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Influence of iron chelation with organic ligands on the growth of red tide phytoplankton

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Abstract: Iron mostly dissolves as complexes with organic ligands in natural seawater. In this study, the growth rates of thirteen species of marine eukaryotic red tide phytoplankton, including harmful species were measured in growth experiments using a chemically-defined synthetic medium with iron complexed with different organic ligands [iron salicylate chelates (Fe–SA) (1 : 1, 1 : 10 and 1 : 100), iron citrate chelates (Fe–CA) (1 : 1, 1 : 10 and 1 : 100), iron ethylenediaminetetraacetate chelates (Fe–EDTA) (1 : 1, 1 : 10 and 1 : 100)] and inorganic iron $FeCl_3$. The experiments were carried out at a temperature of 20°C or 25°C under 45–110 μmol photons $m^{-2} s^{-1}$ fluorescent light with a 14 : 10 h L : D cycle. Our study demonstrated that the iron chelated with different ligands was bioavailable to multiple species of red tide phytoplankton. In Fe–SA medium, growth was observed for the raphidophyte *Heterosigma akashiwo*, the dinoflagellates *Heterocapsa circularisquama* and *Heterocapsa triquetra*, the diatom *Ditylum brightwellii*, the cryptophyte *Rhodomonas ovalis*, the green alga *Oltmannsiellopsis viridis*, and the coccolithophorid *Cricosphaera roscoffensis*. In Fe–CA medium, we also found growth of the raphidophyte *Fibrocapsa japonica* in addition to that of the above-mentioned seven red tide species. Thirteen red tide species were able to grow in Fe–EDTA medium. The differences in concentration of organic ligands in each medium affected the growth (yields and rates) of red tide phytoplankton. These results therefore suggest that bioavailability of iron depends not only on ligands but also on the stability of the complexes. Therefore, iron speciation may play an important role in controlling the uptake by red tide phytoplankton of iron complexed with organic materials that exist in coastal water.

Key words: chelation, growth, organic iron, phytoplankton, red tide

Introduction

Iron is a key element in many crucial biological processes. Its deficiency has been demonstrated to limit the growth of phytoplankton in regions of high-nutrient low-chlorophyll (Martin & Fitzwater 1988; Martin et al. 1994; Coale et al. 1996; Boyd et al. 2000) and in coastal waters (Hutchins & Bruland 1998; Hutchins et al. 1998, 2002). In seawater, bioavailable dissolved iron (colloidal and soluble iron) is present at extremely low concentrations (Bruland et al. 1991; Miller & Kester 1994; Sunda & Huntsman 1995; Johnson et al. 1997), and most of that is shown to bind to organic ligands (Rue & Bruland 1997). These organic ligands dramatically change the physicochemical forms and behavior of iron by either keeping it in solution or enhancing its coagulation, and hence, the bioavailability of iron (Hutchins et al. 1999a; Wells 1999). Electrochemical measurements indicate that there are excess free ligands in seawater, and it is pointed out that iron bound to these ligands accounts for 99% of the dissolved iron (Gledhill & van den Berg 1994; Rue & Bruland 1995; Wu & Luther 1995; Witter & Luther 1998). Accordingly, organic ligands may control dissolved iron in seawater and play an important role in iron bioavailability to phytoplankton and its geochemical cycling.

Organic ligands may include hexadentate siderophores produced by microorganisms such as bacteria in response to iron stress, or the release of other intracellular substances such as tetradentate porphyrin complexes (Neilands 1984).

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Hutchins et al. (1999b) suggested that prokaryotes (cyanobacteria) utilize iron complexed to siderophores, whereas eukaryotes (diatoms) appear to rely on a ferrireductase system that preferentially accesses iron chelated by porphyrins. On the other hand, there are several reports on the production of siderophores under iron limited conditions by eukaryotic phytoplankton (Trick et al. 1983; Benderliev & Ivanova 1994; Naito et al. 2001, 2004). The mechanism of iron uptake by phytoplankton is not yet fully understood because of the intricate iron chemistry of natural seawater (Kuma et al. 1996; Millero 1998). Furthermore, there are difficulties in direct measurement of chemical iron species (Obata et al. 1997) and in differentiation between intra- and surface adsorbed iron pools in phytoplankton (Tovar-Sanchez et al. 2003). The distribution, speciation and transformation of different forms of iron are more dynamic and complicated in coastal water than in oceanic water because of the input, of river water.

The cellular iron requirement for the growth of coastal phytoplankton is generally higher than that of oceanic phytoplankton (Sunda & Huntsman 1995). However, phytoplankton blooms have frequently occurred and sometimes formed red tides with water discoloration in coastal areas where supplies of free Fe ions or inorganic iron are apparently insufficient for the growth of coastal phytoplankton (Anderson et al. 1998; Okaichi 2003). Harmful algal blooms are a significant and expanding threat to human health and fisheries resources in the coastal waters of the world (Smayda 1990; Hallegraeff 1993; Ishida et al. 2000). Environmental and economic impacts of harmful algal blooms have increased in recent decades and it is therefore important to understand the mechanisms of harmful algal bloom outbreaks in coastal areas. However, the important roles of micro nutrients such as iron in the outbreak mechanisms are poorly understood because cultivation of most red tide phytoplankton species has so far been impossible in chemically defined synthetic media. Hence, red tide occurrences under bioavailable iron limitation has many puzzling aspects.

Phytoplankton bloom dynamics are affected by physical, chemical, and biotic conditions (Paerl 1988; Imai & Itakura 1999). In order to elucidate the role of iron uptake mechanisms in red tide occurrences, we investigated the effects of naturally existing organic iron complexes at pH 8 (Stumm & Morgan 1996) on the growth of various marine eukaryotic red tide phytoplankton, such as notorious harmful algal species like *Chattonella antiqua*, *Heterosigma akashiwo*, *Heterocapsa circularisquama* and *Karenia mikimotoi* using a newly developed chemically defined synthetic medium (Imai et al. 2004) that gives good growth for these species.

Materials and Methods

Red tide phytoplankton

Axenic clonal cultures of red tide phytoplankton species were used in this study. These were *Chattonella antiqua* (Hada) Ono, *Chattonella marina* (Subrahmanyan) Hara et Chihara, *Chattonella ovata* Hara et Chihara, *Chattonella verruculosa* Hara et Chihara, *Fibrocapsa japonica* Toriumi et Takano and *Heterosigma akashiwo* (Hada) Hada (Raphidophyceae, Heterokontophyta), *Ditylum brightwellii* (West) Grunow ex van Heurck (Bacillariophyceae, Heterokontophyta), *Heterocapsa circularisquama* Horiguchi, *Heterocapsa triquetra* (Ehrenberg) Stein and *Karenia mikimotoi* (Miyake et Kominami ex Oda) Hansen et Moestrup (Dinophyceae, Dinophyta), *Rhodomonas ovalis* Nygaard (Cryptophyceae, Cryptophyta), *Oltmannsiellopsis viridis* (Hargraves et Steele) Chihara et Inouye (Chlorophyceae, Chlorophyta) and *Cricosphaera roscoffensis* (Dangeard) Gayral et Fresnel (Haptophyceae, Haptophyta).

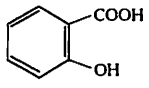
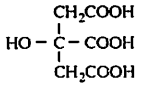
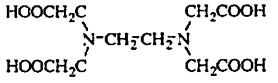
Growth medium

A chemically defined synthetic medium for marine red tide phytoplankton (IHN-medium) (Imai et al. 2004) was modified and used as the basal medium. The pH of the medium was adjusted to 7.8±0.1 with 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and NaOH. Nitrilotriacetic acid, a ligand of metals, was eliminated and HEPES was adopted as a buffer in place of Tris (hydroxymethyl) aminomethane in the IHN-medium. The concentrations of $NaNO_3$ and $NaH_2PO_4 \cdot 2H_2O$ in the medium were increased from 0.6 mM to 2 mM and from 65 μM to 0.1 mM, respectively. The synthetic medium was sterilized by autoclaving at 121°C for 20 min. All reagents used were of the highest purity available. Glass-distilled demineralized water (Milli-Q system, Millipore) was employed.

Preparation of iron complexes with organic ligands

In order to examine the effects of organic iron complexes on the growth of red tide phytoplankton, we selected salicylic acid (SA), citric acid (CA) and ethylenediaminetetraacetic acid (EDTA), which exist in water at pH 7.8 as bidentate, tridentate and hexadentate ligands, respectively (Stumm & Morgan 1996). The structural formulae of these organic ligands are shown in Table 1. The modified IHN-medium without $Na_2EDTA \cdot 2H_2O$ and NaFeEDTA was prepared as the iron-deprived synthetic medium for the growth experiments. SA (Nacalai tesque), CA (SIGMA) and EDTA (Nacalai tesque) in sterilized Milli-Q water were added to the polycarbonate bottles (Nalge Nunc) containing the iron-deprived synthetic media after filtration through a 0.1-μm filter (Millex-VV, Millipore). $FeCl_3 \cdot 6H_2O$ (Nacalai tesque) in 0.017 N HCl, which had been passed through a 0.1-μm filter, was then added to each ligand at 1 : 1, 1 : 10 or 1 : 100 molar ratios in each medium, and allowed to equilibrate for at least 24 h at 20°C. Each medium contained 2 μM Fe and ligands of 2 μM, 20 μM and 200 μM, respectively. The preparation of each medium was done in a clean box to avoid contamination by metals in the air. All equipment ex-

Table 1. Formation constants of Fe(III)–organic ligands complexes and structural formulae of organic ligands.

Organic ligand	Log β[a]		Structual formula
Salicylic acid (SA)	FeL	17.6	
	FeL_2	28.6	
	FeL_3	36.2	
Citric acid (CA)	FeL	13.5	
	$Fe_2(OH)_2L_2$	56.3	
EDTA	FeL	27.7	
	FeHL	29.2	
	FeOHL	33.8	
	$Fe(OH)_2L$	37.7	

[a] Constants are given as logarithms of the overall formation constants, β, for complexes at zero ionic strength and 25°C. From Morel and Hering (1993).

cept for already sterilised instruments were soaked in a detergent solution of neutral pH (Scat 20X-N, Dai-Ichi Kogyo Seiyaku) and then in 4 M HCl, and rinsed with Milli-Q water.

Growth experiments

Polystyrene test tubes (13 mmϕ×100 mm) with screw caps (Fisher brand Co.) were used for the growth experiments. Maintenance cultures were axenically transferred to fresh iron-limited synthetic medium containing 0.2 μM Fe–EDTA and were grown at 25°C (*Chattonella antiqua*, *C. marina*, *C. ovata*, *Heterocapsa circularisquama*) and at 20°C (other nine species). Eighty micro-liters of each of these precultures at the late exponential growth phase were inoculated into 4 ml of each medium in 8-ml volume polystyrene test tubes using an acid-washed micropipette. The experimental cultures were incubated under fluorescent lighting at 45–70 μmol photons $m^{-2}s^{-1}$ for *Karenia mikimotoi* and at 75–110 μmol photons $m^{-2}s^{-1}$ for the other twelve species under a 14 : 10 h light : dark cycle. Algal growths were determined by measuring *in vivo* fluorescence using a fluorometer (Turner Designs 10-AU 005). Growths were compared with those in the modified IHN-medium (at a ratio of 1 : 16 for Fe : EDTA). The growth experiments were performed in quadruplicate.

Results

Raphidophyceae

Four species of the genus *Chattonella* were unable to utilize iron salicylate chelates (Fe–SA), iron citrate chelates (Fe–CA) or $FeCl_3$ (organic ligand-free) for their growth (Fig. 1). *Chattonella antiqua* and *C. ovata* utilized Fe–EDTA (1 : 10) (Fig. 1A, B), while *C. marina* and *C. verruculosa* utilized Fe–EDTA (1 : 10 and 1 : 100) (Fig. 1C, D) for their growth. The growths of *C. antiqua* and *C. ovata* in Fe–EDTA (1 : 10) medium were similar to those in the modified IHN-medium (Fig. 1A, B). The growth of *C. marina* in Fe–EDTA (1 : 100) medium was slower than that in Fe–EDTA (1 : 10) medium (Fig. 1C). The growth in Fe–EDTA (1 : 10) medium was similar to that in the modified IHN-medium, whereas the maximal growth yield in Fe–EDTA (1 : 100) medium was 66% of that in the modified IHN-medium. The growths of *C. verruculosa* were similar in Fe–EDTA (1 : 10) and Fe–EDTA (1 : 100) media, and these were faster than those of the other three species of the genus *Chattonella* (Fig. 1D). The maximal growth yields of *C. verruculosa* in Fe–EDTA (1 : 10 and 1 : 100) media were 50–60% of those in the modified IHN-medium.

Fibrocapsa japonica grew in Fe–CA (1 : 1 and 1 : 10) and Fe–EDTA (1 : 1, 1 : 10 and 1 : 100) media, but did not grow in Fe–SA (1 : 1, 1 : 10 and 1 : 100) or Fe–CA (1 : 100) media (Fig. 2A). Maximal growth yields in Fe–EDTA (1 : 10 and 1 : 100) media were 40–50% of those in the modified IHN-medium. Growth in Fe–CA (1 : 1 and 1 : 10) and Fe–EDTA (1 : 1) media was similar to that in $FeCl_3$ medium. The order of maximal growth yields was: Fe–EDTA (1 : 10 and 1 : 100) > $FeCl_3$, Fe–CA (1 : 1 and 1 : 10), Fe–EDTA (1 : 1). The modified IHN-medium gave better growth of *F. japonica*.

Heterosigma akashiwo was able to grow in Fe–SA, Fe–CA and Fe–EDTA media (Fig. 2B). Maximal growth yields in Fe–CA (1 : 1, 1 : 10 and 1 : 100) and Fe–EDTA (1 : 10) media were >100%, and those in Fe–EDTA (1 : 1) and Fe–EDTA (1 : 100) media were 44% and 73% of those in the modified IHN-medium. The growth in Fe–EDTA (1 : 100) medium was similar to that in $FeCl_3$ medium. The order of maximal growth yields was: Fe–EDTA (1 : 10) > Fe–CA (1 : 10) > Fe–CA (1 : 1 and 1 : 100) > $FeCl_3$, Fe–EDTA (1 : 100) > Fe-EDTA (1 : 1) ≫ Fe–SA (1 : 1, 1 : 10 and 1 : 100). The order of growth rates was: Fe–EDTA (1 : 10) > Fe–CA (1 : 100) ≫ Fe–CA (1 : 10), Fe–EDTA (1 : 1) ≥ $FeCl_3$, Fe–CA (1 : 1), Fe–EDTA (1 : 100) ≫ Fe–SA (1 : 10 and 1 : 100) > Fe–SA (1 : 1).

Dinophyceae

Heterocapsa circularisquama was able to grow in Fe–SA, Fe–CA and Fe–EDTA media (Fig. 3A). Maximal growth yields in each organic Fe medium were >80% of those in the modified IHN-medium. However, the growth yield was highest in $FeCl_3$ medium. The order of maximal growth yields was: $FeCl_3$ > Fe–EDTA (1 : 1 and 1 : 10), Fe–SA (1 : 10) > Fe–CA (1 : 1 and 1 : 10), Fe–SA (1 : 1) > Fe–EDTA (1 : 100), Fe–CA (1 : 100) > Fe–SA (1 : 100). On the other hand, the order of growth rates was: Fe–CA (1 : 1 and 1 : 10) ≥ $FeCl_3$ > Fe–SA (1 : 1 and 1 : 10) > Fe–EDTA (1 : 1) ≥ Fe–CA (1 : 100) ≥ Fe–SA (1 : 100) > Fe–EDTA (1 : 10) > Fe–EDTA (1 : 100).

Heterocapsa triquetra was able to grow in Fe–SA, Fe–CA and Fe–EDTA media with the exception of the

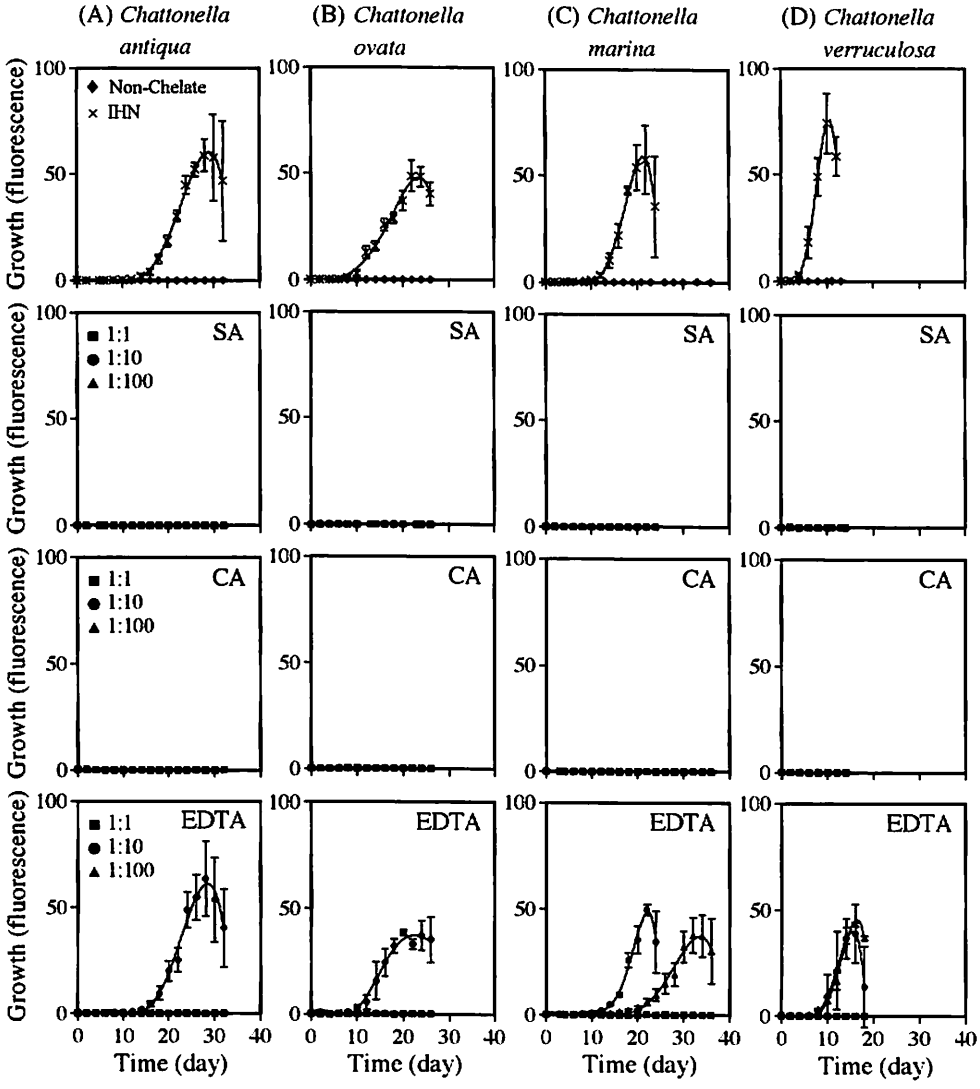


Fig. 1. Growth curves of the species of *Chattonella* (Raphidophyceae) in cultures supplied with iron salicylate (SA), iron citrate (CA), iron ethylenediaminetetraacetate (EDTA), $FeCl_3$ (Non-Chelate) and in the modified IHN-medium. Growth curves are shown for (A) *Chattonella antiqua*, (B) *Chattonella ovata*, (C) *Chattonella marina*, (D) *Chattonella verruculosa*. Data on fluorescence represent mean±SD.

Fe–SA (1 : 100) concentration media (Fig. 3B). *Heterocapsa triquetra* in Fe–SA media started to grow later than in Fe–CA or Fe–EDTA media. The growths of *H. triquetra* in Fe–CA (1 : 10) and Fe–EDTA (1 : 10) media were similar to those in the modified IHN-medium. In Fe–SA media, the fluorescence decreased gradually with increasing concentration of SA, and disappeared at a 1 : 100 molar ratio of Fe : SA. The order of maximal growth yields was: Fe–CA (1 : 10), Fe–EDTA (1 : 10) > Fe–CA (1 : 1 and 1 : 100), Fe–EDTA (1 : 100), $FeCl_3$ > Fe–EDTA (1 : 1) ≧ Fe–SA (1 : 1) > Fe–SA (1 : 10) ≫ Fe–SA (1 : 100).

Karenia mikimotoi was able to utilize Fe–EDTA (1 : 1 and 1 : 10) for growth (Fig. 3C). The growth of *K. mikimotoi* in Fe–EDTA (1 : 10) medium was faster than that in Fe–EDTA (1 : 1) medium. The maximal growth yield in $FeCl_3$ medium was 21% of that in the modified IHN-medium, whereas those in Fe–EDTA (1 : 1) and Fe–EDTA (1 : 10) media were 35% and 63%. *Karenia mikimotoi* did not grow in Fe–SA, Fe–CA or Fe–EDTA (1 : 100) media.

Other red tide species (Bacillariophyceae, Cryptophyceae, Chlorophyceae and Haptophyceae)

Ditylum brightwellii (Bacillariophyceae) was able to grow in Fe–SA, Fe–CA and Fe–EDTA media (Fig. 4A). The growths of *D. brightwellii* in Fe–SA and $FeCl_3$ media were slower than in Fe–CA and Fe–EDTA media. The order of maximal growth yields was: Fe–SA (1 : 10), Fe–EDTA (1 : 1 and 1 : 10), $FeCl_3$ > Fe–CA (1 : 10 and 1 : 100) > Fe–SA (1 : 1) > Fe–CA (1 : 1) ≫ Fe–SA (1 : 100), Fe–EDTA (1 : 100).

A significant increase in the growth of *Rhodomonas*

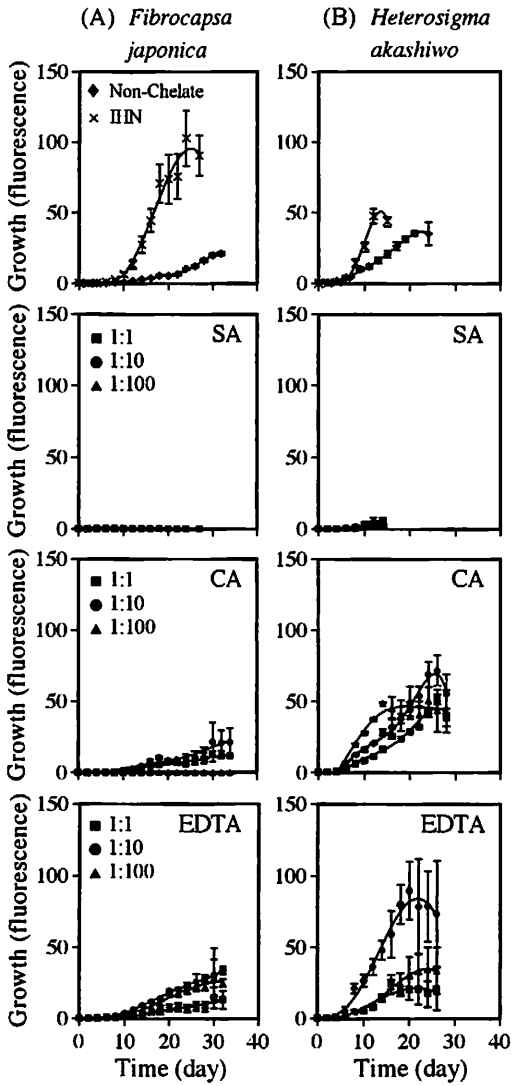


Fig. 2. Growth curves of the raphidophytes (A) *Fibrocapsa japonica* and (B) *Heterosigma akashiwo* in cultures supplied with iron salicylate (SA), iron citrate (CA), Fe–EDTA (EDTA), $FeCl_3$ (Non-Chelate) and in the modified IHN-medium. Data on fluorescence represent mean ± SD.

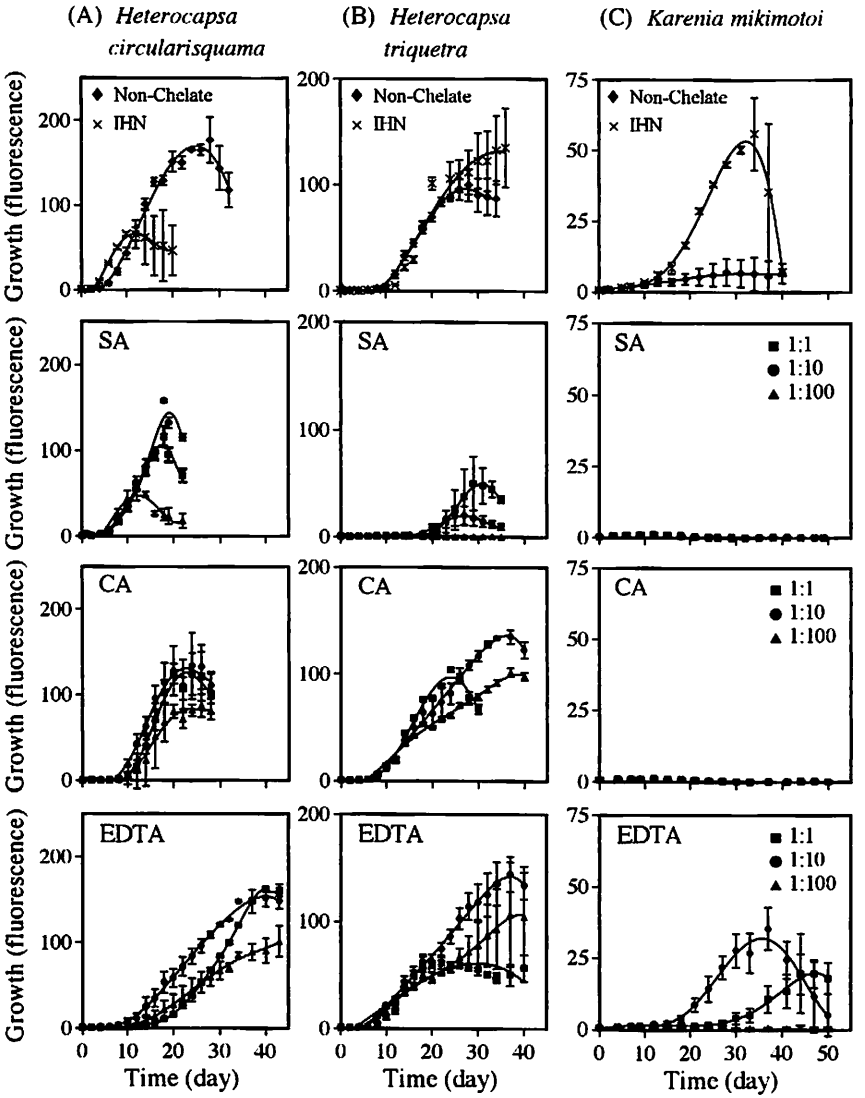


Fig. 3. Growth curves of the red tide dinoflagellate species in cultures supplied with iron salicylate (SA), iron citrate (CA), Fe–EDTA (EDTA), $FeCl_3$ (Non-Chelate) and in the modified IHN-medium. Growth curves are shown for (A) *Heterocapsa circularisquama*, (B) *Heterocapsa triquetra*, (C) *Karenia mikimotoi*. Data on fluorescence represent mean ± SD.

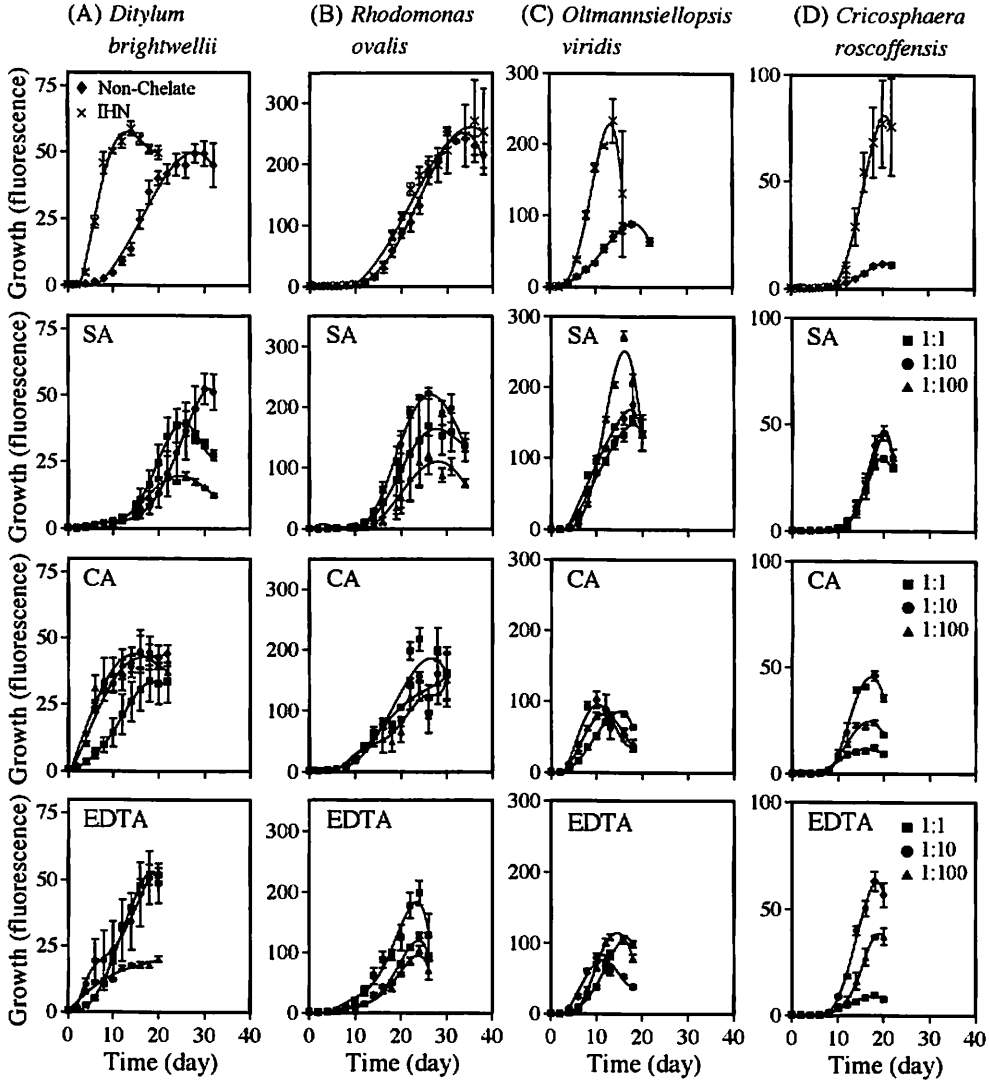


Fig. 4. Growth curves of (A) *Ditylum brightwellii* (Bacillariophyceae), (B) *Rhodomonas ovalis* (Cryptophyceae), (C) *Oltmannsiellopsis viridis* (Chlorophyceae) and (D) *Cricosphaera roscoffensis* (Haptophyceae) in cultures supplied with iron salicylate (SA), iron citrate (CA), Fe-EDTA (EDTA), $FeCl_3$ (Non-Chelate) and in the modified IHN-medium. Data on fluorescence represent mean±SD.

ovalis (Cryptophyceae) was observed in each media, whereas their maximal growth yields were 45–82% of those in the modified IHN-medium (Fig. 4B). The growth was almost the same in $FeCl_3$ and in the modified IHN-medium. The order of maximal growth yields was: $FeCl_3$ > Fe–SA (1 : 10), Fe–CA (1 : 1), Fe–EDTA (1 : 1) > Fe–SA (1 : 1), Fe–CA (1 : 10 and 1 : 100), Fe–EDTA (1 : 10) > Fe–SA (1 : 100), Fe–EDTA (1 : 100). The order of growth rates was: Fe–SA (1 : 10) ≫ $FeCl_3$ > Fe-CA (1 : 1) > Fe–SA (1 : 1), Fe–EDTA (1 : 1) > Fe–SA (1 : 100) > Fe–CA (1 : 10), Fe–EDTA (1 : 10) > Fe–CA (1 : 100), Fe–EDTA (1 : 100).

Oltmannsiellopsis viridis (Chlorophyceae) was able to grow well in all organic Fe media, particularly in the Fe–SA media (Fig. 4C). The order of maximal growth yields was: Fe–SA (1 : 100) ≫ Fe–SA (1 : 10) ≥ Fe–SA (1 : 1) ≫ Fe–CA (1 : 10), Fe–EDTA (1 : 1 and 1 : 100) ≥ $FeCl_3$, Fe–CA (1 : 1 and 1 : 100), Fe–EDTA (1 : 10). The growth rates of *O. viridis* in organic Fe media were faster than in $FeCl_3$ medium.

Cricosphaera roscoffensis (Haptophyceae) was able to grow in Fe–SA, Fe–CA and Fe–EDTA media (Fig. 4D). Of all the organic Fe media, the growth efficiency of *C. roscoffensis* was highest at the ratio of Fe : L = 1 : 10. The order of maximal growth yields was: Fe–EDTA (1 : 10) ≫ Fe–SA (1 : 10 and 1 : 100), Fe–CA (1 : 10) > Fe–EDTA (1 : 100) ≥ Fe–SA (1 : 1) > Fe–CA (1 : 100) > Fe–CA (1 : 1), $FeCl_3$, Fe–EDTA (1 : 1). The order of growth rates was almost identical to that for maximal growth yields.

Table 2. Comparison of availability of iron chelated with organic ligands for red tide phytoplankton (Iron sources: 2 μM $FeCl_3$).

Ligands	Salicylic acid			Citric acid			EDTA			Non-chelate
Ratios (Fe : Ligand)	1 : 1	1 : 10	1 : 100	1 : 1	1 : 10	1 : 100	1 : 1	1 : 10	1 : 100	1 : 0
Chattonella antiqua	−	−	−	−	−	−	−	++++	−	−
Chattonella marina	−	−	−	−	−	−	−	+++	+++	−
Chattonella ovata	−	−	−	−	−	−	−	++	−	−
Chattonella verruculosa	−	−	−	−	−	−	−	+++	+++	−
Fibrocapsa japonica	−	−	−	+	+	−	+	++	++	+
Heterosigma akashiwo	+	−	−	−	++++	−	−	++++	+++	−
Heterocapsa circularisquama	++++	−−+++	+++	++++	+++++	++++	+++++	+++++	++++	+++++
Heterocapsa triquetra	++	+	−	+−	−−++	−++	++	++++	−++	+++
Karenia mikimotoi	−	−	−	−	−	−	++	+++	−	+
Ditylum brightwellii	+++	+−−	++	+++	+−−	+++	+++	−++	++	+++
Rhodomonas ovalis	+++	+++	++	+++	−++	+++	+++	+++	+−	+−−
Oltmannsiellopsis viridis	+++	−++	+−−−	++	++	++	++	−−	++	++
Cricosphaera roscoffensis	++	+++	+++	−	+++	++	−	+++	++	+

The maximal growth yield of red tide phytoplankton in the modified IHN-medium = 100%.
(−) <5%, (+) 5–30%, (++) 30–50%, (+++) 50–100%, (++++) 100–200%, (+++++) >200% of yields in the modified IHN-medium.

Availability of iron chelated with organic ligands for red tide phytoplankton

We estimated the maximal growth yields of red tide phytoplankton in each medium in order to evaluate the availability of three species of organic Fe complexes. Table 2 summarizes the maximal growth yields of red tide phytoplankton in each medium compared to those in the modified IHN-medium. In Fe–SA medium, growth was observed for the raphidophyte *Heterosigma akashiwo*, the dinoflagellates *Heterocapsa circularisquama* and *Heterocapsa triquetra*, the diatom *Ditylum brightwellii*, the cryptophyte *Rhodomonas ovalis*, the green alga *Oltmannsiellopsis viridis* and the coccolithophorid *Cricosphaera roscoffensis*. In Fe–CA medium, we also observed growth of the raphidophyte *Fibrocapsa japonica* in addition to the above-mentioned seven species. All thirteen red tide species examined were able to grow in Fe–EDTA medium. When the growth of red tide phytoplankton in organic Fe medium is lower than in $FeCl_3$ medium (with no organic ligands), these organic ligands can be regarded as inhibitors of growth. Therefore, we concluded that SA stimulates the growths of *O. viridis* and *C. roscoffensis*, and that CA promotes the growths of *H. akashiwo*, *H. triquetra* (only for Fe : L = 1 : 10) and *C. roscoffensis* (for Fe : L = 1 : 10, 1 : 100). EDTA appears to promote the growth of *K. mikimotoi* at Fe : L = 1 : 1. Growth of the species of *Chattonella* (*C. antiqua*, *C. marina*, *C. ovata*, *C. verruculosa*), *F. japonica*, *H. akashiwo*, *H. triquetra*, *K. mikimotoi* and *C. roscoffensis* was also enhanced at Fe : EDTA = 1 : 10, and growth of *C. marina*, *C. verruculosa*, *F. japonica* and *C. roscoffensis* at Fe : EDTA = 1 : 100.

Discussion

The speciation of iron in the chemically defined synthetic medium

The stability of complexes cannot be predicted from complex stability constants alone, and competitive effects of H^+ (with metal ions) and of OH^- (with ligands) need to be considered. The equilibrium constants of complexes with metal (M) and ligand (L) can be expressed according to the following reaction and associated mass action equations:

In the case of mononuclear complexes,

(1) addition of ligand

$$M \xrightarrow[K_1]{L} ML \xrightarrow[K_2]{L} ML_2 \cdots \xrightarrow[K_i]{L} ML_i \cdots \xrightarrow[K_n]{L} ML_n$$

$$\beta_i = \frac{[ML_i]}{[M][L]^i}$$

(2) addition of protonated ligands

$$M \xrightarrow[K_1]{HL} ML \xrightarrow[K_2]{HL} ML_2 \cdots \xrightarrow[K_i]{HL} ML_i \cdots \xrightarrow[K_n]{HL} ML_n$$

$$\beta_i = \frac{[ML_i][H^+]^i}{[M][HL]^i}$$

In the case of polynuclear complexes,

$$\beta_{mn} = \frac{[M_mL_n]}{[M]^m[L]^n}$$

$$\beta_{nm} = \frac{[M_m L_n][H^+]^n}{[M]^m[HL]^n}$$

At low pH, H^+ successfully competes with metal ions for the ligand. At high pH, OH^- successfully competes with the ligand for the coordinative positions on the metal ion. Furthermore, at low and high pH, mixed hydrogen–metal and hydroxide–ligand complexes can be formed. In the case of EDTA, in addition to $FeEDTA^-$, the complexes FeHEDTA, $FeOHEDTA^{2-}$, and $Fe(OH)_2EDTA^{3-}$ have to be considered. Stability constants for the formation of ferric complexes with the organic ligands examined are shown in Table 1. As regards Fe(III) complexation, multidentate ligands have relatively greater stability than monodentate (Stumm & Morgan 1996). In our experiment, three organic ligands (SA, CA, EDTA) have more than 10-times greater stability than chlorine ($\log\beta=1.5$ (FeL), $\log\beta=2.1$ (FeL_2)) for Fe(III) complexation.

Furthermore, because of the competing influence of other cations or anions, the complexing effect cannot be estimated solely from the stability constants. The use of a chemically defined growth medium enabled calculation of Fe speciation with a chemical equilibrium software program. Therefore, we calculated the degree of speciation of iron in each medium using MINEQL+ (ver. 4.0) software (Schecher & McAvoy 1992).

Iron is present as $Fe(OH)_2^+$, $Fe(OH)_3$, $Fe(OH)_4^-$ and $Fe(SA)_2^-$ in the Fe–SA medium (Table 3). With increasing SA, the iron salicylic chelate increases from 0.34% (Fe : L=1 : 1) to 24% (Fe : L=1 : 10), and 97% (Fe : L= 1 : 100) of the dissolved Fe(III) species and the concentration of HSA^- also increases in the Fe–SA medium (Table 3). In the Fe–CA medium, the dominant species is $Fe_2(OH)_2(CA)_2^{2-}$ for iron complexes, but the predominant metal complexed with CA changes from iron to calcium and magnesium with increasing concentrations of CA (Table 3). The citrate chelates exist as $Fe_2(OH)_2(CA)_2^{2-}$ (100%) in Fe–CA (1 : 1) medium, and as free CA (12–14%), $CaCA^-$ (63–69%), $MgCA^-$ (14–16%), and $Fe_2(OH)_2(CA)_2^{2-}$ (1–10%) in Fe–CA (1 : 10 and 1 : 100) media. Iron is present as $Fe(OH)_2^+$, $Fe(OH)_3$, $Fe(OH)_4^-$, $FeOHEDTA^{2-}$ and $FeEDTA^-$ in Fe–EDTA medium (Table 3). In Fe–EDTA medium, the dominant species is $FeOHEDTA^{2-}$ for the iron species, but changes from $FeOHEDTA^{2-}$ (84–85% of EDTA species at Fe : L=1 : 1) to $MnEDTA^{2-}$ (61% of EDTA species at Fe : L=1 : 10), and $CaEDTA^{2-}$ (76% of EDTA species at Fe : L=1 : 100) for EDTA species (Table 3). In all the media examined, the concentration of the free hydrated ion (Fe^{3+}) is very low (<2.0 fM).

The order of stability of the dominant iron complexes with high ligand concentrations is: CA ($Fe_2(OH)_2(CA)_2^{2-}$) > EDTA ($FeOHEDTA^{2-}$) > SA ($Fe(SA)_2^-$) (Tables 1, 3). Two paths of iron uptake by phytoplankton from the organic iron complexes are considered; i.e. direct uptake of organic Fe(III) complexes and the uptake of dissociative free or hydroxide Fe ions (Anderson & Morel 1982; Tessier et al. 1994). A schematic representation of iron uptake from organic Fe(III) complexes by phytoplankton is shown in Fig. 5. It was assumed that phytoplankton favor the utilization of organic iron complexes with high formation constants in the case of direct uptake of organic iron complexes, and to utilize organic iron complexes with low formation constants in the case of indirect uptake of dissociated iron (working hypothesis).

Effects of iron chelation with organic ligands on the growth of red tide phytoplankton

Differences in the ratio of organic ligands to Fe(III) in each medium affected not only the maximal growth yields but also the growth rates of red tide phytoplankton. The seven red tide phytoplankton that grew in Fe–SA medium can be divided into 3 types based on the differences in growth rates. These growth rate patterns were (1) 1 : 100 ≥ 1 : 10 ≥ 1 : 1, (2) 1 : 1 > 1 : 10 > 1 : 100, (3) 1 : 10 ≥ 1 : 1 > 1 : 100 (Fe : SA). *Heterosigma akashiwo*, *Oltmannsiellopsis viridis* and *Cricosphaera roscoffensis* belonged to type (1) (Figs. 2B, 4C, 4D). As the proportion of $Fe(SA)_2^-$ in the Fe–SA medium increased (Table 3), these growth rates also increased. Therefore, SA is considered to accelerate the growth rates of these three algal species. The growth rate of *Heterocapsa triquetra* decreased with an increase in the concentration of SA in the Fe–SA medium (type (2)) and the cells all died at a ratio of 1 : 100 for Fe : SA. Hence, SA is revealed to be an inhibitor of the growth of this alga (Fig. 3B). *Heterocapsa circularisquama*, *Ditylum brightwellii* and *Rhodomonas ovalis* were regarded to be of type (3) (Figs. 3A, 4A, 4B). These algae are considered to utilize not only $Fe(SA)_2^-$ but also hydroxides for iron uptake and SA itself seems to be inhibitive to their growths at ratios of 1 : 10–1 : 100 for Fe : L, because the growth rates were lower in the Fe–SA (1 : 100) medium than in the Fe–SA (1 : 1) medium.

The eight red tide phytoplankton species that grew in Fe–CA medium can be divided into 4 types based on differences in growth rates. These growth rate patterns were (1) 1 : 100 ≥ 1 : 10 > 1 : 1, (2) 1 : 1 ≥ 1 : 10 > 1 : 100, (3) 1 : 10 > 1 : 1 ≫ 1 : 100, (4) 1 : 10 > 1 : 100 > 1 : 1 (Fe : CA). It is considered that *H. akashiwo* and *D. brightwellii*, belonging to type (1), utilize $Fe_2(OH)_2(CA)_2^{2-}$ as an iron source (Table 3) and that CA is a promoter for the growth rates of these two algae (Figs. 2B, 4A). On the other hand, CA was found to inhibit the growth rates of *H. circularisquama*, *H. triquetra* and *R. ovalis*, belonging to type (2) (Figs. 3A, 3B, 4B). *Fibrocapsa japonica* (type (3)) (Fig. 2A) and the two species *O. viridis* and *C. roscoffensis* (type (4)) (Fig. 4C and D) are considered to utilize $Fe_2(OH)_2(CA)_2^{2-}$ as an iron source for iron uptake. However, CA appears to decrease (type (3)) or slow down (type (4)) the growth rates of these three algae at high ratios of CA in the Fe–CA medium. Cit-

Table 3. Ranges of highest percentages of speciated forms of Fe(III) species and organic ligand (SA, CA, EDTA) species in the artificial medium with organic Fe addition (pH 7.8, 20–25°C).

Medium	Speciated forms	% Range	Speciated forms	% Range
Fe–SA (1 : 1)	$Fe(OH)_2^+$	50	HSA^-	96
	$Fe(OH)_3$	26	$CaHSA^+$	4
	$Fe(OH)_4^-$	23	$Fe(SA)_2^-$	0
	$Fe(SA)_2^-$	0.3		
Fe–SA (1 : 10)	$Fe(OH)_2^+$	38	HSA^-	92
	$Fe(OH)_3$	20	$CaHSA^+$	3.5
	$Fe(OH)_4^-$	18	$Fe(SA)_2^-$	4.8
	$Fe(SA)_2^-$	24		
Fe–SA (1 : 100)	$Fe(OH)_2^+$	1.5	HSA^-	94–95
	$Fe(OH)_3$	0.8	$CaHSA^+$	3.6
	$Fe(OH)_4^-$	0.7	$Fe(SA)_2^-$	1.9
	$Fe(SA)_2^-$	97		
Fe–CA (1 : 1)	$Fe(OH)_2^+$	0	CA	0
	$Fe(OH)_3$	0	$CaCA^-$	0
	$Fe(OH)_4^-$	0	$MgCA^-$	0
	$Fe_2(OH)_2(CA)_2^{2-}$	100	$Fe_2(OH)_2(CA)_2^{2-}$	100
Fe–CA (1 : 10)	$Fe(OH)_2^+$	0	CA	12
	$Fe(OH)_3$	0	$CaCA^-$	63
	$Fe(OH)_4^-$	0	$MgCA^-$	14
	$Fe_2(OH)_2(CA)_2^{2-}$	100	$Fe_2(OH)_2(CA)_2^{2-}$	10
Fe–CA (1 : 100)	$Fe(OH)_2^+$	0	CA	13–14
	$Fe(OH)_3$	0	$CaCA^-$	69
	$Fe(OH)_4^-$	0	$MgCA^-$	15–16
	$Fe_2(OH)_2(CA)_2^{2-}$	100	$Fe_2(OH)_2(CA)_2^{2-}$	1
Fe–EDTA (1 : 1)	$Fe(OH)_2^+$	6	$FeOHEDTA^{2-}$	84–85
	$Fe(OH)_3$	3	$FeEDTA^-$	3.8
	$Fe(OH)_4^-$	3	$MnEDTA^{2-}$	5.8
	$FeOHEDTA^{2-}$	84–85	$ZnEDTA^{2-}$	3
	$FeEDTA^-$	4	$CoEDTA^{2-}$	0–2
Fe–EDTA (1 : 10)	$Fe(OH)_2^+$	0.04	$FeOHEDTA^{2-}$	9.5
	$Fe(OH)_3$	0.02	$FeEDTA^-$	0
	$Fe(OH)_4^-$	0.02	$MnEDTA^{2-}$	61
	$FeOHEDTA^{2-}$	95	$ZnEDTA^{2-}$	14
	$FeEDTA^-$	4	$CoEDTA^{2-}$	0
			$CaEDTA^{2-}$	14
Fe–EDTA (1 : 100)	$Fe(OH)_2^+$	0	$FeOHEDTA^{2-}$	0
	$Fe(OH)_3$	0	$FeEDTA^-$	0
	$Fe(OH)_4^-$	0	$MnEDTA^{2-}$	17
	$FeOHEDTA^{2-}$	95	$ZnEDTA^{2-}$	2
	$FeEDTA^-$	4	$CoEDTA^{2-}$	0
			$CaEDTA^{2-}$	76
			$MgEDTA^{2-}$	4

rate chelates ($CaCA^-$, $MgCA^-$ and free CA) may have some effect on the growth of these algae.

The growth rates of the eleven red tide phytoplankton species that grew in Fe–EDTA medium can be divided into 4 types: (1) 1 : 100 ≧ 1 : 10 > 1 : 1, (2) 1 : 1 > 1 : 10 > 1 : 100, (3) 1 : 10 > 1 : 1 ≫ 1 : 100, (4) 1 : 10 > 1 : 100 ≧ 1 : 1 (Fe : EDTA). *Chattonella verruculosa*, *F. japonica* and *O. viridis* (type (1)) are considered to utilize $FeOHEDTA^{2-}$ as an iron source (Table 3) and EDTA is a promoter for the growth rates of these three algae (Figs. 1D, 2A, 4C). It was found that EDTA inhibited the growth rates of three species belonging to type (2), *H. circularisquama*, *D. brightwellii* and *R. ovalis* (Figs. 3A, 4A, 4B). *Karenia mikimotoi* (type (3)) (Fig. 3C) and the 4 species *Chattonella marina*, *H. akashiwo*, *H. triquetra*, and *C. roscoffensis* (type (4)) (Figs. 1C, 2B, 3B, 4D) are considered to utilize $FeOHEDTA^{2-}$ and/or $FeEDTA^-$ as iron sources (Table 3), but EDTA seems to decrease (type (3)) or slow down (type (4)) the

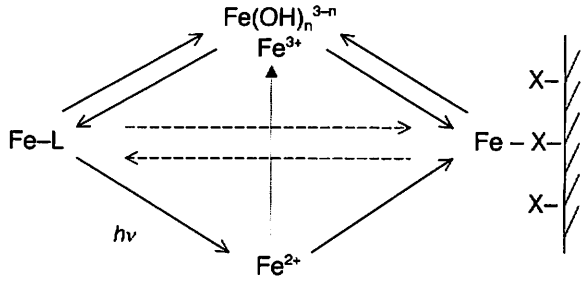


Fig. 5. Schematic representation of iron uptake mechanism from organic Fe(III) complexes by phytoplankton (X: surface transport sites; L: ligand). Modified from Anderson and Morel (1982).

growth rates at 1 : 100 > Fe : EDTA > 1 : 10 in Fe–EDTA medium. The metal-exchange reaction of organic Fe(III) complexes by alkaline-earth metals (Ca and Mg) (Hasegawa et al. 2002) and other trace metals (Mn, Zn and Co) in the medium must be considered. These reactions can significantly affect the growth of these red tide algae.

The results showed differences in growth in Fe–EDTA (1 : 10) medium and in the modified IHN-medium (a ratio of 1 : 16 for Fe : EDTA) for some red tide phytoplankton, despite the similarity of computed percentages of Fe(III) species (Tables 3, 4). This is probably a result of differences in the preparation procedure of the organic iron complexes in the experimental media, because the dissociation of Fe(III) from premixed Fe–EDTA is generally slow (Kuma et al. 1999). This dissociation is also an important process for the supply of biologically available Fe through increasing the dissolved Fe concentration. Furthermore, the effects of the kinetic properties of association and dissociation of iron with organic ligands in the medium must be taken into account when considering the growth of red tide phytoplankton. Accordingly, understanding Fe speciation is also very important for controlling the growth of phytoplankton-causing red tides.

The mechanism of iron uptake by red tide phytoplankton

The dissolved iron species are predominantly the hydrolysis products $Fe(OH)_2^+$, $Fe(OH)_3$ and $Fe(OH)_4^-$ in $FeCl_3$ medium (Table 4). *Heterocapsa circularisquama* and *Rhodomonas ovalis*, which showed good growth in $FeCl_3$ medium, grew better at the higher concentrations of iron hydroxides in each organic Fe medium (Fe : L = 1 : 1 or 1 : 10) (Figs. 3A, 4B). Since the growths of *H. circularisquama* and *R. ovalis* were inhibited by the presence of an excess of organic ligands in each medium, these red tide species are found to prefer inorganic Fe to organic Fe for iron uptake, even when organic Fe is also available. *Heterocapsa triquetra* and *Ditylum brightwellii* also exhibited higher growth in $FeCl_3$ medium and showed the same tendency as the above-mentioned dinoflagellate and cryptophyte. EDTA for *H. triquetra* and CA for *D. brightwellii*

Table 4. Ranges of highest percentages of speciated forms of Fe(III) species in the iron-limited artificial medium with $FeCl_3$ added and in the modified IHN-medium (pH 7.8, 20–25°C).

Medium	Speciated forms	% Range
$FeCl_3$ (ligand-free)	$Fe(OH)_2^+$	50
	$Fe(OH)_3$	26
	$Fe(OH)_4^-$	24
Modified IHN	$Fe(OH)_2^+$	0.02
	$Fe(OH)_3$	0.009
	$Fe(OH)_4^-$	0.008
	$FeOHEDTA^{2-}$	95
	$FeEDTA^-$	4

have promoting effects on their growth (Figs. 3B, 4A). In our previous studies, these four species were able to utilize particulate $FePO_4$ and/or FeS as iron sources for their growth (Naito et al. submitted), and *R. ovalis* may have the ability to produce siderophore, an Fe(III)-specific ligand, under iron limiting conditions (Naito et al. 2001). Therefore, these four red tide species may have iron uptake mechanisms that preferentially uptake soluble and insoluble inorganic Fe by the uptake strategies of "swim" for flagellates and "sink" for diatoms, as is the case for macro nutrients (Smayda 1997). Soluble organic Fe might be taken up by a siderophore-mediated ligand-exchange reaction (Völker & Wolf-Gladrow 1999).

Three species, *Heterosigma akashiwo*, *Oltmannsiellopsis viridis* and *Cricosphaera roscoffensis* grew in each organic Fe medium, and their growth was promoted by SA, CA, or EDTA (Figs. 2B, 4C, 4D). Therefore, it may be easy for these three species to grow where and when organic Fe chelators exist. Here, we analyzed the form of Fe taken up by these three species to test our hypothesis. For *H. akashiwo*, the order of the growth yields was: CA > EDTA > SA at a ratio of 1 : 100 (Fe : L), accordingly, this species may follow the strategy of direct uptake of organic Fe complexes. On the other hand, *O. viridis* and *C. roscoffensis* can be considered to utilize dissociated Fe from organic Fe complexes because the order of the growth yields of these species was: SA > EDTA > CA at a ratio of 1 : 100 (Fe : L).

Red tide species that were unable to grow (genus *Chattonella*) or that exhibited lower growth (*Fibrocapsa japonica* and *Karenia mikimotoi*) in $FeCl_3$ medium had higher growths with the existence of EDTA (high coordination and stable complex), especially, at a ratio of 1 : 10 for Fe : EDTA (Figs. 1, 2A, 3C). For *F. japonica*, the order of the growth yields was: EDTA > CA ≫ SA at the same ratio of Fe : L, and growth in Fe–CA (1 : 1 and 1 : 10) and Fe–EDTA media was similar to that in $FeCl_3$ medium. Therefore, this species may employ an uptake mechanism including ligand-exchange and dissociation of inorganic Fe from organic Fe in the medium or on the cell surface (Fig. 2A). It is considered that the lack of growth of *F. japonica* in Fe–SA medium is

because HSA^- acts as an inhibitor for this species. *Karenia mikimotoi* is thought to favor organic Fe over inorganic Fe because this species grew better in Fe–EDTA medium than in $FeCl_3$ medium (Fig. 3C). The four species in the genus *Chattonella* may utilize organic Fe preferentially because these species grew only in Fe–EDTA medium. The aforementioned six species are considered to follow the strategy of organic Fe uptake by a ligand-exchange reaction (siderophore production etc.).

The association of iron with red tide occurrences

It has been suggested that episodic supplies of Fe through runoff of river water, direct rainfall, and sediment resuspension helps to initiate algal blooms. For example, *Karenia brevis* (formerly *Gymnodinium breve*) blooms in Florida have been correlated with the concentration of Fe and humic substances in rain and river water (Ingle & Martin 1971; Martin & Martin 1973; Kim & Martin 1974; Glover 1978). For several red tide phytoplankton in Osaka Bay or in the Seto Inland Sea, Japan, growths were stimulated by the addition of iron and EDTA (Iwasaki 1979; Takahashi & Fukazawa 1982; Nakamura & Watanabe 1983; Yamochi 1983, 1984). These studies have speculated that Fe input and chelation might be important in controlling bloom initiation.

In recent studies, new discoveries of Fe chelators in seawater have been made due to the development of analytical methods and techniques (Macrellis et al. 2001; Rue & Bruland 2001). During a series of brown tide *Aureococcus anophagefferens* blooms in the Peconic Estuary, NY, the physicochemical speciation of Fe (dissolved, high molecular weight (HMW), low molecular weight (LMW), labile particulate, refractory particulate, organically complexed, and labile dissolved fractions) was measured (Gobler et al. 2002). Decreases in dissolved Fe (organically complexed and HMW fractions) were observed when algal biomass peaked in West Neck Bay. The present study suggested that organic Fe complexes (<0.1 μm) which exist in water at pH 8.0, are available to marine red tide phytoplankton, and that differences in the growths of red tide phytoplankton are due to organic Fe(III) chelators. Moreover, elucidation of the mechanism of iron uptake from organic Fe is presumably an important key to understanding the mechanism of red tide occurrences.

Anthropogenic ligands occur in many natural waters. For example, both EDTA and NTA (nitrilotriacetic acid) were found in a concentration range of 10–100 nM in Swiss rivers (Buffle 1988). In this study, red tide phytoplankton utilized Fe–SA (7 species), Fe–CA (8 species) and Fe–EDTA (all 13 species) as iron sources for their growth. Therefore, reduction of the input of anthropogenic ligands such as EDTA may be an effective way to prevent red tide occurrences. Furthermore, diversity in the uptake mechanisms of macro nutrients such as nitrogen and phosphorus may aid the development and maintenance of harmful phytoplankton blooms in coastal areas (Paerl 1997; Smayda 1997; Fan et al. 2003). The uptake of major nutrients (nitrate, phosphate and silicate) seems to be directly related to Fe availability (Greene et al. 1991; Price et al. 1994; Takeda 1998). Elucidating the biological availability of the organically chelated forms of Fe for red tide phytoplankton will contribute to better understanding of red tide occurrence mechanisms and ultimately of global Fe cycling.

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