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INTERACTIVE EFFECT OF AMMONIA AND NITRATE ON THE NITROGEN UPTAKE BY *Nannochloropsis* sp.

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Abstract: Microalgae are able to assimilate various types of nitrogen. The interaction of nitrogen on their removal by microalgae should be of great concern when microalgae are used as a biological treatment in waste water. This study aims to reveal the preference of nitrogenous compounds by *Nannochloropsis* sp. The microalgae were cultivated in 900 µM nitrate, 900 µM ammonium, and 900 µM nitrate plus 900 µM ammonium. *Nannochloropsis* sp. preferred ammonium rather than nitrate when both compounds were available to the microalgae. Nitrate was utilised in the absence of ammonium. The uptake rate of ammonium was significantly higher than nitrate ($p = 0.004$). There was significant difference in the growth rate of the microalgal with different nitrogen sources ($p < 0.000$). *Nannochloropsis* sp. grew faster in treatment containing ammonia. However, there were no significant difference in the maximum cell density produced by the cultures in different nitrogenous nutrients ($p = 0.224$). *Nannochloropsis* sp. can be used as a biological treatment for waste water. The microalgae will first remove ammonium and remediate ammonia toxicity to most fish. Once ammonium has been removed or brought down to a safer level, nitrate can be removed by the microalgae.

KEYWORDS: Ammonium, *Nannochloropsis* sp., nitrate, removal, waste water

Introduction

Effluents from aquaculture are rich in solids and dissolved nutrients. The nutrients are mainly in the form of inorganic nitrogen and phosphorus (Pillay, 1990). Effluents from aquaculture industry should be treated before discharge into their adjacent aquatic ecosystems. Excessive nutrients discharged into the environment will lead to eutrophication and contamination (Tovar *et al*., 2000a; Tovar *et al*., 2000b; Holmer *et al*., 2002; Peuhkuri, 2002). The composition of nitrogenous compounds in aquaculture water may be varied depending on fish stock, feed, feeding regime, culture system, and water-quality management (Hargreaves, 1998; Nielsen *et al*., 1999; Engin and Carter, 2001; Perera *et al*., 2005; Rafiee and Saad, 2005; Mazon *et al*., 2007; Merino *et al*., 2007; Ahmed *et al*., 2008; Tng *et al*., 2008). Ammonia is the principle nitrogenous waste excreted by fish (Morii *et al*., 1978). In addition to ammonia, nitrite, nitrate and urea are other nitrogenous compounds that are usually found in aquaculture water. Fish could also excrete metabolic nitrogen as urea (Gregory, 1977; Wright and Land, 1998; Kajimura *et al*., 2002). Urea can be hydrolysed into ammonia (Allison and Prosser, 1991) while ammonia can be converted into nitrite and nitrate through nitrification (Abraham *et al*., 2004).

Microalgae are able to assimilate nitrogen from a variety of sources (Paasche and Kristiansen, 1982; Queguiner *et al*., 1986; Lund, 1987; Dortch, 1990; Cochlan and Harrison, 1991; Page *et al*., 1999). Ammonia, nitrite, nitrate and many dissolved organic nitrogens (urea, free amino-acids and peptides) are regarded as the main nitrogen sources for microalgae (Abe *et al*., 2002; Soletto *et al*., 2005; Converti *et al*., 2006). Microalgae have been widely used for nutrient removal in waste water (Hammouda *et al*., 1995; Craggs *et al*., 1997; Hoffmann, 1998; Olguin, 2003; Borges *et al*., 2005). They are considered to be one of the most efficient,

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environmentally-friendly, relatively low-cost and simple treatments compared to other physical and chemical treatments.

Nannochloropsis sp. is an important food source and raw material for feed formulation in aquaculture, especially for live-food organisms such as rotifers and copepods (Suchar and Chigbu, 2006; Milione and Zeng, 2007). It is commonly cultivated to create a ''green-water effect'' in fish larvae tanks. The nutritional value of *Nannochloropsis* sp. makes it well-appreciated in aquaculture (Lubian *et al*., 2000; Krienitz and Wirth, 2006). *Nannochloropsis* sp. is a potential source of EPA due to its high content of eicosapentaenoic acid (EPA, C20:5n3) (Sukenik, 1991; Volkman *et al*., 1993; Krienitz and Wirth, 2006). Besides, *Nannochloropsis* sp. is recognised as a source of commercially-valuable pigments. The interest is related to its availability of a range of pigments such as zeaxanthin, canthaxanthin and astaxanthin, each with high production levels (Lubian *et al*., 2000).

The interaction of nitrogen sources on nitrogen assimilation by microalgae varies among species and is strongly influenced by the environmental condition (Thacker and Syrett, 1972; Admiraal *et al*., 1987; Lund, 1987; Dortch, 1990; Harrison *et al*., 1990; Flynn *et al*., 1997; Yin *et al*., 1998; Page *et al*., 1999; Soletto *et al*., 2005; Converti *et al*., 2006). In order to apply *Nannochloropsis* sp. for nitrogen removal in aquaculture water, understanding on the growth and nitrogen uptake by the microalgae are essential. The uptake of ammonia and nitrate by *Nannochloropsis* sp. was assessed since ammonia is toxic to most aquatic organisms and nitrate is the end product of nitrification. Also, the interaction between ammonia and nitrate on nitrogen uptake by *Nannochloropsis* sp. was determined. The cultivation of *Nannochloropsis* sp. on aquaculture water offers the combined advantages of removing nutrients and, at the same time, produces biomass which can be further exploited as live feed and valuable biochemical source as raw materials.

Material and methods

Nannochloropsis sp. was obtained from marine microalgae stock culture in Universiti Malaysia Terengganu, Malaysia. Natural-filtered (Whatman Protran Nitrocellulose membrane filter, pore sizes 0.45 µm) and pasteurised (95 °C for one hour**)** sea water enriched with Guillard f/2 medium (Smith *et al*., 1993) was used for stock cultures. *Nannochloropsis* sp. was cultivated in 2 L Erlenmeyer flask with continuous illumination under a constant light intensity of 100 µmol m^{-2} s^{-1} from cool-white fluorescent light. The culture was maintained under constant environment at parameters 28 °C, salinity 30 ppt, and pH 8.

An aliquot of exponentially-growing *Nannochloropsis* sp. culture was harvested by centrifugation at 3000 rpm for 15 minutes. Cells densities were determined by spectrophotometer (UV-160, SHIMADZU) at 600 nm. The cells were washed twice with pasteurised sea water and re-suspended in the pasteurised sea water. A selected volume of the concentrated *Nannochloropsis* sp. cell suspension was thoroughly mixed with the f/2 medium with different nitrogen sources. The treatments were applied as ammonia (900 µM of NH_4^+-N), nitrate (900 µM of NO_3^--N), and ammonia plus nitrate (900 µM of NH_4^+-N and 900 µM of NO_3^--N). Sodium nitrate, $NaNO_3$, used in f/2 medium was replaced by ammonium chloride, NH_4Cl as nitrogen source for the microalgae. Experiment was conducted in triplicates under standard culture conditions with an initial cell density of 1 x 10^6 cell/ml. Guillard's f/2 medium with different nitrogen sources but without cell inoculation was used as control.

At each sampling time, 40 ml of water sample was taken for analysis. Cell density was determined spectrophotometrically at 600 nm. Water sample was then centrifuged at 3000 rpm for 15 minutes and the clear supernatant was used to determine nitrogen concentration. Determination of ammonia in water sample was conducted following Parsons *et al*. (1984). Briefly, water sample in triplicates were treated in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside and measured spectrophotometrically at 640 nm.

The method described by Collos *et al*. (1999) was used for the rapid measurement of micromolar concentrations of nitrate. The water sample absorbance was measured at 220 nm in a 1 cm quartz cuvette by using spectrophotometer (UV-160, SHIMADZU). The pH value was recorded with a pH meter (WTW SERIES) and maintained at pH 8 – pH 10 with 0.01 M sterilised HCl.

Growth rate of *Nannochloropsis* sp., (cell/ml/day) was calculated by the equation:

$$Growth\ rate = \frac{(C_x - C_0)}{t_x}$$

where C_x (cell ml^{-1}) is the cell density obtained at the time t_x (days) and C_0 (cell ml^{-1}) is the inoculums density.

Uptake rate of nitrogen, U_N (µM N/cell/day) in any form of ammonia or nitrate was calculated by the equation:

$$U_N = \frac{(N_0 - N_x)}{t_x}$$

where N_0 (µM N/cell) is the initial nitrogen concentration and N_x (µM N $cell^{-1}$) is the nitrogen concentration remaining at time t_x (hour).

A One-way ANOVA was used to compare the maximum number of cells produced, growth rate, and uptake rate among nitrate, ammonia, and ammonia plus nitrate treatment. The differences were significant if $p < 0.05$ and followed by a Tukey' test to verify the differences.

Results

Nannochloropsis sp. preferred ammonia rather than nitrate when both ammonia and nitrate were available. Presence of ammonia suppressed the uptake of nitrate by *Nannochloropsis* sp. (Figure 1). Nitrate was utilised in the absence of ammonia. Ammonia uptake rate was significantly higher than nitrate ($p = 0.004$, Table 1). At the end of the experiment, 50 % of ammonia and 33.24 % of nitrate were removed from the medium. Ammonia and nitrate uptake were retarded when the growth of *Nannochloropsis* sp. approached the stationary phase.

Results showed that there were significant different in the microalgal growth rate cultivated by using different nitrogen sources ($p < 0.000$, Table 1). *Nannochloropsis* sp. grew faster in ammonia than in nitrate (Figure 2). Mean growth rates of *Nannochloropsis* sp. in ammonia and ammonia + nitrate treatments were $9.68 \pm 0.058 \times 10^5$ and $9.92 \pm 0.222 \times 10^5$ cell ml^{-1} day^{-1}, respectively. The exponential growth lasted for five days in these treatments. Mean growth rate of *Nannochloropsis* sp. in nitrate was $8.19 \pm 0.640 \times 10^5$ cell ml^{-1} day^{-1}. The exponential growth lasted for eight days. There were no significant difference in the maximum cell density produced in the cultures ($p = 0.224$, Table 1).

Discussion

In this study, presence of ammonia directly interferes with the nitrate uptake by *Nannochloropsis* sp. The microalgae preferred ammonia as its nitrogen source. The preferential uptake of ammonia and the suppression of nitrate uptake by ammonia availability is well studied (Syrett and Morris, 1963; Losada *et al*., 1970; Herrera *et al*., 1972; Cresswell and Syrett, 1979; Blasco and Conway; 1982; Paasche and Kristiansen, 1982; Admiraal *et al*., 1987; Parker, 1993; Flynn *et al*., 1997; Flynn, 1999; Maguer *et al*., 2007). The inhibition of nitrate uptake may be due to the inactivation of nitrate reductase system by ammonia (Losada *et al*., 1970; Herrera *et al*., 1972; Serra *et al*., 1978) or by the by-product of ammonia assimilation (Syrett and Morris, 1963; Thacker and Syrett, 1972; Cresswell and Syrett, 1979). Ammonia requires no enzymatic reduction for assimilation but nitrate has to be reduced to ammonia before it can be assimilated by the microalgae. The reduction of nitrate to ammonia involves two independent enzymatic steps. Firstly, the reduction of nitrate to nitrite catalysed by $NADH_2$-nitrate reductase, and secondly, the reduction of nitrite to ammonia catalysed by the ferredoxin-nitrite reductase. There is considerable energy requirement for the utilisation of nitrate due to the number of electrons required to reduce nitrate to ammonia (Losada *et al*., 1970; Serra *et al*., 1978).

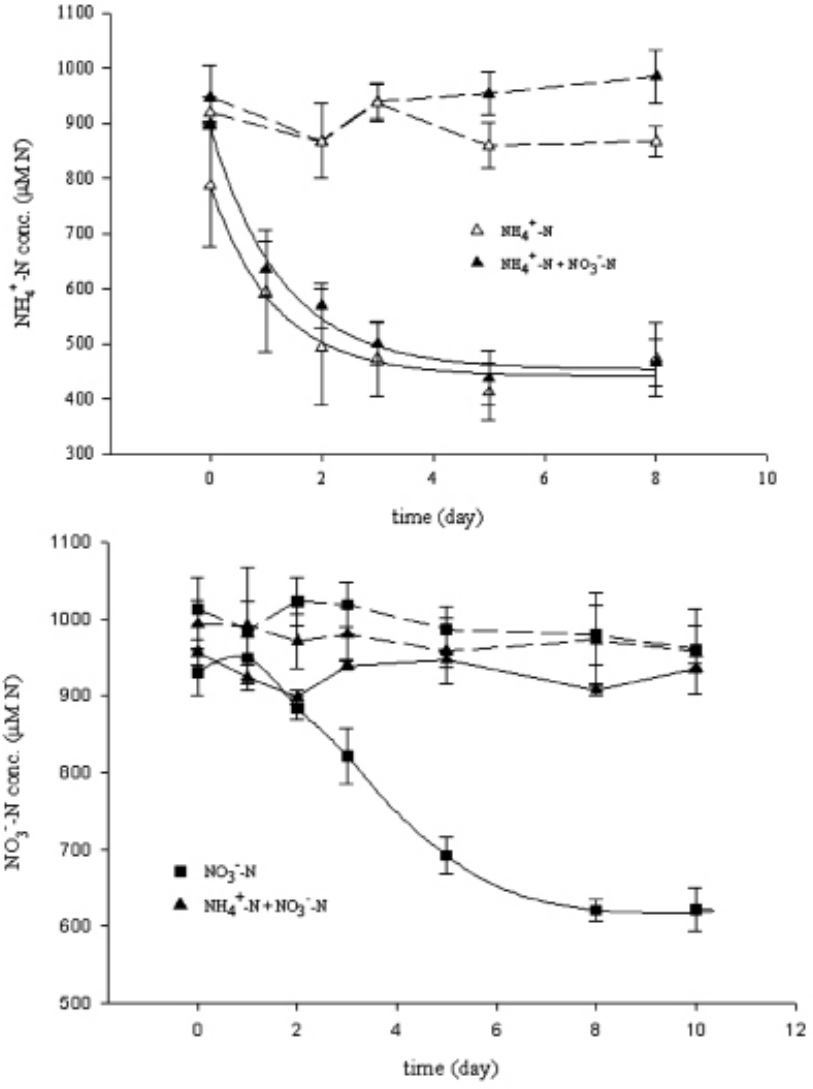

Figure 1. Ammonia and nitrate uptake (solid line) by *Nannochloropsis* sp. inoculated with 1 x 10^6 cell ml^{-1} in f/2 medium enriched with 900 µM NO_3^--N, 900 µM NH_4^+-N, and 900 µM NH_4^+-N + 900 µM NO_3^--N. Control (dash line) was medium without microalgae inoculation. Markers are means and vertical bars showed standard deviation with n = 3.

Ammonia and nitrate exert different effects on their removals as well as on the biomass growth. The result showed that uptake rate of ammonia was higher than nitrate. Similarly, Cochlan and Harrison (1991) reported that the ammonia maximum specific uptake by the eukaryotic picoflagellate, *Micromonas pusilla,* was two times higher than nitrate or urea. Also, ammonia uptake rate of dinoflagellate, *Alexandrium minutum,* was consistently higher than nitrate at any substrate concentration and degree of nitrogen deficiency of the cells (Maguer *et al*., 2007).

In this study, *Nannochloropsis* sp. was able to produce similar cell densities growing on both ammonia and nitrate. Nevertheless, when it grew on ammonia, high cell density was reached more rapidly, presumably because of the higher

Table 1. Summary of the results and one-way ANOVA of batch cultivation of *Nannochloropsis* sp. inoculated with 1 x 10^6 cell ml^{-1} in f/2 medium enriched with different nitrogen species as nitrogen source: 900 µM NO_3^--N, 900 µM NH_4^+-N, and 900 µM NH_4^+-N + 900 µM NO_3^--N. Data are means ± standard deviation of n = 3 incubations.

	Treatment			One-way ANOVA				
	NO_3^--N	NH_4^+-N	NH_4^+-N + NO_3^--N	Source of variation	df	MS	F	p
C_m, cell ml^{-1}	5.00 ± 0.125 x 10^6 [a]	4.84 ± 0.029 x 10^6 [a]	4.96 ± 0.118 x 10^6 [a]	Effect Error	2 6	1.96 x 10^{10} 1.01 x 10^{10}	1.94	0.224
t_m, day	8th day	5th day	5th day					
G_m, cell ml^{-1} hr^{-1}	8.19 ± 0.640 x 10^5 [a]	9.68 ± 0.058 x 10^5 [b]	9.92 ± 0.222 x 10^5 [b]	Effect Error	2 6	2.63 x 10^{10} 9.24 x 10^9	17.10	**0.003**
G_{un}, cell ml^{-1} hr^{-1}	6.22 ± 0.093 x 10^5 [a]	9.68 ± 0.058 x 10^5 [b]	9.92 ± 0.222 x 10^5 [b]	Effect Error	2 6	1.29 x 10^{11} 2.02 x 10^8	639.23	**0.000**
U_m, µM N hr^{-1}	49.42[a] ± 5.986	191.49[b] ± 20.935	263.38[b] ± 48.734	Effect Error	2 6	35564 950	37.45	**0.000**
U_{un}, µM N hr^{-1}	38.68[a] ± 2.246	74.94[b] ± 18.132	92.12[b] ± 9.428	Effect Error	2 6	2233 141	15.85	**0.004**
Uptake efficiency, %	33.24 ± 0.947	47.19 ± 6.725	51.25 ± 5.327					

C_m = Maximum cell density produced, t_m = Time of achievement of maximum cell density attained, G_m = maximum growth rate,
G_{un} = growth rate calculated at t_m, U_m = maximum uptake rate, and U_{un} = uptake rate calculated at t_m.
df = degree of freedom, MS = mean squares, F = Fisher F ratio, p = probability; significant values ($p < 0.05$) are indicated in bold.
[a] Means followed by the same letter are not significantly different for each parameter according to a one-way ANOVA test at $p \leq 0.05$ followed by a Tukey's test.

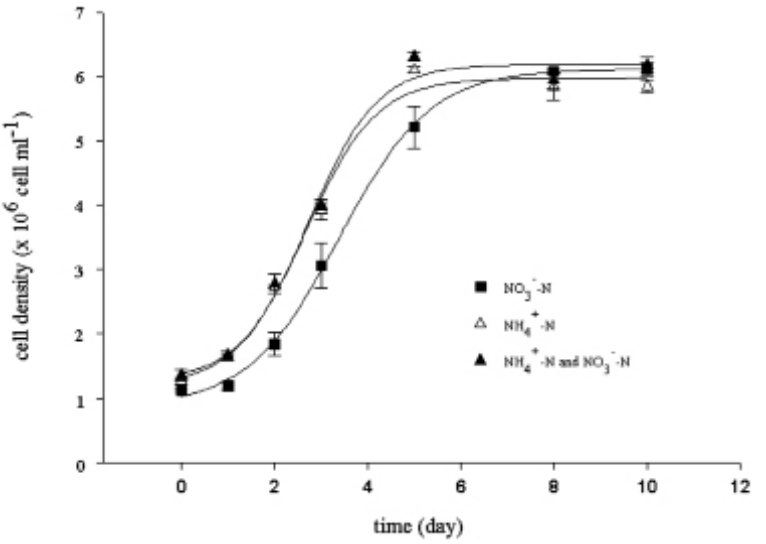

Figure 2. Cell growth of *Nannochloropsis* sp. inoculated with 1 x 10^6 cell ml^{-1} in f/2 medium enriched with 900 µM NO_3^--N, 900 µM NH_4^+-N, and 900 µM NH_4^+-N + 900 µM NO_3^--N. Markers are means and vertical bars showed standard deviation with n = 3.

growth rate. However, *Nannochloropsis* sp. could maintain a population of high cell density for longer duration under nitrate. Ruckert and Giani (2004) showed that cyanobacteria, *Microcystis viridis,* grew faster with ammonia than nitrate. They attributed this pattern to the higher assimilation rate of ammonia than nitrate. On the contrary, there was no significant effect of nitrogen sources on growth rate of marine microalgae, *Isochrysis galbana,* and marine diatoms, *Phaeodactylum tricornutum* and *Chaetoceros muelleri* (Fidalgo *et al*., 1998; Liang *et al*., 2006).

The preferential uptake of ammonia by *Nannochloropsis* sp. is advantageous. Total ammonia-nitrogen, consisting of un-ionised ammonia (NH_3) and ionised ammonia (NH_4^+), is the principle nitrogenous compound in aquaculture water. The un-ionised ammonia is extremely toxic to most fish. Therefore, removal of ammonia is the main concern in aquaculture. On the other hand, nitrate, the end product of nitrification, is relatively nontoxic unless at very high concentration. Nitrate concentration should also be reduced to a safety level before the aquaculture water is discharged. When *Nannochloropsis* sp. is used for nitrogen removal in aquaculture water, the microalgae will first solve the problems of ammonia toxicity to fish. Once ammonia has been removed or down to a safe level, nitrate can be removed by the microalgae.

Conclusions

Nannochloropsis sp. preferred ammonia as its nitrogen source. However, nitrate would be utilised in the absence of ammonia. Uptake rate of ammonia was higher than nitrate. *Nannochloropsis* sp. was able to produce similar cell densities growing on both ammonia and nitrate. The microalgae grew faster in ammonia than nitrate, presumably due to the higher uptake rate. The results showed that *Nannochloropsis* sp. can be a potential biological treatment for aquaculture water. The preferential uptake of ammonia by *Nannochloropsis* sp. solves the problems of ammonia toxicity in the culture system. The microalgae will utilise nitrate from the aquaculture water and bring it down to a safe level before discharge.

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