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PHOTOSYNTHESIS REGULATION OF PHYTOPLANKTON BY ORGANIC COMPLEXATION OF IRON

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Abstract

Biological carbon fixation is considered an efficient and inexpensive method for mitigating rises in atmospheric carbon dioxide. Fertilizing the oceans with iron may help to reduce carbon dioxide in the atmosphere since it stimulate phytoplankton growth in the oceans. In this study, we demonstrate the effect of iron complexation by synthetic chelators on the growth of phytoplankton. Under low-iron conditions, the growth rates were affected by the concentrations of synthetic chelators in the culture media. On the basis of the data obtained, we discuss equilibrium distribution of iron controlling the phytoplankton growth in the cultures.

KEYWORDS: *global warming, iron, fixation of carbon dioxide, phytoplankton*

1. Introduction

Atmospheric concentration of carbon dioxide has increased as a result of human activities such as burning of fossil fuels, land-use change and agriculture (IPCC, 2001). Since fossil fuels are used as a major source of energy, it is difficult to control the overall levels of atmospheric carbon dioxide only by reducing the anthropogenic emissions. The effects of fixation on land are also limited because carbon is circulated in the biosphere within a relatively short time scale. In order to maintain the atmospheric carbon dioxide at the levels which will have no greenhouse effect in future, the final solution would be the sequestration of much of carbon exhausted from human activities out of the biospheric carbon cycle.

Biological fixation of carbon dioxide in the oceans is expected to provide a useful means of carbon sequestration. The oceans are not only a major source but also a sink for atmospheric carbon dioxide, the two processes being balanced in the global ocean cycle. Carbon in the surface layer of the oceans, which is mainly dissolved as carbon dioxide, is fixed into organic matter by phytoplankton through photosynthesis, and a part of the organic matter sinks toward the seabed. Martin and colleagues hypothesized that artificial iron fertilization can control the biomass and

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productivity of phytoplankton in the major nutrient-rich iron-limited regions of the world's oceans (Martin and Gordon, 1988; Martin *et al.*, 1990). More photosynthetic production being exported from the ocean surface would result in more carbon being retained in the oceans and less returned to the atmosphere. This process has the potential to reduce the atmospheric concentration of carbon dioxide and to mitigate the greenhouse effect. The validity of iron limitation was demonstrated by a series of iron fertilization experiments in the surface waters of the equatorial Pacific, subarctic Pacific and Southern Ocean (Martin *et al.*, 1994; Coale *et al.*, 1996; Watson *et al.*, 2000).

Although a great deal of effort has been made on iron limitation (Anderson and Morel, 1982; Brand *et al.*, 1983; Sunda *et al.*, 1991; de Baar *et al.*, 1995; Hutchins, 1995; Wells *et al.*, 1995; Sunda and Huntsman, 1995, 1997), what remains to be resolved is to seek more suitable species of iron for supplying it to phytoplankton. In laboratory cultures, an artificial chelator, ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), was commonly used in order to obtain a constant and controlled supply of iron (Harrison *et al.*, 1980; Muggli and Harrison, 1996; Gerringa *et al.*, 2000). In this paper, we present data on the effects of synthetic chelators, EDTA analogues (Figure 1) on growth of phytoplankton in artificial seawater media, in which we have varied compositions of dissolved iron species. We also discuss the chemical process that affect the phytoplankton growth in the cultures by modeling the speciation of iron at thermodynamic equilibrium.

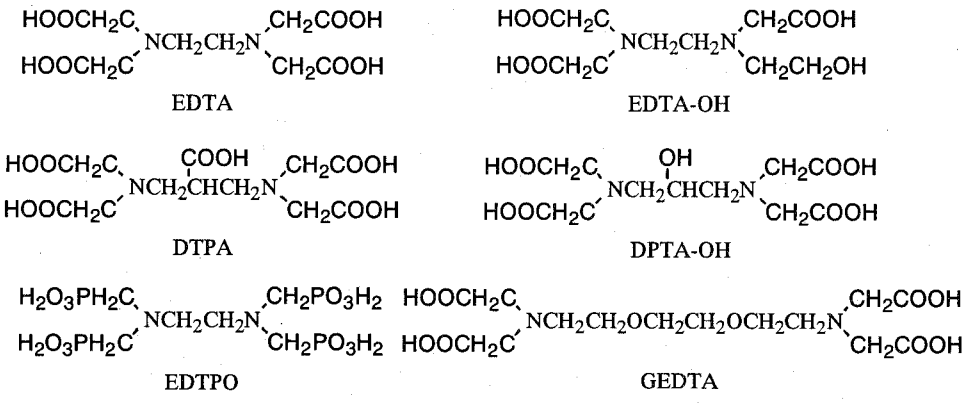

Figure 1. Chemical forms of EDTA analogues.

2. Methodology

2.1. Reagents

Stock solutions (10^{-2} M) of chelating ligands were prepared by dissolving the corresponding compounds (Dojindo Molecular Technologies; ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA), 1,3-diamino-2-hydroxypropane-N,N,N',N'-tetraacetic acid (DPTA-OH), diethylenetriamine-N,N,N',N'',N''-pentaacetic acid (DTPA), N-2-hydroxyethly-ethylenediamine-N,N',N'-triacetic acid (EDTA-OH), ethylenediamine-N,N,N',N'-tetrakismethylenephosphonic acid (EDTPO), O,O'-bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (GEDTA) in 0.1 M sodium hydroxide. A stock

solution of Fe(III) was prepared by dissolving $FeCl_3 \cdot 6H_2O$ (Nacalai Tesque) in 1M HCl and standardized by using inductively coupled plasma atomic emission spectrometry (Japan Jarrel Ash, ICAP-500). They were diluted to the desired concentrations and sterilized by autoclaving. Artificial seawater was prepared according to Fleming and deionized water using a MiliQ-II system (Millipore) was used throughout. Other reagents were of analytical reagent grade or better.

2.2. Culture experiments

An axenic culture of *Cricosphaera roscoffensis* NIES-8 (Haptophyceae) was used. *C. roscoffensis*, classified as coastal red-tide species, was selected for the fast growth rate and the high iron requirement. Experimental cultures were grown at 20 ºC under a 12:12 h L/D photoperiod at a light intensity of 50 μmol m^{-2} s^{-1} provided by cool white fluorescent lights. Before the experiments, the algal cultures were maintained in modified ASP7 media (Table 1) reducing the concentrations of iron (Fe(III); 0.5 μM) and omitting chelating ligands, until cells were at an exponential phase of growth. For culture experiments, all procedures were performed under a clean bench (class 100), and stock solutions were filter sterilized by passing through 0.1-μm filters (Millipore, HA). Twenty-milliliter aliquots of modified ASP7 medium were pipetted into 30-ml capacity acid-washed polycarbonate bottles. After sterilization by autoclaving, 100 μl of stock solutions of chelating ligands and Fe(III) were added. The media were maintained at 20 ºC for 48 hours and inoculated with acclimated exponential phase cells of phytoplankton which resulted in 20 cells mL^{-1}. Cultures were grown for a period of 2-4 weeks.

Phytoplankton growth was followed by measuring spectrophotometrically on a UV-VIS spectrophotometer at 540 nm, and correlated with an established cell density-to-absorbance ratio to estimate cell number. Cell number was counted directly by microscope. The axenic nature was verified frequently by DAPI direct straining and examination under an epifluorescent microscope (Bohlool and Schmidt, 1980).

Table 1. Modified ASP7 medium

NaCl	2.5 g	H_3BO_3	3.39 mg
$MgSO_4 \cdot 7H_2O$	900 mg	$CoSO_4 \cdot 7H_2O$	2.79 μg
KCl	70 mg	$ZnSO_4 \cdot 7H_2O$	140 μg
$CaCl_2 \cdot 2H_2O$	30 mg	$MnCl_2 \cdot 4H_2O$	96 μg
$NaNO_3$	5 mg	$FeCl_3 \cdot 6H_2O$	1.5 or 6.0 μM
$NaH_2PO_4 \cdot 2H_2O$	2 mg	EDTA analogues	1.5-150 μM
$Na_2SiO_3 \cdot 9H_2O$	1 mg		
$VitaminB_{12}$	0.1 μg		
Thiamine HCl	50 μg		
Biotin	0.1 μg		
HEPES	2.38 g		
Distilled water	100 ml		
PH 8			

3. Results and Discussion

3.1. Effects of synthetic chelators on the growth of *C. roscoffensis*

Figure 2 shows growth curves of *C. roscoffensis* in modified ASP7 media containing 1.5 μM of Fe(III) and 1.5-150 μM of EDTA analogues. The growth rates of *C. roscoffensis* were divided into two types, which depended on the contents of the chelating ligands in the media. When the growth media contained less than 1.5 μM of the chelating ligands, the growth rates were almost equal to the rate, 0.38±0.05 d^{-1}, of a growth control in modified ASP7 medium without any chelating ligands. With increasing concentrations of the chelating ligands, the growth rates changed to a depressed type with 0.05±0.02 d^{-1}. The depressed effects of the chelating ligands were observed as follows:

GEDTA, EDTA < EDTA-OH < DTPA < DPTA-OH < EDTPO ····· (1)

In Figure 2, 1.5 μM of EDTPO suppressed the cell growth of *C. roscoffensis*. DPTA-OH, DTPA and EDTA-OH reduced the growth rates when the medium contained more than 7.5, 15 and 150 μM, respectively. On the other hand, no suppression was found in the range of 1.5-150 μM of EDTA and GEDTA.

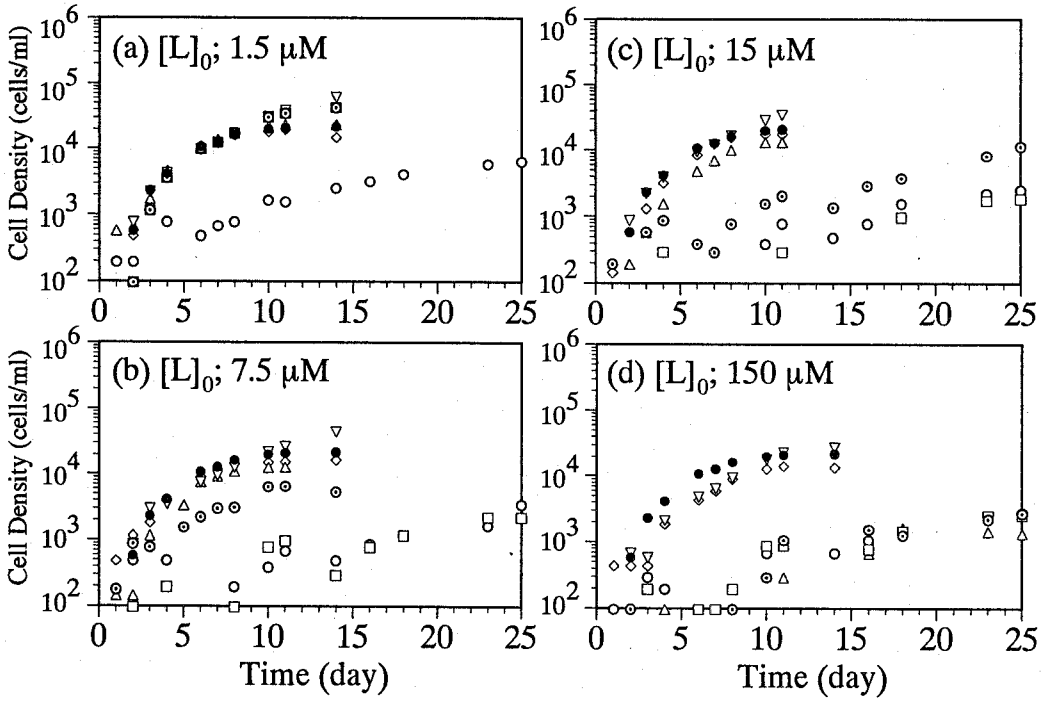

Figure 2. Effects of synthetic chelators on the growth of *C. roscoffensis* in modified ASP7 media containing 1.5 μM of Fe(III). GEDTA; ▽, EDTA; ◇, EDTA-OH; △, DTPA; ⊙, DPTA-OH; □, EDTPO; O, Control; ●.

Among the dissolved components in the culture medium, the iron concentration is related to the variation of the growth curves. So far as the higher growth type is concerned, the cultures of *C. roscoffensis* entered the stationary phase of growth after 6-8 days incubation, by which concentrations of iron decreased less than 100 nM. The decrements of phosphate, nitrate, silica, cobalt, zinc and manganese were within 10% of the initial concentrations throughout the incubation of both growth types. It is suggested that the growth of *C. roscoffensis* was followed by decreases in iron during the exponential phase and that was limited by iron concentration during the stationary phase. In addition, the effect of EDTA analogues on the growth of *C. roscoffensis* also changed with iron concentration in the medium. In the modified ASP7 media containing 6.0 μM of iron, little difference in the growth rates was observed in the range of 1.5-150 μM of the chelating ligands, except the highest cell density at the stationary phase slightly decreased in the order of Eq. (1). The cell growth was not limited by iron in the media containing 6.0 μM of iron since the concentrations of Fe(III) were 3.8-4.9 μM during the stationary phase. Therefore, the chelating ligands would depress the growth of phytoplankton significantly under iron-deficient conditions. Similar results were obtained from other phytoplankton cultures, *e.g.*, *Rhodomonas ovalis*, *Heterosigma akashiwo*, *Oltmannsiellopsis viridis*, *Pleurochrysis carterae* and *Skeletonema costatum* (not shown).

3.2. Iron availability to marine phytoplankton in the medium containing chelating ligands

Effects of chelating ligands and iron speciation on the growth of phytoplankton are examined in the thermodynamic model presented in Figure 3. In modified ASP7 media, dissolved iron is comprised of chelating complexes, FeL, free ferric ion, Fe^{3+}, and inorganic iron complexes, Fe(III)'.

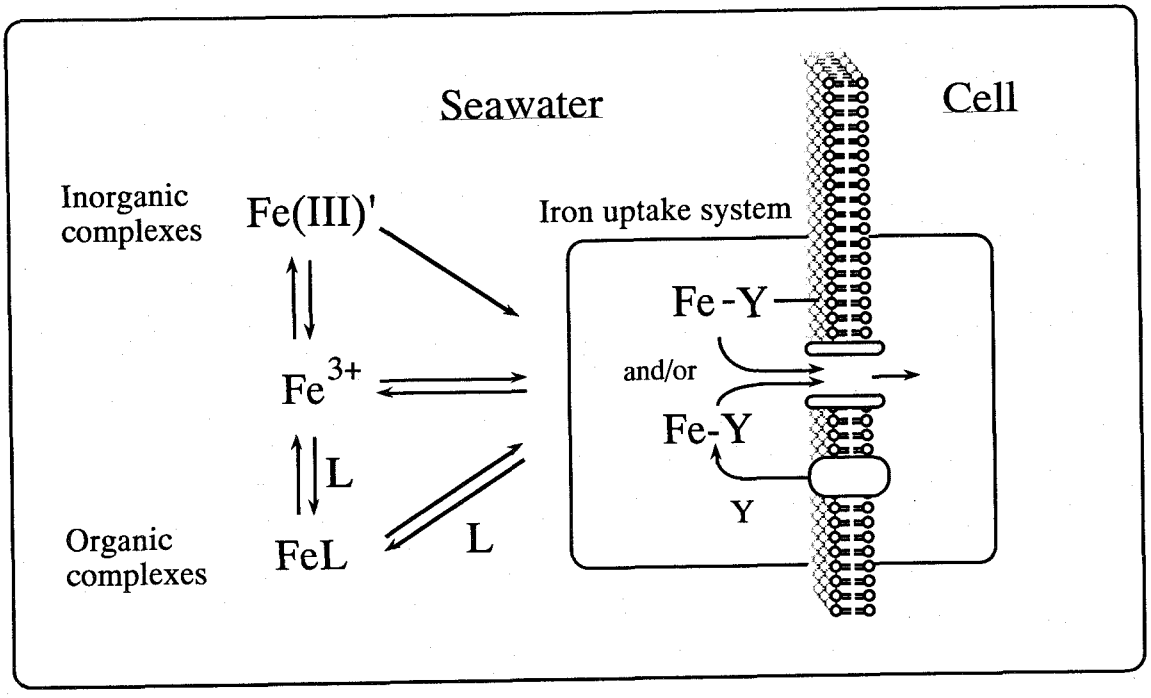

Figure 3. Schematic representation of iron acquisition by phytoplankton in the medium.

The major divalent cations in seawater, Ca^{2+} and Mg^{2+}, play a dominant role on the speciation of the chelating ligands. For iron transport system by phytoplankton, an iron transporter, Y, is defined for simplification. Many studies have been made on the two common strategies for iron acquisitions, biologically mediated reduction of Fe(III) organic complexes by reductases located on the cell surface followed by uptake of iron (Anderson and Morel, 1982; Hudson and Morel, 1990) and excretion of iron transporters such as siderophores that form stable complexes with iron and are subsequently taken up (Raymond et al., 1984; Trick et al., 1983).

We consider the following stability constants with Fe^{3+}, the major divalent cations, M (M=Ca^{2+}, Mg^{2+}) and the chelating ligands, L:

$$K_{FeL} = \frac{[FeL]}{[Fe][L]} \quad \cdots\cdots \quad (2) \qquad K_{FeY} \quad \frac{[FeY]}{[Fe][Y]} \quad \cdots\cdots \quad (3) \qquad K_{ML} = \frac{[ML]}{[M][L]} \quad \cdots\cdots \quad (4)$$

Equation (2)-(4) can be transformed into the following equation:

$$\frac{}{[Y]} \quad \frac{K_{FeY} \cdot K_{ML}}{} \cdot \frac{[FeL][M]}{} \quad \cdots\cdots \quad (5)$$

, where [FeY]/[Y] is considered as an indication of iron availability to phytoplankton. In the medium containing EDTA analogues, iron is mostly present as FeL, and Fe(III)' and Fe^{3+} are negligible. Since Ca^{2+} and Mg^{2+} are in large excess of the chelating ligands, total concentrations of the chelating ligands are approximately equivalent to [ML]. Then, the following equation is obtained from (5):

$$\frac{[FeY]}{[Y]} = K_{FeY}[M] \cdot \frac{[Fe]_t}{[L]_t} \cdot \frac{K_{ML}}{K_{FeL}} \quad \cdots\cdots \quad (6)$$

, which $[Fe]_t$ and $[L]_t$ are total concentrations of iron and the chelating ligands, respectively.

Equation (6) shows that the availability of iron to marine phytoplankton is related to compositions of the culture media ($[Fe]_t/[L]_t$) and chemical properties of the chelating ligands (K_{ML}/K_{FeL}). The factor, $[Fe]_t/[L]_t$, is concentration ratios of iron to chelating ligands in the culture media. This term explains the suppression of the growth rates which occurred as the chelating ligands increased in the culture experiments of Figure 2. The factor, K_{ML}/K_{FeL}, represents effects of stability constants for the chelating ligands. Table 2 shows stability constants for formation of FeL and ML.

Table 2. Stability constants for formation of complexes from EDTA analogues and metals

Chelating ligands	log K_{FeL}	log K_{CaL}[a]	log K_{MgL}[a]
DTPA	28.6	10.74	9.3
EDTA	25.1	10.96	8.69
GEDTA	20.5	11.0	5.21
EDTA-OH	19.8	8.14	7.0
EDTPO	19.6	6.93	5.69
DPTA-OH	-	6.69	5.3

[a] $[Ca^{2+}]$ =10 mM, $[Mg^{2+}]$ =53 mM in modified ASP7 medium.

The high concentrations of Ca^{2+} and Mg^{2+} in seawater affect the complexing abilities of the chelating ligands toward iron, although the stability constants for Ca^{2+} and Mg^{2+} are considerably lower than that for iron. The effect of Ca^{2+} on the complexing ability of the chelating ligand with iron is higher than that of Mg^{2+} because of the stabilities of ML complexes. Stability constants of GEDTA, EDTA and DTPA for Ca^{2+} are of the same order of magnitude. In Figure 2, the cell growth of *C. roscoffensis* was depressed by DTPA which has the highest stability constant for iron. Formation of a more stable complex with iron would reduce the iron availability in the medium. In the presence of GEDTA, EDTA-OH and EDTPO, of which stability constants for iron are in the range of $10^{19.6}$-$10^{20.5}$, the depressed effects of the chelating ligands are in order of their stability constants for Ca^{2+}. It is considered that a decrease in the interactions between the chelating ligands and Ca^{2+} caused an increase in the stability of FeL and a decrease in the availability of iron to phytoplankton in the medium. These results are consistent with Eq. (6).

4. Conclusions

The cell growth of marine phytoplankton depended on the composition of iron species in the culture media containing EDTA analogues. Especially, the chelating ligands affected the growth rates at the exponential phase under iron-deficient conditions. Examination of iron speciation and chemical properties of the chelating ligands using the equilibrium thermodynamic model suggests that the iron availability to marine phytoplankton is related to the ratios of iron to chelating ligands in the media and the abilities of the chelating ligands to complex calcium, magnesium and ferric ions.

Over the last few decades, iron availability to phytoplankton has been the subject of controversy. Anderson and Morel (1982) reported that the iron uptake rate is a function of the activity of free ferric iron in seawater. It is seemingly suggested that free ferric iron is utilized as a sole iron source. However, inorganic and organic iron complexes may also be taken up by way of exchange reactions with iron transporters. In order to evaluate the effect of synthetic chelators on phytoplankton growth exactly, iron availability should be determined not only by the activity of free ferric iron, but also by the equilibrium governing the distribution of iron species in the medium. From that point of view, we determined the iron availability by modeling the thermodynamic equilibrium in the culture experiments. On the other hand, little is known about the characteristics of natural organic chelators in the oceans, although dissolved iron is predicted to occur >99% complexed by strong organic ligands (van den Berg, 1995; Rue and Bruland, 1995). Rrecent studies have shown that a significant fraction of the natural organic chelators contain iron-binding functional groups consistent with biologically produced siderophores (Macrellis *et al.*, 2001). Siderophores facilitate iron uptake by the siderophore producer but limit access to iron by other organisms (Raymond et al., 1984). Our finding provides theoretical approaches for elucidation of the latter mechanism, while the former is due to the specific interaction of siderophores and receptor proteins at the cell surfaces. Hutchins et al. (1999) found that prokaryotic phytoplankton are able to utilize bacterially produced siderophores for promotion of iron acquisition. Some species of phytoplankton may be selected for in evolution because of the ability to outcomplete other species in low-iron regions of the oceans. Utilization of organic iron complexes for iron fertilization may lead to a method for regulating the phytoplankton growth in the oceans efficiently. Further work is needed to elucidate the iron transport system of phytoplankton.

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