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Iron requirements of *Heterosigma akashiwo* (Raphidophyceae), *Heterocapsa circularisquama* (Dinophyceae) and two common centric diatoms

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Abstract

Iron is increasingly recognized to play a key role in the growth of microalgae in various marine systems, but there is a paucity of knowledge about the physiological iron requirements of harmful algal species. This study aimed to elucidate the half-saturation constant for growth (K_s) and the maximum growth rate (μ_{max}) of four phytoplankton species with regard to iron concentrations. The four species were the fish-killing raphidophyte *Heterosigma akashiwo*, the bivalve-killing dinoflagellate *Heterocapsa circularisquama*, and the centric diatoms *Ditylum brightwellii* (large sized) and *Chaetoceros didymus* (small sized). We conducted incubation experiments with axenic cultures of the four species using a chemically defined synthetic medium. Compared with *Heterocapsa circularisquama* (0.59 day^{-1}) and *Heterosigma akashiwo* (0.50 day^{-1}), the two diatom species exhibited high μ_{max} values (0.68 day^{-1}). The smaller diatom had the lowest K_s value of 7.5×10^{-9} M, while the larger diatom had the highest K_s value of 2.1×10^{-7} M. The K_s values of *Heterosigma akashiwo* and *Heterocapsa circularisquama* were 5.1×10^{-8} and 3.1×10^{-8} M, respectively. Smaller-sized phytoplankton showed better affinity to lower concentrations of iron in the sea.

Key words : artificial synthetic medium, half-saturation constant, culture experiment, iron requirement, maximum growth rate

Introduction

Harmful algal blooms (HABs) have shown trends of increasing frequency and scale, becoming a major problem in coastal areas of the world, even in the Arctic zones (Walsh et al., 2006 ; Natsuike et al., 2013 ; Natsuike and Imai, 2016). It has been reported that the occurrence of HABs is influenced by a combination of various factors in a given region, including physical, chemical, and biological conditions (Gowen et al., 2012 ; Imai and Yamaguchi, 2012). In particular, nutrient conditions are believed to be crucial for phytoplankton species to accumulate significant biomass. Thus, to predict and forecast the occurrence of HABs and thus to minimize the damage caused by blooms, it is important to assimilate basic knowledge about the physiological features of HAB-forming phytoplankton, including their nutrient requirements.

Various experiments have been conducted to reveal the physiological requirements of harmful algal species in relation to various macronutrients such as nitrate, phosphate, and silicate (Hallegraeff, 1993 ; Imai et al., 2006). There is also clear evidence that trace metals, particularly iron, may also influence the growth of marine phytoplankton (Martin and Fitzwater, 1988 ; Brand, 1991 ; Sunda, 2006 ; Naito et al., 2008 ; Imai and Yamaguchi, 2012 ; Naito, 2016). Iron is an essential element for the growth of phytoplankton and is one of the limiting factors of phytoplankton production, particularly in vast open ocean areas termed high-nitrate low-chlorophyll (HNLC) regions (Martin and Fitzwater, 1988 ; Martin et al., 1991 ; Boyd et al., 2007, 2010). It has been suggested that iron concentrations in the marine environment

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might influence the species composition of phytoplankton assemblages and the outbreak of red tides (Brand, 1991 ; Wells et al., 1995). For example, Wells et al. (1991) reported that the pulsed iron input via an influx of river water may be a triggering factor for the generation of offshore blooms of the toxic species *Alexandrium tamarense*.

It depends on both the physiological iron requirement of a phytoplankton species, and the concentration of bioavailable iron determines whether iron is the growth limiting factor of the species.

The dissolved iron concentrations of coastal surface water are relatively high, ranging from 10^{-9} to 10^{-7} M, whereas those in oceanic surface waters range from 10^{-12} to 10^{-9} M (de Baar and de Jong, 2001). Estuarine and riverine waters display greater variations in dissolved iron concentrations. For example, in Matsushima Bay and the adjacent Takashiro Estuary in Japan, the iron concentration was found to range from 10^{-8} to 10^{-6} M (Fujii et al., 2006). In Ribble Estuary in the UK, the iron concentration was reported to range from 10^{-7} to 10^{-6} M (van den Berg et al., 1986).

Some coastal species have relatively high iron requirements (Brand, 1991 ; Sunda and Huntsman, 1995). For instance, Timmermans et al. (2001) calculated K_s values of 5.1 $\times 10^{-8}$ and 4.3×10^{-8} M for the coastal diatoms *Thalassiosira pseudonana* and *Thalassiosira weissflogii*, respectively, based on the data reported by Sunda and Huntsman (1995). In contrast, it is reported that the K_s values of four large diatom species in the Southern Ocean ranged between 1.1×10^{-9} M for *Actinocyclus* sp. and 1.9×10^{-10} M for *Fragilariopsis kerguelensis* (Timmermans et al., 2004). The open-ocean small diatom *Chaetoceros brevis* was reported to have a K_s value as low as 0.6×10^{-12} M (Timmermans et al., 2001).

Therefore, it is important to reveal species-specific iron requirements to understand the effect of iron as a controlling factor for the species composition of phytoplankton assemblages and for the outbreak and the maintenance of red tides in coastal areas. But data about the physiological characteristics, such as the half-saturation constants (K_s) for iron utilization and maximum growth rates (μ_{max}), of harmful coastal species remain very limited due to the technical difficulties in the incubation of red tide species in a chemically defined medium, despite iron being increasingly recognized as a critical factor for the growth of phytoplankton in coastal areas (Hutchins et al., 1998 ; Bruland et al., 2001 ; Firme et al., 2003 ; Lewitus et al., 2004 ; Jurgensone and Aigars, 2012).

The IHN medium is a chemically defined artificial medium that was first reported in 2004 and made it possible to culture various phytoplankton species, including HAB-forming species (Imai et al., 2004). Culture experiments using this medium have revealed the effects of iron and its ligands on the growth of red tide algae (Naito et al., 2005b, 2008 ; Fukuzaki et al., 2011). In this study, we employed this synthetic medium for incubation experiments to obtain the half-satura-

tion constants for growth (K_s) and maximum growth rates (μ_{max}) with regard to iron concentrations using axenic cultures of four coastal phytoplankton species : the harmful fish-killing raphidophyte *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara & M. Chihara, the harmful bivalve-killing dinoflagellate *Heterocapsa circularisquama* Horiguchi, and two diatom species, *Ditylum brightwellii* (T. West) Grunow and *Chaetoceros didymus* Ehrenberg, which are important primary producers in coastal environments.

Materials and Methods

Phytoplankton cultures

Four axenic cultures of the phytoplankton species were used in the culture experiments : *Heterosigma akashiwo* 893 (isolated in 1989 from Hiroshima bay) (Imai et al., 1993), *Heterocapsa circularisquama* (isolated in 2004, Uranouchi Inlet) (Shiraishi et al., 2008), *Ditylum brightwellii* (isolated in 1989, Hiroshima Bay) (Yamaguchi, 1994) and *Chaetoceros didymus* (isolated in 2010, Ago Bay). Maintenance cultures were incubated in 50-mL glass Erlenmeyer flasks using normal modified IHN medium (Naito et al., 2005a) (2 μM iron and 32 μM EDTA) and iron deficient modified IHN medium (0.2 μM iron and 3.2 μM EDTA) under a light intensity of 93-145 μmol photons·m^{-2}·s^{-1} using cool-white fluorescent lights with a 14-h : 10-h light : dark photo cycle. The cultures were maintained at 25°C for *H. circularisquama* and at 20°C for the other three species. Bacterial contamination was examined with DAPI staining and direct epifluorescence microscopy (Imai, 1987).

Preparation of the culture medium

To prevent iron contamination of the instruments, all equipment and containers were immersed in 4 M hydrochloric acid for at least 24 h, and then thoroughly rinsed with Milli-Q water. In addition, micropipette tips and the bottles of stock solutions were washed in boiling 1 M nitric acid, hydrochloric acid, and Milli-Q water in succession. The IHN medium composition (Imai et al., 2004) was modified and used for the experiment (Table 1). Iron and its chelator EDTA were excluded in the preparation of the basal medium. The basal medium was autoclaved at 121°C for 15 min. The stock solution of Na$_2$EDTA·2H$_2$O was filter-sterilized using a 0.1-μm mesh PVDF membrane filter (MILLEX-VV, Millipore, Billerica, MA, USA) before being added to the sterilized basal medium. The final EDTA concentration in the culture medium was set as 0.2, 2, 20 and 200 μM. Subsequently, the FeCl$_3$·6H$_2$O solution was filter-sterilized and added to the culture medium, whereas the final concentration of iron was set as 1/10 of iron chelate (EDTA), namely from 0.02 to 20 μM, respectively. For the negative control medium, neither EDTA nor iron was added (Table 1). All culture media were stored in the dark at 20°C for at least 24 h to reach chemical

Table 1. Chemical composition of basal medium.

NaCl	0.43 M	S$_3$–Vitamin mix		P1 metals (except for iron source)	
KCl	9.4 mM	Vitamin B$_{12}$	0.74 nM	H$_3$BO$_3$	1.0 mM
MgSO$_4$ · 7H$_2$O	37.0 mM	Biotin	4.1 nM	MnCl$_2$ · 4H$_2$O	35 μM
CaCl$_2$ · 2H$_2$O	7.5 mM	Thiamine HCl	1.5 μM	ZnCl$_2$	4.0 μM
NaNO$_3$	2 mM	Nicotinic acid	0.81 μM	CoCl$_2$ · 6H$_2$O	0.1 μM
NaH$_2$PO$_4$ · 2H$_2$O	0.1 mM	Calcium pantothenate	0.21 μM	CuCl$_2$ · 6H$_2$O	1.0 μM
Na$_2$SiO$_3$ · 9H$_2$O	0.33 mM	p-aminobenzoic acid	73 nM		
Na$_2$SeO$_3$	2.0 nM	Inositol	28 μM		
KI	0.47 μM	Folic acid	4.5 nM		
Na$_2$MoO$_4$ · 2H$_2$O	0.1 μM	Thymine	24 μM		
HEPES	5 mM				

equilibrium.

Culture experiments

In total, 4 mL of the prepared culture medium was dispensed into gamma-ray sterilized 8-mL polystyrene testing tubes with screw caps (Evergreen Scientific, Los Angeles, CA, USA) by an acid-washed micropipette. Well-grown strains (80 μL) in the late exponential growth phase or early stationary phase in the modified IHN (containing 2 μM iron) were inoculated in the experimental culture medium containing 2, 4, or 20 μM iron. Well-grown strains in iron-deficient modified IHN (containing 0.2 μM iron) were also inoculated in a similar way in the experimental culture medium containing 0 (no iron added plot), 0.02, or 0.2 μM iron. Subsequently, the cells were transferred into the new experimental culture medium containing the same concentrations of iron and EDTA when they reached the late exponential growth phase or early stationary phase, which occurred at least five days after the inoculations. Incubation conditions were the same as those for the maintenance culture mentioned before. The algal growth of each culture was determined by measuring the *in vivo* fluorescence with a fluorometer (10-AU 005 Turner Design Co.) (Brand et al., 1981). Culture experiments were performed in quadruplicate.

Data analyses

The growth rate in each experimental medium was calculated using the following equation for the data collected during the exponential growth phase :

$$\mu = \frac{\ln F_1 - \ln F_0}{t_1 - t_0},$$

where F_1 and F_0 represent fluorescence values and t_0 and t_1 represent the incubation times (days). The parameters μ_{max} and K_s were calculated by nonlinear regression using a computer program (MATLAB, Math Works) based on Monod's equation (Monod, 1949) as follows :

$$\mu = \frac{\mu_{max} \times S}{K_s + S},$$

where μ, μ_{max}, S, and K_s represent the calculated growth rates, the maximum growth rates, the concentration of added iron, and the half-saturation constant, respectively. The cell surface area (A), volume (V) and A/V ratio were calculated by an approximation to simple three-dimensional shapes (Sun and Liu, 2003) based on the cell size parameters obtained by microscopic measurements. The morphologies of the four species were approximated as follows : *Heterosigma akashiwo*, oblate spheroid ; *Heterocapsa circularisquama*, cone + hail sphere ; *D. brightwellii*, prism on a triangle-based girdle ; *C. didymus,* prism on an elliptic-based girdle (Sun and Liu, 2003).

Although we employed clean techniques to avoid iron contamination and the chemical reagents used in the preparation of the medium were the highest purity available, a nanomoler order of iron was considered to exist even in the control medium (no iron and no chelater addition) as background concentration. Therefore, the growth data in control medium should be excluded from the kinetic analysis.

Results

Plankton growth at various iron concentrations

Figure 1 shows the growth of the four phytoplankton species in the modified IHN medium containing various iron concentrations. The growth of all examined species was limited in the culture medium containing <0.2 μM iron. In the culture medium without iron addition, *Heterosigma akashiwo* did not grow, and transferred cells died after a few days, whereas *Heterocapsa circularisquama*, *C. didymus*, and *D. brightwellii* survived or grew slowly. Those species are considered to utilize background concentration of iron. Both the growth rates and maximal growth yields of the four species were proportional to the added iron concentrations in the range of 0.02-2 μM (Figs. 1 and 2).

The growth curve of *Heterosigma akashiwo* in the presence of 4 μM iron was comparable to that in the medium containing 2 μM iron. The growth rate of *D. brightwellii* in the 4 μM iron-containing medium was comparable to that in the
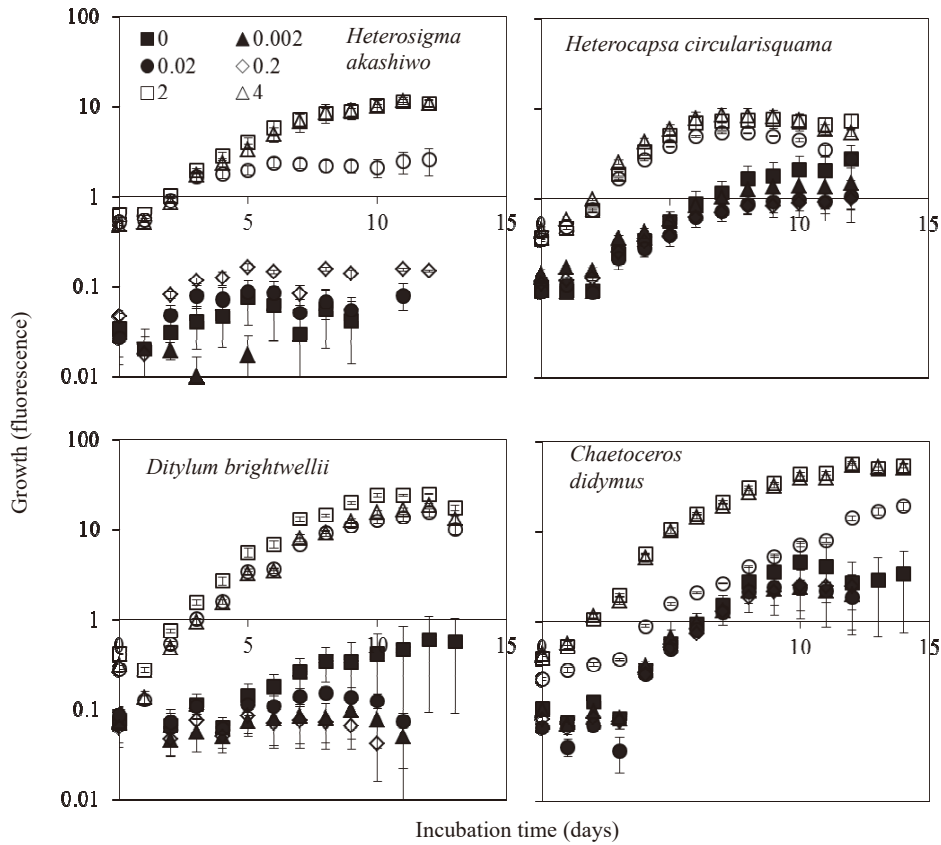
Fig. 1. Growth of phytoplankton species cultured in modified IHN medium with various concentrations of iron (0–20 μM). The symbols and iron concentrations (μM) are indicated in the result of *Heterosigma akashiwo*, and those are the same in all four graphs.
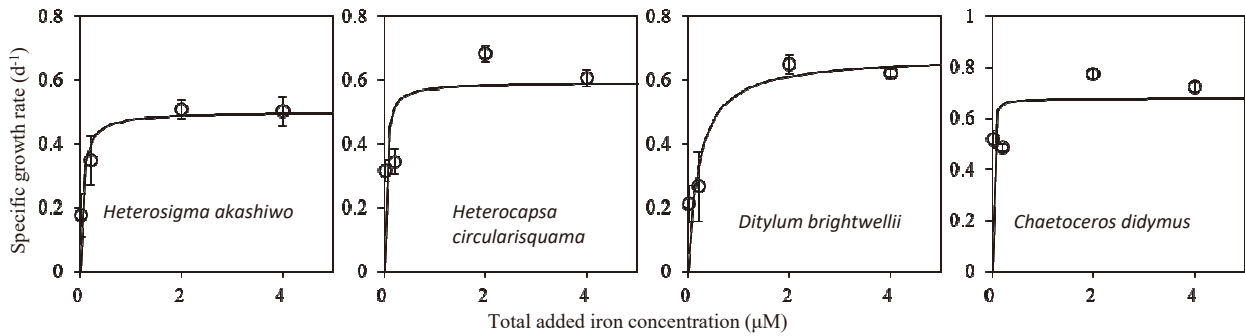

Fig. 2. Specific growth rates (d^{-1}) of the phytoplankton species in relation to total added iron concentrations (μM).

2 μM iron–containing medium ; however, the maximum yield was lower in the 4 μM iron–containing medium than in the 2 μM iron–containing medium. The growth rates of *Heterocapsa circularisquama* and *C. didymus* were slightly lower in the 4 μM iron–containing medium than in the 2 μM iron–containing medium, although the maximum yields were comparable in the two media (Figs. 1 and 2). The addition of 20 μM iron to the media significantly reduced both the growth rates and maximum yields of all examined species (Fig. 1). Hence, the data for 20 μM iron–supplemented medium were excluded from the subsequent analyses.

Growth Parameters (K_s and μ_{max})

The half saturation constant (K_s) for growth, μ_{max}, and morphological parameters, i.e., the surface area to volume ratios (A/V) of the tested species, are summarized in Table 2. The two diatom species *C. didymus* and *D. brightwellii* had relatively high μ_{max} values (0.68 day^{-1}) compared to *Heterocapsa circularisquama* (0.59 day^{-1}) and *Heterosigma akashiwo* (0.50 day^{-1}). *C. didymus* had the lowest K_s value (7.5 × 10^{-9} M), while *D. brightwellii* had the highest K_s value (2.1 × 10^{-7} M) (Table 2). The cell volume was positively corre-

Table 2. Estimated half-saturation constants (K_s) with respect to dissolved total Fe and maximum growth rates (μ_{max}), growth temperatures (T), surface area (A), volumes (V), and surface to volume ratios (A/V) in cultures of *Heterosigma akashiwo*, *Heterocapsa circularisquama*, *Ditylum brightwellii*, and *Chaetoceros didymus*.

	K_s (M)	μ_{max} (d^{-1})	T (°C)	A (μm^2)	V (μm^3)	A/V (μm^{-1})
Heterosigma. akashiwo	5.1×10^{-8}	0.50	20	613	1,374	0.45
Heterocapsa. circularisquama	3.1×10^{-8}	0.59	25	519	1,001	0.52
Ditylum brightwellii	2.1×10^{-7}	0.68	20	8,977	60,795	0.15
Chaetoceros didymus	7.5×10^{-9}	0.68	20	419	517	0.81
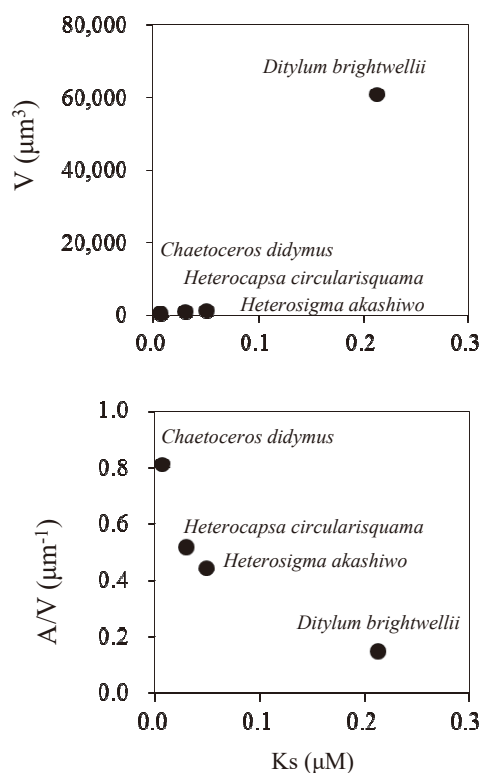
Fig. 3. Half saturation constants (K_s) of the phytoplankton species in relation to total added iron concentration (μM) and volume (μm^3) (upper panel) and A/V ratios (μm^{-1}) (lower panel). A, surface area ; V, volume of phytoplankton.

lated with the K_s values of the species ($R = 0.983, p = 0.017$) (Fig. 3 upper panel). The species with higher A/V ratios tended to have lower K_s values ($R = -0.909, p = 0.091$) (Fig. 3 lower panel).

Discussion

What limits the growth at high iron concentrations ?

The addition of 20 μM iron to the culture media inhibited the growth of all examined phytoplankton species. It has been reported that the growth of the diatom *Thalassiosira pseudonana* was significantly inhibited in the presence of 10 μM total iron, but maximal growth was observed in the presence of 1 μM total iron (Sunda and Huntsman, 1995). This

phenomenon may be attributed to the toxicity of high iron concentrations or the presence of excess EDTA. Kalis et al. (2006) suggested the first possible mechanism and stated that competition occurs between metal irons (Fe, Cu, and Pb vs. Cd, Mn, Zn, and Ni) with respect to metal binding sites on the plant root surface. In media with high iron concentrations, the uptake of essential trace elements might be inhibited because the binding sites on the cell surface are occupied by iron. The second possible mechanism is an inhibitory effect caused by the presence of excess EDTA. For example of the case of Ni, excess EDTA forms a kinetically inert complex i.e., Ni (EDTA)$_2^-$, which reduces the bioavailability of Ni ions (Beck et al., 2002). It was not possible to conclude the dominant mechanism based only on the current results.

Iron requirements of coastal red tide algae (diatoms vs. dinoflagellates and raphidophytes)

The μ_{max} values of *Heterosigma akashiwo* and *Heterocapsa circularisquama* were lower compared to the two diatoms. *Heterosigma akashiwo* and *Heterocapsa circularisquama* exhibited higher K_s values compared to *C. didymus*. It is suggested that *Heterosigma akashiwo* and *Heterocapsa circularisquama* are at a disadvantage in an iron-depleted natural environments compared to *C. didymus*. Moreover, it has been reported that the K_s value of the dinoflagellate *Akashiwo sanguinea* (formerly *Gymnodinium sanguineum*) for iron were two orders of magnitude higher than those of the coastal diatoms (Doucette and Harrison, 1990). We should note that the μ_{max} values we obtained for the four species were somewhat lower than the previously reported values of those species. For example, the μ_{max} values are 0.9 d^{-1} in *Heterosigma akashiwo* (Herndon and Cochlan, 2007), 0.9 d^{-1} in *Heterocapsa circularisquama* (Yamaguchi et al., 1997), 0.8-1.0 d^{-1} in *C. didymus* (Yamaguchi, 1994), and 0.9-1.0 d^{-1} in *D. brightwellii* (Yamaguchi, 1994). A possible reason for the differences is that the gas exchange rate was not enough to sustain their maximal growth because we employed test tubes with screw caps to avoid bacterial and iron contamination (Yamamoto and Nakahara, 2005). Still, it should be reasonable to compare the relative growth responses and the μ_{max} and K_s values, considering that the treatment conditions were the same among the four phytoplankton species.

Flagellates have nutrient-retrieval migration strategies to

compensate for the higher nutrient uptake K_s values compared to diatoms (Smayda, 1997). These strategies for nutrient uptake may also be applied to iron. As a strategy to compensate for relatively high K_s values, *Heterosigma akashiwo* and *Heterocapsa circularisquama* utilize macronutrients in deep water of the bottom layer through diurnal vertical migration (Yamochi and Abe, 1984 ; Shiraishi et al., 2007). These two species probably utilize iron in the bottom layer, where oxygen tends to be depleted and iron is replete because of reductive elution from the bottom sediments. It has been demonstrated that *H. akashiwo* and *H. circularisquama* are able to grow in a medium supplemented with particulate $FePO_4$ or FeS, which also suggests that those species utilize iron in the bottom layer (Naito et al., 2005a).

The formation of resting stage cells of the diatom *Thalassiosira nordenskioeldii* has been induced by iron deficiency (Sugie and Kuma, 2008). A potentially favorable environment would be present for phytoflagellates to bloom with iron obtained in the bottom layer when diatom blooms are terminated by iron limitation in the surface layer and diatom cells settle as resting stage cells. To test this hypothesis, it is needed to determine the ability of phytoflagellates to proliferate with iron obtained in iron replete environments after they move to the iron deplete surface layer. Some diatom species are able to accumulate iron in excess of the minimum quantity required to achieve maximal growth, which is a process termed "luxury uptake" (Iwade et al., 2006 ; Marchetti et al., 2009). The coastal dinoflagellate *Prorocentrum minimum* is able to uptake iron at a 2-fold higher level than the reported necessary level ; however the number of possible cell divisions in an iron-depleted environment remains unknown (Sunda and Huntsman, 1995). Studies of the luxury uptake of iron by other red tide species remain limited, but are urgently required to understand the mechanisms regulating the occurrence and maintenance of blooms.

We observed a strong positive correlation between cell volume and K_s values (Fig. 3 upper panel), and also a tendency for species with higher A/V ratios to have lower K_s values (Fig. 3 lower panel). It is considered that smaller cells sequester iron more efficiently due to their larger cell surface area per volume and would survive better in iron-depleted environments. The K_s values were well explained by the cell size parameters in this study.

Estuarine and coastal environments are more dynamic and turbid compared to offshore environments because of frequent disturbances, such as rainfall and the subsequent influx of river water or the vertical mixing and circulation of bottom water.

Conclusion

The raphidophyte *Heterosigma akashiwo* and the bivalve-killing dinoflagellate *Heterocapsa circularisquama* both had relatively high K_s values and low μ_{max} values compared with the small diatom *Chaetoceros didymus*, indicating that the phytoflagellates are at a disadvantage in iron-depleted natural environments. As a strategy to compensate for this disadvantage, the phytoflagellates are assumed to utilize iron present in the bottom layer and/or bottom sediments via diurnal vertical migration. Further studies are needed to determine the association of phytoflagellates with the "luxury uptake" of iron. Compared to the oceanic species reported in previous studies, the coastal species examined in this study had higher K_s values. In particular, the large diatom *D. brightwellii* had the highest K_s value ; however this species also had the highest μ_{max} value, indicating that it may have an advantage in coastal areas, where frequent environmental disturbances occur.

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