Effects of the substances secreted from *Closterium aciculare* (Charophyceae, Chlorophyta) on the growth of freshwater phytoplankton under iron-deficient conditions

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**Abstract:** Iron is an especially essential element for the growth of phytoplankton and its deficiency is well known to suppress primary productivity in both freshwater and marine ecosystems. We discovered iron-complexing ligands secreted by a freshwater eukaryotic phytoplankton, *Closterium aciculare* in an iron-deficient chemically defined medium. To investigate the character of the siderophore-like substance secreted from *C. aciculare*, growth experiments were carried out for several phytoplankton species using the culture filtrate (<0.4 μm) of *C. aciculare* after incubation under iron-deficient conditions. Addition of the culture filtrate of *C. aciculare* enhanced growth in the green algae *Closterium aciculare*, *Pediastrum simplex*, *Staurastrum paradoxum* and the diatom *Aulacoseria granulata*. However, addition of the filtrate suppressed that of the green alga *Cosmocladium constrictum*, and did not affect growth in the cyanobacteria *Anabaena spiroides* or *Microcystis wesenbergii*. These results suggest that the substances secreted from *C. aciculare* have species-specific growth-promoting effects and may control phytoplankton growth under iron-deficient conditions.

**Key words:** *Closterium aciculare*, freshwater phytoplankton, growth, iron-complexing ligand, iron-deficient conditions

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**Introduction**

In aquatic ecosystems, phytoplankton play an important role in biogeochemical cycles of elements due to their role as primary producers. Nutrients (macro and micro nutrients) and other limiting factors such as light and temperature give interactive effects on phytoplankton growth, and the correlation is very complicated (Schindler 1977; Hein & Riemann 1995; Maldonado & Price 1996; Sunda & Huntsman 1997; Huang et al. 1999; Alam et al. 2001; Kagami & Urabe 2001; Noiri et al. 2005). In particular, the effects of trace elements are not well understood compared with macro nutrients such as nitrogen and phosphorus.

Among the trace elements, iron is one of the most important elements for phytoplankton in aerobic environments, because it is essential for many enzymes concerning redox reactions in the cytoplasm and is necessary in large amounts for growth as compared to other trace elements (Beck 1978). Iron is also important in metabolisms where it is required for photosynthetic and respiratory electron transport, nitrate reduction, chlorophyll synthesis, and detoxification of reactive oxygen species (Weinberg 1989). However, the chemical complexities of iron and its potential interactions with microbes in natural waters may obfuscate the relationship between phytoplankton physiology and iron concentrations (Geider 1999; Durham et al. 2002). Iron dissolves as free ions, inorganic complexes and organic complexes in natural water, and most dissolved iron has been shown to bind organic ligands (Rue & Bruland 1997; Aldrich et al. 2001; Nagai et al. 2004). In the surface waters of oceans and lakes, dissolved iron may be present at ex-

In high-nutrient and low-chlorophyll (HNLC) areas in open oceans, iron has been confirmed as a limiting nutrient for primary production (Martin et al. 1990; Harrison et al. 1999; Bowie et al. 2001; Gervais et al. 2002; Tsuda et al. 2003). Iron limitation in freshwater is suggested to occur mainly via complexation of iron by organic matter (Imai 1999) and sulfate clusters (Rozan et al. 2000). Phytoplankton has been shown to be limited by low availability of iron in hard-water lakes and saline lakes (Schelske 1962; Schelske et al. 1962; Wetzel 1966; Evans & Prepas 1997). To survive under low iron availability conditions, many eukaryotic phytoplankton appear to acquire iron in a manner similar to ‘strategy I’ (Römheld 1987) for higher plants, in which iron limitation leads to an increase in plasma membrane ferric chelate reductase activity (Weger et al. 2002). An iron acquisition mechanism similar to strategy I was demonstrated for some green algae under iron-deficient conditions (Keshtacher-Liebson et al. 1995, 1999; Lynnes et al. 1998; Schwarz et al. 2003). On the other hand, many prokaryotes acquire iron via a siderophore-based system. Murphy et al. (1976) reported that a siderophore-mediated iron-transport system enables cyanobacteria to dominate other phytoplankton. Hutchins et al. (1999) suggested that uptake of bound iron by cyanobacteria may involve specific cell-surface siderophore receptors, whereas diatoms are thought to use a non-specific cell-surface enzyme (ferrireductase) to liberate inorganic iron(II) from porphyrin complexes. Some eukaryotic phytoplankton may also use a siderophore-mediated iron-transport system (Trick et al. 1983; Benderliev & Ivanova 1994; Naito et al. 2001, 2004; Hasegawa et al. 2004), but there is an extreme paucity of reports that eukaryotic phytoplankton use a siderophore-mediated iron-transport system.

Iron distribution in lakes is affected by the chemical composition of lake water, iron inputs and removal processes, and internal cycling (Davison 1993; Hamilton-Taylor et al. 1996; Inaba et al. 1997). In many lakes, the minimum concentration of total dissolved iron (<0.2 \(\mu \text{M}\)) in the epipelagic zone is usually lower than 10 \(\text{nM}\). For example, the concentration of dissolved iron is 0.030–3200 \(\text{nM}\) in Lake Biwa (Mito et al. 2004), 0.5–73 \(\text{nM}\) in Lake Ontario (Nriagu et al. 1996), 0.64–27 \(\text{nM}\) in Lake Superior (Nriagu et al. 1996), 2.1–90 \(\text{nM}\) in Lake Erie (Nriagu et al. 1996; Twiss et al. 2000), 8–57 \(\text{nM}\) in Lake Kinneret (Shaked et al. 2004). Furthermore, iron availability depends on the existence of organic matter associated with iron in lakes (Imai et al. 1999; Sun et al. 2005).

We have discovered that the green alga Closterium aciculare secretes iron-complexing ligands like siderophores to uptake iron in iron-deficient chemically-defined media (Naito et al. 2004). In the present paper, substances secreted from C. aciculare were investigated for their effects on the growth of C. aciculare and other various phytoplankton species under iron-deficient conditions. Based on the results obtained, the characteristics of the secreted matter and the possibility of growth control of phytoplankton in freshwater ecosystems were discussed.

### Materials and Methods

#### Organisms

Axenic clonal cultures of the following phytoplankton species were used in this study: Closterium aciculare, Cosmocladium constrictum NIES-248, Staurastrum paradoxum NIES-528 (Charophyceae), Pediasstrum simplex NIES-215 (Chlorophyceae), Aulacosera granulata var. angustissima f. spiralis NIES-333 (Bacillariophyceae), Anabaena spiroides f. spiroides NIES-78 and Microcystis wesenbergii NIES-104 (Cyanophyceae). The axenic clonal cultures of Closterium aciculare were established by H. Nakahara (Graduate School of Agriculture, Kyoto University). The others were supplied by the National Institute for Environmental Studies, Japan.

#### Reagents and equipment

All reagents used were of the highest purity available. Glass-distilled demineralized water (Milli-Q system, Millipore) was employed. Nucleopore filters were cleaned in a mixed acid solution (0.5 M HNO₃/1 M HCl/0.5 M HF) at 100°C for 2 h and then in hot Milli-Q water 4 times on a hot plate to reduce the potential iron contamination. Narrow-mouth square polycarbonate bottles (Nalge Nunc) were used for incubation of the phytoplankton. The bottles were soaked in a detergent solution of neutral pH (Scat 20 X-N, Nacalaitesque) and then in 4 M HCl overnight, before being rinsed thoroughly with Milli-Q water. The concentration of iron in the iron-deficient medium was measured directly with a high resolution inductively-coupled plasma mass spectrometer (JMS-PLASMAX 1, JEOL). The detection limit for iron was 48 \(\text{pM}\) (n=8). Absorbance of polycarbonate bottles containing cultures was measured with a UV-VIS recording spectrophotometer (UV-2200, SHIMADZU).

#### Growth medium

The modified C medium (Provasoli & Pintner 1960; Ichimura 1971) was employed to cultivate all freshwater species excluding A. granulata. The composition of the modified C medium is shown in Table 1. With 0.5 M NaCl, the pH of the medium was adjusted to 8.1 ± 0.1. Potassium dihydrogen phosphate (KH₂PO₄) was added as a phosphorus source in place of β-sodium glycerophosphate. For zinc and iron, ZnCl₂ and FeNaEDTA were used. The CSI medium was prepared for A. granulata by adding 0.35 mM Na₂SiO₃·9H₂O to the modified C medium. Each medium was freshly prepared and autoclaved (121°C, 30 min) the day before use.
Preparation of medium for growth experiments

The modified C medium (150 ml) without NaFeEDTA and Na₄EDTA was sterilized by autoclaving. Cells of *Closterium aciculare* in the early stationary growth phase were collected on a 0.2 μm pore nuclepore filter by gentle filtration under 20 cm Hg pressure and were washed 2 times with Milli-Q water by filtration. These washed cells were transferred to an iron-free and EDTA-free modified C medium. After 5 days of incubation the cells were removed from the culture medium by gentle filtration with 0.4 μm pore nuclepore filters. The filtrate was used for growth experiments as the medium including secretions from *C. aciculare*. An aliquot of the filtrate was subjected to the determination of the iron-complexing ligands secreted from *C. aciculare* (Schwyn & Neilands 1987; Naito et al. 2004).

Preparation of secretions from *Closterium aciculare*

The modified C medium (150 ml) without NaFeEDTA and Na₄EDTA (medium 1) and the medium including the secretions from *C. aciculare* described above (medium 2). The concentration of dissolved iron (through a 0.2 μm filter) in the freshly prepared medium 1 was 6.9 ± 5.4 nM (n = 8) and 6.1 ± 3.3 nM (n = 3) for medium 2. Twenty milliliters of these media were put into 30 ml square polycarbonate bottles (38 × 38 × 64 mm) and then autoclaved. For iron-replete growth experiments, modified C and CSI media (medium 3, 1.5 μM Fe) were employed. Chemical equilibrium of ferric iron in each of the media 1, 2 and 3 were determined using MINQUEL+ software (version 4.0, Schecher & McAvoy 1992).

Preculture of freshwater phytoplankton for growth experiments

Maintenance cultures were axenically transferred to fresh modified C and CSI media and were grown in an incubator at 20°C. Illumination (24–40 μmol photons m⁻² s⁻¹) was supplied with 18 W fluorescent lamps (light : dark = 12 h : 12 h).

Growth experiments

Two hundred micro-liters of the preculture during the late exponential growth phase were harvested with a micropipette and inoculated into 20 ml of each medium in 30 ml polycarbonate bottles. Each culture was prepared in triplicate for each run. The cultures were incubated for about one month at 20°C under 117–144 μmol photons m⁻² s⁻¹ (light : dark = 12 h : 12 h). The absorbance of the bottles was directly measured at 750 nm every day. The growth was followed by ΔA₇₅₀ (absorbance at 750 nm of sample−absorbance at 750 nm of blank). The blank was the medium without phytoplankton and its optical density was 0.12 ± 0.02 (n = 24). The absorbance was converted to cell density using a calibration curve. The cell number was counted directly using a microscope (CH40, OLYMPUS).

Determination of nutrients

Concentrations of phosphate, nitrite and nitrate were determined colorimetrically just after the growth experiments by spectrophotometry (Murphy & Riley 1962; Matsunaga & Nishimura 1974; Kitamura et al. 1982). Samples were filtered through a 0.22-μm hydrophilic polysulfone membrane filter (Millex-GP25, Millipore).

Axenic check

After the growth experiments, axenic checks were made by using a liquid medium for freshwater bacteria (Ishida et al. 1980). The medium was composed of 0.5 g trypticase peptone (BBL) and 0.05 g yeast extract (Difco) in 1 liter of filtered (GF/C glass fiber filter) lake water. Further checks were made by 4,6-diamidino-2-phenylindole (DAPI) staining and epifluorescence microscopy (Porter & Feig 1980; Imai 1987).

Results

Growth of *Closterium aciculare* in the iron-deficient and iron-replete media

Figure 1 shows the growth of *Closterium aciculare* in the iron-deficient and iron-replete media. The maximum cell density at the stationary growth phase in the iron-deficient medium (medium 1) (5.7 × 10⁵ cells ml⁻¹) was one-twentieth of that in the iron-replete medium (medium 3) (1.1 × 10⁶ cells ml⁻¹). According to Table 2, the dissolved quantities of ferric iron species, except EDTA-complexes in medium 1, exceeded those in medium 3. Fe(OH)⁺², Fe(OH)₃ and Fe(OH)₄⁻ existed as 23%, 34% and 43% of the total dissolved iron (6.9 nM) in medium 1. In medium 3, Fe(OH)₂EDTA⁻² and FeEDTA⁻ comprised 94%.

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Table 1. Composition of the Modified C medium (M)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>6.35 × 10⁻⁴</td>
</tr>
<tr>
<td>KNO₃</td>
<td>9.89 × 10⁻⁴</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>4.63 × 10⁻⁵</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>1.62 × 10⁻⁴</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>7.38 × 10⁻¹¹</td>
</tr>
<tr>
<td>Biotin</td>
<td>4.09 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>2.96 × 10⁻⁸</td>
</tr>
</tbody>
</table>

FeNaEDTA            | 1.53 × 10⁻⁶  |
MnCl₂·4H₂O          | 5.46 × 10⁻⁷  |
ZnCl₂               | 2.31 × 10⁻⁷  |
CoCl₂·6H₂O          | 5.04 × 10⁻⁸  |
Na₂MoO₄·2H₂O        | 3.10 × 10⁻⁸  |
Na₂EDTA·2H₂O        | 8.06 × 10⁻⁶  |
HEPES               | 5.04 × 10⁻⁴  |

²-(4-2-Hydroxyethyl)-1-piperazinyl-ethanesulfonic acid.
and 5.9% of total dissolved iron (1.5 μM) (Table 2).

### Table 2. Concentrations of ferric iron in each medium 1, 2, 3 calculated using MINEQL+ software (version 4.0) (M)

<table>
<thead>
<tr>
<th>Species</th>
<th>medium 1</th>
<th>medium 2</th>
<th>medium 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{3+}$</td>
<td>4.69×10$^{-20}$</td>
<td>4.15×10$^{-20}$</td>
<td>3.42×10$^{-21}$</td>
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<tr>
<td>FeOH$^{2+}$</td>
<td>3.81×10$^{-14}$</td>
<td>3.37×10$^{-14}$</td>
<td>2.78×10$^{-15}$</td>
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<td>Fe(OH)$_3^+$</td>
<td>1.59×10$^{-9}$</td>
<td>1.41×10$^{-9}$</td>
<td>1.16×10$^{-10}$</td>
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<tr>
<td>Fe(OH)$_2$</td>
<td>2.35×10$^{-9}$</td>
<td>2.08×10$^{-9}$</td>
<td>1.71×10$^{-10}$</td>
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<tr>
<td>Fe(OH)$_4$</td>
<td>2.96×10$^{-9}$</td>
<td>2.62×10$^{-9}$</td>
<td>2.16×10$^{-10}$</td>
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<tr>
<td>Fe$^{2+}$</td>
<td>3.74×10$^{-19}$</td>
<td>3.30×10$^{-19}$</td>
<td>2.73×10$^{-20}$</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>5.45×10$^{-20}$</td>
<td>4.82×10$^{-20}$</td>
<td>3.98×10$^{-21}$</td>
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<tr>
<td>Fe(OH)$_2$EDTA$^{3-}$</td>
<td>—</td>
<td>—</td>
<td>1.45×10$^{-6}$</td>
</tr>
<tr>
<td>Fe$^{3+}$EDTA$^{3-}$</td>
<td>—</td>
<td>—</td>
<td>2.88×10$^{-9}$</td>
</tr>
<tr>
<td>FeEDTA</td>
<td>—</td>
<td>—</td>
<td>2.88×10$^{-14}$</td>
</tr>
<tr>
<td>Fe$^{3+}$EDTA$^{2-}$</td>
<td>—</td>
<td>—</td>
<td>9.12×10$^{-8}$</td>
</tr>
<tr>
<td>Total dissolved iron</td>
<td>6.90×10$^{-9}$</td>
<td>6.10×10$^{-9}$</td>
<td>1.54×10$^{-6}$</td>
</tr>
</tbody>
</table>

**Effects of the secretion from C. aciculare on the growth of various phytoplankters**

The effects of substances secreted from *C. aciculare* on the growth of seven species of freshwater phytoplankton were examined in the iron-deficient medium with and without the filtrate of *C. aciculare* (media 2 and 1). The concentration of free ferric iron in medium 1 (4.7×10$^{-20}$M) was slightly higher than that in medium 2 (4.2×10$^{-20}$M). Hydroxides occupied most of the species (>99%) in media 1 and 2 (Table 2). The secretion from *C. aciculare* was CAS-reactive, and the concentration was 25–50 nM.

Growth curves of *C. aciculare* in the medium with and without the secreted matter are shown in Fig. 2. When the filtrate of *C. aciculare* was added to the culture in the iron-deficient medium (medium 2), a higher cell yield was observed as compared to the case without the filtrate (medium 1) (Fig. 2). The optical density at the stationary growth phase in medium 2 was 4-fold higher or more than that in medium 1 (ANOVA, P<0.001). Consequently, the secreted matter from *C. aciculare* enhanced significantly its own growth under iron-deficient conditions.

The cell densities of *Pediastrum simplex* (P<0.02) and *Aulacosira granulata* (P<0.001) were significantly higher with the addition of filtrate under iron-deficient conditions (Figs. 3A, 3B). The growth of *Staurastrum paradoxum* in the medium with the secreted matter always showed higher cell density than in medium 1 (without secretion), although the differences were not so clear between with and without the filtrate (P<0.05) (Fig. 3C).

When the filtrate was added to the *Cosmocladium constrictum* culture, the growth of *C. constrictum* was hindered (Fig. 3D). The final cell yield in medium 2 (with secretion) was about one-fourth of that in medium 1 (P<0.002, Table 3).

Growth of the cyanobacteria *Anabaena spiroides* and *Microcystis wesenbergii* did not change significantly with addition of the filtrate and no statistically significant differences were observed between growth in media 1 and 2 (P>0.05, Figs. 3E, 3F).

The pH values of samples, including the controls (*C. aciculare*, *P. simplex*, *S. paradoxum*, *A. granulata*, *C. constrictum*, *A. spiroides*, *M. wesenbergii* and medium containing no plankton) at the end of the growth experiments were 7.9±0.2, 8.0±0.9, 7.6±0.2, 7.8±0.1, 7.6±0.2, 7.5±0.1, 7.5±0.2 and 7.6±0.2, respectively, in the iron-deficient medium.

The axenic check was performed for all samples and blanks after the growth experiments. After the samples and blanks after the growth experiments. After the samples and blanks after the growth experiments. After the samples and blanks after the growth experiments.
blanks were inoculated into the media for the bacterial contamination check, no contamination was observed. Also, with DAPI-staining and observation by epifluorescence microscopy, no bacteria were observed in any of the samples or blanks.

**Nutrients**

The concentrations of soluble reactive phosphate (SRP) in media 1 and 2 were 52.7±1.6 and 51.5±4.1 μM. For all algal species, SRP in each culture medium remained at more than 85% of that in each control. The concentrations of nitrate were 2.2±0.3 mM (medium 1) and 2.3±0.2 mM (medium 2). Nitrate in each culture medium remained at more than 83% of that in each control for all algal species. Hence, phosphate and nitrate were not extensively used for the growth of phytoplankton in any of the samples. The concentrations of nitrite were 2.8±1.6 μM (medium 1) and 3.2±1.2 μM (medium 2). Concentrations of nitrite in all samples were negligible compared to those of nitrate, and no transformation to nitrite occurred in any of the samples. In conclusion, there should have been no nutrient limitation
the secretion of phytoplankton examined. These results clearly indicate that the order of medium 1 bioavailable for phytoplankton in each medium was in the Table 4. Iron speciation in media 1 and 2 considering the concentrations of components carried over from the preculture of $C. \text{aciculare}$. Values represent the mean±SD (n=3)

<table>
<thead>
<tr>
<th>Species</th>
<th>medium 1</th>
<th>medium 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{3+}$</td>
<td>$2.27\times10^{-20}$</td>
<td>$2.07\times10^{-20}$</td>
</tr>
<tr>
<td>FeOH$^{2+}$</td>
<td>$9.89\times10^{-15}$</td>
<td>$9.01\times10^{-15}$</td>
</tr>
<tr>
<td>Fe(OH)$_2$$^+$</td>
<td>$4.57\times10^{-10}$</td>
<td>$4.17\times10^{-10}$</td>
</tr>
<tr>
<td>Fe(OH)$_3$</td>
<td>$6.34\times10^{-10}$</td>
<td>$5.78\times10^{-10}$</td>
</tr>
<tr>
<td>Fe(OH)$_4$$^-$</td>
<td>$8.51\times10^{-10}$</td>
<td>$7.76\times10^{-10}$</td>
</tr>
<tr>
<td>FeHPO$_4$$^2-$</td>
<td>$10.8\times10^{-20}$</td>
<td>$9.87\times10^{-20}$</td>
</tr>
<tr>
<td>FeSO$_4$$^2-$</td>
<td>$1.16\times10^{-20}$</td>
<td>$1.06\times10^{-20}$</td>
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<td>Fe(OH)$_2$EDTA$^{2-}$</td>
<td>$4.15\times10^{-23}$</td>
<td>$3.78\times10^{-23}$</td>
</tr>
<tr>
<td>Fe(OH)$_2$EDTA$^{3-}$</td>
<td>$7.77\times10^{-9}$</td>
<td>$7.18\times10^{-9}$</td>
</tr>
<tr>
<td>Fe(EDTA)$^{2-}$</td>
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<td>$1.98\times10^{-11}$</td>
</tr>
<tr>
<td>Fe(EDTA)$^{3-}$</td>
<td>$1.19\times10^{-16}$</td>
<td>$1.10\times10^{-16}$</td>
</tr>
<tr>
<td>Total dissolved iron</td>
<td>$4.03\times10^{-10}$</td>
<td>$3.72\times10^{-10}$</td>
</tr>
<tr>
<td>With secretion</td>
<td>Without secretion</td>
<td>$A$ with secretion/$A$ without secretion</td>
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<table>
<thead>
<tr>
<th>Phytoplankton</th>
<th>Optical density $A$</th>
<th>Growth ratio $\mu$</th>
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<tr>
<td>Chlorophyta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Charophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Closterium aciculare</td>
<td>0.222±0.019</td>
<td>0.057±0.006</td>
</tr>
<tr>
<td>Staurastrum paradoxum</td>
<td>0.139±0.036</td>
<td>0.087±0.014</td>
</tr>
<tr>
<td>Cosmocladium constrictum</td>
<td>0.010±0.004</td>
<td>0.044±0.003</td>
</tr>
<tr>
<td>Chlorophyceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pediasstrum simplex</td>
<td>0.112±0.026</td>
<td>0.056±0.003</td>
</tr>
<tr>
<td>Heterokontophyta</td>
<td></td>
<td></td>
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<tr>
<td>Bacillariophyceae</td>
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<td></td>
</tr>
<tr>
<td>Aulacoseria granulata</td>
<td>0.096±0.005</td>
<td>0.033±0.008</td>
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<tr>
<td>Cyanophyta</td>
<td></td>
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<tr>
<td>Cyanophyceae</td>
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<td></td>
</tr>
<tr>
<td>Anabaena spiroides</td>
<td>0.046±0.006</td>
<td>0.034±0.006</td>
</tr>
<tr>
<td>Microcystis wesenbergii</td>
<td>0.050±0.015</td>
<td>0.033±0.001</td>
</tr>
</tbody>
</table>

to the growth of phytoplankton in this study.

Discussion

It is well known that the growth of phytoplankton can be limited by iron (Brand et al. 1983; Yagi et al. 1987; Martin et al. 1991; Chang et al. 1992). Our results (Fig. 1) also indicated that the cell density at the stationary growth phase of Closterium aciculare in the iron-deficient medium (6.9 nM) was depressed to one-20th of that in the iron-replete medium (1.5 μM). Nutrients (phosphate and nitrate) were supplied in excess for seven different freshwater phytoplankton species in iron-deficient media. Accordingly, it can be said that growth experiments in the iron-deficient medium were performed under the conditions of iron-limitation but no limitation by macro-nutrients such as nitrogen and phosphorus.

When the filtrate of Closterium aciculare was added to iron-deficient $C. \text{aciculare}$, the optical density (final yield) of $C. \text{aciculare}$ was 3.89-fold that without the filtrate added (Table 3). The ratio of its growth with and without the addition of the filtrate of $C. \text{aciculare}$ was the highest of all the phytoplankton examined. These results clearly indicate that the secretion of $C. \text{aciculare}$ had the greatest effect on its own growth. The concentrations of trace metals remaining in the preculture at the inoculation (at the late exponential growth phase) of $C. \text{aciculare}$ were 517 nM (Mn), 324 nM (Fe), 59 nM (Co), 131 nM (Zn) and 37 nM (Mo). Considering the concentrations of components carried over from the preculture of $C. \text{aciculare}$ (e.g. 3.24 nM for Fe), the iron speciation in media 1 and 2 should have changed as shown in Table 4. The concentration of free ferric ions directly bioavailable for phytoplankton in each medium was in the order of medium 1>medium 2>medium 3 (Tables 2, 4). In media 1 and 2, hydroxides and EDTA-complexes occupied 20% and 80% of total dissolved iron, respectively (Table 4). We obtained the same percentages of iron species for A. spiroides and M. wesenbergii. These results imply that the growth of phytoplankton does not depend on the quantity of free ferric ion but rather on the quantity of total dissolved iron and the existence of secretions from $C. \text{aciculare}$ under iron-limiting conditions (Fig. 2), the secreted matter from $C. \text{aciculare}$ seems to include extracellular ferric-specific chelating agents (siderophore), which enhance iron uptake during periods of low iron availability (Matzanka 1991). These ferric ion-specific chelating agents would act through ligand-exchange mechanisms to uptake iron from its hydroxides and EDTA-complexes.

The addition of secreted matter from $C. \text{aciculare}$ had
various effects on the growth of other phytoplankton under iron-deficient conditions. For the chlorophyte *Pediasstrum simplex*, the charophyte *Staurastrum paradoxum* and the diatom *Aulacosiera granulata*, the secreted matter was effective at promoting growth of these phytoplankton under iron-deficient conditions (Figs. 3A–C). The optical densities during the stationary growth phase of *P. simplex*, *S. paradoxum* and *A. granulata* with the secreted matter added were 2.0, 1.6 and 2.9-fold of those without the secretion, respectively (Table 3). As is the case of *C. aciculare*, the secreted matter had a growth-stimulating effect like siderophore on these phytoplankton. On the other hand, the chlorophyte *Cosmocladium constrictum* grew at a reduced rate and total estimated cell number after supplementation of secreted matter under iron-deficient conditions (Fig. 3D). The optical density during the stationary phase of *C. constrictum* with the secretion of *C. aciculare* added was about one-fourth of that without the secretion (Table 3). The secreted matter appears to act an inhibitor against the uptake of Fe for the growth of *C. constrictum*. For the cyanobacteria *Anabaena spiroides* and *Microcystis wesenbergii*, the secreted matter from *C. aciculare* had no significant effect on the growth of these species under iron-deficient conditions (Figs. 3E, 3F). Cyanobacteria preferentially utilize available iron by producing hydroxamate siderophores, which suppress the growth of other phytoplankton under low iron conditions (Murphy et al. 1976). Because cyanobacteria have a strategy for overcoming iron limitation, these species may undergo no effect on growth due to the presence of secretions from *C. aciculare*.

Naito et al. (2004) reported that *C. aciculare* secretes iron-complexing ligands, presumably siderophores, under iron-deficient conditions. It is reasonable to consider that the growth of phytoplankton can be much facilitated or inhibited by this secreted matter under iron-deficient conditions. It has been reported that the prokaryotic phytoplankters *Anabaena* sp. and *Microcystis* sp. release hydroxamate-type siderophores during periods of low iron availability (Simpson & Neilands 1976; Imai et al. 1999). However, our results show that secretions from *C. aciculare* have no promoting effects on the growth of *A. spiroides* or *M. wesenbergii* under iron-deficient conditions (Figs. 3E, 3F). The substances secreted from *C. aciculare* most probably differ from siderophores released by cyanobacteria. There is a possibility that stability constants for Fe(III) of siderophores from the cyanobacteria are higher compared to those from *C. aciculare*.

Some microbes produce antibiotics that are structural homologs of competitors' siderophores (Sussman 1974). These antibiotics bind iron and are inducible by low concentrations of iron. Because these antibiotics are actively taken up by the siderophore-mediated uptake mechanism and negatively affect intracellular reactions, the toxicity can be reduced or eliminated by the addition of another siderophore (Neillands & Valenta 1985). When siderophores from cyanobacteria have stronger affinities for Fe(III), it appears to be easier to reduce their toxicity. If two species of cyanobacteria produce siderophores, the lack of any effects due to addition of the secretion from *C. aciculare* on these cyanobacteria can be explained by this mechanism.

The processes controlling uptake of iron in aquatic ecosystems are not well-understood. Iron has the potential to control the growth and composition of phytoplankton communities (Murphy et al. 1983; Chang et al. 1992; Pollingher et al. 1995; Evans & Prepas 1997; Sunda & Huntsman 1997; Hyenstrand et al. 1999; Schmidt & Hutchins 1999; Twiss et al. 2000; Shaked et al. 2004; Noiri et al. 2005). This study demonstrated that the secretion from *C. aciculare* showed some patterns of selectivity in its effect on phytoplankton growth. Natural substances secreted from phytoplankton such as *C. aciculare* may play an important role in regulating the growth of other phytoplankton. The next step in our study is to determine the structure and function of the substances secreted by *C. aciculare*. Further studies are needed in iron-limited, low-chlorophyll areas in freshwater systems.

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**References**


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