Limnology (2019) 20:39–53
https://doi.org/10.1007/s10201-018-0542-6

SPECIAL FEATURE

Ecological and limnological bases for management of overgrown macrophytes

The existence of cyanobactericidal bacteria and growth-inhibiting bacteria on water plants in Lake Ohnuma, Japan

Yohei Miyashita[1] · Takumi Hagiwara[1] · Ichiro Imai[1]

Received: 29 November 2017 / Accepted: 31 January 2018 / Published online: 2 March 2018

Abstract

Microorganisms such as bacteria are considered to be control agents against toxic cyanobacterial blooms such as *Microcystis aeruginosa*. We investigated cyanobactericidal bacteria and growth-inhibiting bacteria against *M. aeruginosa* in the biofilm on the surface of the water plants *Trapa japonica*, *Myriophyllum verticallatum* and *Utricularia vulgaris* at three sites in Ohnuma Quasi-National Park during the period from July to October 2012. Bacteria were isolated using nutrient agar plates, and killing abilities and growth-inhibiting abilities of isolated bacteria were examined for *M. aeruginosa* with co-culture experiments. Effective bacteria (sums of cyanobactericidal bacteria and growth-inhibiting bacteria) were confirmed from the leaves and water roots of *T. japonica* with densities of 3.6×10^5 CFU g^{-1} wet weight—1.2×10^7 CFU g^{-1} wet weight and 1.5×10^6 CFU g^{-1} wet weight—1.4×10^8 CFU g^{-1} wet weight, respectively. *M. verticillatum* and *U. vulgaris* harbored effective bacteria with densities of 2.5×10^6 CFU g^{-1} wet weight—1.1×10^7 CFU g^{-1} wet weight and 2.3×10^6 CFU g^{-1} wet weight—9.2×10^6 CFU g^{-1} wet weight, respectively. Effective bacteria were also detected from water samples collected from all three sites and the most numerous values were detected at Sansui with abundant water plants such as *T. japonica* and *M. verticillatum*. Densities of *M. aeruginosa* tended to be fewer at Sansui and were more abundant at Ohnuma Park with almost no water plants. The results of PCA suggested that the absence and/or lower densities of *M. aeruginosa* was closely related to the abundant presence of effective bacteria detected from the water and biofilms of water plants such as *T. japonica* at Sansui. The present findings provide new insights on the ecology of cyanobactericidal bacteria and growth-inhibiting bacteria, and suggest that water plants provide an environment that influences the abundance of cyanobacterial blooms in freshwater ecosystems.

Keywords Cyanobactericidal bacteria · Growth-inhibiting bacteria · *Microcystis* · Cyanobacterial blooms · Water plants · *Trapa japonica*

Introduction

Widespread eutrophication and climate change have caused occurrences of harmful algal blooms with increasing frequency and scale in aquatic ecosystems in the world (Paerl et al. 2011; Anderson et al. 2012). In freshwater ecosystems such as lakes, ponds and drinking water reservoirs, cyanobacterial blooms have occurred in almost every part of the world, and have caused a wide range of social, economic and environmental problems (Codd 2000; Cronberg and Annadotter 2006). The cyanobacterium *Microcystis aeruginosa* is one of the most toxic bloom-forming species that causes the deterioration of water quality to give negative impacts on animals and human beings, and decreasing aesthetic value of affected water (Carmichael et al. 1985; Herry et al. 2007). Therefore, the development of mitigation strategies is urgently needed for preventing and/or reducing the occurrences of cyanobacterial blooms in the world. As environment-friendly tools for the control of toxic cyanobacterial blooms, cyanobactericidal bacteria have been proposed as promising agents since they are abundant in aquatic ecosystems, rapidly proliferate, effectively kill cyanobacteria

Handling Editor: Maiko Kagami.

✉ Yohei Miyashita
y.miyashita0601@fish.hokudai.ac.jp

[1] Plankton Laboratory, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

and are sometimes prey species-specific (Daft et al. 1975; Manage et al. 2001; Imai et al. 2014; Imai 2015).

In marine ecosystems, numerous cyanobactericidal bacteria were found in the biofilms on the surfaces of seaweeds and seagrasses, and in adjacent surrounding waters in coastal areas such as the Seto Inland Sea, Japan (Imai et al. 2002; Imai 2015) and Puget Sound, USA (Inaba et al. 2017). Hence, artificial restoration and/or developments of seaweed and seagrass beds can be proposed as prevention strategies for harmful algal blooms in coastal environments.

Cyanobactericidal bacteria (algicidal bacteria) were newly found in the biofilm on the surface of submerged reed stems and adjacent surrounding waters in the coastal areas of Lake Biwa (Imai 2010) and Lake Ohnuma, Hokkaido, Japan (Imai et al. 2014; Kojima et al. 2016). Bacteria possessing cyanobactericidal and growth-inhibiting activities against *M. aeruginosa* and the odor-producing *Dolichospermum crassum* (Shimizu et al. 2017) were isolated and confirmed from lake waters (Manage et al. 2000; Yang et al. 2013) and from the biofilm of the submerged reed stems (Kojima et al. 2016) and the water plant *Egeria densa* (Imai et al. 2013). Since there is a serious paucity of studies on cyanobactericidal bacteria (CBs) and growth-inhibiting bacteria (GIBs) associated with water plants, more efforts should be concentrated on the studies on CBs and GIBs of water plants to develop control strategies for nuisance cyanobacterial blooms in freshwater ecosystems. In the present study, we examined the numbers of CBs and GIBs against *M. aeruginosa* in the biofilms of the water plants *Trapa japonica*, *Myriophyllum verticillatum* and *Utricularia vulgaris*. Seasonal fluctuations were investigated on the densities of these active bacteria in the biofilm of water plants and in adjacent waters on the occurrences of *M. aeruginosa*. Control strategies for cyanobacterial blooms were discussed taking the existence of CBs and GIBs on the surface of water plants into consideration.

Materials and methods

Sampling sites

The Ohnuma Quasi-National Park has several lakes such as Lake Ohnuma, Lake Konuma and Junsainuma Lake, etc. Three sampling sites are shown in Fig. 1. Sansui (SS) is a spot of small inner lake where water plants are abundant and there is a small inflow of spring water. SS is connected to the main lake of Ohnuma with a small tunnel water way (diameter of 30 cm). The water plants *Trapa japonica*, *Nuphar pumilum*, *Phragmites australis* and *Utricularia vulgaris*, etc., were observed. A large part of the water surface was covered with *T. japonica* and SS was surrounded all around by *P. australis*. Junsainuma Lake (JL) possesses the reed *P. australis* communities and moderately grown water

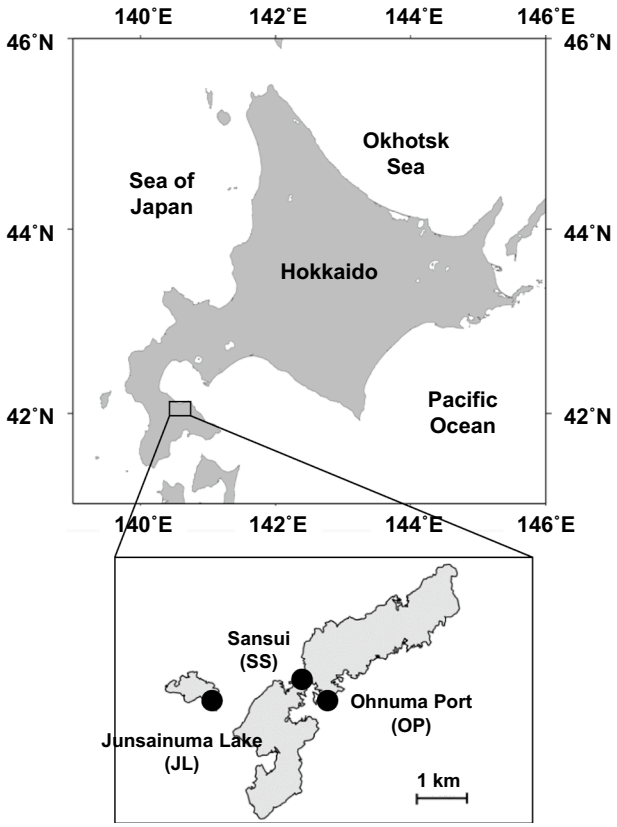

Fig. 1 Location of three sampling stations of Sansui (SS), Junsainuma Lake (JL) and Ohnuma Port (OP), in Ohnuma Quasi-National Park, Hokkaido, Japan

plant community, and has no experience of cyanobacterial blooms. The site of Ohnuma Port (OP) has a port and a pier for a sightseeing boat, and has little communities of reed and water plants there. Water plants were most abundant at SS.

Sample collections were made for the water plants *T. japonica* and *U. vulgaris* at SS, and *T. japonica* and *M. verticillatum* at JL. One plant body was sampled for each species of water plant using autoclaved forceps and sterilized bottles. Water plant samples were put into bottles (500 ml volume) containing 200 ml autoclaved-sterilized distilled water. The lake water samples were also collected at SS and JL at the same time. Lake water samples were also taken from the pier at OP where water plants were not observed.

Samplings were conducted once a month from June to November in 2012. For *T. japonica*, pinnate roots in water and leaves in surface waters were separately treated after the sampling in June. The *T. japonica* sample collected in June was treated without separating water roots and leaves of the plant on the whole. It is noted that samplings were not feasible for the pinnate roots of *T. japonica* at JL in October, and both the pinnate water roots and leaves of *T. japonica* were not able to be collected at either sampling site in November due to the disappearance of *T. japonica* toward the winter

season. Lake water samples were collected from the surface for analyses of nutrients and phytoplankton, and measurements of temperature, and pH were carried out at the sampling times.

Treatments of water samples

Lake water samples were filtered using GF/F glass-fiber filters to obtain samples for nutrient analyses. Nutrient analyses were conducted to examine limiting factors for the growth of phytoplankton. Each nutrient was determined using an autoanalyzer (Quatro, Bran Luebbe). The samples from OP for nutrient analyses were unfortunately lost and unavailable this time.

Collected water samples were fixed with glutaraldehyde to a final concentration of 1% (v/v). Observations were made for identification and counting of phytoplankton using an inverted microscope.

Sample treatments for bacteria

Bacteria attached to the surfaces of water plants were detached and enumerated as follows. To isolate bacteria from water plants in bottles with 200 ml of sterilized distilled water, these bottles were shaken 600 times by hand to detach the biofilm from the surface of the aquatic plants. Then the aquatic plants were taken from the bottle, and those wet weights were measured. The resulting suspensions were serially diluted with sterilized distilled water to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution levels. For each dilution level, 0.1 ml samples were spread onto $LT10^{-1}$ agar medium (Ishida et al. 1986), in triplicate. These plates were cultured for 2 weeks in the dark at room temperature (20–25 °C), and the colonies formed were counted (100–500 colonies) as viable bacteria.

Lake water samples were decimally diluted stepwise with sterilized distilled water to obtain the dilution levels of 10^{-1}, 10^{-2} and 10^{-3}. One milliliter of each sample was filtered using a sterilized 3.0-μm pore size Nuclepore filter to collect particle-associated bacteria (PAB), and the bacteria in the filtrates (< 3.0 μm) were treated as free-living bacteria (FLB). Those filters were put onto $LT10^{-1}$ agar medium and cultured for 2 weeks in the dark. The number of heterotrophic bacteria of PABs was determined by counting the colonies formed on the filters. Since small bacterial colonies consisting of multiple cells on detrital particles would be retained on the filter and form single colonies, the number of PABs were probably underestimated to some extent. Concerning FLBs, 0.1 ml of filtrate for each dilution level was spread onto $LT10^{-1}$ agar medium, cultured for 2 weeks in the dark, and the formed colonies counted. Forty-eight randomly selected colonies of PAB and FLB isolates for each sample were picked, with sterilized toothpicks, inoculated onto each well containing $LT10^{-1}$ agar medium and cultivated at room temperature for 2 weeks in the dark.

Culture of cyanobacteria

An axenic cyanobacterium *Microcystis aeruginosa* (strain MA17, provided by Dr. S. Tsujimura) was used in this investigation. The cyanobacterial cultures were maintained in CT medium (Watanabe and Ichimura 1977) at a temperature of 25 °C under a light intensity of about 100 μmol photon m^{-2} s^{-1} using a 14 h light:10 h dark photo-cycle.

Cyanobactericidal bacteria and growth-inhibiting bacteria against *Microcystis aeruginosa*

PAB and FLB isolates were examined on cyanobactericidal and growth-inhibiting activities with monoaxenic co-culturing experiments. Well-grown axenic *M. aeruginosa* cultures in CT medium were diluted with the same medium to a cell density of about 1.0×10^5 cells ml^{-1}, and 0.8 ml aliquots were inoculated into wells of sterilized 48-well microplates. A small portion of each colony of PAB and FLB isolates was aseptically picked using a sterile toothpick and inoculated onto the cyanobacterial cultures in each well, and then incubated under the same conditions as maintenance of culture for 2 weeks. The number of bacteria which could be picked by a sterile toothpick was about 10^5–10^6 cells ml^{-1}. Four wells with no addition of bacteria were employed as bacteria-free controls in each microplate, and each well was observed after 2 weeks of incubation with an inverted microscope (Nikon ECLIPSE TE200). Using microscopic observations, the wells in which 90% or more *M. aeruginosa* cells were killed as compared with control (no addition of bacteria) were judged as having cyanobactericidal activity (Fig. 2). The wells in which the numbers of cyanobacterial cells were significantly reduced and sank to the bottom, compared with the control, were recognized to have growth-inhibition.

The numbers of cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) against *M. aeruginosa* were calculated by the following formula:

$$B_K = B_C \times S_A / S_T,$$

where B_K is the number of bacteria showing cyanobactericidal activity and growth-inhibiting activity against *M. aeruginosa* (CFU g^{-1} wet weight), B_C is the number of heterotrophic bacteria (CFU g^{-1} wet weight), S_A is the number of bacterial strains showing cyanobactericidal activity or growth-inhibiting activity, and S_T is the total number of strains of heterotrophic bacteria used for co-culture experiments (usually 30 strains). The proportions of CBs and GIBs among the number of viable bacteria were calculated.

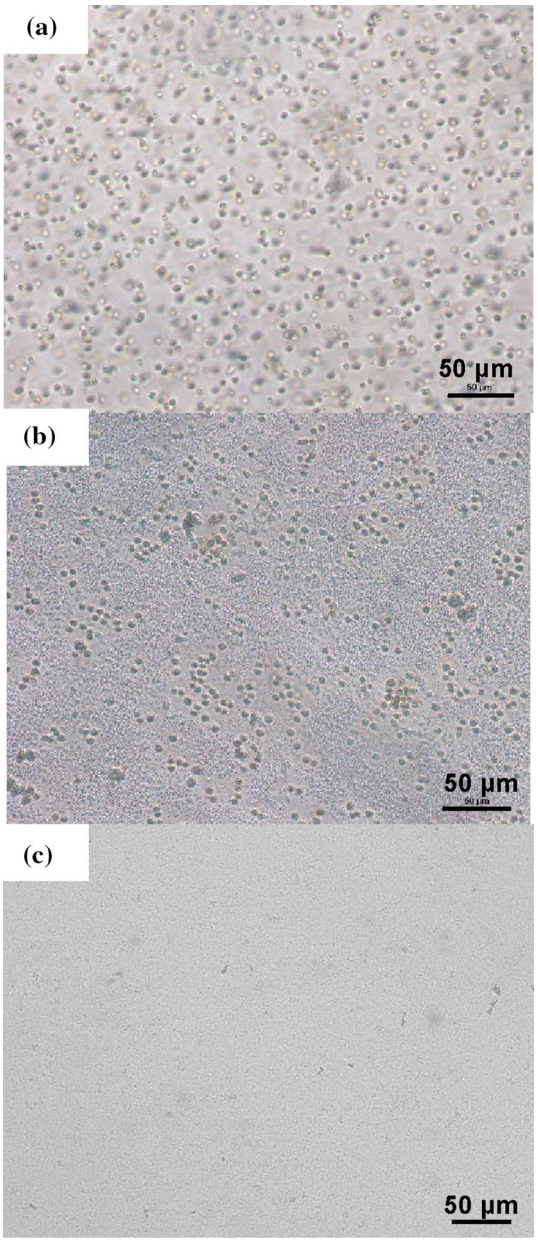

Fig. 2 Microscopic observations on cyanobactericidal activities and growth-inhibiting activities for tested bacterial strains against *Microcystis aeruginosa*. **a** Control (no addition of bacteria), **b** growth-inhibition, and **c** cyanobactericidal activity

Statistical analyses

To show the variation across the sampling sites with respect to the physicochemical characteristics and to estimate the effects of the presence or absence of cyanobactericidal bacteria (CB), growth-inhibiting bacteria (GIB) and nutrients on the cell densities of *M. aeruginosa*, principal component analysis (PCA) was performed with eight environmental variables, i.e., water temperature (WT), DIN, PO_4–P, DIN:DIP ratio, SiO_2–Si, cell densities of *Microcystis aeruginosa*, CBs + GIBs detected from *Trapa japonica* and CBs + GIBs detected from lake water. Because the units were different, PCA was done on standardized variables divided by the standard deviation.

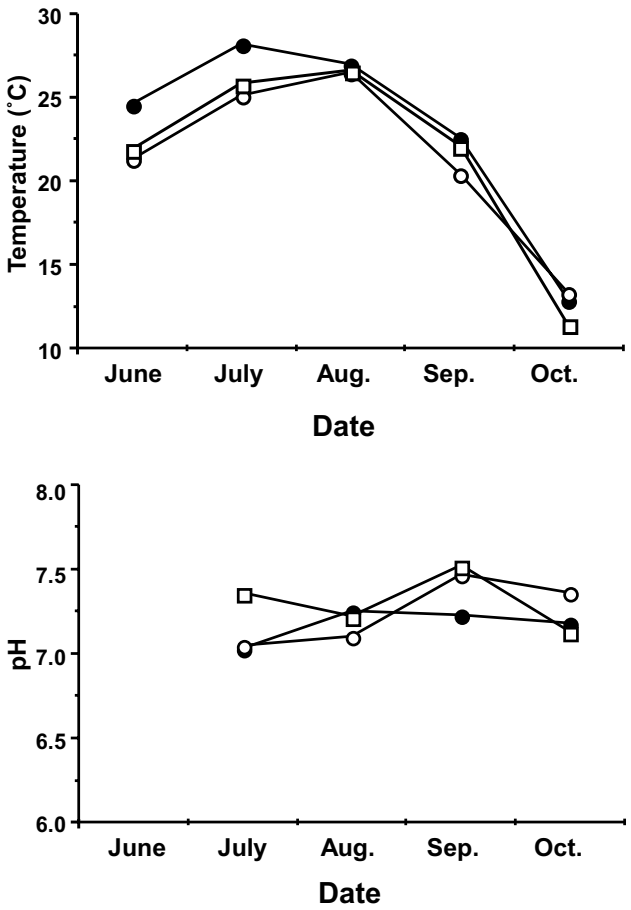

Fig. 3 Temporal changes of water temperature (°C) and pH at Sansui: SS (*open circles*), Junsainuma Lake (JL) (*filled circles*) and Ohnuma Port: OP (*open squares*) from June to November in 2012

Results

Environmental parameters

Changes in temperature at Junsainuma Lake (JL), Sansui (SS) and Ohnuma Port (OP) from June to October in 2012 are shown in Fig. 3. The highest temperature at each station was recorded in July at JL (28.2 °C) and in August at SS (26.5 °C) and OP (26.6 °C). The pH changed in the range of 7.05–7.47 in SS, 7.03–7.25 in JL and 7.13–7.32 in OP, respectively, from July to October. The highest pH was observed in September at SS and OP, and in August at JL. All sites had high pH values in the summer and thereafter

tended to decrease. The variation of pH value was small in JL during the study period.

Figure 4 shows the changes in nutrients from June to November in 2012 at SS and JL. For NH_4–N, it varied between 0.57 and 2.62 µM at SS, tended to decrease from June to October, and sharply increased in November. JL had very low concentrations of NH_4–N, below the detection limit, except in November. NO_2–N was in the range of 0.09–1.06 µM in SS and 0.08–0.33 µM in JL. Both sites showed maximum values in July. The change of NO_3–N showed a range from under the detection limit to 10.5 µM at SS, and from under the detection limit to 0.65 µM at JL. In SS, it was below the detection limit from July to September and increased from October to November, showing the maximum value (10.5 µM). In JL, it showed values below the detection limit from June to October, and slightly increased to as low as 0.65 µM in November. DIN (dissolved inorganic nitrogen: NH_4–N + NO_2–N + NO_3–N) varied between 1.24 and 13.7 µM at SS and 0.11–0.87 µM at JL. At SS, DIN slowly declined from June to September and then sharply increased from October to November (maximum value of 13.7 µM). On the other hand, JL always showed a low concentration, and the maximum value was 0.87 µM, recorded in November. The concentration of PO_4–P varied between 0.13 and 0.94 µM at SS and 0.04–0.095 µM at JL. At SS, PO_4–P increased from June to July (maximum 0.94 µM), and then gradually decreased. As for JL, PO_4–P was consistently very low as compared with SS.

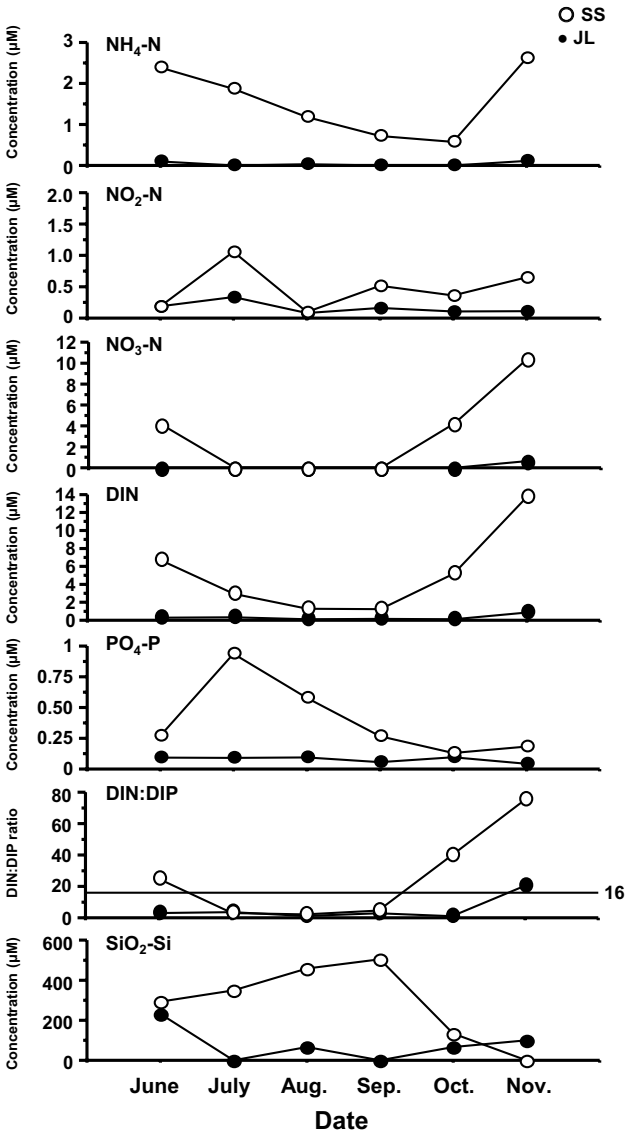

Fig. 4 Temporal changes in nutrients NH_4–N, NO_2–N, NO_3–N, DIN, PO_4–P, SiO_2–Si, and DIN:DIP ratio at Sansui: SS (*open circles*) and Junsainuma Lake: JL (*filled circles*) from June to November in 2012. DIN = NH_4–N + NO_2–N + NO_3–N

The DIN:DIP ratio in SS exceeded the value of 16 (Redfield ratio) in June, October (39.9) and November, and that value was lower during the period from July to September. DIN:DIP at JL was always less than 16 from June to October, and it slightly increased in November (20.4). Regarding the silicate, concentrations ranged from being under the detection limit (November) to 505.0 µM (September) at SS, and under the detection limit (July and September) to 231.9 µM (June) at JL.

Fluctuations of phytoplankton

Figure 5 shows the changes in cell densities (total phytoplankton and *Microcystis aeruginosa*) and taxa compositions of phytoplankton at three points from June to October. In Junsainuma Lake (JL) (Fig. 5a), diatoms dominated in June, and the proportion was about 70%. Cyanobacteria rapidly increased in July and occupied about 80% in abundance of phytoplankton, and the domination of cyanobacteria continued until September. Then the cyanobacteria disappeared, and diatoms increased again in October. *M. aeruginosa* recorded a maximum density of 5.1×10^3 cells ml^{-1} in July, followed by 4.1×10^3 cells ml^{-1} in August. It declined sharply in September with a density of 5.2×10^2 cells ml^{-1}, and disappeared in October.

At Sansui (SS) point (Fig. 5b), diatoms (mainly pennate diatoms) tended to dominate and always occupied over 40% of the phytoplankton community. The proportion of cyanobacteria was basically less dominant than diatoms. The maximum total cell number recorded was 1.3×10^4 cells ml^{-1} in August, and cyanobacteria (mainly *Dolichospermum* spp.) predominated (about 40%). *M. aeruginosa* showed relatively low cell densities at SS as compared with other sites. *M. aeruginosa* was not detected in August and September, and the highest density (3.4×10^3 cells ml^{-1}) of *M. aeruginosa* was observed in October, after the water plants died.

As for Ohnuma Port (OP) (Fig. 5c), the total cell densities of phytoplankton were always higher than the other

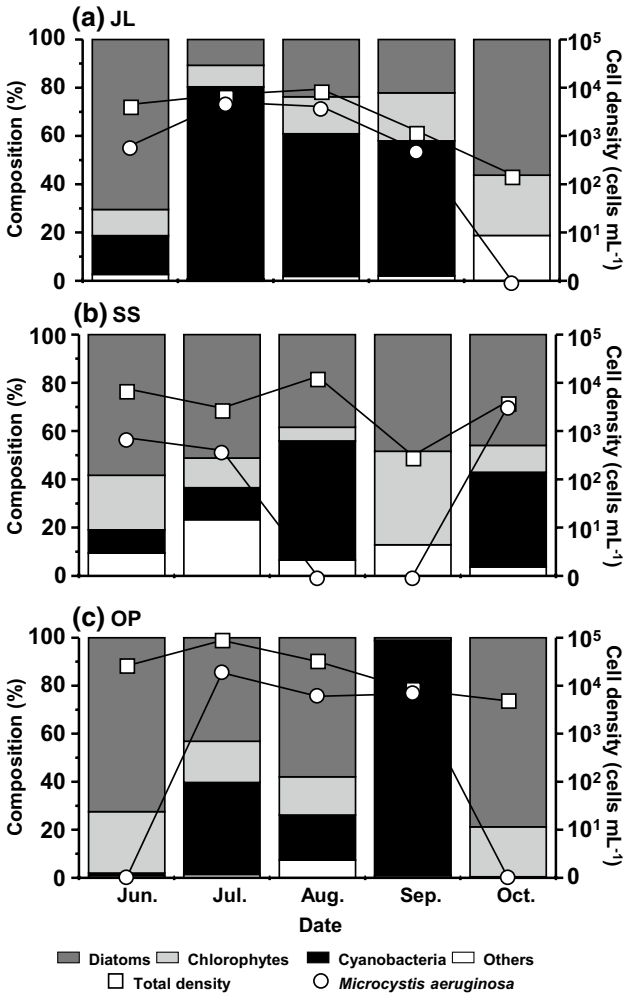

Fig. 5 Changes in cell densities (cells ml^{-1}) and taxa composition histograms of phytoplankton in Junsainuma Lake: JL (**a**), Sansui: SS (**b**) and Ohnuma Port: OP (**c**) in 2012

two sites. Diatoms accounted for more than 70% of the total phytoplankton in June. The proportion of cyanobacteria increased to 40% in July and *M. aeruginosa* attained the highest cell density of 1.9×10^4 cells ml^{-1} in July. *M. aeruginosa* maintained relatively high cell densities of 6.0×10^3 cells ml^{-1}, and 6.8×10^3 cells ml^{-1} in August and September, respectively. Especially in September, cyanobacteria occupied about 99% of the total phytoplankton community, most of which was *M. aeruginosa*. In October *M. aeruginosa* disappeared and diatoms and chlorophytes dominated in the phytoplankton community.

Proportion of cyanobactericidal bacteria and growth-inhibiting bacteria against *Microcystis aeruginosa*

Figure 6 depicts the proportion of cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) isolated from the water plant *Trapa japonica*. The sample of *T. japonica* collected in June was treated without separating water roots and leaves of the plant on the whole. Concerning the proportions of CBs and GIBs detected from water leaves of *T. japonica* in Sansui (SS) (Fig. 6a), CBs were recorded at 6.7% in June and July, 3.3% in August, and GIBs were recorded at 10% in June, 6.7% in July and 13.3% in September, respectively. As for the proportions of CBs and GIBs detected from water roots of *T. japonica* in SS (Fig. 6b), CBs were recorded at 6.7% in June and 13% in August. GIBs were recorded at 10% in June, 3.3% in July, 10% in August, 3.3% in September and 6.7% in October, respectively.

On leaves of *T. japonica* in Junsainuma Lake (JL) (Fig. 6c), CBs were recorded at 16.7% in June, 3.3% in July, August, and September, respectively, and GIBs were recorded at 23.4% in June, 6.7% in July, August, and September, respectively (Fig. 6c). The proportions of CBs detected from water roots of *T. japonica* from JL were recorded at 16.7% in June, 3.3% in July and August, and that of GIBs were recorded at 23.4% in June, 6.7% in July and August and 3.3% in October (Fig. 6d).

Figure 7 shows the proportion of CBs and GIBs isolated from *U. vulgaris* in SS and *M. verticillatum* in JL. No CBs were detected from *U. vulgaris* in SS (Fig. 7a). GIBs were recorded at 6.7% in June and July, and 3.3% in August, respectively. As for the proportions of CBs and GIBs detected from *M. verticillatum* in JL (Fig. 7b), CBs were recorded at 6.7% in June and 3.3% in July, and GIBs were recorded at 6.7% in June and August.

Figure 8 illustrates the proportion of CBs and GIBs isolated from water samples. From the PAB of the water sample at SS (Fig. 8a), CBs were recorded at 3.3% in June and 26.7% in August, and GIBs were recorded at 6.7% in June and October, 13.3% in July and September, and 3.3% in August, respectively. For the FLBs of water samples at SS (Fig. 8b), the percentage of CBs detected in October was recorded at 4.7%, GIBs detected in September and October were recorded at 9.5%.

As for the proportions of CBs and GIBs detected from the PAB of the water sample at JL (Fig. 8c), CBs were recorded at 13.3% in June and 3.3% in August, respectively; and GIBs were recorded at 23.3% in June, 6.7% in July, and 3.3% in August and September. For the FLBs of the water samples at JL (Fig. 8d), the proportion of CBs detected in August was recorded at 3.3%, and GIBs detected in June was recorded at 10%.

No CBs or GIBs of PABs were detected during the entire study period at Ohnuma Port (OP) (Fig. 8e). For the FLBs of the water samples from OP (Fig. 8f), no CBs were detected here. GIBs were recorded at 6.7% in June and October, 24.6% in August and 10% in November, respectively. It was generally shown that about 10% of viable bacteria were CBs

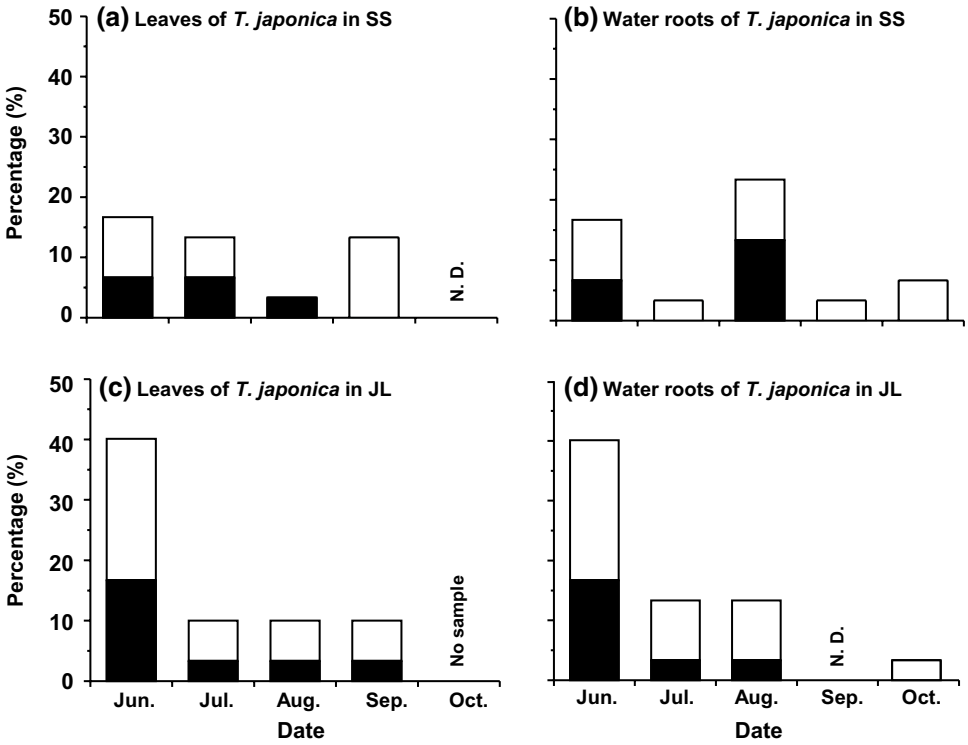

Fig. 6 Proportion of cyanobactericidal bacteria (*black*) and growth-inhibiting bacteria (*white*) isolated from leaves of *Trapa japonica* in Sansui: SS (**a**), water roots of *T. japonica* in SS (**b**), leaves of *T. japonica* in Junsainuma Lake: JL (**c**), and water roots of *T. japonica* in JL (**d**). *ND* not detected

and GIBs in the biofilm on the surface of water plants and in lake water.

Bacterial densities detected from the water plant *Trapa japonica*

Figure 9 depicts the number of viable bacteria, cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) against *Microcystis aeruginosa* detected from leaves and water roots of the water plant *Trapa japonica* at the Sansui (SS) and Junsainuma Lake (JL) sites. The densities of viable bacteria detected from the leaves of *T. japonica* changed, with a tendency to increase from June to August, and showed a maximum value of 1.1×10^8 CFU g^{-1} wet weight in August (Fig. 9a). For *T. japonica* collected from SS in June, two strains of CBs and three strains of GIBs were detected without separating water roots and leaves of the plant on the whole. For the leaves of *T. japonica* at SS, the CB density in June was 1.5×10^5 CFU g^{-1} wet weight and GIBs was 2.2×10^5 CFU g^{-1} wet weight, respectively. Densities of CBs were 2.5×10^6 CFU g^{-1} wet weight in July and 3.6×10^6 CFU g^{-1} wet weight in August for *T. japonica*. No CBs were detected from floating leaves of *T. japonica* in September and October. The GIB densities showed a maximum value of 1.2×10^7 CFU g^{-1} wet weight in September (Fig. 9a). Neither CBs nor GIBs were detected in October from the leaves of *T. japonica* at SS. The sums of those bacteria (CBs + GIBs) tended to increase from June to September, and the maximum value was recorded in September (1.1×10^7 CFU g^{-1} wet weight).

The number of viable bacteria, CBs and GIBs detected from the water roots of *T. japonica* collected at SS are shown in Fig. 9b. The densities of viable bacteria changed similarly to the leaves of *T. japonica*, and showed a maximum value of 5.8×10^8 CFU g^{-1} wet weight in August. CBs were detected twice, in June and August, and showed a maximum value of 7.8×10^7 CFU g^{-1} wet weight in August. GIBs also revealed a maximum density of 5.8×10^7 CFU g^{-1} wet weight in August, and then 9.0×10^6 CFU g^{-1} wet weight in September and 2.1×10^7 CFU g^{-1} wet weight in October. The sums of those bacteria (CBs + GIBs) recorded a maximum value in August (1.4×10^8 CFU g^{-1} wet weight). This was about 400 times greater as compared with the density of those bacteria in June (3.5×10^5 CFU g^{-1} wet weight).

Viable bacteria, CBs and GIBs were detected from *T. japonica* at JL in June when bacteria were collected without separating roots and leaves (Fig. 9c). The densities of viable bacteria were 3.8×10^6 CFU g^{-1} wet weight, and the CBs and GIBs were 6.3×10^5 CFU g^{-1} wet weight and 8.9×10^5 CFU g^{-1} wet weight, respectively.

The densities of viable bacteria changed with a similar tendency to those in SS, and recorded a maximum value in September (9.5×10^7 CFU g^{-1} wet weight). CBs and GIBs were always detected in July and later from the leaves collected at JL, and the maximum value of CBs was 3.2×10^6 CFU g^{-1} wet weight in September and that of GIBs was 6.3×10^6 CFU g^{-1} wet weight in September.

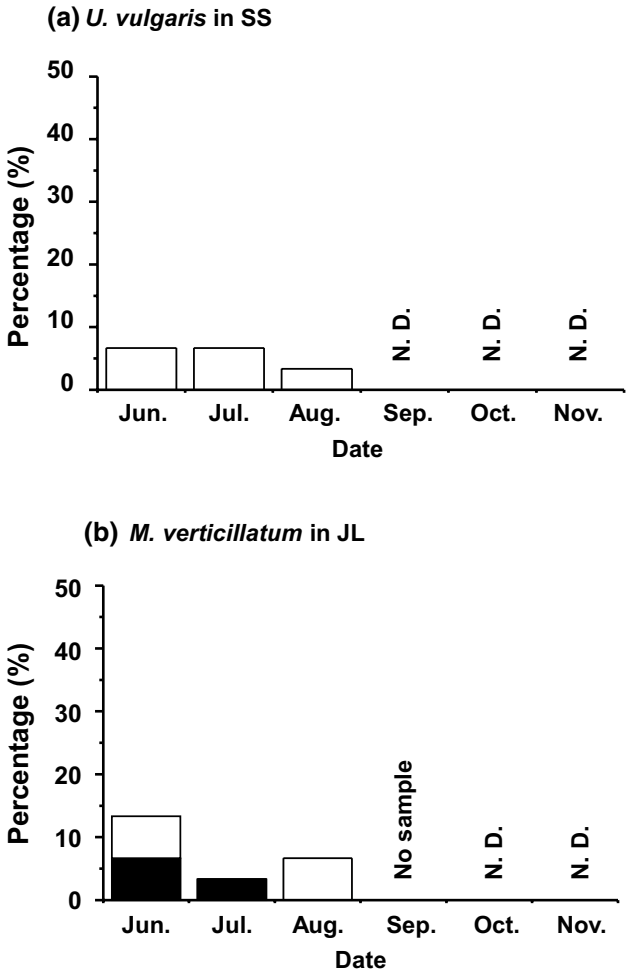

Fig. 7 Proportion of cyanobactericidal bacteria (black) and growth-inhibiting bacteria (white) isolated from the water plant *Utricularia vulgaris* in Sansui: SS (**a**) and the water plant *Myriophyllum verticillatum* in Junsainuma Lake: JL (**b**). *ND* not detected

The effective bacteria (CBs + GIBs) against *M. aeruginosa* increased from June and recorded a maximum density of 9.5×10^6 g^{-1} wet weight in September.

Viable bacteria, CBs and GIBs were also detected from the water roots of *T. japonica* collected at JL (Fig. 9d). Viable bacteria detected from water roots also showed a similar pattern to those of leaves at JL. The densities were around 10^8 CFU g^{-1} wet weight during the summer and autumn seasons in this study.

The maximum values of CBs (4.1×10^6 CFU g^{-1} wet weight) and of GIBs (1.2×10^7 CFU g^{-1} wet weight) were both found in August. The sum of those bacteria (CBs + GIBs) recorded a maximum value in August (1.7×10^7 g^{-1} wet weight).

CBs isolated from both the leaves and roots of *T. japonica* at both sites were detected more frequently from June to August. GIBs were detected with relatively higher frequencies. Effective bacteria (CBs + GIBs) tended to be detected with higher densities in summer, together with the occurrences of cyanobacteria with higher densities.

Cyanobactericidal bacteria and growth-inhibiting bacteria from the water plants *Utricularia vulgaris* and *Myriophyllum verticillatum*

Figure 10a shows the numbers of viable bacteria and growth-inhibiting bacteria (GIBs) detected from *U. vulgaris* sampled from SS. Viable bacteria changed with a tendency to increase from June to October, and recorded a maximum value in October (1.1×10^9 CFU g^{-1} wet weight). No cyanobactericidal bacteria (CB) were detected, and GIBs were detected in June (two strains), July (two strains) and in August (one strain). These GIB densities were calculated to be 2.3×10^6 CFU g^{-1} wet weight in June, 6.2×10^6 CFU g^{-1} wet weight in July, and 9.2×10^6 CFU g^{-1} wet weight in August, respectively. No CBs or GIBs were detected in September or later.

Figure 10b depicts the numbers of viable bacteria, CBs and GIBs detected from the water plant *M. verticillatum* collected at JL. Viable bacteria were around 10^8 CFU g^{-1} wet weight from June to October, and it dropped sharply in November (1.8×10^6 CFU g^{-1} wet weight). Two strains of CBs were detected in June and one strain in July. The densities of CBs were 5.4×10^6 in June and 2.5×10^6 CFU g^{-1} wet weight in July, respectively. CBs were not detected in August or later. GIBs were detected in June and August, and the densities were 5.4×10^6 CFU g^{-1} wet weight in June and 1.0×10^7 CFU g^{-1} wet weight in August. Effective bacteria (CBs + GIBs) against *M. aeruginosa* were observed with relatively higher densities from June to August, but were not detected in September or October.

Cyanobactericidal bacteria and growth-inhibiting bacteria detected from water samples

Figure 11 shows the numbers of viable bacteria, cyanobactericidal bacteria (CBs) and growth-inhibiting bacteria (GIBs) in water samples collected at Sansui (SS), Junsainuma Lake (JL), and Ohnuma Port (OP). Viable bacteria from particle-associated bacteria (PAB) in samples taken at SS (Fig. 11a) changed, with a tendency to decrease from a maximum of 4.4×10^5 CFU ml^{-1} during this study, to a minimum value in November (3.6×10^4 CFU ml^{-1}). CBs and GIBs from PABs in samples taken at SS were detected in June and August, and the CB densities were calculated to be 1.5×10^4 and 3.2×10^4 CFU ml^{-1}, respectively. CBs in PABs were not detected in July or September or later. GIBs in PABs were detected every month until October (Fig. 11a). The densities of GIBs ranged between 4.0×10^3 and 2.9×10^4 CFU ml^{-1}. The densities of effective bacteria (CBs + GIBs) against *M.*

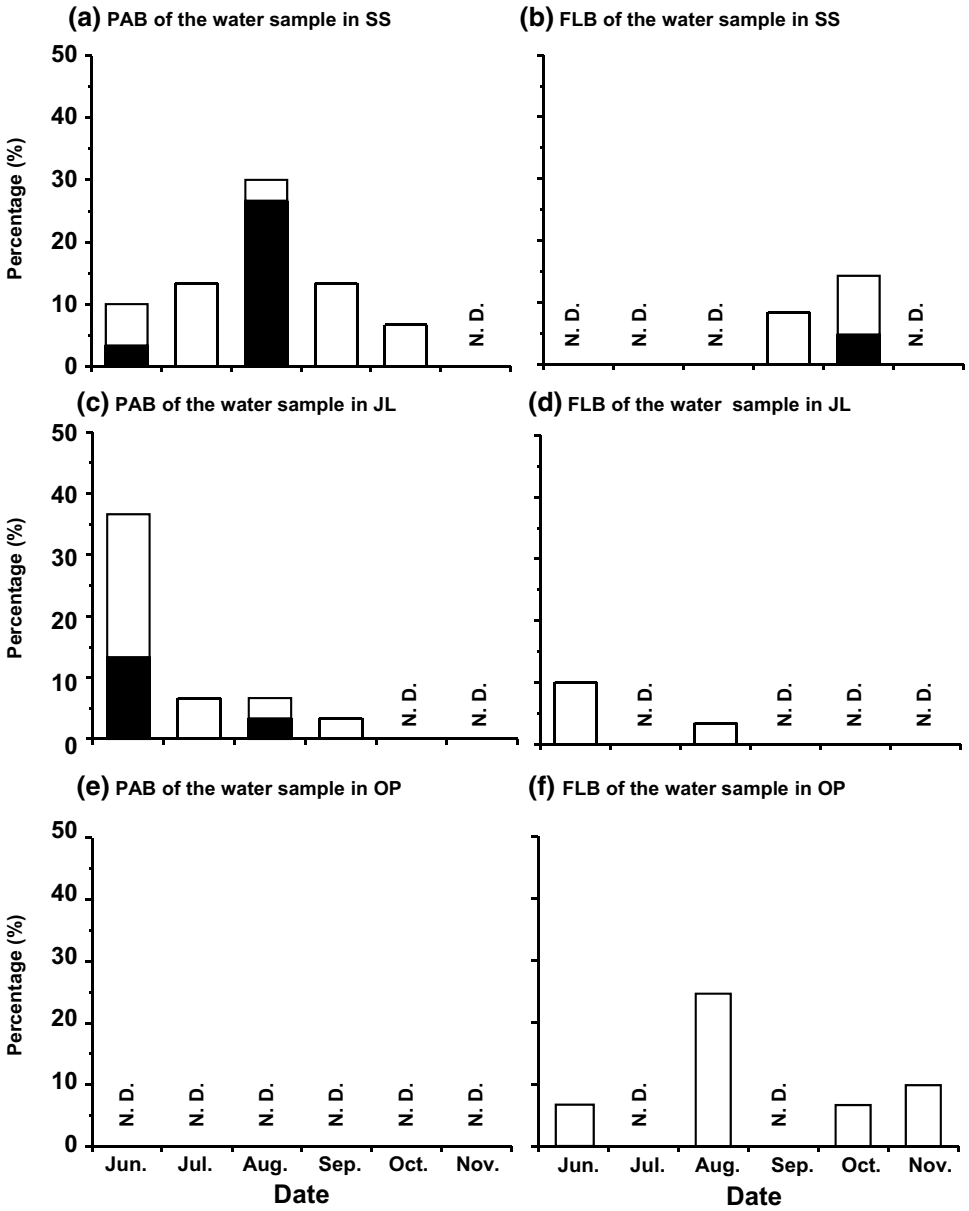

Fig. 8 Proportion of cyanobactericidal bacteria (black) and growth-inhibiting bacteria (white) detected from the water in Sansui: SS (**a**, **b**), Junsainuma Lake: JL (**c**, **d**), and Ohnuma Port: OP (**e**, **f**). Bacteria in water samples were fractionated into particle-associated bacteria (PAB, > 3.0 µm) and free-living bacteria (FLB, < 3.0 µm). *ND* not detected

aeruginosa were higher than 10^4 cells ml^{-1} from June to September. Regarding free-living bacteria (FLB) from SS (Fig. 11b), the numbers of viable FLBs showed a maximum value (3.1×10^5 CFU ml^{-1}) in July, and then decreased until November. CBs were detected in October, and the bacterial density was 6.2×10^2 CFU ml^{-1}. GIBs were found in September and October, and the bacterial densities were 4.5×10^3 and 1.2×10^3 CFU ml^{-1}, respectively.

In JL, viable PAB were recorded at a maximum density of 1.8×10^4 CFU ml^{-1} in June, and fluctuated around 10^4 CFU ml^{-1} until November (Fig. 11c). CBs of PABs were detected in June and August, and CBs of FLBs in August. The bacterial densities were 2.3×10^3 CFU ml^{-1} in June (PAB) and 1.0×10^2 CFU ml^{-1} in August (PAB), respectively. GIBs of PABs were found in June, July, August, and September. The bacterial densities were 4.1×10^3, 7.6×10^2, 1.0×10^2, and 2.3×10^2 CFU ml^{-1}, respectively. Effective bacteria (CBs + GIBs) against *M. aeruginosa* tended to decrease consistently, and the maximum density was 6.4×10^3 CFU ml^{-1} in June. They disappeared in October and November. The viable FLBs from JL (Fig. 11d) changed from around 10^4 CFU ml^{-1} from June to October, and dropped sharply in November (4.8×10^2 CFU ml^{-1}). CBs of FLBs were detected once in August and the density was 3.7×10^2 CFU ml^{-1}. GIBs of FLBs were detected in June, and the density was 1.1×10^3 CFU ml^{-1}.

In OP, the numbers of viable PABs recorded a maximum value of 5.5×10^5 CFU ml^{-1} in June, showed a minimum value in August (1.1×10^4 CFU ml^{-1}), and then increased once more (Fig. 11e). No CBs or GIBs of PAB were

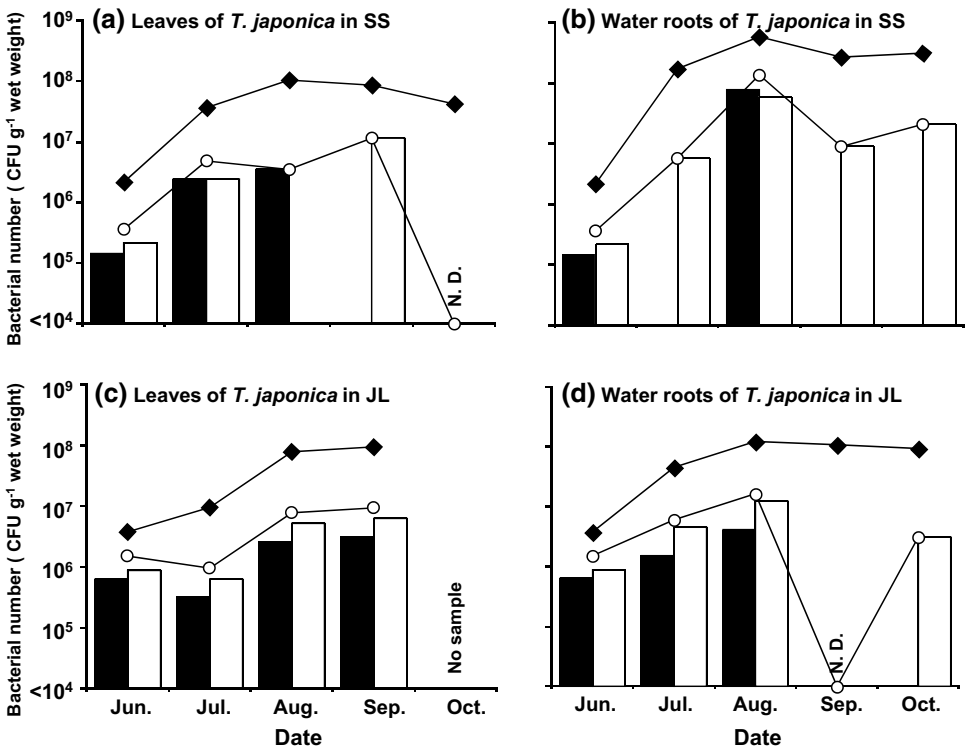

Fig. 9 Changes in the numbers of cyanobactericidal bacteria (black bars), growth-inhibiting bacteria (white bars), sums of those bacteria (open circles) and viable bacteria (filled diamonds) detected from floating leaves of *Trapa japonica* in Sansui: SS (**a**), water roots of *T. japonica* in SS (**b**), floating leaves of *T. japonica* in Junsainuma Lake: JL (**c**) and water roots of *T. japonica* in JL (**d**). *ND* not detected, *CFU* colony forming units

detected during the entire study period. Viable FLBs at OP were found at densities of around 10^4 CFU ml^{-1} (Fig. 11f). GIBs of FLBs were detected in June, August, October and November.

Water plants were more abundant at SS than JL, and OP had almost no water plants. The CBs and GIBs tended to show higher densities at the sites with abundant water plants (SS > JL).

Relationships among cyanobactericidal bacteria, growth-inhibiting bacteria, and *Microcystis aeruginosa*

Table 1 shows the factor loadings of principal component and cumulative contribution ratios (%) to *Microcystis aeruginosa* for eight variables measured at Sansui (SS) and Junsainuma Lake (JL). The first principal component (PC1) of the eight environmental variables measured at JL and SS explained 39.49% of variation in the data. It mainly accounted for cyanobactericidal bacteria (CB) + growth-inhibiting bacteria (GIB) [lake water, SiO_2–Si, PO_4–P, CB + GIB (*Trapa*)] and cell densities of *Microcystis aeruginosa*. The second principal component (PC2) explained 28.81% of the variation. It mainly accounted for the DIN:DIP ratio, DIN and water temperature (WT). The third component (PC3) explained 14.33% of the variation. It mainly accounted for cell densities of *Microcystis aeruginosa*, water temperature (WT), SiO_2–Si.

Figure 12 shows the individual points of the cell densities of *Microcystis aeruginosa* in the PCA. Concerning SS, the points of cell densities of *M. aeruginosa* were plotted in various places on the graph, and the point of the highest density (3360 cells ml^{-1}) in SS was plotted in the upper part of the graph. It seemed that the increasing of cell densities of *M. aeruginosa* tended to be higher with high DIN and DIN:DIP ratio. The two points of no detection of *M. aeruginosa* were plotted in the lower right quadrant. It was indicated that the absence of *M. aeruginosa* in August was closely related to the abundant presence of CBs and GIBs in SS. And the points with fewer *M. aeruginosa* cells (< 1000) also reasonably related with the existence of CBs and GIBs in SS (Fig. 12).

The points indicating cell densities of *M. aeruginosa* in JL were plotted in the lower left quadrant on the graph. Higher densities of *M. aeruginosa* tended to be plotted in lower regions of the graph, compared with the plots of lower cell densities. Accordingly, it was considered that abundances of *M. aeruginosa* were influenced by the warming of water in JL. The point showing absence of *M. aeruginosa* to the left of the PC1 axis on the graph appears to indicate no association of *M. aeruginosa* with CBs and GIBs in JL.

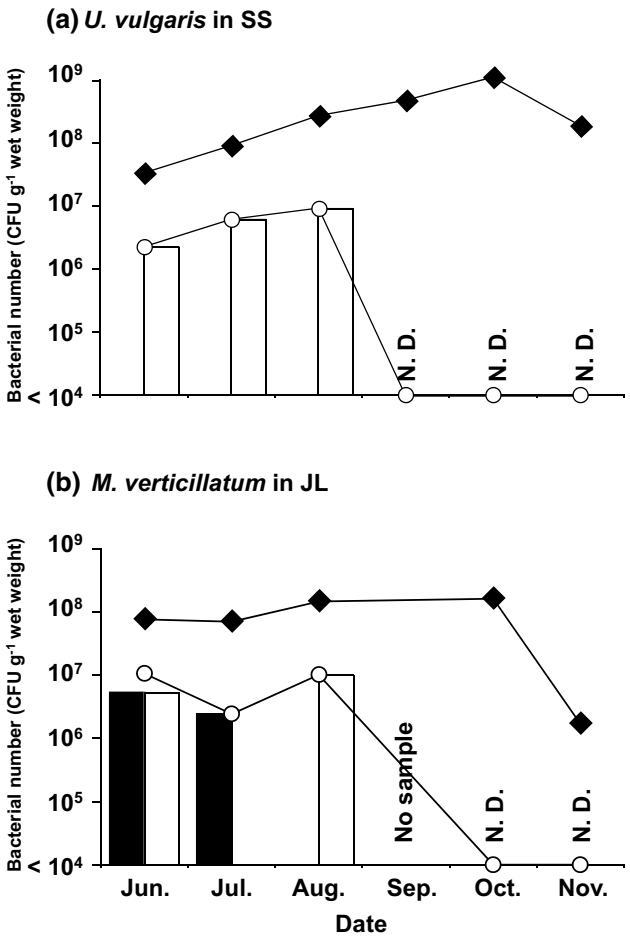

Fig. 10 Numbers of cyanobactericidal bacteria (black bars), growth-inhibiting bacteria (white bars), sums of those bacteria (open circles) and viable bacteria (filled diamonds) detected from the water plant *Utricularia vulgaris* in Sansui: SS (**a**) and the water plant *Myriophyllum verticillatum* in Junsainuma Lake: JL (**b**). *ND* not detected, *CFU* colony forming units

Discussion

Environmental conditions

There was a slight difference in water temperature among Sansui (SS), Junsainuma Lake (JL) and Ohnuma Port (OP), and the highest temperature exceeded over 25 °C (maximum 28.2 °C in JL, Fig. 3). The water temperature of the three surveyed sites exceeded 20 °C from June to September. Yagi et al. (1984) reported that *Microcystis* strains grow at 20–40 °C, and the maximum specific growth rate of *Microcystis* strains was observed at 30–35 °C. The water temperatures appeared not to be optimal but somewhat suitable for the growth of *Microcystis* at studied sites during the study period. Regarding the pH values, OP and SS recorded the highest values in September, and JL in August. A small-scale bloom (about 10^4 cells ml^{-1}) of cyanobacteria was observed at OP in September, accompanying a higher pH environment.

Regarding nutrients, JL generally showed lower concentrations than SS (Fig. 4), ranging between 0.0015 and 0.012 mg N l^{-1}. Nitrogen could be the limiting nutrient for phytoplankton in both sites during the summer season (July–September). However, it was reported that *Microcystis aeruginosa* multiplies under a nitrogen concentration as low as 0.1 mg N l^{-1} (Takahashi et al. 2012). It was also reported in laboratory growth experiments, comparing with the diatom *Cyclotella* sp., that the half saturation constant for nitrogen (K_N) of *M. aeruginosa* was 0.05 mg N l^{-1}, which was a lower K_N than for *Cyclotella* sp. (Takahashi et al. 2012). *M. aeruginosa* probably has an ability to utilize and exhaust nutrients (e.g., nitrogen) reaching as low as 0.01 mg N l^{-1} or lower. Therefore, it must be relatively easy for *M. aeruginosa* to grow even in low nitrogen environments (Takahashi et al. 2012) such as JL, as shown in Fig. 4.

Relationships among cyanobactericidal bacteria, growth-inhibiting bacteria and phytoplankton composition

Relatively higher cell densities of cyanobacterial blooms were observed in Ohnuma Port (OP) from July to September, and the proportion was as high over 99% in September (Fig. 5). During this study, no cyanobactericidal bacteria (CBs) or growth-inhibiting bacteria (GIBs) were detected from particle-associated bacteria (PAB) in water samples from OP, and also no CBs were detected from free-living bacteria (FLB) (Fig. 11). Cell densities of *Microcystis aeruginosa* were rather abundant in OP, and the absence of CBs and GIBs probably contributed to the abundance of *M. aeruginosa*. But the presence of GIBs in the FLB fraction appeared to suppress *M. aeruginosa* to certain extent at OP.

On the other hand, in water at Sansui (SS), CBs and GIBs against *M. aeruginosa* were detected with higher frequencies in almost every month and with higher densities than Junsainuma Lake (JL) and OP (Fig. 11). The water plants, including *T. japonica*, were much more abundant in SS than in JL or OP. Concerning the abundance and composition of phytoplankton in SS, the cell densities of *M. aeruginosa* were as low as undetectable in August and September despite the suitable temperature and unlimited nutrients (Figs. 3, 4, 5). Although several studies have suggested that aquatic plants have allelopathic effects on cyanobacteria (Nakai et al. 1997; Lurling et al. 2006), the results of this study newly suggest a possibility that CBs and/or GIBs suppressed *M. aeruginosa* and controlled the occurrences of its bloom in places such as SS with abundant water plants.

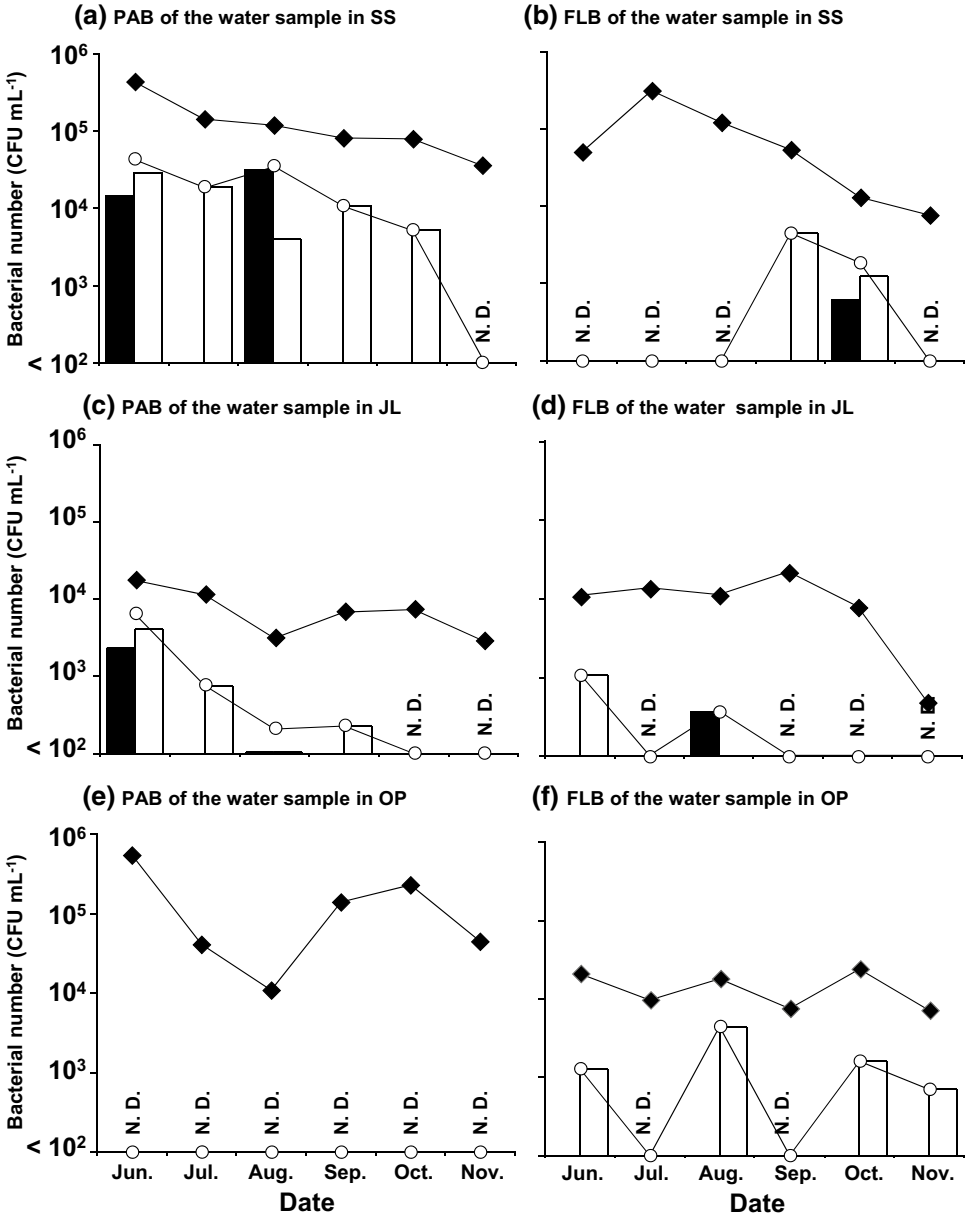

Fig. 11 Numbers of cyanobactericidal bacteria (black bars), growth-inhibiting bacteria (white bars), sums of those bacteria (open circles) and viable bacteria (filled diamonds) detected from the water in Sansui: SS (**a**, **b**), Junsainuma Lake: JL (**c**, **d**), and Ohnuma Port: OP (**e**, **f**). Bacteria in water samples were fractionated into particle-associated bacteria (PAB > 3.0 μm) and free-living bacteria (FLB < 3.0 μm). *ND* not detected, *CFU* colony forming units

Relationships among cyanobactericidal bacteria, growth-inhibiting bacteria and aquatic plants

The highest cell density of *Microcystis aeruginosa* was observed in water at Ohnuma Port (OP) during the summer season (Fig. 5). Junsainuma Lake (JL) showed relatively lower cell densities of phytoplankton. JL was surrounded by a zone of the reed *Phragmites australis* communities and there were communities of submerged and floating plants. At Sansui (SS), the cell densities of *M. aeruginosa* were lower than OP despite the high cell densities of pennate diatoms. Cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) were detected in the waters collected at SS with higher abundance than OP. CBs and GIBs tended to be detected with high frequency and high densities from the waters of SS where aquatic plants grow abundantly (Fig. 11). In addition, high densities of CBs and GIBs also tended to be detected from the biofilm on the surface of leaves and water roots of the water plant *Trapa japonica* (Fig. 9). As a result of correlation analysis, a positive relationship was found between the total number of CBs + GIBs detected from the biofilm of *T. japonica* and those bacteria detected from lake water, even though it was not statistically significant ($r = 0.45$, $p = 0.19$). It is presumed that the detachment of biofilms from the surface of water plants needs some disturbance of water by strong winds and wave action. CBs and GIBs in biofilms are thought to be released to surrounding water from the surface of water plants after some kind of

Table 1 Factor loadings of principal components and cumulative contribution ratio (%) for eight variables at Sansui and Junsainuma Lake

	PC1	PC2	PC3
Water temperature (WT)	0.050	− 0.455	0.584
DIN	0.283	0.536	0.209
PO_4–P	0.434	− 0.154	0.088
DIN:DIP	0.075	0.621	0.197
SiO_2–Si	0.502	− 0.088	− 0.116
Microcystis aeruginosa	− 0.337	0.105	0.695
CB + GIB (*Trapa*)	0.313	− 0.260	0.214
CB + GIB (lake water)	0.510	0.102	0.165
Proportion of variance (%)	39.49	28.81	14.33
Cumulative contribution ratio (%)	39.49	68.30	82.63

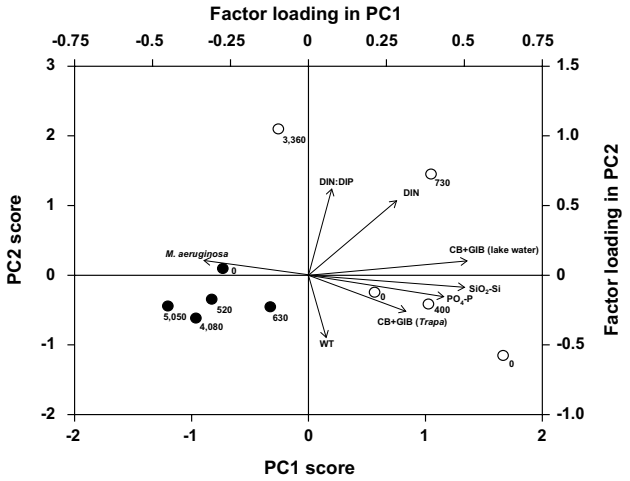

Fig. 12 Plots of data for cell densities of *M. aeruginosa* against values for the first two principal components, PC1 and PC2 at Sansui (open circles) and Junsainuma Lake (filled circles). *WT* water temperature, *DIN* dissolved inorganic nitrogen, *PO_4–P* phosphate phosphorus, *DIN:DIP* DIN DIP ratio, *SiO_2–Si* silicate–silicon, *M. aeruginosa* cell densities of *M. aeruginosa*, *CB + GIB (Trapa)* sum of the number of cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) detected from the biofilm of *Trapa japonica*, *CB + GIB (lake water)* sum of the number of cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) detected from lake water

meteorological, limnological or biological event. More data are needed to reach a conclusion.

It is reported that *T. japonica* has a strong ability to absorb nutrients (Takamura et al. 2003; Iamchaturapatr et al. 2007; Miyashita et al. 2015), and has allelopathic effects against green algae (Akiyama and Kunii 1989). The nutrient absorption ability of diatoms inhabiting the biofilm on the surface of *T. japonica* was also recently reported (Miyashita et al. 2015). These characteristics of *T. japonica*, together with CBs and GIBs in the biofilm on the surface of *T. japonica* probably contribute to controlling not only the blooms of green algae but also cyanobacteria such as *M. aeruginosa*. These possibilities might be the reason why there were fewer *M. aeruginosa* at SS with more *T. japonica*.

CBs were also detected from aquatic plants other than *T. japonica*, such as *Egeria densa* (Imai et al. 2013). A possibility of controlling the cyanobacterial blooms was also revealed in the submerged plants such as *Utricularia vulgaris* and *Myriophyllum verticillatum* (Fig. 10). Furthermore, submerged plants were also reported to have allelopathic effects against phytoplankton (Nakai et al. 1997; Lurling et al. 2006). CBs and GIBs were also detected from the biofilm of submerged stems of *Phragmites australis* that forms communities on the shore (Imai 2010; Kojima et al. 2016). Therefore, all three types of water plants, such as emerged, floating and submerged plants would be useful for controlling the nuisance cyanobacterial blooms such as *M. aeruginosa* by the effects of CBs and GIBs.

Cyanobactericidal bacteria and growth-inhibiting bacteria detected from lake water

In marine costal environments, it has been reported that most algicidal bacteria were isolated from the particle-associated fraction as particle-associated bacteria (PAB) in seawater of the Seto Inland Sea (Park et al. 2010). It was recently observed in seagrass systems in the Seto Island Sea and in Puget Sound, USA, that strains of algicidal bacteria were detected abundantly from the biofilm of *Zostera marina* (Imai et al. 2016; Inaba et al. 2017), and the same bacterial strains were also detected from the seawater of seagrass beds and off-shore water (Sakami et al. 2017).

In this study, cyanobactericidal bacteria (CB) and growth-inhibiting bacteria (GIB) were detected from lake water, especially at Sansui (SS) and Junsainuma Lake (JL). Ohnuma Port (OP) showed lower abundance and frequency of CBs and GIBs than SS. The lake water samples tended to have more CBs and GIBs against *M. aeruginosa* in the PAB than in the FLB fraction. It was also confirmed that many CBs and GIBs exist as PABs in lake water. Since many more CBs and GIBs were isolated and confirmed from the biofilm of aquatic plants, it is considered that CBs and GIBs of the PAB fraction in lake water probably originated from the biofilm derived from aquatic plants.

Importance of cyanobactericidal bacteria and growth-inhibiting bacteria based on PCA

Looking at the relationship between *Microcystis aeruginosa*, cyanobacterial bacteria (CB) and growth-inhibiting bacteria (GIB), it seemed that the cell densities of *M. aeruginosa* tended to be higher with fewer effective bacteria (CB + GIB) (Fig. 12). The maximum cell density was

at most 3360 cells ml^{-1} at SS despite no severe nutrient limitation (Fig. 4). Consequently, CBs and GIBs could be considered to control the blooming of *M. aeruginosa* to a certain extent.

The cell densities of *M. aeruginosa* in JL were plotted in the lower left quadrant on the graph (Fig. 12). It seems that these differences were caused by the biomass of water plants in each site. The water surface of SS was rather largely occupied with water plants, but a part of the water surface was covered with water plants in JL. Unfortunately, we have no quantitative data for the biomass of water plants at both sites. It is suggested that CBs and GIBs depend on the biomass of water plants, although detailed studies measuring water plant biomass quantitatively, together with CBs and GIBs are needed in the future.

Overview: lake management using water plants and effective bacteria

The "Sato-Umi" concept (Yanagi 2008) appears to be helpful for the control of nuisance cyanobacterial blooms in lake systems by using water plants. Sato-Umi inspires management of coastal areas where human activities promote higher productivity and biodiversity of coastal ecosystems. Seagrass beds are important components for promoting the Sato-Umi initiative plan for sustainable management in coastal sea areas.

The amounts of aquatic plants and phytoplankton biomass are reported to be in a competitive relationship (van Donk and van de Bund 2002; Ruggiero et al. 2003; Hilt and Gross 2008, etc.). The present study revealed abundant amounts of CBs and GIBs in the biofilms of water plants. Hence, it is suggested that in such a competition, CBs and GIBs are plausible candidates in helping water plants dominate.

The restoration and/or creation of water plant zones and reed communities are thought to contribute to preventing occurrences of nuisance cyanobacterial blooms and to maintain the higher productivity and biodiversity of lake systems. However, overgrowing of water plants may cause deterioration of water quality including the reduction of dissolved oxygen. Appropriate management and control (harvesting of excess water plants) should be exercised on the biomass, distribution and species composition of available water plants.

Acknowledgements We would like to express our sincere gratitude to Professor Kuniaki Tanaka of the Environmental Science Course, Hokkaido University of Education, Hakodate Campus for helpful advice during this research. We express our sincere thanks to those at Ohnuma Boating Co., Ltd. for overall cooperation with this research. We are grateful to Mr. Tsukasa Miyazaki of Ohnuma Fisheries Cooperative Association for his helpful information on Lake Ohnuma. We appreciate Messrs. Keigo Sekiguchi, Taku Kosugi and Akira Kobayashi of Nanae Town Office for their diligent cooperation with this study.

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