

Effects of Biological Control Agents on Nuisance Cyanobacterial and Diatom Blooms in Freshwater Systems

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Biological control agents such as bacteria and ciliates were tested alone and in combination for their abilities to decrease densities of the warm-weather cyanobacterium, *Microcystis aeruginosa*, and the cold-weather centric diatom, *Stephanodiscus hantzschii*. The growth of *Microcystis aeruginosa* was effectively suppressed by the algicidal bacterium, *Streptomyces neyagawensis*, and the predator heterotrich ciliate, *Stentor roeselii*. However, co-treatment increased the algal density rather than decreasing it, suggesting that *Streptomyces neyagawensis* and *Stentor roeselii* have an antagonistic relationship. Additional experiments revealed that the density of *Stephanodiscus hantzschii* was effectively reduced by the bacterium, *Pseudomonas putida*, and by the abovementioned strain of *Stentor roeselii*. Co-treatment had a greater antialgal effect than treatment with either bio-agent alone, indicating that the two act synergistically. These results suggest the potential antialgal efficacy of treatments with multiple biological control agents, providing new insight that may be useful for the bioremediation of nuisance algal blooms in freshwater.

Key words: algicidal bacteria, ciliate, cyanobacteria *Microcystis*, diatom *Stephanodiscus*

There have been many attempts to control nuisance algal blooms through the direct application of chemicals such as cupric-sulfate, smazine, dichromate, ozone and L-lysine^{8,15,21}. However, these agents can cause irreversible damage to aquatic ecosystems by killing off beneficial plankton and fish^{17,18}. Thus, researchers are currently seeking to develop environmentally friendly bio-agents for controlling algal density, in a manner consistent with current pan-ecological and environmental approaches to lake water conservation^{1,3,7,12}.

Microorganisms such as viruses, bacteria, fungi, and amoebae have been shown to kill cyanobacteria^{20,22}. Among these, antagonistic bacteria are potentially useful agents for algal control, as they are simple to culture and manipulate^{16,20}. We have previously investigated a number

of aquatic bacteria, zooplanktons, and ciliates for their abilities to control algal blooms in Korea^{10,11}. Here, we sought to identify bio-agents capable of managing two target algae: the cyanobacterium *Microcystis* and the diatom *Stephanodiscus*. In the Pal'tang Riverine of Korea, *Microcystis* is often found during the warm season (water temperatures above 20°C)¹⁰, while *Stephanodiscus* is more common during the cold season (water temperatures below 20°C)¹¹. Together, these two organisms are representative of many naturally occurring algal blooms.

Several previous reports have identified algicidal bacteria capable of killing cyanobacteria such as *Microcystis aeruginosa*^{13,22}, but there have been few previous reports of bio-agents capable of managing the diatom *Stephanodiscus hantzschii*⁹. Here, we investigated the ability of several bacterial and ciliate bio-agents, alone and in combination, to decrease the density of these two representative algae. This is the first investigation of combined treatment with both

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bacteria and ciliates to control cyanobacterial and diatomal blooms *in situ*, and our findings provide the first evidence that the ciliate, *Stentor roeselii*, is algicidal to both *Microcystis* and *Stephanodiscus*.

Materials and Methods

Algal culture

The cyanobacterium, *Microcystis aeruginosa* NIES-44, was obtained from the National Institute for Environmental Studies, Tsukuba, Japan and cultured in CB medium (http://www.nies.go.jp/biology/mcc/strainlist_j.htm) at $27\pm 2^\circ\text{C}$, with shaking at 120 rpm, and a light-dark cycle of 12:12 h and light intensity of $40\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$. The small centric diatom, *Stephanodiscus hantzschii* UTCC 269, was obtained from the University of Toronto Culture Collection of Algae and Cyanobacteria (UTCC, Toronto, ON, Canada) and cultured in DM medium²⁾ at $20\pm 2^\circ\text{C}$ with a 12L:12D photo-cycle and $50\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$. Algal cells in the exponential stage were used in all experiments, and determined by direct counting of cells using a Sedgwick-Rafter chamber under a light microscope (Nikon, Tokyo, Japan).

Algicidal bacteria culture

The bacterium *Streptomyces neyagawensis* HYJ0209-MK50, previously reported by Choi *et al.*¹⁹⁾, was isolated using the plaque method or a soft-agar overlayer technique. In brief, algal lawns were prepared in Petri dishes containing axenic cultures of *Microcystis aeruginosa* NIES-44 on nutrient broth (NB) medium. These were inoculated with 1 ml of stream water filtered through a Nucleopore membrane (Millipore). Cultures were incubated at 25°C with a 12L:12D photo-cycle and $40\text{--}50\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ for 6–8 days. Potential antialgal or algicidal bacteria were picked from clear zones where the *Microcystis aeruginosa* cells had lysed. These bacteria were purified, isolated, grown to the logarithmic growth phase, and inoculated into an axenic culture of *Microcystis aeruginosa* in NB. This gram-negative bacterium grows at $35\text{--}45^\circ\text{C}$, pH 6–8; optimal growth occurs at 40°C , pH 7. The isolates were axenically maintained under optimal growth conditions on nutrient agar (NA) plates, cryopreserved at -76°C in NB medium containing 20% glycerol. The bacterial biomass of *Streptomyces neyagawensis* was determined by dry weight, measured as the weight difference of membrane filters (0.2- μm pore size, 25-mm diameter; Whatman) before and after cultivation with the target alga. The dry weight contribution of *Microcystis* was ignored because it was approximately 1000-fold smaller than that of *Streptomyces neyagawensis*.

Pseudomonas putida HYK0203-SK02, which is capable of inhibiting the growth of *Stephanodiscus hantzschii*, was isolated by Kang *et al.*⁹⁾ using the above method, except that the algal lawns were prepared with an axenic culture of *Stephanodiscus hantzschii* UTCC 267 on NB. This strain of *Pseudomonas putida* grows at $20\text{--}30^\circ\text{C}$ and pH 5–9; optimal growth occurs at 30°C and pH 7. The bacterium was maintained in the dark on NA plates containing 1.5% agar, at 30°C and pH 7, or cryopreserved at -76°C in NB containing 20% glycerol. For enumeration of bacteria, water samples were fixed with formalin (2% v/v final concentration) immediately after sampling, stained with 4',6-diamidino-2-phenylindole (DAPI, $1\ \text{mg ml}^{-1}$), filtered through isopore membrane filters (0.2 μm , Millipore, Ireland) and counted at 1000x magnification using an epifluorescence microscope (Nikon, Tokyo, Japan).

Antialgal ciliate culture

For the isolation of the heterotrich ciliate, *Stentor roeselii*, the waterway was screened with a plankton net (pore size 40 μm) near the marginal portion of the waterway, where aquatic plants flourished. In the laboratory, the plankton samples were size fractionated with a net (0.1–4 mm) and *Stentor* cells were directly picked under an inverted microscope. These cells were concentrated in a depression glass, rinsed three times with filtered water, and inoculated into a multi-well chamber containing different prey species and mixtures thereof. Samples were maintained at 23°C , with a 12L:12D photo-cycle and $2\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$. To investigate changes in ciliate density in the presence of algal prey, subsamples were fixed immediately with Bouin's solution (3% v/v final concentration), filtered with a cellulose nitrate membrane filter (0.45 μm ; Sartorius, Germany) and stained with protargol stain. The ciliates were enumerated using a Sedgwick-Rafter chamber under a light microscope (Nikon, Tokyo, Japan).

Algicidal activity test with different agents

Cultured *M. aeruginosa* maintained in CB medium (http://www.nies.go.jp/biology/mcc/strainlist_j.htm) was diluted to $2\text{--}6\times 10^6$ cells/ml with the same medium, and 95-ml aliquots were inoculated into duplicate 250-ml Erlenmeyer flasks. To investigate the antialgal effects of the *Microcystis*-lysing bacterium, *Streptomyces neyagawensis*, the bacterium was incubated in liquid NB medium at 37°C for 48 h. When cell density reached $1\text{--}2\times 10^8$ cells/ml, the culture was centrifuged at 14,000 g for 20 minutes, and the pellet was washed twice with sterilized NB medium. Five-milliliter (5% v/v) aliquots were inoculated into the algal cultures at a final

concentration of $1\text{--}2 \times 10^4$ cells/ml. Duplicate control cultures were prepared by adding 5 ml of NB medium to 95 ml of *Microcystis* culture. The flasks were incubated at $27 \pm 2^\circ\text{C}$ with a 12L:12D photo-cycle and $40\text{--}50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

To first test the antialgal effect of the *Stephanodiscus*-lysing bacterium, *Pseudomonas putida*, we grew the bacterium to the logarithmic growth phase and diluted the samples with CB medium to 1.5×10^3 cells/ml or approximately 10^5 cells/ml. We inoculated 10 ml of exponentially growing *Stephanodiscus* cells into 90 ml of the bacterial culture, for a final algal concentration of 1%. The samples were incubated as described above. Bacteria-free cultures served as the controls.

To investigate the algicidal effect of each of these organisms on *Stephanodiscus*, we used the same methods described above for the *Microcystis* assays, except that the culture flasks were incubated at 20°C with a 12L:12D photo-cycle and $40\text{--}50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The initial concentration of the diatom (1.5×10^3 cells/ml) was similar to that encountered in nature during the bloom period. Algal cells were enumerated daily under light microscopy, as described above.

To examine the removal activity of the ciliate, *Stentor roeselia*, on *Microcystis* and/or *Stephanodiscus*, six-well plates (Falcon, USA) were loaded with 5 cells/ml of three-day starved ciliates in 5 ml of filtered water/well. The algal concentration was 2×10^6 cells/ml for *Microcystis* and/or 1.5×10^3 cells/ml for *Stephanodiscus*. Algal densities and ciliate numbers were calculated daily under the light microscope after fixation with a glutaraldehyde solution.

To examine the combined algicidal effects of the tested organisms on algal growth, pairs of bio-agents (bacteria or ciliates) were introduced into culture flasks containing *Microcystis* and/or *Stephanodiscus*. The initial concentrations of bacteria and starved ciliates, and the final culture volumes were very similar to those described above. Cultures were maintained at 20°C , with a 12L:12D photo-cycle and light intensity of $2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Ciliates and algae were counted using a Sedgwick-Rafter chamber under an inverted microscope (Nikon, Tokyo, Japan).

To further investigate the competitive relationship between the bacterium and the ciliate, the bio-agents were co-cultured in filtered freshwater under the same conditions. As the bacteria grew similarly under dark conditions and $2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, we were able to ignore the influences of light in our calculations. Cell densities of *Streptomyces*, *Pseudomonas* and *Stentor* populations were measured or enumerated daily for 6–7 days.

Data analysis

The algicidal activity (AA) of the bacteria or ciliate on *Microcystis aeruginosa* and *Sephanodiscus hantzschii* was calculated using the formula $AA = (1 - D/Do) \times 100$, where Do and D are the cell densities in the control and treated groups, respectively, at the end of each experiment. The AA increase (indicating an effective algicide) or decrease (indicating an ineffective algicide) of target algae *Microcystis aeruginosa* and *Stephanodiscus hantzschii* in the presence of biological control agents was analyzed by the Student's *t* test.

Results

The bacterium *Streptomyces neyagawensis* effectively suppressed the growth of *Microcystis aeruginosa* cultured in filtered water, without any apparent change in the biomass of *Streptomyces neyagawensis* (Fig. 1 upper). The lack of change in the bacterial biomass may be attributed to

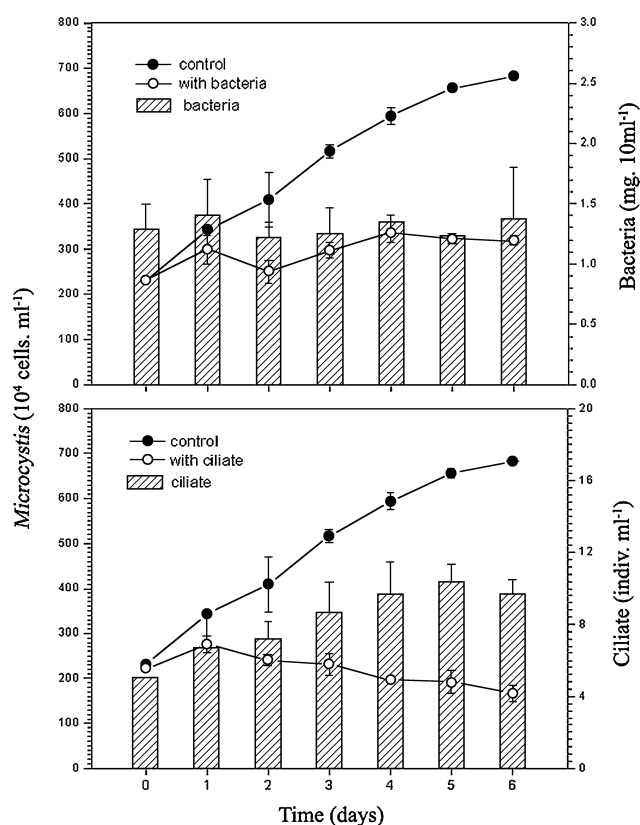


Fig. 1. Antialgal effect of the bacterium *Streptomyces neyagawensis* (upper) and the ciliate *Stentor roeselia* (lower) on the cyanobacterium *Microcystis aeruginosa* in filtered water.

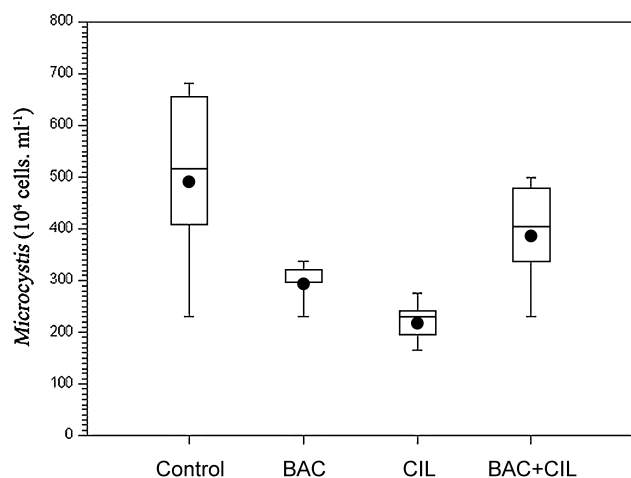


Fig. 2. Comparison of single and combined antialgal effects of the bacterium *Streptomyces nagayawensis* and the ciliate *Stentor roeselii* on the cyanobacterium *Microcystis aeruginosa* in filtered water. The heights of the blank boxes represent the growth of *M. aeruginosa* and its inhibition or stimulation by the treatments. BAC+CIL, co-treatment with bacteria (BAC) and ciliates (CIL).

the differences in optimal temperature and pH ranges between the bacteria and the algae. The ciliate, *Stentor roeselii*, gradually inhibited the growth of *Microcystis* cultured in filtered water beginning one day after cultivation, while its abundance increased slightly over the first five days of cultivation, and declined thereafter (Fig. 1 lower). Unexpectedly, combined treatment with bacteria and ciliates did not effectively inhibit the growth of *Microcystis* (Fig. 2), suggesting possible competition between the bacteria and ciliates. However, the cell densities and growth of *Microcystis aeruginosa* in the combined treatment cultures were lower than those in control cultures. In mixed bacterial/ciliate cultures without nutrient supplementation, the growth of the ciliate was severely limited (decreased to 80% of the control) on the first day, with less than 100% mortality by the second day (Fig. 3).

The bacterium *Pseudomonas putida* effectively inhibited the growth of the diatom *Stephanodiscus hantzschii* in filtered water, decreasing its population to 80% of control levels (Fig. 4 upper). The bacterial population gradually decreased with time, and the density of the diatom declined to about 14% of the control level after eight days of cultivation. The ciliate *Stentor roeselii* similarly inhibited growth of *Stephanodiscus hantzschii*, although its effect was weaker than that of *Pseudomonas putida* (Fig. 4 lower), and all ciliates died within four days of cultivation. In contrast to our finding of decreased anti-algal effects following co-treatment of *Microcystis*, the treatment of *Stephanodiscus*

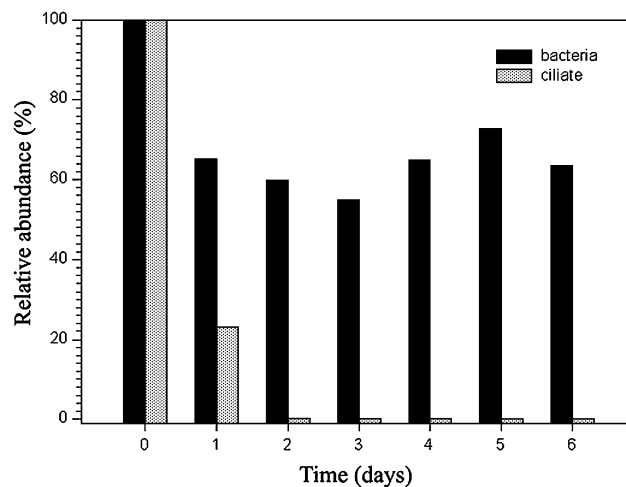


Fig. 3. Changes in the relative abundances of ciliate (*Stentor roeselii*) and bacteria (*Streptomyces nagayawensis*) in mixed cultures.

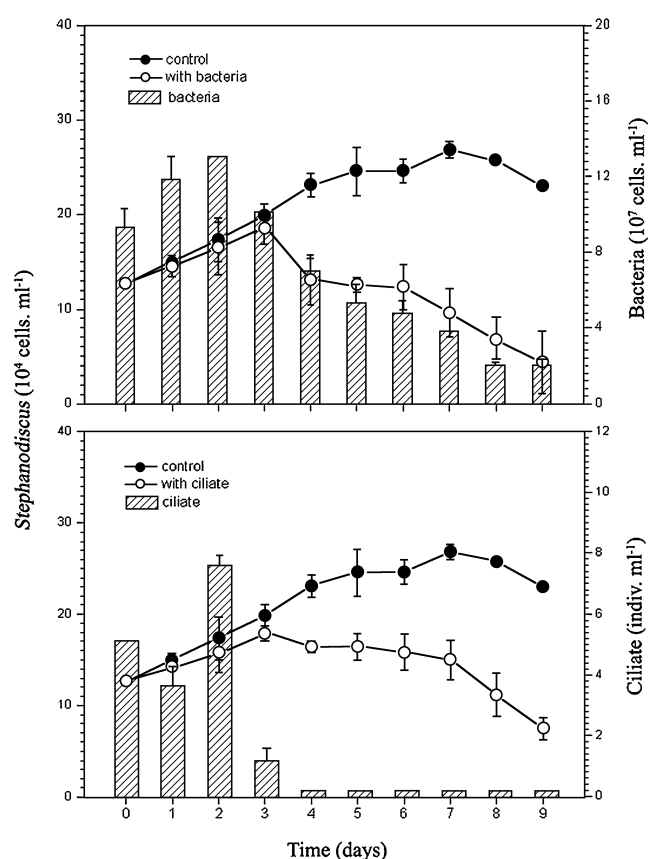


Fig. 4. Antialgal effect of *Pseudomonas putida* (upper) and the ciliate *Stentor roeselii* (lower) on the diatom *Stephanodiscus hantzschii* in filtered water.

hantzschii with *Pseudomonas putida* plus *Stentor roeselii* was more effective than either of the single treatments alone

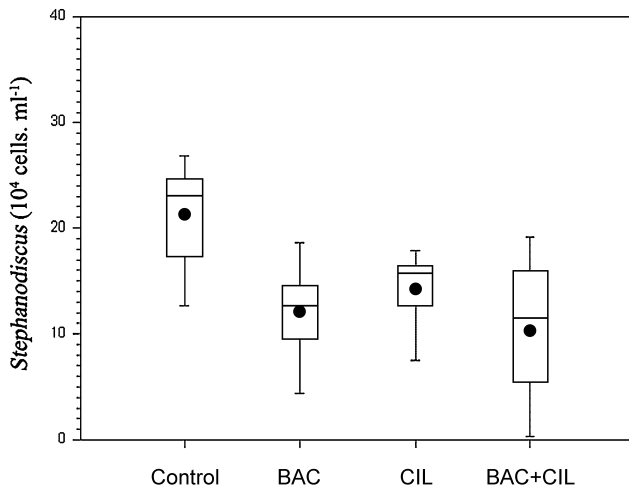


Fig. 5. Comparison of single and combined antialgal effects of the bacterium *Pseudomonas putida*, the ciliate *Stentor roeselii*, and combined treatments on the diatom *Stephanodiscus hantzschii* in filtered water. The heights of the blank boxes represent the growth of *S. hantzschii* and its inhibition or stimulation by each treatment. BAC+CIL, co-treatment with bacteria (BAC) plus ciliates (CIL).

(Fig. 5). Following the combined treatment, *Stephanodiscus hantzschii* levels reached zero within nine days of cultivation.

The treatment of *Stephanodiscus hantzschii* with *Pseudomonas putida* plus *Stentor roeselii* decreased the algal cell density, whereas treatment of *M. aeruginosa* with the bacterium *Streptomyces neyagawensis* plus the ciliate did not effectively inhibit algal growth (Table 1).

Discussion

Our results clearly show that the efficacies of bio-agents alone and combination vary depending on the target algae and the agents themselves. In the presence of the *Micro-*

cystis-lysing bacterium, *Streptomyces neyagawensis*⁴⁾, the density of *Microcystis* fluctuated near the average value, whereas control cultures lacking the lytic bacterium showed a steady increase in *Microcystis* cell density (AA=0.05), indicating that the bacterium had a suppressive effect on the proliferation of *Microcystis*. In the presence of the *Stephanodiscus*-lysing bacterium, *Pseudomonas putida*⁹⁾, the cell density of the diatom *Stephanodiscus hantzschii* gradually decreased over time (AA=−0.12). In terms of bacterial growth patterns, the density of *Streptomyces neyagawensis* fluctuated near the initial density in the presence of *Microcystis aeruginosa*, while the density of *Pseudomonas putida* increased during the first two days of diatom culture and decreased sharply thereafter. Our observation of feeble or nonexistent bacterial growth in the presence of algae is perhaps due to differences between the conditions optimal for algal growth used in our experiments and those preferred by the bacteria. The growth of *Microcystis* and *Stephanodiscus* in the presence of the ciliate, *Stentor roeselii*, showed a similar pattern to that observed in the presence of the two bacteria. However, during the co-treatments, the ciliate showed different growth patterns depending on the bacteria; the *Stentor* cell density declined to zero after four days of cultivation with *Stephanodiscus hantzschii*, whereas the ciliate levels gradually increased in the presence of *Microcystis aeruginosa*.

Surprisingly, we found that the co-treatment of *Microcystis*-containing cultures with *Streptomyces neyagawensis* and *Stentor roeselii* had a smaller algicidal effect than treatment with either agent alone, perhaps due to some sort of antagonistic effect. Our results regarding growth patterns (Fig. 5) showed that *Streptomyces neyagawensis* and ciliates grown in nutrient-free filtered water of the sampling stations decreased in number simultaneously; however, this observation was insufficient to explain all of the observed differences. In the presence of *Streptomyces neyagawensis*, ciliate

Table 1. Decreases in *Microcystis aeruginosa* and *Stephanodiscus hantzschii* biomass following individual or combined treatment with bacteria and ciliates, and their algicidal activities (AA) on both algae

Target algae	Treatments	AA	r
<i>Microcystis aeruginosa</i>	<i>Streptomyces nagayawensis</i> SM02	0.05	0.97**
	<i>Stentor roeselii</i>	−0.04	0.85*
	<i>Streptomyces</i> + <i>Stentor</i>	0.10	0.92**
<i>Stephanodiscus hantzschii</i>	<i>Pseudomonas putida</i> SK02	−0.12	0.97***
	<i>Stentor roeselii</i>	−0.06	0.98***
	<i>Pseudomonas</i> + <i>Stentor</i>	−0.42	0.96***

* P<0.05, ** P<0.01, *** P<0.001

levels decreased more severely than did *Streptomyces neyagawensis* levels, with total ciliate mortality occurring within four days of cultivation. This phenomenon might suggest that the bacterium may even have an anti-ciliate property, similar to the anti-nematodal and antibiotic properties seen in other bacteria^{5,6}. Further study will be required to determine the relationship between co-applied bio-agents in an aquatic ecosystem.

We also investigated the effect of a second bacterium, *Pseudomonas putida*, and the ciliate, *Stentor roeselii*, on the diatom, *Stephanodiscus*. We observed widespread ciliate death in the presence of *Stephanodiscus*, possibly due to a lack of nutrients. We used the same nutrient conditions as found in the lake, and seeded cultures with less than 10⁵ diatoms/ml, which is consistent with the diatom load found in nature. The *Microcystis aeruginosa* NIES-44 used in this study is not a toxic strain and does not produce microcystin, in contrast to the previously reported *Microcystis aeruginosa* NIES-298²³. Although the target algae were inhibited by treatment with these bio-agents, the co-culture conditions did not seem to be optimal for growth of the bacteria and/or ciliate. However, our results revealed that co-treatment of *Stephanodiscus* with *Pseudomonas putida* and *Stentor roeselii* more effectively inhibited the diatom than did treatment with each agent alone, suggesting some kind of synergistic interaction.

Collectively, these results indicate that the efficacies of bio-agents alone and in combination vary depending on the target algae and the agents themselves. More work will be required to investigate the use of bio-agents such as algicidal bacteria and ciliates to control cyanobacterial and diatomaceous blooms. A better understanding of the interactions among bio-agents will allow researchers to select relevant predators and/or bacteria within each freshwater ecosystem. Here, our preliminary data show the possibility of synergistic and antagonistic effects of bio-agents during co-treatments, providing new insight that may be useful for the future bioremediation of diatomaceous and cyanobacterial blooms.

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