

# Effect of iron complexation with dissolved organic matter on the growth of cyanobacteria in a eutrophic lake

Takashi Nagai<sup>1,\*</sup>, Akio Imai<sup>2</sup>, Kazuo Matsushige<sup>2</sup>, Takehiko Fukushima<sup>1</sup>

<sup>1</sup>Environmental Modeling and Creation, Integrative Environmental Sciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennoudai, Tsukuba, Ibaraki 305-8572, Japan

<sup>2</sup>National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-0053, Japan

**ABSTRACT:** Dissolved organic matter (DOM) in lake water can have a harmful effect on algal growth through either direct toxicity or iron limitation induced by its complexation with iron. We assessed the effect of iron complexation with DOM on algal growth in the eutrophic Lake Kasumigaura, Japan, using 2 species of cyanobacteria, *Microcystis aeruginosa* and *Planktothrix agardhii*, by combining the algal growth potential (AGP) test and pretreatment of DOM decomposition by UV irradiation. We also determined which nutrient limited the growth of the 2 species in the lake water using the AGP test: iron limited the growth of the 2 species, as did nitrogen and phosphorus in Lake Kasumigaura. Moreover, the growth of the 2 species was inhibited by iron complexation with DOM. This is consistent with our results regarding iron speciation in Lake Kasumigaura by cathodic stripping voltammetry: most (>99.9%) of the dissolved iron was present as organic species in the lake water. Furthermore, data from our AGP test suggest that iron requirement or iron availability between the 2 species are different. This difference in growth characteristics between algal species would be an important determinant of the dominance of specific algal species.

**KEY WORDS:** Dissolved organic matter · DOM · Cyanobacteria · Iron · Organic complexation

Resale or republication not permitted without written consent of the publisher

## INTRODUCTION

Succession and diversity of algal species in natural aquatic systems are affected by various complexing factors. Gradual accumulation of recalcitrant dissolved organic matter (DOM) in several lakes in Japan has been observed (Imai 2002), and the effects of DOM accumulation on lake ecosystems are of much concern. DOM, which contains a substantial amount of toxic organic compounds, may reduce algal growth through its direct toxic effects. In addition, colored DOM can affect algal growth by changing light availability or light spectral quality. In contrast, DOM can inhibit algal growth by its indirect effect of iron complexation, which induces iron limitation on the growth. Inorganic iron (Fe<sup>3+</sup>, the sum of free hydrated and hydrolyzed ferric iron species) is available for uptake, whereas

organically complexed iron is kinetically inert and is thus thought to be unavailable (Hudson & Morel 1993). Several studies have suggested that iron and its availability are important determinants of algal growth in natural lake waters (Clasen & Bernhardt 1974, Lin & Schelske 1981, Evans & Prepas 1997, Hyenstrand et al. 1999, Twiss et al. 2000). DOM can also have a positive effect on phytoplankton growth by complexing toxic metals, such as copper, into their harmless forms. Studies on the effect of metal complexation with DOM on algal growth in natural aquatic systems are therefore very important in understanding the succession and diversity of algal species.

In Lake Kasumigaura, a eutrophic lake in Japan, *Microcystis* blooms were frequently observed until 1986, but they disappeared in 1987, and thereafter *Planktothrix* spp. were dominant instead for a long

\*Email: nagai@ies.life.tsukuba.ac.jp

time. From 2001 onward, *Planktothrix* spp. became uncommon when the presence of diatoms such as *Cyclotella* spp. and *Skeletonema* spp. increased (CGER 2004). The change in dominance of species in Lake Kasumigaura is quite difficult to explain rationally. Takamura et al. (1992) discussed the above-mentioned change from the viewpoint of the nitrogen:phosphorus ratio (N:P ratio). Before 1986, the N:P ratio was <10, but exceeded 20 after 1987. They suggested that the shift from *Microcystis* spp. to *Planktothrix agardhii* around 1987 was related to the change of limiting nutrient from nitrogen to phosphorus in Lake Kasumigaura. However, *Microcystis* spp. did not become dominant as expected by their explanation when the N:P ratio decreased to <10 after 1992 (CGER 2004).

It has been suggested that iron may also limit the growth of bloom-forming cyanobacteria (Yagi et al. 1987, Aizaki & Aoyama 1995). Imai et al. (1999) reported that the ambient level of fulvic acid in Lake Kasumigaura significantly inhibited the growth of *Microcystis aeruginosa* in artificial growth media because of complexation of Fe(III) with fulvic acid. Furthermore, Nagai et al. (2004) determined the speciation of dissolved iron in Lake Kasumigaura by cathodic stripping voltammetry (CSV) and showed that >99.9% of the dissolved iron is present as organic species. These studies suggest that the iron limitation in Lake Kasumigaura is caused by the low availability of iron. However, Imai et al. (1999) used artificial culture media, not natural water samples, and investigated the effect of iron on the growth of only one species (*Microcystis aeruginosa*). In the study by Nagai et al. (2004), the relationship between iron speciation in lake water and its biological response was not investigated. In order to understand natural aquatic ecosystems, it is therefore necessary to connect iron speciation in natural water samples to its effect on the algal growth.

We hypothesized that the growth of cyanobacteria, such as *Microcystis* spp. and *Planktothrix* spp., is inhibited by iron limitation in Lake Kasumigaura due to iron complexation with DOM. Our aims in the present study were to assess the effects of iron complexation with DOM on algal growth in Lake Kasumigaura to understand the mechanisms of dominance of particular algal species. Although the effects of DOM on algal growth have been assessed using the algal growth potential (AGP) test (Sudo et al. 1981), no studies have distinguished a direct effect of DOM from an indirect effect of DOM due to its iron complexation. We used an approach combining the AGP test, pretreatment of DOM decomposition by UV (ultraviolet) irradiation, and nutrient addition to separately assess the direct toxic effect of DOM and the effect of iron complexation.

## MATERIALS AND METHODS

**Study site and sample collection.** Lake Kasumigaura is the second largest lake in Japan and is located 50 km northeast of Tokyo. It has a surface area of 171 km<sup>2</sup>, a mean depth of 4 m, and a maximum depth of 7.3 m. The lake is so shallow that vertical stratification is easily destroyed by a moderately strong wind.

Sampling was performed every 3 mo at the center of the lake on 10 July 2002, 9 October 2002, 8 January 2003, and 10 April 2003. Surface-water samples were collected directly into 2 l polycarbonate bottles. The samples were stored in an ice cooler and immediately brought back to the laboratory. The samples were filtered through precombusted (450°C for 4 h) glass fiber filters (GF/F, Whatman). The filtrates were used for the AGP test and for chemical analyses, with the exception of iron analysis. Separated samples for iron analysis were filtered through a 0.2 µm pore-size polycarbonate membrane filter (Nuclepore, Whatman). A portion of the filtrates was stored at 3°C in Teflon vials after acidification to pH 2.5 with HCl for the determination of total dissolved iron (TDFe). The remaining filtrates were stored frozen (–30°C) in high-density polyethylene bottles until analysis of the iron speciation. Before use, the high-density polyethylene bottles were cleaned by soaking in 3 M HCl for 3 d and then rinsing with Milli-Q water (resistance 18.3 MΩ; Millipore). The Teflon vials were cleaned by soaking in 3 M HCl for 3 d, then soaking in 2 M HNO<sub>3</sub> for 3 d, and finally rinsing with Milli-Q water.

Water temperature and pH were measured at the sampling sites. The chlorophyll *a* (chl *a*) concentration was determined spectrophotometrically after overnight extraction with 100% methanol (Marker et al. 1980). Inorganic nutrient (ammonium, nitrite, nitrate, and phosphate) concentrations were measured with an autoanalyzer (TRAACS 800, Bran+Luebbe). Total dissolved nitrogen and total dissolved phosphorus were measured with the autoanalyzer after digestion by potassium peroxodisulfate. Dissolved Ca and Mg concentrations were determined by inductively coupled plasma-atomic emission spectrometry (ICAP-750, Nippon Jarrell-Ash). Dissolved organic carbon (DOC) was measured with a total organic carbon analyzer (TOC-5000, Shimadzu) equipped with a Pt catalyst on quartz wool.

**Assessing the effect of DOM.** Our basic idea for evaluating the effect of DOM on algal growth was to remove all DOM in the water sample without substantially changing the other chemical constituents of the sample; AGP tests were then conducted for the original and pretreated samples, and the effect of DOM on algal growth was evaluated by comparing their AGPs. For this purpose, UV irradiation is very attractive, as it

can decompose DOM in the water sample without adding any chemicals. Both toxic organic compounds and iron-complexing organic ligands are destroyed by UV irradiation, and consequently iron availability is increased. The effects of DOM can be assessed by comparing the maximum growth of algae in AGP tests between UV-irradiated and unirradiated samples. Water samples need to be acidified during UV irradiation to prevent iron from precipitating out. Furthermore, before pH is adjusted to the original value for AGP tests, an addition of chelator (EDTA, ethylenediamine tetraacetic acid) is essential to keep iron dissolved near neutral pH.

Total concentrations of nitrogen, phosphorus, and iron are not varied by UV irradiation. However, nitrate, ammonia, and phosphate are solubilized from decomposed DOM after UV irradiation. Therefore, it is impossible to determine whether the differences in AGPs between UV-irradiated and unirradiated samples are a result of the effect of DOM or simply result from the increase in inorganic nutrient concentrations. To overcome this drawback, we used an approach whereby the effect of DOM is assessed by comparing the AGPs in UV-irradiated and unirradiated samples after nutrient addition; thus, any effects due to changes in composition of inorganic nitrogen and phosphorus are minimized.

Yagi et al. (1995) reported that the substances limiting the growth of bloom-forming cyanobacteria (*Microcystis*, *Planktothrix*, *Phormidium*, and *Anabaena*) in Lake Kasumigaura were nitrogen (N), phosphorus (P), iron (Fe), and EDTA (they suggested that EDTA had the effect of alleviating heavy metal toxicity or promoting iron acquisition). Therefore, the direct toxicity of DOM on algal growth can be assessed by comparing the AGPs in UV-irradiated and unirradiated samples after the addition of N + P + Fe + EDTA. The amount of nutrients added should be sufficient for algal growth and be the same between UV-irradiated and unirradiated samples. When enough N and P are added to the samples, the limiting nutrient should be only iron, and thus we can assess the effect of DOM (direct toxicity + iron complexation) by comparing the AGPs in UV-irradiated and unirradiated samples after the addition of N+P.

It should be noted that the assessment of the effect of DOM through iron complexation cannot be made under exactly the same conditions for comparing the AGPs in UV-irradiated and unirradiated samples, because we could not avoid adding EDTA in UV samples to keep iron dissolved. However, inorganic (bioavailable) iron concentrations in UV-irradiated samples were much higher than in unirradiated samples (see 'Results' and Table 2), even if dissolved iron was complexed with EDTA in UV-irradiated samples.

Thus, it is still probable that we can evaluate the effect of DOM due to iron complexation on algal growth through our approach.

It is very important to know which nutrients limit algal growth in AGP tests, since our approach for assessing the effect of DOM is based on an assumption that the limiting nutrients are N, P, and Fe only. Therefore, the algal growth is determined after each addition of N, P, or Fe. Furthermore, no effect of heavy metal toxicity was verified by addition of EDTA only to the sample.

**AGP test.** Axenic unialgal cultures of *Microcystis aeruginosa* strain NIES-44 and *Planktothrix agardhii* strain NIES-204, obtained from the National Institute for Environmental Studies (NIES) Microbial Culture Collection (Kasai et al. 2004), were used for our AGP test. The 2 strains were isolated from Lake Kasumigaura, and are not able to fix nitrogen. *M. aeruginosa* NIES-44 has lost the ability to aggregate; *P. agardhii* NIES-204 can be suspended well in liquid culture without attaching to the wall of the culture tube. Stock cultures were maintained at 25°C under a photon flux of 20  $\mu\text{E m}^{-2} \text{s}^{-1}$  continuous light in a modified CB medium of the following composition:  $6.35 \times 10^{-4}$  M  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ,  $9.89 \times 10^{-4}$  M  $\text{KNO}_3$ ,  $2.30 \times 10^{-4}$  M  $\text{K}_2\text{HPO}_4$ ,  $8.11 \times 10^{-5}$  M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $3.07 \times 10^{-3}$  M Bicine (N, N-bis[2-hydroxyethyl]glycine),  $1.00 \times 10^{-5}$  M  $\text{Na}_2 \cdot \text{EDTA} \cdot 2\text{H}_2\text{O}$ ,  $2.00 \times 10^{-6}$  M  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $5.46 \times 10^{-7}$  M  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $4.84 \times 10^{-7}$  M  $\text{ZnCl}_2$ ,  $5.04 \times 10^{-8}$  M  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , and  $3.10 \times 10^{-8}$  M  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ; the pH was adjusted to 9.0 (Kasai et al. 2004).

Part of the filtrate of lake water samples for the AGP test were UV irradiated to decompose the DOM. The samples were filtered through a 0.2  $\mu\text{m}$  pore-size polycarbonate membrane filter (Nuclepore, Whatman) and were acidified to pH 2.5 with HCl before UV irradiation. The samples were then placed in acid-washed quartz tubes and UV irradiated with a 400 W low-pressure Hg lamp for 60 min. Details of the UV irradiation system have been described previously (Yokoi et al. 1999). DOM in water from Lake Kasumigaura was completely digested by UV irradiation within 60 min under these conditions (Nagai et al. 2004).  $\text{Na}_2 \cdot \text{EDTA} \cdot 2\text{H}_2\text{O}$  was then added to the UV-digested samples (final conc. 0.5  $\text{mg l}^{-1}$ ). Finally, the pH was adjusted to the original value (see Table 1) with  $\text{Na}_2\text{CO}_3$ . Note that the algae did not grow at all when the pH was adjusted with NaOH. This is probably because dissolved inorganic carbon in the sample escaped as carbon dioxide to the air during UV irradiation. The samples after UV treatment were called 'UV samples', while 'control samples' did not receive UV treatment.

The control and UV samples were sterilized by passage through a 0.22  $\mu\text{m}$  pore-size membrane filter (Sterivex, Millipore). Sterilization by autoclaving is unacceptable for assessing the effects of DOM on algal

growth because the autoclaving destroys thermosensitive DOM and precipitates phosphorus together with iron (Lukavsky 1992). Filter sterilization is more appropriate than autoclaving. Although it could eliminate particulate nutrients (N, P, and Fe) that are potential resources for algal growth, we ignored this potential of particulate nutrients in this study.

Each sterilized sample (10 ml) was then pipetted into sterilized glass tubes (25 mm diameter). The control samples in the glass tubes were enriched with (1) nothing (control), (2) N (10 mg N l<sup>-1</sup> as KNO<sub>3</sub>), (3) P (1 mg P l<sup>-1</sup> as K<sub>2</sub>HPO<sub>4</sub>), (4) Fe (0.1 mg Fe l<sup>-1</sup> as FeCl<sub>3</sub> · 6H<sub>2</sub>O), (5) EDTA (0.5 mg Na<sub>2</sub> · EDTA · 2H<sub>2</sub>O l<sup>-1</sup>), (6) both N + P, or (7) N + P + Fe + EDTA, all from filter-sterilized stock solutions. The UV samples were enriched in a similar way, as follows (1) N + P or (2) N + P + Fe. Modified CB medium was prepared as a positive control.

Cells for the inoculum were washed twice by centrifugation (5000 rpm [4600 × g], 15 min) and suspension in Milli-Q water and then incubated in Milli-Q water for 2 d to starve them. The starved cells were inoculated into each glass culture tube to achieve an absorbance of 0.001 at a wavelength of 750 nm. Absorbance was measured with a spectrophotometer (UV-2200, Shimadzu) using a 10 mm pass length glass cell. Growth experiments were run in triplicate for 2 wk under continuous light (20 μE m<sup>-2</sup> s<sup>-1</sup>) at 25°C. The culture tubes were shaken by hand once a day. Two weeks later, growth was measured as absorbance at 750 nm, and the AGP was defined as the value of the absorbance. We verified a linear relationship between absorbance at 750 nm and counted cell or filament density: *Microcystis aeruginosa*, cell density (cells ml<sup>-1</sup>) = 6.80 × 10<sup>6</sup> × absorbance (absorbance ranged from 0.005 to 0.3, r = 0.98, n = 8); *Planktothrix agardhii*, filament density (filaments ml<sup>-1</sup>) = 2.95 × 10<sup>5</sup> × absorbance (absorbance ranged from 0.007 to 0.07, r = 0.93, n = 10). All glass culture tubes were cleaned by soaking in 3 M HCl for 3 d and then rinsing with Milli-Q water before use.

#### Determination of dissolved iron and its speciation.

The concentration of total dissolved iron (TDFe) was determined by CSV, as described in Nagai et al. (2004). Filtrate samples for analysis of TDFe were acidified to pH 2.5 and UV irradiated for 60 min to decompose the DOM. UV-irradiated samples were diluted 10 times with Milli-Q water. 1-nitroso-2-naphthol (NN; 20 μM), NaCl (50 mM), tris (hydroxymethyl) aminomethane (10 mM), and potassium bromate (10 mM) were added to the diluted samples to achieve the final concentrations shown in parentheses (final pH, 8.1). Deposition was carried out for 30 s, and a voltammetric scan was performed in differential pulse stripping mode with a hanging mercury drop electrode (303A, Princeton Applied Research). The concentrations of TDFe

were quantified by the standard addition method in triplicate.

The concentrations of natural iron-complexing organic ligands (C<sub>L</sub>) and the conditional stability constants (K'<sub>FeL</sub> = [FeL]/([Fe<sup>3+</sup>][L'])) were determined by a competitive ligand equilibration/CSV method (Gledhill & van den Berg 1994, Nagai et al. 2004). Iron titration was conducted in 9 increments without UV irradiation, in triplicate. The added iron, NN, and natural organic ligands were allowed to equilibrate overnight. The iron complexed with NN was then determined by CSV. The voltammetric procedure was the same as that used for the TDFe analysis, except that the NN concentration was 50 μM. C<sub>L</sub> and K'<sub>FeL</sub> were calculated by linear least-squares regression of the data fitted to the following equation (Gledhill & van den Berg 1994):

$$[\text{Fe labile}]/[\text{FeL}] = [\text{Fe labile}]/C_L + (\alpha'_{\text{Fe}} + \alpha'_{\text{FeNN}})/(C_L K'_{\text{FeL}}) \quad (1)$$

where [Fe labile] is the concentration of iron complexed by the added NN as well as inorganic iron, and [FeL] is the concentration of iron complexed by the natural organic ligand, L. Values for α'<sub>Fe</sub> and α'<sub>FeNN</sub> (the α-coefficients for inorganic complexation of iron and complexation of Fe<sup>3+</sup> by NN) of 10<sup>12.3</sup> and 10<sup>16.7</sup> were used, respectively (Nagai et al. 2004). The concentration of inorganic iron (Fe') originally present in the sample was calculated from α'<sub>Fe</sub>, TDFe, C<sub>L</sub>, and K'<sub>FeL</sub> on a thermodynamic equilibrium basis. The concentration of organically complexed iron is calculated as TDFe – [Fe'].

Inorganic iron concentrations in the UV samples were calculated using the MINEQL+ computer program (Schecher & McAvoy 1992). The components used in the calculations were H<sub>2</sub>O, H<sup>+</sup>, Fe<sup>3+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup>, EDTA<sup>4-</sup>, and PO<sub>4</sub><sup>3-</sup>.

## RESULTS

### Water quality

The basic properties of the surface water samples are shown in Table 1. DOC concentrations ranged from 0.262 to 0.293 mM (from 3.1 to 3.5 mg C l<sup>-1</sup>) and did not differ markedly among samples. Nitrate concentrations ranged from <0.1 to 40.3 μM (~564 μg N l<sup>-1</sup>) and were very low in July 2002 and April 2003. Phosphate concentrations ranged from 0.1 to 1.6 μM (from 4 to 51 μg P l<sup>-1</sup>). Concentrations of TDFe ranged from 24 to 69 nM. Concentrations of C<sub>L</sub> ranged from 33 to 96 nM and varied similarly with TDFe. The log values of K'<sub>FeL</sub> were 25.6 to 26.2, and there was no distinctive variation. According to calculations of the chemical speciation of iron, most (>99.9%) dissolved iron was present as

Table 1. Basic properties of lake water samples, including dissolved organic carbon (DOC), total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), total dissolved iron (TDFe), ligand concentration ( $C_L$ ), conditional stability constant ( $K'_{FeL}$ ), and inorganic iron concentration ( $Fe'$ ). ND: not detected ( $<0.1 \mu\text{M}$ )

	Jul 2002	Oct 2002	Jan 2003	Apr 2003
Temp. ( $^{\circ}\text{C}$ )	24.8	21.3	3.9	12.3
pH	7.6	7.3	7.6	8.1
Chl a ( $\mu\text{g l}^{-1}$ )	34.2	25.7	32.2	60.6
DOC (mM)	0.262	0.293	0.279	0.271
Ca (mM)	0.514	0.494	0.509	0.481
Mg (mM)	0.374	0.370	0.374	0.358
TDN ( $\mu\text{M}$ )	27.1	59.9	60.2	15.9
$\text{NO}_3$ ( $\mu\text{M}$ )	2.7	40.3	39.6	ND
$\text{NO}_2$ ( $\mu\text{M}$ )	1.5	0.1	0.4	ND
$\text{NH}_4$ ( $\mu\text{M}$ )	3.9	0.8	0.2	ND
TDP ( $\mu\text{M}$ )	0.6	2.0	0.6	0.3
$\text{PO}_4$ ( $\mu\text{M}$ )	0.3	1.6	0.3	0.1
TDFe (nM)	44	69	48	24
$C_L$ (nM)	68	96	80	33
$\log K'_{FeL}$ ( $\text{M}^{-1}$ )	25.8	26.2	25.9	25.6
$\log Fe'$ (M)	-13.3	-13.5	-13.4	-12.9

organic complexes in all samples. Inorganic iron concentrations ranged from  $10^{-13.5}$  to  $10^{-12.9}$  M (0.03 to 0.13 pM) and were very low compared with TDFe. Chl a concentrations ranged from 25.7 to 60.6  $\mu\text{g l}^{-1}$ . Few cyanobacteria were found during the study period, and instead diatoms were the dominant phytoplankton in all samples; chlorophytes were also dominant phytoplankton in July 2002, and cryptophytes in October 2002 and January 2003.

We compared the properties of UV-treated and untreated (control) samples (Table 2). Concentrations of DOC decreased after UV treatment. Note that the DOC in the UV samples included organic carbon derived from the added EDTA. After UV treatment, the concentrations of nitrate and phosphate increased slightly, and ammonia concentrations increased sub-

Table 2. Comparisons of water properties of untreated samples (control sample) and samples subjected to UV irradiation (UV sample). DOC of UV samples includes organic carbon derived from added EDTA. ND: not detected ( $<0.1 \mu\text{M}$ )

	Jul 2002		Oct 2002		Jan 2003		Apr 2003	
	Control	UV	Control	UV	Control	UV	Control	UV
DOC (mM)	0.262	0.044	0.293	0.052	0.279	0.058	0.271	0.057
$\text{NO}_3$ ( $\mu\text{M}$ )	2.7	8.4	40.3	42.9	39.6	42.5	ND	3.9
$\text{NO}_2$ ( $\mu\text{M}$ )	1.5	0.1	0.1	0.2	0.4	0.1	ND	ND
$\text{NH}_4$ ( $\mu\text{M}$ )	3.9	14.6	0.8	13.0	0.2	11.5	ND	10.4
$\text{PO}_4$ ( $\mu\text{M}$ )	0.3	0.5	1.6	1.7	0.3	0.4	0.1	0.2
TDFe (nM)	44	44	69	69	48	48	24	24
$\log Fe'$ (M)	-13.3	-9.5	-13.5	-9.9	-13.4	-9.5	-12.9	-8.8

stantially owing to dissolution from decomposed DOM. The  $Fe'$  concentrations of the UV samples were 4 orders of magnitude higher than those of the control samples, indicating that dissolved iron in the UV samples was much more bioavailable than that in the control samples.

### AGP test

The AGP of *Microcystis aeruginosa* in lake water samples collected in July 2002 did not increase with the addition of N+P (bar 6 in Fig. 1a) or the addition of Fe (bar 4) compared with the control (no addition, bar 1), but it increased greatly with the addition of N+P+Fe+EDTA (bar 8), to the same level as in the CB medium (bar 10). These results show that nitrogen, phosphorus, and iron simultaneously limited growth in the water. Thus, we proceed to assess the direct toxicity of DOM and the indirect effect through iron complexation on algal growth by comparing the AGPs of control samples and UV samples after the addition of N+P+Fe+EDTA (toxicity) or N+P (toxicity + complexation). AGPs did not differ between control and UV samples after the addition of N+P+Fe+EDTA (bars 8 and 9, Fig 1), clearly indicating no direct toxic effect of DOM. In contrast, the AGP of the UV sample after the addition of N+P (bar 7) was higher than that of the control sample after addition of N+P (bar 6), indicating that DOM inhibited growth through iron complexation.

The AGPs of *Microcystis aeruginosa* in water collected in October 2002 showed the same pattern as those in water collected in January 2003 (Fig. 1a). In both cases the AGPs increased with the addition of N+P+Fe+EDTA (bar 8), indicating that the limiting nutrients were nitrogen, phosphorus, and iron. Furthermore, the AGPs did not differ between the control and UV samples (bars 6 and 7 vs. bars 8 and 9), indicating no effect of DOM (toxicity or iron complexation) on algal growth. In April 2003 there was almost no nitrate in the lake water (Table 2), and the growth of *M. aeruginosa* was limited primarily by N and secondarily by P and Fe (Fig. 1a). No direct toxic effect of DOM was observed (bars 8 and 9), but an effect of DOM through iron complexation was clearly visible (bars 6 and 7). The addition of EDTA had no influence on the growth of *M. aeruginosa* in all samples (bar 5), indicating no toxic levels of metals in the samples.

The growth pattern in the AGP tests using *Planktothrix agardhii* was differ-

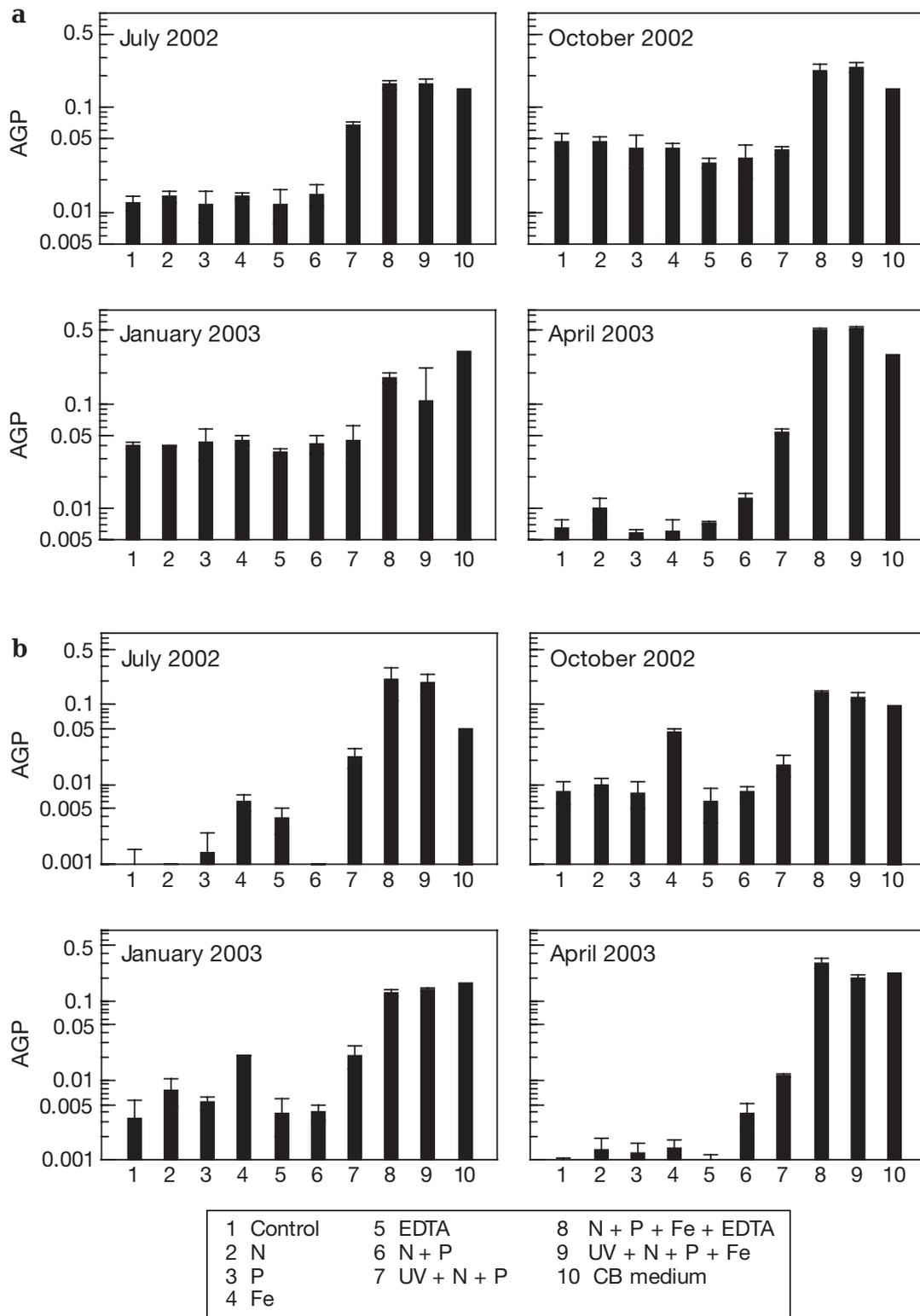


Fig. 1. Algal growth potentials (AGPs) of (a) *Microcystis aeruginosa* and (b) *Planktothrix agardhii* with nutrient enrichment. Mean values and standard deviations (error bars) are shown. Treatments (additions) were as follows: (1) Control (no addition); (2) N ( $10 \text{ mg N l}^{-1}$  as  $\text{KNO}_3$ ); (3) P ( $1 \text{ mg P l}^{-1}$  as  $\text{K}_2\text{HPO}_4$ ); (4) Fe ( $0.1 \text{ mg Fe l}^{-1}$  as  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ); (5) EDTA ( $0.5 \text{ mg Na}_2 \cdot \text{EDTA} \cdot 2\text{H}_2\text{O l}^{-1}$ ); (6) N+P; (7) N+P after UV treatment; (8) N+P+Fe+EDTA; (9) N+P+Fe after UV treatment; or (10) modified CB medium as a control

ent from that in the tests using *Microcystis aeruginosa* (Fig. 1b). The AGPs of *P. agardhii* in the lake waters collected in July 2002, October 2002, and January 2003 increased with the addition of Fe compared with control samples (bars 1 and 4, Fig. 1b), and they increased still more with the further addition of N + P + EDTA to the Fe (bar 8). These results show that the limiting nutrients were primarily Fe and secondarily N and P. In April 2003, the limiting nutrients were primarily N and P, and secondarily Fe (Fig. 1b). In all samples, DOM had no direct toxic effect (bars 8 and 9) but we found an indirect effect of DOM through iron complexation (bars 6 and 7) on the growth of *P. agardhii*. No effect of EDTA addition on the growth of *P. agardhii* was observed in water collected in October 2002, January 2003, and April 2003 (bar 5), but the AGP in water collected in July 2002 increased with the addition of EDTA (bar 5). The increase was likely to be caused by promoting iron acquisition by algae, because the AGP also increased with iron addition (bar 4, Fig. 1b). These results also indicate no toxic levels of metals on the growth of *P. agardhii* in all samples.

## DISCUSSION

### Effect of iron complexation on algal growth

The effects of DOM and limiting nutrients on the growth of the cyanobacteria *Microcystis aeruginosa* and *Planktothrix agardhii* are summarized in Table 3. The inhibition of algal growth by DOM in Lake Kasumigaura, Japan, was due not to direct toxicity but to complexation of DOM with iron and subsequent prevention of iron acquisition. When the effect of iron complexation was substantial and iron was the primary limiting nutrient, it can be said that DOM inhibited the growth of *M. aeruginosa* and *P. agardhii* by complexa-

tion of iron with DOM in Lake Kasumigaura. That is, algal growth and was inhibited by iron complexation in the lake water collected in July 2002 for *M. aeruginosa* and in July 2002, October 2003, and January 2003 for *P. agardhii*. This is consistent with our results on iron speciation determined by CSV, i.e. that most of the dissolved iron is present as organic species in the lake water.

It is now well documented that iron limits primary production in the open ocean (e.g. Martin et al. 1994). However, there are only a few studies about iron limitation in lake water, as iron concentration in lake water is generally much greater than that in seawater. Dissolved iron concentrations in the oceans are reported to range from 0.05 to 2 nM (Achterberg et al. 2001), while in the present study in Lake Kasumigaura they ranged from 24 to 69 nM, much greater than those in the oceans. However, inorganic iron concentrations in Lake Kasumigaura determined in our study (from  $10^{-13.5}$  to  $10^{-12.9}$ ) were similar to those in the oceans (from  $10^{-14}$  to  $10^{-9.3}$ , Nagai et al. 2004). Moreover, our AGP test data showed that algal growth in Lake Kasumigaura was limited by iron limitation due to iron complexation with DOM. Thus, the effects of iron on algal growth cannot be disregarded even in a eutrophic lake that contains a high concentration of TDFe.

Clear effects of iron complexation with DOM on the growth of *Microcystis aeruginosa* were observed in the lake waters collected in October 2002 and January 2003, but there was no such effect in July 2002 and April 2003. This difference suggests that the characteristics of DOM should vary among the water samples in Lake Kasumigaura, because DOC concentrations did not vary markedly among the samples (Table 1). Therefore, physicochemical characteristics of DOM in lake water may be a key factor for the dominance of specific algal species. Characterizations of natural DOM and organic ligands of iron in aquatic environments are required for further study.

Table 3. *Microcystis aeruginosa* and *Planktothrix agardhii*. Effect of DOM and limiting nutrients on the growth of 2 species of cyanobacteria in Lake Kasumigaura, Japan. ✓: effect found; -: effect not found

		Effect of DOM		Limiting nutrient	
		Toxicity	Iron complexation	Primary	Secondary
<i>M. aeruginosa</i>	Jul 2002	-	✓	N, P, Fe	
	Oct 2002	-	-	N, P, Fe	
	Jan 2003	-	-	N, P, Fe	
	Apr 2003	-	✓	N	P, Fe
<i>P. agardhii</i>	Jul 2002	-	✓	Fe	N, P
	Oct 2002	-	✓	Fe	N, P
	Jan 2003	-	✓	Fe	N, P
	Apr 2003	-	✓	N, P	Fe

### Difference in growth characteristics between species

Limiting nutrients differed between *Microcystis aeruginosa* and *Planktothrix agardhii* (Table 3). Nitrogen, phosphorus, and iron simultaneously limited the growth of *M. aeruginosa* in the lake waters collected in July 2002, October 2002, and January 2003. In contrast, primarily iron and secondarily nitrogen and phosphorus limited the growth of *P. agardhii* in the same waters. Differences in species-specific

requirements for nitrogen, phosphorus, and iron would be responsible for these differences in limiting nutrients: the results suggest that *M. aeruginosa* has a higher nitrogen and phosphorus requirement than *P. agardhii*, or that *P. agardhii* has a higher iron requirement than *M. aeruginosa*. In our previous study, we reached the same conclusion from observations of differences in the growth characteristics of *M. aeruginosa* and *P. agardhii* (Nagai et al. 2005). Another explanation is that a difference in iron uptake ability between the 2 species is responsible for the difference in limiting nutrients. Although the growth of *P. agardhii* was inhibited by complexation of DOM with iron in all samples, growth of *M. aeruginosa* was not affected in the lake waters in October 2002 and January 2003 (Table 3). According to Imai et al. (1999), *Oscillatoria* (*Planktothrix*) spp. may be able to survive in Lake Kasumigaura by scavenging iron from iron-fulvic acid complexes because *Oscillatoria* (*Planktothrix*) *tenuis* produces multiple siderophores: both hydroxamate- and catechol-type siderophores (Brown & Trick 1992). However, *Planktothrix* spp. disappeared in 2001. Our results suggest that *Microcystis* spp. are rather superior to *Planktothrix* spp. under iron limitation.

#### Algal species succession in Lake Kasumigaura

In Lake Kasumigaura, iron was a limiting nutrient, together with nitrogen and phosphorus for *Microcystis aeruginosa* and *Planktothrix agardhii*. Our results support the hypothesis that the growth of cyanobacteria such as *Microcystis* and *Planktothrix* is inhibited by iron limitation in Lake Kasumigaura due to iron complexation with DOM. However, the inability of *Microcystis* spp. to grow in Lake Kasumigaura from 1987 may be caused by other factors as well as iron limitation, because *Microcystis* spp. would be superior to *Planktothrix* spp. under iron limitation. Moreover, we do not know which species wins when iron competition occurs between cyanobacteria and diatoms. According to Brand (1991), cyanobacteria have a relatively higher cellular iron requirement than do other phytoplankton. However, the relationship between iron and phytoplankton growth is highly complicated and is not understood thoroughly. Recently, the availability of organically complexed iron with low concentrations of inorganic iron has been discussed. Hutchins et al. (1999) suggested that cyanobacteria and diatoms possess different uptake strategies for organically complexed iron, and therefore iron competition between cyanobacteria and diatoms may depend on the chemical nature of the iron complexes. Investigations into algal iron requirements and the availability of several iron species to each algal species are

required to obtain solid evidence on the effect of iron on algal species succession in lakes such as Lake Kasumigaura.

**Acknowledgements.** This study was partly supported by JSPS Research Fellowships for Young Scientists to T.N., by a Grant-In-Aid for JSPS Research Fellows (No. 17-7257, 2005), and by a Grant-In-Aid for Scientific Research to A.I. (No. 17310013) from the Ministry of Education, Science, Sports and Culture, Japan. Sampling was supported by the GEMS/Water Trend Monitoring Project at Lake Kasumigaura. We thank all members participating in the project for their cooperation.

#### LITERATURE CITED

- Achterberg EP, Holland TW, Bowie AR, Mantoura RFC, Worsfold PJ (2001) Determination of iron in seawater. *Anal Chim Acta* 442:1–14
- Aizaki M, Aoyama K (1995) Changes of phytoplankton composition on the cyanobacterial blooms forming experiment using large outdoor continuous flow-through ponds. In: Fukushima T, Aizaki M (eds) Aoko (water-blooms of blue-green algae): measurement, occurrence, and factors on its growth. *Natl Inst Environ Stud F-72-'95/NIES*. National Institute for Environmental Studies, Ibaraki, p 105–114 (in Japanese)
- Brand LE (1991) Minimum iron requirements of marine phytoplankton and the implications for the biogeochemical control of new production. *Limnol Oceanogr* 36: 1756–1771
- Brown CM, Trick CG (1992) Response of the cyanobacterium, *Oscillatoria tenuis*, to low iron environment: the effect on growth rate and evidence for siderophore production. *Arch Microbiol* 157:349–354
- CGER (Center for Global Environmental Research) (2004) Lake Kasumigaura database. National Institute for Environmental Studies, Center for Global Environmental Research, <http://www-cger2.nies.go.jp/moni-e/gems/database/kasumi/index.html>
- Clasen J, Bernhardt H (1974) The use of algal assays for determining the effect of iron and phosphorus compounds on the growth of various algal species. *Water Res* 8:31–44
- Evans JC, Prepas EE (1997) Relative importance of iron and molybdenum in restricting phytoplankton biomass in high phosphorus saline lakes. *Limnol Oceanogr* 42:461–472
- Gledhill M, van den Berg CMG (1994) Determination of complexation of iron(III) with natural organic complexing ligand in seawater using cathodic stripping voltammetry. *Mar Chem* 47:41–54
- Hudson RJM, Morel FMM (1993) Trace metal transport by marine microorganisms: implications of metal coordination kinetics. *Deep-Sea Res I* 40:129–150
- Hutchins DA, Witter AE, Butler A, Luther GW III (1999) Competition among marine phytoplankton for different chelated iron species. *Nature* 400:858–861
- Hyenstrand P, Rydin E, Gunnerhed M (1999) Response of pelagic cyanobacteria to iron additions—enclosure experiment from Lake Erken. *J Plankton Res* 22: 1113–1126
- Imai A (2002) Accumulation of recalcitrant dissolved organic matter in lake water. *Aquabiology* 140:203–208 (in Japanese)
- Imai A, Fukushima T, Matsushige K (1999) Effect of iron limitation and aquatic humic substances on the growth

- of *Microcystis aeruginosa*. Can J Fish Aquat Sci 56: 1929–1937
- Kasai F, Kawachi M, Erata M, Watanabe MM (2004) NIES-collection, list of strains, 7th edn. Natl Inst Environ Stud R-182–2004. National Institute for Environmental Studies, Ibaraki
- Lin CK, Schelske CL (1981) Seasonal variation of potential nutrient limitation to chlorophyll production in southern Lake Huron. Can J Fish Aquat Sci 38:1–9
- Lukavsky J (1992) The evaluation of algal growth potential (AGP) and toxicity of water by miniaturized growth bioassay. Water Res 26:1409–1413
- Marker AFH, Nusch EA, Rai H, Riemann B (1980) The measurement of photosynthetic pigments in freshwaters and standardization of methods: conclusions and recommendations. Arch Hydrobiol Ergeb Limnol 14:91–106
- Martin JH, Coale KH, Johnson KS, Fitzwater SE and 40 others (1994) Testing the iron hypothesis in ecosystems of the equatorial Pacific Ocean. Nature 371:123–129
- Nagai T, Imai A, Matsushige K, Yokoi K, Fukushima T (2004) Voltammetric determination of dissolved iron and its speciation in freshwater. Limnology 5:87–94
- Nagai T, Imai A, Matsushige K, Fukushima T (2005) Limiting nutrients on the growth of bloom-forming cyanobacteria with special focus on iron speciation. Verh Int Verein Limnol 29:949–952
- Schecher WD, Mcavoy DC (1992) MINEQL+: a software environment for chemical equilibration modeling. Comp Environ Urb Syst 16:65–76
- Sudo R, Tai S, Yagi O, Okada M, Hosomi M, Yamane AN (1981) Determination of algal growth potential by algal assay procedure. Natl Inst Environ Stud R-26-'81/NIES. National Institute for Environmental Studies, Ibaraki (in Japanese)
- Takamura N, Otsuki A, Aizaki M, Nojiri Y (1992) Phytoplankton species shift accompanied by transition from nitrogen dependence to phosphorus dependence of primary production in Lake Kasumigaura, Japan. Arch Hydrobiol 124: 129–148
- Twiss MR, Auclair JC, Charlton MN (2000) An investigation into iron-stimulated phytoplankton productivity in epipelagic Lake Erie during thermal stratification using trace metal clean techniques. Can J Fish Aquat Sci 57:86–95
- Yagi O, Hagiwara T, Takamura Y, Sudo R (1987) Limiting nutrients of algal growth in Lake Kasumigaura. Jpn J Water Pollut Res 10:115–122 (in Japanese)
- Yagi O, Hagiwara T, Takamura N, Sakurai M, Takamura Y (1995) Limiting nutrients of several blue-green algal species in Lake Kasumigaura. In: Fukushima T, Aizaki M (eds) Aoko (water-blooms of blue-green algae): measurement, occurrence, and factors on its growth. Natl Inst Environ Stud F-72-'95/NIES. National Institute for Environmental Studies, Ibaraki, p 51–61 (in Japanese)
- Yokoi K, Yakushiji M, Hatanaka M, Kubono K, Koide T (1999) Novel photolytic decomposition method of organic compounds with a high output low-pressure mercury lamp for voltammetric trace metal analysis. Fresenius J Anal Chem 365:364–367

Editorial responsibility: Edna Granéli,  
Kalmar, Sweden

Submitted: January 7, 2006; Accepted: July 11, 2006  
Proofs received from author(s): September 11, 2006