

Full Length Research Paper

Growth and proximate composition of tropical marine *Chaetoceros calcitrans* and *Nannochloropsis oculata* cultured outdoors and under laboratory conditions

Sanjoy Banerjee¹, Wei Ee Hew², Helena Khatoun¹, Mohamed Shariff^{1,2*} and Fatimah Md. Yusoff¹

¹Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.

²Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia.

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The growth and proximate composition of two marine microalgae, *Chaetoceros calcitrans* and *Nannochloropsis oculata*, cultured outdoors under shade (24 to 36°C, 140 µmol/m²/s) and laboratory conditions (environmental chamber, 23°C for *C. calcitrans* and 20°C for *N. oculata*, 150 µmol/m²/s) were compared. Outdoor cultures of both *C. calcitrans* and *N. oculata* had significantly higher ($p < 0.05$) biomass, cell count, optical density and specific growth rate compared to the cultures grown under laboratory conditions. Lipid content was significantly higher in *C. calcitrans* grown outdoors, whereas, protein and carbohydrate composition did not show any significant differences ($p > 0.05$) between the outdoor and laboratory cultures. In the case of *N. oculata*, no significant differences ($p > 0.05$) were found in protein and lipid composition, but carbohydrate was significantly higher ($p < 0.05$) in the outdoor culture. In addition, the results showed that both *C. calcitrans* and *N. oculata* cultures grew faster outdoors, producing more biomass within a shorter period of time. This study illustrated that outdoor culture of microalgae was viable despite the fluctuating environmental conditions.

Key words: Growth, proximate composition, *Chaetoceros calcitrans*, *Nannochloropsis oculata*, outdoor culture.

INTRODUCTION

Microalgae are the primary food source for a large number of aquatic organisms and play a key role in aquaculture development. Among the several strains available, *Chaetoceros* spp. and *Nannochloropsis* spp. are widely used in marine hatcheries as food sources as well as to maintain water quality (Riquelme and Avendaño-Herrera, 2003; Khatoun et al., 2007). Microalgae are photoautotrophic organisms that need light as their main energy source. The effects of light intensity, temperature, salinity and media nutrients on the growth and proximate composition of microalgae have been widely explored (Thompson et al., 1992; Brown et al., 1997). Numerous investigations have been made on the optimal growth conditions for microalgae in the laboratory. Currently, microalgae are grown in photobioreactors, which are able to provide optimal conditions for unialgal culture. How-

ever, the use of a photobioreactor to maintain controlled conditions for microalgal growth is expensive due to the need for artificial light and controlled temperature. In addition, indoor controlled culture requires expertise often not found in the hatchery or farm.

In tropical countries, microalgae for larval feeding are produced in hatcheries indoors, which are supposed to improve the quality and reliability of production but at high cost or outdoors where they are mass cultured at lower cost but with high seasonal variability. Therefore, culturing microalgae outdoors under ambient conditions is one way of decreasing the cost of production. However, culturing algae outdoors can expose the cells to severe and rapid fluctuations in light and temperature. Inadvertently, the climate where the algae are grown is often suboptimal, being either too cold or too hot or lacking sufficient light due to cloud cover during the rainy season. All these parameters influence the growth and proximate composition of the microalgae (Thompson et al., 1992). Therefore, the aim of this study was to compare the growth and proximate composition of indigenous

*Corresponding author. E-mail: shariff@vet.upm.edu.my. Fax: +60 3 8947 2101.

isolated tropical marine *Chaetoceros calcitrans* (UPMAA-HU10) and *Nannochloropsis oculata* (UPMAAHU20) grown outdoors under natural conditions and indoors under controlled laboratory conditions.

MATERIALS AND METHODS

Microalgal cultures

Tropical marine *C. calcitrans* (UPMAAHU10) and *N. oculata* (UPMAAHU20) were obtained from the Aquatic Animal Health Unit, Universiti Putra Malaysia. The microalgae were isolated from Port Dickson (2°31'N 101°48'E) and acclimatized for a year according to their respective environmental conditions as follows: (1) microalgae were kept outdoors under shade (O) at a temperature range of 24 to 36°C, natural daylight (average 140 $\mu\text{mol}/\text{m}^2/\text{s}$, LD 12:12 cycle) and was provided with aeration but no carbon dioxide (CO_2); (2) microalgae maintained under laboratory conditions (L) were kept in environmental chambers (Sanyo, Japan); the temperatures (23°C for *C. calcitrans* and 20°C for *N. oculata*) were chosen on the basis of reports by Yanase and Imai (1968) and Brown et al. (1998), respectively. The cultures were provided with light (150 $\mu\text{mol}/\text{m}^2/\text{s}$, LD 12:12 cycle (12 hours light alternating with 12 hours darkness) as well as aeration and CO_2 .

The cultures were grown in filtered and autoclaved seawater (30 ppt, 8.0 pH) using Conway medium (Tompkins et al., 1995). The sterilized medium was kept for 2 days before inoculating the microalgae to allow sufficient time for CO_2 equilibration.

Experimental design

In the experiment, *C. calcitrans* and *N. oculata* were grown outdoors under shade and indoors under laboratory conditions. Both treatments had four replicates. The experiment was gradually scaled up from an initial starter culture volume of 20 ml to 1L for both *C. calcitrans* and *N. oculata*. Initially, 20 ml of microalgal stock cultures were mixed with 30 ml medium in each flask (total culture volume 50 ml), with batch cultures of increasing volume (250, 500 ml and 1L) as inocula for the next step until a 1 L culture was obtained. The cultures were transferred in their log-phase of growth as determined by biomass, cell count and optical density (OD). The transfer of the microalgae from one flask to another was done inside a laminar flow unit to prevent any contamination to the culture. Aeration was provided to each flask through filters (0.45 μm , Sartorius, Germany) and flasks were also hand-agitated three times a day. The outdoor cultures were kept under a shade at diurnal temperature range of 24 to 36°C and average light intensity of 140 $\mu\text{mol}/\text{m}^2/\text{s}$ with aeration and no CO_2 addition throughout the experiment. Laboratory cultures were kept in an environmental chamber (Sanyo, Japan) at 23°C for *C. calcitrans* and 20°C for *N. oculata*, with light intensity of 150 $\mu\text{mol}/\text{m}^2/\text{s}$, LD 12:12 cycle and aeration. Carbon dioxide was added to adjust the pH of the culture when the pH reached 9.0.

During the experimental period, physical parameters such as temperature and pH were measured daily each morning. For daily determination of cell count, biomass, OD and pH, 4 ml of aliquots were collected aseptically from all the flasks starting from the 250 ml culture. The experiment was terminated on day 17 when the microalgae were harvested; at this point the 1L culture had reached the stationary phase as determined by biomass, cell count and OD.

Analysis of growth parameters

Microalgal growth was measured using biomass, cell count, specific

growth rate and OD. Biomass was estimated using 2 ml microalgal samples filtered through precombusted (100°C, 4 h) and pre-weighed glass fibre filters (Advantec, Japan). After filtration, *C. calcitrans* and *N. oculata* samples were rinsed with 2 ml of 0.5 M ammonium formate. The filtrates were dried at 100°C for 4 h, cooled in a desiccator and then weighed. The dry biomass concentration in the culture was calculated by dividing the difference between the weights of the dried filter paper (after and before filtration) by the filtered volume (Lavens and Sorgeloos, 1996).

Cell numbers were determined daily by placing an aliquot of well-mixed culture suspension on a Neubauer haemocytometer (Assistant, Germany). The cells were counted in five small squares in the centre block. The cell number in the culture was calculated by dividing the number of cells counted by the volume and the dilution. The specific growth rate (SGR) of microalgae was calculated by the following equation:

$$\text{SGR/day} = \ln (X_2/X_1)/t_2-t_1$$

Where, X_1 is the biomass concentration at the beginning of the selected time interval; X_2 is biomass concentration at the end of the selected time interval; t_2-t_1 is the selected time (in days) for the determination of biomass of microalgal species.

The OD for all the cultures was determined daily using a spectrophotometer (UV-VIS 1601, Shimadzu, Japan). The wavelengths used were 750 nm for *C. calcitrans* and 540 nm for *N. oculata* (Rocha et al., 2003).

Proximate composition

Protein and carbohydrate were analysed according to the methods of Lowry et al. (1951) and Dubois et al. (1956), respectively, using 5 to 6 mg freeze-dried microalgal culture. Lipid was analysed using 1 ml of fresh microalgal culture following the carbonization method of Marsh and Weinstein (1966) using tripalmitin as the standard.

Statistical analysis

The collected data were analyzed using one-way analysis of variance (ANOVA). Significant differences among the different treatments were determined using the Duncan multiple range test at 0.05 level of probability. Protein, lipid and carbohydrate percentages were arcsine transformed before statistical analysis. All statistical analysis was done using the statistical analysis system computer package (SAS, 2002).

RESULTS

pH and temperature

The temperature (°C) and pH ranges for *N. oculata* and *C. calcitrans* cultured outdoors were 25 to 36 and 8.4 to 9.4, respectively, while under laboratory conditions were 20 to 23 and 7.9 to 8.5, respectively.

Growth of *C. calcitrans* and *N. oculata*

There were no significant differences ($p > 0.05$) between the outdoor and laboratory grown cultures during the first 5 days in terms of biomass, cell count and OD for 250 ml *C. calcitrans* cultures. The 250 ml cultures from both

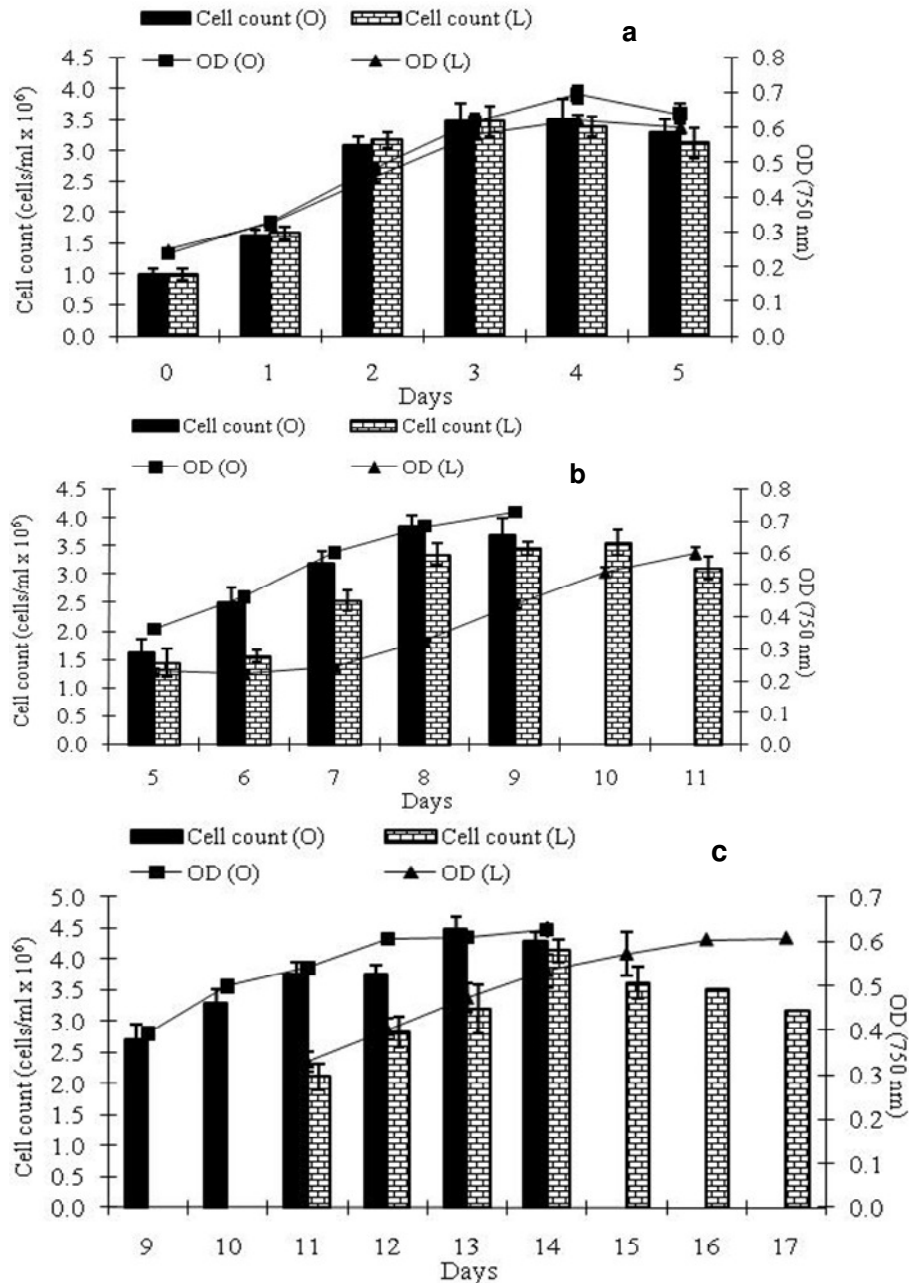


Figure 1. Cell count and optical density (OD) of *C. calcitrans* in (a) 250 ml, (b) 500 ml, and (c) 1 L cultures. Values are mean \pm standard error (n=4).

treatments were scaled up to 500 ml on day 5. For 500 ml culture, the highest biomass, cell count and OD were achieved on day 8 outdoors whereas, for laboratory conditions it was on day 10. Culture grown outdoors (500 ml) reached the stationary phase on day 9 and therefore, scaled-up to 1 L 2 days earlier than the cultures grown in the laboratory. Cultures grown outdoor reached the stationary phase earlier and had shorter exponential phase, higher cell count, OD and biomass when compared with laboratory cultures and therefore, were harvested 3 days earlier (Figures 1 and 2; Table 1).

N. oculata cultures grown outside had significantly higher ($p < 0.05$) biomass, cell count and OD. Culture in 250 ml and 1 L flask grown outdoors reached stationary phase 3 days and 1 day earlier respectively, when compared with the laboratory conditions, whereas, in the 500 ml culture, both outdoors and laboratory cultures reached stationary phase in 6 days. Thus, the total culture period was reduced by 4 days since the *N. oculata* grown outdoors was harvested on day 16 when compared with 20 days for the laboratory culture (Figures 3 and 4; Table 1).

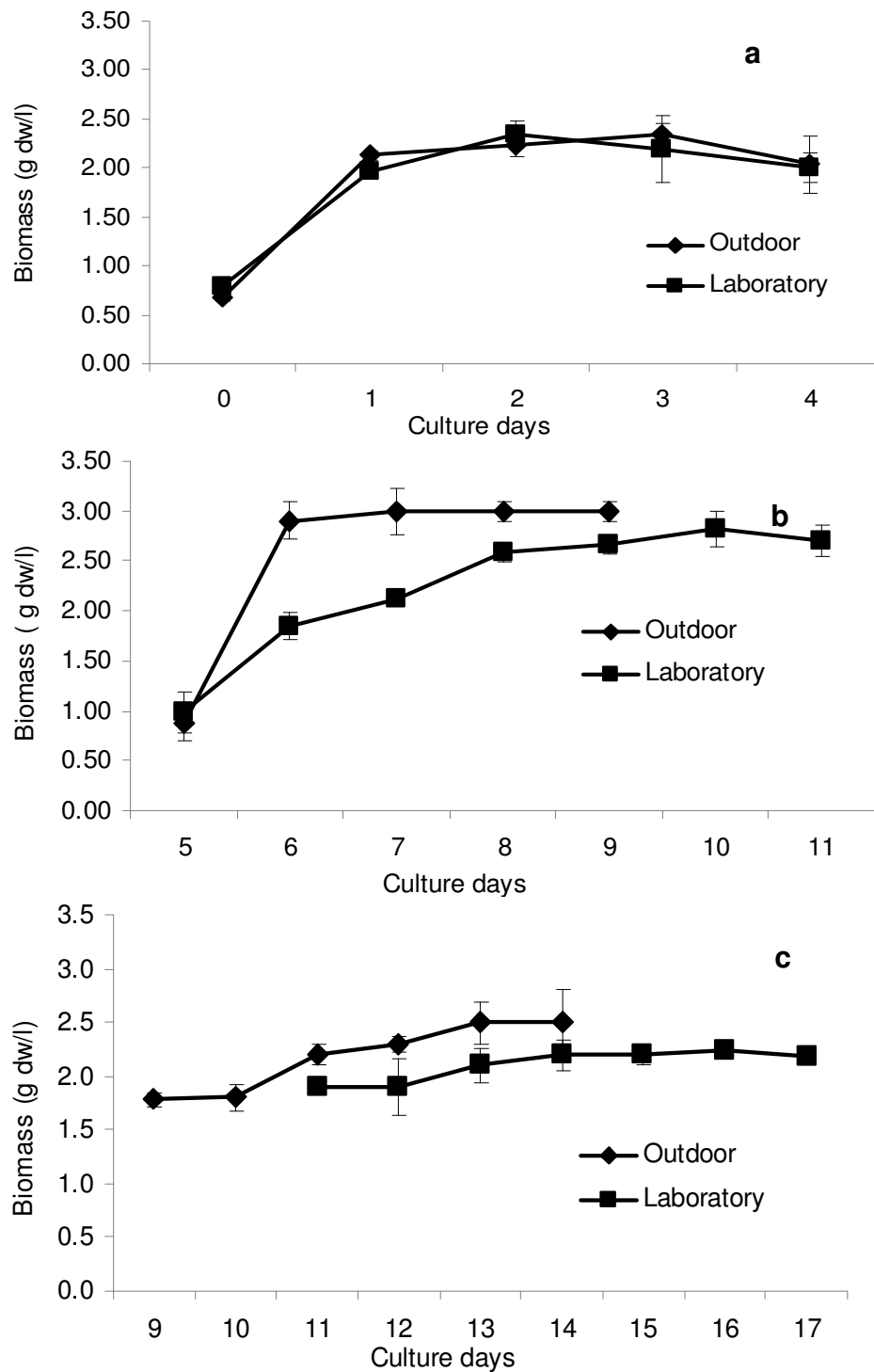


Figure 2. Biomass of *C. calcitrans* in (a) 250 ml (b) 500 ml (c) 1 L cultures. Values are mean \pm standard error (n=4).

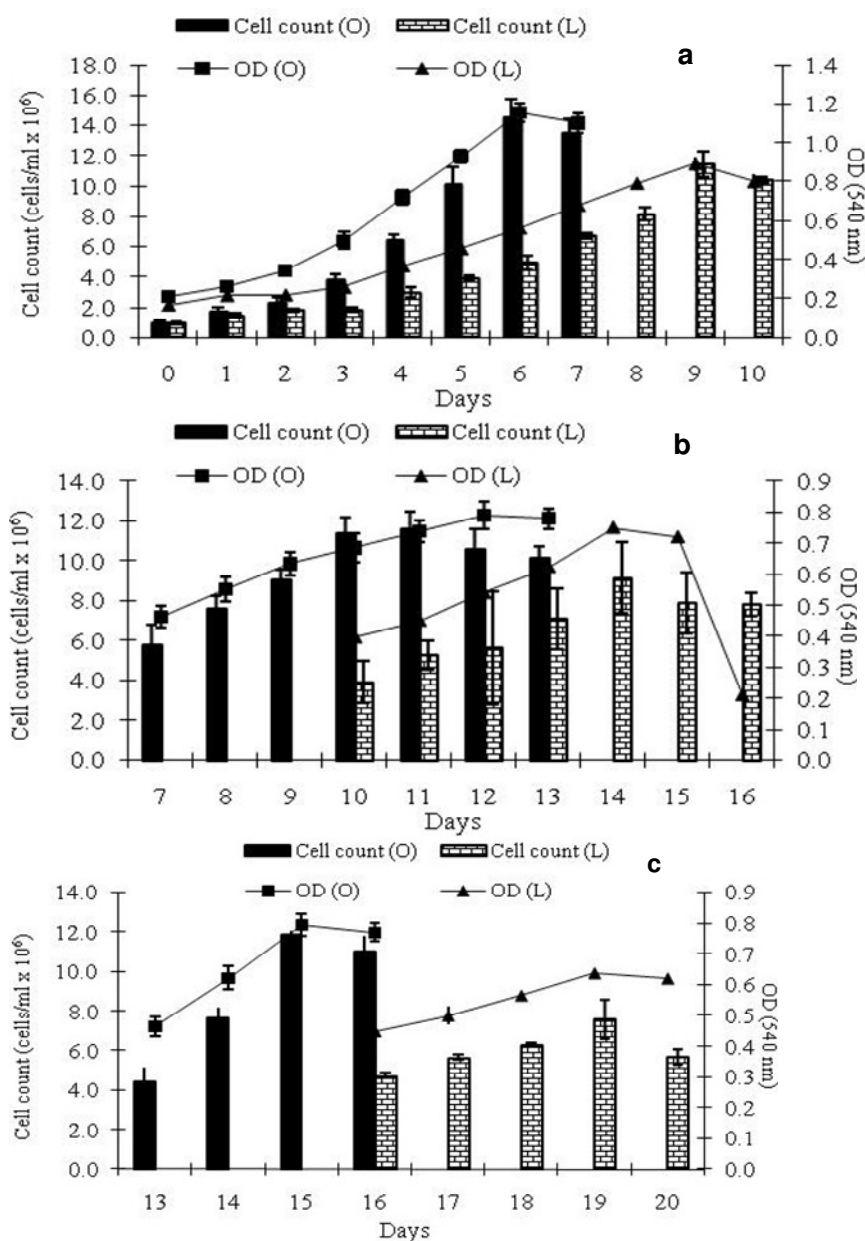
At the end of the experiment, the specific growth rate of both *C. calcitrans* and *N. oculata* was found to be significantly higher ($p < 0.05$) when cultured outdoors when compared with the culture under laboratory conditions (Table 1).

Proximate composition

Protein and carbohydrate composition for *C. calcitrans* did not show any significant differences ($p > 0.05$) between the outdoor and laboratory cultures. However, lipid

Table 1. Biomass, cell count, proximate composition and time period at harvest of culturing marine *C. calcitrans* and *N. oculata* outdoors under shade and under laboratory conditions (n=4).

Parameter	<i>C. calcitrans</i>		<i>N. oculata</i>	
	Outdoor (O)	Laboratory (L)	Outdoor (O)	Laboratory (L)
Biomass (g/l dw)	2.50 ± 0.20	2.20 ± 0.10	2.11 ± 1.70	1.6 ± 0.20
Cell count (cells/ml × 10 ⁶)	4.30 ± 0.60	3.20 ± 0.40	11.12 ± 0.71	5.7 ± 0.40
SGR (/day)	0.05 ± 0.01	0.03 ± 0.01	0.01 ± 0.02	0.004 ± 0.01
Protein (% dw)	41.60 ± 4.20	43.10 ± 1.30	32.82 ± 3.11	34.11 ± 0.70
Lipid (% dw)	26.80 ± 5.20	11.71 ± 6.01	13.02 ± 0.91	12.51 ± 1.01
Carbohydrate (% dw)	8.70 ± 1.20	6.62 ± 1.51	26.13 ± 2.70	19.81 ± 2.02
Time period (days) (scaling up from 250 ml to 1L)	14	17	16	20

**Figure 3.** Cell count and optical density of *N. oculata* in (a) 250 ml, (b) 500 ml, and, (c) 1 L cultures. Values are mean ± standard error (n=4).

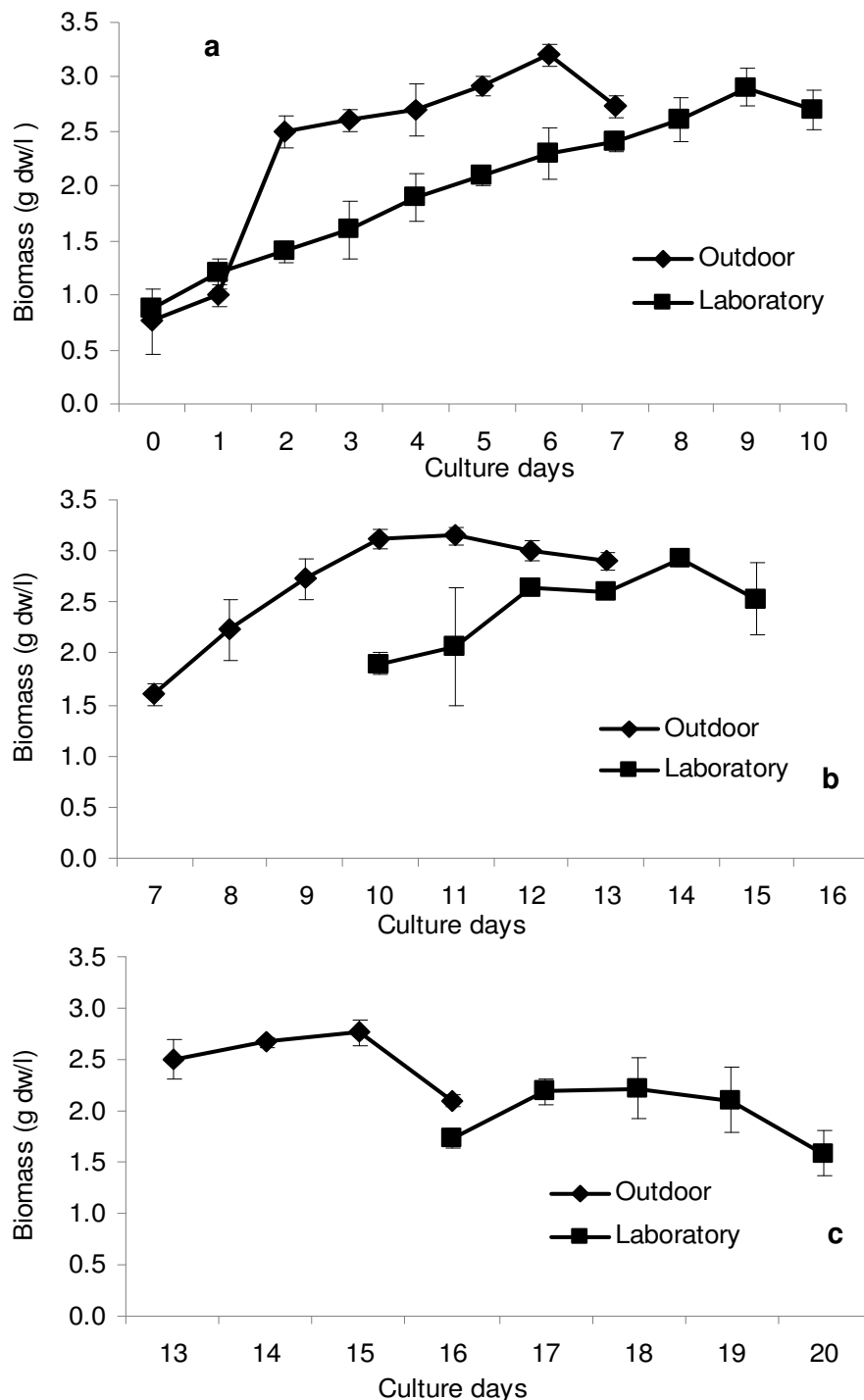


Figure 4. Biomass of *N. oculata* in (a) 250 ml (b) 500 ml and, (c) 1 L cultures. Values are mean \pm standard error (n=4).

was significantly higher ($p < 0.5$) in outdoor culture when compared with laboratory culture (Figure 5). For *N. oculata*, no significant differences ($p > 0.05$) were found in the protein and lipid composition when compared with carbohydrate, which was significantly higher ($p < 0.05$) in outdoor culture (Figure 6).

DISCUSSION

In this experiment, the growth of microalgae was monitored through the measurement of the biomass, cell count and OD because each technique has its own limitations. The biomass results can be affected by the

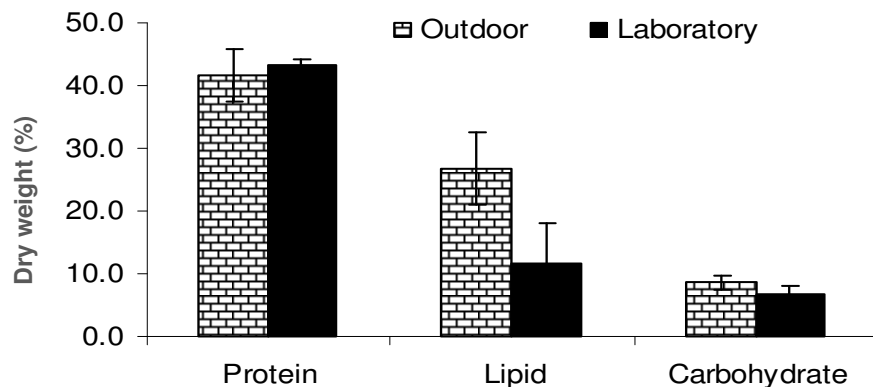


Figure 5. Proximate composition of *C. calcitrans*. Values are mean \pm standard error (n=4).

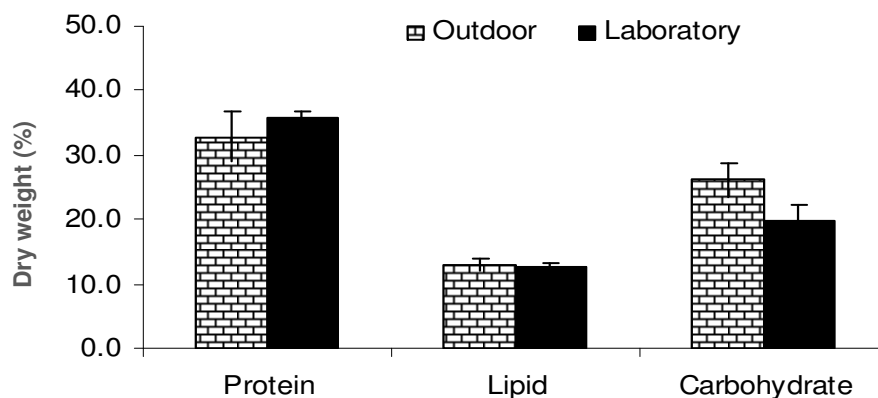


Figure 6. Proximate composition of *N. oculata*. Values are mean \pm standard error (n=4).

amount of salts absorbed on the cell surface and present in the intercellular water in marine microalgae (Zhu and Lee, 1997). To prevent this error, the microalgae were rinsed with ammonium formate. The cell count technique has the disadvantage of being time consuming. In addition, similar-sized fragments like microalgal cells that fail to separate during cell division can cause incorrect counts. The other option is to use OD to evaluate the cell density, a method that is easy, practical and user friendly. The experiment showed that there was a positive correlation between biomass, cell count and OD.

In this study, although the microalgal species were acclimatized according to their respective conditions, the higher biomass seen in the outdoor cultures could be due to reduced irradiance to the cultures grown under shade outdoors. Studies by Ugwu and Aoyagi (2008) showed that, shading of photobioreactor surfaces helped to get higher biomass productivity. Vonshak and Richmond (1988) also reported that shading of cultures could reduce the adverse effect of irradiation. The growth of the microalgae under laboratory conditions was significantly lower when compared with the growth observed under natural conditions, most probably due to artificial illumi-

nation causing stress to the cultured microalgae. According to Rocha et al. (2003), artificial light can cause heating and difficulty in dissipation of energy to the atmosphere.

Moreover, the spectral composition of light can influence the physiological and biochemical changes in plants (Voskresenskaya, 1972; Senger, 1987). According to Sánchez-Saavedra and Voltolina (1996) and Mercado et al. (2004), different light sources can vary the microalgal composition. In fluorescent lighting, the light is concentrated in a few preferred colors while sunlight has all the wavelengths in equal amount. In this study, the different spectral composition of fluorescent lights used in the incubators for indoor culture may not exactly match the spectral distribution of the sun which might explain the better growth of the cultures grown outdoors.

Cultures of *C. calcitrans* grown outdoors had higher growth in terms of biomass, cell count and OD. This significantly higher growth rate could also be due to the suitable outside temperature, which ranged from 24 to 35°C and was within the optimal temperature as reported by McGinnis et al. (1997). Even *N. oculata* cultured outdoors had significantly higher cell count and optical

density than those grown under controlled conditions in the laboratory. This result is in accordance with Cho et al. (2007) who reported that *N. oculata* had high maximum density at high temperature (30°C) instead of low temperature (15 to 20°C).

According to Brown et al. (1997), microalgal composition varies by species in the proportion of protein (6 to 52%), lipid (7 to 23%) and carbohydrate (2 to 23%). In this experiment, the protein and carbohydrate in *C. calcitrans* and *N. oculata* cultured outdoors and under laboratory conditions were within the ranges reported by Brown et al. (1997). However, the lipid content of *C. calcitrans* was two times higher when cultured outdoors when compared with the laboratory-grown culture. This result is in accordance with López-Elías et al. (2005), who reported that *C. muelleri* had higher lipid content when cultured outdoors when compared with the indoors. Opute (1974) reported that, extreme high or low temperatures could reduce microalgal lipid production. Based on this, in this experiment the cultures grown outdoors under shade could possibly be in the optimal temperature range, which resulted in significantly higher lipid production when compared with the laboratory-grown cultures. In *N. oculata*, the composition of protein, lipid and carbohydrate were also within the range as reported by Brown et al. (1997). However, there was no significant difference in the lipid composition between the treatments as seen in *C. calcitrans*. The difference in temperature did not affect the lipid composition for this species and according to Thompson et al. (1990) there is no consistent relationship between temperature and the total lipid content. According to Thompson et al. (1990), changes in the amount of essential fatty acids and growth appears to be species specific. Likewise, in this experiment, *C. calcitrans* cultured outdoors had higher total lipid content whereas, no difference in *N. oculata* was found under both conditions.

In the experiment, microalgae grown outdoors showed qualitatively equal or in some cases better production than those grown under controlled conditions in the laboratory. López-Elías et al. (2005) have also shown that microalgae cultured outdoors are safe and reliable, with similar or better monthly yields and reduction in operating cost compared to indoors. Therefore, there are advantages of culturing microalgae outdoors, which could give higher growth rates without compromising on the quality of the microalgae. In addition, microalgae grown outdoors were harvested earlier and there was no necessity for the use of carbon dioxide.

Conclusion

There is a high demand for microalgae as live feed for aquaculture. However, the cost of production is a major constraint. Cultures grown using artificial lighting and controlled temperature increase the cost of production. Thus, an alternative option for microalgal production is

needed to overcome these limitations. The study indicated that, culturing *C. calcitrans* and *N. oculata* outdoors under natural conditions can save energy and costs without a decrease in the quality and the composition of the microalgae cultured. This experiment was done on a small scale. Since large quantities of these microalgae are needed for marine hatcheries, further studies are necessary to determine if similar results are obtained when microalgae are grown at a commercial scale.

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