

Physiological responses of coastal phytoplankton (Visakhapatnam, SW Bay of Bengal, India) to experimental copper addition

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Abstract:

Trace amount of copper (Cu) is essential for many physiological processes; however, it can be potentially toxic at elevated levels. The impact of variable Cu concentrations on a coastal phytoplankton community was investigated along a coastal transect in SW Bay of Bengal. A small increase in Cu supply enhanced the concentrations of particulate organic carbon, particulate organic nitrogen, biogenic silica, total pigment, phytoplankton cell and total bacterial count. At elevated Cu levels all these parameters were adversely affected. $\delta^{13}\text{C}_{\text{POM}}$ and $\delta^{15}\text{N}_{\text{POC}}$ reflected a visible signature of both beneficial and toxic impacts of Cu supply. *Skeletonema costatum*, the dominant diatom species, showed higher tolerance to increasing Cu levels relative to *Chaetoceros* sp. Cyanobacteria showed greater sensitivity to copper than diatoms. The magnitude of Cu toxicity on the phytoplankton communities was inversely related to the distance from the coast. Co-enrichment of iron alleviated Cu toxicity to phytoplankton.

Key words: Coastal; Phytoplankton; Diatoms; Cyanobacteria; Copper; Iron; Toxicity; Bay of Bengal.

Introduction:

Cells use some trace metals like iron (Fe), zinc (Zn) and copper (Cu) to stabilize protein structure, to help electron transfer reactions and to catalyze enzymatic reactions (Ash and Stone 2003). Green et al. (1939) documented the role of Cu in plant photosynthesis more than 6 decades ago. Later, the role of Cu in photosynthetic electron transport chain reactions and respiratory cytochrome oxidase was reported (Stryer 1988). The special electron configuration of Cu permits the straight interaction with spin-restricted molecular oxygen and hence Cu often participates as a protein co-factor in many fundamental redox reactions. Under oxidative stress, most of the eukaryotes utilize the enzyme superoxide dismutase containing Cu and Zn moiety to detoxify superoxide radicals (Chadd et al. 1996). Moreover, several eukaryotic microorganisms, including diatoms, possess a Cu-dependent high affinity iron acquisition system and utilize Cu to overcome stress caused by Fe limitation. (Biswas et al. 2013).

Conversely, at high levels, Cu can be potentially toxic due to its ability to make strong complexes with biomolecules (Letelier et al. 2005). High Cu concentrations have been found to reduce marine phytoplankton growth rate (Manimaran et al. 2012), inhibit photosynthesis (Nguyen-Deroche et al. 2009), decrease nitrate uptake (Harrison et al. 1977), hinder silicic acid uptake (Morel et al. 1978; Rueter et al. 1981) and change phytoplankton community composition (Brand et al. 1986). Cu toxicity is mediated by interactions with thiol sites on proteins and the production of reactive oxygen species (ROS) which promotes lipid peroxidation chain reactions (De Vos et al. 1989). Nevertheless, the magnitude of toxicity can be species specific (Levy et al. 2008). Interestingly, Cu toxicity has been shown to be alleviated by other metals like iron (Fe) and manganese (Stauber and Florence, 1985).

Metal toxicity is not only dependent on its concentration and input rate, but also largely controlled by some physicochemical parameters such as pH, salinity, dissolved organic carbon contents, etc. In addition, Cu bioavailability is strongly controlled by its chemical speciation, given that almost 99.9% of available Cu is complexed to strong organic ligands (Sunda and Huntsman 1998) which are mostly phytoplankton exudates, or degradation product from organic matter or from synthetic compounds from industrial effluents. Without organic complexation, Cu bioavailability and toxicity would have been much higher in the aquatic systems.

The coastal waters receive significant amount of copper from both natural or anthropogenic sources (Morillo et al. 2004). Atmospheric deposition of Cu enriched aerosol is also of great concern in coastal areas and can alter phytoplankton community structure and chlorophyll concentrations (Jordi et al. 2012). However, the coastal phytoplankton are likely to develop some adaptations to

tolerate higher metal concentrations relative to the offshore communities. Additionally, the demand for essential metals can be very high for the coastal species as they grow in a metal enriched environment. When living permanently in a metal enriched environment, some marine phytoplankton can control their internal Cu pool well below the toxic levels (Brand et al. 1986). Increasing anthropogenic activities in coastal areas is likely to increase metal input and thus can considerably affect growth and physiology of phytoplankton communities. Debelius et al. (2010) reported that in addition to algal type and cell size, geographic location also controls the toxic impact of Cu on marine phytoplankton.

The present study area, Visakhapatnam coast (SW Bay of Bengal), a heavily anthropogenically perturbed system, was chosen to study the impacts of different Cu concentrations on the natural phytoplankton communities using microcosm experiments. We show that the phytoplankton community composition can be highly vulnerable to high Cu exposure. In our earlier study using a diatom isolate from this coast (Biswas et al. 2013), we noticed that Cu enhanced the growth up to a certain level beyond which it became inhibitory. Moreover, Cu addition helped in reducing the stress induced by Fe limitation. In the present study, we investigated the impacts of variable Cu, Fe and Fe+Cu enrichment on the natural phytoplankton communities from Visakhapatnam coast in different time and at different sites. We aimed to determine the physiological responses of the natural phytoplankton communities to changing Cu concentrations in the coastal waters of Visakhapatnam.

2. Material and methods:

2.1 Study area:

The study area (Visakhapatnam coast) is a coastal embayment of the Bay of Bengal, situated between 17°44'N and 83°23'E, almost 7 km north from the Visakhapatnam Port (Fig. 1). Extensive shipping and fishing activities take place in this area and the adjacent coastal water probably receives considerable amount of trace metals. Phytoplankton community is mostly dominated by diatoms in this coastal area (Paul et al. 2007; Shaik et al., 2015; Bandyopadhyay et al., 2017). This area is largely influenced by the Indian monsoon systems and receives a significant amount of freshwater via river discharge. Therefore, the coastal water experiences tremendous fluctuations in physicochemical properties which in turn also influence phytoplankton ecology (Shaik et al., 2015).

2.2 General experimental planning:

Surface seawater containing the natural plankton community was collected using a clean Niskin bottle (and sometimes with an acid cleaned plastic beaker/bucket) and dispensed into the acid cleaned (overnight) 20L Nalgene bottles (USA). The seawater was pre-filtered with a 200µm mesh

to avoid the entry of mesozooplankton (to reduce grazing pressure). All bottles used for this experiment were washed thoroughly first with acid (overnight) and followed by MQ water and kept inside zip lock plastic covers before use. Sample water was evenly distributed into pre-cleaned 2L Nalgene polycarbonate bottles. Cu was added as CuCl_2 (Merck, Germany) solution (without any artificial chelator like EDTA) to each bottle according to the target concentrations (in duplicate) (Table 1) and immediately closed with the screw caps. A clean bench was used for Cu addition and also to set the entire experiment. Extra care was taken to avoid any contamination from any other trace metals. We could not measure the actual concentrations of dissolved Cu in the ambient waters; the responses of phytoplankton communities to a particular Cu level were monitored in this study. According to the earlier reports, total dissolved Cu concentration in Visakhapatnam coastal water was $25 \pm 6 \text{ nM}$, (Rejomon et al. 2008, 2010) which has been considered as a base line for Cu addition during the present experiment.

2.3 Incubation condition:

The bottles were mixed carefully and kept for 3 days inside a water bath under natural day and light cycle to allow phytoplankton growth (please see Biswas et al., 2013). In our earlier experiment, it has been observed that the phytoplankton community from this coastal area showed the highest growth rate within 48-72 hours depending on the concentrations of macro nutrients. Under a moderate nutrient concentration (like the present experiment) the phytoplankton community showed a specific growth rate of $\approx 1.55 (\mu_{\text{Cell}} \text{ d}^{-1})$ which is corresponding to almost 2.2 generations per day and hence, more than 6 generation in 3 days. A black nylon mesh was used to mimic the light intensity at 0.5m water depth to avoid photo-inhibition. The water temperature in the bath varied within $26.7\text{-}29.5^\circ\text{C}$ and the water was exchanged three times a day to avoid temperature variation. Each bottle was mixed gently everyday at 10am to avoid biomass settlement at the bottom. After 3days of incubation the bottles were brought back to the laboratory for cell harvesting and further analysis.

A set of three individual microcosm experiments (will be addressed as EXP-I, EXP-II, EXP-III A and EXP-IIIB in the text) were conducted in March, June, November and January using natural phytoplankton communities collected from different distance from the coast. The aims of these four consecutive experiments were not similar. Different Cu concentrations were used depending on the sampling location (since Cu sensitivity was expected to change with varying distance from the coast).

Experiment I (EXP-I):

The first experiment (EXP-I) was conducted in March 2011. The goal of this experiment was to understand the level of sensitivity of the phytoplankton community inhabiting close to the coastline. The experimental seawater was collected from a place approximately 100m away from the coastline (S1a; Fig. 1) following the above mentioned method. Three concentrations of Cu (10,100 and 1000 nM) were considered for enrichment experiment; unenriched seawater served as the control. All sets were kept in duplicate. After 3 days of incubation, the concentrations of *Chla* and other pigments, number of phytoplankton cells and total bacteria were estimated for each treatment and the control. The details are given in the section 2.3.

Experiment-II (EXP-II):

The second experiment (EXP-II) was conducted in June'11 and seawater samples were collected from the previous location (S1; Fig. 1). This was planned almost like EXP-I, but considering a larger range of Cu concentrations (5, 50, 100, 150, 200, 400 and 800 nM) and additional physiological parameters. The concentrations of particulate organic carbon (POC), particulate organic nitrogen (PON) and biogenic silica (BSi) along with $\delta^{13}\text{C}_{\text{POM}}$ and $\delta^{15}\text{N}_{\text{POM}}$ were measured during this experiment.

EXP-III:

The third experiment (EXP-III) includes two sub-experiments (EXP-IIIA and EXP-IIIB) and was aimed to at understanding the responses of the natural phytoplankton communities to experimental Cu, Fe and Cu+Fe additions along a coastal transect in Visakhapatnam. The natural phytoplankton samples were collected from three locations [1km (S1), 6km (S2) and 10km (S3) far from Visakhapatnam coast] (Fig.1) in November (EXP-IIIA) and two stations [1km (S1) and 10km (S3)] in January (EXP-IIIB). In EXP-IIIA similar Cu levels (50 nM, 100 nM and 150nM) were chosen to be added for both coastal and offshore phytoplankton communities. But it was noticed that these Cu levels were highly toxic for the phytoplankton communities present in the offshore stations. Hence, relatively lower Cu levels (5, 10, 25 50nM Cu and Fe for the coastal station; 2, 4, 6 10nM Cu and Fe for the offshore station) were considered in the repeat experiment (EXP-IIIB) in January (Table 1). In EXP-IIIA, additional nitrate was added to all samples to a final concentration of ~ 10 μ M; but not in EXP-IIIB. Only *Chla* a, an indicator of phytoplankton biomass, was measured during these experiments. HPLC analysis was done for EXP-IIIB. The added Fe was complexed to ethylene diamine tetraacetic acid (EDTA) before addition. The analytical grade FeCl_3 , $6\text{H}_2\text{O}$ and EDTA disodium salt were procured from the Merck chemicals (Germany) and the Fe stock solution (400 μ M) was prepared in 1:5 molar ratio (Fe:EDTA).

2.3 Sample analysis:

For total chlorophyll *a* (Chl *a*) estimation, 100 -200 mL water sample was filtered from each treatment onto GF/F filters and kept at -20°C until analysis. Later, the filters were soaked overnight in 5 mL DMF (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). Major nutrients (dissolved inorganic nitrogen, DIN; dissolved inorganic phosphate, DIP and dissolved inorganic silicate, DSi) were analyzed using the standard colorimetric method of Strickland and Parsons (1972). Water samples for pH measurement were preserved with HgCl₂ in capped glass bottles with the caps wrapped with a parafilm strip. pH was measured using a 794 Basic Titrino from Metrohm, and standard buffer from Merck, Germany. Samples of δ¹³C and δ¹⁵N of particulate organic matter (δ¹³_{CPOM}; δ¹⁵_{NPOM}) were analyzed using the Delta V Plus, Isotopic Ratio Mass Spectrometer. An aliquot of 100-200mL water sample was filtered through the pre-combusted glass fiber (GF/F) filters, followed by overnight drying at 40 °C. Filters were exposed to HCl fumes to remove the particulate inorganic carbon (if any) and measured using PDB standard, following the formula:

$$\delta^{13}_{CPOM} = [\{^{13}C/^{12}C \text{ (sample)}/^{13}C/^{12}C \text{ (standard)}\} - 1] \times 1000$$

An aliquot of the same sample was kept without acidifying it and used to measure δ¹⁵_{NPOM} using the following formula:

$$\delta^{15}_{NPOM} = [\{^{15}N/^{14}N \text{ (sample)}/^{15}N/^{14}N \text{ (standard)}\} - 1] \times 1000$$

POC and PON were analyzed using an elemental analyzer attached to the Isotopic Ratio Mass Spectrometer (Sharp, 1999).

For Biogenic silica (BSi) analysis, 100-200 mL water sample was filtered using 0.2µm polycarbonate filters (GTPP from Millipore) and kept inside individual plastic petri dishes after folding in quarters and oven dried at 60°C overnight. The filters were digested in 0.2N NaOH inside a water bath at 85°C for 1 hour. Later the samples were brought to room temperature and 0.2N HCl was used to neutralize the samples. One aliquot of this solution (preferably 1mL) was taken and diluted to 25 mL (Paasche 1973) with the help of Milli-Q water. Silicate concentration was measured using the colorimetric methodology of Strickland and Parsons (1972). A blank filter paper was also digested and treated in the same way as samples to measure the reagent blank.

For HPLC pigment analysis, 100-500mL of water was filtered onto 25 mm GF/F filters using a vacuum pump (Rocker 600 from Tarsons, India) keeping the pressure below 200mb. The filtration

was done under minimum light to avoid any degradation/conversion of pigments. Immediately after the filtration, the filter paper was folded and wrapped inside an aluminum foil and kept at -20°C until analysis. Pigment extraction was done by soaking the filter in 3-5 mL 90% acetone (HPLC grade from Merck chemicals) overnight inside an amber coloured bottle to prevent any 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. An HPLC from Agilent Technology (Series 1200) was used to quantify the pigments following the method proposed by Heukelem and Thomas (2001) using manual injector mode. The concentrations of pigments were calculated using the standard pigments procured from Sigma Aldrich (USA) and DHI (Netherlands).

The number of total bacteria was quantified following the method of Hobbie et al. (1977). The samples were collected in 2 mL Eppendorf tubes, preserved with 4% formalin and kept refrigerated. 250µl of water was incubated with 40µl of 4,6-diamidine-2 phenyl indole (DAPI) for 10 min and filtered on a black polycarbonate 0.2µm filter. The entire filter paper was mounted on a glass slide in oil immersion and examined under 1500 magnification (91500) under the microscope (Olympus BX51 Epi-Fluorescence) using UV.

For phytoplankton cell density and enumeration, 500mL water sample was fixed with Lugol's iodine (1%; w/v) solution. The Samples were stored in plastic bottle, in dark at low temperature and a settling and siphoning procedure was followed to concentrate the samples to 25mL. One mL of concentrated samples was examined microscopically (OLYMPUS BX 53) in a Sedgewick-Rafter plankton counting chamber at 400× magnification. Phytoplankton enumeration was done following the manuals of Desikachary (1987) and Hasle et al. (1997). Total phytoplankton abundance was calculated in terms of the number of phytoplankton cells observed in 1L of water sample and was expressed in terms of relative percentage for each phytoplankton genus identified.

For some parameters correlations coefficients were calculated using Microsoft Excel 2007.

3. Results:

3.1 EXP-I:

HPLC pigment analysis showed that Fucoxanthin (Fuco) was the dominant marker pigment in the cell extracts of the experimental phytoplankton community. Additionally, chlorophyll c_2 (Chl c_2), diadinoxanthin (DD), diatoxanthin (DT) and β -carotene were also present in addition to chlorophyll a (Chl a). Zeaxanthin (Zea) was present in very small quantity ($< 0.1\mu\text{g L}^{-1}$) relative to

Fuco. The concentrations of all pigments together represent the total pigment (TP) concentrations. Compared to the control, a significant increase in TP (30-44%) and Chla (83-93%) concentrations were noticed in response to 10nM and 100nM Cu addition (Fig. 2a, Fig. 2b). Further increase in Cu addition (1000nM) caused a significant reduction in both TP (3.88times) and Chla (4.77 times) concentrations coupled with a marked increase in the concentration Chla degradation product (allomer/epimer of Chla). The % of Chla degradation product relative to Chla values were calculated (Fig. 2c) and the results showed that the values deferred slightly ($15\pm 2\%$) from the control till 100nM Cu level; however, increased to 77% at 1000nM Cu level indicating strong negative impact of Cu on Chla.

Compared to the values of the control, Fuco concentrations (Fig. 2d) increased $\approx 32\%$ in response to 10 -100nM Cu addition followed by a drastic decline (2.8 times) at 1000nM Cu level. Chlc₂ concentrations did not show any noticeable change from the control to 100nM Cu addition; but a 26 times reduced values was noticed at 1000nM Cu level (Fig. 2e). This observation suggested that chlorophyll synthesis is more sensitive to increasing Cu stress than the carotenoids. DD concentrations remained similar in both the control and 10nM Cu treated samples (Fig. 2f) followed by a significant decrease ($>34\%$) at 100nM Cu treatment. Fifteen times reduction in DD concentration was observed under 1000nM Cu treatment. Furthermore, the DT-index [DT/(DT+DD)] showed its minimum value in the control and maximum value at 1000nM Cu level (Fig. 2h).

Phytoplankton cell counts (Fig. 2i) increased gradually in response to increasing Cu level (till 100 nM Cu) followed by a 20% decline at 1000nM Cu relative to the control. Total bacteria count (TBC) showed a very similar variability (Fig. 2j). Microscopic analysis revealed that there was a noticeable change in phytoplankton community composition under different Cu treated samples (Fig. 3). In the control, the community was dominated by mainly *Chaetoceros* (69%) followed by *Skeletonema costatum* (22.4%) with negligible contribution of dinoflagellates (Fig. 3a). The relative abundance of *Chaetoceros* sp. remained unchanged ($\approx 60\%$) till 100nM Cu level and decreased to 51% at 1000nM Cu level. Compared to the control, the relative abundance of *Skeletonema costatum* increased almost 28% till 100nM Cu treatment and 39% at 1000nM Cu level. In the initial samples, *Thalassiosira decipiens* was present in very low quantity (0.39%) and proliferated in all incubated samples (up to 5.5% relative abundance), except at 1000nM Cu level. The relative abundances of *Chaetoceros* sp. and *Skeletonema costatum* varied inversely and showed a significant negative linear correlation ($R^2 = 0.96$; $p < 0.05$).

3.2 EXP-II:

In EXP-II also Cu acted as growth promoting agent till a certain concentration and became toxic thereafter (Fig. 4). The concentrations of POC increased initially with increasing Cu level. From the control, a 40% and 78% enhancement in POC concentration was observed in response to 25nM and 50nM Cu addition, respectively (Fig. 4a). POC showed its minimum value at 800nM Cu level (2.8 times less relative to the control). PON showed an almost similar pattern (Fig. 4b). At 25nM and 50nM Cu treatment, PON concentrations increased by 58% and 84%, respectively. A slight reduction in the concentrations of PON was noticed between 50 and 400nM Cu level (on average $45.9 \pm 2.7 \mu\text{molL}^{-1}$), with a maximum reduction (1.4 times reduction) at 800nM Cu treatment relative to the control. The ratios of C:N were close to the classical Redfield ratios (6.63) for phytoplankton (Fig. 4c). C:N ratio increased slightly at 100-150nM Cu level and decreased steadily thereafter reaching its minimum value to 800nM Cu level. BSi concentrations did not vary widely, except at 800nM Cu treatment (Fig. 4d) where it became almost half than the value noticed in the control. BSi and POC concentrations showed a significant positive correlation ($n=8$, $p > 0.001$), indicating a similar pattern.

In the control treatment, $\delta^{13}\text{C}_{\text{POM}}$ value was -23.78‰ which is typical for the marine diatoms (Fig. 4e) and this value declined gradually till 50nM Cu level (-25.24‰) followed by slight increase at 100 -150nM Cu level. Interestingly, between 200 -800nM Cu treatment a significant depletion in $\delta^{13}\text{C}_{\text{POM}}$ value was noticed. The minimum value (-31.90‰) was noticed at 800nM Cu level. The first three values of $\delta^{13}\text{C}_{\text{POM}}$ (in the control, 25 and 50nM Cu level) revealed a significant negative logarithmic correlation with the concentrations of POC ($R^2 = 0.999$, $p < 0.02$); however, the relation became positive when the values from 100-800nM Cu treatments were correlated ($R^2 = 0.891$, $p < 0.05$). The average $\delta^{15}\text{N}_{\text{POM}}$ values were much higher (Fig. 4f) relative to normal phytoplankton grown on nitrate. The trend of $\delta^{15}\text{N}_{\text{POM}}$ showed a slight increase from the control till 100nM Cu level and decreased thereafter reaching the minimum at 800nM Cu. The corresponding difference between the control and the highest Cu treated $\delta^{15}\text{N}_{\text{POC}}$ was almost 9.25‰. The first three values of PON and $\delta^{15}\text{N}_{\text{POM}}$ were linearly correlated ($R^2 = 0.999$, $p < 0.02$); a positively logarithmic correlation ($R^2 = 0.9347$, $p < 0.02$) was obtained between the PON concentrations and $\delta^{15}\text{N}_{\text{POM}}$ values from 100-800nM Cu treated cells.

3.3.1 EXP-III A-November 2011:

In EXP-III A, Chl*a* concentrations decreased with increasing distance from the coast (Fig. 5) indicating lower phytoplankton abundance in the offshore stations. In response to 50nM Cu addition

at S1, a 24.4% enhancements in *Chla* concentration was noticed (Fig 5a) than that of the control. However, a 6.5 time decrease in *Chla* concentration was noticed at 150nM Cu level. The addition of Fe did not elicit a growth enhancement and the *Chla* values were lower than the control (Fig. 5b). However, when various Cu concentrations were added in the presence of 100nM Fe, all treatments showed adverse effects on *Chla* values (Fig. 5c).

At S2 station, the concentrations of *Chla* decreased with increasing Cu addition (Fig. 5d) and reduced almost 12.7 times at 150nM Cu level. Unlike S1 station, at this station, various Fe addition significantly promoted growth (Fig. 5e) and the highest *Chla* value was detected at 50nM Fe addition. Addition of Cu in the presence of Fe boosted *Chla* concentration more than 5 times, on average, in all treatments (Fig. 5f). At S3 station, *Chla* values decreased drastically to almost 3.7 times in all Cu levels relative to the control (Fig. 5g). At this station too, the supply of Fe seemed to enhance growth; the highest *Chla* value was noticed at 50nM Fe level (Fig. 5h). Interestingly, Cu enrichment increased *Chla* concentration in a concentration-dependent manner at fixed (100 nM) concentration of Cu. At this station, the magnitude of growth enhancement was significantly higher than that of station S2. The highest *Chla* value ($7.84 \pm 1.7 \mu\text{g L}^{-1}$) was detected at the highest Cu level (150nM Cu+100nMFe).

3.3.2 EXP-IIIB-January 2012:

In EXP-IIIB (January) the added Cu and Fe levels were much lower than that of EXP-IIIA (November), and thus, phytoplankton growth responses was also different (Fig. 6). At S1 station, *Chla* concentration increased almost 60% in response to 25nM Cu addition; however, decreased by 2.14 times at 50nM Cu level (Fig. 6a). Fe addition slightly decreased *Chla* concentrations (Fig. 6b). The samples supplied with both Cu and Fe also did not show any noticeable variation from the control; however, a 8.22% higher *Chla* values were noticed in all Cu treatments except at 5nM+10nM Fe (Fig. 6c). At S3 station, *Chla* concentrations were always higher in all Cu treated samples; however, the magnitude was different (Fig. 6d). *Chla* values increased 1.6 and 2.11 times at 2nM and 4nM Cu levels, respectively than that of the control. The *Chla* values started to decrease at 6 and 10nM Cu level. The samples treated with only Fe (Fig. 6e) showed almost 29% higher *Chla* values on average than that of the control in the first three treatments (2nM, 4nM, 6nM) and reduced by 18% at 10nM Fe level. *Chla* value at 2nM Cu+6nM Fe treatment ($0.32 \mu\text{g L}^{-1}$) slightly decreased than the control ($0.45 \pm 0.04 \mu\text{g L}^{-1}$) and increased in the rest of the treatments with Cu+Fe. The maximum *Chla* value ($1.22 \pm 0.61 \mu\text{g L}^{-1}$) was noticed at 6nMCu+6nM Fe level (Fig. 6f) and compared to the control this value was almost 98% higher.

At S1 station, HPLC pigment analysis showed the presence of Fuco and Zea among the diagnostic pigments. There was no Chl*b* present along with Zea indicating that the probable origin of Zea was cyanobacteria. At S1 station, Fuco/Chl*a* ratio (Fig. 7a) did not show any clear trend in response to 5nM -50nM Cu addition. Zea/Chl*a* ratio showed noticeable decrease from the control to all other Cu treatments (Fig. 7a). Fuco/Chl*a* ratio showed almost 47% increase in response to 10nM Fe addition (Fig. 7b); however, the ratio decreased with further increase in Fe level, but remained comparable with the ratio observed in the control. A 2-3 times reduction in Zea/Chl*a* ratio was observed in the Fe added samples than the control. Under Fe+Cu addition (Fig. 7c), nearly 20% increase in Fuco/Chl*a* ratio was noticed at the highest Cu level (10nM Fe+50nM Cu); Zea/Chl*a* ratio showed no clear pattern till 25nM Cu+10nM Fe level and a sharp decrease was noticed at 50nM Cu+10nM Fe treatment.

HPLC pigment analysis showed that at S3 station too both Fuco and Zea were the major contributing diagnostic pigments. In the control, Zea/Chl*a* ratio was higher than the Fuco/Chl*a* ratio (Fig. 7d) indicating higher abundance of Zea containing algae relative to Fuco. Almost 50% decrease in Zea/Chl*a* ratio than that of the control was noticed in response to Cu addition (2-10nM). Fuco/Chl*a* ratio showed a gradual increase in response to Cu addition and became more than double at 4nM Cu level. The enhancement remained almost 47% higher in other Cu concentrations relative to the value seen in the control. In response to Fe addition (Fig. 7e), the ratio of Fuco/Chl*a* increased gradually from 2nM till 6nM and decreased slightly at 10nM Fe level. Zea/Chl*a* ratio showed a gradual increase in response to Fe addition and almost 54% increase was noticed under 10nM Cu level than that of the control. 2nMCu+6nM Fe addition resulted in a clear enhancement in both Fuco/Chl*a* and Zea/Chl*a* ratios from the control (Fig. 7f). Fuco/Chl*a* ratio decreased when Cu levels increased; however, it did not decrease below the ratio noticed in the control. Zea/Chl*a* ratio showed a significant decrease under 10nM Cu+6nM Fe.

4. Discussion:

4.1.1 General growth response to Cu addition

Cu addition clearly impacted phytoplankton pigment signature, biomass (POC and PON), BSi, $\delta^{13}\text{C}_{\text{POM}}$, $\delta^{15}\text{N}_{\text{POM}}$, cell density and species composition. However, the magnitude of response differed significantly depending on the community, sampling time and distance from the coast. The concentration of total dissolved Cu can vary on a wide range when moving away from the coast. Strong horizontal gradients in dissolved metal concentrations have been observed for Zn, Fe, Mn. The concentrations of these metals can be several hundred folds higher in the coast than the open ocean (Reviewed by Bruland et al. 1991). Rejomon et al. (2010) reported clear spatial trends in the

concentrations of dissolved metals and zooplankton in the Bay of Bengal during summer monsoon. The authors also noticed higher Cu concentrations in the coastal and upwelling regions; while, lower levels were detected in the offshore warm core eddies. Similarly, a coastal transect along the Visakhapatnam coast showed much higher dissolved Cu concentrations (20nM) relative to the offshore water (8.5nM). A similar gradient was observed in the concentrations of metals present in zooplankton and it was attributed to absorption from phytoplankton (Rejomon et al. 2010). The enhanced Cu sensitivity of the phytoplankton community with increasing distance from the coast could be attributed to the decreasing Cu concentration in the experimental transect.

4.1.2 Impact of Cu on phytoplankton pigments

The phytoplankton community in both EXP-I and III showed that Cu addition increased Chl*a* concentrations up to a certain level. A similar result was also reported during our earlier study (Biswas et al., 2013). Peers et al. (2006) reported a doubling in Chl*a* content in response to 2nM Cu addition in the phytoplankton communities from Bering sea. However, Cu induced Chl*a* enhancement in natural phytoplankton community could also be due to inhibited grazing by microzooplankton (Coale 1991) which are highly sensitive to Cu toxicity (Stoecker et al. 1986). Likewise, during the present study at S1 station, the observed Cu-induced Chl*a* enhancement may not be solely due to Cu enrichment. Since, microzooplankton analysis was not done, this fact remains inconclusive. The sharp decrease in Chl*a* content at 150nM Cu level indicated toxic impact of Cu. In our earlier experiments, 150nM Cu did not show such a strong inhibition and higher background Cu level could be the reason for such result. In November, both dissolved and free metal concentrations were high due to the impact of SW monsoonal river runoff, which brings a huge amount of metal enriched freshwater to this bay. The combined enrichment of Cu and Fe did not promote growth in the phytoplankton at S1 station thereby suggesting that none of the trace metals were limiting in the system during the experimental period.

Cu showed significant deleterious impact on phytoplankton pigment signature at higher concentrations. Cid et al. (1995) noticed in a marine diatom that Chl*a* concentration increased initially when Cu level increased below 0.1mg L⁻¹ and severely decreased thereafter. Interestingly, the magnitude of Cu-induced decrease in chlorophylls (Chl*a* and Chl*c*) was higher than that of carotenoids. In higher plants, pigment accumulation, integration of chlorophyll molecules to pigment system II (PSII) (Capsi et al. 1999) and PSII functioning (Barón et al. 1995) can be largely hampered by Cu stress. In the present experiment, the significant increase in Chl*a* degradation product, particularly allomer and epimer, under 1000nM Cu treatment also indicated a malfunction of chlorophyll assembly in the coastal phytoplankton. One common mechanism responsible for

decreased Chl *a* value due to heavy metal contamination is the replacement of Mg⁺² ions with other divalent metals within the porphyrin ring of chlorophyll molecule (Küpper et al. 1996). These heavy-metal-replaced-chlorophyll (hmr-Chl) molecules behave differently than the normal chlorophyll and are not capable of light harvesting. It has been observed in-vitro that Cu-replaced chlorophyll (Cu-Chl) showed significant lower or no fluorescence quantum yield relative to Mg-Chl (Watanabe and Kobayshi 1988). It is likely that inhibition in the synthesis, accumulation and function of chlorophyll molecules by Cu is the first responsible step for reduced growth in autotrophs.

Inhibited chlorophyll synthesis under higher Cu levels could be also due to low nitrate uptake rate. During the present study, under 800nM Cu level significant reduction in $\delta^{15}\text{N}_{\text{POM}}$ and PON values showed considerable negative impact on nitrogen metabolism. It has been shown that Cu toxicity can significantly lower nitrate uptake in marine phytoplankton (Harrison et al. 1977) and as a consequence chlorophyll synthesis can be largely hindered under low nitrogen availability.

Cu ions may generate oxidative stress by producing reactive oxygen species (ROS) which are actively scavenged by certain enzymes (superoxide dismutase, glutathione reductase, catalase). In the present study, oxidative stress was indicated by increased DT-index under higher Cu levels. Diatoms usually possess a xanthophyll cycle where DD is enzymatically converted to DT for dissipating the extra energy as heat (non-photochemical quenching) in order to avoid photo-inhibition (Lavaud et al. 2002). Our earlier studies showed that a coastal diatom increased DT production with increasing Cu concentrations (Biswas et al. 2013). Cu is well known to elicit oxidative stress in marine diatoms (Rijstenbil et al. 1994; Morelli and Scarano 2004) and its expression has been shown at the genetic level (Davis et al. 2006). However, Bertrand et al. (2001) showed that higher concentrations of cadmium did not impact the conversion of DD to DT (de-epoxidation) in a marine diatom; but, slowed down the reverse conversion of DT to DD (epoxidation). In the present experiment, the observed decrease in DD concentration at 1000nM Cu level could be due inhibited epoxidation. As a consequence, there was an increase in DT relative to DD concentration resulting in a high DT index.

4.1.3 Impact of Cu addition on cellular stoichiometry

Initial increase in the concentrations of POC, PON and BSi in EXP-II clearly demonstrated that Cu addition up to a certain level was growth promoting for the coastal phytoplankton community. Decreased POC values with increasing Cu toxicity showed inhibited photosynthesis. Gustavson et al. (1999) reported that the coastal phytoplankton community from the Isefjord, Denmark, showed significant decrease in primary productivity. However, some authors (Stauber and Florence, 1987; Cid et al. 1995) noticed that the inhibitory effects of Cu on photosynthesis and growth are not coupled; growth rate can be inhibited on a higher magnitude than photosynthesis

because Cu inhibits the process of cell division and thus slows down the production of new cellular material.

There is almost no data showing the impact of Cu toxicity on phytoplankton isotopic signature. However, the observed trend could be influenced by multiple physiological factors. The initial depletion in $\delta^{13}\text{C}_{\text{POM}}$ values from the control to 50nM Cu level could be attributed to higher growth rate and this was confirmed by the high linearity between POC and $\delta^{13}\text{C}_{\text{POM}}$ values. When autotrophs including marine phytoplankton fix CO_2 , the heavier isotope ^{13}C is being largely discriminated against the lighter isotope ^{12}C , resulting in a depleted values of $\delta^{13}\text{C}_{\text{POM}}$ (Rau et al. 1996). The level of discrimination can be highly correlated (negative) with dissolved CO_2 levels (Laws et al. 1995). $\delta^{13}\text{C}_{\text{POM}}$ values did not show further depletion till 200nM Cu level suggesting inhibited carbon uptake and growth at this Cu level.

Interestingly, the $\delta^{13}\text{C}_{\text{POM}}$ values at 400 and 800nM Cu level reached its minimum and depletion of more than 5‰ in $\delta^{13}\text{C}_{\text{POM}}$ is highly unusual in marine diatoms. This could be linked to several other factors. Phytoplankton fractionate more ^{12}C over ^{13}C leading to a depleted $\delta^{13}\text{C}_{\text{POM}}$ values during a slow growth phase and simultaneous reduction in POC values also supported this view. Laws et al. (1997) reported a depleted value of -39.99‰ at a pH of 8.26 in a slow growing nitrogen limited diatom. Morel et al. (1994) noticed that when a marine diatom was grown under atmospheric CO_2 and limited Zn, carbonic anhydrase (CA) activity was significantly low which hampered HCO_3^- uptake and photosynthetic activity. As a result, a highly depleted (< -34‰) values of $\delta^{13}\text{C}_{\text{POM}}$ was noticed. Cu has been shown to interfere with cell permeability or the binding of essential metals (Sunda and Huntsman 1983) and can enter into the cell where it binds quickly to the enzyme's active sites making them non-functional (Florence and Stauber 1986). Likewise, Cu stress might have hampered both bicarbonate uptake and CA activity leading to a slow growth and thus a depletion in $\delta^{13}\text{C}_{\text{POM}}$ values. A similar depletion was also noticed in a coastal diatom *Chaetoceros gracilis* in response to Cu stress from the study area (Biswas et al. unpublished data).

Cu induced change in biochemical composition of phytoplankton cells may be another reason for depleted $\delta^{13}\text{C}_{\text{POM}}$ values. Henley et al. (2012) noticed a strong depletion in $\delta^{13}\text{C}_{\text{POM}}$ values due to the dominance of a particular diatom species (*Proboscia*) which produces a specific set of long-chain lipids 1,14-diols (<-32‰) and 12-hydroxy methyl alkanates <- (34 %) (Sinninghe Damsté et al. 2003). Likewise, any change in relative synthesis of cellular protein, carbohydrate and lipid pools could also result in such depleted values of $\delta^{13}\text{C}_{\text{POM}}$ (Laws 1991). Lipids are isotopically lighter by 5-6‰ than proteins and carbohydrates; hence, relative increase or decrease in lipid production could

also lead to a significant change in $\delta^{13}\text{C}_{\text{POM}}$ values. It is possible that the coastal diatoms produce some phytochelatin with enriched $\delta^{13}\text{C}_{\text{POM}}$ to combat against Cu toxicity and that may cause a depletion in $\delta^{13}\text{C}_{\text{POM}}$.

The $\delta^{15}\text{N}_{\text{POM}}$ values in the first three Cu treatments clearly indicated that within the growth promoting phase, PON increase was well coupled with linear enrichment of $\delta^{15}\text{N}_{\text{POM}}$. Richoux and Froneman (2009) reported a similar result from a subtropical convergence zone where PON and $\delta^{15}\text{N}_{\text{POM}}$ exhibited positive correlation. The rate of decrease in PON production at elevated Cu level indicated that nitrogen metabolism was sensitive to Cu stress. Nitrogen metabolism can be hampered in two ways: due to inhibition in 1) nitrate uptake rate and 2) conversion of nitrate into amino acids. Proteins are relatively more sensitive to Cu stress than carbohydrates by the virtue of their thiol /amino groups which are usually being attacked at first by free Cu^{++} ions (Letelier et al. 2005). The enzyme nitrate reductase, the main enzyme involved in nitrate reduction, is highly sensitive to Cu stress (Harrison et al. 1976). A significant down regulation of this enzyme was noticed in a marine diatom when exposed to high level of Cu (Davis et al. 2006). The drop in $\delta^{15}\text{N}_{\text{POM}}$ values at 800nM Cu level (almost half that of the control) indicated a strong inhibition in nitrate uptake rate which has been reported earlier (Harrison et al. 1977). However, $\delta^{15}\text{N}_{\text{POM}}$ values seen in this experiment were relatively higher than the normal values observed in the coastal phytoplankton communities grown under nitrate. A possible reason could be the nitrate salt used to enrich the culture as a nitrogen source which showed such high isotopic signature and presumably led to a higher $\delta^{15}\text{N}_{\text{POM}}$.

BSi concentration also showed a similar response like POC. Silicate uptake rate can be significantly inhibited by increasing Cu supply and has been seen in our earlier experiment too (Biswas et al., 2013). As mentioned earlier, Cu can inhibit silicate uptake rate in marine diatoms because Cu and Si ions compete for the same protein transporter during the cellular transport process (Morel et al. 1978; Rueter et al. 1981). Moreover, during the first experiment, the microscopic analysis revealed that the *Skeletonema costatum* chains were longer under higher Cu levels relative to the control. This suggested that inhibited silicate uptake rate probably hindered the process of new frustules formation and therefore remained in the same chain

4.1.4 Impact of Cu on phytoplankton community structure

The sensitivity and responses of phytoplankton community to increasing Cu concentrations could be related to their community composition (Gustavson et al. 1999; Pérez et al. 2010). Both HPLC pigment and microscopic analysis revealed that the experimental phytoplankton community from the coastal stations were diatom dominated. A clear shift in the experimental phytoplankton

community structure in EXP-I indicated dissimilar sensitivity of different diatom species to Cu toxicity. With increasing Cu concentrations *Skeletonema costatum* dominated over *Chaetoceros* sp. which seemed to be sensitive to increasing Cu level. Whereas, *Skeletonema costatum* showed higher tolerance under maximum Cu level. *Thalassiosira decipiens* showed higher sensitivity and its relative abundance reduced significantly at 1000nM Cu level. Davis et al. (2006) reported that another species of *Thalassiosira* showed high sensitivity to Cu stress and possesses several cellular mechanisms to counteract copper toxicity. Usually the occurrences of *Skeletonema costatum* bloom after heavy river discharge has been observed from the coastal Bay of Bengal (Shaik et al. 2015) and during this time significant amount of heavy metals enter the coastal systems. During a coastal water pollution monitoring program along the Visakhapatnam coast, *Skeletonema costatum* was found to be present even in highly polluted water inside the harbour (Biswas et al. unpublished data). Morel et al. (1978) showed in a laboratory experiment that *Skeletonema costatum* was insensitive to Cu stress. It was observed by Croot et al. (2000) that *Skeletonema costatum* showed no-linearity with ligand production and external Cu stress which was attributed to its tolerance to high Cu level. In another study by Nassiri et al. (1997) it was reported that *Skeletonema costatum* showed 50% growth inhibition at 700nM Cu level.

The dominance of *Zea* over *Fuco* at S3 station in January indicated cyanobacterial abundance over diatom. The significant decrease in *Zea/Chla* ratios coupled with enhanced *Fuco/Chla* ratios with increasing Cu levels both at S1 and S3 stations showed greater sensitivity of cyanobacteria to Cu stress relative to diatoms. Brand et al. (1986) investigated the sensitivity of 38 marine phytoplankton species in vitro and noticed that the prokaryotic cyanobacteria were the most sensitive to Cu stress; whereas, diatoms were the least sensitive. Coccolithophores and dinoflagellates showed moderate sensitivity. Cyanobacteria evolved more than 2 billion years ago when the oceans were highly reducing and the concentrations of Cu were extremely low due to the low solubility of copper sulfides. Hence, the organism did not develop any efficient biochemical means of resisting Cu toxicity. In contrast, when the other three major eukaryotic phytoplankton groups, particularly, diatoms, evolved the oceanic waters were oxygenated and Cu concentrations increased. Probably, the phytoplankton groups evolved lately in the course of evolution, developed an efficient mechanism to counteract Cu toxicity (Brand et al. 1986). Furthermore, eukaryotic algae like diatoms exhibit advanced cell structure with membrane bound cell organelles which are highly efficient in accumulating and immobilizing toxic metal ions within the cell. In contrast, cyanobacterial simple prokaryotic cellular structure could be disadvantageous to reduce metal toxicity (Gadd and Griffiths, 1978).

Fe addition in January resulted in increased *Zea/Chla* ratio at S3 station indicating that Fe addition increased cyanobacterial abundance. *Fuco/Chla* also increased to some extent showing diatom growth and declined thereafter. It is possible that there was a shift within the community from diatom to cyanobacteria with increasing Fe levels and therefore *Chla* concentration did not show any net growth promoting trend. The addition of Fe+Cu did not show any particular change in phytoplankton biomass at S1 station. There was a clear shift from cyanobacteria (*Zea/Chla*) to diatom abundance (*Fuco/Chla*) at the highest Cu level. This could be due to the competitive efficiency of diatom over cyanobacteria under Fe enrichment and Cu toxicity. Significant increase in both diatoms and cyanobacteria in response to the addition of 2nMCu in the presence of 6nM Fe indicated that Cu was slightly limiting at S3 station. The presence of Fe also could help the cyanobacterial community to produce more chelating ligands (Stauber and Forence, 1985). Moreover, the magnitude of increase was much larger compared to only Cu addition. The presence of Fe seemed to be beneficial at this station and helped to utilize Cu efficiently. It has been observed that in open ocean regions, cyanobacteria produce very high level of strong chelating ligand to reduce Cu toxicity (Moffet and Brand 1996).

Sensitivity of heterotrophic bacterial community to heavy metals has also been reported by some authors (Jonas 1989). In the present study, the observed reduction in TBC was probably due the sensitivity of the total community to increasing Cu level. Goulder et al. (1979) showed that Cu and arsenic contamination inhibited bacterial growth in an estuarine water. Nevertheless, it was also shown that the resistance of the bacterial isolates from coastal waters to increasing Cu concentrations can be significantly high (Beleneva et al. 2011). However, the observed increase in TBC numbers following the same trend of phytoplankton cell density could be due to more availability of labile organic carbon from phytoplankton origin.

4.1.3 Interactive impacts of Cu and Fe

Fe addition alone at S1 station did not show any growth enhancement neither in November, nor in January suggesting that they were not limited by Fe probably due to their proximity to the coast. The significant increase in phytoplankton growth at S3 station (In January) in response to increasing Cu+Fe addition indicated the co-limitation of these metals. Coastal phytoplankton may not be limited by the essential trace metals like Fe, Cu or Zn; however, our earlier studies (Biswas et al. 2013; Biswas and Bandyopadhyay 2013) show that this may not be always valid. Hutchins and Bruland (1998) showed that the diatoms in coastal upwelling region can also be iron limited. The activity of these trace metals is largely dependent on the free metal ion concentrations, which are further controlled by the quantity and quality of the organic ligands and the seawater pH (Sunda and Huntsman 1998). The presence of high levels of organic ligands in coastal waters can readily

scavenge free metal ions and can reduce their bioavailability. Moreover, the added iron may also help the cells to counteract Cu toxicity. Phytoplankton cells can be more sensitive to Cu toxicity under Fe limitation and Cu toxicity can be alleviated by Fe addition (Stauber and Forence, 1985; Nielsen and Nielsen, 1970). High concentrations of colloidal ferric hydroxide in the Fe replete medium probably bound to the organic layer surrounding the diatom siliceous frustules and adsorb Cu^{++} ions (Davies 1966) and thus prevented toxic Cu entry inside the cell. Hence, the supply of Cu and Fe together to the coastal waters is likely to have completely different impacts on the coastal phytoplankton communities.

5. Conclusions:

The present study showed that the phytoplankton communities from the coastal waters of Visakhapatnam can be highly sensitive to increasing Cu levels. The physiological responses of phytoplankton clearly demonstrated that increasing Cu concentration hampers pigment signature, biomass production, biogenic silica production and also the stable isotopic signature of carbon and nitrogen. All these may have considerable influence on the overall primary production and major nutrient utilization efficiency in marine phytoplankton. Phytoplankton community composition also showed marked changes in response to Cu toxicity which may have significant impact on the food web. The co-enrichment experiment clearly indicated that both Fe and Cu were probably limiting at the offshore stations and Fe seemed to help in counteracting Cu toxicity to phytoplankton. Therefore, simultaneous addition of Cu and Fe will impact the phytoplankton community differently compared to individual enrichments of these elements. Further research is needed to understand the biochemical changes in phytoplankton cells in response to increasing Cu stress. Additionally, a detailed study on phytoplankton community response to Cu toxicity is also required to be done.

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Table 1: The concentrations of Cu used during different experiments. Please note that this is the added Cu concentrations and not the measured values.

Experim ent ID	Sampling time	Stations	Added Cu concentrations (nM)							
			Control	1st	2nd	3rd	4th	5th	6th	7th
EXP-I	March 2011	S1a*	0	10	100	1000	-	-	-	-
EXP-II	June 2011	S1a*	0	25	50	100	150	200	400	800
	Novemer 2011	S1	0	50	100	150	-	-	-	-
		S2	0	50	100	150	-	-	-	-
EXP-III	January 2012	S3	0	50	100	150	-	-	-	-
		S1	0	5	10	25	50	-	-	-
		S3	0	2	4	6	10	-	-	-

*S1a= samples were collected from <100m from the coast. S1=1 km from the coast; S2 = 6 km from the coast; S3 = 6 km from the coast; S4 = 10 km from the coast.

Table 2: Nutrient concentrations during different experiments in relation to variable Cu concentrations. The values here are the mean from duplicates. S1=1km from the coast; S2 = 6km from the coast; S3 = 10km from the coast.

Experiments	Sampling time	Station	Silicate	Phosphate	DIN	pH
			($\mu\text{mol L}^{-1}$)	($\mu\text{mol L}^{-1}$)	($\mu\text{mol L}^{-1}$)	
EXP-I	March 2011	S1a*	4.78±0.09	1.26±0.17	8.8.±0.39	8.15±0.02
EXP-II	June 2011	S1a*	26.82±0.27	6.84±0.07	26.78	8.17±0.04
		S1	16.32±0.82	0.52±0.02	9.59	8.13±0.015
	November	S2	11.85±3.71	0.30±0.08	10.72	8.22±0.05
EXP-III	2011	S3	4.80±0.04	0.33±0.015	10.58	8.26±0.025
	January 2012	S1	7.67±0.35	0.98±0.03	11±0.6	8.15±0.02
		S3	4.54±0.29	0.33±0.03	3.44±0.49	8.21±0.02

*S1a= samples were collected from <100m from the coast; S1=1 km from the coast; S2 = 6 km from the coast; S3 = 10 km from the coast.

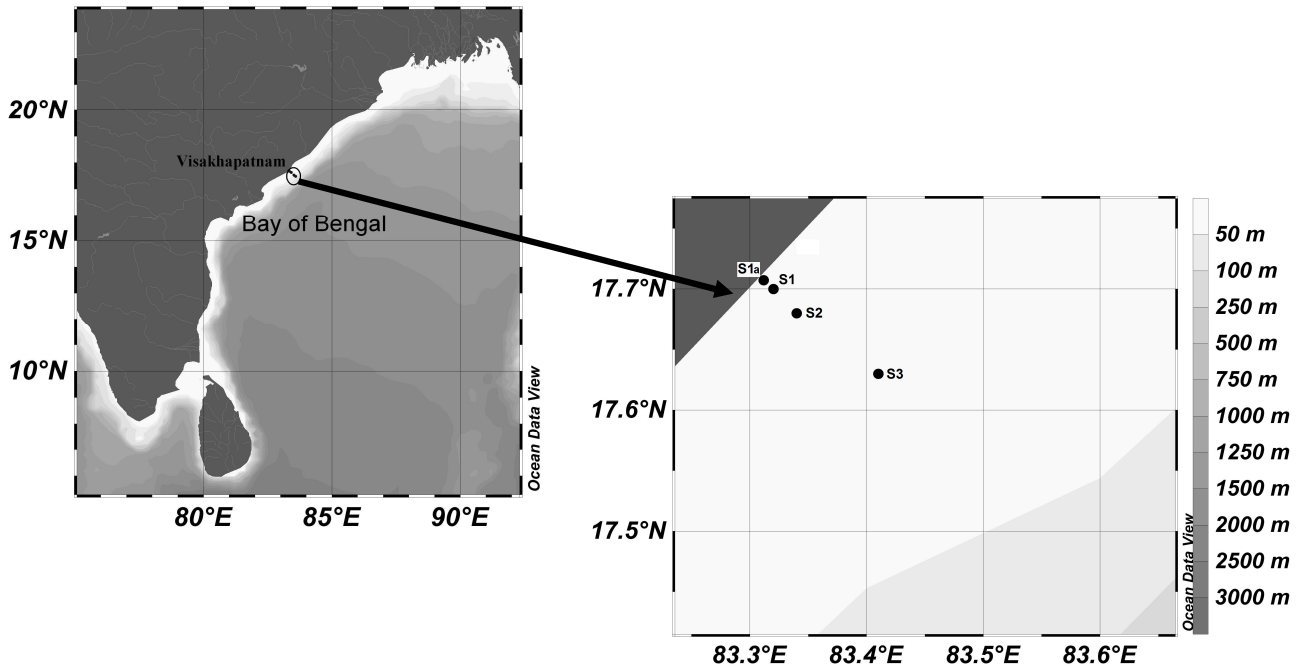


Fig. 1: Map showing the sampling locations. S1a =<100m from the coast; S1 =1km from the coast; S2 =6km and S3 =10km.

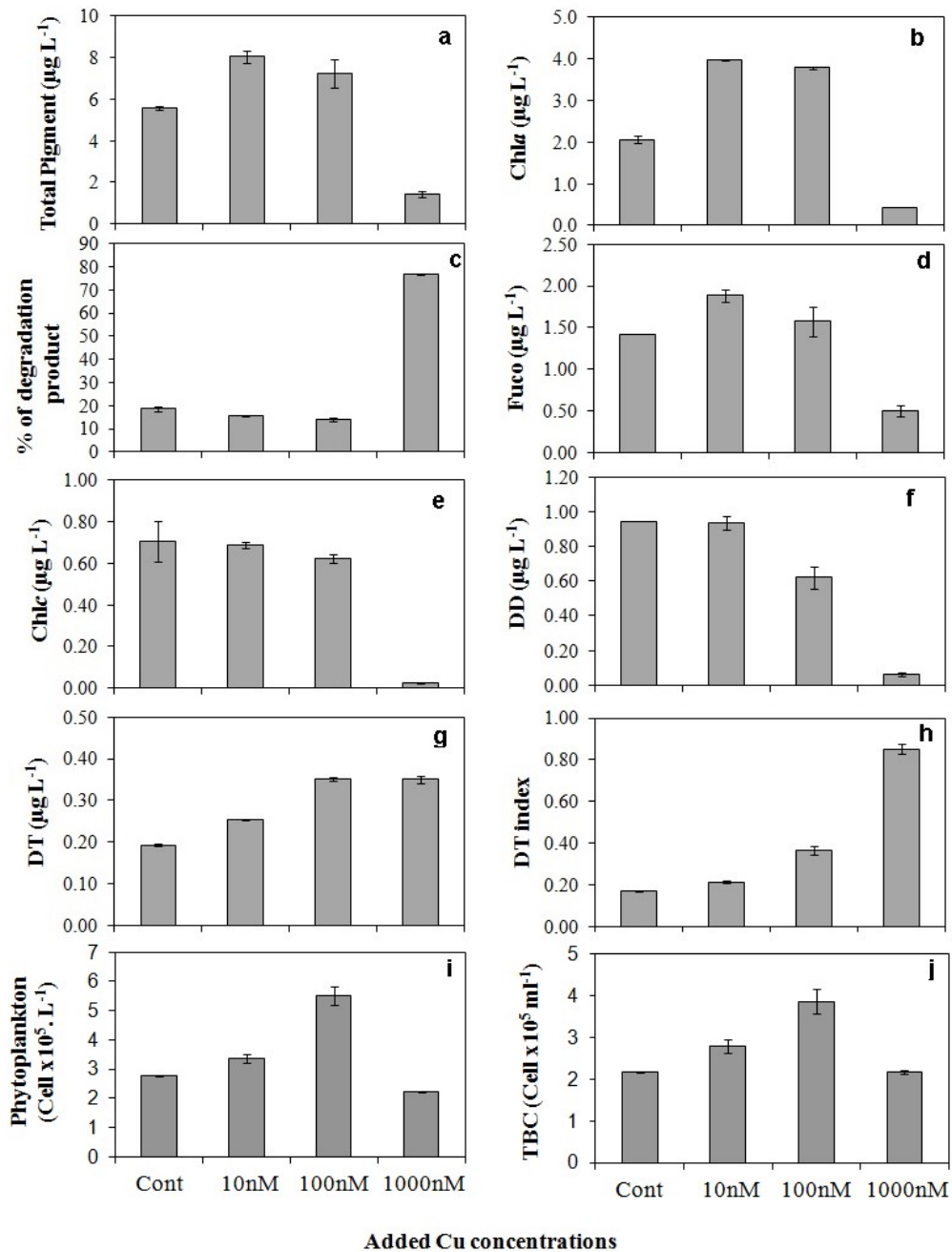


Fig.2: Results of EXP-I (March 2011). The Concentration of (a) total cellular pigment detected in HPLC analysis; b) Chla; (c) % of degradation product relative to Chla detected in HPLC analysis; (d) Fuco; (e) Chlc; f) DD; g) DT and h) DTindex; i) phytoplankton cell counts and j) total bacterial count under the experimental Cu addition. The values are from duplicate analysis (mean \pm SD).

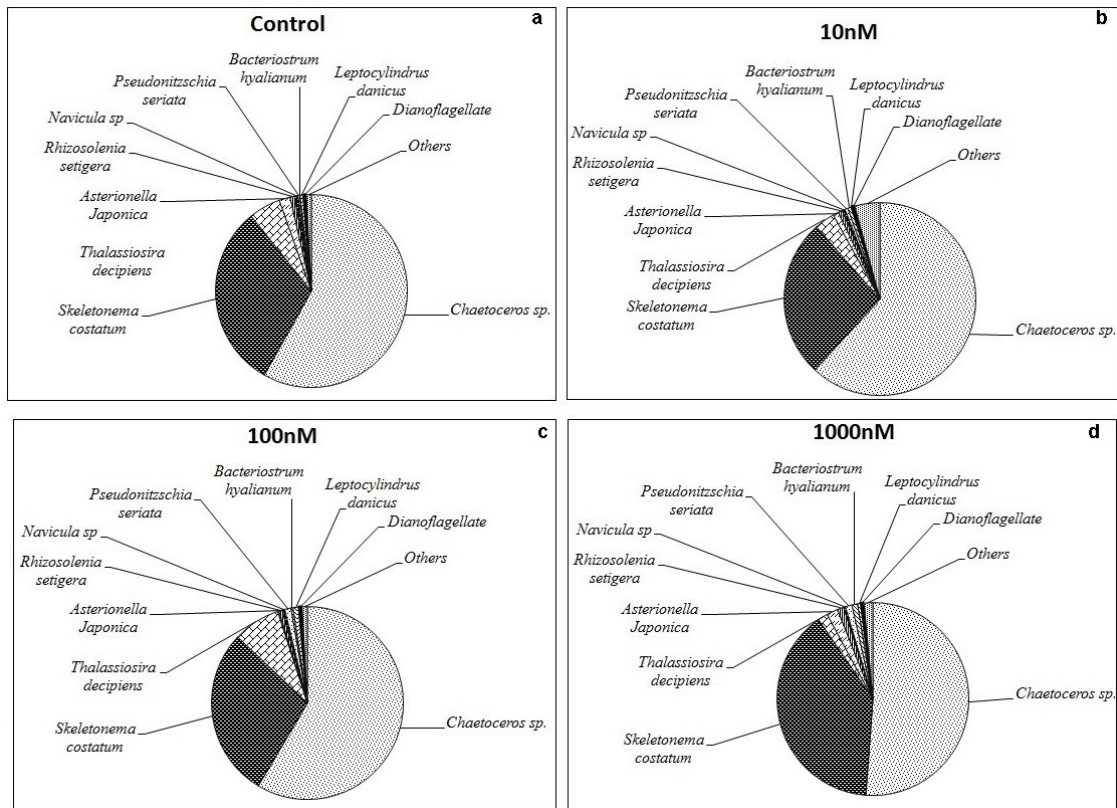


Fig.3: The results of EXP-I (March 2011). Relative abundance (%) of different phytoplankton species in a) Control; b) 10 nM; c) 100 nM and d) 1000 nM experimental Cu treatments in March 2011.

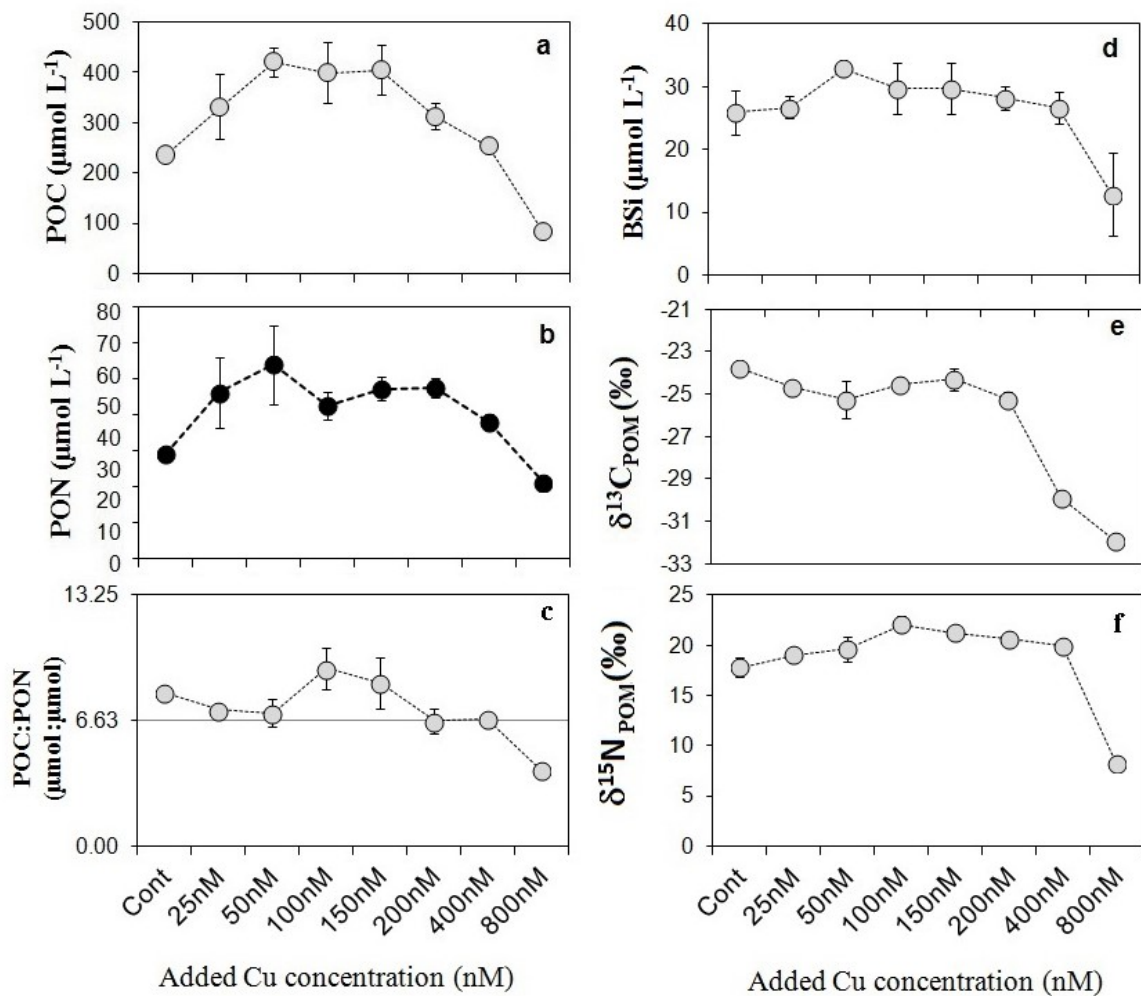


Fig. 4: The results of EXP-II (June 2011). The concentrations of (a) particulate organic carbon (POC); b) particulate organic nitrogen (PON); c) the ratios of POC:PON (the gray line indicated the classical Redfield ratio 6.63); d) the concentrations of biogenic silica (BSi); e) $\delta^{13}\text{C}_{\text{POM}}$ and f) $\delta^{15}\text{N}_{\text{POM}}$ under various experimental Cu addition (the values are from duplicate measurements; mean \pm SD).

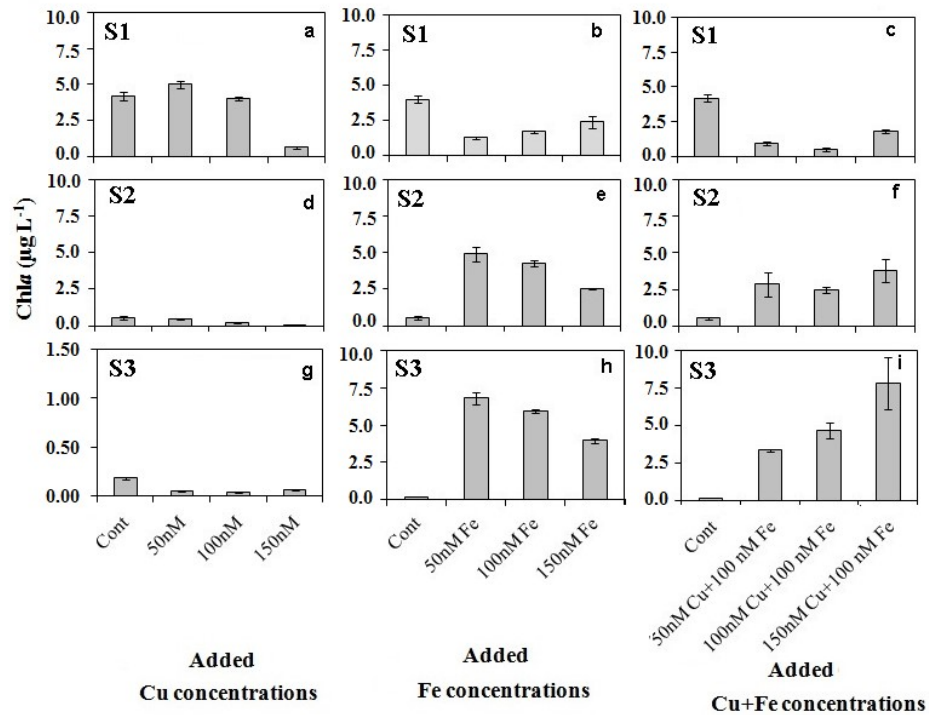


Fig. 5: Results of EXP-III A (November 2011). The concentrations of Chla at S1 station in response to a) Cu addition, b) Fe addition and c) Cu+Fe addition; the concentrations of Chla at S2 station in response to d) Cu addition, e) Fe addition and f) Cu+Fe addition; the concentrations of Chla at S3 station in response to g) Cu addition, h) Fe addition and i) Cu+Fe addition. The controls were without any metal addition. The values given are from the duplicate analysis (Mean±SD). S1=1km from the coast; S2 = 6km from the coast; S3 = 10km from the coast.

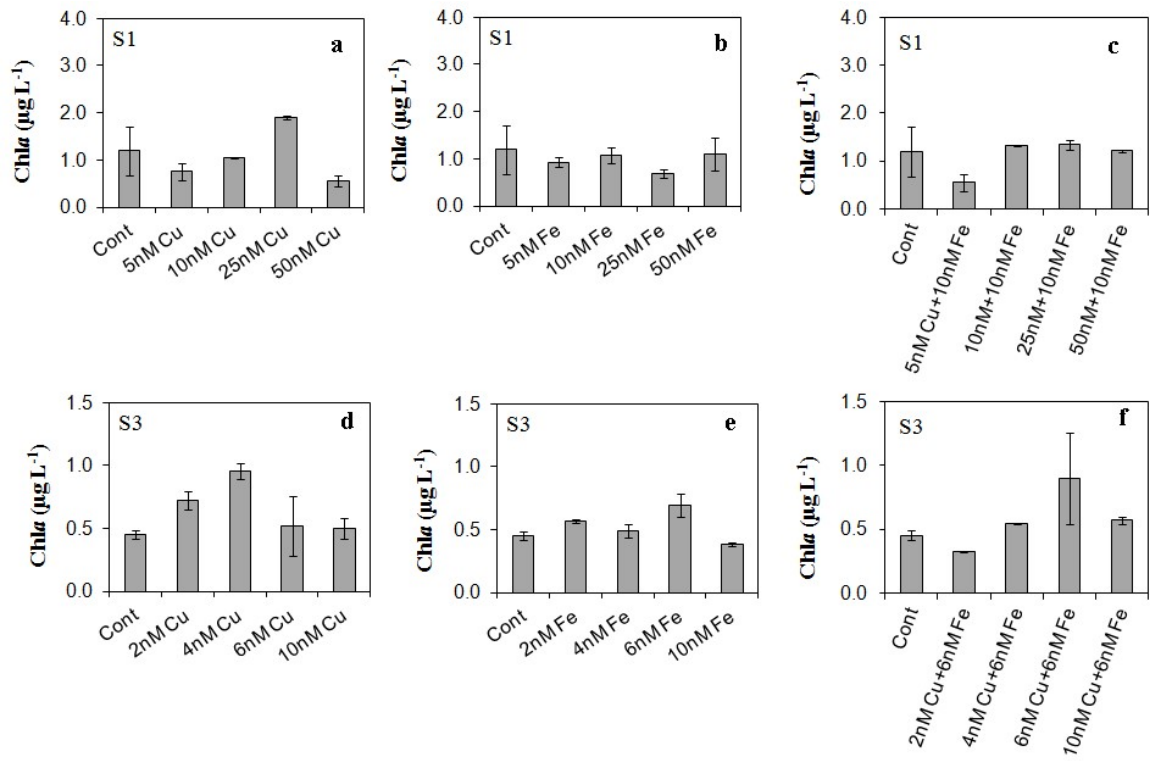


Fig. 6: The results of EXP-IIIB (January 2012). The concentrations of Chla at S1 in response to a) Cu addition; b) Fe addition and c) Fe+Cu addition; the concentrations of Chla at S3 in response to d) Cu addition; e) Fe addition and f) Fe+Cu addition. The controls were without any Cu addition. The values given are from the duplicate analysis (Mean±SD). S1=1km from the coast; S3 = 10km from the coast. It should be noted that different scales are used for S1 and S3 samples.

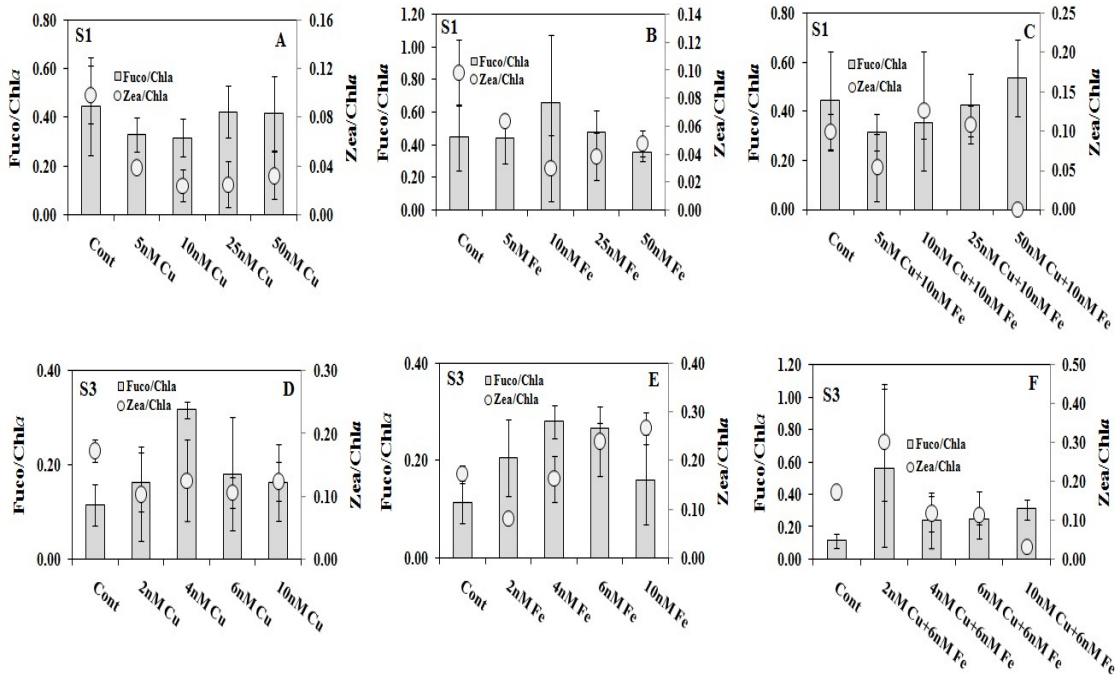


Fig. 7: The ratios of Fuco:Chla and Zea:Chla at S1 station in response to a) Cu addition; b) Fe addition and c) Fe+Cu addition; the ratios of Fuco:Chla and Zea:Chla at S3 station in response to variable a) Cu addition; b) Fe addition and c) Fe+Cu addition. The values given are from the duplicate analysis (Mean±SD). S1=1km from the coast; S3 = 10km from the coast.