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 km^2) 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-HNO_3) 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 7500cx inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, USA) multi-element standards (10 mg L^{-1}, Nos. 2A, USA) and internal standards (including 100 mg L^{-1} ^{45}Sc, ^{72}Ge, ^{103}Rh, ^{115}In, and ^{209}Bi) were used for trace elements determination (Li et al., 2013). $NaNO_3$, Na_2HPO_4, $Fe_2(SO_4)_3$, EDTA-Na_2, NH_4Cl, NaCl, H_2O_2, ammonium molybdate, ascorbic acid, sulfanilic acid, and N-(1-naphthyl) ethylenediamine dihydrochloride were analytical reagent grade (Sigma, USA). HCl and HNO_3

were TMG (Fisher Scientific, USA). N, P and Fe standard solutions were prepared from stock standard solutions of $NaNO_3$ (N, 10 mM), Na_2HPO_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 was 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 ℃ for 15 min. Immediately, while still hot, the solution was transferred to a 250 mL Telfon 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

105 extracted again with 4 mL of 1, 2-dichloroethane. In each extraction, the organic phase was
106 discharged and aliquots of the reagent were collected. The clean oxalate solution was then
107 transferred to LDPE bottle.

108 *2.2 Instrumentation*

109 Agilent ICP-MS (Agilent Technologies, USA) was used for metal content in samples. The
110 operational parameters applied were listed in Table 1 (Shraim et al., 2011).WHG-102A2-based
111 flow injection hydride generator (John Manufacturing, Beijing, China) was used to measure the
112 N and P concentrations. MK-III-based fiber optic pressure controlled closed microwave digestion
113 system (Shanghai Branch Microwave Digestion Test New Technology Institute, China) was used
114 to microwave digested trace metals test sample. The UV-3200PCS UV-Vis spectrophotometer,
115 provided by Shanghai Spectrum Instruments Co., Ltd. (China) was used to determinate the
116 absorbance of test sample. The double-sided clean bench was purchased from Suzhou
117 Purification Equipment Co., Ltd. (China) and used to alga vaccination. The SPX-300 IC
118 Microcomputer artificial climate chamber (Shanghai Bo Xun Industrial Co., China) was used to
119 culture the algal medium. The Leica DM LB2 microscope Leica (Leica Instruments, Germany)
120 was used to count algal density.

121 *2.3 Seawater Sample*

122 Coastal seawater was collected from Zhangzhou Bay, Fujian Province, China. The salinity
123 of the seawater was 33.1±0.05 psu. The N and P concentrations were measured three times using
124 a flow injection analyzer (FIA) (Li et al., 2014; Zuo and Deng, 1998), and the background
125 concentrations were 47.5 μmol L^{-1} for N (as nitrate) and 0.250 μmol L^{-1} for P (as reactive P). The
126 background concentrations of Fe were 0.40 μmol L^{-1} measured by ICP-MS, similar to that
127 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 remoted from anthropogenic activities.

All the seawater was stored at 4℃ 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 *S. costatum* and *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 $Na_2SiO_3 \cdot H_2O$, but without trace metals) at different N (added as $NaNO_3$) and P (added as Na_2HPO_4) concentrations with different species of Fe at 19℃ 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

151 illuminaton on the surface and the bottom of marine phytoplankton suspension could be ignored.

152 *2.5 Iron absorption and adsorption by the bloom-forming species under different nutrient*
153 *regimes*

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

166 After cultured for 4 days, iron absorption, adsorption, and uptake by the diatom species, *S.*
167 *costatum* and *T. weissflogii* were measured, and the cell density was counted microscopically.
168 The cell diameter of *S. costatum* was 6-18 μm. The cell length and width of *T. weissflogii* was
169 15-22 μm and 9-14 μm, respectively. The cells contained in 600 mL of the medium were
170 collected on a 3.0 μm membrane filter, rinsed with clean natural seawater with 0.16 nmol L^{-1} Fe
171 twice, resuspended into 25 mL of trace metals clean reagent, stirred for 1 h to remove
172 surface-bound Fe, and filtered on a 3.0 μm membrane filter. The filtrate was added into a closed
173 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. 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 (Kannamkumarath et al., 2004; Zhang and Katayama, 2012). 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 data for the ultrafilters used in this study were in the range 92-110%. The recovery was calculated as:

R% = {((particulate Fe) + (colloidal Fe) + (dissolved Fe))/ (total Fe in culture medium)} ×100

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 (HNO_3:H_2O_2, v : v=2:1), microwave digested for 7 min at 1.01×10^6 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 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 0.165 nmol 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 g^{-1}) and NASS- 5 (0.240±0.035 ng g^{-1}). The method described was applicable for the determination of low levels (ng g^{-1} or μg L^{-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^{-1} could stimulate cell growth and such stimulating effect was the most significant at 32.0 μmol L^{-1} N. However, algal growth could be inhibited by 64.0 μmol L^{-1} N, the influence trends were reverse. When P concentrations from 1.0 to 2.0 μmol L^{-1}, the influence on growth of *T. weissflogii* and *S. costatum* wasn't significant, but under high concentration of P (>2.0 μmol L^{-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^{-1}, Fe adsorption by *T. weissflogii* per cell was increased with increasing macronutrient concentration of N concentration from 8 to 32 μmol L^{-1}. The minimum (0.07 fmol $cell^{-1}$) and the maximum (1.96 fmol $cell^{-1}$) adsorption of Fe was observed at concentrations of 8

243 μmol L^{-1} N/2.5 μmol L^{-1} P and 32 μmol L^{-1} N/1 μmol L^{-1} P, respectively. The maximum
244 adsorption was 28 times of the minimum. The influence of macronutrient concentration on the Fe
245 adsorption by *S. costatum* per cell was more significant. The maximum adsorption (7.74 fmol
246 $cell^{-1}$) was 38.7 times of the minimum (0.2 fmol $cell^{-1}$). Thus, the influence of macronutrient
247 addition on the adsorption of Fe was obviously dependent on algal species.

248 Marine algae adsorb and coordinate Fe with basic functional groups on their cell surface
249 (Zuo and Hoigné, 1993; Li et al., 2009). Iron adsorption by *S. costatum* per cell was higher than
250 that *T. weissflogii* per cell under various macronutrient regimes, although the surface area *S.*
251 *costatum* per cell (28-254 μm^2) was less than that of *T. weissflogii* (154-804 μm^2). The Fe
252 speciation in the culture solution was controlled by the species of marine phytoplankton and the
253 concentrations of N and P. The amount of basic functional groups on the cell's surface and the
254 cell size are both affected by the concentrations of N and P (Zuo and Hoigné, 1992; Liu et al.,
255 2014). Iron diffusion decrease (or increase) with decreasinge (or increasing) cell size,
256 respectively. The degree of influence of macronutrient additions on the cell size of *S. costatum*
257 was more significant than that of *T. weissflogii*, so the effect of macronutrient additions on Fe
258 adsorption by *S. costatum* was more obvious than that by *T. weissflogii*. Fe adsorption was most
259 likely to be affected by the following five factors: 1) the amount of surface basic groups on the
260 cell surface, 2) the cell size, 3) the species and concentration of Fe, 4) the affinity constant
261 between Fe and surface basic groups on the cell surface, and 5) the concentrations of N and P
262 through their effects on the above four factors.

263 *3.4 Influence of N and P addition on iron absorption by the cells of T. weissflogii and S. costatum*

264 Different species of marine algae have different, growth rate and biochemical composition
265 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 $\geq$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

289 and Wolf-Gladrow, 1999). In the marine environment, eukaryotic phytoplankton utilizes mainly a
290 reductive strategy to absorb iron (Shaked and Lis, 2012). The rates of iron reduction are
291 inversely proportional to the ratio of the stability constants of their Fe (III) and Fe(II) complexes.
292 The stability constants of iron complexes in the medium are different for different species of
293 marine alga because the ligands of organically bound iron complexes are controlled by the
294 excretion of marine phytoplankton (Wells and Trick, 2004). The activities of cell surface ferric
295 reductases can be relevant to phytoplankton nutrition (Hutchins et al., 1998).

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

303 *3.6 Total sorption under different nutrient regimes*

304 Total Fe uptake by marine phytoplankton, including Fe absorption and adsorption by all of
305 the algal cells, is important for depletion of Fe in seawater. Total Fe uptake, adsorption, and
306 absorption by all *T. weissflogii* and *S. costatum* cells under different nutrient regimes are shown
307 in Fig. 3. With increasing N concentration from 8 to 32 μmol L^{-1}, the total Fe uptake, adsorption,
308 and absorption by *S. costatum* cells were more than that by *T. weissflogii* cells. It was mainly due
309 to the difference between the cell densities achieved by *S. costatum* and *T. weissflogii*. The
310 influence of the species of marine phytoplankton, including the diatom species, on the depletion
311 of Fe in seawater was obvious. Total Fe uptake by *T. weissflogii* cells was increased with

312 increasing N concentration from 8 to 32 μmol L^{-1}. A similar result was observed in *S. costatum*
313 cells, that was, total Fe uptake was increased with increasing N concentration from 8 to 32 μmol
314 L^{-1} and decreased with increasing P concentration from 1 to 2.5 μmol L^{-1}. Macronutrient
315 enrichment in coastal ecosystems could cause an increase in the depletion of Fe in seawater by
316 the non-toxic coastal diatom (Li et al., 2013).

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

321 The *P*-value was 0.530 for total iron uptake, 0.0348 for total iron adsorption, and 0.541 for
322 total iron absorption, so the influence of different species of marine alga on the total adsorption
323 was statistically significant, but total iron uptake and absorption was statistically non-significant.
324 While the influence of different concentrations of macronutrient on total iron uptake, adsorption,
325 and absorption was statistically non-significant, because the *P*-value 0.858 for total iron uptake,
326 0.268 for total iron adsorption, and 0.855 for total iron absorption.

327 *3.7 Distribution of iron species in seawater under different nutrient regimes*

328 Marine algae produce extracellular organic compounds including polysaccharides, proteins,
329 peptides, and small organic acids (Zuo and Hoigné, 1992; Chen and Wang, 2001). Some of these
330 organic molecules may form stable complexes with iron. Several studies have indeed observed
331 extremely high conditional stability constants (log *K*FeL=20~23) for iron and organic ligands
332 during an algal bloom, especially in the final stage of a phytoplankton bloom in estuarine and
333 coastal waters at similar salinities (Gobler et al., 2002; Rose and Waite, 2003; Rijkenberg et al.,
334 2006). On the other hand, macronutrient addition may affect the production of extracellular

335 organic compounds and their composition by marine algae, and subsequently influence the
336 complex formation, speciation and solubility of iron (Li et al., 2013). Thus, it is important to
337 examine the effects of macronutrients on the solubility of iron in the algae cultures. To test these
338 effectes, $Fe_2(SO_4)_3$ salt was added into the culture medium as a compensating daily addition.
339 The results are shown in Table 2.

340 With N concentration from 8 to 32 μmol L^{-1}, its influence on the concentration of colloidal
341 and particulate Fe from *T. weissflogii* was similar, i.e., decreasing for colloidal Fe and particulate
342 Fe. The effect of P addition from 1.5 to 2.5 μmol L^{-1} on the concentration of dissolved and
343 particulate Fe from *T. weissflogii* was reverse. However, the concentration of dissolved and
344 colloidal Fe from *T. weissflogii* was similar when N concentration from 8 to 64 μmol L^{-1}. The
345 three species of Fe from *S. costatum* was increased with increasing P concentration from 1.5 to
346 2.5 μmol L^{-1}.

347 According to *P*-value analyis, the influence of algal species on dissolved Fe was extremely
348 statistically significant (P=1.92×10^{-5}), and on colloidal Fe was also statistically significant
349 (P=0.0162), but on particulate Fe was statistically non-significant. However, the influence of
350 macronutrient addition on the three iron species was statistically non-significant. The date is
351 shown in Table 3.

352 There were significant inter-species relationships between different algal species, although
353 these patterns were not always consistent (Table 4). There was a significantly positive
354 correlation between colloidal and particulate Fe from *S. costatum* (r= 0.995). Dissolved and
355 colloidal/particulate Fe from *S. costatum* (r= 0.883/0.873) were also positively correlated in
356 different macronutrient additions. In contrast, between dissolved and particulate Fe from *T.*
357 *weissflogii* had negatively correlations with macronutrient addition.

358 *3.8 Influence of the additions of macronutrient and different species of iron on the growth of T.*
359 *weissflogii and S. costatum*

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

365 All species of Fe could be bio-available for *S. costatum* or *T. weissflogii*. The growth of *T.*
366 *weissflogii* and *S. costatum*, including the cell density and growth period, was affect by the
367 addition of macronutrient, the size fractions and the source of Fe. Under all of nutrient regimes
368 studied by our experiments, the influence of $Fe(OH)_3$ on *T. weissflogii* and *S. costatum* growth
369 was the severest in eight species of Fe, the second was Fe(Ⅲ)-EDTA; the growth of *T.*
370 *weissflogii* could be limited by all dissolved, colloidal and particulate Fe from *S. costatum*.
371 Except at the concentration of N 8 μmol L^{-1} and P 2.5 μmol L^{-1}, the influence of particulate Fe
372 from *S. costatum* on the growth of *T. weissflogii* was the severest in six species of Fe from *T.*
373 *weissflogii* and *S. costatum*, followed by dissolved and colloidal Fe. So, the growth of *T.*
374 *weissflogii* could be limited by the coexistence of *S. costatum*. The lack of growth by *T.*
375 *weissflogii* in *S. costatum* medium could be released by the addition of dissolved, colloidal and
376 particulate Fe, so it was caused by the unavailable Fe, not by some other substance excreted into
377 the medium by *S. costatum*. Under high P ($\geq$1.5 μmol L^{-1}) regimes, as a source of Fe, colloidal
378 Fe from *T. weissflogii* was the best species for the growth of *T. weissflogii*, followed by
379 particulate and dissolved Fe from *T. weissflogii*, i.e., the secretions from *T. weissflogii* could
380 enhance its growth; but under low N (8 μmol 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 prometed 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 μmol L^{-1}) regimes; but under high P ($\geq$1.5 μmol L^{-1}) regimes, the growth of *S. costatum* entered the stationary phase after 3 days culture for P 1.5 μmol L^{-1}, and 2 days culture for P 2.0 and 2.5 μmol 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 μm) in the culture medium of *T. weissflogii* and *S. costatum* under different N and P concentration (μmol L^{-1}) could be calculated from the results listed in Table 4. 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 64.0μmol 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 μmol 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

404 of N and P addition on cell density was observed. According to the value of C2/C1, C3/C4,
405 C3/C1, C4/C2, and C3/C2, algal exudates could promote diatom growth itself, such promotion
406 on *S. costatum* was more obvious than that on *T. weissflogii*, which would be beneficial to *S.*
407 *costatum* during an interspecific competition. According to *P*-value analyis, iron species and
408 macronutrient addition could extremely significantly effect the growth of both *T. weissflogii* and
409 *S. costatum*, and the influence of Fe species was more significant than macronutrient addtion.
410 The data is showed in Tables 5 and 6.

411 *3.9 Effect of macronutrient additions on the interspecific competition between T. weissflogii and*
412 *S. costatum*

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

422 According to *P*-value analyis, the influence of Fe species on algal cell density was more
423 significant than macronutrient. As Fe complexing ligands, algal exudates can promote diatom
424 growth itself and such promotion on *S. costatum* was more obvious than that on *T. weissflogii*.
425 There were not significant correlation between cell density ratio and iron requirement, including
426 iron adsorption and absorption per cell, iron adsorption and absorption by all algal cells. Iron

427 species was a key determinant on interspecific competition of coastal diatom, and the degree of
428 bioavailability was described as follows: dissolved iron from own exudates > colloidal iron from
429 own exudates > particulate iron from own exudates > particulate iron from another algal
430 exudates > colloidal iron from another algal exudates >dissolved iron from another algal
431 exudates > Fe (III)-EDTA> $Fe(OH)_3$.

432 **Acknowledgment**

433 This work was supported by the National Natural Science Foundation of China (41206096,
434 40506020, and 21175115), the Program for New Century Excellent Talents in University
435 (NCET-11 0904), Outstanding Youth Science Foundation of Fujian Province, China
436 (2010J06005) and the Science & Technology Committee of Fujian Province, China
437 (2012Y0065).

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Table 1 ICP-MS operating parameters.

Instrument	
Sampler	7500cx
Skimmer	Ni (standard)
Nebulizer	Ni (standard)
Plasma torch	Micromist (standard)
Intergration time (s × points)	
Mg	0.05 × 3
Cr, Ni, As, Cd. Hg	1.00 × 3
Mn, Fe, Co, Cu, Zn, Rb, Sr, Ag, Sn, Ba, Pb	0.10 × 3
Se	5.00 × 3
Tune parameters	
RF power (W)	1500
Sample depth (mm)	7.6 (8.3 for Hg)
Carrier gas (L min^{-1})	0.95
Makeup gas (L min^{-1})	0.21
Extract 1(V)	4.4
Extract 2(V)	-89
Energy discrimination (V)	2
Reaction gas (He, mL min^{-1}): Mg, Cr, Mn, Fe, Co, Ni, Cu, Zn, and As. off for Hg, Rb, Sr, Ag, Cd, Sn, Ba, Hg, and Pb	4
% Oxide (156/140)	1.84
% Doubly charged (70/140)	3.88
% RSD for m/z: 7, 59, 89, 205	<2
Spray chamber temperature（℃）	2
Nebulizer pump (rps)	0.1

Table 2. Influence of N and P addition on the distribution of Fe species in seawater by coastal alga

T. weissflogii

Fe species	N and P concentration (μmol L^{-1})						
(fmol $cell^{-1}$)	8:2.5	8:2	8:1.5	8:1	16:1	32:1	64:1
Dissolved	42.0±0.4	37.8±0.3	24.1±0.2	34.4±0.3	43.7±0.4	29.7±0.3	30.9±0.3
Colloidal	17.4±0.1	20.9±0.2	12.7±0.1	16.1±0.1	4.05±0.1	1.43±0.1	6.43±0.1
Particulate	6.15±0.1	12.0±0.1	14.2±0.1	5.70±0.1	5.15±0.1	4.77±0.1	2.15±0.1

S. costatum

Fe species	N and P concentration (μmol L^{-1})						
(fmol $cell^{-1}$)	8:2.5	8:2	8:1.5	8:1	16:1	32:1	64:1
Dissolved	17.6±0.1	5.74±0.1	5.36±0.1	10.7±0.1	6.50±0.1	0.94±0.1	1.73±0.1
Colloidal	13.2±0.1	2.85±0.16	2.44±0.1	2.08±0.1	1.60±0.1	0.41±0.1	0.82±0.1
Particulate	19.9±0.1	3.86±0.1	3.51±0.1	2.24±0.1	3.61±0.1	0.45±0.1	0.96±0.1

Table 3 Statistically significant analysis of variance by distribution of Fe species under different algal species and macronutrient addition.

Cell density	*P-value*	
	N:P	Algal species
Dissolved	0.0780	1.92×10^{-5}
Colloidal	0.127	0.0162
Particulate	0.467	0.481

Table 4 Correlations between Fe species under different algal species and macronutrient addition.

		T.weissflogii			*S. costatum*		
		Dissolved	Colloidal	Particulate	Dissolved	Colloidal	Particulate
T.weissflogii	Dissolved		0.191	-0.297	0.564	0.476	0.520
	Colloidal			0.555	0.621	0.519	0.466
	Particulate				0.0542	0.0542	0.0387
S. costatum	Dissolved					0.883	0.873
	Colloidal						0.995

Table 5. Theoretic value of the cell density of *T.weissflogii* and *S. costatum* under different nutrient regimes. Data are mean ± SD (*n*=3).

	Cell density (10^3cell mL^{-1})				Cell density ratio					
	T.weissflogii		*S. costatum*							
N and P concentration (μmol L^{-1})	Fe from *T.weissflogii* C1	Fe from *S. costatum* C2	Fe from *T.weissflogii* C3	Fe from *S. costatum* C4	C1/C2	C3/C4	C3/C1	C4/C2	C3/C2	(C3+C4)/ (C1+C2)
8:1	56.7±0.6	47.0±0.5	208±1.2	447±1.0	1.21	2.15	3.67	9.52	4.43	6.32
16:1	77.4±0.8	59.7±0.6	264±1.3	499±1.5	1.30	1.89	3.41	8.36	4.42	5.57
32:1	119±1.1	79.7±0.8	420±1.2	723±1.4	1.50	1.72	3.52	9.07	5.27	5.74
64:1	110±1.0	66.0±0.7	371±1.3	607±1.2	1.67	1.64	3.38	9.20	5.62	5.57
8:1.5	43.0±0.5	32.7±0.4	169±1.2	347±0.8	1.32	2.05	3.93	10.6	5.17	6.82
8:2	33.4±0.4	22.6±0.4	152±1.3	223±1.1	1.48	1.47	4.55	9.88	6.73	6.70
8:2.5	30.1±0.4	16.7±0.4	126±1.0	203±1.0	1.80	1.61	4.19	12.2	7.55	7.03

Table 6 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;).

Cell density	*P-value*	
	N:P	Iron
T.weissflogii	5.56×10^{-12}	4.53×10^{-12}
S. costatum	2.81×10^{-6}	8.98×10^{-9}
C1-C2	1.73×10^{-3}	9.39×10^{-3}
C1-C3	6.66×10^{-2}	9.01×10^{-4}
C2-C4	2.85×10^{-1}	9.03×10^{-4}
C3-C4	4.09×10^{-3}	1.19×10^{-3}

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})

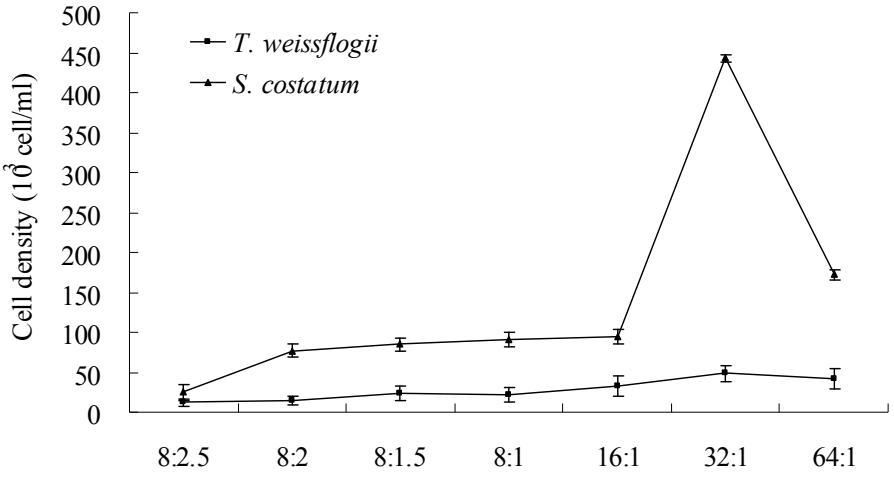

Fig. 1. Influence of the additions of nitrate and phosphate on growth of *T. weissflogii* and *S. costatum*. Data are mean ± SD (n=3). (Fe concentration 1.8 μmol L^{-1})

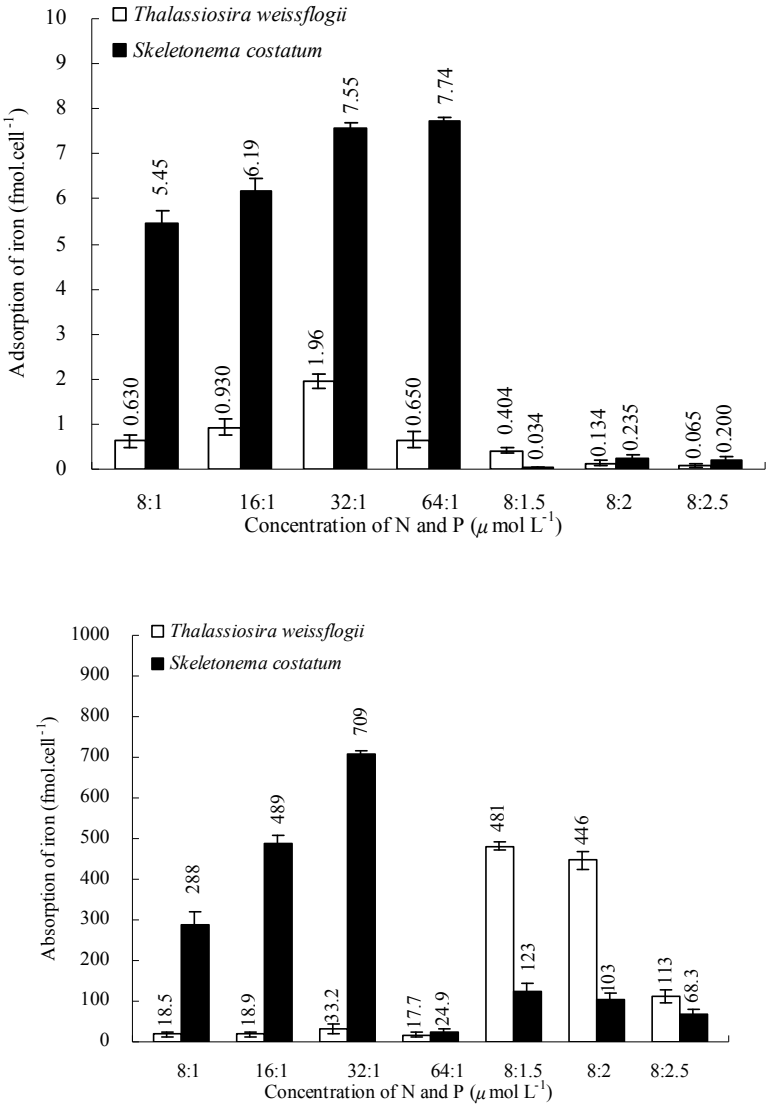

Fig. 2. Influence of N and P concentration on Fe adsorption and absorption by the cells of *T. weissflogii* and *S. costatum*. Data are mean ± SD (n=3).

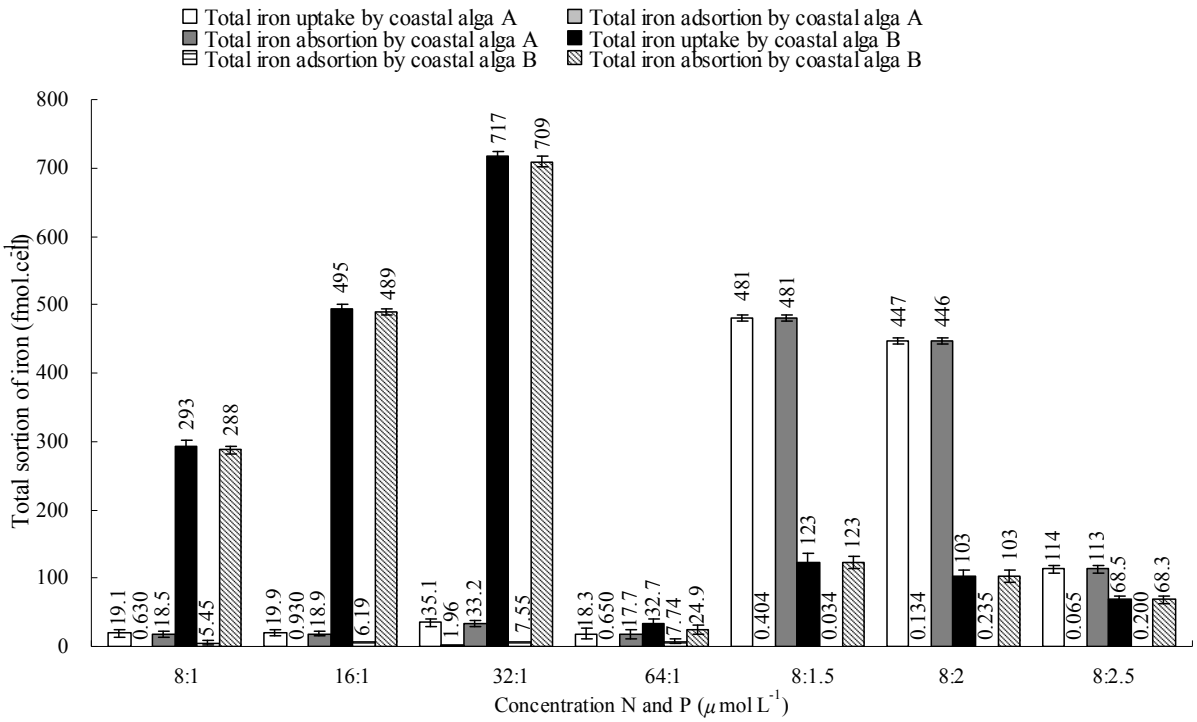

Fig. 3. Influence of the concentration of N and P on total Fe uptake, absorption, and adsorption by coastal alga A (*T. weissflogii*) or B (*S. costatum*). Data are mean ± SD (n=3).

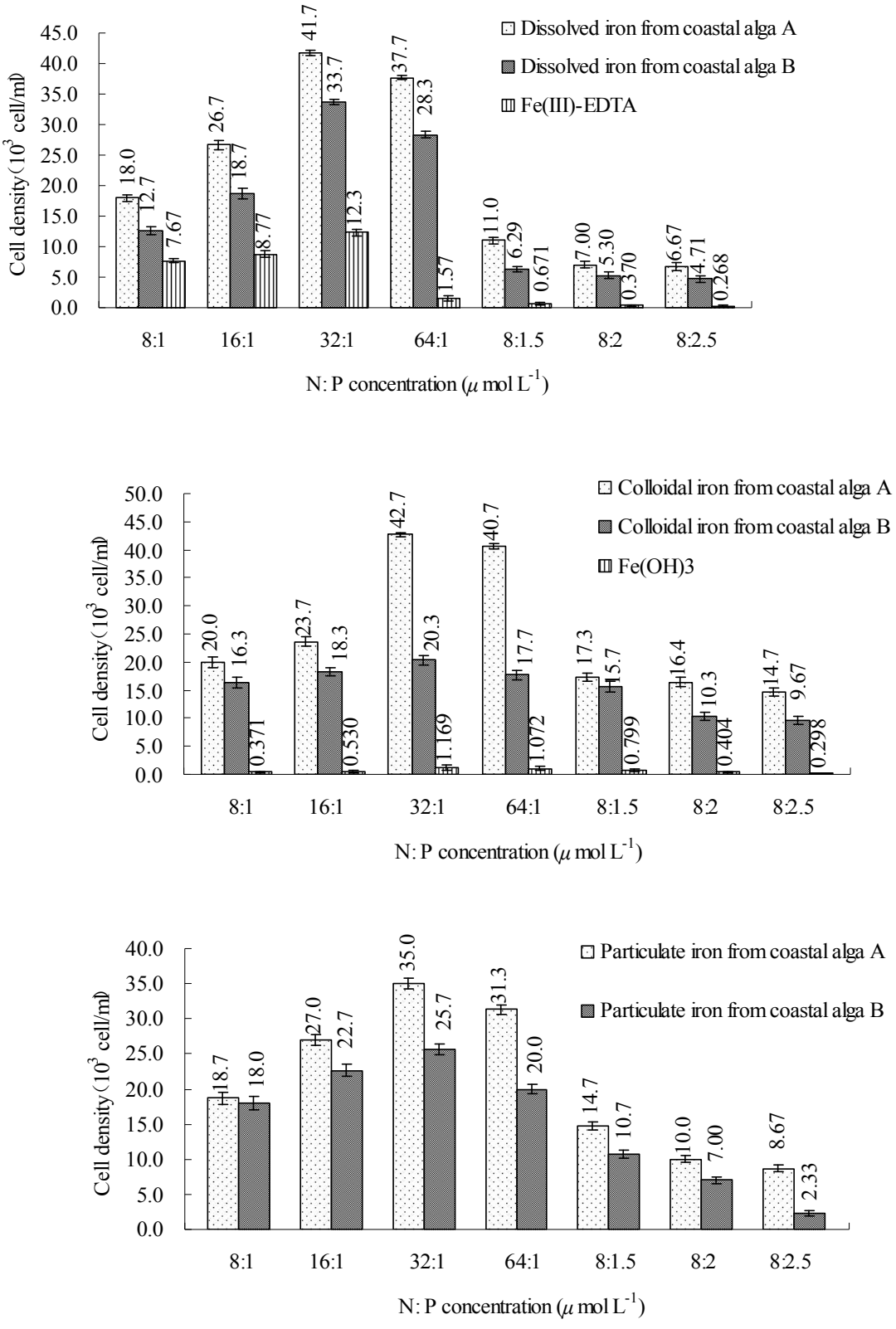

Fig. 4. Influence of the additions of nitrate, phosphate and different species of Fe on growth of *T. weissflogii*. The coastal alga A and B is *T. weissflogii* and *S. costatum*, respectively. Data are mean ± SD (n=3). (Fe concentration 1.8 μmol L^{-1})

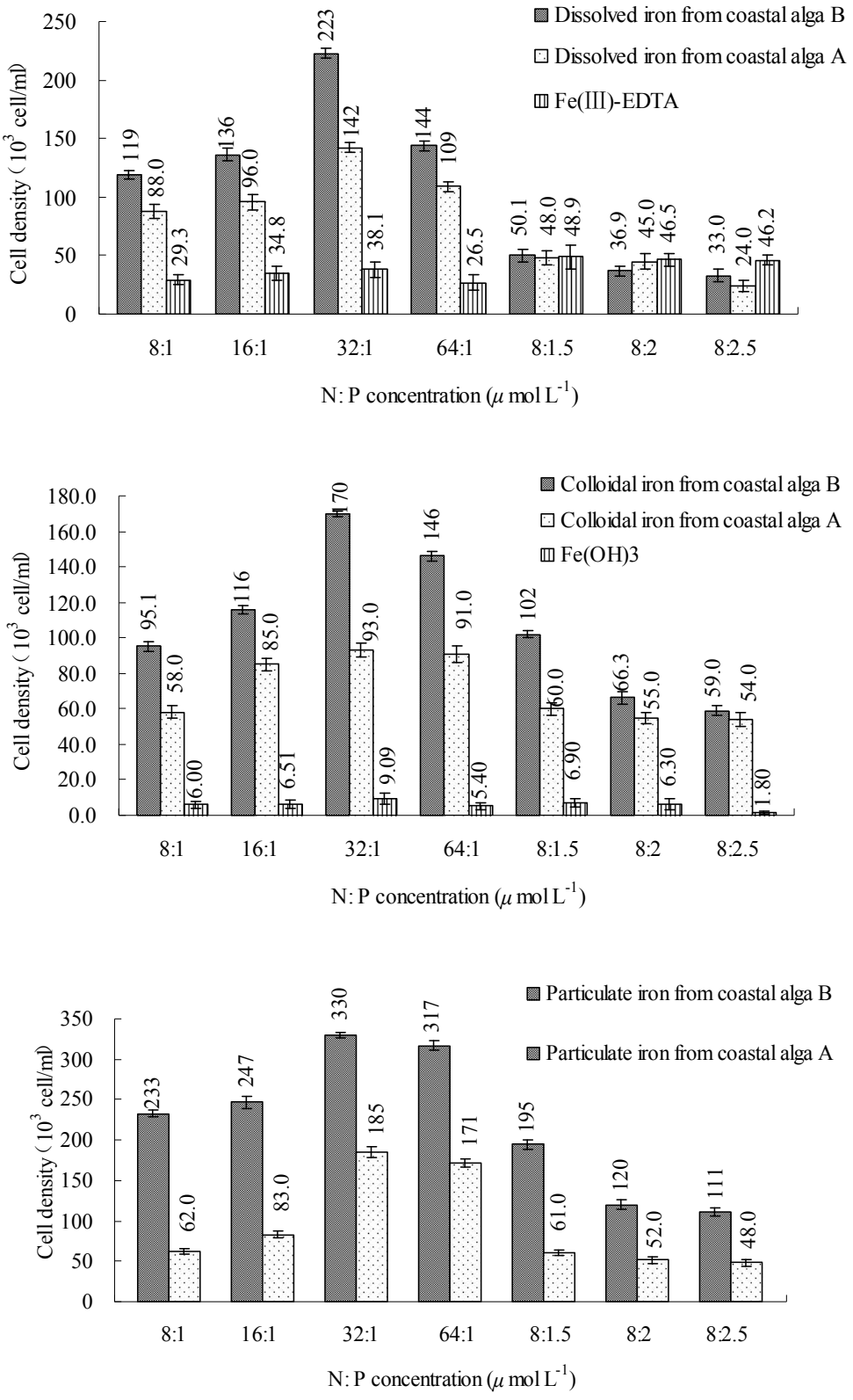

Fig. 5. Influence of the additions of nitrate, phosphate and different species of Fe on growth of *S. costatum.* The coastal alga A and B is *T. weissflogii* and *S. costatum*, respectively. Data are mean ± SD (n=3). (Fe concentration 1.8 μmol L^{-1})