INTRODUCTION

Blooms of the cyanobacterium *Microcystis aeruginosa* are common in many eutrophic lakes and reservoirs where they may cause serious environmental problems such as a decreased recreational value, increased cost of water treatment and increased production of toxic and malodorous compounds. The physiology and ecology of *M. aeruginosa* have been extensively investigated, and many of the bloom-initiating mechanisms have been clarified (Nakano et al. 2001a). However, the factors acting against the proliferation of *M. aeruginosa* are less well understood.

In addition to environmental changes, such as decrease in water temperature and nutrient depletion (Paerl 1983), *Microcystis* populations may be subjected to 2 biological loss processes: grazing (Beveridge et al. 1993, Klaveness 1995, Zhang et al. 1996) and viral infection (Fuhrman & Suttle 1993, Suttle 1994, Tucker & Pollard 2005). Grazing by higher trophic level organisms appears to be a major contributor to the loss of *Microcystis* blooms (Dryden & Wright 1987). Many organisms graze on *Microcystis* spp., including fish (Miura 1990, Northcott et al. 1991), zooplankton (Hanazato & Yasuno 1984) and protozoa (e.g. Dryden & Wright 1987). Among these, the highly abundant protozoa may be the most important grazers of *Microcystis* spp. Previous laboratory studies have demonstrated that some species of flagellates, including the heterotrophic flagellate *Colloidicyon triciliatum* and the mixotrophic flagellate *Poterioochromonas* sp. (Nishibe et al. 2002, Ou et al. 2005), and rhizopods (Nishibe et al. 2004) graze on *Microcystis* populations. While amoeboid grazing is relatively well understood there is less information regarding flagellate grazing on *Microcystis* populations.

We isolated a flagellate grazing on *Microcystis aeruginosa* from a hypereutrophic pond in Japan and
examined the ecophysiology of this flagellate on its prey using comparisons of grazing behavior on toxic and non-toxic Microcystis strains, along with other algal strains, which represent different food types and qualities.

MATERIALS AND METHODS

Isolation and culture of the heterotrophic flagellate. Furuike Pond (33° 49’ 21’’ N, 132° 48’ 04’’ E; altitude 40 m) is an impoundment located in Sancho, Matsuyama City, Ehime Prefecture, Japan. It has a surface area of 7400 m² and a maximum depth of 1.5 m. The pond is hypereutrophic due to anthropogenic loading from the watershed (Nakano et al. 2001b) and is heavily populated by cyanobacteria, such as Microcystis aeruginosa and M. wesenbergii, from early summer to fall each year (Manage et al. 1999).

For isolation of a flagellate capable of grazing on Microcystis species, Microcystis aeruginosa NIES-298 was incubated in Cytophaga broth (CB) medium (MCC-NIES; www.nies.go.jp/biology/mcc/home_j.htm) and mixed with a pond water sample that had been filtered through a Nitex net (mesh size, 154 µm) for removal of larger zooplankton. This mixture was incubated at 25°C in the dark for 3 to 4 d. Flagellates were isolated using a Pasteur micropipette under a light microscope. We established a monoclonal flagellate culture using a repetitive serial isolation process consisting of enrichment, dilution and single cell isolation steps. The isolated flagellate was maintained using a commercial strain of Chlorella™ (Aquanet) as prey. Water was collected from the Pal’tang Reservoir, Korea, filtered through a Whatman GF/F filter and sterilized through a Nitex net (mesh size, 154 µm) for removal of larger zooplankton. This mixture was incubated at 25°C in the dark for 3 to 4 d. Flagellates were isolated using a Pasteur micropipette under a light microscope. We established a monoclonal flagellate culture using a repetitive serial isolation process consisting of enrichment, dilution and single cell isolation steps. The isolated flagellate was maintained using a commercial strain of Chlorella™ (Aquanet) as prey.

Chlorella species, Cytophaga aeruginosa NIES-298 was incubated in Cytophaga broth (CB) medium (MCC-NIES; www.nies.go.jp/biology/mcc/home_j.htm) and mixed with a pond water sample that had been filtered through a Nitex net (mesh size, 154 µm) for removal of larger zooplankton. This mixture was incubated at 25°C in the dark for 3 to 4 d. Flagellates were isolated using a Pasteur micropipette under a light microscope. We established a monoclonal flagellate culture using a repetitive serial isolation process consisting of enrichment, dilution and single cell isolation steps. The isolated flagellate was maintained using a commercial strain of Chlorella™ (Aquanet) as prey.

To identify the flagellate, we used light and epifluorescence microscopy and 18S rDNA sequencing. Morphological observations, including the presence of 2 long apical and equal flagella inserting near the anterior end at the top of the ventral groove formed by the curving lateral margins of the cell, a cell dimension of 15 × 20 µm (subject to change during feeding), and feeding observations such as free movement or rotation during feeding via the ventral groove, identified the flagellate as Diphylella rotans (Massart 1920, Brugerolle & Patterson 1990). This finding was confirmed by 18S rDNA sequencing (authors’ unpubl. data), which showed 99.8% homology with D. rotans Massart (Accession No. AF420478).

Cyanobacteria and diatom strains used as prey in this study. Five strains of Microcystis spp. were used, comprising 4 toxic strains (Microcystis aeruginosa NIER-10001, M. aeruginosa NIES-298, M. viridis NIES-102, and M. wesenbergii NIES-104) and 1 non-toxic strain (M. aeruginosa NIES-101) (Table 1). M. aeruginosa NIER-10001 was obtained from the National Institute of Environmental Research (NIER), Korea, while the latter 4 cyanobacteria were obtained from the National Institute for Environmental Studies (NIES), Japan. The cells of these Microcystis strains were spherical and had diameters ranging from 3.7 to 5.7 µm and volumes ranging from 27 to 97 µm³ (Table 1). All strains were maintained at 25°C in CB media under a light intensity of 40 to 48 µE m⁻² s⁻¹ with a 12:12 h light:dark cycle. Cultures with organisms in the exponential growth phase were used for feeding experiments.

Two centric diatoms, similar in morphology, were used for the evaluation of food selectivity and quality. The small diatom Stephanodiscus hantzschii UTCC 267 was obtained from the University of Toronto Culture Collection of Algae and Cyanobacteria, Canada (UTCC), while the larger diatom Cyclotella sp. HYK0210-A1 was first isolated by Kang et al. (2005) using the sample from the Gyeonggan stream in Korea. The 2 diatom species were cultured and maintained at 15°C in DM media under a light intensity of 50 µE m⁻² s⁻¹ with a 12:12 h light:dark cycle. Cultures in the exponential growth phase were used for feeding experiments.

Table 1. Mean cell diameter (µm), cell volume (µm³), and microcystin-LR concentration (µg mg⁻¹ dry cells) of toxic and non-toxic strains of Microcystis spp. (n > 300)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean cell diameter</th>
<th>Mean cell volume</th>
<th>Toxin (LR) concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. aeruginosa NIER-10001</td>
<td>4.1 ± 0.39</td>
<td>36</td>
<td>1.438</td>
<td>This study</td>
</tr>
<tr>
<td>M. aeruginosa NIES-298</td>
<td>4.3 ± 0.49</td>
<td>41</td>
<td>0.168</td>
<td>Yasuno et al. (1998)</td>
</tr>
<tr>
<td>M. aeruginosa NIES-101</td>
<td>3.7 ± 0.37</td>
<td>27</td>
<td>0</td>
<td>Yasuno et al. (1998)</td>
</tr>
<tr>
<td>M. viridis NIES-102</td>
<td>5.5 ± 0.93</td>
<td>87</td>
<td>0.287</td>
<td>Yasuno et al. (1998)</td>
</tr>
<tr>
<td>M. wesenbergii NIES-104</td>
<td>5.7 ± 0.57</td>
<td>97</td>
<td>0.004</td>
<td>Yasuno et al. (1998)</td>
</tr>
</tbody>
</table>
Measurement of microcystins. The microcystin concentration of *Microcystis aeruginosa* NIER-10001 was determined as described by the ELISA method of Nagata et al. (1995a,b). The anti-microcystin M8H5 MAb used in the ELISA recognized almost equally the major microcystin derivatives such as microcystin-LR, microcystin-RR and microcystin-YR. We chose microcystin-LR as the standard for the ELISA as it is the most widely studied microcystin (C_{49}H_{74}N_{10}O_{12} = 995.17, 250 µg, Wako). The water specimens to be analyzed were treated twice by freeze-thawing followed by filtration over glass filters (Whatman GF/C, 25 mm diameter). The samples or the standards were mixed with an appropriate dilution of M8H5 MAb and added to a 96-well microtiter plate (Coaster) that had been coated with a microcystin-LR bovine serum albumin conjugate. After washing, the bound MAb was detected with horseradish peroxidase-labeled goat anti-mouse IgG (TAGO 4550) and its substrate (0.1 mg H2O2 in 0.1 M acetate buffer [pH 5]). The microcystin concentration was determined from the standard competitive curve of microcystin-LR. For the remaining 4 *Microcystis* strains we used the microcystin contents reported by Yasuno et al. (1998).

Feeding experiments. Based on the concentration of chlorophyll *a*, *Microcystis aeruginosa* NIER-10001 (1 × 10^6 cells ml⁻¹), *Chlorella*TM (1 × 10^6 cells ml⁻¹), *Stephanodiscus hantzschii* UTCC 267 (1 × 10^4 cells ml⁻¹) and *Cyclotella meneghiniana* HYL0210-A1 (1 × 10^4 cells ml⁻¹) were used as prey to evaluate food selectivity or quality of the prey fed to the flagellate. All algae were cultured to the exponential growth phase, harvested by centrifugation and separately resuspended in an Erlenmeyer flask containing 300 ml of FSW. We added *Diphyllleia rotans* that had been starved for 2 d to minimize possible residual growth resulting from ingestion of prey during batch culture, or added no flagellates as a control. The initial prey density was 10^4 to 10^6 cells ml⁻¹, while that of *D. rotans* was 1 × 10^3 cells ml⁻¹. Duplicate cultures were incubated at 20 to 25°C under 40 to 48 µE m⁻² s⁻¹ with a 12:12 h light:dark cycle for 5 d. To monitor the changes in algal and flagellate cell density over time, 5 ml of culture suspension was withdrawn from each flask every 12 h. These samples were fixed with 25% buffered glutaraldehyde at a final concentration of 1% and prey density was enumerated using a haemocytometer under a light microscope. Flagellates were counted under an epifluorescence microscope following ultraviolet excitation by the primulin method (Caron 1983).

Because the highest growth of the flagellate was detected with a diet of *Microcystis aeruginosa* NIER-10001 in preliminary experiments, we cultured *M. aeruginosa* NIER-10001 under various temperatures (15, 20, 25 and 30°C) or pH levels (5, 6, 7, 8, 9 and 10) to examine the growth requirements of *Diphyllleia rotans*. Changes in flagellate cell density were monitored as described above.

For quantitative examination of the responses of *Diphyllleia rotans* to 5 different *Microcystis* prey—4 toxic strains (*Microcystis aeruginosa* NIER-10001, *M. aeruginosa* NIES-298, *M. viridis* NIES-102, and *M. wesenbergii* NIES-104) and 1 non-toxic strain (*M. aeruginosa* NIES-101)—we conducted feeding experiments using different densities of the *Microcystis* strains (0, 2, 4, 6, 8, 10, 30 and 50 × 10^5 cells ml⁻¹ for each strain) in the presence of *D. rotans* at 1 × 10^3 cells ml⁻¹. All *Microcystis* cells were grown to the exponential growth phase, harvested by centrifugation and resuspended at various densities in Erlenmeyer flasks containing 300 ml of FSW. These suspensions were incubated with the flagellate and monitored for flagellate and *Microcystis* cell densities, as described above.

The specific growth rate (µ, d⁻¹) of *Diphyllleia rotans* was calculated as:

\[ µ = \frac{(\ln F_t - \ln F_0)}{t} \]

where \( F_0 \) is the initial density of *D. rotans*, and \( F_t \) is the final density after time \( t \).

Ingestion rate (\( I, \text{prey HF}^{-1} \text{d}^{-1} \)) was calculated using the equations of Frost (1972) and Heinboekel (1978) as:

\[ I = p \times C \times 24 \]

where \( p \) is the mean prey concentration and \( C \) is the clearance rate (volume grazer⁻¹ h⁻¹). \( I \) and \( \mu \) of *Diphyllleia rotans* were analyzed using the Michaelis-Menten equations, respectively, as previously described (Jeong et al. 2002).

RESULTS

*Diphyllleia rotans* showed active growth when feeding on *Microcystis aeruginosa* (Fig. 1A) and lower (but significant) growth on *Chlorella*TM (Fig. 1B). The food vacuole of the flagellate contained 1 to 3 cells of *Microcystis* or *Chlorella*TM, and the ingested cells seemed to be decomposed within a few minutes after ingestion.

In contrast, we did not observe any significant flagellate proliferation when fed *Cyclotella meneghiniana* or *Stephanodiscus hantzschii*, perhaps due to the difficulty in ingesting the rigid cell, even though the food vacuole of the flagellate contained one cell of *C. meneghiniana* or *S. hantzschii* (Fig. 1C,D).

Growth of *Diphyllleia rotans* was most active when the flagellate was incubated at 25°C, whereas the flagellate showed a low growth rate at 15°C (Fig. 2A). The flagellate grew actively at a pH range of 6 to 9, with less active growth observed at pH 5 to 10 (Fig. 2B).
Diphylleia rotans showed different responses to the various Microcystis strains examined in the present study (Figs. 3 & 4). The maximum numerical specific growth rate ($\mu_{\text{max}}$) of $D. \ rotans$ was 1.88 d$^{-1}$ for $M. \ aeruginosa$ NIER-10001, 1.43 for NIES-298, 1.18 for NIES-101, 1.06 for $M. \ viridis$ NIES-102 and 1.01 for $M. \ wesenbergii$ NIES-104 (Table 2). In terms of the functional responses, the maximum ingestion rate of each Microcystis strain by $D. \ rotans$ was 27.0 prey HF$^{-1}$ d$^{-1}$ for $M. \ aeruginosa$ NIER-10001, 30.8 for NIES-298, 4.5 for NIES-101 and 15.1 for $M. \ viridis$ NIES-102 (Table 2). The functional response on $M. \ wesenbergii$ NIES-104 did not follow the Michaelis-Menten equation.

**DISCUSSION**

Mischke (1994) showed that Diphylleia rotans could graze on Chlorella cf. neustonica, Chlamydomonas sp., Monoraphidium griffithii and Stephanodiscus sp. Here, we showed that $D. \ rotans$ was not only capable of grazing on both Microcystis aeruginosa and Chlorella$^{\text{TM}}$ (Fig. 1), but the growth of $D. \ rotans$ was higher using $M. \ aeruginosa$ as prey than when Chlorella$^{\text{TM}}$ was used. However, using $M. \ aeruginosa$ as a food is inferior to many common species of green algae, such as Chlorella spp. or Scenedesmus spp., probably due to its toxic nature (Gulati & Demott 1997, Alva-Martínez et al. 2004). However, our findings suggest that despite the toxic nature of $M. \ aeruginosa$, this algal food may be of better quality and more favorable than Chlorella$^{\text{TM}}$ for $D. \ rotans$. To our knowledge this is the first report showing that $D. \ rotans$ is capable of grazing on the toxin-producing $M. \ aeruginosa$.

Under natural conditions Microcystis aeruginosa is a dominant species at temperatures between 20 and 30°C and at a pH range of 8 to 10. Our results revealed that Diphylleia rotans grows actively at 25°C and at a pH range of 6 to 9 (Fig. 2). Thus, although the flagellate prefers slightly more acidic conditions than the alga, the ranges overlap. As the flagellate did not show active growth at pH 10, which is when $M. \ aeruginosa$ is flourishing, our results suggest that the flagellate may put substantial grazing pressure on $M. \ aeruginosa$ at the beginning of its bloom.
Diphylleia rotans grew more actively on Microcystis aeruginosa than on Chlorella TM, Cyclotella meneghiniana or Stephanodiscus hantzschii (Fig. 1), and the maximum growth rate of D. rotans (Fig. 3) was detected when the flagellate fed on M. aeruginosa NIER-10001, which is the most toxic of the tested strains (Table 1). Similarly, the maximum ingestion rate of M. aeruginosa NIER-10001 by D. rotans was high relative to that of other Microcystis strains (Fig. 4). This suggests that D. rotans may have a high tolerance to microcystin, or may show microcystin-mediated growth, or both. Although toxic cyanobacteria are usually harmful to zooplankters (Yasuno et al. 1998), some previous studies have reported active flagellate growth on toxic Microcystis spp. Ou et al. (2005) noted that the mixotrophic flagellate Poterioochromonas sp. grew well in the presence of increasing concentrations of microcystin or abundant M. aeruginosa, or both, and had the ability to degrade microcystin. Thus, our novel findings suggest that future studies are warranted to measure the changes of microcystin concentrations in algal cells and culture media during grazing experiments as a means to better understand the relationships among microcystin concentrations, flagellate growth and possible microcystin use by heterotrophic nanoflagellates.

While we could calculate the maximum growth rate of the flagellate on Microcystis wesenbergii NIES-104 (Table 2), we were unable to calculate the ingestion rate because we could not detect a clear decrease in M. wesenbergii NIES-104 cell density in the feeding experiment. In general, M. wesenbergii NIES-104 provided a lower concentration of microcystin-LR than that of other strains such as M. aeruginosa NIES-298 and M. viridis NIES-102 (Yasuno et al. 1998), although they are bigger in cell volume. Why a large-sized and less-toxic strain (LSLT) did not induce a higher growth rate of the flagellate compared with a small-sized and more-toxic strain (SSMT) remains unknown. Therefore, although we cannot fully explain why, among 5 Microcystis strains, M. wesenbergii NIES-104 showed relatively high growth even in the presence of Diphylleia rotans and we failed to detect a predation-based decrease in the cell density, this result indicates that toxic M. wesenbergii NIES-104 may not be relevant as a food for grazing flagellates. Further study is required to generalize the food selectivity of flagellates like D. rotans, and to understand the survival strategy of LSLT and SSMT phytoplankton in freshwater systems.

In natural lakes Microcystis populations usually form colonies that cannot be eaten by most zooplankters, due to toxin production by the cyanobacteria (Fulton & Pearl 1987) or the large size of the colonies, or both, which exceed the ranges of size-selective grazing by the zooplankters (Gliwicz & Siedlar 1980, Thompson et al. 1982). We showed that Diphylleia rotans appears highly tolerant of microcystin, suggesting that the flagellate is capable of over-
Fig. 3. Diphylleia rotans. Specific growth rates during feeding on (A) *Microcystis aeruginosa* NIER-10001, (B) NIES-298, (C) NIES-101, (D) *M. viridis* NIES-102, and (E) *M. wesenbergii* NIES-104. Symbols represent treatment means ± 1 SE. Curves were fitted by a Michaelis-Menten equation using all treatments.

Fig. 4. Diphylleia rotans. Maximum ingestion rates during feeding on (A) *Microcystis aeruginosa* NIER-10001, (B) NIES-298, (C) NIES-101, and (D) *M. viridis* NIES-102. Symbols represent treatment means ± 1 SE. Curves were fitted by a Michaelis-Menten equation using all treatments. HF: heterotrophic flagellate.
coming the limitation that could be produced by the toxin. With regard to size-selective grazing by the flagellate, Mischke (1994) noted that D. rotans could feed on algal cells like Monoraphidium griffithii, which is larger than the flagellate, suggesting that the genus Diphylella may have the ability to ingest large particles. However, we found that D. rotans could not grow on Cyclotella meneghiniana (733 µm$^3$) or Stephanodiscus hantzschii (98 µm$^3$), because their cell walls were made of siliceous matter. Although this seems to suggest that the flagellate could not prey on the larger algae due to size-selective feeding or food quality, or both, we noted that the flagellate grew more actively on M. aeruginosa compared with Chlorella$^TM$, which has a smaller individual cell size (6 µm$^3$) than M. aeruginosa. Although further studies are required to examine size-selective grazing by D. rotans, we hypothesize that the higher growth of the flagellate on the more toxic M. aeruginosa strain compared with that on other prey tested was due to toxin-mediated growth.

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