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# Enhancing Lipid Production of *Dunaliella tertiolecta* by Manipulating the Interactive Effect of Salinity and Nitrogen

**M. Rizwan,[a,b] G. Mujtaba,[a,c] N. Rashid,[d] and K. Lee[a,*]**

[a]Department of Environmental Engineering and Energy, Myongji University, Yongin, Gyeongki, 17058, Republic of Korea

[b]Department of Environmental Sciences, University of Haripur, 22620, Haripur, Pakistan

[c]Department of Energy and Environment Engineering, Dawood University of Engineering and Technology, Karachi, 74800, Pakistan

[d]Department of Chemical Engineering, COMSATS Institute of Information Technology, 55180, Lahore, Pakistan

Low lipid yield is a major bottleneck towards the scale-up application of microalgae for biodiesel production. The focus of this study was to improve the lipid production of *Dunaliella tertiolecta* (a marine microalga) by altering the concentration of salinity and nitrogen (as nutrients). The unique aspect of this study was to investigate the interactive effect of nitrogen and salinity on lipid production and their correlation with biomass yield and nitrogen uptake. In this experiment, microalgae were grown under various combinations of salinity and nitrogen. At first, lipid production was observed under varied nitrogen concentration (0–75 mg $L^{-1}$ as $NaNO_3$) and fixed salinity, 37.7 Practical Salinity Unit (PSU). The maximum cell growth rate of 288.4 mg $L^{-1}$ $d^{-1}$ and lipid production of 29.3 % were achieved at 75 mg $L^{-1}$ of $NaNO_3$. Now, this concentration of nitrogen was fixed and the effects of salinity concentrations were observed. The lipid production increased to 34 %, and cell growth rate decreased to 201.3 mg $L^{-1}$ $d^{-1}$ at 3.77 PSU of salinity. However, further reduction of the nitrogen concentration down to 18.75 mg $L^{-1}$ of $NaNO_3$ increased the lipid production to 42 % and decreased biomass to 0.64 g $L^{-1}$. It was also found that lipid production was linearly correlated with nitrogen uptake. Microalgae cells consumed all of the nitrogen in the first 24 hours of acclimation; however, lipid yield did not change much over time.

*Key words:*
marine microalgae, nitrogen, salinity, biomass, lipid

## Introduction

Overuse of fossil fuels has led to finding renewable, environmentally friendly, and cost-effective energy resources.[1] Since conventional energy resources are neither cheap nor environmentally friendly. Conventional energy resources produce $CO_2$ (greenhouse gas), which is a potential threat to the environmental recipients. In this perspective, biofuels production has emerged as a potential alternative. Among biofuels, biodiesel is the most attractive choice, inherited with the traits of sustainability. A number of feedstocks can be used to produce biodiesel.[2] However, extraction of biodiesel from microalgae offers unique advantages over other feedstocks. Microalgae offer fast growth rate, high carbohydrate, lipid, and have the ability to grow under harsh conditions.[3]

Besides the unique features of microalgae, their scale-up applications for biofuels production is as yet uneconomical.[4] Actually, the current level of lipid production (30–40 % of cell biomass) is much lower than its potential.[5] Thus, there is a need to improve the lipid production of microalgae. A number of strategies have been applied in the past to improve lipid production.[6–8] However, nutrients control is considered a key to improving biomass and lipid yield. Rate of nutrients uptake in microalgae is different at different stage of microalgal growth. In lag phase, they require low nutrients due to low cell density. In exponential phase, they consume maximum nutrients to develop their cell biomass. However, in late-exponential or stationary phase, they shift their mechanism from biomass development to lipid and carbohydrate production. A number of studies have revealed that microalgae cells produce more lipids when exposed to nutrient-starved conditions in late exponential or stationary phase. Among

*Corresponding author: e-mail: kisay@mju.ac.kr;
Tel: +82-31-330-6689, Fax: +82-31-336-6336.

nutrients, nitrogen is a key element, which plays a major role in microalgal growth. Microalgae during nitrogen-rich conditions produce more protein than lipids; whereas, during nitrogen-starved conditions, the cells cannot synthesize protein due to less availability of nitrogen (a protein source). Thus, microalgae start to accumulate lipids. It is also reported that marine microalgae growth largely depends on salinity concentration along with nitrogen. Studies have demonstrated the effect of nitrogen and salinity individually. However, we hypothesized that nitrogen and salinity might have an interactive effect on microalgae growth. Optimization of this interaction might return high biomass and lipid yield. Thus, the scope of this study was to investigate the interactive effect of nitrogen and salinity. The approach of this work is different from typical studies available, which focus on investigating the effect of nitrogen, and salinity (as an independent element) instead of studying their interactive effect.

The purpose of this study was to assess the dependence of lipid production in *Dunaliella tertiolecta* (a marine microalga) upon different concentrations of salinity and nitrogen. The interactive effect of these two factors on lipid production, biomass yield, and nitrogen uptake was also investigated. A two-phase cultivation system was adopted; cells were actively grown in phase-1 under optimal environmental conditions. The cells in early stationary phase were transferred to phase-2, where cells were exposed to various concentration combinations of nitrogen and salinity.[6,8]

# Materials and methods

## Algal strain and culture conditions

Marine microalga *Dunaliella tertiolecta* (UTEX LB999) was obtained from the culture collection of the University of Texas, Austin, USA. Sterilized f/2 medium having 75 mg L$^{-1}$ of NaNO$_3$, 4.32 mg L$^{-1}$ NaH$_2$PO$_4$, 0.023 mg L$^{-1}$ ZnSO$_4$·7H$_2$O, 0.217 mg L$^{-1}$ MnSO$_4$·5H$_2$O, 0.0073 mg L$^{-1}$ Na$_2$MoO$_4$·2H$_2$O, 0.014 mg L$^{-1}$ CoSO$_4$·7H$_2$O, 0.0068 mg L$^{-1}$ CuCl$_2$·2H$_2$O, 3.15 mg L$^{-1}$, FeCl$_3$·6H$_2$O, 4.4 mg L$^{-1}$ Na$_2$EDTA·2H$_2$O and vitamins, supplemented with artificial seawater (MBL) having 24.72 g L$^{-1}$ of NaCl, 0.67 g L$^{-1}$ KCl, 1.36 g L$^{-1}$ CaCl$_2$·2H$_2$O, 4.66 g L$^{-1}$ MgCl$_2$·6H$_2$O, 6.29 g L$^{-1}$ MgSO$_4$·7H$_2$O, 0.18 g L$^{-1}$ of NaHCO$_3$ and 0.606 g L$^{-1}$ of Tris.[9] At first, seed culture was grown in 100 mL of f/2 medium using 250 mL flask in shaking incubator under light intensity of 80–100 μE m$^{-2}$ s$^{-1}$ at 25 °C. When stationary phase was reached, the cells were shifted to glass bubble-column photo bioreactor.

Cells were grown photoautotrophically at 25 °C in 1 L f/2 medium in a bubble-column photo-bioreactor (internal diameter 6.5 cm; height, 37 cm).[10]

Air mixed with CO$_2$ (5 %) after passing through 0.2 μm PTFE membrane filter was supplied to the cells at a rate of 0.2 vvm. A continuous white fluorescent light (100 μE m$^{-2}$ s$^{-1}$) was supplied to the cells.

# Experimental

Two-phase cultivation system was adopted in this study. In the first phase, the cells density of 1.5–2.0 g L$^{-1}$ was achieved at the optimal growth conditions in a glass bubble-column photo-bioreactor. In phase-2, the cells in stationary phase were transferred to a nutrient-deprived medium in glass bubble-column photo-bioreactor (according to the experimental design). Afterward, the cells were harvested by centrifugation at 3000 rpm for 5 min, and separated from the spent medium. The collected cells were washed several times with sterilized distilled water for lipids extraction.

## Analyses

### Biomass concentration

A correlation between growth rate and cell density (OD$_{680}$) was developed. The optical density of the cells was measured regularly at 680 nm using UV/VIS spectrophotometer (DR-4000U, Hach, USA). For conversion of OD$_{680}$ values into biomass, calibration curve between OD$_{680}$ and dry cell weight was used by determining the conversion factor (i.e., 0.428 OD$_{680}$ equals approximately 0.96 CDM g L$^{-1}$). The cell dry mass was (CDM) calculated by filtering the cells through acetate membrane filter (0.7 μm pore size, 47 mm in diameter, Whatman, UK) and drying in an oven at 80 °C for 12 hours.

### Lipid analysis

Total cellular lipids were extracted using 40 mL of suspended cells. These cells were harvested by means of centrifugation (3,000 rpm for 5 min). For the extraction of lipid from harvested cells, 7.6 mL mixture of chloroform/methanol/water (1/2/0.8, v/v/v) was used. Sonication of mixture was carried out at 100 W and 20 kHz for 1 minute (VCX 130, Sonics and Materials Inc., USA), and later the mixture was vortexed for 30 sec. In order to make the final ratio of chloroform/methanol/water: 1/1/0.9 (v/v/v), 2 mL of chloroform and water each were added to the above mixture, and vortexed again for 30 sec. This mixture was centrifuged again at 3,000 rpm for 5 min in order to fractionate the mixture into three layers. The uppermost layer of methanol was separated, while the bottom layer of chloroform containing lipid was collected in a new tube. The upper layer of methanol was extracted twice using the above process. The chloroform layers were combined and evaporated in a drying oven at
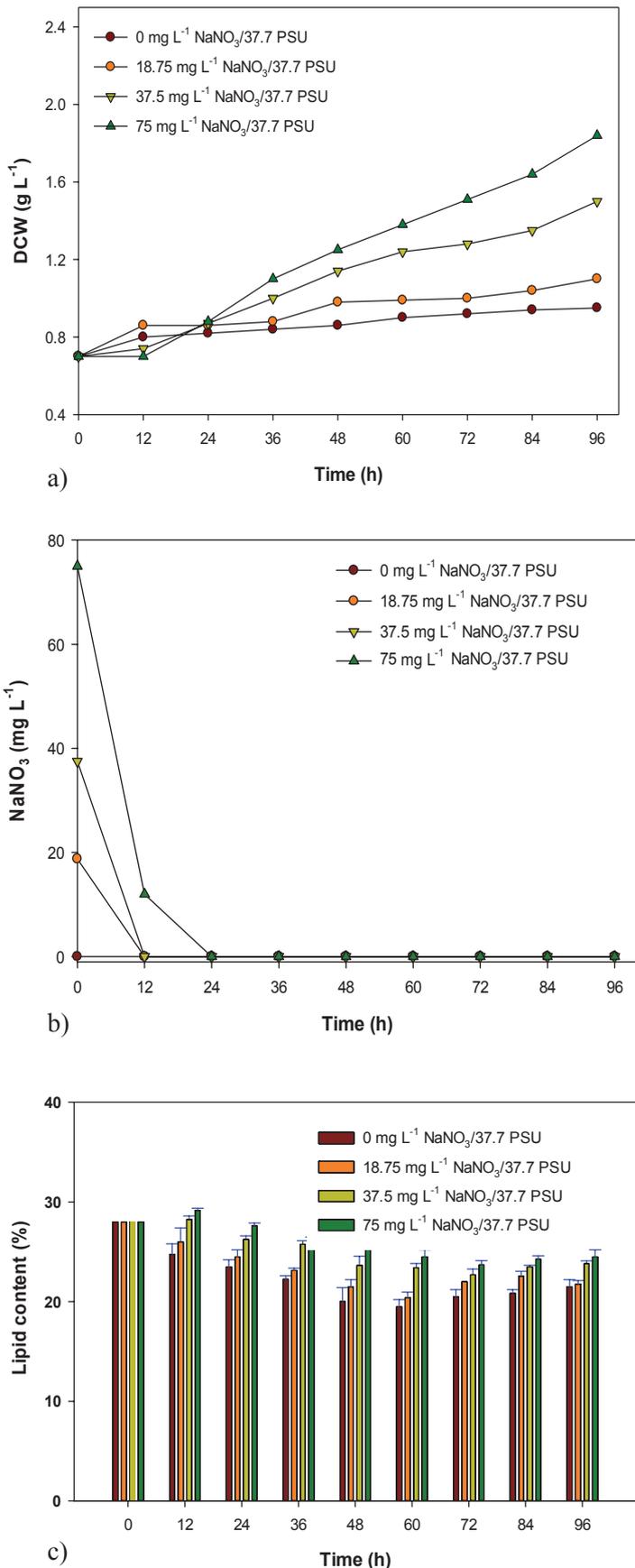
a)



b)



c)

Fig. 1 – *Effect of nitrogen variation on (a) cell dry mass, (b) nitrogen consumption, and (c) lipid content under normal salinity (37.7 PSU)*

80 °C for 24 h. The total lipid contents were expressed as the % of cell dry mass (CDM).

*Lipid productivity*

Lipid productivities were calculated from the initial cell dry mass and final cell dry mass from the equation used by Griffiths and Harrison:[11]

$$\frac{\left\{\left(CDM_t \cdot Lipid_t\right) - \left(CDM_{t_0} \cdot Lipid_{t_0}\right)\right\}}{\left(t - t_0\right)} \qquad (1)$$

where $CDM_t$ and $Lipid_t$ are the final CDM and lipid contents, respectively, while $CDM_{t_0}$ and $Lipid_{t_0}$ are initial CDM and lipid content, respectively. Similarly, $t$ and $t_0$ are final and initial time, respectively.

*Nitrate*

Standard method (APHA, 1995) of reading multi-wavelength UV absorbance was used for nitrate measurements.[12]

## Results and discussion

### Effect of nitrogen concentration

Inorganic nitrogen is an essential element for the growth of microalgae.[13] Microalgae use nitrogen for their metabolic activities. During nitrogen-rich conditions, the cells use nitrogen to develop biomass. However, under nitrogen stress, they change the metabolism from biomass development to lipid or carbohydrate production, and they fix carbon through photosynthesis.[14] In order to unravel the effect of nitrogen, *D. tertiolecta* was subjected to various concentrations of $NaNO_3$ at fixed level of salinity (37.7 PSU). The growth was linearly correlated with a change in initial concentration of $NaNO_3$ (Fig. 1a). The highest growth was achieved with 75 mg L$^{-1}$ of $NaNO_3$. The lowest biomass was observed when nitrogen was depleted from the medium. The growth of *Dunaliella* in the absence of nitrogen might be due to the release of internal nitrogen from the cells.[15]

The nitrogen uptake was observed in *D. tertiolecta* with time (Fig. 1b). Microalgae consumed all $NaNO_3$ within 12 hours, except for 75 mg L$^{-1}$ dose. The cells with this concentration also consumed most of the nitrogen in the first 12 hours; however, it took another 12 hours (24 in total) to consume all the nitrogen. Nitrogen is one of the most important nutrients affecting lipid metabolism in microalgae. It has been reported that several microalgal species increase the accumulation of lipids under nitrogen-deprived conditions.[14] However, Fig. 1c shows that nitrogen stress did not result in lipid accumulation in *D. tertiolecta*. The lipid content did not change much with time. Nevertheless, there was a
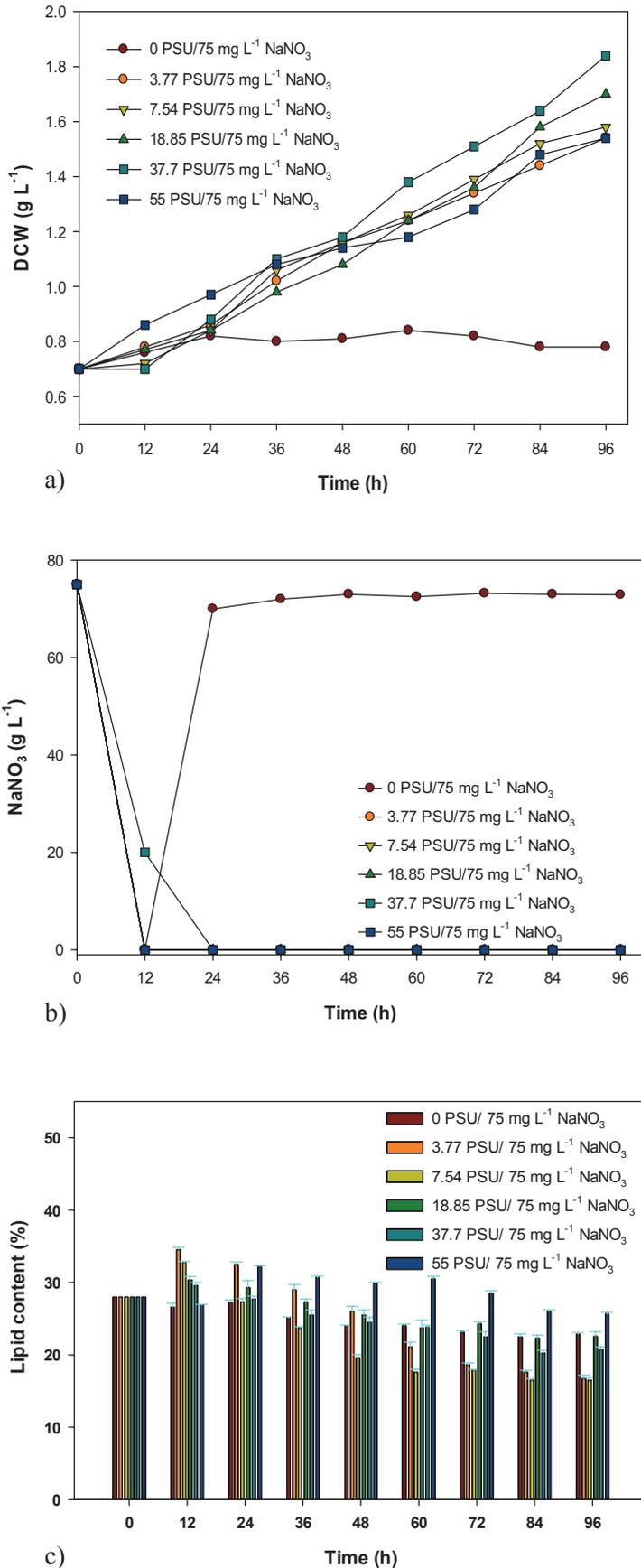
a)



b)



c)

F i g . 2 – *Effect of salinity variation on (a) cell dry mass, (b) nitrogen consumption, and (c) lipid content under 75 mg L$^{-1}$ of NaNO$_3$*

slight increase in lipid content after 12 hours at 75 mg L$^{-1}$ of NaNO$_3$. Both the lipid content (29 %) and lipid productivity (61 mg L$^{-1}$ d$^{-1}$) were maximal at 75 mg L$^{-1}$ of NaNO$_3$, which was the highest initial nitrate concentration used in this study. These results were supported by a previous study on *Dunaliella* where nitrogen stress alone did not result in lipid enhancement.[16] Thus, we investigated whether the combined effect of salinity and nitrogen might induce any positive influence on lipid production in *D. tertiolecta*.[14]

### Effect of salinity

The effect of different concentrations (0–55 PSU) of salinity was compared when the concentration of nitrogen was fixed at 75 mg L$^{-1}$ of NaNO$_3$. Fig. 2a shows that the decrease in biomass production was observed with decreasing salinity from 37.7 PSU. A negative impact of salinity was found above 37.3 PSU, the normal seawater salinity. It is pertinent to mention that the optimum level of salinity (37.7 PSU) was equivalent to the salinity level of one dose of the f/2 medium. The lipids production was also high at 37.7 PSU in the early stage of cultivation, but higher salinity resulted in better lipid production in a later stage, after 36 h (Fig. 2c).

It was observed that the salinity above or below the optimal range (37.7 PSU) posed a negative impact on the lipids synthesis. Similar results were found in a study on *Nannochloropsis* sp by Khatoon.[17] Jahnke and White observed that low salinity conditions changed *Dunaliella* to have enlarged cell size, decreased carbonate pool, and increased production of α-tocopherol and total glutathione.[18] Our results are in agreement with the previous studies on *Dunaliella* where increased salinity had resulted in slow growth.[17–22] In fact, increased salinity could inhibit photosynthesis in microalgae.[19,23] The mechanism of this inhibition is the destruction of repair cycle of photo-damaged PS-II via suppression of protein de novo synthesis, especially D1 protein of the PS II at high salinity.[25] Meanwhile, the absence of salt in the medium disabled the microalgae to grow and uptake nutrients.[23,6] The presence of salts in the medium is essential for marine microalga growth and lipid synthesis.

Fig. 2b shows that almost all of the nitrogen was consumed between 12–24 hours. The maximum lipid content was observed between 12~24 hours of cultivation (Fig. 2c). After the depletion of nitrogen from the medium, the lipid contents decreased slowly overall as time passed. It is noted that the salinity > 37.7 PSU tended to give increased lipid content than that in 37.7 PSU, which implied that extreme salinity conditions acted as a stress to enhance lipid synthesis. Salinity stress affects several physiological and biochemical mechanisms of
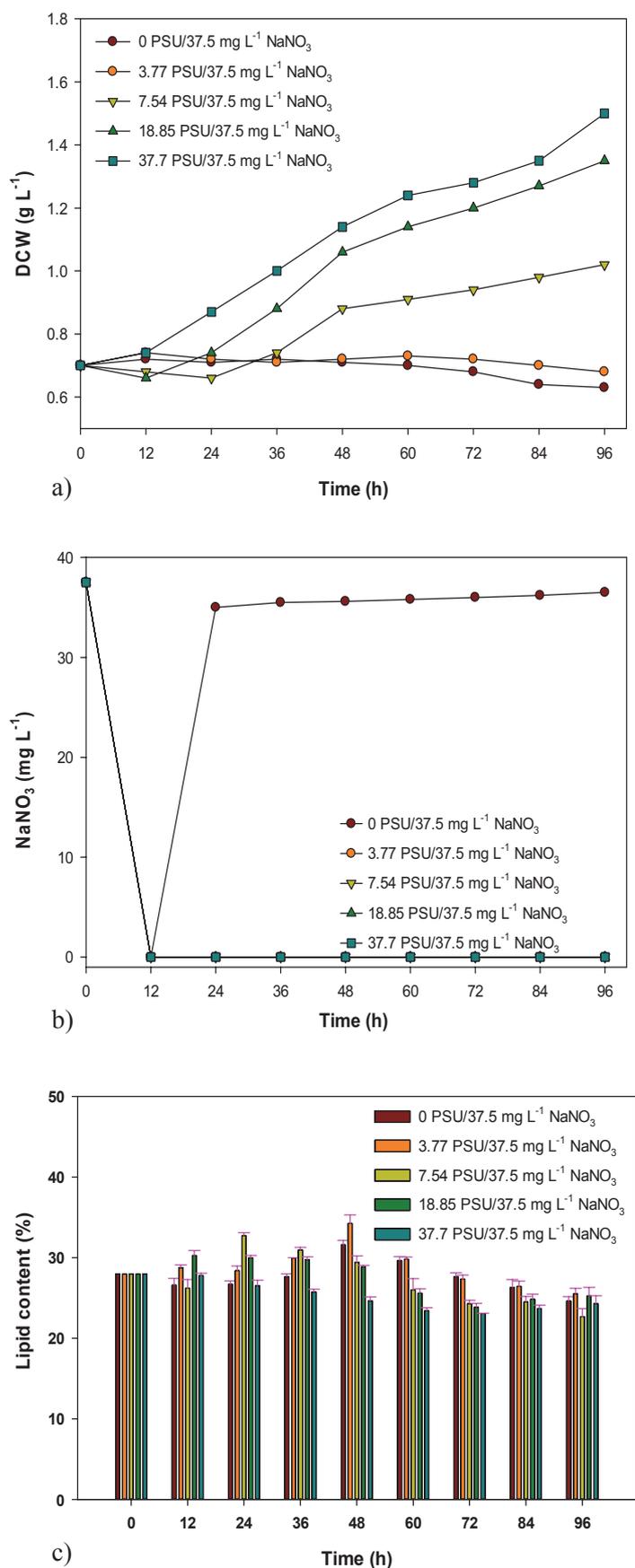
a)



b)



c)

Fig. 3 – *Effect of salinity variation and fixed nitrogen (37.5 mg L⁻¹) on (a) dry cell weight, (b) nitrogen consumption rate, (c) lipid content*

microalgae. Salinity stress also causes changes in the fatty acid metabolism of microalgae, which ultimately results in lipid enhancement.[26] It is also known that a high salinity can induce saturation of fatty acid, which can change the properties of produced biofuels.[27,28]

## Interactive effect of salinity and nitrogen

Fig. 3 shows the effect of salinity on biomass, and lipids contents by adjusting the nitrogen level at 37.5 mg L⁻¹. The biomass increased with an increase in salinity (Fig. 3a) while at low salinity (3.77 PSU), the biomass initially decreased. However, in the absence of salts, biomasses gradually decreased and the cells died. Marine microalgae grow best at salinity equals to marine water. Therefore, any change in salinity level negatively affects microalgal growth. Our results are supported by previous studies on marine microalgae where low salinity level adversely affects growth.[17,18] The maximum biomass was achieved at the highest salinity level of 37.7 PSU. However, this increase in biomass was lower compared to the previous experiment which was performed under salinity variation alone. This shows that this concentration of NaNO₃ was not optimum for normal cell growth.

Fig. 3b shows that, under all salinity conditions, nitrogen was consumed within 12 hours. However, due to the cell death in the medium without salts, nitrogen was released back into the medium. The same was the case when salinity variation experiment was performed alone, which shows that the presence of the optimum level of salt in the medium is necessary for cell survival. The presence of salts in the medium is essential for nutrients uptake and cells survival.[23,26]

Fig. 3c shows that the lowest salinity, i.e., 3.77 PSU, and intermediate level of NaNO₃ (37.5 mg L⁻¹) resulted in the maximum lipid content, i.e., 34 %. Although lipid content slightly increased in our previous experiment at the lowest salinity level (3.77 PSU) and N-replete conditions, an increase in lipid content was higher in this case. These results show that the combined salinity and nitrogen stress was essential for lipid enhancement in *Dunaliella tertiolecta*. Our results are supported by Dan Cheng *et al.* study, which also suggested that combined application of multiple stress factors could be more effective in improving biofuel yield than the application of single stress factor.[14] Lipid content increased in our previous experiment of salinity stress in the presence of an intermediate level of NaNO₃ (37.5 mg L⁻¹). Previous results revealed that lowering the concentration of NaNO₃ governs high lipid productivity. With this in mind, further experiments were carried out in which NaNO₃ concentration was
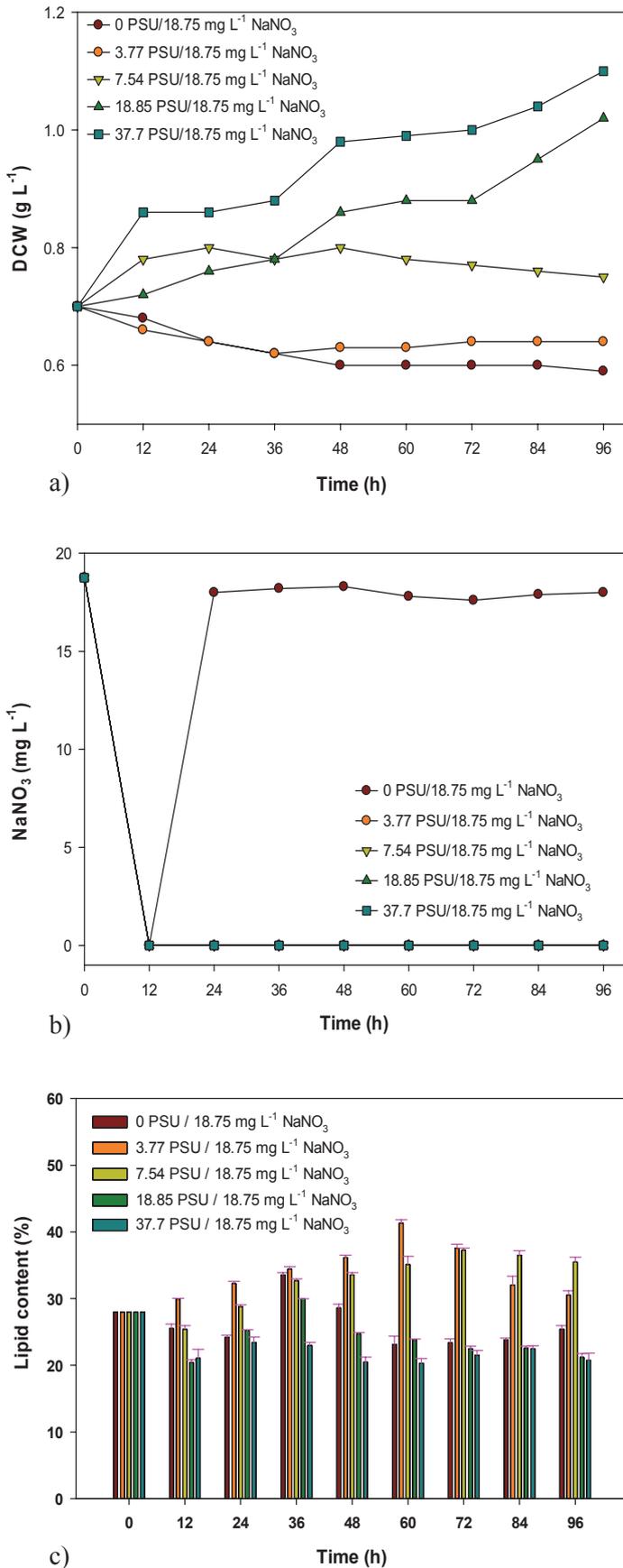
a)



b)



c)

Fig. 4 – *Effect of salinity variation and fixed nitrogen (18.75 mg L⁻¹) on (a) dry cell weight, (b) nitrogen consumption rate, (c) lipid content*

further reduced to 18.85 mg L$^{-1}$; varied concentrations of salinity were employed. Fig. 4a shows that biomass in all cultures increases except for the cultures without salt and with the lowest salinity, i.e., 3.77 PSU. However, due to the presence of low nitrogen level in all cultures, biomass increase was less than that intermediate fixed levels, 37.5 mg L$^{-1}$ and high 75 mg L$^{-1}$ of NaNO$_3$. This was due to an insufficient concentration of NaNO$_3$ in the medium. Several studies on marine microalgae had shown that low salinity resulted in reduced cell growth.[17,23] Jahnke *et al.* also reported a decrease in marine microalga growth at low salinity level.[18]

Fig. 4b shows the nitrogen consumption in salinity stress experiment. All the cultures completely consumed nitrogen within 12 hours. However, the culture without salts released nitrogen back into the culture medium due to cell death. Similar results were reported by Hellebust *et al.*[23] In their study, microalgae did not grow at low salinity, while in the absence of salts they showed low nutrient uptake. As for marine microalgae, salinity is one of the most important factors affecting their growth, lipid content, and biochemical composition.[15,16]

Fig. 4c shows lipid content increase in the cultures having low salinity, i.e., 3.77 PSU and 7.54 PSU. The maximum lipid content of 42 % was achieved at 3.77 PSU of salinity. The lipid contents under this condition were the highest compared to previous salinity variation experiments, which were performed under N-replete condition. This shows that at low salinity level and low N in the medium, lipid content can be enhanced. Our results are in agreement with the findings of Pal *et al.*, where lower salinity improved lipid production at high light and nitrogen stress conditions.[15] Yao *et al.* also found starch accumulation enhanced in the presence of nitrogen stress and low salinity.[29] It appeared that salinity alone cannot be considered as a manipulation factor for enhanced lipid accumulation in *D. tertiolecta*. The next salinity variation experiment was performed in the absence of nitrogen. There was no significant increase in biomass and lipid content at all salinities in the absence of nitrogen (data not shown). Adams *et al.* also reported that only a certain degree of nitrogen stress on microalgae could result in lipid accumulation.[30] It shows that this specie prefers low salinity and low nitrogen level for lipid enhancement.

## Comparison of lipid productivity

Experimental results in Figs. 1 and 2 show that *D. tertiolecta* grew well in normal seawater salinity and under high nitrate conditions. Cellular lipid content enhanced under high nitrate conditions and at high salinity conditions. Since lipid content devi-
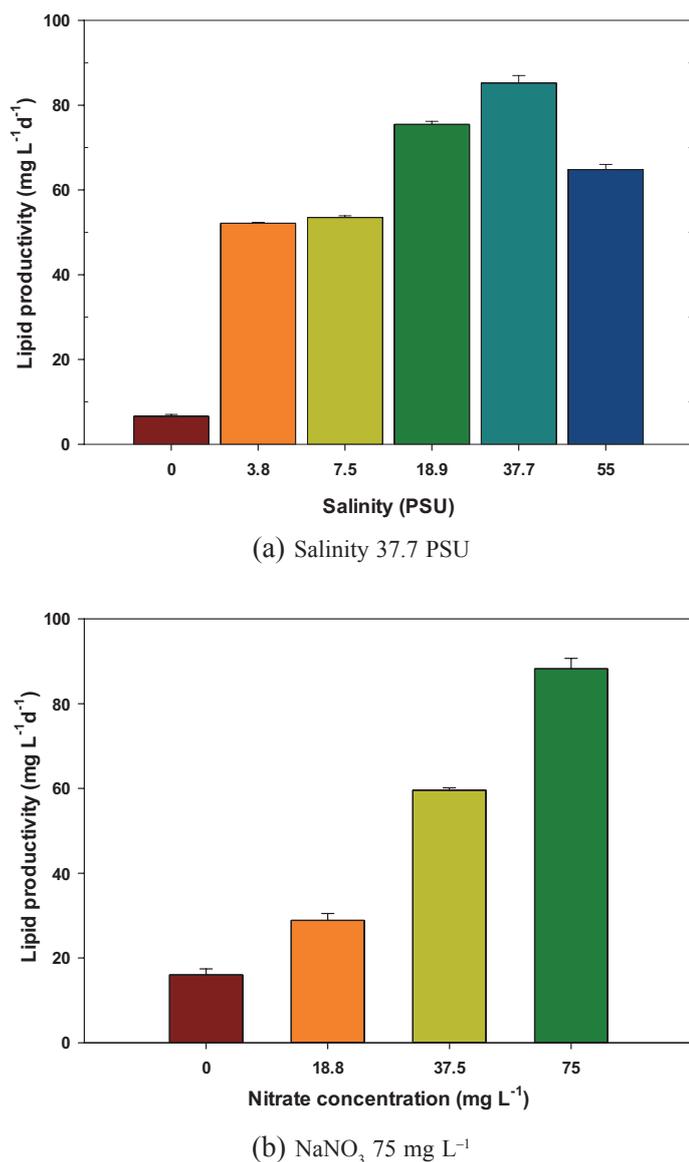
(a) Salinity 37.7 PSU



(b) NaNO₃ 75 mg L⁻¹

F i g . 5 – *Comparison of lipid productivity depending upon different nitrate concentrations and salinity conditions*

ation was not largely dependent upon salinity or nitrate variations, it was not easy to deduce an optimal condition for lipid production directly from Figs. 1 and 2. Therefore, lipid productivity was calculated based upon the average cell growth rates and cellular lipid content. Lipid productivity (as mg $L^{-1}$ $d^{-1}$) can be calculated from the product of cell growth rate (as mg $L^{-1}$ $d^{-1}$) and lipid content (as w/w %).

Table 1 (as supplementary material) shows the cell growth rates, cellular lipid contents, and the estimated lipid productivity values at different salinity and nitrate concentrations. Comparisons of the resulting lipid productivity were made in Fig. 5. Cell growth rate and cellular lipid content were enhanced when initial nitrate concentration was increased.

The same was valid when the salinity was increased up to 37.7 PSU. It is noted that, although lipid content was the highest at high salinity conditions (55 PSU), lipid productivity was best at normal salinity 37.7 PSU, because cell growth rate was reduced at the high salinity condition.

In the presence of 75 mg $L^{-1}$ of NaNO₃ lipid productivity was the maximum. As the concentration of NaNO₃ was decreased in the medium, it also resulted in a decrease in lipid productivity. In case of salinity variation, 3.77 PSU showed the maximum lipid productivity (140 mg $L^{-1}$ $d^{-1}$). Salinity variation under intermediate nitrogen level resulted in the maximum lipid productivity of 54.1 mg $L^{-1}$ $d^{-1}$ in the presence of 18.85 PSU. While salinity variation in the presence of low nitrogen returned the maximum lipid productivity of 38.33 mg $L^{-1}$ $d^{-1}$ in the presence of 7.54 PSU. The lipid productivity of *Dunaliella tertiolecta* under different nitrogen and salinity variations was better than the lipid productivity of most of the 30 microalgal strains studied by Rodolfi *et al.*[30] Similarly, lipid productivity of our specie was better than that of *Dunaliella* sp as reported by Mata *et al.*[32]

## Conclusions

Nitrogen starvation is popularly used to induce lipid synthesis in many microalgae species; however, the present marine microalga *D. tertiolecta* was an exception, showing reduced lipid content under low nitrogen concentrations. Lipid content did not increase even when all nitrogen was depleted due to the consumption by cell growth. Lipid synthesis was induced under high salinity stress; meanwhile, cell growth was the best at normal seawater salinity. The optimized conditions for the highest lipid productivity was 75 mg $L^{-1}$ $d^{-1}$ of NaNO₃, which are the normal nitrogen level of f/2 medium and normal seawater salinity.

## S u p p l e m e n t a r y   M a t e r i a l s

T a b l e   1 – *Effect of nitrogen variation on biomass, lipid content, and lipid productivity*

| NaNO₃ (mg $L^{-1}$) | Maximum biomass (g $L^{-1}$) | Maximum lipid content (%) | Maximum lipid productivity (mg $L^{-1}$ $d^{-1}$) |
|---|---|---|---|
| 75 | 1.84 | 29 | 61.4 |
| 37.7 | 1.5 | 28 | 40.5 |
| 18.75 | 1.1 | 25 | 38 |
| 0 | 0.95 | 24 | 0.88 |

Table 2 – *Effect of salinity variation on biomass, lipid content, and lipid productivity*

| Salinity PSU | Maximum biomass (g L$^{-1}$) | Maximum lipid content (%) | Maximum lipid productivity (mg L$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| 55 | 1.54 | 32 | 114.1 |
| 37.7 | 1.84 | 29 | 52.7 |
| 18.85 | 1.7 | 30 | 70 |
| 7.54 | 1.58 | 32.5 | 76 |
| 3.77 | 1.54 | 34 | 140 |
| 0 | 0.78 | 27 | 25.4 |

Table 3 – *Effect of salinity variation and intermediate nitrogen (37.5 mg L$^{-1}$) on biomass, lipid content, and lipid productivity*

| Salinity PSU | Maximum biomass (g L$^{-1}$) | Maximum lipid content (%) | Maximum lipid productivity (mg L$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| 37.7 | 1.5 | 28 | 40.5 |
| 18.85 | 1.35 | 30 | 54.1 |
| 7.54 | 1.02 | 32 | 29.16 |
| 3.77 | 0.68 | 33 | 22.6 |
| 0 | 0.63 | 31 | 12.7 |

Table 4 – *Effect of salinity variation and low nitrogen (18.75 mg L$^{-1}$) on biomass, lipid content, and lipid productivity*

| Salinity PSU | Maximum biomass (g L$^{-1}$) | Maximum lipid content (%) | Maximum lipid productivity (mg L$^{-1}$ d$^{-1}$) |
|---|---|---|---|
| 37.7 | 1.1 | 25 | 10.40 |
| 18.85 | 1.02 | 30 | 24.81 |
| 7.54 | 0.75 | 37 | 38.33 |
| 3.77 | 0.64 | 42 | 26.68 |
| 0 | 0.59 | 33 | 6.9 |

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