon *et al.* 2015). However, cyanobacteria have evolved different forms of defence. Some of them are considered as constitutive, such as their filamentous or colonial morphology (Gliwicz and Siedlar 1980; DeMott, Gulati and Van Donk 2001) or the presence of a mucilage matrix, capsules and sheaths (Dodds, Gudder and Mollenhauer 1995; Trabeau *et al.* 2004; Camacho and Thacker 2006).

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The more effective, cost-saving mechanism is inducible defence, which is evoked only in the presence of a predator or a grazer (Tollrian and Harvell 1999). The first report on the inducible defence of cyanobacteria described the ability of *Phormidium* sp. to accelerate the production of sheaths in the presence of a ciliate grazer specialising in filament ingestion. As a direct response to ciliate attacks, cyanobacterium filaments withdrew inside the sheath and thus became inaccessible to the grazers (Fiałkowska and Pajdak-Stós 1997). Cyanobacteria such as *Microcystis aeruginosa* and *Planktothrix agardhii* are able to increase their toxin production in the presence of grazers such as *Daphnia magna* and *Moina macrocopa* (Jang, Jung and Takamura 2007). However, the ciliate *Nassula* sp. was found to graze effectively on a microcystin-producing strain of *Pl. agardhii* and to grow on this type of diet for a prolonged period of time (Combes *et al.* 2013). The density of microcystin-producing *Planktothrix rubescens* is significantly reduced by the protists *Nuclearia delicatula* and *Nuclearia thermophila* (Dirren *et al.* 2017).

In response to ciliate attacks, cyanobacteria are also able to form more compact mats resembling clumps. The acceleration of mucilage production in the presence of grazers helps them to form a barrier to protect the cyanobacterial trichomes that are entangled inside the mat from ingestion by ciliates specialising in the ingestion of filamentous cyanobacteria (Pajdak-Stós, Fiałkowska and Fyda 2001). Similarly, the cyanobacterium *Mi. aeruginosa* was subjected to grazing by flagellates and it responded by synthesising and secreting exopolysaccharides to surround the cyanobacterial cells in the newly formed colonies (Yang *et al.* 2008).

The rotifer *Lecane inermis* was found to feed on biofilms successfully (Sobczyk *et al.* 2013), so we decided to explore if they are also able to graze the mucilage produced by cyanobacteria to defend themselves against ciliate consumers. In a series of experiments, we tested the hypothesis that *Lecane* rotifers weaken the defence of *Phormidium* cyanobacterium against the ciliate grazers *Pseudomicrothorax dubius* and *Furgasonia blochmanni* by grazing on exopolysaccharides. The 'strength' of the inducible defence was assessed on the basis of the mat compactness and the percentage of filament endings covered with empty sheaths.

MATERIALS AND METHODS

Cyanobacteria, ciliate and rotifer cultures

In all the experiments, we used an undetermined strain of *Phormidium* sp. Ph2 cyanobacterium that was described in detail in our previous papers (Fiałkowska and Pajdak-Stós 1997, 2002). For the grazers, we used *Ps. dubius* and *F. blochmanni*, ciliates equipped with a cytopharyngeal basket that specialise in ingesting cyanobacterial filaments. As a potential grazer of cyanobacterial mucilage, the obligatory asexual rotifer *L. inermis*, strain 1.A2.15, was used. As noted in a pilot experiment, this rotifer is not able to feed on filamentous cyanobacteria.

Clonal populations of cyanobacteria and ciliates were obtained from a single filament (*Phormidium*) and single cells (*Ps. dubius* and *F. blochmanni*) isolated from an aquarium maintained at the Institute of Environmental Sciences at Jagiellonian University, Kraków. A culture of the *L. inermis* strain 1.A2.15 rotifer was obtained from an activated sludge sample taken from a municipal wastewater treatment plant located in the Upper Silesia region of Poland.

The *Phormidium* strain was cultured in BG11 medium (Stanier *et al.* 1971) that was prepared according to a protocol obtained from CCAP (Ambleside, UK), and under a 12L:12D light regime. The ciliates were cultured in Żywiec® spring water and fed once a week with small pieces of a *Phormidium* mat and maintained under a natural light cycle. The rotifers were kept in the dark, in Żywiec spring water, and fed once a week with NOVO nutrition powder prepared according to the patented formula (Pajdak-Stós *et al.* 2017). All the cultures were kept at 20 ± 1°C.

All the cyanobacteria, ciliate and rotifer cultures are constantly maintained for future studies within the culture collection of the Aquatic Ecosystems Group at the Institute of Environmental Sciences, at Jagiellonian University.

Experimental setup

To check the influence of *L. inermis* rotifers on the inducible defence of *Phormidium* sp. Ph2 against ciliate grazers, a factorial design using the factors ciliates density (high-500 ind. vs. low-200 ind. vs. absent) and rotifers (present/absent) was prepared. Five replicates per factor-level combination were conducted. The experiments were performed on two different ciliate species (*Ps. dubius* and *F. blochmanni*).

The experiments were conducted in four 24-cell culture test plates, two plates per ciliate species. Twenty wells of each plate were inoculated with small, ~1 × 1 mm pieces of cyanobacterial mat cut with a coverslip from 3–4 week-old *Phormidium* mats. The mats were left undisturbed for ~1 h to ensure their attachment to the bottom. Half of the plates were designated for the experiment with a higher density (500 individuals per mL) and the other half for a lower (200 individuals per mL) density of ciliates. The *Ps. dubius* ciliates taken from a dense culture (~1120 ind./mL) were transferred to an adequate volume of Żywiec (446 μL for higher and 179 μL for lower ciliate densities) in 10 wells per plate. *F. blochmanni* individuals were transferred from a dense culture (~2000 ind./mL) in an adequate volume of Żywiec (250 μL in higher and 100 μL in lower ciliate densities) into 10 wells per test plate. Similarly, *L. inermis* rotifers taken from a dense culture (~4000 ind./mL) were transferred in a volume of 50 μL into 10 wells per plate (five wells were inoculated earlier with ciliates and five contained only mat pieces). Żywiec water and BG11 medium were added to each well to reach a total of 0.5 mL of water and 0.5 mL of BG11. The initial density of each culture was determined by counting the individuals in a volume of 10 μL using an inverted microscope. For each culture, counting was repeated three times.

The experimental plates were kept at 20 ± 1°C for 4 days at a light intensity of 25 μmol m^{-2} s^{-2} under a 12L/12D light regime. Starting from the first day of the experiment, images of the initial cyanobacterial mat were taken every 24 h using the 4× objective of an inverted Olympus IX71 microscope with fluorescence, which was equipped with a PixelLink digital camera and an NIS Elements image analysis system. For the best visualisation of the cyanobacterial mats, chlorophyll autofluorescence of *Phormidium* sp. filaments after green-light excitation was applied. Exposure was set manually on camera settings at the 300 ms level to ensure comparable results. When the dispersion of filaments from the cyanobacterial mat is limited as a response to ciliate attacks, the density of the mat increases and the mat's autofluorescence is more pronounced. To reflect the range of the mat's compactness, we used the mean brightness value for the manually marked area in the initial piece of mat that was measured using the ImageJ image analysis program (Analyse/Histogram).

This software measures the average light intensity of an arbitrarily chosen area from an image and expresses it in absolute values on a scale from 0 for black objects to 255 for white ones. To ensure precision, every measurement was repeated twice.

Additionally, every day, the ciliates among 50 observed individuals that had traces of food in their vacuoles were counted and the percentage of satiated ciliates was calculated. The percentage of satiated ciliates was treated as an indirect measure of cyanobacterial filament availability for the grazers.

On the third day (after 72 h), the percentage of filament endings covered with an empty sheath was calculated based on the direct observation of 50 endings. All the observations were made *in vivo* using an inverted Olympus IX71 microscope with interference contrast.

To compare the abundance of trichomes outside the initial pieces of mats between treatments with ciliates and with or without rotifers on the fourth day of the experiment, the numbers of filaments crossing the edges of the field of view (3850 × 3078 μm) under the 4x objective were counted and the resulting value was called the density factor (DF).

In the experiment with a higher *F. blochmanni* density, no filaments were crossing the edges of the field of view, and when the density of ciliates was lower, the abundance of filaments was so high that it was impossible to count them reliably.

Additionally, the trials using a lower *F. blochmanni* density were prolonged to 11 days because, until the fourth day, almost all the ciliates had visible traces of food in their vacuoles and no differences could be observed in the percentage of satiated ciliates between the treatments with and without rotifers.

At the end of the experiments, the ciliates and rotifers were fixed with a drop of acidic Lugol solution that was added to each well. All the rotifers, ciliates and cysts of ciliates were then counted.

The significance of differences in the numbers of rotifers, ciliates and cyanobacterium trichomes in the trials described above was tested by factorial or one-way ANOVA depending on the number of factors under consideration. Data concerning the percentage of satiated ciliates were arcsine-transformed prior to the analyses. The STATISTCA 12.5 package (StatSoft 2014) was used for the calculations.

RESULTS

At 24 h after the start of the experiment, there were already visible significant differences in cyanobacterial mat compactness as reflected in the mean brightness of the initial piece of mat ($F_{9,64} = 13.59$, $P < 0.001$) with the higher ciliate density (~500 ind. of *Ps. dubius*/mL). The lowest brightness of the mat was noted in the treatment in which only rotifers were introduced into the wells containing Ph2 mats. In this treatment, the dispersal of cyanobacterial trichomes was the most pronounced, and it was even more so than for the cyanobacterial mat alone (Fig. 1A). The filaments were most entangled under the pressure of *Ps. dubius* alone, which displayed slightly brighter fluorescence than that of the mat under the simultaneous pressure of ciliates and rotifers (Fig. 1A). The mean brightness of the cyanobacterial mat decreased gradually in the *Ps. dubius* + *L. inermis* treatment, and on the fourth day of the experiment, it was significantly lower in comparison with the treatment with ciliates alone (Fig. 1A). The compactness of the cyanobacterial mat in the presence of *Lecane* was significantly lower than that of the mat subjected to ciliate pressure. At the end of the experiment, the mean autofluorescent brightness of all the mats except the *Ps. dubius* + *L. inermis* treatment increased slightly, most likely due to the growth of cyanobacteria inside the mat.

At the high *F. blochmanni* density (500 ind./mL), similar to the experiments on *Ps. dubius*, the highest autofluorescent brightness of the initial piece of cyanobacterial mat was noted in the treatment on mats and ciliates alone, and this value gradually increased during the days that followed (Fig. 1B). By contrast, in the treatment involving ciliates and rotifers, the mat compactness reflected in the mean autofluorescent brightness was already weaker after 1 day and the density of the mat decreased gradually until the end of the trial. The trichomes in the undisturbed cyanobacterial mat (control) and mats containing rotifers alone dispersed during the first 24 h, and consequently, the mean brightness of the cyanobacterial mat was significantly lower in comparison with the two treatments involving ciliates, for which the trichome dispersion was limited. In the following days, the mean brightness levels of the cyanobacterial mat in the *L. inermis* and control treatments gradually increased (Fig. 1B). The differences between treatments were significant at $F_{3,64} = 200.86$, $P < 0.001$.

The pronounced dispersion of trichomes outside the mat in the presence of *L. inermis* was reflected in the percentage of satiated *P. dubius* ciliates, which, in the experiment with a high ciliate density (500 ind./mL), was ~25% higher in the *Ps. dubius* + *L. inermis* treatment after 24 h than in the treatment in which the cyanobacterial mats were subjected to ciliates alone ($F_{3,32} = 22.42$, $P < 0.001$). On the second day (after ~48 h), the percentage of ciliates with a visible presence of food inside the vacuoles decreased radically, and in the *Ps. dubius* treatment, the percentage remained at a similar level until the end of the experiment. In the treatment in which rotifers were introduced, only solitary ciliates remained satiated at the end of the experiment (Fig. 2A).

The availability of cyanobacterial trichomes as food for *F. blochmanni* ciliates in the 500 ind./mL treatment was reflected in the percentage of satiated individuals and was highest on the first day of the experiment. The percentage of satiated ciliates was significantly higher ($F_{3,32} = 10.60$, $P < 0.001$) in the treatment with rotifers than in the treatment with ciliates alone (Fig. 2B). After 2 days, the percentage of ciliates with food visible inside their vacuoles dropped rapidly, and in the treatment with rotifers only, solitary satiated individuals remained until the end of the experiment. On the fourth day of the experiment, the number of satiated ciliates in the treatment without rotifers started to increase, reaching ~18% on average after a short decline on the third day of the experiment (Fig. 2B).

In the treatment involving *Ps. dubius* (500 ind./mL) and *L. inermis*, only solitary trichomes remained inside the mucilage of the initial piece of the cyanobacterial mat until the end of the experiments. However, in the treatment involving ciliates alone, not only a dense clump of cyanobacterial mat, but also dispersed filaments hidden inside the rigid sheaths were observed until the end of the experiment. An average number of filaments crossing the edges of the field of view in the *Ps. dubius* trial exceeded 60, whereas no such filaments were present in the treatment with ciliates and rotifers. Approximately 90% of the trichomes in the *Ps. dubius* treatment were covered with empty sheaths. At the end of the experiment, the mean number of active *Ps. dubius* ciliates in the treatment with rotifers exceeded 400 and was almost 20% higher than the *Ps. dubius* treatment, and the difference was significant ($F_{1,8} = 17.33$, $P < 0.05$). Similarly, the numbers of active *Lecane* were significantly higher in the treatment with ciliates and rotifers combined than in the treatment with rotifers alone ($F_{1,8} = 47.35$, $P < 0.001$). The mean numbers of active rotifers were

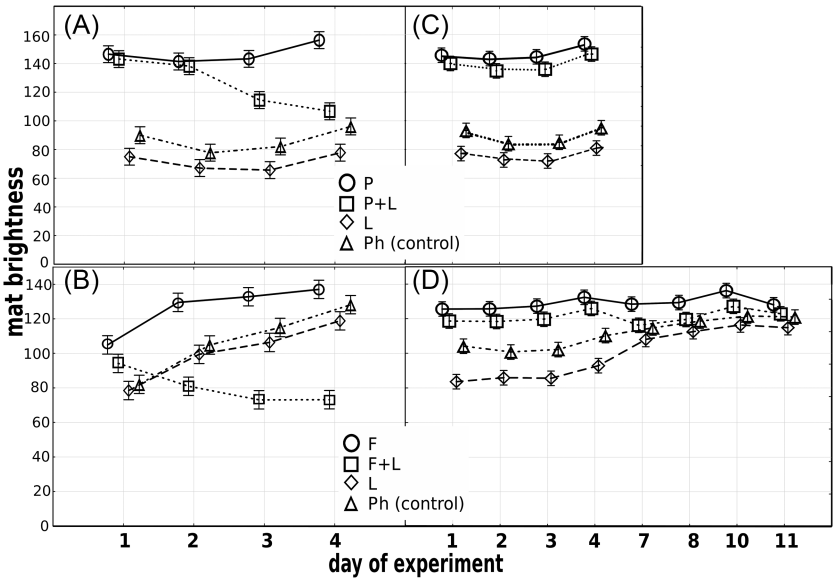

Figure 1. The mean brightness of cyanobacterial mats in the experiment where mats were subjected to **(A, B)** high (500 ind./mL) and **(C, D)** low (200 ind./mL) ciliates pressure. P: *Ps. dubius*; F: *F. blochmanni*; L: *L. inermis*; Ph: *Phormidium* sp. Whiskers represent standard deviations.

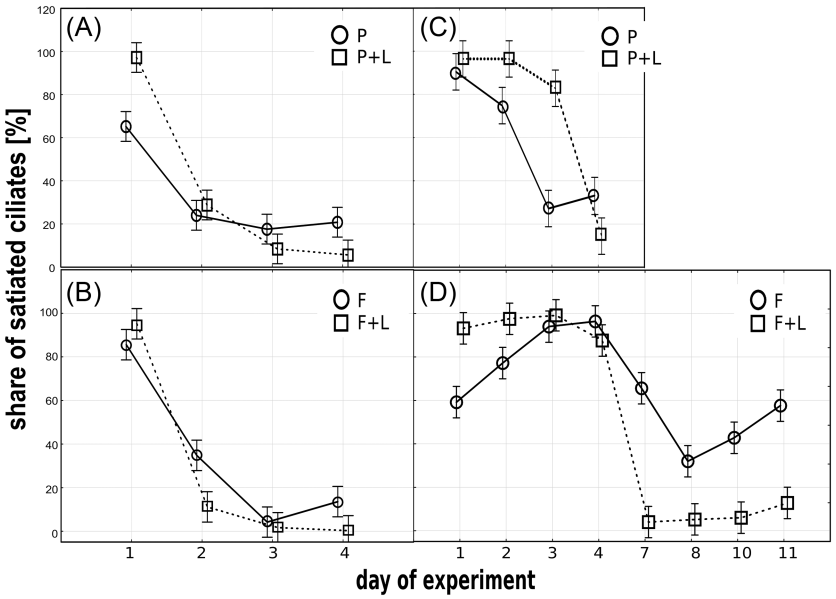

Figure 2. The mean share of satiated ciliates in the experiment where mats were subjected to **(A, B)** high (500 ind./mL) and **(C, D)** low (200 ind./mL) ciliates pressure. P: *Ps. dubius*; F: *F. blochmanni*; L: *L. inermis*. Whiskers represent standard deviations.

equal to 279 and 178, respectively. In both treatments, some ciliates underwent encystment; in the *Ps. dubius* treatment, an average of 24 ciliates were encysted, whereas in treatments containing rotifers, there were only 13 per well.

As a consequence of the limited trichome availability, the number of active ciliates in the high density *F. blochmanni* treatment (500 ind./mL) at the end of the experiment was lower than that at the start, and in the ciliates and rotifers treatment, it was significantly higher (mean 491 ind./well) in comparison with the treatment with ciliates alone (mean 342 ind./well) and the difference was significant ($F_{1,8} = 15.03$, $P < 0.005$). In the treatment without rotifers, significantly more ciliates ($F_{1,8} = 25.79$, $P < 0.001$) were encysted. At the end of the experiment, the number of active rotifers in the *F. blochmanni* + *L. inermis* treatment was slightly higher (226) in comparison with the beginning, whereas in *L. inermis*, it was slightly lower (196), but the difference between the treatments was not significant.

At the beginning of the experiment with the lower *Ps. dubius* density (200 individuals/mL), the pattern in the cyanobacterial mat compactness was similar to that of the experiment with the higher ciliate density. The highest autofluorescent brightness reflecting the highest compactness was noted in the case of mats under ciliate pressure alone, although after 24 h, the difference between treatments involving ciliates alone and with ciliates and rotifers was not statistically significant (Fig. 1C). The undisturbed cyanobacterial mats and mats subjected to *Lecane* rotifers started to disperse shortly after the beginning

of the experiment, which was already reflected in the significantly decreased brightness of an initial piece of mat after 1 day (Fig. 1C). The most dispersed were the cyanobacterial mats with rotifers because their mean brightness was significantly lower in comparison with all the other treatments. The mean brightness pattern in the cyanobacterial mats was almost unchanged until the end of the experiment. Two days after the start, the difference in the mean brightness of cyanobacterial mats between the *Ps. dubius* and *Ps. dubius* + *L. inermis* treatments became more pronounced and remained statistically significant ($F_{3,64} = 1784.2$, $P < 0.001$) until the end of the experiment.

In the experiment with the lower density of *F. blochmanni* (200 ind./mL), the pattern of changes in the compactness of the cyanobacterial mat was similar to that described in the earlier experiments. At the start, the most pronounced dispersion was observed in the *L. inermis* treatment followed by the control (Fig. 1D). The most compact ones were the mats that were subjected to ciliates alone. The mean autofluorescent brightness of the initial piece of the mat was significantly higher than that of the mat that was additionally disturbed by rotifers ($F_{3,64} = 273.98$, $P < 0.001$) (Fig. 1D). The pattern was repeatable for 4 days, and then in the second week, the mean brightness of cyanobacterial mats in the *L. inermis* treatment and control started to increase gradually and at the end reached values similar to those observed in both treatments involving *F. blochmanni*.

In the experiment with the lower density of *Ps. dubius* (200 ind./mL), after the first day of the experiment, the percentages of satiated ciliates in the *Ps. dubius* and *Ps. dubius* + *L inermis* treatments were almost similar. After 2 days, the percentage of satiated ciliates in the treatment involving rotifers reached almost 100% and was significantly higher ($F_{3,32} = 18.49$, $P < 0.001$) in comparison with the treatment in which the cyanobacterial mats were subjected to ciliates alone (Fig. 2C). The discrepancy was even more pronounced on the third day of the experiment when ~3-fold fewer ciliates were satiated in the treatment without rotifers in comparison with the treatment in which they were present. The relation was reversed on the fourth day. In the treatment involving ciliates alone, the percentage of satiated ciliates remained at a similar level as the day before, whereas the percentage of satiated ciliates in treatments with rotifers dropped rapidly to less than 20% (Fig. 2C).

Initially, the percentage of *F. blochmanni* ciliates with the trace of food was significantly ($F_{3,32} = 6.85$, $P < 0.005$) higher in treatments in which rotifers were introduced (Fig. 2D). The value exceeded 90% after 1 day, remained almost unchanged over 4 days, then rapidly decreased. From the seventh day forward, only solitary *F. blochmanni* individuals remained satiated until the end of the experiment in the *F. blochmanni* + *L. inermis* treatment, whereas in the treatment containing ciliates alone, the percentage of *F. blochmanni* with a trace of food in their vacuoles increased gradually until the fourth day, then dropped significantly ($F_{7,64} = 33.66$, $P < 0.001$), and stabilised at ~30% on the following days (Fig. 2D).

In the experiment with a lower ciliate *Ps. dubius* density, the cyanobacterial trichomes mostly remained inside dense clumps, but some of them dispersed out of the initial piece of mat. Starting from the second day, the density of the trichomes was significantly higher in the treatment without rotifers. At the end of the experiment, the mean DF for the treatment with ciliates alone exceeded 150, whereas in the treatment containing ciliates and rotifers, the DF was less than 20. It is worth emphasising that in the P + Ph2 treatment, almost all the trichomes (97%) were hidden inside rigid sheaths, whereas in the treatment involving

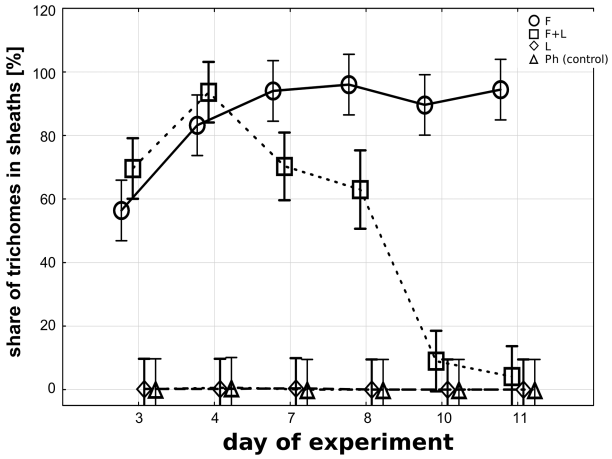

Figure 3. The percentage of filaments ending in empty sheaths in the prolonged experiment where mats were subjected to the lower (200 ind./mL) pressure of ciliates *F. blochmanni*. F: *F. blochmanni*, L: *L. inermis*, Ph: *Phormidium* sp. Whiskers represent standard deviations.

rotifers, the percentage of trichome endings covered by sheaths was significantly lower and did not exceed 75%.

Because the pressure of *F. blochmanni* ciliates in the treatment with a lower density (200 ind./mL) from the start of the experiment was relatively weak, many trichomes started to disperse in all directions and even grow during the prolonged experiment. As a consequence, it was impossible to distinguish solitary trichomes and to count their crossings over the edge of the field of view in the treatment with ciliates alone. However, in the treatment involving rotifers, almost all the dispersed trichomes were ingested by the *F. blochmanni* ciliates and none of them crossed the edges of the field of view. The availability of the trichomes depended on the absence of sheaths. The percentage of filament endings covered with empty sheaths increased gradually until the eighth day in the treatment with ciliates alone and remained at almost the same level until the end of the experiment (Fig. 3). The differences between treatments with and without the ciliates were significant at $F_{3,116} = 109.15$, $P < 0.001$. In the treatment involving ciliates and rotifers, the percentage of filament endings covered by sheaths also initially increased, but starting from the seventh day, they decreased sharply (Fig. 3). As in some experimental wells in the *F. blochmanni* + *L. inermis* treatment, the number of trichomes visible outside the mat was lower than 50 and the percentages were calculated as per all of these filaments.

At the end of the low *Ps. dubius* density experiment, the average number of active ciliates was significantly higher ($F_{1,8} = 32.95$, $P < 0.001$) in the treatment involving *Lecane* and reached almost 500. In the treatment with ciliates alone, the mean number of active *Ps. dubius* reached ~200. The number of cysts in the *Ps dubius* and *Ps dubius* + *L. inermis* treatments at the end of the experiment averaged 72 and 5, respectively. The number of rotifers in both treatments decreased equally during the experiment, and at the end of the trial, they barely exceeded 150 for each treatment.

In the prolonged experiment on *F. blochmanni*, it was impossible to count all the ciliates and cysts at the end of the experiment, and as in the treatment with ciliates alone, an enormous number of them were entangled within the cyanobacterial filaments.

DISCUSSION

The production of a mucilage matrix, exopolysaccharides and sheaths surrounding the filaments are believed to protect cyanobacteria against protozoan and metazoan grazers (Pajdak-Stós, Fiałkowska and Fyda 2001; Reynolds 2007). Sheaths covering the filaments and mucilage matrix of the colonies often significantly reduce the palatability of cyanobacteria. The presence of different forms of exopolysaccharide capsules, slimes and sheaths is treated as a form of constitutive or inducible defence, depending on the cyanobacteria and grazer species (Dodds, Gudder and Mollenhauer 1995; Pajdak-Stós, Fiałkowska and Fyda 2001; Yang *et al.* 2008). This type of defence forms a physical barrier, protecting filaments or cells from ingestion by micrograzers such as flagellates, amoebae, ciliates and rotifers. As a consequence, cyanobacteria can reach extremely high levels of abundance, which results in troublesome blooms.

Our experiments have shown that the protective function of mucilage or sheaths is not always sufficiently effective. Apparently some organisms, even those not feeding on cyanobacterial cells, may play an important role just by making the cyanobacteria more accessible to grazers. In the experiment in which the higher grazer pressure was applied and rotifers were also introduced, almost all the trichomes were ingested by ciliates (Fig. 4). At the end of the experiment, only solitary filaments remained inside the remnants of the mucilage from the initial piece of mat. As shown in the film (Vid. 1), *L. inermis* can graze on exopolysaccharide mucilage and thereby loosen the matrix and uncover the trichomes. As a consequence, it is easier for a ciliate to pull the entire trichome out of the mat (Vid. 2). If a trichome is too long and still entangled inside the mat, the rate of ingestion is highly limited. The effect of the presence of *L. inermis* is clearly visible when we compare the mean autofluorescent brightness of the mat with and without rotifers (Fig. 1A). During the experiment, the density of the cyanobacterial filaments gradually decreased in the presence of ciliates and rotifers, which was reflected in the diminishing brightness of chlorophyll autofluorescence in a frame of the initial piece of the mat (Fig. 1A).

The mechanism responsible for this phenomenon is most likely 2-fold. The presence of rotifers not only leads to changes in the structure of the mucilage or sheaths but also somehow enhances the trichome dispersion, increasing the availability of cyanobacterial filaments to ciliate grazers. This effect is visible when we compare the compactness of the cyanobacterial mat without any ciliate pressure with (*L. inermis*) and without (control) rotifers. The mean autofluorescent brightness was lowest in treatments in which the cyanobacterial mat was disturbed exclusively by rotifers that are not able to ingest trichomes. This finding indicates that in this treatment more trichomes were dispersed out of the initial piece of mat. This situation was repeatable for almost all of the experiments except for the trial with a higher density of *F. blochmanni* (Fig. 1A–D). However, for that experiment, there were no statistically significant differences between the treatment with *L. inermis* alone and the control in the autofluorescent brightness of the cyanobacterial mats, and so its trend was similar to the other cases (Fig. 1B). An analogous situation was observed in the experiment in which the chemical stimuli evoking the cyanobacterial defences were investigated (Fiałkowska and Pajdak-Stós 2014). When the *Euplotes octocarinatus* ciliate, which is unable to ingest filamentous cyanobacteria, was introduced over the filter, the trichome dispersion out of the initial piece of mat placed under the filter was more pronounced in comparison with the control (Fiałkowska and Pajdak-Stós 2014). In the present experiment, the rotifers had direct contact with the cyanobacterial mat, and we cannot exclude the possibility that the mechanical stimuli exerted by rotifers crawling on the mat evoked accelerated chaotic trichome motility that was consequently reflected in the lower mean autofluorescent brightness of the initial piece of the mat. The other possible explanation is that rotifers feeding on cyanobacterial mucilage release nutrients and thus modify the cyanobacteria behaviour. In their minireview, Gerphagnon *et al.* (2015) presented examples of how the grazing and parasitism of cyanobacteria contribute to nutrient cycling.

The most compact and entangled examples were cyanobacterial mats under the exclusive pressure of ciliates specialising in the ingestion of filamentous cyanobacteria (Fig. 5). The pronounced mat compactness trend was reflected in the observation in which the highest fluorescent brightness values for the mats were similar for all the experiments involving *Ps. dubius* and *F. blochmanni* at higher and lower densities (Fig 1A–D). These results are in accordance with the results of our earlier research showing that cyanobacteria reacted to the pressure of ciliate grazers in the form of limited dispersion and the formation of dense clumps of trichomes (Fiałkowska and Pajdak-Stós 2002; Fyda, Fiałkowska and Pajdak-Stós 2010).

Similar to our earlier experiment (Fiałkowska and Pajdak-Stós 2002), almost all the trichomes in the *Ps. dubius* treatment and all the trichomes in the *F. blochmanni* treatment in the experiment with a higher density of ciliates were entangled in the initial piece of mat, which was reflected by a very low or 0 DF. With the lower ciliate abundance, the numbers of filaments crossing the edges of the field of view were higher. In the treatments in which *Lecane* was introduced into the wells with the ciliates, almost all of the dispersed trichomes were ingested, and as a result, almost no filaments crossing the edges of the fields of view were noted. This is another indicator that rotifers can make cyanobacterial trichomes available to ciliates indirectly. The other known example of when one organism makes cyanobacteria more accessible for other grazers is the feeding behaviour of calanoid copepods. These organisms can cut cyanobacteria filaments, shortening them to the size available for smaller grazers such as cladocerans (Kâ *et al.* 2012; Ger, Hansson and Lürling 2014 and the literature within).

Interestingly, the percentage of filament endings covered with empty sheaths was also higher in the treatments with ciliates alone. This difference could be explained by the rotifers' ability to graze on the exopolysaccharide material produced by cyanobacterial trichomes and/or the chaotic movement of trichomes disturbed by rotifers, resulting in a delay in sheath production. It is worth emphasising that similar to earlier results, almost no sheath production was noted in treatments without ciliates (Fiałkowska and Pajdak-Stós 1997, 2002).

As trichomes became more available for ciliates in the presence of rotifers, a higher number of ciliates was recorded at the end of both experiments involving *Ps. dubius* and at the end of the experiment with the higher *F. blochmanni* abundance. Notably, in the experiments with the higher ciliate abundance, the number of rotifers was also higher in treatments in which they shared a niche with protists feeding on cyanobacteria. It is possible that the presence of ciliates accelerated the production of mucilage used as a source of food by rotifers. In this way, ciliates and rotifers could gain mutual benefits. Because mutualism is defined by interactions between species in which each has a net positive impact on the fitness of the other (Bronstein 2015), we could treat the relation between *Lecane* and ciliates specialising in the ingestion of filamentous cyanobacteria as a

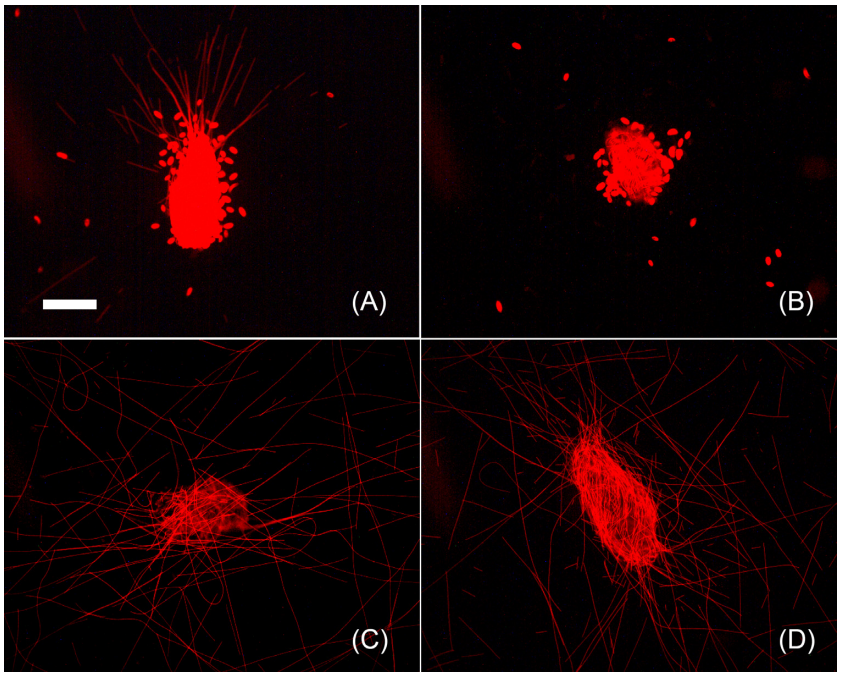

Figure 4. Comparison of cyanobacterial mats in autofluorescence 24 h from the start of the experiment, where mats were subjected to the higher (500 ind./mL) pressure of ciliates *F. blochmanni*. **(A)** A tightly entangled cyanobacterial mat subjected exclusively to the pressure of ciliates *F. blochmanni*, **(B)** a cyanobacterial mat under the pressure of ciliates *F. blochmanni* and rotifers *L. inermis*, with the trichomes already heavily exploited by ciliates, **(C)** trichomes dispersed from a cyanobacterial mat in the presence of rotifers *L. inermis* and **(D)** trichomes dispersed from an undisturbed cyanobacterial mat of *Phormidium* sp. strain Ph2. In (A) and (B) there are visible red ciliates with autofluorescently luminescent cyanobacteria inside their vacuoles. The scale bar represents 0.5 mm.

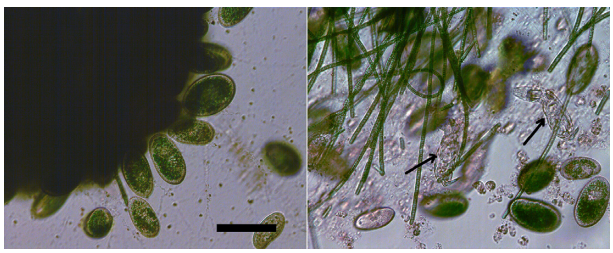

Figure 5. Comparison of the tightly entangled cyanobacterial mat of *Phormidium* sp. strain Ph2 under the exclusive pressure of ciliates *F. blochmanni* (left) with the loose mat under the pressure of ciliates *F. blochmanni* and rotifers *L. inermis* (right). The arrows indicate rotifers *L. inermis* feeding on the cyanobacterial mucilage. The scale bar represents 100 μm.

new example of this phenomenon. To date, only one example of rotifers remaining in a mutualistic relationship was described, and it concerned the bdelloid rotifer *Philodina roseola* feeding on the jelly coat covering the eggs of the fish ectoparasite *Argulus bengalensis*, which enable the larvae to hatch (Banerjee *et al.* 2016).

When we look at the graphs concerning the percentage of satiated ciliates, we note the repeatable pattern in which ciliates in the presence of rotifers exploited a source of food much more rapidly, whereas in treatments with ciliates alone, a form of balance was established at the end of experiments (Fig. 2A–C). When the pressure of ciliate grazers decreased due to encystment, the trichomes started to leave the shelter and became available again. In this way, the inducible defence stabilises the relation between predator and prey, which is in accordance with the earlier hypotheses and results (Verschoor, Vos and Van Der Stap 2004; Vos *et al.* 2004).

This relation is also visible in the prolonged experiment on *F. blochmanni* (Fig. 2D). When the percentage of the filament endings covered by sheaths stabilised at the high level, the percentage of satiated ciliates at the end of the experiment remained at a stable, low level (Figs 2D and 3). Interesting results were obtained in the treatment involving *Lecane*, in which the percentage of trichomes covered with sheaths initially increased and then rapidly decreased, but there were large differences between replicates (Fig. 3). Although there were many naked trichomes protruding from the initial piece of mat, only solitary ciliates were satiated (Fig. 2D). We cannot exclude that cyanobacteria produce metabolites that repel grazers when, due to rotifer activity, trichomes remain unprotected for a prolonged period of time. However, this issue requires further investigation using a more biochemical approach.

When cyanobacteria apply various forms of defence and it is hard to limit their expansion, every report on the possibility of weakening their defence is important. Even though our research concerns relationships between mat-forming benthic cyanobacteria and their potential grazers, it cannot be discounted that interactions similar to those shown in our research can also occur among planktonic organisms. As far as we are aware, this is the first report on the ability of

rotifers to graze on the mucilage produced by cyanobacteria and thereby make cyanobacterial trichomes available to ciliate grazers.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

REFERENCES

Banerjee A, Poddar S, Manna S *et al*. Mutualistic association of rotifer *Philodina roseola* with the branchiuran fish ectoparasite *Argulus bengalensis* at its embryonic stage. *Biol Lett* 2016;**12**:20151043.

Bronstein JL. Introduction. In: Mutualism JL (ed.). *Bronstein*. Oxford, UK: Oxford University Press, 2015, 28–46.

Camacho FA, Thacker RW. Amphipod herbivory on the freshwater cyanobacterium Lyngbya wollei: chemical stimulants and morphological defenses. *Limnol Oceanogr* 2006;**51**: 1870–5.

Carmichael WW. The cyanotoxins.In: *Advances in botanical research*. Waltham, MA: Academic Press, 1997, 211–56.

Combes A, Dellinger M, Cadel-six S *et al*. Ciliate *Nassula* sp. grazing on a microcystin-producing cyanobacterium (*Planktothrix agardhii*): impact on cell growth and in the microcystin fractions. *Aquatic Toxicol* 2013;**126**:435–41.

DeMott WR, Gulati RD, Van Donk E. Daphnia food limitation in three hypereutrophic Dutch lakes: Evidence for exclusion of large-bodied species by interfering filaments of cyanobacteria. *Limnol Oceanogr* 2001;**46**:2054–60.

Dirren S, Pitsch G, Silva MO *et al*. Grazing of *Nuclearia thermophila* and *Nuclearia delicatula* (Nucleariidae, Opisthokonta) on the toxic cyanobacterium *Planktothrix rubescens*. *Eur J Protistol* 2017;**60**:87–101.

Dodds WK, Gudder DA, Mollenhauer D. The ecology of Nostoc. *J Phycol* 1995;**31**:2–18.

Fialkowska E, Pajdak-Stós A. Dependence of cyanobacteria defense mode on grazer pressure. *Aquat Microb Ecol* 2002;**27**:149–57.

Fiałkowska E, Pajdak–Stós A. Inducible defence against a ciliate grazer *Pseudomicrothorax dubius*, in two strains of Phormidium (cyanobacteria). *Proc R Soc Lond B Biol Sci* 1997;**264**: 937–41.

Fiałkowska E., Pajdak-Stos A. Chemical and mechanical signals in inducing Phormidium (Cyanobacteria) defence against their grazers. *FEMS Microbiol Ecol* 2014;**89**: 659–69.

Fyda J, Fiałkowska E, Pajdak-Stós A. Dynamics of cyanobacteria–ciliate grazer activity in bitrophic and tritrophic microcosms. *Aquat Microb Ecol* 2010;**59**:45–53.

Ger KA, Hansson LA, Lürling M. Understanding cyanobacteria-zooplankton interactions in a more eutrophic world. *Freshw Biol* 2014;**59**:1783–98.

Ger KA, Naus-Wiezer S, De Meester L *et al*. Zooplankton grazing selectivity regulates herbivory and dominance of toxic phytoplankton over multiple prey generations. *Limnol Oceanogr* 2019;**64**:1214–27.

Gerphagnon M, Macarthur DJ, Latour D *et al*. Microbial players involved in the decline of filamentous and colonial cyanobacterial blooms with a focus on fungal parasitism. *Environ Microbiol* 2015;**17**:2573–87.

Gliwicz ZM, Siedlar E. Food size limitation and algae interfering with food collection in Daphnia. *Archiv für Hydrobiologie* 1980;**88**:155–77.

Huisman J, Matthijs CP, Visser PM. Harmful Cyanobacteria. *Aquatic Ecology Series*. Dordrecht, The Netherlands: Springer, 2005.

Jang M-H, Jung J-M, Takamura N. Changes in microcystin production in cyanobacteria exposed to zooplankton at different population densities and infochemical concentrations. *Limnol Oceanogr* 2007;**52**:1454–66.

Kâ S, Mendoza-Vera JM, Bouvy M *et al*. Can tropical freshwater zooplankton graze efficiently on cyanobacteria? *Hydrobiologia* 2012;**679**:119–38.

Liu Z, Hu J, Zhong P *et al*. Successful restoration of a tropical shallow eutrophic lake: Strong bottom-up but weak top-down effects recorded. *Water Res* 2018;**146**:88–97.

Paerl HW, Fulton RS, Moisander PH *et al*. Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *Sci World J* 2001;**1**:76–113.

Pajdak-Stós A, Fiałkowska E, Fyda J. Phormidium autumnale (Cyanobacteria) defense against three ciliate grazer species. *Aquat Microb Ecol* 2001;**23.3**:237–44.

Pajdak-Stós A, Fiałkowska E, Fyda J *et al*. A method of mass culture of Lecane rotifers. 2017; International Patent EP14731401.7.

Reynolds CS. Variability in the provision and function of mucilage in phytoplankton: facultative responses to the environment. *Hydrobiologia* 2007;**578**:37–45.

Sobczyk M, Fiałkowska E, Pajdak-Stós A *et al*. Effect of *Lecane inermis* rotifers grazing on wastewater bacteria biofilms. 2013 2nd Young Scientists Conference, World Water Day, Poznań. https://doi.org/10.13140/RG.2.1.4080.5361.

Stanier RY, Kunisawa M, Mandel M *et al*. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol Rev* 1971;**35**:171–205.

StatSoft, Inc. STATISTICA (data analysis software system), version 12. www.statsoft.com, 2014.

Tollrian R, Harvell CD. The evolution of inducible defenses: current ideas. *The Ecology and Evolution of Inducible Defenses*. Princeton, NJ: Princeton University Press, 1999, 306–21.

Trabeau M, Bruhn-Keup R, McDermott C *et al*. Midsummer decline of a Daphnia population attributed in part to cyanobacterial capsule production. *J Plankton Res* 2004;**26**:949–61.

Verschoor AM, Vos M, Van Der Stap I. Inducible defences prevent strong population fluctuations in bi-and tritrophic food chains. *Ecol Lett* 2004;**7**:1143–8.

Vos M, Verschoor AM, Kooi BW *et al*. Inducible defenses and trophic structure. *Ecology* 2004;**85**:2783–94.

Yang Z, Kong F, Shi X *et al*. Changes in the morphology and polysaccharide content of *Microcystis aeruginosa* (Cyanobacteria) during flagellate grazing. *Journal of Phycology* 2008;**44**:716–20.