Laboratory mesocosm studies on the response and potential effects of marine diatom *Nitzschia* sp. to Ocean acidification

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Present study was carried out to know the response and effects of Ocean acidification in the growth of diatom *Nitzschia* sp. and its biochemical constituent viz. protein, lipid and carbohydrate. Ocean acidification is one of the most well-known ocean perturbations. Diatoms are non calcifiers they were able to survive and grow without their cell wall (frustules) was being disrupted by the acidified medium. Increase in photosynthetic activity and growth rate have been observed with increase in CO$_2$ activity. Biochemical profile of diatom showed high protein (4.8 µg/ml), high lipid (14%) whereas lower carbohydrate content (0.68 µg/ml) in acidified medium when compared with control group. Thus, atmospheric CO$_2$ increase could potentially promote phytoplankton productivity and diatoms grown in the acidified cultures were capable of compensating the pH change and they survived showing high productive results.

[Keywords: Ocean acidification, Diatoms, *Nitzschia* sp., Atmospheric CO$_2$.]

**Introduction**

The oceans cover 70% of the Earth’s surface and contain about 50 times more soluble inorganic carbon than the atmosphere. Increasing atmospheric CO$_2$ is causing unprecedented changes in seawater chemistry. Rising carbon dioxide (CO$_2$) concentrations in the atmosphere due to human activity are causing the ocean to become more acidic. Atmospheric increases of carbon dioxide are positively correlated with the amount of fossil fuels being burned. Global warming due to increased carbon dioxide concentration in the atmosphere is receiving a great deal of attention. The oceans are taking up over one million tons of CO$_2$ per h and have been acidified by 30% since the industrial revolution, and will be further acidified by 150% (pH drop to 7.8) by the end of this century, leading to ocean acidification. Typical chemical changes associated with the ocean acidification are increased concentrations of pCO$_2$, H$^+$ and HCO$_3$ and decreased concentration of CO$_3^{2-}$ and the CaCO$_3$ saturation state.

Marine plankton is an important part of the marine food chain upon which all other life in the ocean depends. Phytoplankton also underpin a range of important global biogeochemical processes such as nutrient cycling and carbon storage. The responses of microalgae to ocean acidification could therefore have wide ranging global ramifications. Changes in seawater pH may affect microalgal cells via a number of possible mechanisms. In fact, pH controls such a wide variety of processes that pH is referred to as the “master” variable for physical and biological processes in the ocean.

There is a growing consensus that the ongoing increase in atmospheric carbon dioxide, CO$_2$, as a result of anthropogenic activities will lead to a variety of physical, chemical and physiological effects on marine phytoplankton. Microalgae are the most promising production facilities. CO$_2$ fixation by photo-autotrophic algal cultures has the potential to diminish the release of CO$_2$ into the atmosphere, helping alleviate the trend toward global warming. Generally, phototrophic microalgal growth requires a supply of carbon dioxide as a carbon source. CO$_2$ supply contributes to control the pH of the culture. Biological CO$_2$ fixation has been extensively investigated as part of efforts to solve the global warming problem.

Within all these species only few taxonomic groups are responsible for most of the primary production of the system. One of these so-called phytoplankton functional groups is represented by siliciers. Diatoms are a diverse and ecologically very important group contributing up to 40% of the oceans primary production. Diatoms evolved during the Mesozoic era and have gradually become major actors in the oceanic cycles of
Carbon-concentrating mechanisms (CCM) enable most marine phytoplankton species to accumulate intracellular inorganic carbon either as CO$_2$ or HCO$_3^-$ or both. The ubiquitous enzyme carbonic anhydrase (CA) plays a key role in CCM of marine algae. Most diatoms operate CO$_2$ concentrating mechanisms (CCMs) to supply CO$_2$ to the proximity of Rubisco, which exhibits active transport of CO$_2$ and/or HCO$_3^-$ across the cellular membrane. Different algal species or growth conditions show differential preferences to CO$_2$ or HCO$_3^-$, especially in comparison to other phytoplankton taxa, and these processes are strongly regulated as a function of CO$_2$ supply. Despite the global biogeochemical significance of primary production by diatoms, little is known about how they will respond to the decreasing pH of the ocean. It is therefore very difficult to predict which groups of organisms will profit from the changing environmental conditions and which will turn out to be the losers. Ocean acidification is of course not the only consequence of increased CO$_2$. The objective of this research was to examine the effects of CO$_2$ driven acidification on the growth, photosynthesis, biochemical metabolism and structural deformities in a common marine diatom Nitzschia sp.

Materials and Methods

Algal samples were collected from Muthukuda backwaters (Lat 9.8° N and Long. 79.1° E) using phytoplankton net of 25μm. A stock culture of the Bacillariophyceae Nitzschia sp. was then isolated in the laboratory and cultivated in f/2 enriched seawater medium (Plate 1). The seawater used for the growth medium was sterile filtered, enriched with the full set of nutrients for the f/2 growth medium and stored in a cool and dark place until required for the algae cultures. All of the algae in this experiment were incubated in sub samples of this medium. Replicate cultures were grown and kept at 25°C in a thermostatically controlled room, illuminated with white florescence lamps with 12:12 h of light/ dark regime. The temperature and salinity was maintained in the range of 23 to 25°C and 28 to 30 ppt.

The following treatment series were maintained for the CO$_2$ acidification studies on diatom, Treatment 1 – Control (No CO$_2$ fertilized) – flasks covered with a cotton wad. Treatment 2 - CO$_2$ fertilized – flasks covered with a cotton wad made airtight by wrapping it in a transparent film with a straw inserted in the middle for CO$_2$ fertilized by blowing. CO$_2$ bubbling was performed in the treatment 2, with the air blown into the culture for two minutes four times a day (Plate 2).

Each experiment typically lasted for 12 days. Data was collected every 3 days once and 5 days once for: Chlorophyll and algal cell density. Whereas monitoring of pH and estimation of carbonate and bicarbonate were carried out on daily basis. The pH was measured using an ELICO grip pH meter, Chlorophyll ‘a’ and ‘b’ was estimated by the standard method, estimation of carbonate and bicarbonates by Titrimetry. For growth analysis, sampling was done (10 ml from each replicates) of cultures was measured by checking the absorbance at 660 nm in spectrophotometer. The algae growth was monitored by measuring the solution optical density (absorbance) at 660 nm

Estimation of total lipid, carbohydrate and protein content was analyzed quantitatively, definite volume of cultures of the diatom species were harvested at the mid log phase by centrifugation. The cells were oven dried and stored for further biochemical analysis.
For Electron Microscopy study the specimens were cleaned by adopting hot HCl and KMnO₄ method (recommended technique of acid digestion). The samples were coated using gold sputtering unit Q150R S, Quorum Technologies, England. The samples were then examined under FESEM (Quanta 250 FEG, Czech Republic) and digital images were taken using the system with an acceleration of 30 kV.

The data were expressed as the mean values ± standard deviation (SD). Statistical correlation tests were employed to make statistically significant conclusions about the data using SPSS Package 14.0

Results
In the CO₂ acidification study of diatoms, the recorded optimum pH in control was 8.48±0.39 whereas it was 7.27±0.22 in CO₂ fertilized group. A low pH value in CO₂ fertilized group indicates the acidified condition of the cultures when compared to the control groups (Fig.1A). The control and CO₂ fertilized group were strongly correlated r (11) = 0.615, p<0.05 and they are significant at 0.05 level (Fig.1B).

Nitzschia sp. biomass in terms of chlorophyll ‘a’ and ‘b’ showed that the diatom strains obtained the maximum amount of chlorophyll pigment in CO₂ fertilized group when compared with the control. During the exponential period of growth the chlorophyll ‘a’ content was higher 0.09±0.03 mg/ml in CO₂ fertilized whereas in control it was lesser 0.06±0.02 mg/ml (Fig.3A). The control and CO₂ fertilized group were correlated r (2) = 0.985, p<0.05 and they are significant at 0.05 level (Fig.3B).
Chlorophyll ‘b’ also showed similar results on the growth period, where higher 0.09±0.03 mg/ml chlorophyll ‘b’ was found at CO₂ fertilized whereas in control it was 0.06±0.02 mg/ml (Fig.4A). The control and CO₂ fertilized group were correlated r (2) = 0.980, p<0.05 and they are significant at 0.05 level (Fig.4B).

In the present study the carbonate concentration in the control and CO₂ fertilized were 18.54 (mg/ml) and 0.6 (mg/ml) which showed higher amount of carbonate availability in control group (Fig.5). In contrast the bicarbonate concentration was lower in control 0.54 (mg/ml) whereas higher 47.69 (mg/ml) in CO₂ fertilized group (Fig.6).
The total protein, carbohydrate and lipid in the control were lesser when compared with the biochemical constituents of CO₂ fertilized group. Total protein concentration in control and CO₂ fertilized was 3.20 (µg/ml) and 4.82 (µg/ml) (Fig.7) and Total lipid concentration in control and CO₂ fertilized was 10% and 14% (Fig.9). Both protein and lipid showed high concentration in experiment when compared to control whereas total carbohydrate was comparatively less in experiment. In control the total carbohydrate was 0.72 (µg/ml) and in CO₂ fertilized it was 0.69 (µg/ml) (Fig.8) respectively.

The topography of diatoms cells was observed and its structural features were viewed in scanning electron microscope (Fig.10, 11). There were no structural deformities observed in the diatom frustules. The diatoms were found intact.
Discussion

Research into the responses of marine photoautotrophs to CO₂ enrichment is much less advanced, and there are currently no reliable estimates of how global ocean productivity will change in relation to high CO₂/low pH. With respect to the process of silification, diatoms also do not appear to be particularly CO₂ sensitive. Photosynthesis of diatoms is likely to be stimulated by increased availability of CO₂, lower pH might enhance their respiration too, which would down-regulate their contribution to the marine biological CO₂ pump. Different algal species or growth conditions show differential preferences to CO₂ or HCO₃⁻. However, other studies show insignificant effects on photosynthesis, growth or primary productivity in diatoms or phytoplankton community. Since the diatoms are non calcifiers they were able to survive and grow without their cell walls being disrupted by the acidified medium. This concludes that there are no structural deformities in diatoms unlike coccolithophores and other calcifying plankton. Diatom frustules where seen to be structurally intact with the results of scanning electron micrographs (Fig.11A and 11B).

The present results showed low pH values through the experimental period which indicates the acidified condition due to CO₂ blowing. Variation in pH can affect algal growth in a number of ways. It can change the distribution of carbon dioxide species and carbon availability, alter the availability of trace metals and essential nutrients, and at extreme pH levels potentially cause direct physiological effects. As per the reports responses of phytoplankton to reduced pH in a high CO₂ ocean are likely to be species specific, with potential “winners” and “losers”. The growth rate of Phaeodactylum tricornutum was enhanced by 5.2% under high CO₂ and low pH conditions, the response in photosynthetic carbon fixation was more pronounced (+12%).

Some diatoms showed enhanced growth rate with enriched CO₂ and acidity like the results have been observed with the presently studied diatom Nitzschia sp. There may be indirect benefits of increasing concentrations of CO₂ for those species that possess CCM. The operation of CCM require energy expenditure, therefore CCM capacity may be down-regulated by elevated CO₂ allowing algae to reallocate energy for other purposes. Different types of CCM are present in almost all algal groups. Therefore it is believed that rising CO₂ will affect different species to varying degrees potentially resulting in drastic community shifts.

From both the plant physiology and marine biology literature, there is now substantial evidence that many different species, including marine diatoms, have the ability to concentrate free CO₂ internally. Process is believed to occur either by the facilitated conversion of HCO₃⁻ to CO₂ externally, followed by transport of CO₂ into the cell, or by the active uptake of HCO₃⁻ into the cell, followed by conversion internally to CO₂. The seemingly widespread ability of marine phytoplankton to utilize HCO₃⁻, in principle, allows full utilization of the reservoir of inorganic carbon in oceanic waters. In accordance with the above results, the present findings also support the fact that high bicarbonate concentration of diatoms Nitzschia sp. might enhance the physiological process of conversion of CO₂ in its inorganic form.

Increases in growth rate have been observed in other microalgae species, with carbon dioxide addition. Thus, atmospheric CO₂ increase could potentially promote phytoplankton productivity. Our results show that the cell density was rather high, doubled CO₂ increased the availability of DIC, thus the growth of the marine diatom would be stimulated in the stationary phase. The enhanced growth of the marine diatom should be related to the increase of photosynthesis at elevated CO₂ which has been shown with increased chlorophyll ‘a’ and ‘b’ contents. Operation of cellular mechanisms involved in pH homeostasis may be affected by seawater chemistry alterations.

Lipids and carbohydrates are considered cellular fuel, besides their important function as structural constituents of membranes. Proteins play crucial roles in virtually all biological processes in diatoms as well as other cells. Nearly all biochemical reactions are catalyzed by specific protein enzymes. Proteins are also involved in transport and storage of metabolites and control of growth. The carbohydrate glucan is the primary storage compound in diatoms for carbon fixed during photosynthesis. Biochemical composition of microalgae can change with their growth rates and/ or environmental conditions and with the phase of their life cycle. Energy costs are associated with maintaining an internal pH necessary for cell function under changing external pH conditions.
In the diatom cell wall, an organic casing with structural polysaccharides coats the siliceous frustule. This casing consists of thin coating layers and the diatotepic layer on the inside of the frustule. The acidified condition in diatoms would have made the diatoms to exude a portion of carbohydrate to the external surrounding which has resulted in lower carbohydrate content in the experimental group of acidified diatoms. In *Phaeodactylum tricornutum*, protein content was increased with carbon dioxide addition. Probably the cells were investing the excess of carbon assimilated much more in protein synthesis and growth than in lipids and carbohydrates that are reserve substances in microalgae.

Several diatom species have also been shown to be relatively insensitive to changes in pH and in some experiments diatoms have shown little response CO$_2$ to enrichment. It has therefore been assumed that the effect of CO$_2$ on photosynthesis and growth in diatoms may be small, especially when compared with other taxa. More recently, however, it has been argued that CO$_2$ enrichment may benefit diatoms through down regulation of CCM capacity and the reduced energy costs of carbon fixation. This notion helps explain those studies that have observed positive responses of diatoms to CO$_2$ enrichment. Doubling of ambient CO$_2$ would save around 20 % of the CCM expenditure, decreasing the amount of energy expended on carbon fixation by 3 to 6 % and increasing primary production. This has important ecological ramifications along with the potential to influence biogeochemical cycles and oceanic feedbacks to increasing atmospheric CO$_2$.

**Conclusion**

Ocean acidification is a rapidly emerging field of research and the responses of marine micro algae to elevated CO$_2$ are highly variable and complex. The projected CO$_2$/pH-related changes in seawater carbonate chemistry are likely to induce a species shift within the diverse group of diatoms, which may have consequences for the operation of the biological pump and thus for oceanic feedbacks to rising atmospheric CO$_2$. Our current understanding of the impacts of elevated CO$_2$ on marine diatom, however, is relatively limited as it is mostly derived from simplified, artificial experimental set-ups and is hindered by the results that the diatoms grown in the acidified cultures were capable of compensating the pH change and they survived showing high productive results.

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