The Combined Influences of Salinity, Light and Nitrogen Limitation on The Growth and Biochemical Composition of *Nannochloropsis* sp. and *Tetraselmis* sp

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Abstract:

In the present study, two microalgae species "*Nannochloropsis* sp. and *Tetraselmis* sp." isolated from Penang National Park coastal waters, Malaysia; were cultivated under combined various laboratory conditions "salinity, light, nitrogen limitation and starvation". Growth rate, dry weight, chlorophyll *a* content, total lipid and protein contents, were estimated at mid exponential growth phase. Both *Nannochloropsis* sp. and *Tetraselmis* sp. showed remarkable decrease in growth rate, chlorophyll *a* content and protein content companied with increase in lipid content under nitrogen limitation and starvation conditions. Maintaining *Nannochloropsis* sp. under salinity 15 ‰ caused only significant decrease in total protein content; while *Tetraselmis* sp. grown at the same salinity caused decrease in the growth rate, chlorophyll *a*, dry weight and total protein content only when nitrogen was available.

Keywords:
Biochemical composition; light; Microalgae; nitrogen limitation; salinity.

I. Introduction:

NANNOCHLOROPSIS sp. and *Tetraselmis* sp. are considered amongst the high nutritional value marine microalgae, which have been widely used as a food supply in the aquaculture industry for hatchery grown herbivores such as larval and juvenile bivalves [1]. Either in the laboratory or in nature, phytoplankton are exposed to lots of factors such as temperature, light, salinity and nutritional factors, that definitely influence the growth, physiological activities and biochemical composition. Light, salinity and nutrition limitation are among the most important factors that affect the growth and metabolism of phytoplankton [2]. Previous studies investigated the independent influence of salinity [2], light period [3], nutrition limitation [4] on the growth and biochemical composition of the phytoplankton. Responses of phytoplankton to the influence of each of these factors independently might be in a different way, if they were exposed to the combined impact of all these factors at the same time.

The aim of the present study is to understand the effect of overlapping of some physical factors (salinity 29 ‰ and 15 ‰; light period, 24 h light and 12: 12 h light:dark cycle), as well as nitrogen limitation and starvation conditions, on the growth, dry weight, chlorophyll *a* content, total lipid content and total protein content of the investigated species.

II. Materials and methods:

A. Culturing:

Nannochloropsis sp. and *Tetraselmis* sp. isolated from Penang National Park coastal waters, Malaysia; were grown in batch cultures, conical flasks 250 ml, each containing 150 ml working solution. Natural filtered autoclaved sea water, supplied with F/2 medium [5] was used as a culture medium. For nitrogen-free media (0.0% nitrogen starvation), media were prepared by omitting $NaNO_3$ from the original formula. For nitrogen limitation, the concentration of the $NaNO_3$ was reduced to 10%. Other media were prepared as described in the original formula (100%), [5]. Two folded of triplicates from each nitrogen concentrations implemented under different salinity, 29 ‰ (natural sea water) and 15 ‰ (natural filtered sea water diluted with distilled water and adjusted at salinity 15 ‰), and maintained under two light regimes, 24 h light and 12:12 h light:dark cycle.

B. Growth rate, Chlorophyll a content and Dry weight:

Growth was daily monitored by cell counting, using Bright lined improved Neubauer Haemocytometer (Germany), and after Lugol solution was added. Growth rate calculated according to the equation:

$\mu = \ln (F1 - F0)/t1 - t0$.

Where F1 is the number of cells at *t1* (time1), *F0* is the number of cells at *t0* (time0).

Aliquots were collected when cultures reached the mid exponential growth phase, centrifuged at 3000 rpm for 15 min; the pellet resuspended in distilled water and recentrifuged as described. For extraction of chlorophyll *a*, acetone 90% was added to the pellet, kept in dark overnight at 2±1˚C, chlorophyll *a* estimated according to Jeffery and Humphrey [6]. The pellet dried in an oven at 105±2˚C, and weighted for the dry weight.

C. Biochemical composition:

For total protein content, freeze-dried (lyophilized) and weighed samples were suspended in 0.5 M NaOH and incubated in a water bath at 100±1˚C for 30 minutes with occasional stirring, protein content was measured following the Lowery method [7]. Bovine Serum Albumin (BSA) stock solution (1mg/mL) was used as a standard solution. Total lipid content was extracted in methanol: chloroform:water (10:5:4), and estimated gravimetrically according to Blight and Dyer [8].

D. Statistical analysis:

Data were statistically analyzed using factorial ANOVA, following the general linear model procedure by SPSS, 20. Simple main effects analysis followed by Tukey multiple comparison test (Tukey's HSD, honestly significant difference test), were utilized when appropriate as post-hoc procedures to follow up on significant main effect and interactions. A $p < 0.05$ value was considered significant.

III. Results:

The results obtained for *Nannochloropsis* sp. and *Tetraselmis* sp. are summarized in Tables 1 and 2 respectively. The highest growth rate, chlorophyll *a* content and dry weigh, were recorded when both *Nannochloropsis* sp. and *Tetraselmis* sp. maintained under 24 h light regime in complete nitrogen supplemented media (as in the original formula recipe), at salinity 29 ‰ for *Tetraselmis* sp. (Table 2), and at both salinity levels for *Nannochloropsis* sp. (Table 1). Growth rate of both species, significantly increased when cultures maintained at 24 h light regime compared to 12:12 L:D cycle when nitrogen was supplemented in adequate amount. Regardless of salinity level and light duration that cultures were grown under, significantly increase in total lipid content was observed when the tested species maintained under nitrogen limitation and starvation conditions, as well as when the cultures grown under 12:12 light:dark regime (Tables 1, 2). Growth rate, chlorophyll *a* content and dry weight were remarkably decreased when *Tetraselmis* sp. grown under salinity 15 ‰, only in nitrogen-replete media (Table 2). *Nannochloropsis* sp. showed higher total lipid content (ranged 19.4-24.34%) (Table 1) comparable with *Tetraselmis* sp. (ranged 15.33-19.51%) (Table 2), when both of the species grown under nitrogen limitation and starvation conditions, while no significant differences in total lipid content was recorded once the species maintained under nitrogen-replete conditions. Total protein content was always higher under nitrogen-replete conditions, and drastically decreased under nitrogen depletion conditions. Protein also increased as salinity increased from 15 ‰ to 29 ‰, this was more pronounced under nitrogen-replete conditions (Tables 1, 2).

Table (1). *Nannochloropsis* sp. Growth rate, Chlorophyll *a* Content, Dry Weight, Total Lipid Content and Total Protein Content Under Different Culture Conditions

Factors		Growth rate Division/day	Chlorophyll *a* (μg/ml)	Dry weight (mg/l)	Lipid (%)	Protein (%)
24 light regime,	salinity 29 ‰					
	nitrogen 0.0%	0.18 ± 0.03	0.73 ± 0.04	62 ± 4.1	22.51 ± 1.4	27.33 ± 1.7
	10%	0.27 ± 0.01	1.4 ± 0.04	86 ± 3.1	24.34 ± 1.1	32.77 ± 1.3
	100%	0.91 ± 0.02	3.88 ± 0.03	298 ± 5	10.45 ± 0.5	44.56 ± 1.5
	salinity 15 ‰					
	nitrogen 0.0%	0.16 ± 0.03	0.70 ± 0.06	58.3 ± 3.3	19.4 ± 2.2	23.13 ± 0.8
	10%	0.27 ± 0.03	1.61 ± 0.01	74 ± 4.6	22.96 ± 1.9	29.65 ± 1.3
	100%	0.88 ± 0.01	3.51 ± 0.05	281 ± 4.3	12.10 ± 0.9	36.82 ± 0.9
12:12 L:D cycle.	salinity 29 ‰					
	nitrogen 0.0%	0.16 ± 0.02	0.70± 0.05	59 ± 1.7	20.81 ± 1.6	25.94± 1.4
	10%	0.23 ± 0.03	1.36 ± 0.09	88.6 ± 3.3	19.69 ± 1.7	32.91± 0.7
	100%	0.58 ± 0.04	2.12 ± 0.09	211 ± 4.6	14.17 ± 0.7	42.7 ± 1.6
	salinity 15 ‰					
	nitrogen 0.0%	0.19 ± 0.01	0.68 ± 0.03	56.6 ± 2.2	23.61 ± 3	25.15 ± 1.1
	10%	0.25 ± 0.02	1.19 ± 0.05	78.66 ± 5.2	20.08 ± 1.1	27.11 ± 0.8
	100%	0.61 ± 0.06	1.56 ± 0.02	198.6 ± 4.3	14.97 ± 0.3	34.21 ± 0.8

± Standard error values

Table (2). *Tetraselmis* sp. Growth rate, Chlorophyll *a* Content, Dry Weight, Total Lipid Content and Total Protein Content Under Different Culture Conditions

Factors		Growth rate Division/day	Chlorophyll a (μg/ml)	Dry weight (mg/l)	Lipid (%)	Protein (%)
24 light regime.	salinity 29 ‰					
	nitrogen 0.0%	0.13 ± 0.01	0.63 ± 0.04	62 ± 4.1	19.51 ± 1.4	23.93 ± 0.7
	10%	0.29 ± 0.01	1.4 ± 0.04	86 ± 3.1	16.34 ± 1.1	26.86 ± 1.1
	100%	0.86 ± 0.02	3.18 ± 0.03	228 ± 5	11.29 ± 0.5	42.82 ± 0.9
	salinity 15 ‰					
	nitrogen 0.0%	0.16 ± 0.02	0.63 ± 0.03	58.4 ± 4.3	17.97 ± 0.7	23.2 ± 1.4
	10%	0.26 ± 0.01	1.34 ± 0.01	77.6 ± 4.3	15.33 ± 0.9	27.5 ± 1.2
	100%	0.57 ± 0.01	2.62 ± 0.03	168.6 ± 5.5	10.37 ± 0.3	36.3 ± 1.5
12:12 L:D cycle.	salinity 29 ‰					
	nitrogen 0.0%	0.13 ± 0.02	0.70± 0.05	59 ± 1.7	18.81 ± 1.6	21.24± 0.9
	10%	0.27 ± 0.03	1.36 ± 0.09	88.6 ± 3.3	18.69 ± 1.7	25.99± 0.9
	100%	0.68 ± 0.04	2.92 ± 0.09	201 ± 4.6	13.17 ± 0.7	40.7 ± 1.2
	salinity 15 ‰					
	nitrogen 0.0%	0.19 ± 0.01	0.61 ± 0.02	57.3 ± 4.1	16.2 ± 0.92	23.9 ± 0.7
	10%	0.23 ± 0.02	1.22 ± 0.03	82.6 ± 3.5	17.89 ± 0.6	22.5 ± 0.8
	100%	0.44 ± 0.03	2.24 ± 0.01	140.7 ± 4	14.11 ± 0.8	38.14 ±0.6

± Standard error values

IV. Discussion:

Although *Tetraselmis* sp. growth rate dramatically decreased under salinity 15 ‰, no alteration in its biochemical composition was recorded; it was reported that microalgae can tolerate a wide range of salinities [9]. It has been reported that *Tetraselmis* sp. achieved the highest growth and cell density at salinity ranging 20-35‰ [1]. As mentioned in the results, *Tetraselmis* sp. and *Nannochloropsis* sp. growth rate, dry weight and chlorophyll *a* content were obviously decreased when cells maintained under 12:12 L:D cycle, only under nitrogen-replete conditions, while the total lipid content significantly increased. According to Fábregas et al. [10] lipid suffered a strong increase between the last hour of the dark and the first hour of the light and then increased continuously during the 12 h of light, where the highest lipid content where achieved. Previous study [11] has shown increase in PUFA content (weight %), once the diatom *Thalassiosira pseudonana* grown under 12:12 l:d light regime, compared with those grown under 24 h light regime. Under nitrogen limitation and starvation conditions, *Tetraselmis* sp. and *Nannochloropsis* sp. growth rate, dry weight and chlorophyll *a* content drastically decreased, while the total lipid content significantly increased, either under 24 h light or 12:12 l:d regime. In addition to the well known importance of the nitrogen as macronutrient, the decreasing in the N/P ratio will result in decreasing in the yield biomass, consequently chlorophyll *a* content and the dry weight will be decreased. It has been reported that, *Tetraselmis* sp. growth, was determined once the N/P ratio becomes below 20, as the lipid content decreased with increasing the N/P ratio above 20 [12]. The recorded increase in total lipid content under nitrogen limitation and starvation conditions, may attributed to the accumulation of lipid as the growth slows down because of the shortage in nitrogen, and there is no requirement for the synthesis of new membrane compounds; increasing in total lipid content of different microalgae species under nitrogen limitation and starvation conditions was reported [13-15]. The increasing in the storage products (lipids and carbohydrates), under nitrogen limitation conditions has been reported [4]. It has been also reported that microalgae lipid content may increase up to two-folded, under nutrient limitation conditions, when growth slows down and there no requirement for the synthesis of new membrane compounds [4].

Conclusion: Among the studied factors, nitrogen had the major effect on the tested species, fluctuation in salinity and light duration had no significant impact under nitrogen limitation and starvation conditions, since the growth and biochemical composition are being controlled by nitrogen availability. *Nannochloropsis* sp. and *Tetraselmis* sp. showed different responses to the fluctuation in salinity, *Nannochloropsis* sp. can tolerate changes in salinity, with no significant difference in the measured parameters.

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