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Ability of marine eukaryotic red tide microalgae to utilize insoluble iron

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Abstract

Iron is an essential trace metal and a limiting factor for microalgal growth, but bioavailable dissolved iron concentrations in seawater are low. Microalgal blooms have frequently occurred in coastal areas under such iron limitation accompanied by mass mortalities of fish and bivalves. Their massive growth despite physiological iron-deficiency has long been an enigma, because most of them cannot grow in chemically defined artificial media. We developed a feasible artificial medium for the culture of many species of red tide microalgae modified for investigation of iron utilization. Here, we report on the ability of marine eukaryotic red tide microalgae to utilize insoluble iron. Some microalgal species could utilize particulate $FePO_4$ and FeS for growth. Particulate $FePO_4$ was available for the growth of the raphidophyte *Heterosigma akashiwo*, the dinoflagellate *Heterocapsa triquetra* and the diatom *Ditylum brightwellii*. The dinoflagellates *Heterocapsa circularisquama* and *Karenia mikimotoi*, and the cryptophyte *Rhodomonas ovalis* utilized both particulate $FePO_4$ and particulate FeS for growth. In contrast, particulate FeO(OH) and Fe_2O_3 did not support the growth of any of the red tide microalgae examined. Except for *Chattonella* species (Raphidophyceae), the growth of red tide microalgae were confirmed in the medium with very easily soluble $FeCl_3$ added. The order of bioavailability of tested iron-source species were Fe–EDTA > $FeCl_3$ > $FePO_4$ > FeS > FeO(OH), Fe_2O_3 for most of microalgae examined, although for *H. circularisquama* the utilization of $FeCl_3$ was higher than that of Fe–EDTA. The results suggest that red tide microalgae show different patterns of specific strategies for the utilization of various iron sources. The occurrence of red tides in coastal areas may depend on the combination of microalgal species and insoluble iron species present.

Keywords: Chemically defined artificial medium; Eukaryotic microalgae; Growth; Insoluble iron; Iron uptake mechanism; Red tide

1. Introduction

In marine ecosystems, microalgae play an important role in biogeochemical cycle of elements as the main primary producers (Morel and Price, 2003). The

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growth of microalgae is affected by light, temperature, nutrients (mainly N, P) and trace elements (Maldonado and Price, 1996; Sunda and Huntsman, 1997; Schmidt and Hutchins, 1999). Among trace elements, iron is one of the most essential elements required by microalgae because it is involved in fundamental enzymatic reactions such as oxygen metabolism, electron transfer processes, nitrogen assimilation, and DNA, RNA and chlorophyll synthesis (Weinberg, 1989; Hutchins, 1995). Demand for iron changes with irradiance, growth rate and the relative importance of ammonium and nitrate in marine microalgae (Flynn and Hipkin, 1999). Iron deficiency has been demonstrated to limit the growth of microalgae in high nutrient environments, both in oceanic (Martin and Fitzwater, 1988; Martin et al., 1994; Coale et al., 1996; Boyd et al., 2000) and coastal waters (Hutchins and Bruland, 1998; Hutchins et al., 1998, 2002). In general, the ''bioavailable'' fraction of iron is dissolved iron (colloidal and soluble forms), which is present at extremely low concentrations in seawater (Bruland et al., 1991; Miller and Kester, 1994; Sunda and Huntsman, 1995; Johnson et al., 1997), and thereby marine microalgae are physiologically vulnerable to iron-deficient stress.

Microalgal blooms frequently form red tides in coastal areas (Anderson et al., 1998; Okaichi, 2003). Red tides are often harmful and are a significant and expanding threat to human health and fishery resources throughout the world (Smayda, 1990; Hallegraeff, 1993). Environmental and economic impacts of harmful algal blooms have increased in recent decades. Therefore, urgent elucidation of the mechanism of red tide occurrences is necessary. However, the outbreak mechanisms are poorly understood and little is known about the mechanisms of Fe transport in eukaryotic microalgae; hence, the occurrences of red tide under iron limitation remain an enigma. The relationship between iron uptake mechanisms and red tide occurrences have not been investigated in laboratory culture experiments because of difficulties in achieving growth in chemically defined artificial media. Since the concentration of total iron is rather high in coastal water (Betzer and Pilson, 1970), we evaluated the role of insoluble iron as a potentially important growth factor.

In natural seawater, iron originates from volcanism, hydrothermal activity, weathering and diagenesis, and inputs from rivers and atmospheric deposition (rainwater, etc.). Partially insoluble iron (the solubility at 20 ± 5 °C is lower than 0.1 g for 1 l of H_2O) exists in the form of oxide, oxyhydroxide, phosphate and sulfide (Mill, 1980; Millero et al., 1995; Achterberg et al., 2001). In general, particulate Fe_2O_3 exists in hematite and particulate FeO(OH) is deposited in sediments. Particulate $FePO_4$ and FeS result from the reaction of ferric ion with PO_4^{3-} and H_2S in sediments, respectively (Krom and Berner, 1981; Canfield, 1989; Kostka and Luther, 1994; Rozan et al., 2002). We chose these four naturally existing insoluble iron species and investigated the ability of various axenic marine eukaryotic red tide microalgae including the harmful algal species *Chattonella antiqua*, *Heterosigma akashiwo*, *Heterocapsa circularisquama* and *Karenia mikimotoi*, to utilize these insoluble iron using a newly developed and chemically defined artificial medium (Imai et al., 2004).

2. Materials and methods

2.1. Cultures of microalgae and growth medium

Axenic clonal cultures of 13 microalgal species were used in this study (Table 1). In order to examine the effects of individual iron species on the growth of these species, a chemically defined artificial medium (IHN-medium) (Imai et al., 2004) was modified and used as a basal medium for maintenance cultures. The composition of the modified IHN-medium is shown in Table 2. 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 to 2 mM and from 65 μM to 0.1 mM, respectively. The growth of thirteen species of red tide microalgae in the modified IHN-medium was almost equal to that in the modified SWM-3 medium (Chen et al., 1969; Imai et al., 1996) prepared with natural seawater (data not shown). The artificial medium was freshly prepared and autoclaved (121 °C, 20 min). All reagents used

Table 1
Red tide microalgal species examined

Heterokontophyta	
Raphidophyceae	
Chattonella antiqua (Hada) Ono	
Chattonella marina (Subrahmanyan) Hara et Chihara	
Chattonella ovata Hara et Chihara	
Chattonella verruculosa Hara et Chihara[a]	
Fibrocapsa japonica Toriumi et Takano	
Heterosigma akashiwo (Hada) Hada	
Bacillariophyceae	
Ditylum brightwellii (West) Grunow ex van Heurck	
Dinophyta	
Heterocapsa circularisquama Horiguchi	
Heterocapsa triquetra (Ehrenberg) Stein	
Karenia mikimotoi (Miyake et Kominami ex Oda) Hansen et Moestrup	
Cryptophyta	
Rhodomonas ovalis Nygaard	
Chlorophyta	
Oltmannsiellopsis viridis (Hargraves et Steele) Chihara et Inouye	
Haptophyta	
Cricosphaera roscoffensis (Dangeard) Gayral et Fresnel	

[a] *Chattonella verruculosa* was recently proposed to be transferred to Dictyochophyceae, Heterokontophyta, based on the analyses of 18S rRNA gene sequence and ultrastructure (Honda, personal communication).

were of the highest purity available. Glass-distilled demineralized water (Milli-Q system, Nihon Millipore, Tokyo, Japan) was employed for the preparation of the medium.

2.2. Preparation of the medium for culture experiments

The modified IHN-medium, excluding $Na_2EDTA{\cdot}2H_2O$ and NaFeEDTA, was prepared as the iron-limited artificial medium for culture experiments. The particles of the four almost insoluble iron FeO(OH), $FePO_4{\cdot}4H_2O$, FeS (Aldrich, Milwaukee, WI, USA), α-Fe_2O_3 (99.9%, Wako, Osaka, Japan) species used were powdered with a mortar, and the solutions of almost insoluble iron species and $FeCl_3{\cdot}6H_2O$ (Nacalai tesque, Kyoto, Japan) then added to the iron-limited artificial medium, and their final total iron concentration was adjusted to 2 μM Fe. The preparation of each medium was performed in a ''clean box'' to avoid contamination of metals from the air. Each medium was put into polycarbonate bottles (Nalge Nunc, Rochester, NY, USA) covered with double polymethylpentene wraps and then autoclaved. All equipment except the pre-sterilized instruments were soaked in detergent solution of neutral pH (Scat 20X-N, Dai-Ichi Kogyo Seiyaku, Kyoto, Japan) and then in 4 M HCl, and rinsed with Milli-Q water. Chemical equilibrium of dissolved Fe(III) or Fe(II) in each medium was calculated using version 4.0 of MINEQL + software (Schecher and McAvoy, 1992).

2.3. Culture experiments

Polystyrene tubes (13 mm × 100 mm) with screw caps (Fisher Co., Pittsburgh, USA) were used for culture experiments. Maintenance cultures were transferred to freshly prepared iron-limited artificial medium containing 0.2 μM Fe–EDTA and grown in

Table 2
Composition of the modified IHN-medium

		S_3 Vitamin mix		PI metals	
NaCl	0.43 M	Vitamin B_{12}	0.74 nM	H_3BO_3	1.0 mM
KCl	9.4 mM	Biotin	4.1 nM	$Na_2EDTA{\cdot}2H_2O$	30 μM
$MgSO_4{\cdot}7H_2O$	37 mM	Thiamine HCl	1.5 μM	NaFeEDTA	2.0 μM
$CaCl_2{\cdot}2H_2O$	7.5 mM	Nicotinic acid	0.81 μM	$MnCl_2{\cdot}4H_2O$	35 μM
$NaNO_3$	2 mM	Calcium pantothenate	0.21 μM	$ZnCl_2$	4.0 μM
$NaH_2PO_4{\cdot}2H_2O$	0.1 mM	*p*-Aminobenzoic acid	73 nM	$CoCl_2{\cdot}6H_2O$	0.1 μM
$Na_2SiO_3{\cdot}9H_2O$	0.33 mM	Inositol	28 μM	$CuCl_2{\cdot}6H_2O$	1.0 nM
Na_2SeO_3	2 nM	Folic acid	4.5 nM		
KI	0.47 μM	Thymine	24 μM		
$Na_2MoO_4{\cdot}2H_2O$	0.1 μM				
HEPES	5 mM				

an incubator set at 25 °C (*C. antiqua*, *C. marina*, *C. ovata*, *H. circularisquama*) or at 20 °C (other nine species). All culture strains were axenic. From these precultures, 80 μl taken with an acid-washed micropipet during the late exponential growth phase and inoculated into 4 ml of medium in 8 ml volume polystyrene tubes. The experimental cultures were incubated under fluorescent lighting at 58 ± 13 μmol photons $m^{-2} s^{-1}$ (*K. mikimotoi*) and at 91 ± 15 μmol photons $m^{-2} s^{-1}$ (other twelve species) on a 14 h:10 h light:dark photo-cycle. Growth was determined by measuring in vivo fluorescence of cultures (Brand and Guillard, 1981; Imai et al., 1993) using a Turner Designs 10-AU 005 fluorometer (Sunnyvale, California, USA). Availability of very soluble inorganic iron $FeCl_3$ for the growth of red tide microalgae was also examined. Growth achieved was compared with that for artificial organic iron Fe–EDTA (at a ratio of 1: 16 for Fe:EDTA). The culture experiments were performed at least in quadruplicate.

2.4. Statistical analysis

Iron availability to each red tide microalga was compared statistically by analysis of variance (all data were $P < 0.05$ for Kruskal–Wallis test) followed by Scheffe multiple comparison tests ($P \leq 0.05$ being considered significant).

3. Results

3.1. Raphidophyceae

In the growth experiments of the *Chattonella* species in the artificial medium with Fe–EDTA addition, three species (*C. antiqua*, *C. marina* and *C. ovata*) showed similar behavior (Fig. 1A–C), whereas *C. verruculosa* exhibited rapid growth in comparison with the other *Chattonella* species (Fig. 1D). Their fluorescence intensities (i.e. growth) in the four species of insoluble Fe tested and in the $FeCl_3$ media reached the levels less than 2.3-fold of their initial values. Hence, the four species of the genus *Chattonella* did not utilize insoluble Fe and even $FeCl_3$ for growth (Fig. 1A–D). *Fibrocapsa japonica* did not utilize insoluble Fe species for growth, but grew in $FeCl_3$ medium (Fig. 1E). The growth in $FeCl_3$ medium increased to 129-fold of the initial value. *H. akashiwo* utilized particulate $FePO_4$ as an iron source for growth (Fig. 1F). Growth in $FePO_4$ and $FeCl_3$ media increased to 30- and 491-fold of the initial values, respectively. *H. akashiwo* did not grow in media with particulate FeO(OH), Fe_2O_3 and FeS addition. Growth of *F. japonica* and *H. akashiwo* was slower in $FePO_4$ and/or $FeCl_3$ medium than in Fe–EDTA medium ($P < 0.02$) (Fig. 1E and F).

3.2. Dinophyceae

All three red tide species belonging to the class Dinophyceae could utilize insoluble Fe species for the growth (Fig. 2). *H. circularisquama* utilized both insoluble FeS and $FePO_4$ for growth (Fig. 2A), and reached its population the maximum earlier in the order: FeS, Fe–EDTA > $FeCl_3$ > $FePO_4$. Growth in FeS, $FePO_4$ and $FeCl_3$ media increased to 6.5-, 67- and 269-fold of the initial values, respectively. *H. circularisquama* did not utilize particulate FeO(OH) and Fe_2O_3 for growth. *Heterocapsa triquetra* utilized $FePO_4$, but not insoluble FeO(OH), Fe_2O_3 and FeS for growth (Fig. 2B). *H. triquetra* started to grow and reached the maximum at the same time in $FePO_4$, $FeCl_3$ and Fe–EDTA enriched media. Growth in $FePO_4$ and $FeCl_3$ media increased to 20- and 198-fold of the initial values, respectively. *K. mikimotoi* utilized both FeS and $FePO_4$ as insoluble iron sources for growth (Fig. 2C). *K. mikimotoi* started to grow immediately after inoculation and reached the fluorescence maximum earlier in the following order: FeS, $FePO_4$ » $FeCl_3$ > Fe–EDTA. Growth in FeS, $FePO_4$ and $FeCl_3$ enriched media increased to 2.7-, 5.0- and 10-fold of the initial values, respectively. *K. mikimotoi* did not utilize particulate FeO(OH) and Fe_2O_3 for the growth.

3.3. Bacillariophyceae, Cryptophyceae, Chlorophyceae and Haptophyceae

Ditylum brightwellii (Bacillariophyceae) could utilize particulate $FePO_4$ for growth (Fig. 3A). Its growth in $FePO_4$ and $FeCl_3$ media increased to 39- and 130-fold of the initial values, respectively, and was slow in comparison with that in Fe–EDTA medium ($P < 0.001$) (the length of time reaching the maximum: $FePO_4$ > $FeCl_3$). *D. brightwellii* did not utilize

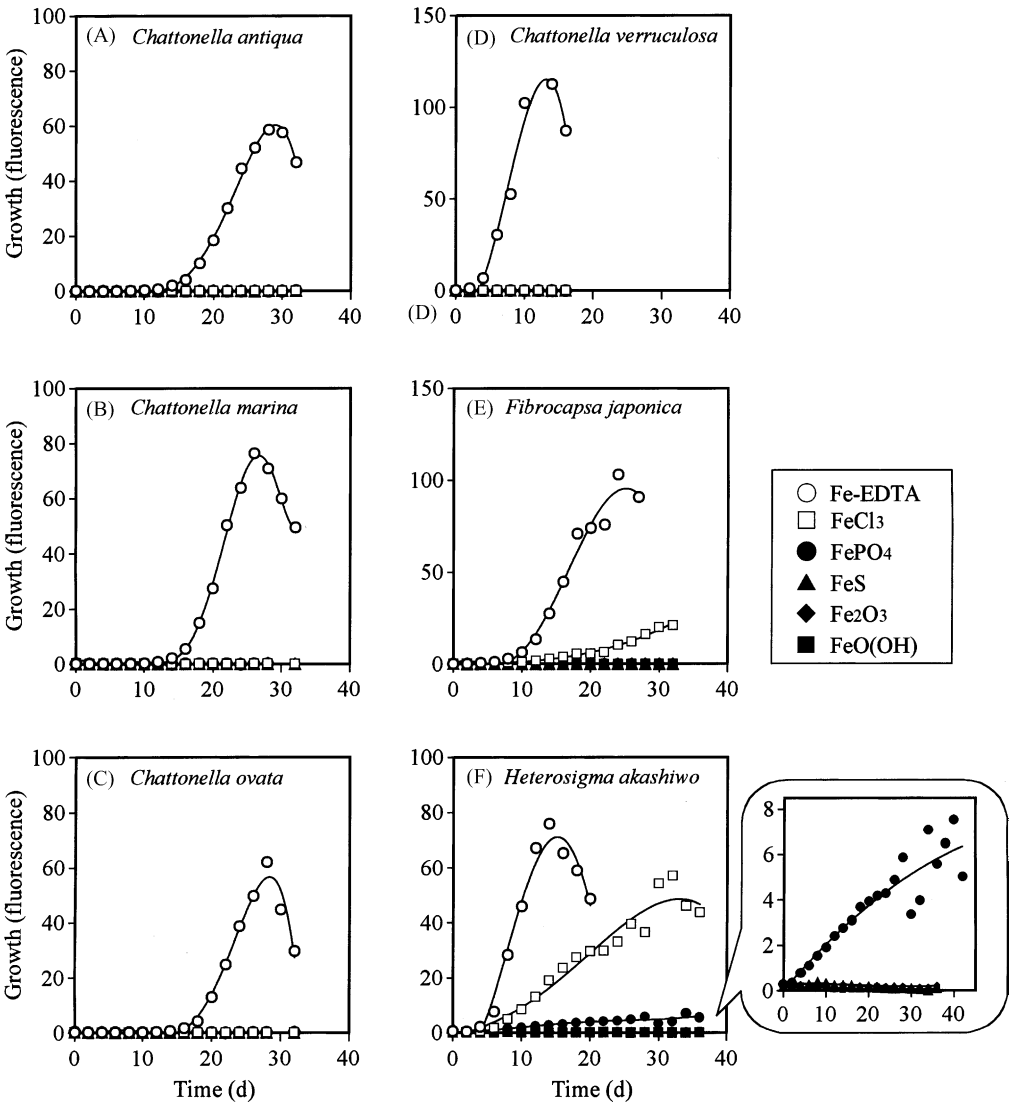

Fig. 1. Raphidophyceae. Growth (changes in fluorescence) in the iron-limited, modified IHN-medium with four insoluble iron species (FeO(OH), α-Fe_2O_3, FeS, $FePO_4{\cdot}4H_2O$), soluble inorganic iron ($FeCl_3{\cdot}6H_2O$), and artificial organic iron (Fe–EDTA, at a ratio of 1:16 for Fe:EDTA) added. Growth curves are for (A) *Chattonella antiqua*, (B) *Chattonella marina*, (C) *Chattonella ovata*, (D) *Chattonella verruculosa*, (E) *Fibrocapsa japonica* and (F) *Heterosigma akashiwo*. Data on fluorescence represent mean of $n = 3$–5 replicates.

particulate FeO(OH), Fe_2O_3 and FeS. *Rhodomonas ovalis* (Cryptophyceae) utilized both FeS and $FePO_4$ as insoluble iron sources for growth (Fig. 3B). Its growth in FeS, $FePO_4$ and $FeCl_3$ media increased to 32-, 179- and 848-fold of the initial values, respectively. *R. ovalis* did not utilize particulate FeO(OH) and Fe_2O_3 for growth. *Oltmannsiellopsis viridis* (Chlorophyceae) and *Cricosphaera roscoffensis* (Haptophyceae) could not utilize the insoluble Fe species for growth, but grew in $FeCl_3$ medium (Fig. 3C and D). Growth of *O. viridis* and *C. roscoffensis* in $FeCl_3$ medium increased to 197- and 55-fold of their initial values, respectively. Growth of *O. viridis* in $FeCl_3$ medium was slow compared with that in Fe–EDTA medium ($P < 0.01$) (Fig. 3C), whereas *C. roscoffensis* started to grow and reached the maximum

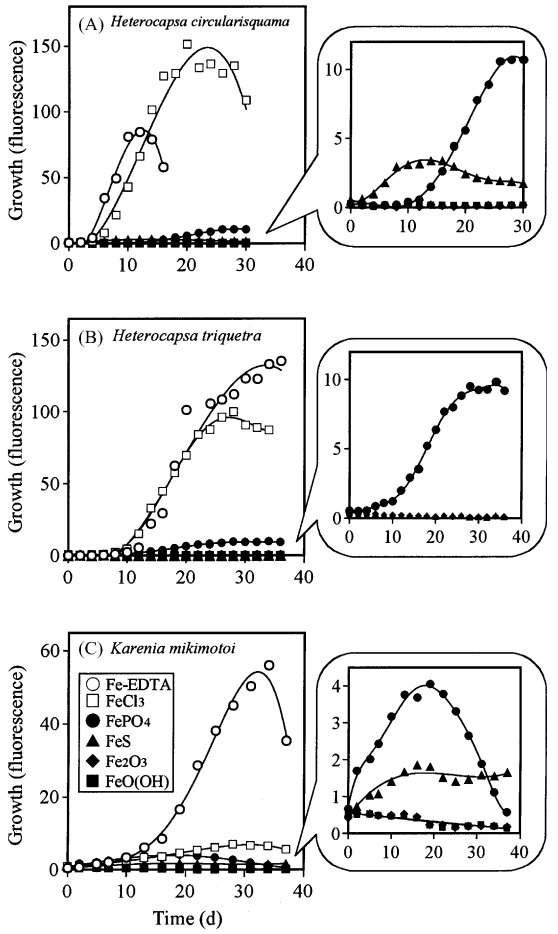

Fig. 2. Dinophyceae. Growth in the iron-limited, modified IHN-medium with FeO(OH), α-Fe_2O_3, FeS, $FePO_4{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$ and Fe–EDTA added. Growth curves are for (A) *Heterocapsa circularisquama*, (B) *Heterocapsa triquetra* and (C) *Karenia mikimotoi*. Data on fluorescence represent mean of $n = 3$–5 replicates.

at almost the same time in $FeCl_3$ and Fe–EDTA media (Fig. 3D).

3.4. Iron availability to red tide microalgae

In order to compare the iron availability among red tide microalgae, their maximal growth yields in FeO(OH), Fe_2O_3, FeS, $FePO_4$, $FeCl_3$ and Fe–EDTA media were summarized in Fig. 4. The 13 species of microalgae could be classified into four groups based on availability of Fe source species. First, inorganic Fe species were unavailable for the growth of the species belonging to the genus *Chattonella* (Fig. 4A–D). Second, soluble inorganic Fe ($FeCl_3$ as iron source) was available for the growth of some microalgae: *F. japonica*, *O. viridis* and *C. roscoffensis* (Fig. 4E–G). The yields with $FeCl_3$ were 21, 55 and 15% of those of Fe–EDTA, respectively. There were significant differences between responses to $FeCl_3$ and Fe–EDTA in *F. japonica* and *C. roscoffensis* ($P < 0.001$) (Fig. 4E and G), but not for *O. viridis* ($P > 0.1$) (Fig. 4F). Third, insoluble $FePO_4$ and soluble $FeCl_3$ were available for the growth of *H. akashiwo*, *H. triquetra* and *D. brightwellii* (Fig. 4H–J). The yields with $FePO_4$ and $FeCl_3$ were 9.9 and 75% of that of Fe–EDTA for *H. akashiwo*; there was a significant difference between $FePO_4$ and $FeCl_3$ ($P < 0.005$), but not between $FeCl_3$ and Fe–EDTA ($P > 0.1$) (Fig. 4H). The yields of $FePO_4$ and $FeCl_3$ were 7.3 and 74% of that of Fe–EDTA for *H. triquetra*; there was a significant difference between $FePO_4$ and $FeCl_3$ ($P < 0.001$), but not between $FeCl_3$ and Fe–EDTA ($P > 0.1$) (Fig. 4I). The yields of $FePO_4$ and $FeCl_3$ were 21 and 84% of that of Fe–EDTA for *D. brightwellii*; there were significant differences between $FePO_4$ and $FeCl_3$ ($P < 0.0001$), between $FeCl_3$ and Fe–EDTA ($P < 0.05$) (Fig. 4J). Fourth, insoluble FeS and $FePO_4$ and soluble $FeCl_3$ were available for the growth of *H. circularisquama*, *K. mikimotoi* and *R. ovalis* (Fig. 4K–M). For *H. circularisquama*, yields of FeS, $FePO_4$ and $FeCl_3$ were 4.0, 13 and 178% of that on Fe–EDTA (Fig. 4K); the order was $FeCl_3 >$ Fe–EDTA $> FePO_4 \geq$ FeS. There were significant differences between $FeCl_3$ and Fe–EDTA ($P < 0.0001$) and between Fe–EDTA and $FePO_4$ ($P < 0.0001$), but not between $FePO_4$ and FeS ($P > 0.1$). In *K. mikimotoi*, yields of FeS, $FePO_4$ and $FeCl_3$ were 3.3, 7.2 and 12% of that of Fe–EDTA (Fig. 4L); the order was Fe–EDTA $> FeCl_3 \geq FePO_4 \geq$ FeS. There was a significant difference between Fe–EDTA and $FeCl_3$ ($P < 0.0005$), but not between $FeCl_3$ and $FePO_4$ ($P > 0.5$) and between $FePO_4$ and FeS ($P > 0.5$). In *R. ovalis*, yields of FeS, $FePO_4$ and $FeCl_3$ were 12, 26 and 77% of that of Fe–EDTA (Fig. 4M), and the order was Fe–EDTA $\geq FeCl_3 > FePO_4 \geq$ FeS. There was a significant difference between $FeCl_3$ and $FePO_4$ ($P < 0.05$), but not between Fe–EDTA and $FeCl_3$ ($P > 0.1$) or between $FePO_4$ and FeS ($P > 0.5$). In the species which utilized insoluble Fe for growth (Fig. 4H–M), there was not a significant difference between FeS and Fe_2O_3 ($P > 0.5$), $FePO_4$ and Fe_2O_3

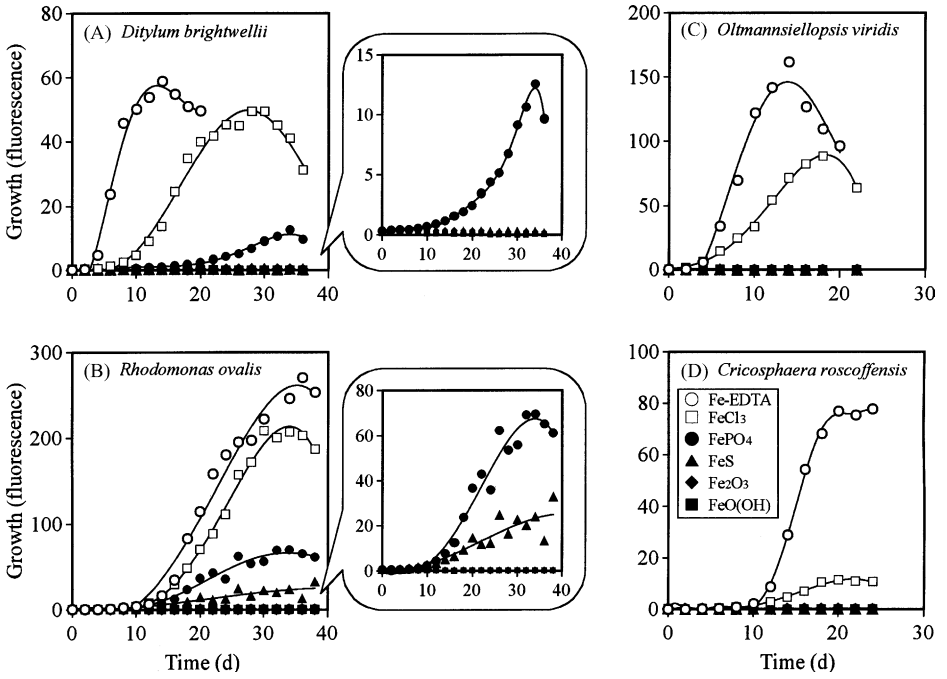

Fig. 3. Bacillariophyceae, Cryptophyceae, Chlorophyceae and Haptophyceae. Growth in the iron-limited, modified IHN-medium with FeO(OH), α-Fe_2O_3, FeS, $FePO_4{\cdot}4H_2O$, $FeCl_3{\cdot}6H_2O$ and Fe–EDTA added. Growth curves are for (A) *Ditylum brightwellii*, (B) *Rhodomonas ovalis*, (C) *Oltmannsiellopsis viridis* and (D) *Cricosphaera roscoffensis*. Data on fluorescence represent mean of $n = 3$–5 replicates.

($P > 0.2$, but $P < 0.01$ for *D. brightwellii*), FeS and FeO(OH) ($P > 0.5$), or between $FePO_4$ and FeO(OH) ($P > 0.2$, but $P < 0.01$ for *D. brightwellii*).

4. Discussion

Flagellates can potentially access nutrients in the water column through their motility and nutrient-retrieval migration (Smayda, 1997). The raphidophyte *H. akashiwo* (Figs. 1F and 4H) has a behavior of diurnal vertical migration by swimming and can reach the sea bottom at night in shallow coastal areas such as Tanigawa Fishing Port (about 3 m depth) and Sano Harbor (about 8 m depth) (Yamochi and Abe, 1984). The dinoflagellate *K. mikimotoi* (Figs. 2C and 4L) also shows a marked diurnal vertical migration behavior, reaching a depth of 20 m or deeper at night (Yamaguchi, 1994; Koizumi et al., 1996). The characteristics of these species would suggest that *H. akashiwo* and *K. mikimotoi* have an ability to utilize particulate $FePO_4$ and/or FeS found at the surface of sea bottom. A strategy of vertical migration by a group of non-motile, oceanic diatoms by buoyancy changes as a means to access new nitrogen in deep waters has been documented (Villareal and Lipschultz, 1995; Villareal et al., 1999). The present results suggest the possibility that some marine eukaryotic red tide microalgae have a strategy of vertical migration to allow uptake of the micro nutrient Fe, as well as macro nutrients such as N and P.

The dinoflagellate *H. circularisquama* (Figs. 2A and 4K) has tended to form red tides after mixing events in the sea (Matsuyama et al., 1995). Therefore, *H. circularisquama* might utilize particulate $FePO_4$ and FeS supplied from the benthic boundary layer after the mixing events such as storms. The cryptophyte *R. ovalis* (Figs. 3B and 4M) was confirmed to produce a siderophore, Fe(III)-binding organic ligand, to uptake Fe under dissolved-iron limitation (Naito et al., 2001). *R. ovalis* presumably has a siderophore-mediated mechanism to utilize the insoluble Fe species.

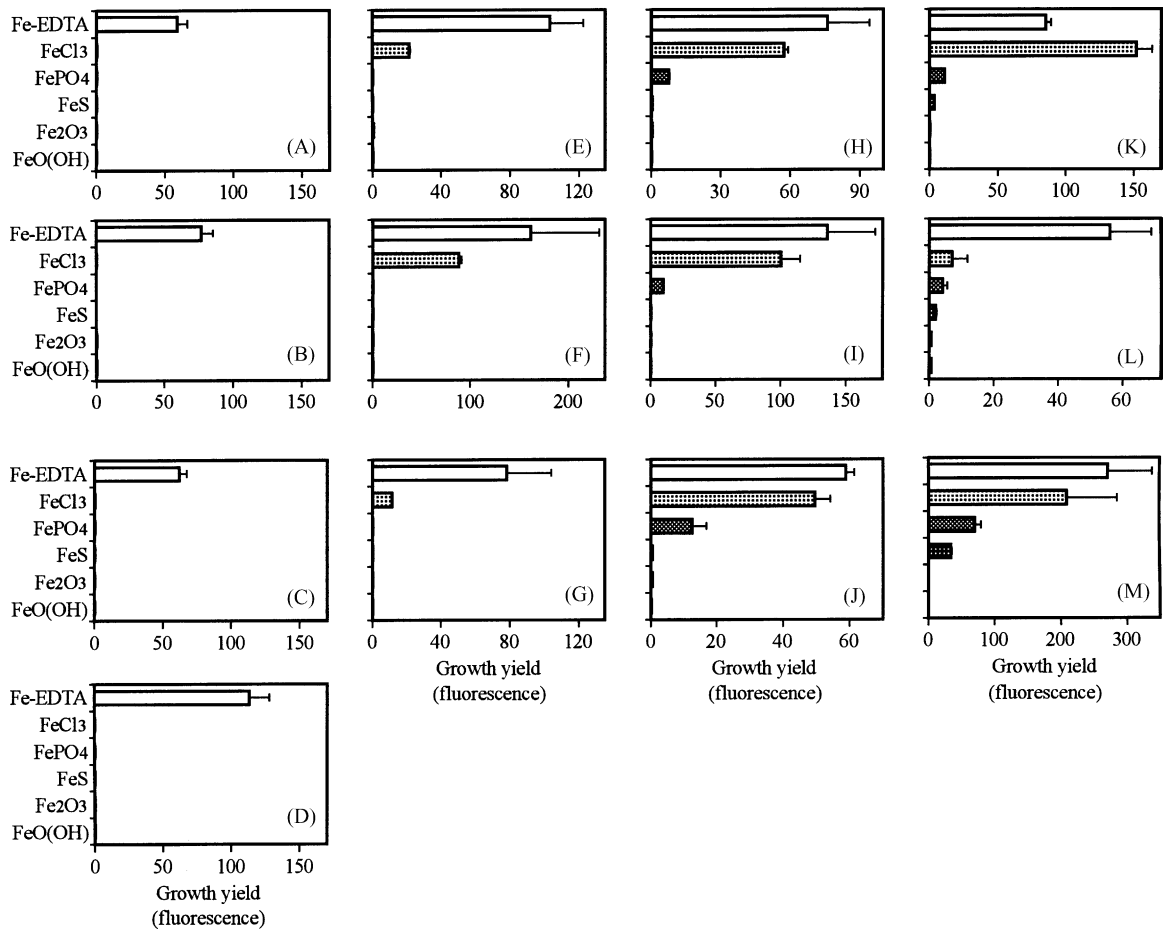

Fig. 4. Comparison of the ability to utilize insoluble and soluble iron species by red tide microalgae. Growth yields are shown for (A) *Chattonella antiqua*, (B) *Chattonella marina*, (C) *Chattonella ovata*, (D) *Chattonella verruculosa*, (E) *Fibrocapsa japonica*, (F) *Oltmannsiellopsis viridis*, (G) *Cricosphaera roscoffensis*, (H) *Heterosigma akashiwo*, (I) *Heterocapsa triquetra*, (J) *Ditylum brightwellii*, (K) *Heterocapsa circularisquama*, (L) *Karenia mikimotoi* and (M) *Rhodomonas ovalis*. Error bars represent standard deviations.

Experimental studies of iron availability for diatoms have a long tradition. Some previous works demonstrated that particles or colloids of $FePO_4$, FeO(OH) and Fe_2O_3 can be utilized for the growth of several diatoms (Harvey, 1937; Goldberg, 1952; Davies, 1967). However, β-FeO(OH) colloids do not support a diatom *Thalassiosira weissflogii* growth (Rich and Morel, 1990). In this study, the diatom *D. brightwellii* could utilize particulate $FePO_4$ but particulate FeO(OH) and Fe_2O_3 were unavailable for the growth (Figs. 3A and 4J). Large species like *D. brightwellii* probably tend to adsorb particles of $FePO_4$ on the cell surface.

The yields with particulate $FePO_4$ and FeS for red tide microalgal growth were not so high (<27% of that of Fe–EDTA) as compared with those with dissolved inorganic and organic Fe species (Fig. 4H–M). Here, we take into account the effects of trace quantities of dissolved Fe from particulate Fe species on growth. A calculation using MINEQL + software indicates that dissolved Fe(III) species from dissolution of particulate $FePO_4$ exist as $Fe(OH)_2^+$ (50%), $Fe(OH)_3$ (26%) and $Fe(OH)_4^-$ (24%) in $FePO_4$ medium. Dissolved Fe(II) species by dissolution of particulate FeS exist as Fe^{2+} (47%), $FeCl^+$ (42–43%) and $FeSO_4$ (9.4–10%) in FeS medium under anoxic conditions, but can be

converted immediately into Fe(III) species due to the rapid chemical oxidation of Fe(II) under aerobic conditions. These dissolved iron species ($\leq$8.1 pM) presumably have some positive effects on the growth of red tide microalgae. However, sufficient time and reaction would be needed for the dissolution of Fe to reach a concentration level supportive of the growth observed in each medium with $FePO_4$ and FeS added.

Particulate FeO(OH) and Fe_2O_3 were unavailable for the growth of all red tide microalgae examined in this study (Fig. 4). It has been reported that iron oxyhydroxide particles and iron colloids are not directly available to some microalgae (Wells et al., 1983; Rich and Morel, 1990; Kuma and Matsunaga, 1995). These results (Fig. 4) might imply that particles and colloids of iron oxide and oxyhydroxide must be solubilized thermally or photochemically (Rich and Morel, 1990), or by phagotrophy (Barbeau and Moffett, 2000; Nodwell and Price, 2001) to be taken up to support the growth of red tide microalgae.

In a comparison between soluble inorganic and organic Fe, the growth of red tide microalgae in $FeCl_3$ medium was almost equal to or lower than that in Fe–EDTA medium (Figs. 1E and F, 2 and 3). These results are considered to depend on the difference of the transformation into dissolved Fe species in each medium ($Fe(OH)_2^+$ (50%), $Fe(OH)_3$ (26%) and $Fe(OH)_4^-$ (24%) in $FeCl_3$ medium, whereas $Fe(OH)EDTA^{2-}$ (95%) and $FeEDTA^-$ (4.2–4.3%) in Fe–EDTA medium). In both insoluble and soluble inorganic Fe media, the speciation of dissolved Fe(III) hydroxides must also play an important role in the growth of microalgae.

Considering the overall results obtained in this study, we present the hypothesis that the insoluble Fe in the bottom water and/or at the surface of bottom sediment plays a significant role in the massive growth of microalgae as well as macro nutrients such as N and P following the scenario of the role of iron in the occurrences of red tides in coastal areas presented in Fig. 5. H_2S in sediments reacts with $FePO_4$, and FeS is then formed under anaerobic conditions (Krom and Berner, 1981; Canfield, 1989; Kostka and Luther, 1994; Rozan et al., 2002). Particulate $FePO_4$ and FeS liberated from the sea bottom as suspended Fe can become bioavailable by dissolution (Davison, 1985; Ishio et al., 1986) or by some mechanism on cell surface of some red tide microalgae. Red tide species possessing this ability to utilize particulate $FePO_4$ and/or FeS are found in the species belonging to Dinophyceae, Bacillariophyceae, Cryptophyceae and Raphidophyceae (*H. akashiwo*) (Fig. 4H–M). Species of Chlorophyceae, Haptophyceae and Raphidophyceae (*F. japonica*) have the ability to utilize of soluble inorganic Fe for the growth (Fig. 4E–G). There are several reports on the production of siderophore by eukaryotic microalgae under iron limitation (Trick et al., 1983; Benderliev and Ivanova, 1994; Naito

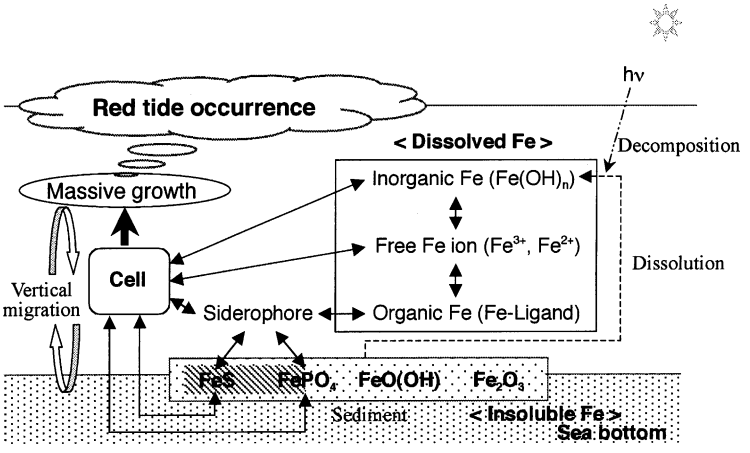

Fig. 5. Schematic diagram for iron uptake by marine eukaryotic red tide microalgae. Insoluble iron species and dissolved iron species are enclosed by a dashed line and a solid line, respectively.

et al., 2001, 2004a,b), and hence the species of the genus *Chattonella* are thought to produce organic ligands such as siderophores to uptake organic Fe in seawater (Fig. 4A–D). Additionally, *C. antiqua* has a glycocalyx layer composed of polysaccharides on the cell surface (Yokote and Honjo, 1985), and Okaichi et al. (1989) suggested that colloidal and particulate Fe would be absorbed to the layer where it can be solubilized by the cell. Therefore, the genus *Chattonella* may have a strategy via the absorption of colloidal Fe to the surface layer.

Increased Fe concentrations associated with river runoff preceded a dinoflagellate bloom in Maine coastal waters (Glover, 1978), and addition of soluble Fe (as Fe–EDTA) stimulated growth of red tide flagellates in bioassay experiments (Iwasaki, 1973; Yamochi, 1984; Nakamura, 1990; Hosaka, 1992). Thus, the role of Fe in red tide outbreaks is considered to be potentially important. Nakamura (1990) suggested that the levels of soluble Fe in the Seto Inland Sea be insufficient to support the maximum growth rate of *C. antiqua* except in bloom years and Fe availability might be different in non-bloom years. It is becoming increasingly clear that natural colloidal Fe (Nishioka and Takeda, 2000; Chen et al., 2003) and organic Fe (Kuma et al., 1999) are available to marine diatoms. Our finding that many species of marine eukaryotic red tide microalgae utilize insoluble Fe expounds this insight. An important, question unresolved is the mechanism that liberates Fe from insoluble Fe compounds for biological use.

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