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The response of a natural phytoplankton community from the Godavari River Estuary to increasing CO₂ concentration during the pre-monsoon period

Running title: Response of a phytoplankton community to elevated CO₂

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Abstract: This paper reports for the first time upon the effects of increasing CO₂ concentrations on a natural phytoplankton assemblage in a tropical estuary (the Godavari River Estuary in India). Two short-term (5-day) bottle experiments were conducted (with and without nutrient addition) during the pre-monsoon season when the partial pressure of CO₂ in the surface water is quite low. The results reveal that the concentrations of total chlorophyll, the phytoplankton growth rate, the concentrations of particulate organic matter, the photosynthetic oxygen evolution rates, and the total bacterial count were higher under elevated CO₂ treatments, as compared to ambient conditions (control). $\delta^{13}\text{C}$ of particulate organic matter (POM) varied inversely with respect to CO₂, indicating a clear signature of higher CO₂ influx under the elevated CO₂ levels. Whereas, $\delta^{13}\text{C}_{\text{POM}}$ in the controls indicated the existence of an active bicarbonate transport system under limited CO₂ supply. A considerable change in phytoplankton community structure was noticed, with marker pigment analysis by HPLC revealing that cyanobacteria were dominant over diatoms as CO₂ concentrations increased. A mass balance calculation indicated that insufficient nutrients (N, P and Si) might have inhibited diatom growth compared to cyanobacteria, regardless of increased CO₂ supply. The present study suggests that CO₂ concentration and nutrient supply could have significant effects on phytoplankton physiology and community composition for natural phytoplankton communities in this region. However, this work was conducted during a non-discharge period (nutrient-limited conditions) and the responses of phytoplankton to increasing CO₂ might not necessarily be the same during other seasons with high physicochemical variability. Further investigation is therefore needed.

Key words: tropical estuary, Godavari River, pre-monsoon period, rising CO₂, phytoplankton, community composition, diatom, cyanobacteria.

Abbreviations:

DIC: Dissolved inorganic carbon
DIN: Dissolved inorganic nitrogen
DIP: dissolved inorganic phosphate
CCM: Carbon Concentrating Mechanism
POM: Particulate organic matter
POC: Particulate organic carbon
PON: Particulate organic nitrogen

1. Introduction:

The projected rise in atmospheric CO₂ has the potential to affect the marine ecosystem in various ways and at different magnitudes (Wolf-Gladrow et al., 1999; Doney, 2009). The global ocean is a significant sink for atmospheric CO₂, currently absorbing almost one-third of it (Sabine, 2004). As a consequence, surface ocean carbonate chemistry is considerably affected (direct impact), resulting in redistribution of inorganic carbon species (Brewer, 1997, Raven, 2005). On the other hand, increasing mean global temperature is likely to cause enhanced sea surface temperature (SST), resulting in warmer surface seawater mass, redistribution of isotherms, reduced upwelling, and greater light penetration (indirect impact) (Raven, 2005). Thus, either directly or indirectly, there is a strong possibility that the projected changes will affect marine phytoplankton physiology (primary production, nitrogen fixation, calcification etc.), taxonomic composition (floristic change) and geographic distribution (Boyd et al., 2010, Bopp et al., 2001, Boyd and Doney, 2002; Raven 2005).

The responses of marine phytoplankton to increasing CO₂ are not yet fully understood. The marine phytoplankton community comprises diverse groups from different geographic locations, each possessing its own unique ecophysiology and biogeography. During the last decade, numerous discrete studies have been conducted on marine phytoplankton (laboratory pure cultures, shipboard and field experiments) to test the effects of increasing CO₂ and a wide range of responses have been observed (Riebesell, 2004; Riebesell, 2008; Doney, 2009). Whilst increased primary production under high CO₂ conditions has been observed in marine phytoplankton assemblages from many different regions of the world's oceans (Hein and Sand-Jensen, 1997; Chen and Gao, 2003; Leonardos and Geider, 2005, Hare et al., 2007; Tortell et al., 2008, Feng et al., 2009), numerous other studies have shown no such enhancement (Yoshimura et al., 2009, Delille et al., 2005). The non-heterocystous marine nitrogen-fixer *Trichodesmium* has been found to show considerably higher carbon and nitrogen fixation rates when incubated under elevated pCO₂ levels (Levitan et al., 2007; Hutchins et al., 2007; Barcelos et al., 2007). In contrast, the heterocystous nitrogen-fixing cyanobacteria *Nodularia spumigena* (from the Baltic Sea) has shown reduced cell division and nitrogen fixation rates when supplied with higher DIC (Czerny et al., 2009). Reduced calcification in marine calcifying phytoplankton is a well-established response to reduced pH (Riebesell et al., 2009).

Shifts in phytoplankton community composition in response to rising CO₂ have been shown to be significant but highly varied across a diverse range of geographical regions (Tortell et al., 2002a; Kim et al., 2006; Tortell et al., 2008; Yoshimura et al., 2009). Additionally, in combination with changing carbonate chemistry, presence of the major nutrients also play a vital role in determining the taxonomic composition of marine phytoplankton, depending on their nutritional preference, demand and uptake efficiency (Riebesell, 2004). However, the key reasons for the observed differences in behavioural response of marine phytoplankton to increasing CO₂ are still not clear. They are assumed to be related to carbon metabolism pathways and cellular energy distribution (Giordano, 2005; Rost et al., 2008).

It has been proposed in the past that, in the marine environment, phytoplankton photosynthesis is limited by CO₂ supply, and that open ocean pCO₂ ranges cannot satisfy even half the saturation level of the carbon-fixing enzyme RuBisCO (Riebesell, 1993). Moreover, RuBisCO in general has a very low affinity for CO₂ (Badger et al., 1998), and so to enhance the DIC supply at the carboxylation site of the cell, most marine phytoplankton have developed an active carbon concentration mechanism (CCM), which involves the active uptake of CO₂ and HCO₃⁻, or only HCO₃⁻, (Giordano, 2005) at the cost of energy (Raven and Johnson 1991). In parallel, there is also continuous diffusive loss of CO₂ from the cell. The uptake of CO₂ in groups of phytoplankton without active CCM occurs mainly by diffusive transport across the cell membrane. Under the scenario of increasing CO₂, the enhanced external CO₂ concentration may lead to a simultaneous enhancement in diffusive CO₂ supply inside the cell and decreased diffusive loss of CO₂ from the cell. This may slow down CCM activity, resulting in the saving and reallocation of cellular energy (Fridlyand et al., 1996; Rost et al., 2008; Giordano et al., 2005), and hence faster growth (Tortell et al., 2008). Previous studies have shown that some groups of phytoplankton possess highly efficient CCMs and might not show any alteration in growth and primary production under elevated CO₂ (Rost et al., 2003; Cassar et al., 2004).

In contrast to open-ocean phytoplankton, estuarine phytoplankton production is not limited by nutrients or CO₂, but mainly by light penetration due to heavy suspended sediment load in the estuarine water. Tropical estuaries and coastal regions are places of huge material flux, with high turnover rates of the major elements (C, N, P), mainly because of extensive biological activity (both autotrophic and heterotrophic). Therefore, these areas exert great control over the global carbon cycle. Summer-monsoon-induced heavy precipitation and river discharge

are largely responsible for controlling the biogeochemistry of tropical estuaries. Huge amounts of organic matter, suspended sediment and nutrients from agricultural and anthropogenic sources are delivered into estuarine systems, leading to partial or complete heterotrophic conditions (characterized by high pCO₂ values in the water column) (Biswas et al., 2004, Mukhopadhyay et al., 2001; Sarma et al., 2009, Bouillion et al., 2003). In contrast, non-discharge (pre-monsoon) periods (April–June) are mostly characterized by low levels of nutrients and low pCO₂ (created by enhanced autotrophy due to increased light penetration), which can limit phytoplankton production. Under the scenario of increasing atmospheric CO₂ levels, it is possible that tropical estuarine phytoplankton communities may not show any alteration in growth and primary production when CO₂ is not the limiting factor, i.e. mainly during monsoon and post-monsoon months. In contrast, during the non-discharge pre-monsoon period, when CO₂ can be limiting, phytoplankton communities may show a response to increased CO₂ availability, and this response could potentially have an influence on the biological carbon pump in the estuary.

There is virtually no literature available regarding the above notion, and there is a particular lack of research focusing on the Indian subcontinent, which of course harbours one of the largest river systems in the world. To address this gap, the present study, which was conducted in the Godavari River Estuary in India, aimed to fulfil the following objectives: 1) to investigate whether CO₂ and nutrient supply can limit growth and primary production of the natural phytoplankton community during the pre-monsoon period; and 2) to study the impact of increasing CO₂ on the phytoplankton community.

2. Study site:

The sampling site was chosen in the mixing zone of the Godavari River Estuary, almost 30 km upstream from the Bay of Bengal (Fig. 1). The Godavari River is one of the major rivers in the east coast region of India and supplies a huge amount of nutrients and organic load to the Bay of Bengal (Gupta et al., 1997). Monsoonal run-off and river discharge coupled with turbidity, light penetration and nutrient availability, are the key factors controlling the biogeochemistry of this estuary (Sarma et al., 2009). Most tropical estuaries are eutrophic, acting as a source of atmospheric CO₂. However, as mentioned above, during the non-discharge pre-monsoon period, major nutrients (N, P, Si) and low pCO₂ might limit phytoplankton primary production. Usually, during May–June (pre-monsoon), discharge in the Godavari River ceases. Coupled with no precipitation, the estuary thus becomes an area of negative water balance

(evaporation exceeds precipitation), leading to the occurrence of low-nutrient, high-saline water. Restricted river discharge also limits the supply of organic carbon loads (particulate and dissolved) to the estuary, resulting in bacterial respiration being lower than autotrophic production. Consequently, the rate of dissolved inorganic carbon removal from the surface water by photosynthetic activity exceeds the production of CO₂ by heterotrophic activity, resulting in comparatively low pCO₂ conditions (Sarma et al., 2009, Bouillon et al., 2003). At the same time, high levels of salinity decrease the carbon dioxide solubility in the water. On an annual scale, diatoms are the dominant group of phytoplankton, along with some nitrogen-fixing cyanobacteria, dinoflagellates, and green algae.

3. Materials and methods:

3.1 Sample collection and experimental setup:

As described above, the pre-monsoon season was chosen for this investigation because of the occurrence of high salinity, low nutrients, and low pCO₂ in the water column, which could all limit phytoplankton primary production. During May and June, 2009 (when river discharge had ceased completely), estuarine surface water was collected in 20 L polycarbonate carboys (Nalgene) from Yanam, the mixing zone of the Godavari River (Fig. 1) and taken immediately back to the laboratory. 5.6 L borosilicate screw-capped bottles were used for further incubation. CO₂ levels were manipulated by adding base and acid following the methodology of Riebesell et al. (2010). Among the processes available for manipulating CO₂ levels in seawater, this method has been recommended as the best for small-volume bottle experiments. After adjusting the pCO₂, the bottles were incubated in triplicates for each CO₂ level in a water bath under ambient light conditions for five days. A set of controls was also kept in triplicates without changing the carbon chemistry. Each bottle was mixed very gently every day to avoid any sedimentation of the phytoplankton biomass. Tables 1 and 2 respectively show the initial carbon chemistry and physicochemical parameters for both experimental sets.

In May ambient water pCO₂ was 156 µatm (control). In addition, two other CO₂ levels (244 and 363 µatm pCO₂) were set in order to see if phytoplankton production was limited by CO₂ supply. Nitrate and phosphate were added to promote phytoplankton growth. The water sample was pre-filtered with the help of a 200 µm zooplankton net in order to reduce the grazing pressure by larger zooplankton. A similar approach was used by Rose et al. (2009). In June, the

initial pCO₂ was 225 µatm (control). Attempts were made to set the pCO₂ level at 750 µatm (in order to examine the effects of projected CO₂ levels on the phytoplankton), but manipulation succeeded only at reaching 646 µatm, less than the projected CO₂ value. The CO₂ levels were further increased (1403 and 1860 µatm) to see at which CO₂ level maximum growth and production occurred. No nutrients were added in this experiment, so as to avoid any overlap between nutrient- and CO₂-induced growth of phytoplankton. The larger zooplankton (>200 µm) were also not pre-filtered, so as to observe only CO₂-induced growth of phytoplankton under normal grazing pressure.

3.2 Sample analysis:

After five days of incubation, the bottles were taken to the laboratory for further analysis. DIC and alkalinity samples were taken in 40 ml screw-capped glass bottles fitted with Teflon septums and fixed with HgCl₂ until analysis. DIC was analysed with the help of a coulometric acidification module (CM5 130) and total alkalinity was analysed using a 794 Basic Titrino from Metrohm, following Dickson et al. (2003). pCO₂ was calculated from CO2SYS using alkalinity and DIC (Lewis & Wallace 1998). Nutrients were analysed following the spectrophotometric method introduced by Strickland and Person (1972). Primary production was estimated using light and dark bottles, and dissolved oxygen (DO) was estimated by Winkler's titration (Strickland and Person, 1972). GF/F filter paper was used to filter the total chlorophyll samples from each treatment in duplicate, before being soaked overnight in DMF (N,N-Dimethyl formamide) (Suzuki and Ishimaru, 1990) and measured without acidifying the sample. HPLC pigment analysis was carried out using the HPLC from Agilent Technologies (series 1200) and following the method of Heukelem and Thomas (2001). δ¹³C_{POM} was analyzed using the Delta V Plus, Isotopic Ratio Mass Spectrometer. 500 ml to 1L of sample water was filtered through the pre-combusted glass fiber filter (GF/F), followed by overnight drying at 40°C. Filters were exposed to HCl fumes to remove the particulate inorganic carbon (if any) and then measured using the PDB standard, following the formula:

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

POC and PON were analysed using an elemental analyser attached to the Isotopic Ratio Mass Spectrometer (Sharp, 1975). The total number of bacteria was quantified following the method of Hobbie et al. (1977). Samples were collected from all the bottles in a 2ml Eppendorf

tube and refrigerated after adding formalin until analysis. 250 µl of water were incubated with 40 µl of 4, 6-diamidine-2 phenyl indole (DAPI) for 10 min and filtered through black polycarbonate 0.2 µm filter paper. The entire piece of filter paper was mounted on a glass slide in oil immersion and examined under the microscope (Olympus BX51 Epi-Fluorescence) at magnification 1500 using UV light. Alkaline Phosphatase Activity was measured spectrophotometrically using *p*-nitrophenyl phosphate (PNPP) (LOBA Chemicals, India) as the substrate, following the methodology of Nicholson et al. (2006). The release of yellow-coloured para-nitro-phenol is normally measured as an analogue of the enzyme activity, which is the product of hydrolysis of *p*-nitrophenyl phosphate. The absorbance was measured at a wavelength of 405 nm after 3–5 min centrifugation. A standard curve was prepared using different concentrations of *p*-nitro phenol (PNP) to calculate the final concentration.

3.3 Statistical analysis:

Two sample t-tests were performed using MINITAB (version 15). The average values of different parameters obtained 1) from two individual experiments (with and without nutrient addition); and 2) from different CO₂ levels, were tested to see if the observed differences were statistically significant. The level of significance was considered as $p < 0.05$ and < 0.001 .

4. Results

4.1 Biomass, growth rate and primary production:

A significant enhancement in biomass was observed in both experiments with the concomitant nutrient depletion in all treatments. The growth response was considerably higher in May than in June, and could have been due to nutrient addition. The magnitudes of the observed results from the two individual experiments were found to be statistically different (significant at the 95% level, $p < 0.001$, DF =27). Figures 2a–c represent total chlorophyll, growth rate ($\mu_{chl\ a} d^{-1}$) and net photosynthetic oxygen evolution at different pCO₂ levels. The initial chlorophyll values were $2.34 \pm 0.15 \mu g L^{-1}$ in May and $1.2 \pm 0.043 \mu g L^{-1}$ in June (Table 2). In May, after five days of incubation, this increased to 6.36 ± 1.63 , 7.78 ± 2.3 and $8.9 \pm 1.98 \mu g L^{-1}$ in the 156, 244 and 363 µatm pCO₂ treatments, respectively (Fig. 2a). The growth rates were 20% and 35% higher at 244 and 363 µatm pCO₂ in comparison to the control (Fig. 2b). Similarly, in June, both total chlorophyll and the growth rate doubled under the 646 µatm pCO₂ treatment compared to the

control (225 μ atm), but remained almost the same after 6.28- and 8.26-fold increases in CO₂ levels (1403 and 1860 μ atm).

In May, the net photosynthetic oxygen evolution rate was enhanced 1.58 times from the control to 363 μ atm pCO₂ (a 2.32-fold increase in pCO₂). In June, this enhancement was 1.24, 1.93, and 2.13 times higher at under the 646, 1403 and 1860 μ atm pCO₂ treatments with respect to the control (Fig 2c). This corresponds to a 23.4% enhancement at the 646 μ atm CO₂ level, which is comparable with earlier CO₂ enrichment studies (\approx 20%) (Hein and Sand-Jensen, 1997).

4.2 Elemental composition:

As with the growth rate and total chlorophyll values, POC production also showed a linear increase with increasing CO₂ levels (Fig. 3a). In May, the initial POC concentration was 58.1 \pm 0.96 μ M, which then reached up to 188 \pm 26 μ M at the highest CO₂ level (363 μ atm), followed by 168 \pm 23 μ M at 244 μ atm, and 156 \pm 47 μ M under the 156 μ atm pCO₂ treatment (control). In June, the initial concentration of POC was 33.75 μ M. The final values were 66.2 \pm 4, 100 \pm 5.04, 107 \pm 15, and 105 \pm 7 μ M in the control (225), 646, 1402 and 1860 μ atm pCO₂ treatments, respectively (Fig. 3a). PON values varied within a range of 24.26 \pm 3.67 to 29.56 \pm 3 μ M in May and 10.21 \pm 0.31 to 14.44 \pm 2.8 μ M in June (Fig. 3b), showing a slight enhancement with the increasing CO₂ levels. The lowest values of C:N ratio were observed in the control in both experiments and showed higher values in other CO₂ treatments (Fig. 3c). In May, the C:N ratio in the control was 6.1 \pm 0.29, which then increased to 6.5 \pm 0.23 at 244 μ atm and remained almost the same (6.5 \pm 0.1) at the 363 μ atm pCO₂ level. Where as in June, however, enhancements of 9.3%, 9.64% and 7.65% for C:N values were observed under the 646, 1403 and 1860 μ atm pCO₂ treatments respectively, as compared to the control.

4.3 Chlorophyll:POC ratio:

The Chl:POC ratio (Fig. 4a) also showed a gradual increase with increasing CO₂ levels. In the May experiment, the Chl:POC value was enhanced by 3% and 16% under the 244 and 363 μ atm CO₂ treatments, respectively. In June, enhancements of 25%, 21%, and 15% were observed at the 664, 1403 and 1860 μ atm pCO₂ levels respectively, as compared to the control. The growth rate and Chl:C ratio showed a significant positive correlation ($r^2 = 0.79$) (Fig. 4b), demonstrating the consistency of the observed higher growth rates under the elevated pCO₂ treatments.

4.4 Carbon stable isotopic composition of POM ($\delta^{13}\text{C}_{\text{POM}}$):

The initial values of $\delta^{13}\text{C}_{\text{POM}}$ were $23.94 \pm 0.07\text{‰}$ in May and $24.62 \pm 0.85 \text{‰}$ in June (Table 2). Figure 5a represents the final values of $\delta^{13}\text{C}_{\text{POM}}$ from all CO_2 treatments. It is interesting to see that in both experiments the initial values did not differ significantly from the post-incubation values in the control. A significant negative correlation ($r^2 = 0.86$) with increasing carbon dioxide and $\delta^{13}\text{C}_{\text{POM}}$ was obtained (Fig. 5b). The maximum value of $\delta^{13}\text{C}_{\text{POM}}$ ($-23.74 \pm 0.63 \text{‰}$) was observed at the $156 \mu\text{atm pCO}_2$ level (in May), with a minimum value ($-26.24 \pm 0.13\text{‰}$) at the $1860 \mu\text{atm pCO}_2$ level (in June). The manipulated pCO_2 values in June were much higher than in May. Consistently, the average $\delta^{13}\text{C}$ of POM in May (-24.16‰) was higher than that observed in June (-25.61‰). Two sample t-tests showed this to be statistically significant at the 95% confidence level (t value = 6.97, DF=27, $p < 0.001$).

4.5 Alkaline phosphatase activity:

Alkaline phosphatase activity was measured on the final day of the experiment. Reasonably high activity was observed in all treatments (Fig. 6a), but the average activity was higher in June ($23.4 \pm 1.2 \text{ nmol PNP } (\mu\text{mol POC})^{-1}\text{h}^{-1}$) than in May ($18.17 \pm 7.8 \text{ nmol PNP } (\mu\text{mol POC})^{-1}\text{h}^{-1}$). In May, the POC normalized activity increased by 1.14 times at $244 \mu\text{atm pCO}_2$ and by 2.12 times under the $363 \mu\text{atm pCO}_2$ treatment, as compared to the control. However, in June, the activity did not differ much among the various pCO_2 treatments. In the control, as well as at the $646 \mu\text{atm pCO}_2$ level, alkaline phosphatase activity was almost the same (24 ± 2 and $24.7 \pm 1.2 \text{ nmol PNP } (\mu\text{mol POC})^{-1}\text{h}^{-1}$), but decreased slightly (23.16 and $21.74 \text{ nmol PNP } (\mu\text{mol POC})^{-1}\text{h}^{-1}$) under the 1403 and $1860 \mu\text{atm pCO}_2$ treatments.

4.7 Total bacterial count:

Total bacterial count was only calculated for the June experiment, and the result showed an increasing trend with increasing CO_2 (Fig. 6b). When pCO_2 was increased 2.86-fold from the control, a bacterial count of 1.5 times more was observed. The maximum number ($1.16 \times 10^6 \pm 8.4 \times 10^4$) of cells per ml was found at the $1860 \mu\text{atm pCO}_2$ level, which was 3.3 times higher than observed in the control.

4.8 Phytoplankton community composition by HPLC pigment analysis:

During both May and June the initial pigment analysis revealed a diatom-dominated community, with a greater abundance of fucoxanthin (a marker pigment of diatoms) over zeaxanthin (a marker pigment of cyanobacteria), with slight variation (Table 2). However, after five days of incubation, the distribution of these major marker pigments showed a significant change from the low to high CO₂ levels, and an increasingly prominent dominance of cyanobacteria was observed, replacing the previous abundance of diatoms. The ratios of fucoxanthin to chlorophyll-*a* and zeaxanthin to fucoxanthin are presented in Figs 7a and 7b. A significant negative correlation ($r^2 = 0.65$ in May and 0.91 in June) was observed between the fucoxanthin to chlorophyll-*a* ratio and CO₂ levels (figure not shown). In May, the Fuco/Chl-*a* ratio decreased by 2.76 times under the 363 μatm pCO₂ treatment in comparison to the control. In June, a two-fold decrease was observed at the 646 μatm pCO₂ level, with a minimum value of 0.716 at the highest pCO₂ level. In contrast, the Zea/Fuco ratio showed a significant positive correlation ($r^2 = 0.64$ in May and 0.61 in June) with CO₂ levels.

5. Discussion

5.1 Biomass, growth rate and elemental composition:

During May and June in the Godavari Estuary, the occurrence of nutrient limitation (particularly phosphate) and low pCO₂ are quite common, as this is the post-bloom phase of the phytoplankton community (Sarma et al, 2009). A higher N:P ratio than the classical Redfield value (16:1) was reported for this estuary by Sarma et al. (2010), and phosphate was found to be the limiting factor, ahead of nitrogen. Thus, both low phosphate and CO₂ can slow down phytoplankton growth and primary production. The aims of this study were to examine whether phytoplankton primary production in the Godavari Estuary is limited by CO₂ supply during the pre-monsoon season, and to discover what the effects are of CO₂ enrichment (alone, as well as coupled with nutrient addition).

Our observations clearly indicate a CO₂-dependent enhancement in growth and primary production across a wide range of pCO₂. Despite lower pCO₂ ranges (Table 1) in May than in June, the growth responses were much larger, possibly due to nutrient addition, more initial biomass, and less grazing (due to the removal of mesozooplankton). DIN concentrations were not significantly different between the two experiments (Table 2), but DIP concentration was 5.8

times higher in May. Thus, the observed greater growth response of phytoplankton to increased phosphate concentrations indicates the existence of phosphate-limited conditions during the study period, which is consistent with earlier work (Sarma et al., 2010). A greater level of alkaline phosphatase activity (Fig 6a) in June than in May (29% higher) also supports this view.

In May, the effect of CO₂ enrichment might be partly overshadowed by that of nutrient enrichment, as the differences between the controls and the high-CO₂ treatments were not statistically significant for all parameters. In June, however, noticeable differences were observed (significant at 95% level, $P = <0.001$, $DF = 13$) between the controls (225 $\mu\text{atm pCO}_2$) and the high-CO₂ treatments (646 -1806 $\mu\text{atm pCO}_2$) for all parameters. During this time, the initial biomass was comparatively lower (1.2 $\mu\text{g L}^{-1}$), no nutrients were added, grazing pressure was also normal due to the presence of mesozooplankton, and pCO₂ ranges were much higher than in the control. In this case, the entire enhancement effect could be attributed to CO₂ enrichment. A similar observation was reported by Egge et al. (2009) from a nutrient-enriched mesocosm experiment where primary production did not differ in relation to CO₂ levels under conditions of nutrient depletion and high CO₂. However, when nutrients became limited, there was a prominent increase in phytoplankton primary production (both in bulk and size-fractionated) at elevated CO₂ levels.

In a similar study by Feng et al. (2009) on the North Atlantic phytoplankton bloom, a clear enhancement in POC and growth rate was observed under greenhouse conditions (high temperature and CO₂). The Bering Sea phytoplankton bloom has also been shown to exhibit a similar response under predicted SST and CO₂, with a 2.6–3.5-fold enhancement in the biomass normalized photosynthetic rate having been observed (Hare et al., 2007). Furthermore, the effect of different CO₂ concentrations combined with light availability on the marine diatom *Skeletonema costatum* has been reported by Chen and Gao (2003). Thus, from our study, as well as other previously published work, it is clear that the magnitude of impact of increasing pCO₂ on marine phytoplankton is subject to the availability of other essential factors, such as light and nutrients.

Increasing pCO₂ also showed a strong influence on the elemental composition of phytoplankton from the Godavari Estuary. The C:N ratio increased by 6.8% from the control to the 244 and 363 $\mu\text{atm pCO}_2$ levels in May. Similarly, in June, the C:N value increased by 7.6–9.6% from the control to all other high-pCO₂ treatments. Levitan et al. (2007) reported an 8.27%

increase in the C:N ratio under conditions changing from 250 to 900 ppm of pCO₂ in a study involving *Trichodesmium*, a genus of marine nitrogen-fixing cyanobacteria. Many other authors have reported results from laboratory-based studies on marine phytoplankton in which the C:N:P ratio was found to be very sensitive to increasing CO₂, but also very varied depending on the individual phytoplankton species being studied (Burkhardt and Riebesell, 1997; Burkhardt et al., 1999). In general, enhanced production of POC during a higher growth phase might lead to the increase in the C:N ratio, which is consistent with the present study. Significant deviations from the classical Redfield values have also been observed in many studies, depending on the availability of nutrients (Riebesell et al., 2004).

The Chl:POC ratio (µg/µg) represents the contribution of phytoplankton biomass to the POC biomass, with greater values indicating higher growth rates both in the laboratory and in the field experiments (Bouteiller et al., 2003, Behrenfeld 2005). In this tropical estuary, the Chl:POC ratio has been reported to be comparatively lower than in the open ocean (Bouillon, 2003), possibly due to the contribution of a higher percentage of terrestrial biomass to POC. A considerable positive correlation was obtained between the growth rate and the Chl:POC ratio (Fig. 4b), with a high r² value (0.79) indicating a consistency among the observed higher growth rates in the high-CO₂ samples.

5.2 Variation in δ¹³C of particulate organic matter:

During periods of limited CO₂ supply, the diffusive influx of CO₂ inside the cell might not be enough to support the optimum performance of the carbon-fixing enzyme RuBisCO. To enhance the DIC pool in the carboxylation site, most phytoplankton actively transport HCO₃⁻ across the plasma membrane against the energy (CCM) (Raven and Johnson, 1991). An active HCO₃⁻ transport can contribute to low discrimination of ¹³C (δ¹³C ≈ 0‰) compared to diffusive CO₂ uptake, where δ¹³C ≈ -8‰ (Mook et al., 1974) and is indicated by an unaltered or slightly modified value of δ¹³C_{POM} after incubation at low CO₂ levels (Giordano et al., 2005). In the present experiment, the initial values of δ¹³C_{POM} (Table 2) showed very little change over the course of incubation under ambient conditions (controls) (-23.75‰ in May and -24.81‰ in June) (Fig. 4). The initial pCO₂ values (156 µatm in May and 225 µatm in June) were probably quite low in the controls in order to support the optimum operation of RuBisCO carboxylation (Badger et al., 1998; Riebesell, 2004). The diffusive influx of CO₂ was also limited, which is evident by

the unaltered values of $\delta^{13}\text{C}_{\text{POM}}$. This means that under low CO_2 conditions, the phytoplankton community actively pumped bicarbonate in order to increase the DIC pool inside the cell.

In natural systems, $\delta^{13}\text{C}_{\text{POM}}$ can be influenced by many factors other than CO_2 , and a series of phytoplankton $\delta^{13}\text{C}_{\text{POM}}$ values from the Godavari River Estuary has been described previously by Bouillon and Deharis (2000). Our experimental results showed a similar range of $\delta^{13}\text{C}$ values of POM. The authors described a lowering of $\delta^{13}\text{C}_{\text{POM}}$ values during freshwater input, and this could have been due to the enhanced DIC supply or the introduction of terrestrial organic carbon with lower $\delta^{13}\text{C}$ values. However, the samples in our experiment were taken during the pre-monsoon period, when the influence of allochthonous organic carbon on the system was negligible, with autochthonous phytoplankton production being predominant. Moreover, our experiments were conducted inside closed bottles, where the influence of other factors can be excluded and the entire effect on $\delta^{13}\text{C}$ values of POM could be due to differential uptake of carbon species by phytoplankton.

Phytoplankton assemblages from different areas of the world's oceans have been found to use bicarbonate as one of their major inorganic carbon sources during CO_2 -limited conditions. Cassar et al. (2004) reported that the Southern Ocean phytoplankton community uses mainly bicarbonate as an inorganic carbon source. Tortell et al. (2002b) showed that in the eastern subtropical and equatorial Pacific Ocean, the principal form of inorganic carbon taken up by phytoplankton is bicarbonate. The possibility of bicarbonate uptake during a diatom bloom in the mangrove waters of the Sundarbans was reported by Biswas et al. (2004). The existence of an efficient bicarbonate uptake path in the marine diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* from a laboratory experiment under low CO_2 conditions was shown by Burkhardt et al. (2001). Rau et al. (2001) reported the existence of a low fractionation of carbon stable isotope in the natural phytoplankton assemblage from the California upwelling system due to the presence of active bicarbonate uptake even under high CO_2 water. Thus, from the available literature, as well as our own observed results, it is quite clear that in the marine environment phytoplankton do not depend solely on the passive diffusion of CO_2 to accelerate carbon fixation, but actively uptake bicarbonate due to more availability.

The observed significant negative correlation ($r^2 = 0.87$) between $\delta^{13}\text{C}_{\text{POM}}$ and CO_2 levels (Fig. 5b) is consistent with earlier studies. Many authors have reported the existence of strong

negative correlations between $p\text{CO}_2$ and $\delta^{13}\text{C}$ of POC in surface seawater (Rau et al., 1989, Freeman and Hayes, 1992, Rau et al., 1992). $\delta^{13}\text{C}_{\text{POM}}$ from marine phytoplankton have been considered as a potential indicator of ambient CO_2 concentration because high DIC supply enhances higher discrimination of the heavier isotope ^{13}C against the lighter (^{12}C) (Totell and Morel, 2002; Wolf-Gladrow, 1999). Therefore, a low value of $\delta^{13}\text{C}_{\text{POM}}$ in phytoplankton might be credited to high CO_2 supply and uptake inside the cell. High values of phytoplankton $\delta^{13}\text{C}$ during diatom blooms indicate limited photosynthesis by diffusive CO_2 supply (Deuser, 1970). Considering the cellular energy budget, the active bicarbonate uptake process might be less favourable to a cell owing to its higher energy demand before the diffusive influx of CO_2 (Fridlyand, 1996, Giordano, 2005). During the present experiment, the phytoplankton community probably benefited from the enhanced CO_2 supply. The observed increase in photosynthetic oxygen evolution, growth rate and biomass production (POM and Chl *a*) under elevated CO_2 also supports this view.

5.3 Total bacterial abundance

Total bacterial abundance increased significantly with increasing CO_2 levels (Fig. 6b), which might have been due to the greater availability of substrate, such as DOC. Under elevated CO_2 levels, enhanced photosynthesis could be the potential source of reduced carbon (both dissolved and particulate). It has been reported that almost half of the daily photosynthetic production can be released by different mechanisms as DOC (Nagata et al., 2000; Lopez-Sandoval et al., 2010) and can be available for bacterial consumption. DOC was not analyzed in the present study, and thus we do not have any direct evidence. However, some earlier studies have shown the co-occurrence of high bacterial abundance with reduced DOC concentrations under elevated CO_2 levels (Yoshimura et al., 2009). Higher heterotrophic bacterial production under elevated CO_2 levels has also been reported, from a nutrient-enriched coastal mesocosm experiment (Engel et al., 2004). Since higher rates of photosynthesis at higher CO_2 levels were observed, part of the reduced carbon might have been released as DOC and supported bacterial growth. However, at the same time increased heterotrophic bacterial activity may have increased the demand for, and rate of, nutrient consumption, especially phosphate and nitrate, and could have created strong competition between autotrophs and heterotrophs.

5.4 Phytoplankton community composition and nutrient budget:

A community shift in response to increasing CO₂ levels has also been reported in many earlier studies, and this is assumed to be controlled not only by the carbonate chemistry, but also by nutrient availability and species preference (Riebesell, 2004). In our study, increased cyanobacterial abundance, over that of diatoms, with increasing CO₂ levels were evidenced by the HPLC pigment analysis. In the controls, however, diatoms comprised the dominant group. Cyanobacteria are well known to perform active CCM during limited CO₂ supply (Price et al., 2007) and can carry out greater levels of carbon fixation and growth under elevated CO₂ levels by slowing down the energy cost for CCM (Raven and Beardall, 2003, Rost et al., 2008, Giordano, 2005). On the other hand, the available literature suggests that diatoms and *Phaeocystis* could be less responsive to enhanced CO₂ levels due to the presence of efficient CCMs (Rost et al., 2003). In a recent study (Tortell et al., 2008), enhanced carbon fixation at elevated CO₂ levels was observed in a diatom–*Phaeocystis*-dominated community in the Ross Sea. The authors interpreted this enhancement effect by the down-regulation of energy cost for inorganic carbon transport under the high CO₂ conditions. Therefore, we can assume diatoms can benefit from increased CO₂ levels, but at the same time nutrient availability and preference should also be considered. For example, Tortell et al. (2002) reported from a field incubation experiment in the equatorial Pacific that the abundance of diatoms decreased, alongside enhanced abundance of the *Phaeocystis* population, when pCO₂ was enhanced from 150ppm to 750ppm. The reason was not only differential CO₂ levels, but also nutrient utilization. A high nitrate:silica concentration led to a non-diatom dominated community.

For diatom growth silicate has a vital role, whereas cyanobacteria can grow without it. For a better understanding of the role of nutrients in shifting the community composition under elevated CO₂ levels, a nutrient budget was constructed for the present study. We have calculated the required amount of DIN, DIP and silicate to support the net increase in POC (by deducting the initial POC from the final value), and assuming that the entire biomass was contributed by diatoms (as diatoms were the main constituents of the phytoplankton group at ≈70%). Below, the possible demand for nutrients in order to sustain the observed net increase in POC is discussed individually for DIP, silicate and DIN, for both experimental sets.

In May the initial phosphate concentration was 3.2 μM (after addition), which can support almost 339 μM (106x3.2) of net increase in POC. The highest net increase in POC was

observed at the 363 μatm pCO_2 level, with a value of 130.57 μM , and the amount of supplied DIP was always in excess of the required amount. On the contrary, in June, except in the control (where the DIP requirement was 0.31 μM), the calculated DIP requirements were higher (0.63–0.7 μM) than the initial value (0.55 μM) and could have been insufficient to support growth when pCO_2 was further increased (1403 and 1860 μatm). Phytoplankton and bacteria are well known to efficiently utilize the organic phosphate pool during phosphate-limited conditions by activating their alkaline phosphatase enzyme (Xu et al., 2006). Phosphate concentrations fell below the detection limit at the end of the experiments, indicating phosphate-limited conditions, and the observed high alkaline phosphatase activity (during May and June) was consistent with this; activity in the June experiment was measured as 29% higher than in May (Fig. 6a), as the level of DIP supply was 5.8 times lower. In addition, the phosphorus storage capacity of cyanobacteria appears to have been much larger than in other groups of phytoplankton, and this capacity has been reported to give cyanobacteria a competitive advantage over diatoms and chlorophytes when P is supplied in pulses (Sommer, 1985). Similarly, in the present study, the phosphate uptake rate of cyanobacteria was probably faster than that of diatoms.

Moreover, phosphate uptake by heterotrophic bacteria could also be a major sink. Given that in the present study we have observed an increase in total bacterial count (Fig. 6b) with increasing CO_2 , competition for phosphate uptake is expected. During phytoplankton blooms in eutrophic conditions, both bacteria and phytoplankton can take up phosphate with greater efficiency. In phosphate-deficient conditions, the supremacy of bacteria over phytoplankton for the uptake of phosphate has also been reported by many authors (Trond et al., 2007; Currie and Kalff, 1984). Even diatoms can be out-done by bacteria during such conditions (Brussard and Riegman, 1998). However, the efficiency of bacteria in competing with phytoplankton for phosphate depends on a reduced carbon input into the system, and this has been proven to be directly proportional (Currie and Kalff, 1984). The observed enhanced bacterial abundance and photosynthetic rates under elevated CO_2 levels could be an indication of increased DOC production, which might have augmented the bacterial phosphate demand. Consequently, there must be tight competition between diatoms, cyanobacteria and heterotrophs for the uptake of phosphate. Diatoms were probably out-done by the other two groups, which have a less efficient phosphate uptake/storage capacity.

Silica could also exert significant control over the phytoplankton community composition, where diatoms are a major constituent. A high nitrate:silicate ratio might lead to the dominance of a non-diatom phytoplankton community (Riebesell, 2004). Tortell et al. (2002) found that a diatom-dominated community in the equatorial Pacific changed to being dominated by *Phaeocystis* sp. under increased CO₂ levels, and the apparent reason was a high nitrate:silicate ratio in the ambient water. In the present study, the final concentration of silicate was almost undetectable, indicating complete uptake. The initial silicate concentrations during May and June were 14.8±1.2 μM and 5.2±0.06 μM respectively. This can support maximum increases of 104 and 36 μM of POC respectively, assuming the entire growth was contributed to only by diatoms. Interestingly, in the control experiments during May and June, the net increases in POC were 98.58 and 32.45 μM respectively, close to the above-mentioned values. This indicates that the amount of silica in the controls was not limiting factor for the observed growth. However, in other elevated CO₂ treatments, the net increase in POC was much higher than these values and silica might not have been enough to ensure this growth.

The calculated DIN concentrations during both May (14.87, 16.60 and 19.79 μM for the 156, 244 and 363 μatm pCO₂ treatments, respectively) and June (4.9, 10.12, 11.12 and 10.75 μM for the 225, 646, 1403 and 1860 μatm pCO₂ treatments, respectively) were higher than the observed concentrations (7.58 in May and 6.9 μM in June). This observation shows that only in the June control experiment was the DIN supply sufficient for the observed growth, and not for any of the other CO₂ treatments. It is not clear from these data which nitrogen source supported the growth, but the possibility of nitrogen fixation cannot be excluded, as cyanobacteria abundance was observed. A superior abundance of nitrogen-fixing cyanobacteria (*Nostoc* sp., *Anabaena* sp., *Cylindropermosis* sp., *Trichodesmium* sp.) in the study area is a common phenomenon during high saline and low nutrient conditions (unpublished data), and nitrogen fixation might also support the observed growth to some extent. However, there was no direct evidence to support this view because nitrogen fixation rates and microscopic analysis were not carried out in this study. Cyanobacteria are better adapted to nutrient-poor conditions because of their greater phosphate uptake efficiency and better nitrogen fixing capability, and also as well as their ability in utilizing their alkaline phosphatase capacity to take up organic phosphate. Hence, cyanobacteria can easily compete with diatoms in a mixed population under high CO₂ supply and low silicate concentrations.

Conclusion:

A natural phytoplankton community in the Godavari River Estuary was found to be quite sensitive to increasing CO₂ levels. The present study has shown that during the non-discharge pre-monsoon period phytoplankton production in the Godavari Estuary might be limited by phosphate, as well as CO₂ supply. Low nutrients coupled with changes in carbonate chemistry can influence phytoplankton physiology and community composition. Enhanced abundance of heterotrophic bacteria with increasing CO₂ could influence the turnover rates of the major elements (C, N, P) in this estuary. All these observed results might potentially affect the ecology and biogeochemistry of the entire trophic structure of the study area. No estimations of future increases in primary production due to CO₂ enrichment were attempted based on the observations made in this study. This is because the study was carried out during a non-discharge period with limited nutrients and CO₂ supply. The response of phytoplankton to increasing CO₂ might not necessarily be the same in other seasons and is thus subject to further investigation.

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Figure captions:

Fig. 1: Station location.

Fig. 2: a) Total chlorophyll; b) chlorophyll *a* based growth rate ($\mu_{\text{Chl}a}\text{d}^{-1}$); and c) POC normalized net photosynthetic oxygen evolution in relation to different CO_2 levels (pCO_2). The experimental results from May and June are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Fig. 3: a) POC; b) PON; and c) POC:PON in relation to increasing CO_2 levels (pCO_2). The experimental results from May (high DIP) and June (low DIP) are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Fig. 4: a) Chl:POC in relation to CO_2 levels (pCO_2); b) linear regression line between Chl:POC and CO_2 (pCO_2). The experimental results May (high DIP) and June (low DIP) are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Fig. 5: a) $\delta^{13}\text{C}$ of POM ($\delta^{13}\text{C}_{\text{POM}}$) under different CO_2 treatments; b) linear regression line between $\delta^{13}\text{C}_{\text{POM}}$ and CO_2 (pCO_2). The experimental results from May (high DIP) and June (low DIP) are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Fig. 6: a) POC normalized alkaline phosphatase activity under different pCO_2 treatments; b) total bacterial count (TBC) in relation to CO_2 levels (pCO_2) (TBC was not analyzed during the first experiment in May). The experimental results from May (high DIP) and June (low DIP) are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Fig. 7: a) Ratio of fucoxanthin to Chlorophyll-*a*; and b) zeaxanthin to fucoxanthin in relation to different CO_2 levels (pCO_2). The experimental results from May (high DIP) and June (low DIP) are separated by the dashed vertical line. Values $\pm\text{SD}$, $n=3$ to 6.

Table 1: Carbon chemistry parameters (value \pm SD, n = 3) of the initial day calculated from CO2SYS (Lewis and Wallace 1998) using pH, DIC, alkalinity, phosphate, silicate, temperature, and salinity.

| Month | pCO ₂ (μ atm) | TA (μ mol kg ⁻¹) | pH (NBS) | DIC (μ mol kg ⁻¹) |
|-------|-------------------------------|-----------------------------------|------------------|------------------------------------|
| M | 156 \pm 12 (Control) | 2212 \pm 12 | 8.52 \pm 0.025 | 1722 \pm 8 |
| A | 244 \pm 5 | 2304 \pm 3 | 8.39 \pm 0.007 | 1899 \pm 7 |
| Y | 363 \pm 9 | 2210 \pm 6 | 8.24 \pm 0.011 | 1899 \pm 7 |
| J | 225 \pm 7 (Control) | 1968 \pm 11 | 8.36 \pm 0.005 | 1624 \pm 7 |
| U | 646 \pm 9 | 2100 \pm 1.41 | 8.02 \pm 0.005 | 1928 \pm 4.5 |
| N | 1403 \pm 14 | 1984 \pm 2 | 7.70 \pm 0.004 | 1928 \pm 4.5 |
| E | 1860 \pm 7 | 1955 \pm 4 | 7.58 \pm 0.002 | 1928 \pm 4.5 |

Table 2: Initial physicochemical and biological parameters during the experimental phase

(value \pm SD, n = 3).

| Parameters | May | June |
|-------------------------------------|-------------------|-------------------|
| Temp ($^{\circ}$ C) | 28.76 \pm 1.3 | 29.54 \pm 0.09 |
| Salinity (psu) | 26.73 \pm 0.02 | 24.98 \pm 0.03 |
| DIN (μ M) | 7.58 \pm 0.023 | 6.91 \pm 0.12 |
| DIP(μ M) | 3.2 \pm 0.09 | 0.55 \pm 0.05 |
| Silicate(μ M) | 14.8 \pm 1.2 | 5.2 \pm 0.06 |
| Chl- <i>a</i> (μ g L $^{-1}$) | 2.34 \pm 0.15 | 1.2 \pm 0.043 |
| POC (μ M) | 58.1 \pm 0.96 | 33.75 \pm 1.25 |
| δ^{13} C _{POM} ‰ | -23.94 \pm 0.07 | -24.64 \pm 0.85 |
| C:N (μ M: μ M) | 6.21 \pm 0.29 | 6.12 \pm 0.26 |
| Zea/Fuco | 0.13 \pm 0.03 | 0.17 \pm 0.9 |

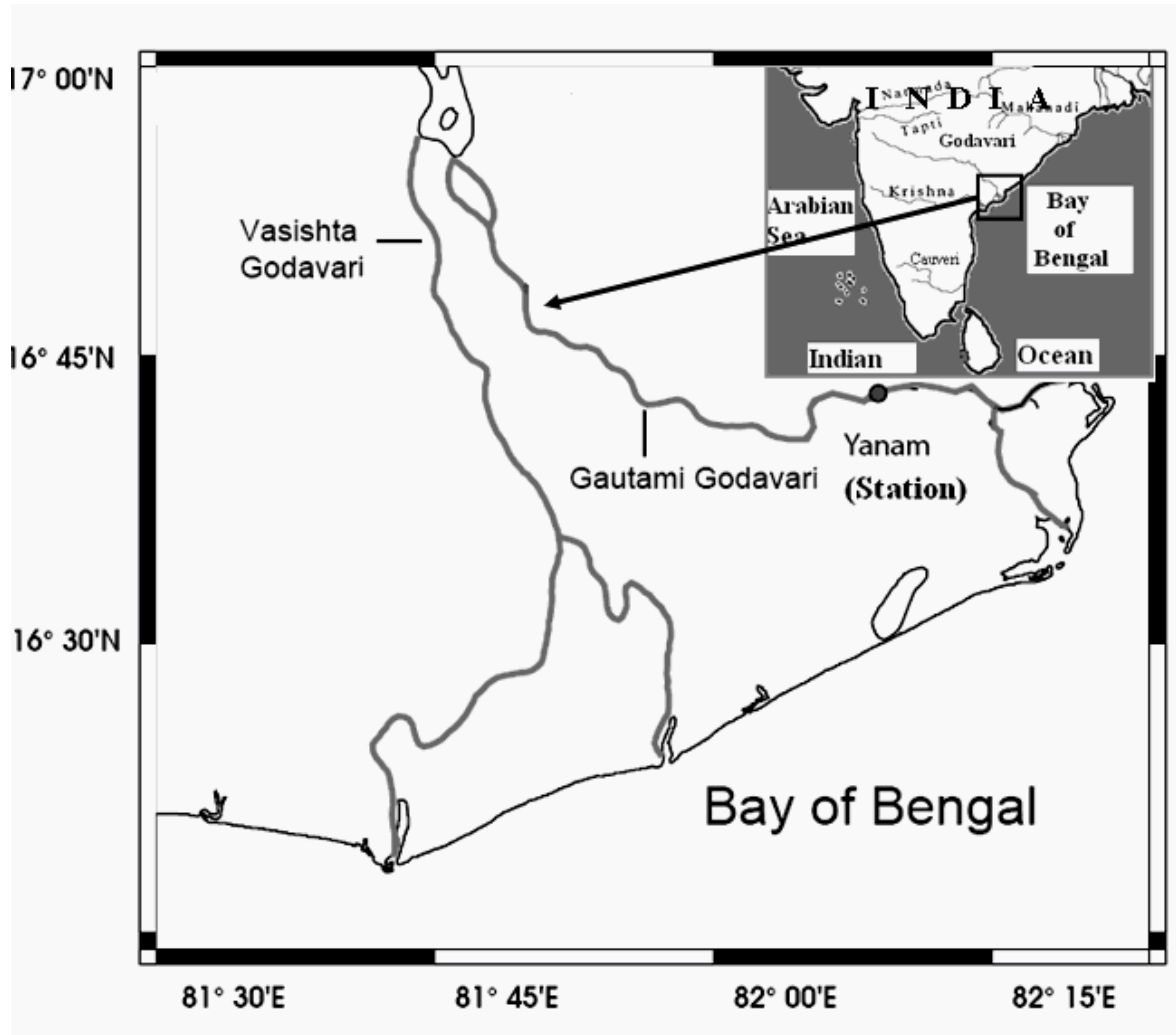


Fig. 1

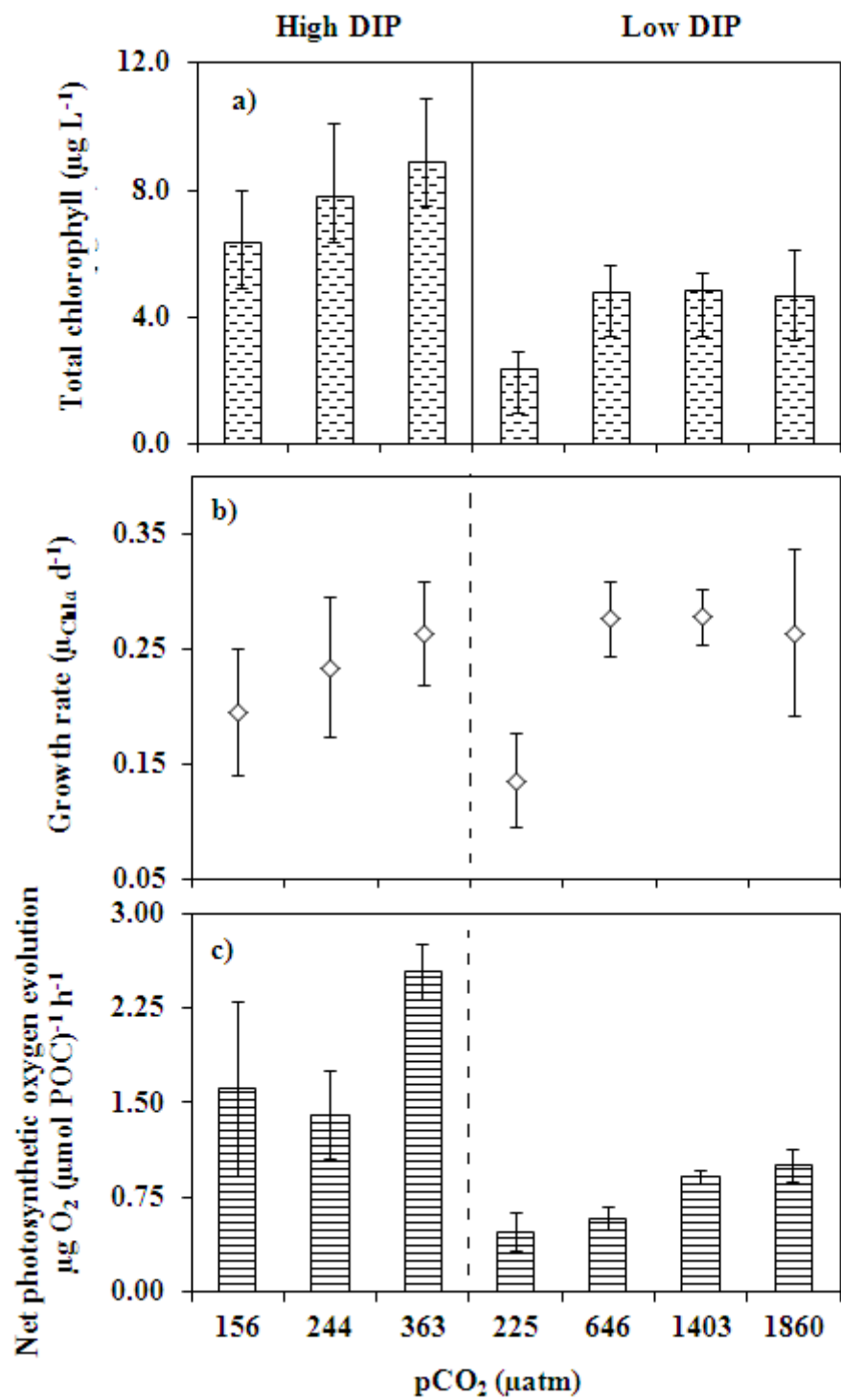


Fig. 2

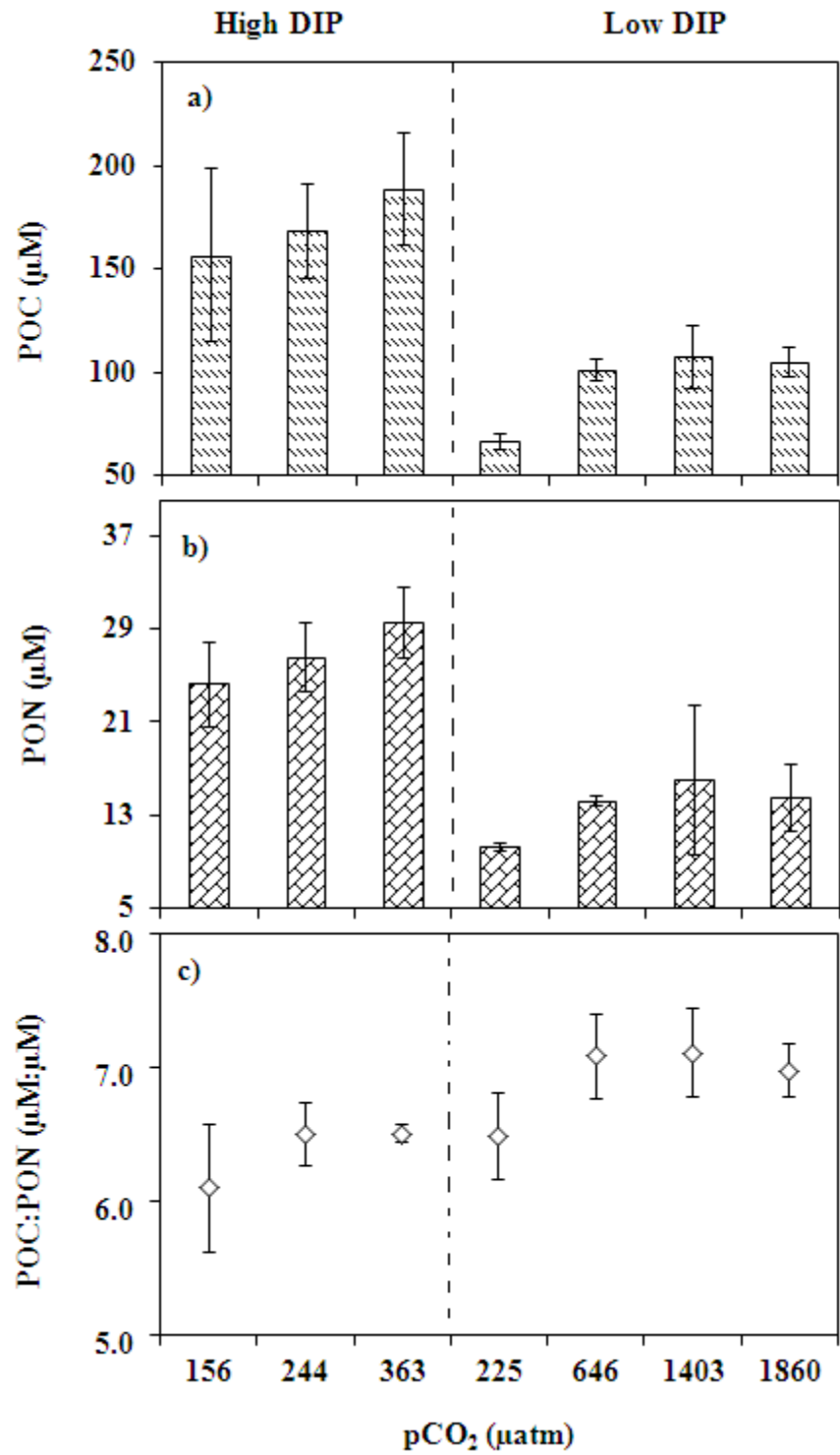


Fig. 3

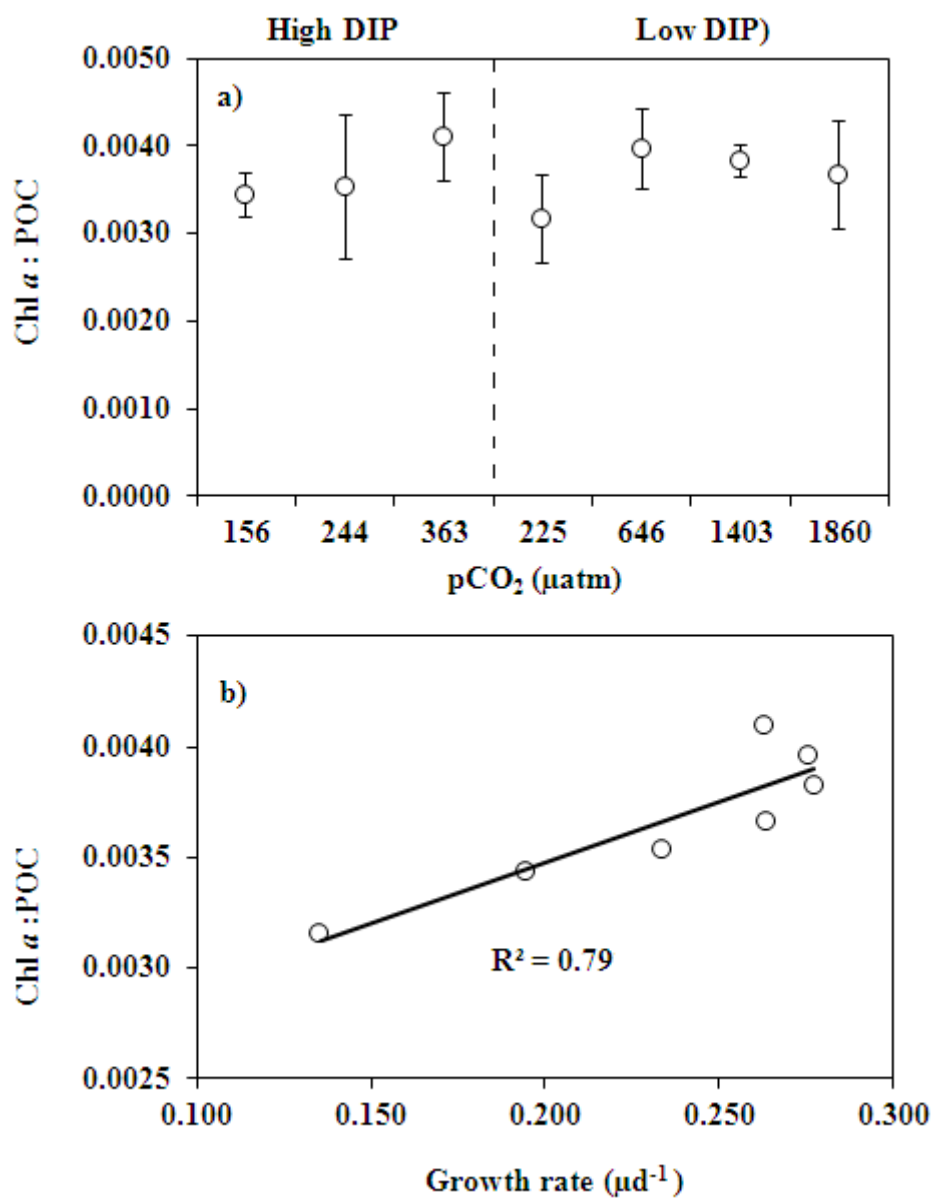


Fig. 4

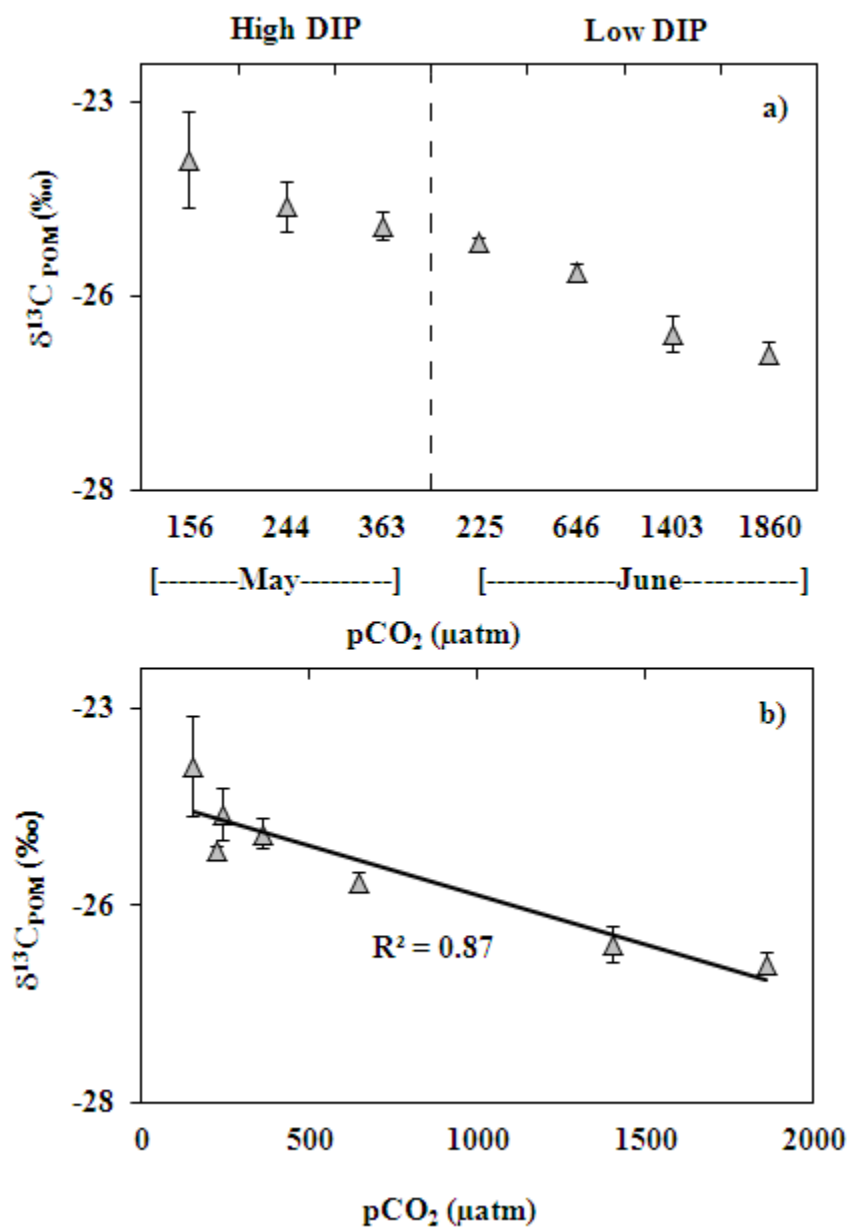


Fig. 5

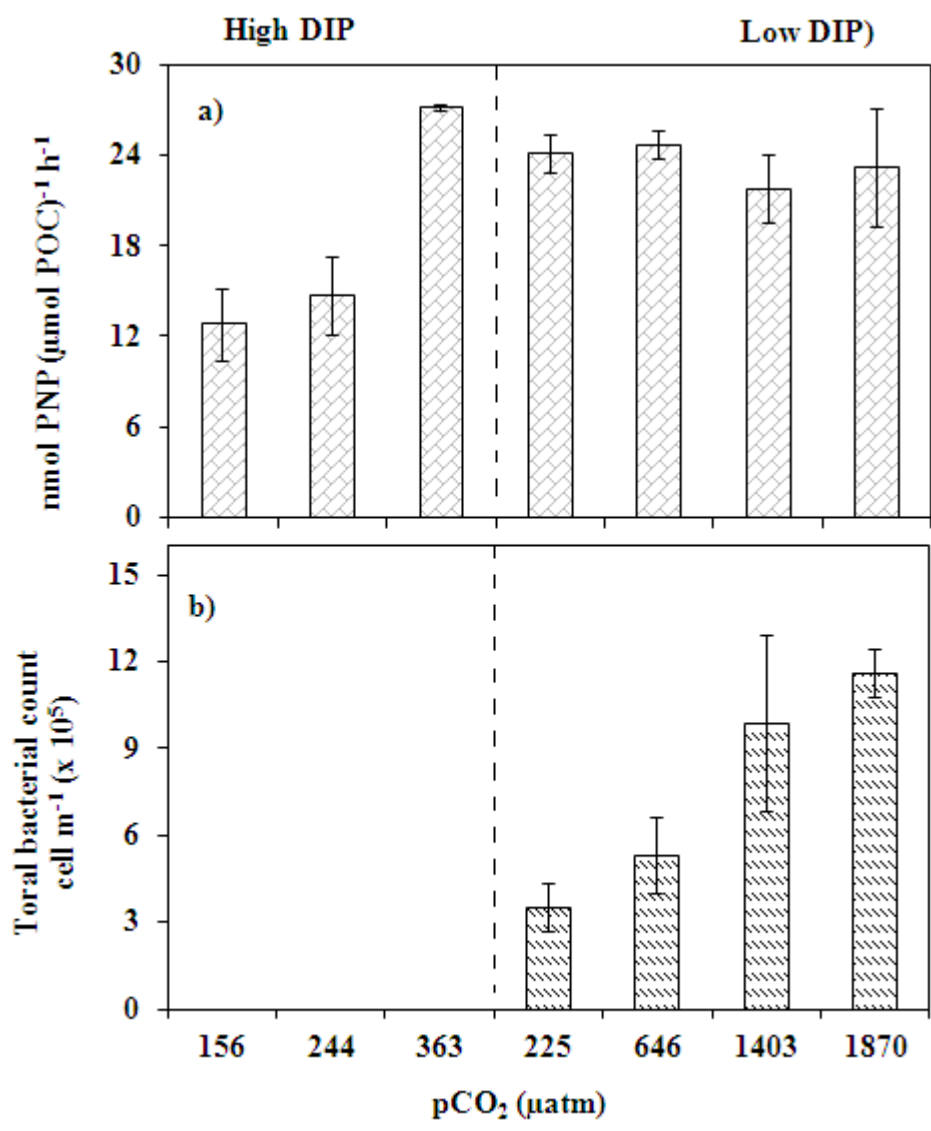


Fig.6

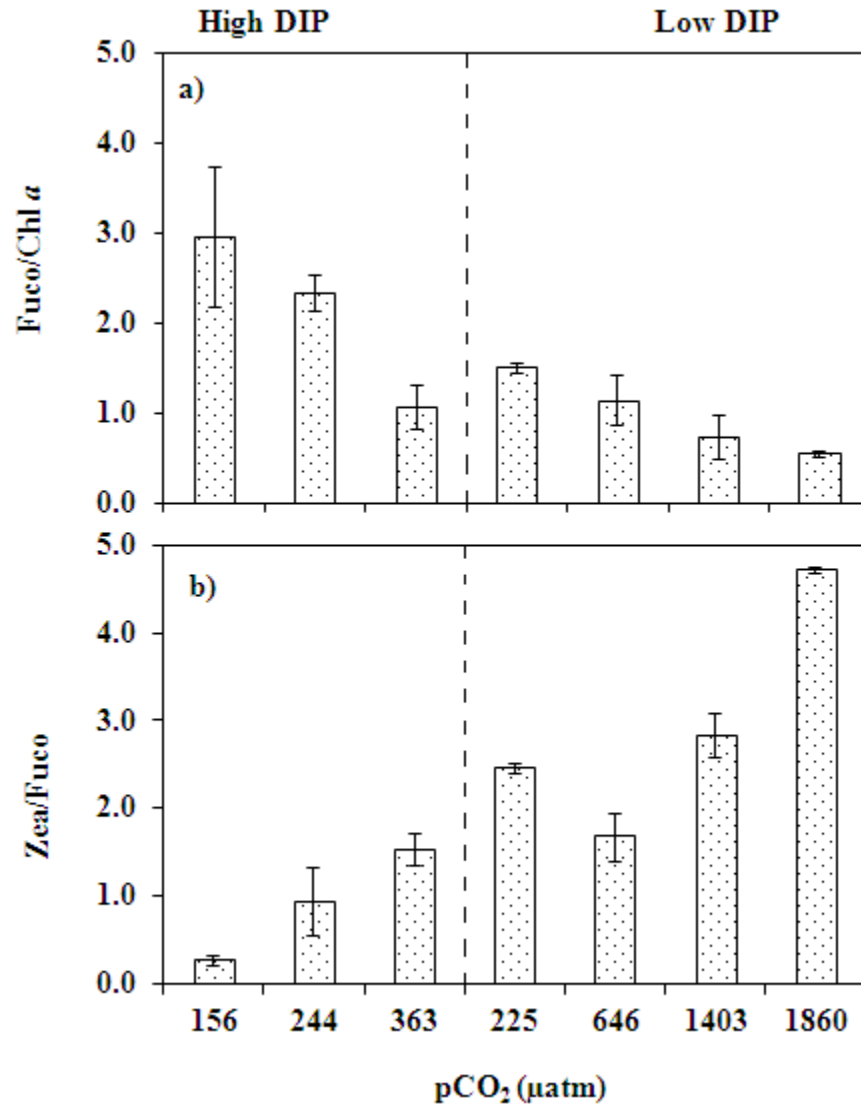


Fig. 7