

Copper addition helps alleviate iron stress in a coastal diatom: response of *Chaetoceros gracilis* from the Bay of Bengal to experimental Cu and Fe addition

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Abstract:

Copper (Cu) is a transition metal with multi-oxidation states, and though it plays numerous roles in vital physiological and biochemical pathways including both photosynthesis and respiration, it can potentially be toxic at high concentrations. Coastal waters receive significant Cu input from a variety of anthropogenic sources which may affect fundamental biological processes including phytoplankton growth. We investigated the responses of the coastal diatom *Chaetoceros gracilis* to variable Cu concentrations using a local isolate from the SW coastal Bay of Bengal. The results suggested that Cu acted as a growth-promoting factor up to concentrations of 125 nM Cu (growth-promoting range), and became inhibitory thereafter (growth-inhibiting range). The cells in the control treatments had high ratios of photoprotective to light harvesting pigments (PP:LH) and high BSi:Chl-*a*, both indicative of Fe stress. Within the growth-promoting range, an increase in Cu supply significantly increased Chl-*a* concentrations, and decreased the ratios of PP:LH and BSi:Chl-*a*. Interestingly, iron (Fe) supplemented cells of *C. gracilis* revealed similar responses. We speculate that *C. gracilis* may utilize Cu to enhance Fe acquisition when Fe levels are inadequate. However, in the presence of Fe, the growth response of *C. gracilis* to variable Cu concentrations was not significant. We infer that, under Fe sufficient conditions, the need for Cu is minimized. Our study suggests that Cu plays a significant role in the physiology of coastal diatoms beyond the simple toxicological effects often investigated.

Key words: diatom, *Chaetoceros gracilis*, Bay of Bengal, phytoplankton, copper, iron, diadinoxanthin, diatoxanthin.

1. Introduction:

Copper (Cu), a redox-active transition metal, plays a complex role in phytoplankton physiology. At higher concentrations, Cu may be toxic (Harrison et al., 1977; Brand et al., 1986, Rijstenbil and Wijnholds, 1991), while at lower concentrations Cu is vital for physiological processes including oxygen chemistry, photosynthetic (and respiratory) electron transport, and other cellular redox reactions (Stryer, 1988; Chadd et al., 1996; Raven et al., 1999). However, our knowledge of Cu requirements by marine phytoplankton and its role in their physiology is still meager. Some eukaryotic microorganisms possess a Cu-dependent Fe transport system with Cu-containing ferroxidase (Askwith and Kaplan, 1997, La Fontaine et al., 2002), including coastal and oceanic diatoms (Maldonado et al., 2006; Guo et al., 2010; Guo et al., 2012). Thus, when Fe is limiting, Cu requirements increase with decreasing Fe availability (Annett et al., 2008), to a point where Fe-starved cells cannot acquire enough Fe to support their growth if Cu is strongly limiting (reviewed by Van ho et al., 2002).

The concentrations of dissolved Fe in many oceanic areas are extremely low (0.05 -2.0 nM) (Martin and Fitzwater, 1988; de Baar and Jong, 2001). Photosynthesis, the principal physiological process in phytoplankton, utilizes almost 80% of the total cellular Fe quota, for the construction and maintenance of photosynthetic electron transport chains (Raven, 1999). For this reason, under Fe-limiting conditions, carbon fixation rates are strongly reduced. Additionally, a significant amount of Fe is also required both to metabolize nitrate (Maldonado and Price, 1996) and to synthesize Chl-*a* (Spiller et al., 1982). It is well documented that some major oceanic areas (Equatorial Pacific, Southern Ocean, subarctic Pacific) characterized with high nutrients but low chlorophyll (HNLC regions) have low primary productivity primarily because of Fe limitation (reviewed by Boyd et al., 2007). To cope with such extreme Fe-limiting conditions, marine phytoplankton have developed some physiological mechanisms to down-regulate their Fe utilization and up-regulate Fe acquisition (Maldonado and Price, 1996). Some open ocean phytoplankton decrease their Photosystem I to Photosystem II ratios in order to reduce their Fe requirement (Strzepek and Harrison, 2004). Fe-starved oceanic diatoms reduce their Fe containing proteins (Greene et al., 1992) and replace them with functionally equivalent non-Fe containing proteins; for example, replacement of ferredoxin by flavodoxin (La Roche et al., 1993) and Cu containing plastocyanin instead of Fe containing cytochrome C6 (Peers and Price, 2006).

However, unlike oceanic isolates, coastal phytoplankton are believed to thrive in a metal-enriched environment, and demonstrate high metal demands for growth. The existence of a specialized high-affinity Fe transport system in such coastal phytoplankton species is therefore debatable. Maldonado et al., (2006) and Guo et al., (2010) reported the presence of both High Affinity and Low affinity Fe Transport systems in the coastal diatom *Thalassiosira pseudonana*; their detailed study based on physiological, phylogenetic and genomic data demonstrated the essential role of Cu in the inducible Fe transport system of coastal marine diatoms. Thus, it is evident that the demand for Cu in both oceanic and coastal phytoplankton can be high under Fe-limiting conditions.

During the last few decades, extensive anthropogenic activities in the coastal areas have significantly increased dissolved Fe and Cu concentrations, resulting in an increasing incidence of possible toxicity. Simultaneously, nitrogen loading to coastal waters has also increased, possibly increasing the total demand for Fe in coastal areas. However, our present knowledge of phytoplankton trace metal physiology in these coastal areas is insufficient to establish whether trace metal concentrations can actually control major elemental cycling (i.e. carbon and nitrogen) in coastal waters. Virtually no phytoplankton isolate from these areas has been examined for the involvement of Cu in their physiology. To fill this gap, we investigated the effects of different Cu concentrations on the coastal diatom *Chaetoceros gracilis*, using a new isolate from the Visakhapatnam coast off eastern India.

The Visakhapatnam coast is a coastal embayment in the Bay of Bengal situated at 17°44'N and 83°23'E, almost 7 km north of the Visakhapatnam Port (Fig. 1). Extensive shipping and fishing activities take place in this area and the adjacent coastal water is expected to be enriched with trace metals. Earlier studies showed very high concentrations of both Cu and Fe in the close vicinity of the port, but with lower concentrations in the adjacent coastal area (Subba Rao, 1973). The region receives a large amount of freshwater discharge from the major monsoon-fed rivers in India, and stratification caused by low salinity as well as low light availability caused by high turbidity are thought to limit primary production in this region during the summer monsoon (Prasanna Kumar et al., 2010) despite high riverine nutrient fluxes into coastal areas. The phytoplankton community in this area is dominated by diatoms, primarily species of *Chaetoceros* and *Skeletonema* (Paul et al., 2007). We chose *Chaetoceros gracilis*, which blooms easily in response to nutrient enrichment.

2. Materials and methods:

2.1 Diatom cell culture:

Chaetoceros gracilis, a common coastal diatom, was isolated from the Visakhapatnam coast (Fig. 1) and grown in 0.2 µm-filtered sterile seawater (also collected from the coast) enriched with standard addition of the macronutrients: nitrate (N) (200 µM NO₃⁻), silicate (Si) (200 µM SiO₃⁻²) and phosphate (P) (15 µM PO₄⁻³). Diatom cells were grown in 1 L polycarbonate bottles (Nalgene, USA). Prior to utilization, all bottles were washed with 10% HCl (overnight), followed by multiple Milli-Q water (MQ) rinses, autoclaved and kept them inside ziplock plastic bags until utilized. The cultures were usually diluted every three days to a chlorophyll-*a* (Chl-*a*) concentration of ≈ 1µg L⁻¹ and allowed to grow until the chlorophyll concentration reached ≈10-15 µg L⁻¹. The cultures were grown inside a water bath under natural sunlight and exposed to natural -light period (12 h LT: 12 h DK). The highest growth rates were observed within 48 -62 hours post-inoculation.

2.2 Experimental setup:

Coastal water was collected from a small boat using pre-cleaned Niskin sampler and carried to the laboratory inside 20 L acid-cleaned (overnight) polycarbonate bottles (Nalgene). These water samples were first filtered through GF/F filters (to remove all particulate biomass) followed by another filtration using 0.2µm polycarbonate filters (to remove bacterial biomass). Initial nutrient concentrations were measured, and final nutrient levels for experiments were adjusted to the levels indicated above. In Experiment 1 (Ex1), diatom cultures were incubated at a wide range of Cu concentrations (15 – 500 nM). In addition to these, one control (CTR) was grown without any Cu addition. For the Cu stock solution, analytical quality CuCl₂, 2H₂O was procured from Merck chemicals (Germany) and dissolved in MQ water to achieve a stock solution of 10 mM. Further dilutions were performed before adding to the incubation bottles. No artificial chelating agents were used for the Cu solution. All pre-cleaned, autoclaved polycarbonate bottles were filled with freshly collected filtered sterilized coastal water, followed by nutrient and Cu addition, and the media were then set aside for 12 hours after mixing well.

In Experiment 2 (Ex2), another five treatments were conducted. In treatment 1 (T1) and 2 (T2) only 50 nM Cu and 200 nM Fe was added, respectively. For the next additional three treatments both Cu

and Fe were added in the following combinations: 50 nM Cu+200 nM Fe (T3), 100 nM Cu + 200 nM Fe (T4) and 150 nM Cu +200 nM Fe (T5) (Table 2). The added Fe was chelated with Ethylenediaminetetraacetic acid (EDTA). The analytical grade $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and EDTA disodium salt were procured from the Merck chemicals (Germany) and the Fe stock solution (400 μM) was prepared in 1:5 molar ratio (Fe:EDTA).

Background Cu or Fe concentrations were not measured during the study period. It should be noted that the control treatments were expected to have natural metal concentrations measured in the sampling area. Previous studies in this area have shown total dissolved concentrations of Cu and Fe of 25 ± 6 nM and 54.28 ± 1.38 nM, respectively (Rejomon et al., 2008; 2010). However, the total dissolved Fe or Cu concentration may not represent the bio-available fraction of these metals. Almost 80-99% of the dissolved Fe (Rue and Bruland, 1995) and 98% of dissolved Cu can be present bound to strong organic complexes (Moffet et al., 1990). Although inorganic dissolved metals are thought to be more bio-available, Maldonado et al., (1999) and Guo et al., (2010) have showed that organically bound Fe and Cu, respectively, could also be assimilated by phytoplankton. Hence, it is difficult to have an accurate estimate of the Fe/Cu availability in this study. However, for our experiment we chose a concentration range of 15-500 nM Cu and 200 nM Fe.

Once the bottles contained target concentrations of Cu and Fe, 1-5 mL of the exponentially growing cultures were added to each experimental bottle, resulting in an initial concentration of Chl-*a* $\approx 1 \mu\text{g L}^{-1}$. This step was performed on a clean bench with extreme care to avoid any trace metal contamination. The bottles were mixed gently and then transported to a water bath for further incubation. The incubated cultures were grown under normal day and light conditions (under natural sunlight, 12 h LT: 12 h DK) inside a water bath. Every day the bottles were mixed very gently at a particular time (10 am) to avoid any sedimentation of biomass within the bottles. Based on earlier growth rate measurements, sampling were executed during the maximum growth phase on day 3 of the incubation. All bottles were returned to the clean laboratory for further sampling and analysis.

2.3 Sample Analysis:

For total Chl-*a* determination, 100 mL of the experimental sample from each treatment was filtered onto GF/F filters and kept in -20°C until analysis. Later, filters were soaked overnight in 5 mL N,N-

Dimethyl formamide following the methodology of Suzuki and Ishimaru, (1990) and the fluorescence was measured using a fluorescence spectrophotometer from Varian (Carry Eclipse). Nutrients (N, Si and P) were analyzed using the standard colorimetric method of Strickland and Parsons, (1972). Chl-*a* based growth rate was calculated as $\mu = (t_2 - t_1)^{-1} \cdot \ln (N_2/N_1)$ where, μ is the net growth rate d^{-1} and N_1 and N_2 are the Chl-*a* concentrations at t_1 and t_2 , respectively.

For biogenic silica analysis, 100 mL of sample was filtered onto a 0.2 μm polycarbonate membrane filter (GTTP Millipore). The membrane filters were washed with 0.2 μm filtered seawater, kept inside individual plastic petri dishes after folding in quarter and oven dried at 60°C overnight. The filters were then digested in 0.2 N NaOH inside a water bath for 5 hours at 80°C. 0.2 N HCl was used to neutralize the sample and one aliquot of this solution (~ 1mL) was diluted to 25 mL (Paasche, 1973). Silicate concentrations were measured using the colorimetric methodology of Strickland and Parsons, (1972). An unused membrane filter was also digested as a sample blank.

For HPLC pigment analysis, 25-50 mL samples were filtered onto 25 mm GF/F filters using a vacuum pump (Rocker 600 from Tarsons, India) under minimum light to avoid any degradation of the pigments, keeping the pressure below 200 mb. Immediately after filtration the filter was folded kept at -20°C until analysis. Filters were then soaked overnight in 3-5 mL 90% acetone (HPLC grade from Merck chemicals) inside an amber colored bottle to prevent photo damage. A tissue homogenizer (Ultra Turrax, Germany) was used to break the cells, followed by cold centrifugation (Eppendorf, Germany). The supernatant was taken and mixed with a buffer composed of 28 mM aqueous tetrabutyl ammonium acetate (TBAA, AR Grade, Fluka) at pH 6.5 and methanol (GC Assay 99.7% pure, Merck) in 90:10 ratios. A HPLC from Agilent Technology (Series 1200) was used to quantify the pigments following the method proposed by Heukelem and Thomas (2001). The concentrations of pigments were calculated using the standard pigments procured from Sigma Aldrich (USA) and DHL (Netherlands).

Ex1 was repeated twice with different additional Cu concentrations included with the 16 core treatment concentrations (control, 25, 30, 40, 50, 70, 80, 100, 125, 150, 200, 250, 300, 350, 400 and 500 nM Cu). Variance in response of core treatments between the two experimental runs has been used here to calculate the average values and standard deviations, representing reproducibility of the results (Table 1). HPLC analysis was done for only the first run of Ex1, hence no replicates were available. The data from the Ex2 are reported with average and SD derived from the replicates.

3. Results:

C. gracilis showed significant sensitivity to increasing Cu concentrations in two distinct concentration ranges: Up to a concentration of 125 nM Cu, *C. gracilis* showed prominent growth enhancement in response to increasing Cu concentrations, while growth inhibition was observed between 150-500 nM Cu. We focus mainly on the growth-promoting responses of *C. gracilis*, while the discussion of the inhibitory effects of Cu on *C. gracilis* is brief.

3.1 Total chlorophyll a contents and specific growth rates within the growth-promoting range:

Total chlorophyll a concentrations (Chl-*a*) (Fig.2a) in the *C. gracilis* cultures varied widely within the Cu growth-promoting range (Table 1). The lowest concentration of Chl-*a* ($10.14 \pm 1.32 \mu\text{gL}^{-1}$) was detected in the control treatments, while the highest value ($33.83 \pm 3.26 \mu\text{gL}^{-1}$) was observed at 125 nM Cu. Chl-*a* was positively correlated ($R^2 = 0.897$, $n = 20$, $p < 0.001$) with Cu concentrations (to 125 nM). At 25 nM Cu level, the concentration of Chl-*a* was enhanced by 86% relative to the control. This value showed further 2.5 X and 3.34 X increase vs the control at 50 nM and 125 nM Cu, respectively. The corresponding Chl-*a* based specific growth rates (Fig. 2b) also revealed a significant positive correlation ($R^2 = 0.876$, $n = 20$, $p < 0.001$) with increasing Cu levels. The average growth rate in the control treatments was $0.89 \pm 0.1 \text{ d}^{-1}$ which increased 28% upon the addition of 25 nM Cu. A further 42% and 54% enhancement in specific growth rate was observed in response to the addition of 50 nM and 125 nM Cu to *C. gracilis*.

3.2 Biogenic silica (BSi) concentrations and BSi:Chl-*a*

The concentrations of cellular biogenic silica (BSi) showed a significant positive correlation ($R^2 = 0.736$, $p < 0.001$, $n = 20$) with increasing Cu concentrations (Fig. 2c). Interestingly, the ratios of BSi:Chl-*a* decreased exponentially in response to increasing Cu concentrations (Fig. 2d) ($R^2 = 0.646$, $n = 20$, $p < 0.01$). The highest BSi:Chl *a* ratio ($4.12 \pm 0.99 \mu\text{mol} \mu\text{g}^{-1}$) was observed in the control treatments and decreased by 81% in the 25 nM Cu treatment. The ratios of BSi:Chl-*a* were quite stable from 10 – 120 nM Cu level, yielding an average value of 2.49 ± 0.36 and the minimum value of 1.92 ± 0.61 at 125 nM Cu, which corresponds to a 114% decrease relative to the control treatments (Table 1).

3.3 Qualitative and quantitative analysis of pigments by HPLC:

HPLC pigment analysis revealed that in addition to Chl-*a* in the cell extracts of *Chaetoceros gracilis*, considerable amounts of fucoxanthin (Fuco), diatoxanthin (DT), diadinoxanthin (DD), chlorophyll *c* (Chl-*c*) and β -carotene were detected in all samples (Fig. 3). Chl-*a*, Fuco, and Chl-*c* constitute the total pool of light harvesting (LH) pigments, whereas DD, DT and β -carotene are considered as photoprotective (PP) pigments (PP = DD+DT+ β -carotene). The ratios of (Fuco+Chl-*c*):Chl-*a* (Fig. 3a) did not reveal any significant trend in response to increasing Cu levels. The ratios of β -carotene:Chl-*a* (Fig. 3b) were negatively correlated ($R^2 = 0.561$, $p < 0.01$, $n = 20$) with Cu concentrations. DT:DD ratios (Fig. 3c) were high (> 1) in the control treatments and decreased significantly ($R^2 = 0.584$, $p < 0.01$, $n = 20$) with increasing Cu concentrations. Overall, (LH) pigment concentrations were highly correlated ($R^2 = 0.881$, $p < 0.001$, $n = 20$) with increasing Cu levels (Fig. 3d), whereas photoprotective (PP) pigments (Fig. 3e) had a significant negative correlation ($R^2 = 0.506$, $p < 0.02$, $n = 20$) with increasing Cu levels. PP:LH pigment ratios showed a significant negative correlation ($R^2 = 0.874$, $p < 0.001$, $n = 20$) with increasing Cu levels (Fig. 3f).

3.4 Growth responses and pigment ratios in response to Cu, Fe and Cu plus Fe addition:

In Ex2, the addition of Fe, both alone and in combination with Cu, showed an interesting growth response by *C. gracilis* (Fig. 4, Table 2). Specific growth rates were increased 42% in T1 (50 nM Cu), comparable to the 38% observed in Ex1 at the same concentration. In T2 (200 nM Fe) specific growth rate was 50% higher than the control treatments. Interestingly, as Cu concentration increased from T3 (50 nM) to T4 (100 nM) and T5 (150 nM) in the presence of 200 nM Fe, the magnitudes of the growth enhancement (+51.5%, +52.4% and +50.5%, in T3, T4 and T5, respectively) remained comparable with those in the T2 treatment (+50%). The differences between the growth rates in T2-T5 were not statistically significant (paired t-test; $t = -0.14$, $DF = 6$, $p > 0.1$), while growth rates in T1 – T5 were significantly different from the control ($t = -24.3$, $p < 0.001$, $DF = 10$).

PP and LH pigment concentrations for Ex2 were highly variable (Fig. 4b, Table 2). Overall, additions of Cu and Fe decreased PP:LH ratios from 1.38 ± 0.34 in the control to 0.38 ± 0.019 in response to 50 nM Cu addition. This ratio was further decreased ~ 7.8 X, on average, in Fe treatments or Fe + Cu

treatments (T2 –T5). The ratios of DT:DD showed a similar trend with a value of 0.98 in the control followed by successive decreases in T1 (~.5 X) and Fe, Fe + Cu-treated cells (T2 –T5) (~5 X).

3.5 Responses of *C. gracilis* within the growth-inhibiting range:

Within the concentration range of 150 – 500 nM, Cu showed considerable inhibitory effects on *C. gracilis*; we refer to these concentrations as “growth-inhibiting”. A significant decreasing trend ($R^2 = 0.832$, $p < 0.01$, $n = 10$) in Chl-*a* (Fig. 5a) concentrations was observed in response to increasing Cu supply. Specific growth rates revealed a decreasing trend with increasing Cu levels (Fig.5b) (0.801 , $p < 0.01$, $n = 10$). Cellular BSi (Fig. 5c) significantly decreased ($R^2 = 0.894$, $p < 0.001$, $n = 10$) with increasing Cu concentrations, confirming a strong inhibitory effect of Cu beyond 125 nM. Interestingly, BSi:Chl-*a* ratios did not change significantly ($R^2 = 0.425$, $p > 0.1$, $n = 10$) with increasing Cu supply (Fig. 5d). This observation suggests both BSi and Chl-*a* contents were inhibited in a similar manner.

Pigment concentrations also showed a decreasing trend with increasing Cu concentrations (Fig. 6). The ratios of Fuco+Chl-*c*: Chl-*a* (Fig. 6a), β -carotene:Chl-*a* (Fig. 6b) showed decreasing trends with increasing Cu levels, though the trends were not highly significant. Interestingly, the ratios of DT:DD (Fig. 6c) increased with increasing Cu level ($R^2 = 0.799$, $p < 0.05$, $n = 7$) indicating that under Cu stress, the conversion of DD to DT was enhanced. The total pool of LH (Fig. 6d) and PP pigments (Fig. 6e) showed drastic drop from 150 nM to 500 nM Cu treatments ($p < 0.001$). PP:LH pigment ratios (Fig. 6f) also decreased with increasing Cu concentration ($R^2 = 0.812$, $p < 0.01$, $n = 7$).

4. Discussion:

Our study showed that the diatom *Chaetoceros gracilis* off the coast of India was likely to be iron (Fe)-limited in the control treatments under nutrient replete condition. Experimentally, we documented the physiological response of *C. gracilis* to subsequent Fe addition, including a decrease in PP:LH pigments ratios, and a decrease in the BSi:Chl-*a*. We also identified copper (Cu) as a growth-promoting factor in diatoms, and provide evidence for Cu-dependent Fe acquisition in *C. gracilis*. We identified two critical Cu concentration ranges, a “growth-promoting range” (< 125 nM Cu) and a “growth-inhibiting range” (> 125 nM Cu) under nutrient replete conditions.

4.1: Growth-promoting range:

4.1.1 Indication of Fe stress in *C. gracilis* and its responses to Fe addition:

A number of observations support the conclusion that the cells are likely to have been Fe-stressed in the untreated controls during the sampling period. The lowest values of total Chl-*a* concentrations and growth rates were observed in the control treatments, despite macronutrient (N, Si and P) replete conditions. Both slow growth rates and low Chl-*a* content under high concentrations of macronutrients (N, P and Si) can be indicative of Fe stress in marine phytoplankton (HNLC regions, reviewed by Boyd et al., 2007). There are a number essential roles of Fe in phytoplankton physiology (e.g., nitrogen metabolism, biosynthesis of Chl-*a*, haeme proteins, cytochromes, ferredoxin, catalase, and peroxidase; Marchetti et al., 2012). To cope with extreme Fe limitation marine phytoplankton grow slowly and down-regulation Fe utilization, up-regulation Fe acquisition, and decrease Photosystem I to Photosystem II ratios (Maldonado and Price, 1996; Strzepek and Harrison, 2004).

The role of Fe in Chl-*a* synthesis has been well established (Kipe-Nolt et al., 1978; Yu and Miller, 1982). The catalysis of Mg-protoporphyrin to protochlorophyllide involves the Fe containing enzyme, coproporphyrinogen oxidase (Spiller et al., 1982). Fe is also found to be directly involved in the biosynthesis of Chl-*a* protoporphyrine precursor, δ -aminolevulinic acid (Kipe-Nolt et al., 1978; Yu and Miller, 1982). Additionally, Chl-*a* synthesis is largely dependent on nitrogen metabolism as the synthesis of tetrapyrrole rings needs high amount of amino groups (Doucette and Harrison, 1991). Phytoplankton cells possess higher Fe uptake rates when growing on nitrate compared to ammonium (Wang and Dei, 2001) as with ammonia amino acids can be directly synthesized. In contrast, the enzymes involved in the nitrate reduction (nitrate reductase and nitrite reductase), require Fe (Maldonado and Price, 1996) and high energy which is normally derived from the Fe-dependent photosynthetic redox reactions. As a result, under Fe stress, decreased nitrate uptake rates have been observed in marine phytoplankton (Price et al., 1994) associated with lower nitrate reductase activity (Timmermans et al., 1994). Recently, Marchetti et al., 2012 conducted a metatranscriptomics study to find out the molecular basis for the physiological responses of marine phytoplankton to different Fe levels. The authors showed that under Fe-replete conditions the genes involved in nitrogen metabolism were well expressed and this indicates that diatoms efficiently utilize the newly acquired Fe for nitrogen metabolism. However, during the present study, despite growing on high nitrate concentrations, *C.*

gracilis revealed low Chl-*a* contents and slow growth rates in the control treatments and it is likely that the dissolved Fe present in the filtered seawater was not enough to assimilate nitrate, hence they grew slowly.

The 50% enhancement in growth rate in response to 200 nM Fe addition confirmed the Fe-limited status of *C. gracilis* in the control treatments. Our study is in agreement with earlier studies where significant enhancement in Chl-*a* synthesis has been observed in diatom when supplied with Fe (Kosakowska et al., 2004; Allen et al., 2008). Kudo et al., (2000) reported that ~50% higher Chl-*a* content in *Phaeodactylum tricornutum* when grown in 2 μ M Fe compared to 2 nM Fe. There are also numerous studies on cyanobacteria and green algae showing significant enhancement in Chl-*a* synthesis when supplemented with Fe (Trick et al., 1995; Suroz and Kosakowska, 1996). Additionally, in all open ocean Fe enrichment studies, enhanced Chl-*a* concentrations and increased primary production (reviewed by Boyd et al., 2007) in response to Fe enrichment indicates that Fe has a strong influence in the process of Chl-*a* biosynthesis in marine diatoms.

Pigment signatures can be important tools to understand Fe stress in photosynthetic autotrophs. In general, diatoms exhibit specific light harvesting antenna complexes with unique accessory pigments; Chl-*a*, Chl-*c*, Fuco are the component of the Fuco- Chl-*a* binding proteins (Olaizola and Yamamoto, 1994). DD and DT are the representatives of a unique xanthophyll pigment cycle in diatoms that functions to protect the organisms against photo-damage. A decline in cellular pigment content in response to Fe stress has been reported in both eukaryotic algae (Doucette and Harrison, 1991; Greene et al., 1992) and cyanobacteria (Guikema and Sherman, 1983). There are several studies conducted on diatoms (Greene et al., 1991; Greene et al., 1992; Davey and Geider, 2001; Kosakowska et al., 2004) reporting changes in photosynthetic pigment signatures in response to Fe stress. Van de Poll et al., (2009) examined the effects of changes in Fe availability under constant light on the Antarctic marine diatom *Chaetoceros brevis* and reported 2 fold differences in the cellular pigment composition under Fe replete conditions compared to the Fe deplete cells.

Fe-stressed phytoplankton show high vulnerability to photo-oxidation because lack of Fe inhibits efficient photosynthetic electron transport (Kosakowska et al., 2004). To protect the cell from surplus light energy and thus photo-damage, algae generally produce more PP pigments relative to LH pigments (Abadia et al., 1989, Morales, 1994; reviewed by Bertrand, 2010). The high ratios of PP:LH pigment in

our control treatments support the argument that the cells were probably Fe stressed. In addition, our study showed decreased PP:LH after iron addition in both Ex1 and Ex2. In the presence of sufficient Fe, Fe-containing electron transporters can use the absorbed light energy efficiently leading to higher growth rates, increased LH pigment content and reduced PP pigment content. The Antarctic diatom *Chaetoceros brevis* also showed a significant increase in PP pigments under Fe stress, and enhanced LH over PP pigments upon the resupply of Fe to the stressed cells Oijen, et al., (2004)

Diatoms also possess a xanthophyll cycle where DD is converted to DT by a single de-epoxidation step (a process of non-photochemical quenching) to dissipate surplus absorbed light energy (Lavaud et al., 2002), specifically observed in *Chaetoceros gracilis* (Kashino and Kuodh, 2003; Ikeda et al., 2008). Fe stressed cells of the Antarctic phytoplanker *Phaeocystis* sp (prymnesiophyceae) also had low Chl-*a* content relative to DT and DD (Van Leeuwe and Stefels 1998). Juhas and Buechel, (2012) observed high DT:DD in a marine diatom *Cyclotella meneghiniana*, under high light and low Fe. In our experiment, the highest ratios of DT:DD were observed in the control treatments, indicating that *C. gracilis* maximized the conversion of DD to DT, probably because of Fe limitation and associated high oxidative stress. In addition, the decrease in DT:DD with Fe addition we observed suggested that in the presence of adequate Fe, the need for converting DD to DT was minimized. Our earlier study on *C. gracilis* (Biswas and Bandyopadhyay, 2013) also supports this observation.

High BSi:Chl-*a* in the control treatments relative to the Cu-treated cells were also indicative of Fe stress. Hutchins and Bruland, (1998) reported the occurrence of high BSi:Chl-*a* ratios (5.8) in Fe deficient coastal waters, and decreased the ratio to 1.3 with addition of 2.5 nM of Fe. Wilken et al., (2011) showed the formation of stronger Si frustules under Fe-limiting growth conditions in six diatom species. Enhanced Fe transport inside the cell has been reported to produce less silicified diatoms (Hutchins and Bruland, 1998; Takeda, 1998; Boyle, 1998). Marchetti and Harrison, (2007) conducted a laboratory experiment on six isolates of marine and coastal *Pseudo-nitzschia* and reported that under Fe deficient conditions, specific growth rates decreased and silicate content per cell increased. However, in some diatoms, silicification does not change in response to Fe stress, whereas, cell morphology changes by altering silica containing valve surface area relative to volume ratios (Marchetti and Casar, 2009). The actual reason for enhanced Si content inside diatoms under low Fe concentration has been proposed to be indirect. The silicate uptake rate is not uniform throughout the cell cycle; just before cell division,

in order to make new frustules for the daughter cells, silicate uptake rates are maximized. Cells grown in low Fe, therefore, possess a prolonged silicate uptake stage as lack of Fe leads to a delay in cell division, resulting in a high silicate content in the cell (Brzezinski, 1992). It is likely that in the control treatments cells were more silicified probably because of Fe limitation. Similarly, our earlier study of *C. gracilis* (Biswas and Bandyopadhyay, 2013) revealed that high values of BSi:Chl-*a* are indicative of Fe stress, and when cells are further supplemented with Fe, these ratios significantly decreased.

4.1.2 Cu as a growth-promoting factor: indication of Cu-dependent Fe acquisition in *C. gracilis*:

We demonstrated that the observed increase in Chl-*a* and growth rate coupled with decrease in the ratios of PP:LH pigments and DT:DD after the Fe addition are indicative of low Fe nutrition in the natural non-amended seawater. Interestingly, pigment contents, growth rates and BSi from the Cu-supplemented cells (Cu concentration of 125 nM) collectively depicted a strong resemblance with those obtained from the Fe-replete cells of *C. gracilis*. High concentrations of Cu have been shown to inhibit silicate uptake in marine diatoms, since Cu and Si compete for the same transporter (Rueter et al., 1981). In the present experiment, Cu additions did not inhibit Si uptake, as within the growth-promoting range of Cu concentrations, Chl-*a* and BSi showed a linear enhancement with increasing Cu concentrations up to 125 nM. . In our previous study (Biswas and Bandyopadhyay, 2013) it was noticed that the ratios of BSi:Chl-*a* in *C. gracilis* were significantly decreased in response to increasing Fe supply, most likely due to enhanced Fe acquisition and concomitant decrease in silicate uptake rate over nitrate consumption. Under Fe replete conditions, the rate of nitrate uptake in marine diatoms is higher than that of silicate hence exhibit high N:Si uptake resulting in thinner frustules (Timmermans et al., 1994). Ex2 indicated that in the presence of Fe, the need of Cu was probably minimized and hence the growth-promoting effects of Cu were not visible.

Our combined results suggest that the coastal diatom probably utilized Cu for acquisition of Fe under Fe stress. Semeniuk et al., (2009) measured Cu uptake by natural plankton community in the subarctic Northeast Pacific Ocean and reported that Cu played an important role in the physiology of natural plankton communities there. Peers et al., (2005) reported a comparable result in the Bering Sea, where addition of 2nM Cu doubled the net growth rate compared to the control. However, in the presence of excess Fe, there was no effect of increased Cu. This observation is in agreement with our results. Guo and co-workers, (2012) demonstrated that under Fe/Cu stress, diatoms had the highest

average Cu quotas among other algal groups suggesting diatoms have a high Cu demand under Fe deficiency. Indeed, *Chaetoceros decipiens* showed 50% higher use efficiency of Cu when Fe starved (Annett et al., 2008) and Fe-starved species of both coastal and oceanic diatoms showed significant growth enhancement in response to Cu addition. However, under Fe sufficient conditions, the Cu enhancement effect was not significant, indicating minimal Cu use under Fe replete conditions. This observation suggests that under Fe stress, coastal and oceanic diatoms may significantly increase their Cu demand in order to acquire Fe.

Laboratory experiments have demonstrated that eukaryotic microorganisms, including yeast, *Saccharomyces Cerevisiae*, *Schizosaccharomyces pombe* (Askwith and Kaplan, 1997), green algae *Chlamydomonas reinhardtii* (Herbik et al., 2002, b; La Fontaine et al., 2002), and a fungal pathogen *Candida albicans* possess a Cu-dependent Fe transport system under Fe-limiting conditions. This specialized Fe transport system is comprised of a 1) putative Fe(III) reductase enzyme 2) a multi Cu oxidase containing enzyme (MCO) and 3) Fe(III) permease (reviewed by Van ho et al., 2002). Consequently, during extreme Fe starvation, the cells cannot acquire enough Fe to support their growth if Cu is not sufficiently available. Thus, the requirement for Cu under Fe-limiting conditions increases with decreasing Fe availability. Short term Fe uptake rate and cellular Fe quota in the oceanic diatom *Thalassiosira oceanica* decreased when external Cu concentrations were reduced, indicating the direct role of Cu in Fe acquisition in marine diatoms (Peers et al., 2005). The authors also observed an enhancement in Chl-*a* content and growth rate in Bering Sea phytoplankton community when enriched with 2 nM Cu. Maldonado et al., (2006) and Guo et al., (2010) reported the presence of a High Affinity Fe Transport system in the coastal diatom *Thalassiosira pseudonana*. Hence, the available literature is consistent with our observation that modest concentrations of Cu can significantly affect growth and primary production of coastal diatoms under iron limited conditions. While our present investigation suggests that *C. gracilis* probably utilize Cu to activate a Cu-dependent Fe acquisition system which can meet their Fe demand, we made no direct measurement of cellular metal contents (Fe/Cu) or uptake rates. Thus the presence of a Cu-dependent Fe acquisition system in *C. gracilis* remains to be confirmed by further investigations.

4.2 Inhibitory effects of Cu on *C. gracilis*:

Inhibitory effects of high Cu (>150 nM) on *C. gracilis*, concentrations in our study included reduced Chl-*a* and accessory pigment concentrations and a reduction in growth rates. The coastal diatom *Ditylum brightwellii*, showed similar responses to increasing Cu levels (Rijstenbil et al., 1994). Excess Cu can inhibit many enzymatic activities hindering vital physiological processes like photosynthesis, protein and pigment synthesis. The most deleterious effect can be blocking of photosynthetic electron transport chain caused by increasing Cu concentrations (Fernandes and Henriques, 1991). Interestingly, within the growth-inhibiting concentration range, the ratios of DT:DD were found to be enhanced by increasing Cu supply, indicating that the conversion of DD to DT was maximized with increasing Cu concentrations. Higher DT vs DD in *C. gracilis* is associated with non-photo-chemical quenching where light energy is dissipated as heat by an enzymatic de-epoxidation process (Kashino and Kudoh, 2003). Within the growth-inhibiting range, an increase in Cu concentration may significantly inhibit photosynthetic electron transport. Under such conditions, *C. gracilis* probably maximized the conversion of DD to DT to reduce oxidative stress.

Nitrate uptake rate can decrease in response to increasing Cu supply in marine diatoms (Harrison et al., 1977). The significant decrease in growth rates in our study indicates that despite high concentrations of available nitrate, *C. gracilis* probably could not metabolize nitrate because Cu toxicity slowed growth rates. BSi contents also showed a linear decrease in response to rising Cu level within the growth-inhibiting range. As indicated earlier, Cu can inhibit silicate uptake rate in marine diatoms because Cu and Si compete for the same transporter, hence with increasing Cu concentration, the rate of inhibition increases (Morel et al., 1978; Rueter et al., 1981).

Levy et al., 2007 investigated Cu toxicity and found big differences in Cu sensitivity across 11 microalgal species, probably due to differences in uptake rates, internal Cu binding and/or detoxification mechanisms (i.e. chelating Cu agents). Earlier studies reported that marine eukaryotic phytoplankton and cyanobacteria are able to release strong organic ligands in response to high Cu stress (Moffet and Brand, 1996; Sunda and Huntsman, 1998; Croot et al., 2000). Slauenwhite and Wangersky, (1991) conducted a study on *Chaetoceros gracilis* and observed that Cu was highly chelated with organic ligands, but remained in the dissolved form. Rijstebil and Wijnholds, (1991) and Ristenbil et al., (1998) reported that Cu toxicity in marine diatoms can be highly dependent on macronutrient availability.

Sufficient nitrogen and phosphorus may accelerate the production of Cu chelating ligands, reducing Cu toxicity.

Rijstenbil and Gerringa, (2002) investigated the effects of long term Cu stress on the marine diatom *Ditylum brightwellii* under nutrient replete conditions and reported that at 126 nM Cu, a slight decrease in the cell division was observed, whereas, at 157 nM Cu, the synthesis of the Cu binding ligand, phytochelatins were observed. Phytochelatin production provides a cellular defense mechanism against toxic metals, and has been used as suitable early-warning indicator for toxic Cu levels in the environment (Ahner and Morel, 1995). We have also observed the first signs of Cu inhibition at 150 nM Cu in *C. gracilis*, making our observations are consistent with this study.

5. Conclusions:

We suggest that under Fe stress, *C. gracilis* utilizes Cu to meet its Fe demand by activating a specialized Fe acquisition system. No direct measurements were done here to confirm this claim. However, possible enhancement of Fe acquisition with increasing Cu supply was supported with results from Chl-*a* contents, growth rates, pigment signatures (the ratios of PP:LH and DT:DD) and the ratios of BSi:Chl-*a*. Coastal waters are generally considered metal enriched, hence trace metal limitation effects on phytoplankton physiology have mostly been overlooked. Our experimental results suggest that Fe can be limiting in coastal areas, and that in addition, Cu can be a vital trace metal for phytoplankton growth where this is the case. The distribution and speciation of Cu in coastal waters can thus be potentially influenced by phytoplankton. *C. gracilis* was found to tolerate a wide range of Cu concentrations; however, Cu can be potentially toxic at higher concentrations. Since the present experiment was conducted under nutrient enriched conditions, direct extrapolation to the natural systems should be made with caution. The Bay of Bengal receives large nutrient loading from river inputs and can naturally enrich coastal waters enhancing the demand for Fe. Under periods of high Fe demand, Cu availability can significantly enhance primary production in the coastal diatoms, and can directly influence nitrogen and carbon biogeochemistry in the study area.

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Table 1: Biochemical and physiological responses of *Chaetoceros gracilis* to the copper (Cu) additions. Dashed line indicates separation between growth-promoting Cu concentrations (< 125 nM) and growth-inhibiting concentrations (≥ 150 nM). Values represent averages and standard deviations of replicate experiments (average \pm SD, n = 2-3).

Cu concentration (nM)	Specific growth rate (μ d ⁻¹)	Chl- <i>a</i> (μ g L ⁻¹)	BSi (μ mol L ⁻¹)	BSi:Chl- <i>a</i> (μ g: μ mol)
Control	0.89 \pm 0.10	10.14 \pm 1.32	42.36 \pm 13.54	4.12 \pm 0.99
25	1.14 \pm 0.09	18.93 \pm 1.63	59.07 \pm 15.16	3.17 \pm 1.07
30	1.20 \pm 0.08	22.40 \pm 1.95	54.99 \pm 12.40	2.49 \pm 0.77
40	1.22 \pm 0.04	22.90 \pm 0.83	62.33 \pm 1.02	2.72 \pm 0.14
50	1.27 \pm 0.14	26.12 \pm 6.35	56.25 \pm 2.79	2.27 \pm 0.72
70	1.37 \pm 0.02	30.80 \pm 0.21	60.68 \pm 4.56	1.97 \pm 0.16
80	1.37 \pm 0.01	30.99 \pm 0.84	65.15 \pm 3.96	2.10 \pm 0.07
100	1.32 \pm 0.08	29.06 \pm 2.99	61.87 \pm 2.42	2.15 \pm 0.32
125	1.37 \pm 0.09	33.83 \pm 3.26	64.11 \pm 14.43	1.92 \pm 0.61
150	1.35 \pm 0.11	29.71 \pm 7.65	53.21 \pm 0.36	1.85 \pm 0.49
200	1.32 \pm 0.12	27.51 \pm 7.76	50.56 \pm 2.53	1.90 \pm 0.44
250	1.32 \pm 0.10	27.26 \pm 6.52	45.35 \pm 9.91	1.67 \pm 0.04
300	1.19 \pm 0.23	25.27 \pm 4.48	41.49 \pm 7.58	1.64 \pm 0.01
350	1.26 \pm 0.12	22.23 \pm 7.50	35.08 \pm 13.31	1.56 \pm 0.17
400	1.07 \pm 0.20	14.02 \pm 8.53	22.74 \pm 16.25	1.56 \pm 0.20
500	0.87 \pm 0.34	9.09 \pm 6.97	13.68 \pm 10.78	1.52 \pm 0.10

Table 2: Physiological responses and changes pigment ratios in response to six treatments (Control + T1 – T5) involving various combinations of copper (Cu) and iron (Fe) addition (Experiment 2; Ex2). Values represent average \pm standard deviations of replicate experiments (n = 2).

Treatments	Added Cu (nM)	Added Fe (nM)	Growth rate (μ d ⁻¹)	DT:DD (μ g: μ g)	PP:LH pigment (μ g: μ g)
Control	0	0	0.94 \pm 0.03	0.98 \pm 0.13	1.38 \pm 0.34
T1	50	0	1.30 \pm 0.02	0.68 \pm 0.22	0.38 \pm 0.02
T2	0	200	1.42 \pm 0.12	0.29 \pm 0.01	0.22 \pm 0.02
T3	50	200	1.42 \pm 0.15	0.18 \pm 0.04	0.15 \pm 0.01
T4	100	200	1.45 \pm 0.15	0.20 \pm 0.07	0.17 \pm 0.02
T5	150	200	1.42 \pm 0.13	0.19 \pm 0.09	0.17 \pm 0.08

Figure Legends:

Fig. 1: Map showing the sampling station location. Note the proximity of several large river systems, which are known to deliver significant macronutrient (N, Si and P) loadings to the continental shelf.

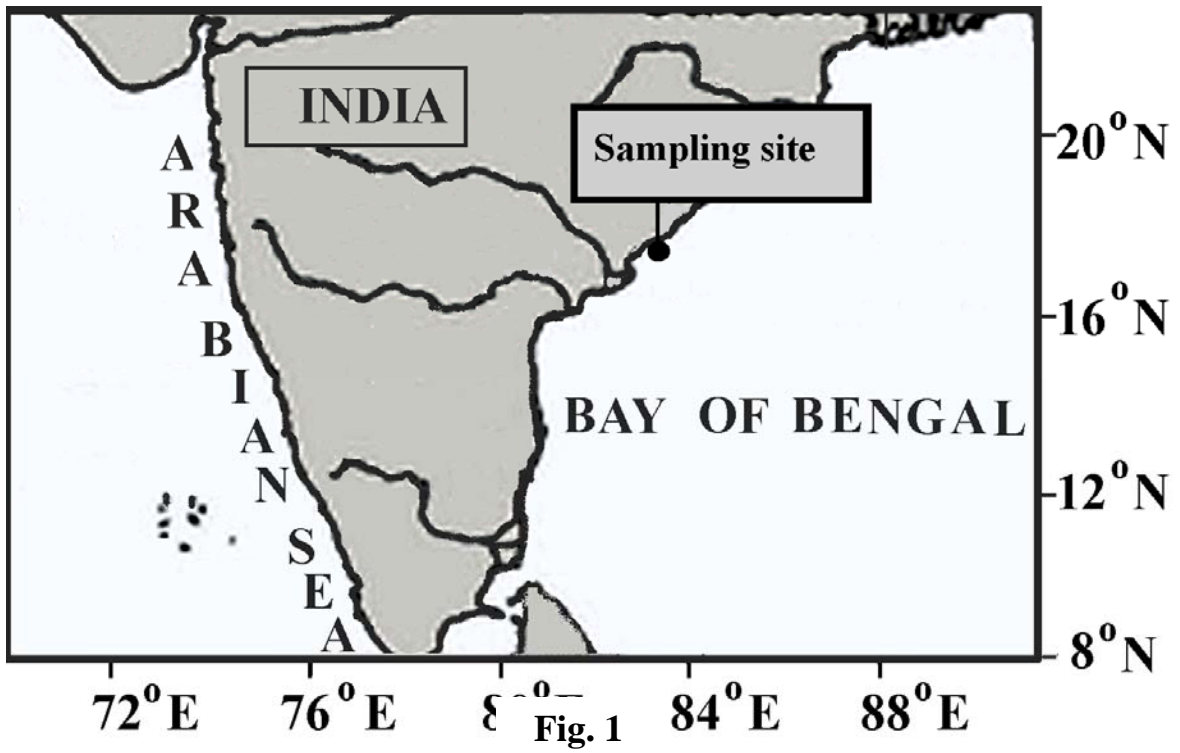
Fig. 2: Experiment 1 (Ex1): The response of the diatom *Chaetoceros gracilis* to increasing copper (Cu) addition within the growth promoting-range (to 125 nM Cu). Note that we believe these cells are likely to be Fe-limited in the control treatments. a) The concentrations of Chl-*a*, b) Chl-*a* specific growth rates, c) BSi contents and d) the ratios of BSi:Chl-*a*. Error bars given for 9 of the 20 experiments executed represent standard deviations of 2-3 replicate experiments under the same conditions (average \pm SD, n = 2-3).

Fig. 3: Experiment 1 (Ex1): Physiological response of the diatom *Chaetoceros gracilis* to copper (Cu)s additions within the growth-promoting range of concentrations (< 125 nM). a) the ratios of (Fuco+Chl-*c*):Chl-*a*, b) the ratios of β -carotene:Chl-*a*, c) the ratios of DT:DD, d) total concentrations of LH pigments, e) total pool of PP pigments, f) the ratios of PP:LH pigments

Fig. 4: Experiment 2 (Ex2): Responses of *Chaetoceros gracilis* to copper (Cu) and iron (Fe) addition. The growth responses suggest a Cu-mediated enhancement of Fe uptake at low (limiting) Fe concentrations: a) Chl-*a* based specific growth rates and b) pigment signatures in response to addition of 50 nM Cu (T1), 200 nM Fe (T2), 50 nM Cu +200 nM Fe (T3), 100 nM Cu + 200 nM Fe (T4) and 150 nM Cu + 200 nM Fe (T5). The values presented here are average \pm SD from two replicate experiments (n = 2).

Fig. 5: Response of *Chaetoceros gracilis* to increasing copper (Cu) concentrations within the growth-inhibiting range (\geq 150 nM Cu).a) The concentrations of Chl-*a*, b) Chl-*a* based specific growth rates, c) BSi contents and d) BSi:Chl-*a* ratios. Where provided, standard deviations indicate averages of replicate experiments (average \pm SD, n = 2-3).

Fig. 6: Response of *Chaetoceros gracilis* to increasing Cu concentrations within the growth-inhibiting range (> 150 nM Cu). a) the ratios of (Fuco+Chl-*c*):Chl-*a*, b) the ratios of β -carotene:Chl-*a* contents, c) the ratios of DT:DD, d) total concentrations of LH and e) PP pigments and f) the ratios of PP:LH pigments.



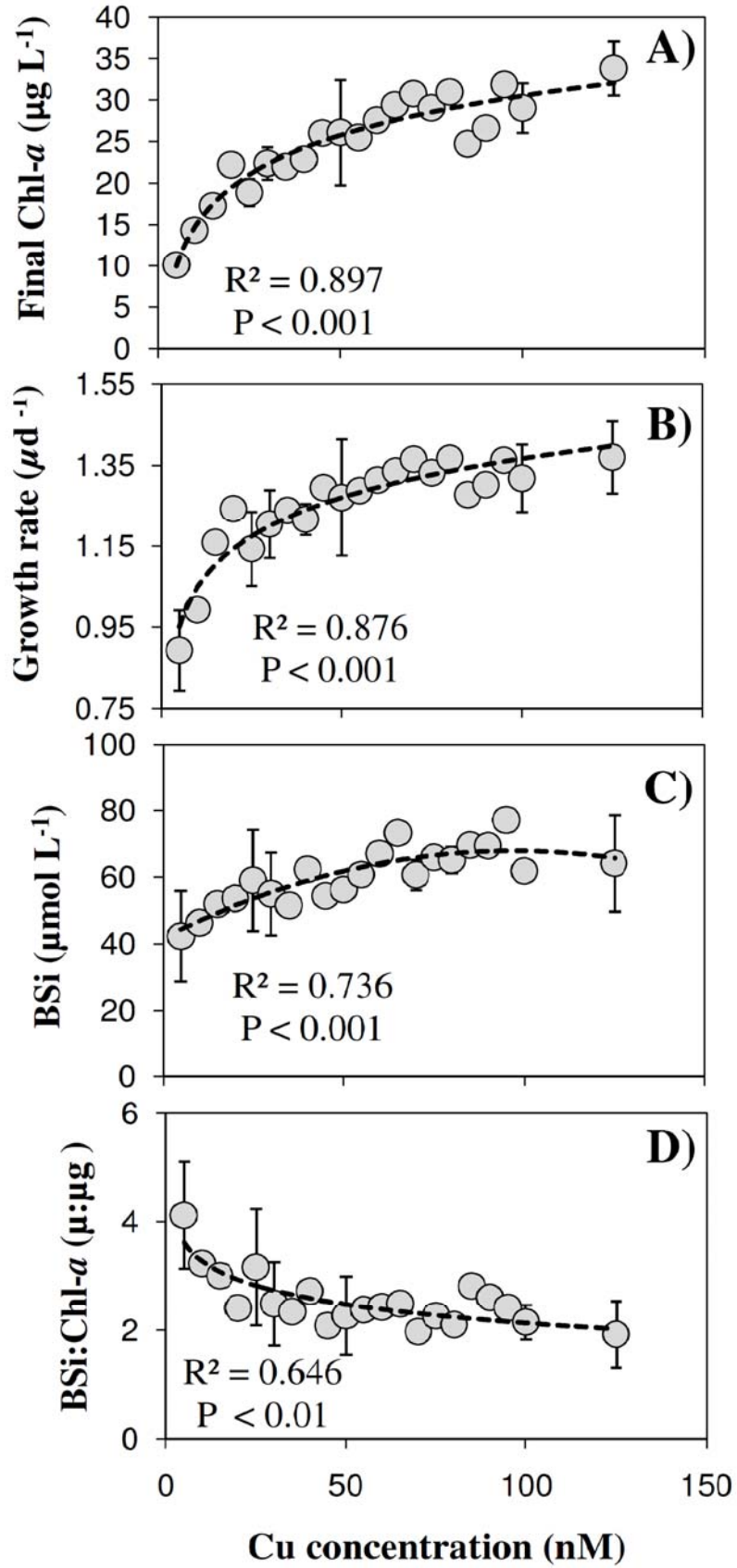


Fig. 2

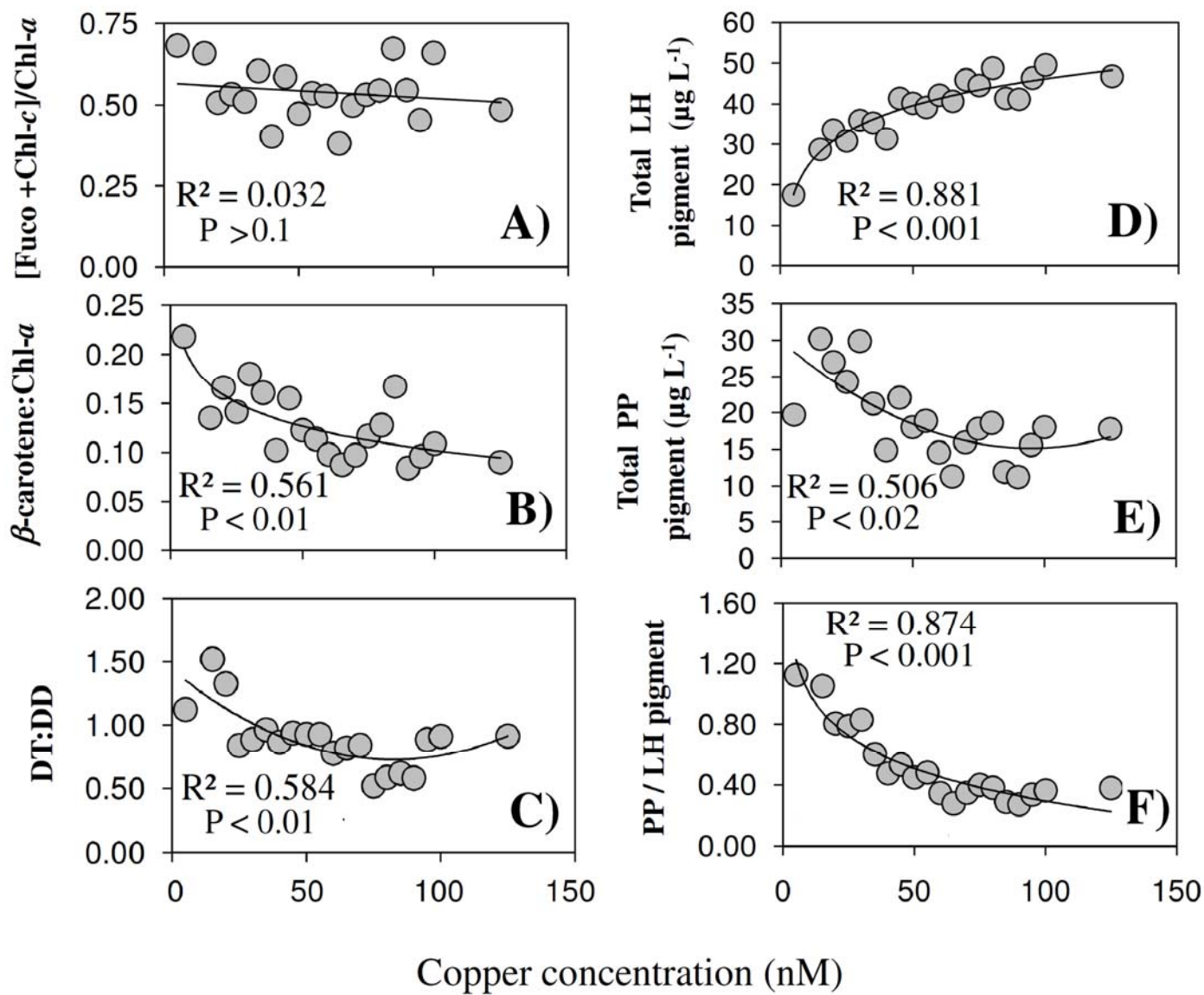


Fig. 3

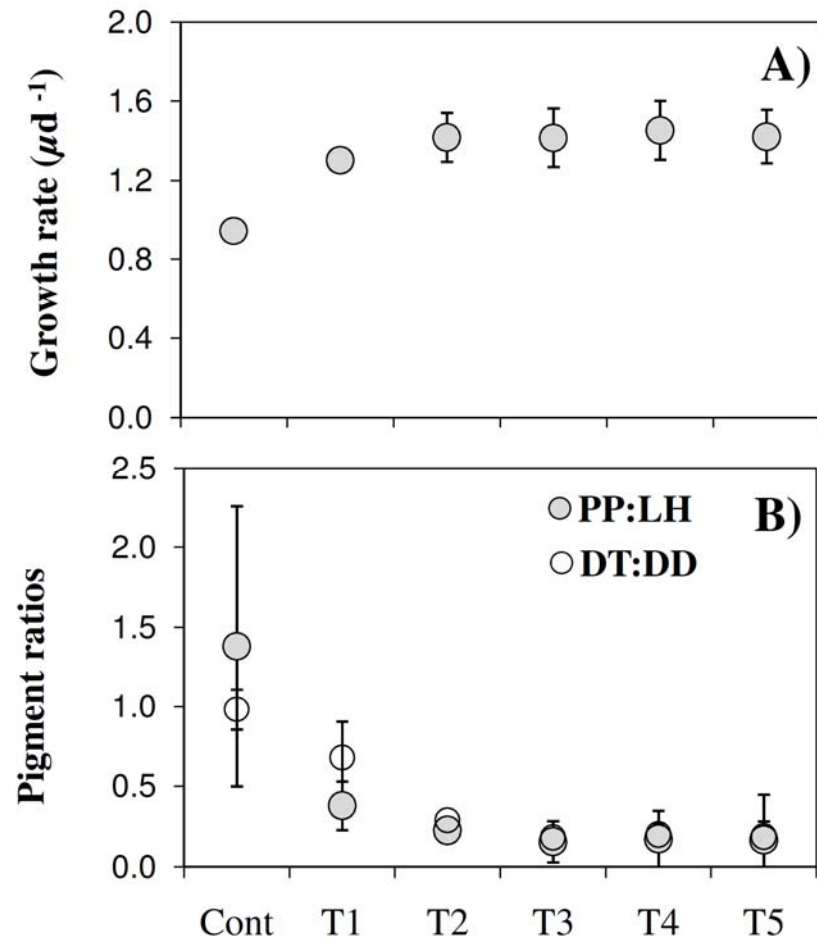


Fig. 4

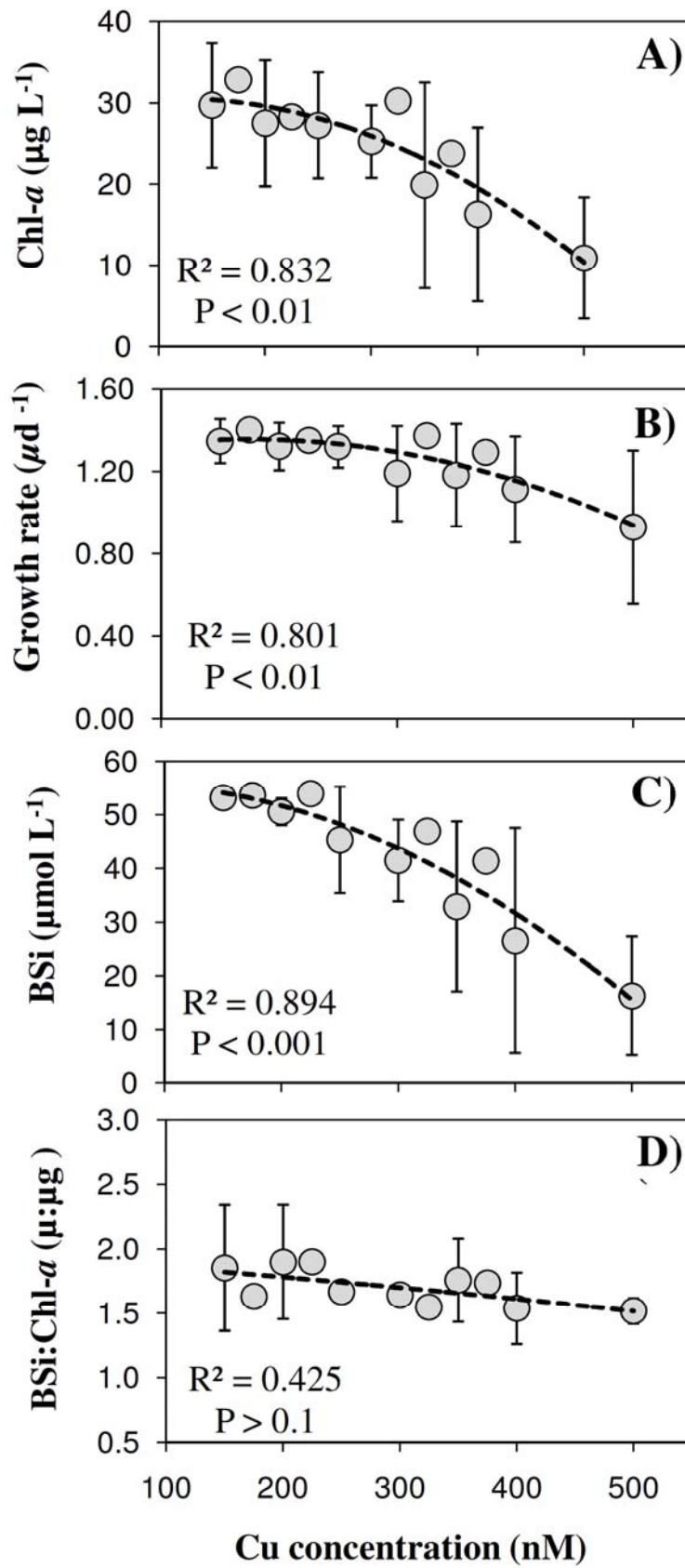


Fig. 5

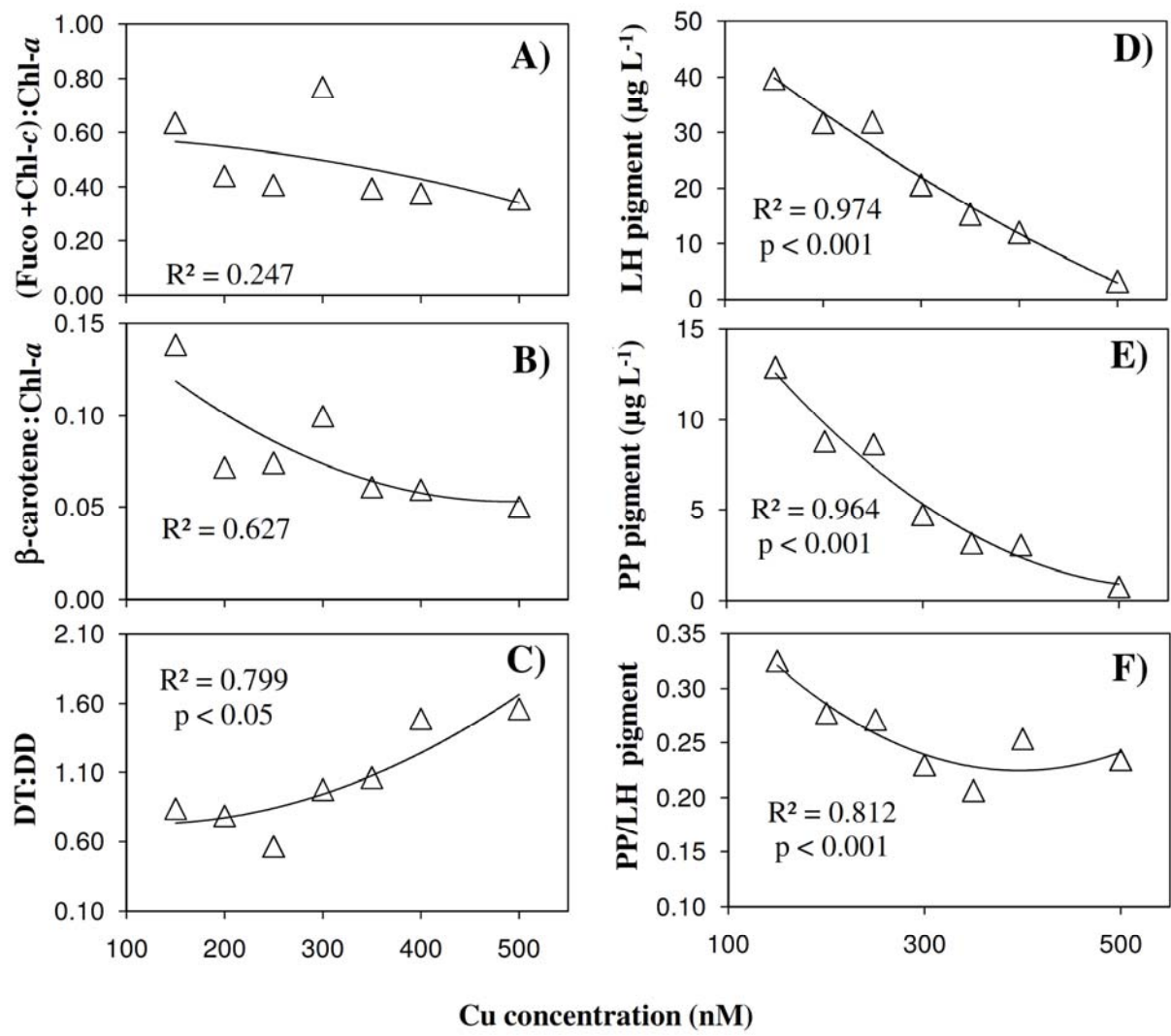


Fig. 6