The role of iron species on the competition of two coastal diatoms, *Skeletonema costatum* and *Thalassosira weissflogii*

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Abstract

Coastal diatoms are often exposed to macronutrient (N and P) and Fe enrichment. However, how these exposures influence on Fe biogeochemical cycle and then on diatom interspecific competition is unknown. In this study, two non-toxic coastal diatoms, *Skeletonema costatum* and *Thalassiosira weissflogii* were exposed to N, P, and Fe enrichment for four-day. The growth of algae was co-controlled by macronutrient and Fe species (Fe (III)-EDTA, Fe(OH)$_3$, dissolved, colloidal, and particulate Fe from culture medium). The influence of Fe species on algal cell density was more significant than macronutrient. When *S. costatum* coexisted with *T. weissflogii*, their cell density ratios were ranged between 5.57-7.03 times, indicating that *S. costatum* was more competitive than *T. weissflogii*. There were not significant correlation between cell density ratio and iron requirement, including iron adsorption and absorption per cell, iron adsorption and absorption by all algal cells. As Fe complexing ligands, algal exudates can promote diatom growth itself and such promotion on *S. costatum* was more obvious than that on *T. weissflogii*. Iron species was a key determinant on interspecific competition of coastal diatom, and the degree of bioavailability was described as follows: dissolved iron from own exudates > colloidal iron from own exudates > particulate iron from own exudates > particulate iron from another algal exudates > colloidal iron from another algal exudates >dissolved iron from another algal exudates > Fe (III)-EDTA > Fe (OH)$_3$.

**Keyword:** Iron; Biogeochemical cycle; Bioavailability; Coastal diatom; Interspecific competition
1 Introduction

Diatoms tend to dominate phytoplankton communities in well-mixed coastal and upwelling regions (Bowler et al., 2008). Marine diatoms greatly influence marine food webs, global climate, atmospheric carbon dioxide concentration, and marine ecosystem function (Armbrust, 2009). Coastal diatoms are often exposed to N, P, and Fe enrichment (Bizsel and Uslu, 2000, Wells and Trick, 2004). An understanding of diatoms species composition in coastal ecosystems and the processes that select for blooms of certain species were still limited, in spite of the importance of diatom competition in coastal ecosystem dynamics.

Macronutrient inputs into coastal waters continue to rise (Turner and Rabalais, 1994, Cai et al., 2011). Coastal eutrophication results in a wide variety of changes in the structure and function of coastal marine ecosystems, metal sorption, bioaccumulation and species distribution (Li et al., 2007, 2009, Li and Zheng, 2011) and protecting these systems from the many adverse effects of eutrophication was extremely important (Smith, 2003).

Dissolved-Fe availability (Sunda and Huntsman, 1997, Takeda, 1998, Hutchins and Bruland, 1998) and siderophore- and porphyrin-complexed Fe (Hutchins et al., 1999) play a critical role in controlling diatom growth, but we have a very restricted knowledge of the role of phytoplankton in controlling Fe species distribution in coastal water, because only phytoplankton blooms have been studied (Nishioka et al., 2001, Christopher et al., 2002, Öztürk et al., 2003), at the same time, it is important to distinguish Fe absorption (intracellular uptake) and adsorption (cellular surface uptake) (Li and Zheng, 2011). Fe bioavailability or toxicity, biogeochemical fates, and ecological effects are quite different between absorbed and adsorbed Fe by marine phytoplankton, because: after absorption (i.e., assimilation) by algal cells, Fe can combine with organic compounds such as proteins and enzymes, and then accumulate through the aquatic food chain;
whereas Fe adsorbed by cell surfaces can be partly desorbed into the seawater, and then exist as inorganic compounds transfer to seawater. There is surprisingly little information available on the quantitative and qualitative effect of N, P, and Fe additions on Fe speciation distribution and it subsequent influence on the species competition.

*Skeletonema costatum*, as a non-toxic coastal diatom, has been responsible for large-scale bloom events (ca.10,000 km²) in Yangtze River estuary and the adjacent East China Sea in recent years (Zhou et al., 2003). *Thalassiosira weissflogii*, a non-toxic coastal centric diatom, is used as a model of marine algae (Qu et al., 2000). In this study, we have investigated the interspecific competition of *S. costatum* and *T. weissflogii* for iron speciation distribution for the first time. These two species of non-toxic coastal diatom were exposed to a four-day macronutrient and iron enrichment. How these exposures influence on Fe biogeochemical cycle, Fe speciation distribution, and competition of diatom were examined.

2 Materials and methods

2.1 Chemicals and Materials

All reagents were made in water purified (>18 MW cm⁻¹) using a Milli-Q (MQ) system (Millipore) and stored at 4 °C. Sub-boiled quartz distilled nitric acid (Q-HNO₃) was produced by a single distillation of trace metal grade (TMG) concentrated nitric acid (Fisher Scientific) in a quartz finger, sub-boiling distillation apparatus. Agilent ICP-MS multi-element standards (10 mg L⁻¹, Nos. 2A, USA) and internal standards (including 100 mg L⁻¹ ⁴⁵Sc, ⁷²Ge, ¹⁰³Rh, ¹¹⁵In, and ²⁰⁹Bi) were used for trace elements determination. NaNO₃, Na₂HPO₄, Fe₂(SO₄)₃, EDTA-Na₂, NH₄Cl, NaCl, H₂O₂, ammonium molybdate, ascorbic acid, sulfanilic acid, and N-(1-naphthyl) ethylenediamine dihydrochloride were analytical reagent grade (Sigma, USA). HCl and HNO₃ were TMG (Fisher Scientific, USA). N, P and Fe standard solutions were prepared from stock
standard solutions of NaNO$_3$ (N, 10 mM), Na$_2$HPO$_4$ (P, 360 mM), and Fe$_2$(SO$_4$)$_3$ (Fe, 1000 µg mL$^{-1}$), respectively. Certified reference materials NIES-03 (green algae, *Chlorella Kessleri*) and NASS-5 (standard seawater) were supplied from the Japanese National Institute of Environmental Studies (Ibaraki, Japan) and National Research Council Canada (Ottawa, Canada), respectively.

Sterile trace element clean techniques were applied for culturing and experimental manipulations (Shi *et al.*, 2010). Reagents for this study were made in acid-washed low-density polyethylene (LDPE) bottles. The acid cleaning procedure for reagent bottles included a 6 M HCl soak for one month and 0.7 M HNO$_3$ storage for another month. Sample bottles (100 mL LDPE, Bel-Art) and eluate bottles (8 mL LDPE, Nalgene) were cleaned by heating overnight in 3 M HCl and then heating again overnight in 4 M HNO$_3$ (Biller and Bruland, 2012). Each new lot of vials was tested before use to ensure that there was no biological or trace element contamination.

Iron was washed from the cell surface using a trace metal clean reagent (Tovar-Sanchez *et al.*, 2003, 2004). The preliminary experiments indicated that iron adsorbed on the cell surface could be removed using a trace metal clean reagent. In the trace metal clean reagent, oxalate was used as the reductant to remove surface adsorbed trace metals from phytoplankton cells and other particles. To the oxalate solution, hydroxylamine, perchlorate, and 1, 10-phenanthroline were added. Next, the pH was adjusted to 8 with 10 mol L$^{-1}$ NaOH and the solution was heated in a water bath to 50 ºC for 15 min. Immediately, while still hot, the solution was transferred to a 250 mL Teflon separating funnel and extracted twice with 6 and then 4 mL of 1, 2-dichloroethane, and then transferred to a trace metal clean Teflon separating funnel and extracted again with 4 mL of 1, 2-dichloroethane. In each extraction, the organic phase was
discharged and aliquots of the reagent were collected. The clean oxalate solution was then transferred to LDPE bottle.

2.2 Instrumentation

Agilent 7500cx inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, USA), WHG-102A2-based flow injection hydride generator (John Manufacturing, Beijing), UV-3200PCS UV-Vis spectrophotometer (Shanghai Spectrum of U.S. Instruments Co., Ltd.), Double-sided clean bench (Suzhou Purification Equipment Co., Ltd.), SPX-300 IC Microcomputer artificial climate chamber (Shanghai Bo Xun Industrial Co., Ltd.), Branson-102C ultrasonic crushing device (Branson Ultrasonic Corporation), Leica DM LB2 microscope Leica (Leica Instruments, Germany), MK-III-based fiber optic pressure controlled closed microwave digestion system (Shanghai Branch Microwave Digestion Test New Technology Institute), and Milli-Q ultrapure water system (the United States, Millipore company) were used in this study.

2.3 Seawater Sample

Coastal seawater was collected from Zhangzhou Bay, Fujian Province, China. The salinity of the seawater was 33.1±0.05 psu. The N and P concentrations were measured three times using a flow injection analyzer (FIA), and the background concentrations were 47.5 $\mu$mol L$^{-1}$ for N (as nitrate) and 0.250 $\mu$mol L$^{-1}$ for P (as reactive P). The background concentrations of Fe were 0.40 $\mu$mol L$^{-1}$ measured by ICP-MS, similar to that reported by Öztürk et al (2003). The detection limit of Fe by ICP-MS was 0.4 nmol L$^{-1}$. The amounts of Fe in seawater were determined three times and the relative standard deviation was 1.1%. Subsequently, this coastal seawater, with both Fe and macronutrient enrichment, could be used for experiments related to Fe sorption by bloom-forming speciation and Fe species distribution in seawater.
Clean seawater used for the experiments on the competition between two dominant bloom-forming species was collected 10 km offshore in Zhangzhou Bay, Fujian State, China. The background concentration of Fe in the seawater was measured using ICP-MS, and the iron concentration was 0.165 nmol L\(^{-1}\). The N and P concentrations were measured using FIA, and the background concentrations were 7.66 µmol L\(^{-1}\) and 0.05 µmol L\(^{-1}\) for N (as nitrate) and P (as reactive P), respectively. The seawater was considered to be remote from anthropogenic activities.

All the seawater was stored at 4°C for about 6 months, filtered through acid-washed Pall Acropak Supor capsule 0.22 µm filters, and sterilized before use.

2.4 Algal culture

Unialgal cultures of \textit{S. costatum} and \textit{T. weissflogii} were obtained from the State Key Laboratory for Marine Environmental Science, Xiamen University. They were maintained in seawater (with 21.1 mmol L\(^{-1}\) Si added as Na\(_2\)SiO\(_3\)·H\(_2\)O, but without trace metals) at different N (added as NaNO\(_3\)) and P (added as Na\(_2\)HPO\(_4\)) concentrations with different species of Fe at 19°C sterile conditions, and with the light illumination of 140 µmol photons m\(^{-2}\) s\(^{-1}\) by a light: dark cycle as 14 hr: 10 hr. The algal suspensions were stirred at 100 rpm during the irradiation experiments and dark controls to simulate the current of seawater and to reduce the adsorption of Fe by vessel. A relatively large volume of culture vessel (5 L) was used to decrease the thickness of the marine phytoplankton suspension for avoiding the light limitation. The difference of light illumination on the surface and the bottom of marine phytoplankton suspension could be ignored.

2.5 Iron absorption and adsorption by the bloom-forming species under different nutrient regimes

Exponentially growing cells of \textit{S. costatum} or \textit{T. weissflogii} cells were filtered and
transferred to new medium every 1-2 days, to ensure that the cells were acclimated to the
experiment. After 4 transfers, the cells were again filtered and added into 1000 mL of filtered
seawater (0.22 µm) in acid-cleaned polycarbonate bottles, at a cell concentration of 1×10^4 cells
mL^-1. For N and P, a two-factor experiment was performed to examine the effect of N and P on
Fe absorption, adsorption and bioconcentration by the cells. The experimental macronutrient
treatments included: total N concentrations of 8, 16, 32, and 64 µmol L^-1, respectively, at a total P
concentration of 1.0 µmol L^-1; and total P concentrations of 1.5, 2.0, and 2.5 µmol L^-1,
respectively, at total N concentration of 8 µmol L^-1. These experiments were replicated (n=3).
The N, P, and Fe concentrations (1.8 µmol L^-1) were maintained in the cultures through
compensating addition daily of NaNO_3, Na_2HPO_4, and Fe_2(SO_4)_3 salt for 3 days after
determination of N, P, and Fe in the medium, i.e., semi-continuous culture was adopted.

After cultured for 4 days, iron absorption, adsorption, and uptake by the diatom species, _S. costatum_ and _T. weissflogii_ were measured, and the cell density was counted microscopically.
The cell diameter of _S. costatum_ was 6-18 µm. The cell length and width of _T. weissflogii_ was
15-22 µm and 9-14 µm, respectively. The cells contained in 600 mL of the medium were
collected on a 3.0 µm membrane filter, rinsed with clean natural seawater with 0.16 nmol L^-1 Fe
twice, resuspended into 25 mL of trace metals clean reagent, stirred for 1 h to remove
surface-bound Fe, and filtered on a 3.0 µm membrane filter. The filtrate was added into a closed
vessel with mixed acid (HNO_3 : H_2O_2, v : v=2:1), microwave digested for 7 min at 1.01×10^6 Pa,
and then used for determining the concentration of Fe adsorbed by _S. costatum_ or _T. weissflogii_
cells. After removing surface sorbed Fe, the _S. costatum_ or _T. weissflogii_ cells were
microwave-digested, and then used for determining the concentration of Fe absorbed by _S.
_S. costatum_ or _T. weissflogii_ cells. Fe adsorption or absorption per cell was calculated. Total Fe
adsorption or absorption by *S. costatum* or *T. weissflogii* cells was the product of Fe adsorption or absorption per cell and the cell density.

### 2.6 Distribution of iron in seawater with *S. costatum* or *T. weissflogii* under different nutrient regimes

After 4-days culture, the cross-flow ultra-filtration devices used in this study were a Millipore Pellicon 2 System. With the Pellicon 2, the filters have cut-offs of 3.0, 0.22µm and 1kDa. The filter material of the 3.0 and 0.22 µm filter was hydrophobic polyvinylidene fluoride. All materials were carefully acid-rinsed, and filters were kept refrigerated before use. Initially, every new filter was rinsed with deionised water and then with a NaOH and HCl rinse programme before use. The same procedure was repeated after every filtration and before every new sampling occasion. All the retentates and permeates were collected and analysed. The recovery was calculated as \( R\% = \left( \frac{\text{(particulate Fe) + (colloidal Fe) + (dissolved Fe)}}{\text{total Fe in culture medium}} \right) \times 100 \). The recovery data for the ultrafilters used in this study were in the range 92-110%.

Particulate Fe (3.0 µm-0.22 µm), colloidal Fe (0.22 µm-1kDa), or dissolved Fe (<1kDa, probably still containing a fraction of smaller colloids) was added into a closed vessel with mixed acid (HNO₃:H₂O₂, v:v=2:1), microwave digested for 7 min at 1.01×10⁶ Pa, and then used for the determination of the concentration of Fe. The concentrations of Fe in different size fractions were determined by ICP-MS. Particulate Fe, colloidal Fe, and dissolved Fe were used for the cultures of *S. costatum* and *T. weissflogii* for examining their influence on the interspecific competition.

To statistically analyse the effects of N, P and different species of Fe additions on the competition between *S. costatum* and *T. weissflogii*, a three-way factorial experimental design
was used. The macronutrient treatments were the same as previous describe and different species of Fe at 1.8 μmol L⁻¹ were added into the clean seawater for the cultures of *S. costatum* and *T. weissflogii*. Six species of Fe were used, including dissolved, colloidal, and particulate Fe from *S. costatum* or *T. weissflogii*. The background concentration of Fe in the clean seawater was only 0.16 μmol L⁻¹, and thus could be ignored. Fe was complexed with EDTA at a ratio of 1:1.1 before spiking into the seawater. Fe (III)-EDTA was chosen to simulate Fe chelation by organic substances such as humic and fulvic acids, which occur naturally in the environment.

2.7 Statistical approaches

Analysis of variance was calculated by using SASPROC MIXED (Littell et al., 1996). For all analyses, significance was assigned at the *P* <0.05 level. Analytical data was tested for homogeneity of variance (Bartlett’s Test). All data was log10 transformed to meet assumptions of normality. Univariate data was analysed using Statistica Version 18.1. Correlations between measured parameters were also performed using Statistica Version 18.1 (Templeman et al., 2010).

3 Results and discussion

3.1 Accuracy and detection limits of iron determination

Iron determination using microwave-assisted digestion and ICP-MS was evaluated by analyzing certified reference materials, including NIES-03 (green algae, *Chlorella Kessleri*) and NASS-5 (standard seawater). Limit of detection (LOD, calculated as three times of the standard deviation of 3 reagent blank replicates analysed at different time intervals between samples) was 2.84 μg g⁻¹; limit of quantification (calculated as 3.3 times LOD) was 9.37 μg g⁻¹. Found value in NIES-03 and NASS-5 were 1.82±0.023 mg g⁻¹ and 0.207±0.023 ng g⁻¹, respectively, the results of these analyses in good agreement with certified concentration in both CRMs (1.85±0.092 mg
and NASS-5 (0.240±0.035 ng g\textsuperscript{-1}). The method described was applicable for the determination of low levels (ng g\textsuperscript{-1} or µg L\textsuperscript{-1}) of Fe in coastal seawater and marine organisms.

3.2 Cell density of T. weissflogii and S. costatum under different nutrient regimes

The influence of the additions of nitrate and phosphate on growth of T. weissflogii and S. costatum has shown in Fig.1. The results indicated that N addition in the range of 8.0 to 64.0 µmol L\textsuperscript{-1} could stimulate cell growth and such stimulating effect was the most significant at 32.0 µmol L\textsuperscript{-1} N. However, algal growth could be inhibited by 64.0 µmol L\textsuperscript{-1} N, the influence trends were reverse. When P concentrations from 1.0 to 2.0 µmol L\textsuperscript{-1}, the influence on growth of T. weissflogii and S. costatum wasn’t significant, but under high concentration of P (>2.0 µmol L\textsuperscript{-1}), algal growth also could be inhibited. So, cell growth rate was controlled by macronutrient concentration, similar results have been reported (Li et al., 2014; Liu et al., 2014). At the same time, the influence of macronutrient on the growth of T. weissflogii was more obvious than S. costatum, i.e., the cell density was lower.

3.3 Iron adsorption on the cells of T. weissflogii and S. costatum under different nutrient regimes

Extracellular adsorption of Fe by T. weissflogii and S. costatum maintained at different N and P concentrations in a semi-continuous culture is shown in Fig.2. Except at the concentration of N 64 µmol L\textsuperscript{-1}, Fe adsorption by T. weissflogii per cell was increased with increasing macronutrient concentration of N concentration from 8 to 32 µmol L\textsuperscript{-1}. The minimum (0.07 fmol cell\textsuperscript{-1}) and the maximum (1.96 fmol cell\textsuperscript{-1}) adsorption of Fe was observed at concentrations of 8 µmol L\textsuperscript{-1} N/2.5 µmol L\textsuperscript{-1} P and 32 µmol L\textsuperscript{-1} N/1 µmol L\textsuperscript{-1} P, respectively. The maximum adsorption was 28 times of the minimum. The influence of macronutrient concentration on the Fe adsorption by S. costatum per cell was more significant. The maximum adsorption (7.74 fmol cell\textsuperscript{-1}) was 38.7 times of the minimum (0.2 fmol cell\textsuperscript{-1}). Thus, the influence of macronutrient
addition on the adsorption of Fe was obviously dependent on algal species.

Marine algae adsorb and coordinate Fe with basic functional groups on their cell surface (Zuo and Hoigné, 1993; Li et al., 2009). Iron adsorption by S. costatum per cell was higher than that of T. weissflogii per cell under various macronutrient regimes, although the surface area S. costatum per cell (28-254 $\mu$m$^2$) was less than that of T. weissflogii (154-804 $\mu$m$^2$). The Fe speciation in the culture solution was controlled by the species of marine phytoplankton and the concentrations of N and P. The amount of basic functional groups on the cell’s surface and the cell size are both affected by the concentrations of N and P (Zuo and Hoigné, 1992; Liu et al., 2014). Iron diffusion decrease (or increase) with decreasinge (or increasing) cell size. The degree of influence of macronutrient additions on the cell size of S. costatum was more significant than that of T. weissflogii, so the effect of macronutrient additions on Fe adsorption by S. costatum was more obvious than that by T. weissflogii. Fe adsorption was most likely to be affected by the following five factors: 1) the amount of surface basic groups on the cell surface, 2) the cell size, 3) the species and concentration of Fe, 4) the affinity constant between Fe and surface basic groups on the cell surface, and 5) the concentrations of N and P through their effects on the above four factors.

3.4 Influence of N and P addition on iron absorption by the cells of T. weissflogii and S. costatum

Different species of marine algae have different, growth rate and biochemical composition of marine alga, including the contents of carbohydrate, protein, chlorophyll, and surface basic groups, and the requirement of Fe (Zuo and Hoigné, 1992). The biochemical composition of marine alga affects iron absorption ability and other bioactivities. Macronutrient addition may influence both the growth rate and biochemical composition of marine algae (Liu et al., 2014). Fig. 3 showed the absorption of iron by T. weissflogii and S. costatum per cell at seven different
N and P concentrations. N addition affected the absorption of Fe by both *S. costatum* and *T. weissflogii* per cell in the same way: 1) the minimum Fe absorption (17.67 fmol cell\(^{-1}\) for *T. weissflogii* and 24.91 fmol cell\(^{-1}\) for *S. costatum*) was at an N concentration of 64 µmol L\(^{-1}\) and an N:P ratio of 64; 2) Fe absorption was increased with increasing N concentration from 8 to 32 µmol L\(^{-1}\), with the maximum absorption of Fe (33.16 fmol cell\(^{-1}\) for *T. weissflogii* and 709.23 fmol cell\(^{-1}\) for *S. costatum*). With increasing P concentration from 1 to 2.5 µmol L\(^{-1}\), Fe absorption was decreased in both *S. costatum* and *T. weissflogii*, so the value of Fe absorption at concentration of 8 µmol L\(^{-1}\) N/2.5 µmol L\(^{-1}\) P was the minimum (113.44 fmol cell\(^{-1}\) and 68.31 fmol cell\(^{-1}\)) for *T. weissflogii* and *S. costatum*, respectively. The content of Fe absorbed by *S. costatum* cells was more than that by *T. weissflogii* under the regimes with N ≥8 µmol L\(^{-1}\), but this situation was reversed when P concentration from 1.5 to 2.5 µmol L\(^{-1}\). Hence, a non-alga-specific influence of N addition on Fe absorption was observed, but the influence of P addition on Fe absorption was species-dependent. Under studied nutrient regimes, the maximum absorption of iron was 27.2 times and 28.5 times of the minimum absorption for *T. weissflogii* and *S. costatum*, respectively.

### 3.5 Comparison of iron adsorption and absorption by *T. weissflogii* and *S. costatum* cells under different nutrient regimes

Iron absorption is not simply diffusion, but results in the internalization of iron by the marine phytoplankton. The iron internalization strategies are highly species-dependent (*Völker and Wolf-Gladrow, 1999*). In the marine environment, eukaryotic phytoplankton utilizes mainly a reductive strategy to absorb iron (*Shaked and Lis, 2012*). The rates of iron reduction are inversely proportional to the ratio of the stability constants of their Fe (III) and Fe(II) complexes. The stability constants of iron complexes in the medium are different for different species of
marine alga because the ligands of organically bound iron complexes are controlled by the excretion of marine phytoplankton (Wells and Trick, 2004). The activities of cell surface ferric reductases can be relevant to phytoplankton nutrition (Hutchins et al., 1998).

Iron absorption might be influenced by the species and concentration of Fe on the cellular surfaces (Li et al., 2013) (i.e., Fe adsorption, e.g. although the influences of N and P addition on Fe absorption and adsorption by T. weissflogii were the same, the bioactivity and cellular biochemical composition of the marine phytoplankton was different (e.g., Fe-transporter or transport systems for Fe (III) siderophore complexes). Because the effect of P addition on Fe absorption and adsorption was alga-specific, the addition of P could affect the Fe internalization strategy.

3.6 Total sorption under different nutrient regimes

Total Fe uptake by marine phytoplankton, including Fe absorption and adsorption by all of the algal cells, is important for depletion of Fe in seawater. Total Fe uptake, adsorption, and absorption by all T. weissflogii and S. costatum cells under different nutrient regimes are shown in Fig. 3. With increasing N concentration from 8 to 32 µmol L⁻¹, the total Fe uptake, adsorption, and absorption by S. costatum cells were more than that by T. weissflogii cells. It was mainly due to the difference between the cell densities achieved by S. costatum and T. weissflogii. The influence of the species of marine phytoplankton, including the diatom species, on the depletion of Fe in seawater was obvious. Total Fe uptake by T. weissflogii cells was increased with increasing N concentration from 8 to 32 µmol L⁻¹. A similar result was observed in S. costatum cells, that was, total Fe uptake was increased with increasing N concentration from 8 to 32 µmol L⁻¹ and decreased with increasing P concentration from 1 to 2.5 µmol L⁻¹. Macronutrient enrichment in coastal ecosystems could cause an increase in the depletion of Fe in seawater by
the non-toxic coastal diatom (Li et al., 2013).

Total adsorption and absorption by all *T. weissflogii* and *S. costatum* cells was increased with increasing N concentration from N concentration from 8 to 32 µmol L\(^{-1}\). With increasing P concentration from 1.5 to 2.5 µmol L\(^{-1}\), total adsorption and absorption by all both *T. weissflogii* and *S. costatum* cells was decreased.

The *P*-value was 0.530 for total iron uptake, 0.0348 for total iron adsorption, and 0.541 for total iron absorption, so the influence of different species of marine alga on the total adsorption was statistically significant, but total iron uptake and absorption was statistically non-significant. While the influence of different concentrations of macronutrient on total iron uptake, adsorption, and absorption was statistically non-significant, because the *P*-value 0.858 for total iron uptake, 0.268 for total iron adsorption, and 0.855 for total iron absorption.

### 3.7 Distribution of iron species in seawater under different nutrient regimes

Marine algae produce extracellular organic compounds including polysaccharides, proteins, peptides, and small organic acids (Zuo and Hoigné, 1992; Chen and Wang, 2001). Some of these organic molecules may form stable complexes with iron. Several studies have indeed observed extremely high conditional stability constants (log \(K_{FeL}=20\sim23\)) for iron and organic ligands during an algal bloom, especially in the final stage of a phytoplankton bloom in estuarine and coastal waters at similar salinities (Gobler et al., 2002; Rose and Waite, 2003; Rijkenberg et al., 2006). On the other hand, macronutrient addition may affect the production of extracellular organic compounds and their composition by marine algae, and subsequently influence the complex formation, speciation and solubility of iron (Li et al., 2013). Thus, it is important to examine the effects of macronutrients on the solubility of iron in the algae cultures. To test these effectes, \(Fe_2(SO_4)_3\) salt was added into the culture medium as a compensating daily addition.
The results are shown in Table 1.

With N concentration from 8 to 32 \( \mu \text{mol L}^{-1} \), its influence on the concentration of colloidal and particulate Fe from \( T. \) weissflogii was similar, i.e., decreasing for colloidal Fe and particulate Fe. The effect of P addition from 1.5 to 2.5 \( \mu \text{mol L}^{-1} \) on the concentration of dissolved and particulate Fe from \( T. \) weissflogii was reverse. However, the concentration of dissolved and colloidal Fe from \( T. \) weissflogii was similar when N concentration from 8 to 64 \( \mu \text{mol L}^{-1} \). The three species of Fe from \( S. \) costatum was increased with increasing P concentration from 1.5 to 2.5 \( \mu \text{mol L}^{-1} \).

According to \( P \)-value analysis, the influence of algal species on dissolved Fe was extremely statistically significant (\( P=1.92 \times 10^{-5} \)), and on colloidal Fe was also statistically significant (\( P=0.0162 \)), but on particulate Fe was statistically non-significant. However, the influence of macronutrient addition on the three iron species was statistically non-significant. The data is shown in Table 2.

There were significant inter-species relationships between different algal species, although these patterns were not always consistent (Table 3). There was a significantly positive correlation between colloidal and particulate Fe from \( S. \) costatum (\( r=0.995 \)). Dissolved and colloidal/particulate Fe from \( S. \) costatum (\( r=0.883/0.873 \)) were also positively correlated in different macronutrient additions. In contrast, between dissolved and particulate Fe from \( T. \) weissflogii had negatively correlations with macronutrient addition.

3.8 Influence of the additions of macronutrient and different species of iron on the growth of \( T. \) weissflogii and \( S. \) costatum

With the addition of macronutrient, eight species of Fe, including Fe(II)-EDTA, Fe(OH)\(_3\), and dissolved, colloidal and particulate Fe from the cultured medium of \( S. \) costatum or \( T. \)
weissflogii, were used to inquire their combined effect on the growth of *T. weissflogii* and *S. costatum* for understanding the interspecific competition between *S. costatum* and *T. weissflogii*. The results are shown in Figs. 4 and 5.

All species of Fe could be bio-available for *S. costatum* or *T. weissflogii*. The growth of *T. weissflogii* and *S. costatum*, including the cell density and growth period, was affect by the addition of macronutrient, the size fractions and the source of Fe. Under all of nutrient regimes studied by our experiments, the influence of Fe(OH)$_3$ on *T. weissflogii* and *S. costatum* growth was the severest in eight species of Fe, the second was Fe(III)-EDTA; the growth of *T. weissflogii* could be limited by all dissolved, colloidal and particulate Fe from *S. costatum*. Except at the concentration of N $8 \mu$mol L$^{-1}$ and P $2.5 \mu$mol L$^{-1}$, the influence of particulate Fe from *S. costatum* on the growth of *T. weissflogii* was the severest in six species of Fe from *T. weissflogii* and *S. costatum*, followed by dissolved and colloidal Fe. So, the growth of *T. weissflogii* could be limited by the coexistence of *S. costatum*. The lack of growth by *T. weissflogii* in *S. costatum* medium could be released by the addition of dissolved, colloidal and particulate Fe, so it was caused by the unavailable Fe, not by some other substance excreted into the medium by *S. costatum*. Under high P ($\geq 1.5 \mu$mol L$^{-1}$) regimes, as a source of Fe, colloidal Fe from *T. weissflogii* was the best species for the growth of *T. weissflogii*, followed by particulate and dissolved Fe from *T. weissflogii*, i.e., the secretions from *T. weissflogii* could enhance its growth; but under low N ($8 \mu$mol L$^{-1}$) regimes, such self-enhanced effect was not obvious.

Under different macronutrient regimes, the growth trends of *S. costatum* were similar to *T. weissflogii* but all the cell densities were high more than *T. weissflogii*. The dissolved, colloidal and particulate Fe from *T. weissflogii* could also limit the growth of *S. costatum*, even the
particulate Fe was depressed obviously, but the particulate Fe from itself was promoted inversely, it might be due to the particulate Fe could enrich the macronutrient from seawater. After 4 days culture, the growth of *S. costatum* still didn’t reach stationary phase under low P (1 $\mu$mol L$^{-1}$) regimes; but under high P ($\geq$1.5 $\mu$mol L$^{-1}$) regimes, the growth of *S. costatum* entered the stationary phase after 3 days culture for P 1.5 $\mu$mol L$^{-1}$, and 2 days culture for P 2.0 and 2.5 $\mu$mol L$^{-1}$.

All the dissolved, colloidal and particulate Fe from *T. weissflogii* and *S. costatum* were used as the sources of Fe. The percentages of dissolved Fe (PDI), colloidal Fe (PCI) or particulate Fe (PPI) over the total Fe (< 3.0 $\mu$m) in the culture medium of *T. weissflogii* and *S. costatum* under different N and P concentration ($\mu$mol L$^{-1}$) could be calculated from the results listed in Table 3.

For the mixed culture of *T. weissflogii* and *S. costatum* were done, the theoretic value of the total cell density of *T. weissflogii* and *S. costatum* (i.e. C1, C2, C3, and C4) under different macronutrient regimes could be calculated obtained from the cell density presented in Figs. 3 and 4 using dissolved, colloidal and particulate Fe. The influence of N and P on cell density of *T. weissflogii* and *S. costatum* was similar, and the growth was controlled by N/P ratio and the concentrations of N and P. However, the growth was affected by Fe speciation not obvious.

With N addition from 8.0 to 64.0 $\mu$mol L$^{-1}$, the value of C1/C2 was increased but C3/C4 was decreased; When P concentration was increased from 1.5 to 2.5 $\mu$mol L$^{-1}$, the value of C1/C2 was increased but the change of the value of C3/C4 wasn’t obvious. Hence, an alga-specific influence of N and P addition on cell density was observed. According to the value of C2/C1, C3/C4, C3/C1, C4/C2, and C3/C2, algal exudates could promote diatom growth itself, such promotion on *S. costatum* was more obvious than that on *T. weissflogii*, which would be beneficial to *S. costatum* during an interspecific competition. According to $P$-value analysis, iron species and
macronutrient addition could extremely significantly effect the growth of both T. weissflogii and S. costatum, and the influence of Fe species was more significant than macronutrient addition. The data is showed in Tables 4 and 5.

3.9 Effect of macronutrient additions on the interspecific competition between T. weissflogii and S. costatum

In coastal environment, S. costatum was coexistence with T. weissflogii, their cell density ratios were 5.57-7.03 times, indicating that S. costatum was more competitive than T. weissflogii. Fe was a key determinant for the interspecific competition, because: 1) under N addition from 8 µmol L\(^{-1}\) to 32µmol L\(^{-1}\), the adsorption and absorption of Fe per cell and total adsorption, absorption and uptake by S. costatum was higher than T. weissflogii; 2) P concentration higher than 1.5 µmol L\(^{-1}\), the absorption of Fe by T. weissflogii was more than S. costatum; 3) the species of Fe in seawater could be affected by the secretions of marine phytoplankton; and 5) all the dissolved, colloidal and particulate Fe from S. costatum and T. weissflogii were available by S. costatum.

According to P-value analysis, the influence of Fe species on algal cell density was more significant than macronutrient. As Fe complexing ligands, algal exudates can promote diatom growth itself and such promotion on S. costatum was more obvious than that on T. weissflogii. There were not significant correlation between cell density ratio and iron requirement, including iron adsorption and absorption per cell, iron adsorption and absorption by all algal cells. Iron species was a key determinant on interspecific competition of coastal diatom, and the degree of bioavailability was described as follows: dissolved iron from own exudates > colloidal iron from own exudates > particulate iron from own exudates > particulate iron from another algal exudates > colloidal iron from another algal exudates >dissolved iron from another algal
Acknowledgment

This work was supported by the National Natural Science Foundation of China (41206096, 40506020, and 21175115), the Program for New Century Excellent Talents in University (NCET-11 0904), Outstanding Youth Science Foundation of Fujian Province, China (2010J06005) and the Science & Technology Committee of Fujian Province, China (2012Y0065).

References


Table 1. Influence of N and P addition on the distribution of Fe species in seawater by coastal alga

### T. weissflogii

<table>
<thead>
<tr>
<th>Fe species</th>
<th>N and P concentration (µmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8:2.5</td>
</tr>
<tr>
<td>Dissolved</td>
<td>42.0±0.4</td>
</tr>
<tr>
<td>Colloidal</td>
<td>17.4±0.1</td>
</tr>
<tr>
<td>Particulate</td>
<td>6.15±0.1</td>
</tr>
</tbody>
</table>

### S. costatum

<table>
<thead>
<tr>
<th>Fe species</th>
<th>N and P concentration (µmol L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8:2.5</td>
</tr>
<tr>
<td>Dissolved</td>
<td>17.6±0.1</td>
</tr>
<tr>
<td>Colloidal</td>
<td>13.2±0.1</td>
</tr>
<tr>
<td>Particulate</td>
<td>19.9±0.1</td>
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Table 2 Statistically significant analysis of variance by distribution of Fe species under different algal species and macronutrient addition.

<table>
<thead>
<tr>
<th>Cell density</th>
<th>P-value</th>
<th>Algal species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N:P</td>
<td></td>
</tr>
<tr>
<td>Dissolved</td>
<td>0.0780</td>
<td>1.92×10⁻⁵</td>
</tr>
<tr>
<td>Colloidal</td>
<td>0.127</td>
<td>0.0162</td>
</tr>
<tr>
<td>Particulate</td>
<td>0.467</td>
<td>0.481</td>
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</table>
Table 3 Correlations between Fe species under different algal species and macronutrient addition.

<table>
<thead>
<tr>
<th></th>
<th>T. weissflogii</th>
<th>S. costatum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dissolved</td>
<td>Colloidal</td>
</tr>
<tr>
<td>Dissolved</td>
<td>0.191</td>
<td>-0.297</td>
</tr>
<tr>
<td>T. weissflogii</td>
<td>Colloidal</td>
<td>0.555</td>
</tr>
<tr>
<td>Particulate</td>
<td></td>
<td>0.0542</td>
</tr>
<tr>
<td>S. costatum</td>
<td>Dissolved</td>
<td></td>
</tr>
<tr>
<td>Colloidal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Theoretic value of the cell density of *T. weissflogii* and *S. costatum* under different nutrient regimes. Data are mean ± SD (n=3).

<table>
<thead>
<tr>
<th>N and P concentration (μmol L⁻¹)</th>
<th>( T.\text{weissflogii} )</th>
<th><em>S. costatum</em></th>
<th>Cell density ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe from</td>
<td>C1</td>
<td>Fe from</td>
</tr>
<tr>
<td>8:1</td>
<td>56.7±0.6</td>
<td>47.0±0.5</td>
<td>208±1.2</td>
</tr>
<tr>
<td>16:1</td>
<td>77.4±0.8</td>
<td>59.7±0.6</td>
<td>264±1.3</td>
</tr>
<tr>
<td>32:1</td>
<td>119±1.1</td>
<td>79.7±0.8</td>
<td>420±1.2</td>
</tr>
<tr>
<td>64:1</td>
<td>110±1.0</td>
<td>66.0±0.7</td>
<td>371±1.3</td>
</tr>
<tr>
<td>8:1.5</td>
<td>43.0±0.5</td>
<td>32.7±0.4</td>
<td>169±1.2</td>
</tr>
<tr>
<td>8:2</td>
<td>33.4±0.4</td>
<td>22.6±0.4</td>
<td>152±1.3</td>
</tr>
<tr>
<td>8:2.5</td>
<td>30.1±0.4</td>
<td>16.7±0.4</td>
<td>126±1.0</td>
</tr>
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</table>
Table 5 Statistically significant analysis of variance by the cell density of *T. weissflogii* and *S. costatum* under different culture medium (C1 and C2 refer to cell density of *T. weissflogii* that Fe from *T. weissflogii* and *S. costatum*, respectively; C3 and C4 refer to cell density of *S. costatum* that Fe from *T. weissflogii* and *S. costatum*, respectively).  

<table>
<thead>
<tr>
<th>Cell density</th>
<th>N:P</th>
<th>Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. weissflogii</em></td>
<td>5.56×10^{-12}</td>
<td>4.53×10^{-12}</td>
</tr>
<tr>
<td><em>S. costatum</em></td>
<td>2.81×10^{-6}</td>
<td>8.98×10^{-9}</td>
</tr>
<tr>
<td>C1-C2</td>
<td>1.73×10^{-3}</td>
<td>9.39×10^{-3}</td>
</tr>
<tr>
<td>C1-C3</td>
<td>6.66×10^{-2}</td>
<td>9.01×10^{-4}</td>
</tr>
<tr>
<td>C2-C4</td>
<td>2.85×10^{-1}</td>
<td>9.03×10^{-4}</td>
</tr>
<tr>
<td>C3-C4</td>
<td>4.09×10^{-3}</td>
<td>1.19×10^{-3}</td>
</tr>
</tbody>
</table>
Fig. 1. Influence of the additions of nitrate and phosphate on growth of *T. weissflogii* and *S. costatum*. Data are mean ± SD (*n*=3). (Fe concentration 1.8 µmol L⁻¹)

Fig. 2. Influence of N and P concentration on Fe adsorption and absorption by the cells of *T. weissflogii* and *S. costatum*. Data are mean ± SD (*n*=3).

Fig. 3. Influence of the concentration of N and P on total Fe uptake, absorption, and adsorption by coastal alga A (*T. weissflogii*) or B (*S. costatum*). Data are mean ± SD (*n*=3).

Fig. 4. Influence of the additions of nitrate, phosphate and different species of Fe on growth of *T. weissflogii*. The coastal alga A and B is *T. weissflogii* and *S. costatum*, respectively. Data are mean ± SD (*n*=3). (Fe concentration 1.8 µmol L⁻¹)

Fig. 5. Influence of the additions of nitrate, phosphate and different species of Fe on growth of *S. costatum*. The coastal alga A and B is *T. weissflogii* and *S. costatum*, respectively. Data are mean ± SD (*n*=3). (Fe concentration 1.8 µmol L⁻¹)
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