Effect of Elevated Carbon Dioxide and Phosphorus Levels on Nitrogen Uptake, Lipid Content and Growth of *Tetraselmis* sp.

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ABSTRACT

The microalga *Tetraselmis* sp. is one of the most widely used species for natural food in aquaculture. The effect of elevated carbon dioxide (CO₂) and phosphorous levels on nitrogen uptake, lipid content and growth of *Tetraselmis* sp. were investigated in order to improve production efficiencies and determine other potential applications of this species. Three levels of CO₂ (0, 5 and 10% v/v of the incoming air for aeration) and three levels of phosphorus added as NaH₂PO₄ (5, 10, 20 mg L⁻¹) in modified F medium with NH₄⁺ as the primary nitrogen source were used as variables in a factorial experiment. Optical density (680 nm) and residual NH₄-N in the culture medium were monitored daily for 6 days. Relative growth rate (k'), nitrogen uptake (μg NH₄-N cell⁻¹ day⁻¹) and lipid content (% dry weight) were calculated and determined at the end of the culture period. No interactive effect between phosphorus and CO₂ was observed in all variables measured (p>0.05). Highest growth rate (k = 1.82±0.04), nitrogen uptake (0.316±0.008 μg NH₄-N cell⁻¹ day⁻¹) and lipid content (10.95±0.15% DW) were exhibited at 10% CO₂ supplementation. Growth and nitrogen uptake were similar in treatments with elevated levels of phosphorus but lipid yield (9.41±0.27% DW) was lowest at 20 mg L⁻¹ of phosphorus supplementation (p<0.05). These results showed the critical role of CO₂ in nitrogen uptake, lipid content and growth of *Tetraselmis* sp. It also indicates the potential of this algal species for waste water remediation, biofuel production and carbon sequestration.

**Key words:** Carbon dioxide, growth rate, nutrient uptake, lipid content, phosphorus, *Tetraselmis* sp.

INTRODUCTION

Microalgae have been an integral part of the aquaculture industry. Its primary function is to serve as natural food for larval stages of finfish, crustaceans and mollusks. Due to the rapid growth of the aquaculture industry within the past decades, increased production of microalgae became imperative and has been addressed through the development of improved mass culture systems. At present, more than 40 species of microalgae have been isolated and cultured as pure strains in intensive production systems (Sen *et al.*, 2005a).
Aside from its use as natural food in aquaculture, microalgae have also gained several applications in the field of biotechnology particularly on waste water remediation, carbon capture systems and the production of bioactive compounds as food supplements and pharmaceuticals. Several species of microalgae have already been found to be suitable candidates for applications in waste water treatment (Abdel Hameed, 2007; Shi et al., 2007). Excessive nutrients in waste water such as ammonia and phosphorus can be easily taken up by algal cells in controlled treatment ponds reducing the possibility of eutrophication and harmful algal blooms in cases where waste water is merely discharged. As an added protocol in intensive aquaculture systems where large volumes of water are used and high concentrations of ammonia are present (Hargreaves and Tucker, 2004), waste water treatment using microalgae could reduce the environmental impacts of these activities and serve as an alternative method of producing natural food.

Microalgal culture has also shown great promise as feed stock in biodiesel production. Research conducted by the National Renewable Energy Laboratory (NREL) of the United States in the 1980s have focused exclusively on biodiesel production from microalgae since these organisms produce more of the right kinds of oils needed for biodiesel. The type of oils of main concern in biodiesel production comes in the form of Triacylglycerides (TAGs), which consist of three long chains of fatty acids attached to a glycerol backbone. Studies done at the NREL have shown species producing TAGs up to 60% of their body weight (Sheehan, 1998).

Although, mass culture systems for microalgae have been widely developed, these have mainly focused on producing microalgae as natural food for aquaculture. In order to make microalgal biotechnology feasible for applications in waste water treatment and biodiesel production, culture protocols and techniques have to be developed in order to further increase nutrient uptake, growth rates and improve algal composition towards the desired compound to be utilized. Such systems should also take advantage of the potential of microalgae to act as carbon sequestration mechanisms in order to maximize the contribution of this green technology in mitigating global warming.

Since microalgal productivity is highly dependent on environmental factors such as nutrient concentrations and carbon source (Sen et al., 2005a), cultivation of these organisms generally involves manipulation of such parameters in order come up with densities higher than those present in the natural environment. Several culture media have already been developed in order to provide the nutrient requirements of various algal species but these have been mainly on macronutrients such as nitrogen and phosphorus. Carbon, which is also considered a macronutrient, is generally provided incidentally in the form of carbon dioxide through the aeration of algal cultures using atmospheric air. The average atmospheric concentration of carbon dioxide is estimated at around 0.033% only hence, tends to become a limiting factor in very dense cultures of microalgae (Coutteau, 1996). The supplementation of pure carbon dioxide to the air supply of algal cultures could thus help in maintaining higher cell densities. Although the addition of pure carbon dioxide may entail additional costs to the production system, coupling microalgal culture systems to existing facilities that emit large volumes of CO₂ such as factories and power plants could eventually diminish this additional cost and at the same time, help reduce the amount of CO₂ levels entering the atmosphere (Jeong et al., 2003; Verma et al., 2010).

Several species of microalgae have already been recognized as potential candidates for microalgal biodiesel production due to their relatively high lipid content and their ability to accumulate lipids under certain conditions. Among the microalgae commonly used in aquaculture,
*Tetraselmis* sp. has been identified as a high lipid producing species (Ferriols and Aguilar, 2008). Like most algal species, studies on the cultivation of *Tetraselmis* sp. were mainly on the effects of salinity, temperature, pH and nutrient concentrations (in the form of nitrogen and phosphorous) on cell densities (Camacho et al., 1990; Ronquillo et al., 1997; Okauchi and Kawamura, 1997; Ouyang et al., 2003; Azma et al., 2010).

Herein, we report the effects of elevated carbon dioxide and phosphorus levels on growth, nitrogen uptake and the subsequent accumulation of lipids in *Tetraselmis* sp. This study thus provides information regarding optimum conditions and protocols for rearing this particular alga for finfish and crustacean hatcheries and for potential lipid production and waste water remediation. It could also serve as a basis for future studies regarding carbon sequestration using *Tetraselmis* sp. in algal biotechnology.

**MATERIALS AND METHODS**

**Experimental design:** The experiment was conducted at the Institute of Aquaculture (IA) Hatchery of the College of Fisheries and Ocean Sciences, University of the Philippines Visayas, Miagao, Iloilo, Philippines. The study followed a 3x3 factorial setup with varying levels of carbon dioxide and phosphorus consisting of nine treatments with three replicates each.

**Algal starters:** Starters of *Tetraselmis* sp. were acquired from the IA’s Phycology Laboratory. Algal starters were inoculated into 6 L plastic vessels at 20% of the final volume of sterilized seawater. Constant aeration and illumination were provided throughout the culture period of 6 days.

**Fertilization rate and CO₂ supplementation:** Fertilization of the culture was done through the addition of a modified Guillard’s F medium (Guillard and Ryther, 1962) at 1 mL L⁻¹ of culture. All components of the F medium were maintained except for the substitution of NaNO₃ with NH₄Cl as the nitrogen source and the addition of varying amounts of phosphorous for treatments at elevated levels with treatment P1 (control) at 5 mg L⁻¹, P2 at 10 mg L⁻¹ and P3 at 20 mg L⁻¹.

Carbon dioxide was added by mixing it with the incoming air used for aeration following the methods of Jeong et al. (2003). CO₂ concentrations were controlled via a regulator attached to a CO₂ tank with the flow rate of incoming air measured with a flow meter. Two treatments and a control were set up in triplicate for each level of phosphorous. Treatments for elevated CO₂ levels were at 5% (C2) and 10% (C3) CO₂ (v/v) while the control (C1) had no supplemental CO₂ in the incoming air for aeration.

**pH and temperature monitoring:** Changes in pH and temperature of the culture media were monitored daily using a pH meter (EcoScan pH 5, Eutech Instruments, U.S.A.). Monitoring of pH and temperature coincided with samplings for growth and nutrient uptake between 9 A.M. and 10 A.M. in the morning.

**Growth measurement:** The growth of microalgae was monitored daily. Fifteen milliliter samples were taken from the culture vessels and their optical density measured with a spectrophotometer at a wavelength of 680 nm (Jeong et al., 2003). Actual cell density was estimated using a standard curve of varying absorbances against known algal densities which were prepared earlier.

The relative growth rate (k') was calculated using Guillard (1973) formula as described by Ronquillo et al. (1997):
where $k'$ is relative growth rate, $N_i$ is concentration of cells in the culture at $t_i$, $N_0$ is concentration of cells at $t_0$, $t_1$ is the final culture time in days, $t_0$ is the initial culture time in days.

**Nitrogen uptake:** Nitrogen species in the form of NH$_4^+$ was monitored daily using an inorganic nitrogen analyzer (Skalar San+ Continuous Flow Analyzer, Netherlands). Prior to analysis, samples were prepared by centrifugation of 15 mL cell suspensions at 3000 rpm using a tabletop centrifuge. The clear supernatant was pipetted out for nitrogen analysis.

Nitrogen uptake rate was calculated through the gradual depletion of nitrogenous species in the culture medium. The following formula modified from Xu et al. (2010) was used in order to calculate microalgal nitrogen uptake rates:

$$N_{\text{uptake rate}} = (N_i - N_f) \times V \times W_0^{-1} \times t^{-1}$$

where, $N_i$ is initial concentration of ammonium, $N_f$ is final concentration of ammonium after time $t$, $V$ is volume of culture media, $W_0$ is initial density of the algal culture, $t$ is time of culture period in days.

**Algal harvesting and lipid analysis:** Cell cultures were harvested by flocculating with alum (Al$_2$(SO$_4$)$_3$) added to reach a final concentration of 200 mg L$^{-1}$. Cells were allowed to settle for 2 h in 15 L conical fiberglass tanks then siphoned out and filtered through ordinary filter paper. Resulting algal cakes were then freeze-dried prior to extraction. The lipid content of the harvested cultures was analyzed through proximate gravimetric methods adapted from Bligh and Dyer (1959).

Approximately 0.5 g of freeze-dried algal samples were transferred into 15 mL test tubes and added with 9.5 mL of a methanol-chloroform-distilled H$_2$O solution at a ratio of 10:5:4. It was then mixed with a vortex mixer and allowed to soak for 12 h. The mixtures were then sonicated for 1 h after which an additional 2.5 mL of chloroform and 2.0 mL of distilled H$_2$O were added to a final MeOH-CHCl$_3$-DH$_2$O ratio of 10:10:4 then mixed again for 1 min. This resulted in the formation of two liquid phases—an upper aqueous phase and a lower organic phase (chloroform containing extracted lipids). The resulting mixtures were centrifuged for 5 min at 2000 rpm for better phase separation. The lower organic phase was then removed using a Pasteur pipette and transferred into a pre-weighed vial and allowed to dry in an oven set at 50°C for 8 h. The resulting residues composed of extracted lipids were weighed and the proximate lipid content of samples was calculated.

**Statistical analysis:** All experimental data were analyzed using two-way ANOVA to test for interactions between the two experimental factors followed by Duncan's Multiple Range Test at a confidence level of 95%. One-way ANOVA was then employed when no interactions were found, pooling samples under each treatment for individual factors. All statistical analysis was done on SigmaStat ver 3.5. Graphs of mean values and their corresponding standard error bars were generated using SigmaPlot ver. 11.0.
RESULTS

Nitrogen uptake: Nitrogen uptake of *Tetraselmis* sp. was significantly affected by elevated CO₂ levels (p<0.05; Fig. 1a) but not by elevated phosphorus levels (p>0.05; Fig. 1b). Nitrogen uptake revealed no interaction between increased levels of CO₂ and phosphorus (p>0.05). Algal cultures supplied with 10% CO₂ (C3) in the incoming air for aeration exhibited the highest nitrogen uptake at 0.390±14.000 μg NH₃-N cell⁻¹ day⁻¹. An increasing trend in nitrogen uptake was observed with increasing CO₂ levels in all treatments and significant differences (p<0.05) were found between each increment (Fig. 1a).

Levels of residual NH₃-N in the culture medium decreased throughout culture period (Fig. 2a, b). The largest decrease in residual NH₃-N was observed during 1st and 2nd day for treatment with elevated CO₂ levels (Fig. 2a). A slower decrease subsequently followed from the 3rd to the last day of culture. For treatments with no added CO₂, decreases in residual NH₃-N were

![Fig. 1(a-b): Mean±SEM of nitrogen uptake rates for *Tetraselmis* sp. cultured under varying levels of (a) CO₂ (C1-natural air; C2-5% CO₂ added; C3-10% CO₂ added) and (b) Phosphorus (P1-5 mg L⁻¹; P2-10 mg L⁻¹; P3-20 mg L⁻¹) Values with the same superscript are not significantly different (p>0.05)](image)

![Fig. 2(a-b): Residual concentrations of NH₃-N in the culture media throughout the 6 day culture period under varying levels of (a) CO₂ (C1-natural air; C2-5% CO₂ added; C3-10% CO₂ added) and (b) Phosphorus (P1-5 mg L⁻¹; P2-10 mg L⁻¹; P3-20 mg L⁻¹)](image)
much smaller during the first few days but no sudden drops were observed throughout the culture period. Daily mean concentrations of NH₃-N markedly decreased with increasing CO₂ levels (p<0.05) on the 1st and 2nd day of culture with lowest values (p<0.05) at 10% CO₂ supplementation. A significant difference (p<0.05) was also observed during the last day of culture with treatments at 10% CO₂ having the lowest concentrations of NH₃-N. Increasing levels of phosphorus did not result in significant changes in nitrogen uptake (p>0.05; Fig. 2b).

**Lipid content:** Elevated levels of CO₂ and phosphorus significantly affected the lipid content of *Tetraselmis* sp. (p<0.05; Fig. 3a, b) but no interaction was detected between the two factors (p>0.05). Addition of 10% CO₂ resulted in higher lipid content (p<0.05) at 10.96±0.15% (Fig. 3a). But this influence was not significant at lower CO₂ supplementation. The reverse was observed with phosphorus addition (Fig. 3b). Lipid yield was lowest (p<0.05) at 20 mg L⁻¹ phosphorus supplementation at 9.41±0.27% DW. Higher lipid yields were observed at lower phosphorus levels (p>0.05).

**Growth:** The increased growth rate of *Tetraselmis* sp. was directly proportional to the concentrations of CO₂ (p<0.05; Fig. 4a, b) but no marked response was observed with increasing levels of phosphorus (p>0.05). Interaction between increasing CO₂ and phosphorus concentrations was not evident in this study (p>0.05). Growth which was highest at 10% CO₂ supplementation (k' = 2.04±0.02), showed an increasing trend with increasing levels of CO₂ (Fig. 4a).

Cell densities throughout the 6 days of culture showed marked increases with addition of CO₂. These were observed notably starting on the 4th day until the end of culture (p<0.05; Fig. 5a). A completely opposite trend was observed with addition of phosphorus although cell densities did not differ significantly among treatments (p>0.05; Fig. 5b). In this study, influence of CO₂ addition on cell densities showed no interaction with phosphorus supplementation.

With no interactive effect between CO₂ and phosphorus observed for all variables measured, the individual effect of each factor became the main focus in analyzing experimental data. Between the factors tested in this study, elevated levels of CO₂ had the greater effect on all variables.

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**Fig. 3(a-b):** Mean±SEM of lipid content (%DW) for *Tetraselmis* sp. cultured under different levels of (a) CO₂ (C1-natural air; C2-5% CO₂ added; C3-10% CO₂ added) and (b) Phosphorus (P1-5 mg L⁻¹; P2-10 mg L⁻¹; P3-20 mg L⁻¹) Values with the same superscript are not significantly different (p>0.05)
Fig. 4(a-b): Mean±SEM of growth rate (k’) for Tetraselmis sp. cultured under different levels of (a) CO₂ (C1-natural air; C2-5% CO₂ added; C3-10% CO₂ added) and (b) Phosphorus (P1-5 mg L⁻¹; P2-10 mg L⁻¹; P3-20 mg L⁻¹). Values with the same superscript are not significantly different (p>0.05).

Fig. 5(a-b): Cell density of Tetraselmis sp. throughout the 6 day culture period with varying levels of (a) CO₂ (C1-natural air; C2-5% CO₂ added; C3-10% CO₂ added) and (b) Phosphorus (P1-5 mg L⁻¹; P2-10 mg L⁻¹; P3-20 mg L⁻¹)

measured namely nitrogen uptake, lipid content, growth rate and final cell density. Elevated levels of phosphorus only had a significant effect on the lipid content. Growth rate, final cell density and lipid content were the most affected (p<0.001) by CO₂ addition showing directly proportional changes with increasing CO₂ levels. Elevated levels of phosphorus on the other hand seemed to particularly inhibit lipid production.

DISCUSSION

The present study demonstrated the effects of elevated levels of CO₂ and phosphorus on the growth, nitrogen uptake and lipid content of the microalga Tetraselmis sp. The concentrations which are higher than what is normally used in established protocols for producing the algae as a natural food for aquaculture were tested to also explore the potential of this species for other applications such as waste water remediation and biofuel production. Results showed the clear
influence of CO$_2$ addition on nitrogen uptake, lipid content and growth of *Tetraselmis* sp. but this was not very apparent in the case of phosphorus except for lipid yield. The present study indicates that the responses of *Tetraselmis* sp. are independently affected by CO$_2$ and phosphorus and that CO$_2$ influenced algal growth, nitrogen uptake and lipid content more than phosphorus.

**Influence of CO$_2$ and phosphorus on nitrogen uptake:** As a generalized assumption for plants including algae, carbon and nitrogen metabolism are interconnected since they share organic carbon and energy sourced directly from photosynthetic electron transport and CO$_2$ fixation (Huppe and Turpin, 1994). The highest nitrogen uptake rates at 10% CO$_2$ (Fig. 1) in this study have also been reported on other species of algae indicating that in mass culture systems for algae, CO$_2$ could have a limiting effect and its supplementation could significantly increase the uptake rates of other nutrients (Amory *et al.*, 1991; Kaya *et al.*, 1996; Xu *et al.*, 2010).

CO$_2$ supplementation in this study results in higher nitrogen uptake rates, which in turn, leads to significant growth increments at increasing levels of the nutrient. Carbon assimilated by microalgae (mainly sourced from CO$_2$ fixation through photosynthesis) is generally directed towards the production of carbon skeletons for amino acid synthesis. Nitrogen assimilation in the form of NH$_4^+$ or NO$_3^-$ is directly affected by the availability of carbon and has been demonstrated to have a photosynthetic CO$_2$ requirement and reductions of either light or CO$_2$, drastically decreased nitrogen uptake (Grant, 1968; Boussiba *et al.*, 1984; Guerrero and Lara, 1987). This could therefore account for the higher nitrogen uptake rates with increased levels of CO$_2$ in this study.

Varying rates of nitrogen uptake observed throughout the culture period follow typical responses of microalgae to different concentrations of nutrients. The drastic decrease in residual NH$_4$-N observed between the 1st and 2nd day (Fig. 2) of culture exhibits an algal response known as "surge uptake" wherein upon introduction into a new medium (i.e., the transfer of the inoculums into the modified F medium), nitrogen uptake far exceeds instantaneous growth rates predicted using simple Michealis-Menten kinetics (Gilbert and Goldman, 1981). This generally occurs in nitrogen starved cells inoculated into a new medium with a fresh supply of nitrogen (Capone, 2008). The rate at which residual NH$_4$-N decreased during the first few days of culture also suggests that assimilation by algae, rather than volatilization of ammonia, could largely account for the reduction in residual NH$_4$-N concentrations. Although volatilization of ammonia could have contributed to reductions in NH$_4$-N concentrations, pH levels and temperatures during the culture period suggest that only less than 15% of the NH$_4$-N present in the system could have volatilized into the overlying air of the culture vessel. Even in uncovered algal ponds for waste water remediation, ammonia volatilization has been observed to be minimal and negligible (Zinnum *et al.*, 2000; Babu, 2011).

Elevated levels of phosphorus beyond those usually used in microalgal production for aquaculture did not have a significant effect on the nitrogen uptake of *Tetraselmis* sp. even at higher CO$_2$ concentrations (Fig. 1b). Earlier works (Rodriguez *et al.*, 2009) showed that lower N:P ratios brought about by higher phosphorus concentrations did not necessarily increase the amount of nitrogen assimilated into algal cells. In the natural environment, phosphorus tends to be a limiting factor due to its reduced availability compared to other nutrients. In the case of this study though, the lowest concentrations of phosphorus were already well above the limiting concentrations in the natural environment at a magnitude of approximately 30-36 times greater (Zeebe and Wolf-Gladrow, 2001). Higher concentrations of nutrients above those for optimum algal growth could induce a phenomenon called "luxury uptake" wherein excessive nutrients are taken
up for storage without necessarily translating them into growth. This phenomenon could lead to depressed or constant nutrient uptake rates for microalgae (Gordon et al., 1981; Buapet et al., 2008; Rodriguez et al., 2009). In this study, excess phosphorus tended to depress growth (Fig. 5b) which could have affected lipid yield in groups receiving high phosphorus supplementation (Fig. 3b).

In situations wherein phosphorus was present in limiting amounts though, it was found that the addition of phosphorus increased nitrogen assimilation by algae (Xu et al., 2010). This is because sufficient amounts of phosphorus are needed for various chloroplast functions, including the generation of ATP to provide energy to reduce NO\textsubscript{3}\textsuperscript{-} to NH\textsubscript{4}\textsuperscript{+} when NO\textsubscript{3}\textsuperscript{-} is assimilated as the primary source of nitrogen (Zer and Ohad, 2003). Results of this study therefore point towards the necessity for further investigations using lower concentrations of phosphorus not only to minimize costs but to reduce overall nutrient loading in cultures as well.

As a potential mechanism for waste water remediation from aquaculture ponds, the addition of CO\textsubscript{2} in treatment ponds inoculated with Tetraselmis sp. could significantly reduce the amount of ammonia present due to excess feeds and metabolic byproducts. Results of this study show improved nitrogen uptake rates at higher CO\textsubscript{2} concentrations for this algal species leading to the possibility of developing new strategies not only for waste water management but carbon sequestration as well.

**Lipid yields under elevated CO\textsubscript{2} and phosphorus levels:** Unlike terrestrial plants, lipid accumulation in algae occurs in the same cells wherein photosynthesis occurs. This means that microalgal cells have the potential to be compact targets for manipulation towards the production of higher lipid yields since carbon fixation and triacylglycerol synthesis are modulated within a single cell (Lembi and Waaland, 1988). The highest lipid content at elevated CO\textsubscript{2} levels in this study (Fig. 3) was similar to the results of Zhila et al. (2005) and Gouveia et al. (2009) for other species of microalgae. The common trend in the accumulation of storage lipids in microalgae is that lipid yields generally increase during periods of nutrient starvation such as low nitrogen or phosphorus environments (Reitan et al., 1994; Kilham et al., 1997; Coleman et al., 1988; Khizin-Goldberg and Cohen, 2006). In this experiment, the higher nitrogen uptake rates at elevated CO\textsubscript{2} levels eventually led to lower nitrogen concentrations in the culture media during the last few days of culture (Fig. 2) resulting in a potentially nitrogen limited environment. This could account for the higher accumulation of storage lipids in Tetraselmis sp. cultures at high CO\textsubscript{2} levels. Aside from the actual depletion of nitrogen in the culture media, changes in the C:N ratio throughout the culture period could have also influenced the increased formation of storage lipids. Increases in the C:N ratio of culture media have been shown to shift nutrient limitations from carbon to nitrogen for the microalgae Chlorella sorokiniana (Chen and Jahn, 1991). At higher C:N ratios, carbon utilization of microalgae shifts from being used for growth to lipid synthesis (Roessler, 1990). Although, shifts in carbon utilization from growth to lipid accumulation have been observed to be the common trend in algae, the presence of excess carbon substrates in the culture medium have also been reported to support good growth and substantial lipid synthesis in other algal species (Moheimani, 2013). The observations on Tetraselmis sp. in this study thus provides evidence that elevated CO\textsubscript{2} levels could improve both the growth and lipid synthesis for this species over an adequate culture period.

Regardless of CO\textsubscript{2} levels, the addition of phosphorus to cultures of Tetraselmis sp. in this study also resulted in a significant difference among treatments with the lowest lipid yields observed in treatments with 20 mg P L\textsuperscript{-1}. The relatively low lipid yield observed at higher phosphorus
concentrations reflects the typical reaction of other microalgae to nutrient sufficient conditions (Ratledge, 2004). This could also be related to the apparent growth depression of *Tetraselmis* sp. at excessive phosphorus levels in the present study. Although, it is clear that higher phosphorus levels reduce lipid yields, its mechanisms are not well understood. It is therefore important to further investigate the relationships of phosphorus concentrations with lipid production under lower levels than those commonly used for aquaculture production. Understanding this can lead to cost reduction and improvement in lipid yields for this species.

**Effect of CO₂ and phosphorous on the growth of *Tetraselmis* sp.**: Improving overall growth and biomass production has been one of the common aims in microalgal production both in aquaculture or biotechnological applications. Improved algal growth rates beyond those currently attained in various industries could also address issues regarding land and water utilization. Results of this study showed that higher growth rates and final cell densities increased with increasing levels of CO₂ (Fig. 4a and 5a). These results corroborate with the observations on other species of microalgae grown on elevated levels of CO₂ (Jeong et al., 2003; Sen et al., 2005a, b). At elevated CO₂ concentrations under constant illumination, peak cell densities for *Chlorella vulgaris* and *Isochrysis galbana* experienced 12-fold and 4-fold increases, respectively compared to treatments with no CO₂ supplementation (Sen et al., 2005a, b). Along with the uptake of nitrogen, carbon is also a precursor to algal growth with carbon skeletons for protein and amino acid synthesis derived from photosynthetic CO₂ fixation (Amory et al., 1991). The supplementation of elevated levels of CO₂ could therefore account for the higher growth rates and final cell densities observed in this study.

It must be noted though that the pH of the culture medium remained relatively constant in all treatments of CO₂ supplementation ranging between 8.0-8.5. This is probably due to the relatively high buffering capacity of seawater (Pytkowicz and Atlas, 1975) and the additional buffering effect of phosphate (Froelich, 1988) added through the culture media. Within this pH range, the equilibrium between HCO₃⁻ and free CO₂ leans towards HCO₃⁻ becoming the dominant inorganic carbon species. Majority of aquatic plants however can utilize HCO₃⁻ as a source of CO₂ through CO₂ concentrating mechanisms (Raven et al., 1990; Giordano et al., 2005). Rigobello-Masini et al. (2003) was able to demonstrate the extra and intracellular activity of carbonic anhydrase in *Tetraselmis gracilis* indicating the capability of this alga to utilize HCO₃⁻ through a possible carbon concentrating mechanism. This could explain the higher growth rates and final cell densities in *Tetraselmis* sp. used in this study despite the constantly high pH throughout the culture period.

In treatments with elevated levels of phosphorus, no significant difference was observed in terms of growth rate and final cell density (Fig. 4b and 5b). Although phosphorus loading in natural waters has been correlated with eutrophication and massive algal blooms, this generally occurs in waters where phosphorus was initially at limiting concentrations. The unaffected growth rates and final cell densities observed in this study are in agreement with earlier reports showing that higher phosphorus concentrations did not necessarily translate into higher algal growth rates (Condit, 1972; Watson et al., 1992; Langrudi et al., 2010). At excessively high phosphorus concentrations, microalgae have been shown to exhibit luxury uptake wherein phosphorus is assimilated and stored as polyphosphates (Vance et al., 2003). These function as a reserve for phosphorus which algal cells could later tap during phosphorus limited conditions (Stewart, 1974; Van den Hoek et al., 2001). Xu et al. (2010) also reported that the growth rate of algae cultured at high phosphorus levels were mainly dependent on photosynthetic carbon utilization and
nitrogen metabolism which was similar to what was observed for Tetraselmis sp. in this study. The depressed uptake of phosphorus at excessive levels of this nutrient reported in other algal species (Gordon et al., 1981; Buapat et al., 2008; Rodriguez et al., 2009) may have also operated in this study thus, resulting in apparently lower growth at high phosphorus supplementation.

CONCLUSION
This study showed that elevated levels of CO₂ and phosphorus above those currently used in aquaculture practices independently affected nitrogen uptake, lipid content and growth of Tetraselmis sp. Increasing CO₂ levels resulted in proportional increments in nitrogen uptake, growth, cell density and lipid yield indicating the potential of CO₂ addition in improving natural food production for aquaculture (due to increased growth rate) and other biotechnological applications such as waste water remediation (due to increased nitrogen uptake) and biofuel production (due to improved lipid yields). Phosphorus supplementation at high levels on the other hand, tended to depress algal growth and positively affect nitrogen uptake but not in significant increments. Excessive phosphorus levels significantly reduced lipid production. Possible effects of lowering phosphorus levels in order to lower costs and improve lipid production merit further investigation.

As possible directions for future studies, the effect of CO₂ addition on the fatty acid composition of lipids in Tetraselmis sp. could also be investigated with the aim of improving its nutritional value as a natural food source in aquaculture. Aside from this, studies focusing on the carbon uptake rates of Tetraselmis sp. should also be conducted to further elucidate on its potential for carbon sequestration leading to optimized protocols for CO₂ addition. Actual applications of Tetraselmis sp. using flue gas from factories and waste water from aquaculture also merit further investigations to determine the overall feasibility of using this alga in large scale operations.

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