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INTERACTIVE EFFECT OF AMMONIUM AND NITRATE ON THE NITROGEN UPTAKE AND BIOMASS PRODUCTION BY *Chlorella vulgaris* IN DIFFERENT ENVIRONMENTAL CONDITIONS

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ABSTRACT

Microalgae assimilate different types of nitrogen by interacting in different areas. The interactive effect of uptake rate and biomass production of *Chlorella vulgaris* was investigated under summer and fall environmental conditions. *C. vulgaris* was cultivated in a media containing 1 mM NH_4Cl, 0.5 mM NH_4Cl + 0.5 mM KNO_3, and 1 mM KNO_3 as the nitrogen source. Nitrate and ammonium was more rapidly utilized when both were available. *C. vulgaris* preferred ammonium to nitrate in the fall condition, but both nitrate and ammonium were completely assimilated in the summer condition. The uptake rate of nitrate and ammonium in the summer condition was significantly higher ($p < 0.01$) than in the fall condition and the summer/fall ratio for nitrate uptake (290%) was significantly higher ($p < 0.01$) than for ammonium (120%). In all experiments, biomass production in the summer condition was significantly higher than fall condition ($p < 0.01$). The highest biomass was during the summer condition (5 g/l (f wt)) from the medium containing NO_3^- as the nitrogen source. *C. vulgaris* recorded faster growth in the NO_3^- and (NO_3^- + NH_4^+) media than in the NH_4^+ medium. The summer/fall ratio of biomass production was 235%, 300%, and 265% for the media containing NO_3^-, NH_4^+, and (NO_3^- + NH_4^+), respectively.

Key words: nitrate, ammonium, uptake rate, biomass production, *Chlorella vulgaris*

INTRODUCTION

Study of the interactive effect of nitrate and ammonium is important for microalgal biology and algal-based industries. The focus in biology is on the type of nitrogen transporters and the inhibitory effect of ammonium on nitrate uptake (Dortch 1990). Algae are mainly used in wastewater treatment to remove nutrients in stabilization ponds (Eaton *et al.,* 2005) in which ammonium is the major form of nitrogen which biochemically converts to nitrate in later stages (Thakur and Kumar 1999). The rate of nitrogen removal in stabilization ponds is a function of time and nitrogen type (Chen *et al.,* 2012; Chinnasamy *et al.,* 2010; Perez Garcia *et al.,* 2010; Wang *et al.,* 2010).

For mass production of algae, achieving maximum biomass requires optimization of the cultivation media and environmental conditions. The interaction of ammonium and nitrate must be considered to increase biomass (Hadi *et al.,* 2008; Hosseini-Tafreshi and Shariati 2009). The interaction of nitrate and ammonium is also important in aquaculture where the effluent is rich in urea and ammonium (Abraham *et al.,* 2004). Microalgae can be used to treat the effluent before it is discharged into the aquatic ecosystem.

Studies on the interaction between ammonium and nitrate uptake in phytoplankton have shown that nitrate uptake decreased in the presence of ammonium (Dortch 1990; Hii *et al.,* 2011; Yan *et al.,* 2013). The preference for ammonium uptake is indicated by the higher V_{max} and lower K for ammonium. Thakur and Kumar (1999) studied nitrate and ammonium uptake by *Dunaliella salina* and showed that the uptake rate was 44% for nitrate and 42.2% for ammonium after 36 hr. *Chlorella vulgaris* was able to remove half of the nitrogen concentration by 48 h after a 24 h lag-phase period (Kim *et al.,* 2010). Although ammonium was the preferred nitrogen source for uptake, the growth rates when nitrate was utilized usually equaled or exceeded those for ammonium.

Hii *et al*. (2011) studied the uptake of ammonia and nitrate from aquaculture effluent by *Nannochloropsis sp*. They reported that *Nanno-*

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chloropsis sp. preferred ammonium uptake to nitrate, but that there was no significant difference in maximum biomass on the basis of nitrogen source.

Regardless of the type of industry, algal performance was strongly affected by environmental conditions. The present study examined the interactive effect of nitrate and ammonium uptake rate and biomass production by *Chlorella vulgaris* under summer and fall conditions.

MATERIALS AND METHODS

Microalgae

Chlorella vulgaris (UTEX 265) was obtained from the University of Texas at Austin. Bristol medium was used for stock cultures.

Experimental design

Modified Bristol media (Davis and Wilcomb 1967) was used as base medium $MgSO_4$ (0.5 mM); KH_2PO_4 (0.05 mM); $FeCl_3 + Na_2 – EDTA$ (0.4 μM + 0.01 μM); $NaHCO_3$ (0.25 mM). A completely randomized design with a factorial matrix and 3 replications was used (Table 1). The factors studied were nitrogen source (3 levels) and environmental condition (2 levels). Three nitrogen combinations ((1mM NH_4Cl), (0.5 mM NH_4Cl + 0.5 mM KNO_3), and (1mM KNO_3)) having identical amounts of total nitrogen were designed to evaluate the interactive effect of nitrate and ammonium (Table 1). These media were designated as NH_4^+ medium (1mM NH_4Cl), ($NH_4^+ + NO_3^-$) medium (0.5 mM NH_4Cl + 0.5 mM KNO_3), and NO_3^- medium (1mM KNO_3).

Variations in pH affect the conversion of ammonium to ammonia and may affect the results. To control for this, pH was kept at about 7 by adding sterilized HCl as necessary. Two environmental conditions were simulated in the growth chamber as representative of summer [14 h light (35°C)/10 h dark (25°C)] and fall [10 h light (15°C)/14 h dark (8°C)] in Iran (Table 2). A 250 ml Erlenmeyer flask was used for the cultures. Each flask was inoculated with *Chlorella vulgaris* under aseptic conditions until initial biomass reached 0.5 g/l.

Nitrate and ammonium concentrations and biomass production were monitored daily for 7 d. At each sampling time, the biomass was calculated by measuring the optical density at 750 nm (Gomez and Gonzalez 2005) with a spectrophotometer (Shimadzu UV160A). To determine the cell population, the number of cells was counted using a hemocytometer. The media samples were then centrifuged at 3000 rpm for 15 min and the supernatant was used to determine the removal rates for nitrate and ammonium.

Nitrate was measured using by the ultraviolet technique described by Eaton *et al.* (2005). Briefly, 0.1 ml HCl is added to 5 ml of sample, mixed thoroughly and read in 220 nm. A second measurement at 275 nm was be used to correct the nitrate value. Ammonium was determined based on the method recommended by Smith (Jiménez *et al.,* 2009). The blue color produced from the reaction of ammonium, phenol and hypochlorite was read at 640 nm.

Uptake rate and specific uptake rate

Nitrate and ammonium uptake rate is calculated as given in Eq. (1)

$$1) \text{ uptake rate } (r_{su}) = \text{removal rate} = \frac{[S_2] - [S_1]}{t_2 - t_1}$$

where r_{su} was nutrient uptake rate, t_1 and t_2 represent the first and second time, $[S_1]$ and $[S_2]$ shows the concentration in t_1 and t_2. The slope of time versus efuent concentration at time gives the uptake rate (r_{su}). The ammonium uptake rate (SAUR) and the nitrate uptake rate (SNUR) was determined by dividing the uptake rate of nitrate and ammonium to the biomass as given in Eq. (2) and (3)

2) $SAUR = r_{su}$ /biomass

3) $SNUR = r_{su}$ /biomass

Statistical analysis

Experiment was carried out in complete randomized design in a factorial matrix. 2 factors in 3 replications were considered. Nitrogen sources in 3 levels and environmental condition in 2 levels

Table 1. Different nitrogen sources and environmental conditions used in experimental design

Factor	Conventionally named in the text	
Nitrogen source	(1mM NH_4Cl)	*NH_4^+ media*
	(0.5mM NH_4Cl + 0.5 mM KNO_3)	*($NH_4^+ + NO_3^-$) media*
	(1mM KNO_3)	*NO_3^- media*
Environmental condition (photoperiod + termoperiod)	14h light (35°C) / 10h dark (25°C)	Summer condition
	10h light (15°C) / 14h dark (5°C)	Fall condition

were assigned. Minitab software was used to analyze the one-way ANOVA to compare the uptake rate of ammonium and nitrate, biomass production, and the growth rate from uptake. The Tukey test was performed to verify the differences and the difference was considered significant at $p < 0.05$.

RESULTS

Nitrate completely removed in the NO_3^- and (NH_4^+ + NO_3^-) media on day 7 in the summer condition (Fig. 1A). Fig. 1B shows that maximum nitrate uptake (0.31 mM NO_3^- day^{-1}) was obtained at 4 d in the NO_3^- medium. The maximum uptake was 0.16 mM NO_3^- day^{-1} at 3 d for the (NH_4^+ + NO_3^-) medium. Specific nitrate uptake rate (SNUR) in the NO_3^- and (NH_4^+ + NO_3^-) media showed similar patterns (Fig. 1C). Maximum SNUR (0.32 mM NO_3^- g^{-1} biomass d^{-1}) was obtained at 2 d.

In the fall condition, 32% of nitrate was removed from the NO_3^- medium after 7 d (Fig. 2A). The nitrate concentration in the medium was kept unchanged in the presence of ammonium. In the

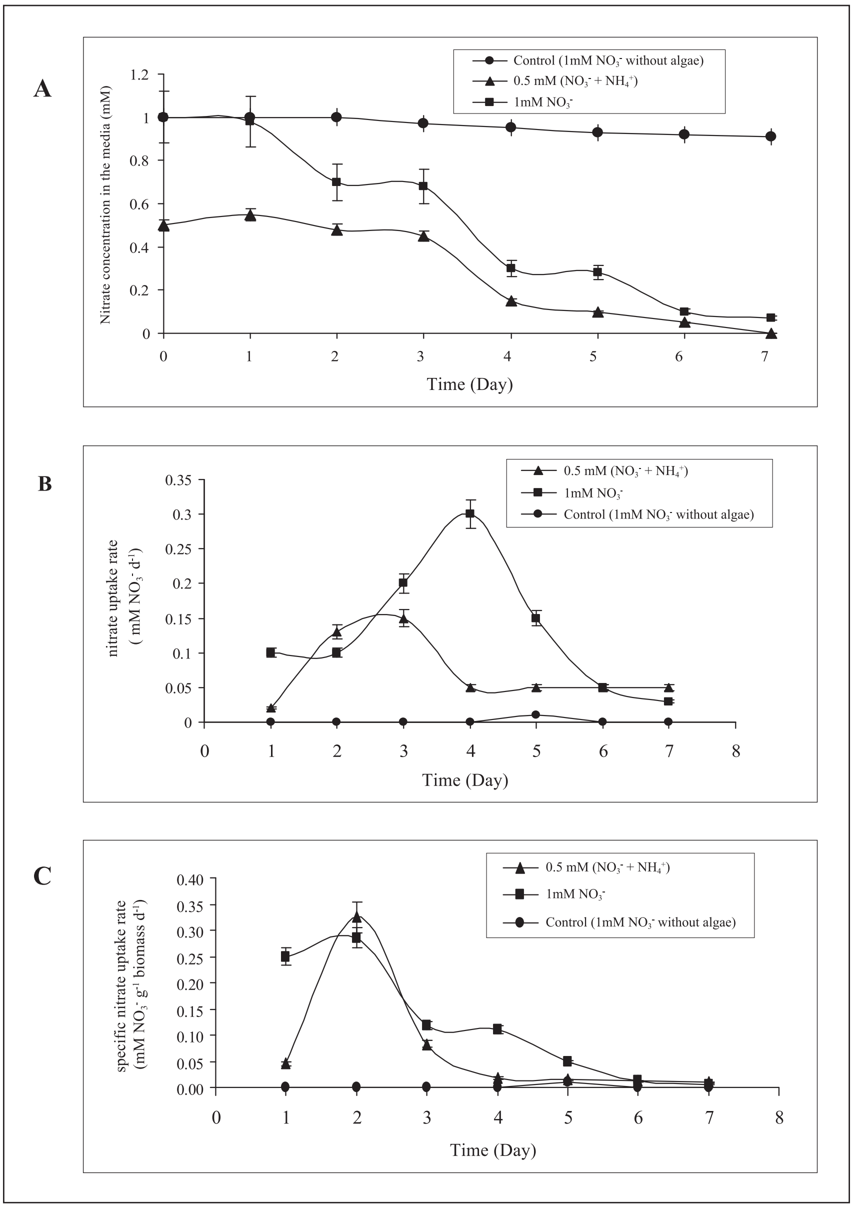

Fig. 1. A) Change in nitrate concentration over time by *Chlorella vulgaris* in summer weather condition. B) Removal rate of nitrate over time by *Chlorella vulgaris* in summer weather condition. C) Specific removal rate of nitrate over time by *Chlorella vulgaris* in summer weather condition.

A, B, and C) summer weather condition 14h light (35ºC)/10h dark (25ºC). Artificial wastewater with different nitrogen source was used as the media. The values are mean of 3 replications. A) The control contained 1mM nitrate without microalgae inoculation.

($NH_4^+ + NO_3^-$) medium, the maximum nitrate uptake (0.11 mM NO_3^- day^{-1}) was obtained at 6 day after treatment (Fig. 2B). This decreased to 0.04 mM NO_3^- day^{-1} at 4 d. SNUR for the fall condition is shown in Fig. 2C. In ($NH_4^+ + NO_3^-$) medium, the maximum SNUR (0.06 mM NO_3^- g^{-1} biomass d^{-1}) was observed at 3 d and decreased thereafter.

Fig. 3 shows the change in ammonium concentration in the media containing $NH_4^+ + NO_3^-$ and NH_4^+ alone. In the summer condition, 85% of ammonium was removed after 7 d in the NH_4^+ medium (Fig. 3A). In ($NH_4^+ + NO_3^-$) medium, all ammonium had been removed at 7 d. The ammonium uptake rate for the NH_4^+ medium increased from days 1 to 6 (0.3 mM NH_4^+ d^{-1}) with an average of 0.135 mM NH_4^+ d^{-1} (Fig. 3B). In ($NH_4^+ + NO_3^-$) medium, the rate of ammonium uptake decreased over the 7 d of testing.

The maximum SAUR (0.35 mM NO_3^- g^{-1} biomass d^{-1}) was observed in ($NH_4^+ + NO_3^-$) medium on day 1 and was near zero at 5 d (Fig. 3C). In NH_4^+ medium, SAUR was 0.08±0.02 mM NO_3^- g^{-1}

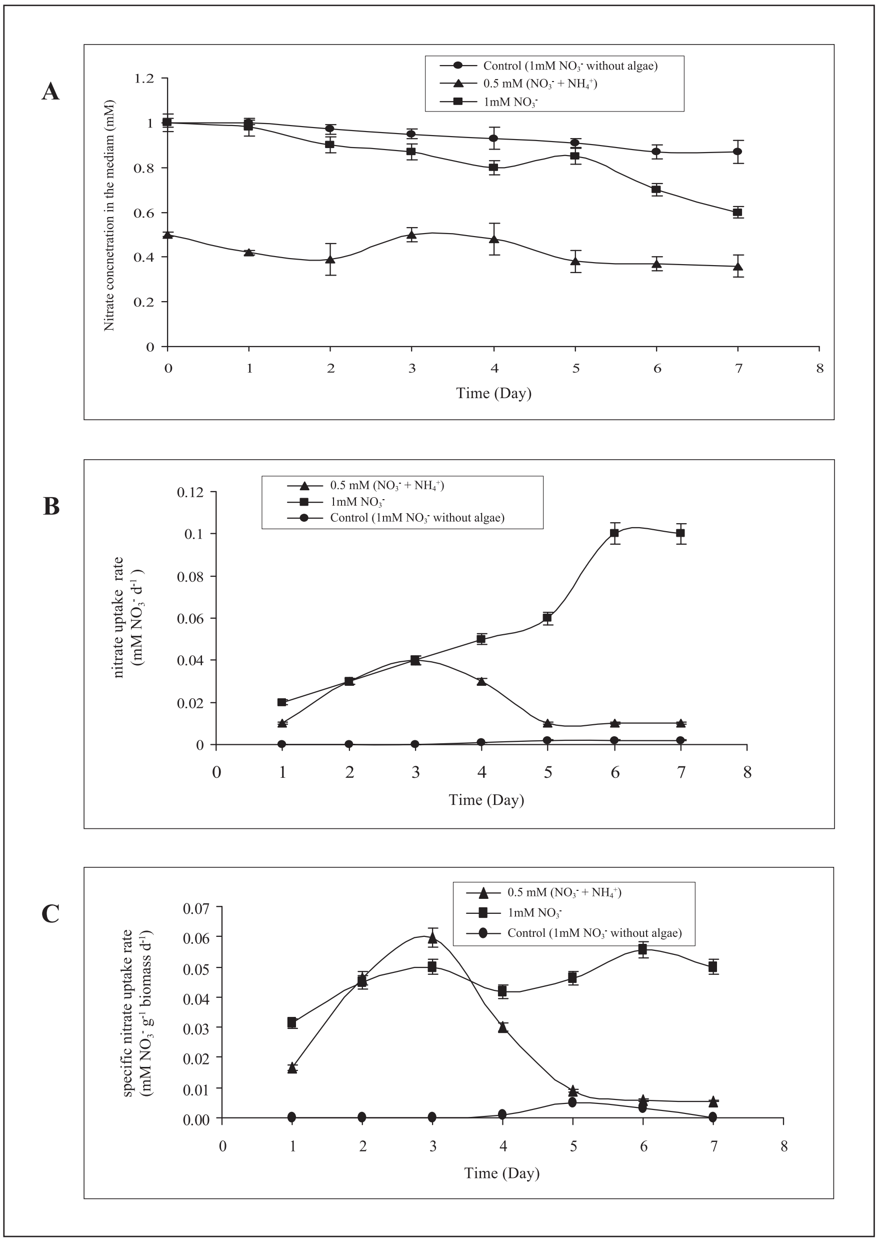

Fig. 2. A) Change in nitrate concentration over time by *Chlorella vulgaris* in fall weather condition. B) Removal rate of nitrate over time by *Chlorella vulgaris* in fall weather condition. C) Specific removal rate of nitrate over time by *Chlorella vulgaris* in fall weather condition.

A, B, and C) fall weather condition 10 h lights (15ºC)/14h dark (8ºC). Artificial wastewater with different nitrogen source was used as the media. The values are mean of 3 replications. A) The control contained 1mM nitrate without microalgae inoculation.

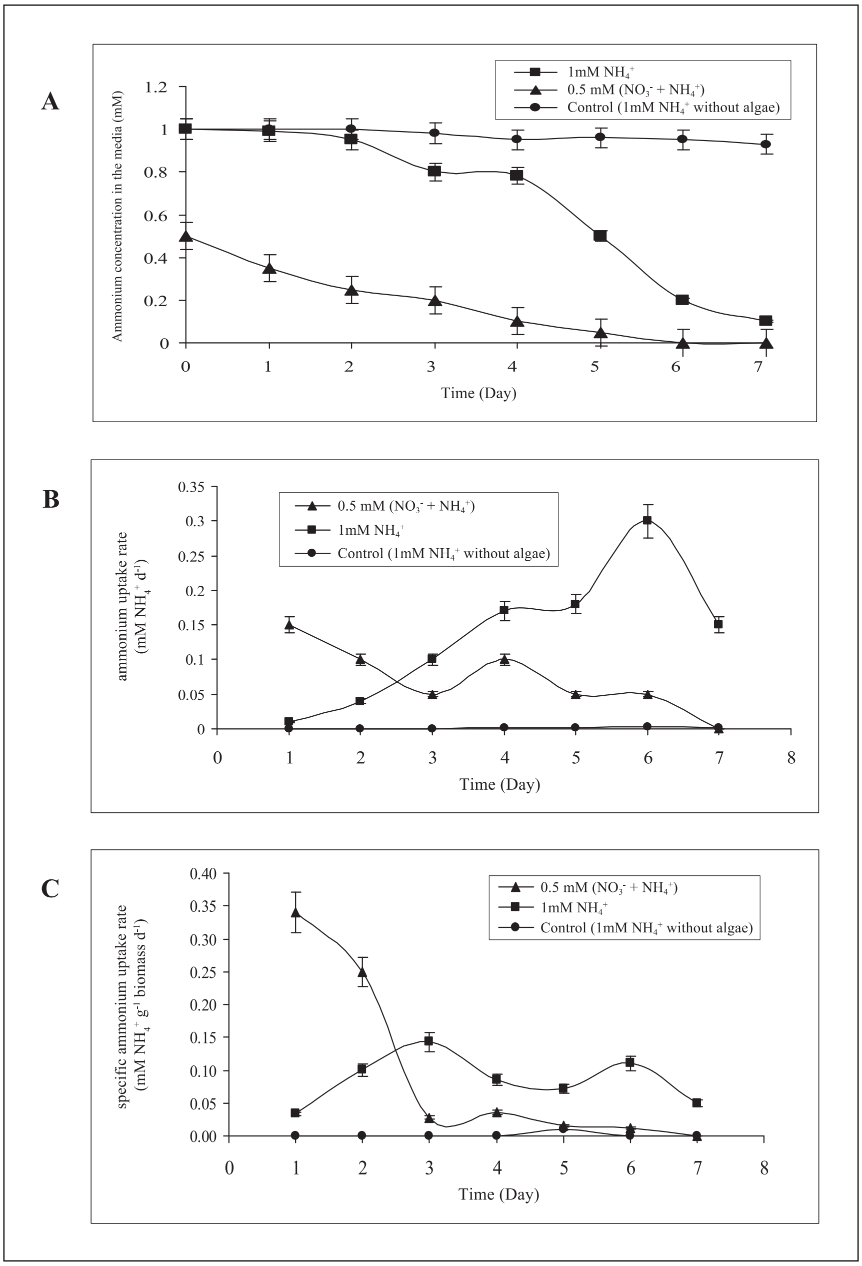

Fig. 3. A) Change in ammonium concentration over time by *Chlorella vulgaris* in summer weather condition. B) Removal rate of ammonium over time by *Chlorella vulgaris* in summer weather condition. C) Specific removal rate of ammonium over time by *Chlorella vulgaris* in summer weather condition.

A, B, and C) summer weather condition 14h light (35ºC)/10h dark (25ºC). Artificial wastewater with different nitrogen source was used as the media. The values are mean of 3 replications.

A) The control contained 1mM ammonium without microalgae inoculation.

biomass d^{-1}. In the fall weather condition (Fig. 4), 0.62% of ammonium was removed from the NH_4^+ medium and 0.86% was removed from the (NH_4^+ + NO_3^-) medium (Fig. 4A). Ammonium uptake in the NH_4^+ medium was approximately constant at 0.03 mM NH_4^+ d^{-1} for 4 d, but increased afterward and reached a maximum (0.3 mM NH_4^+ d^{-1}) after 7 d (Fig. 4B). For the (NH_4^+ + NO_3^-) medium, ammonium uptake increased slightly during the initial days. Fig. 4C shows the average ammonium uptake at 0.057 mM NH_4^+ d^{-1}.

SAUR for the NH_4^+ medium showed a different pattern from those of the other media. In this medium, the SAUR of 0.1 mM NH_4^+ g^{-1} biomass d^{-1} after 1 d increased to the maximum of 0.3 mM NH_4^+ g^{-1} biomass d^{-1} after 7 d. The uptake rate of nitrate and ammonium in the summer condition was significantly higher than fall condition ($p < 0.01$).

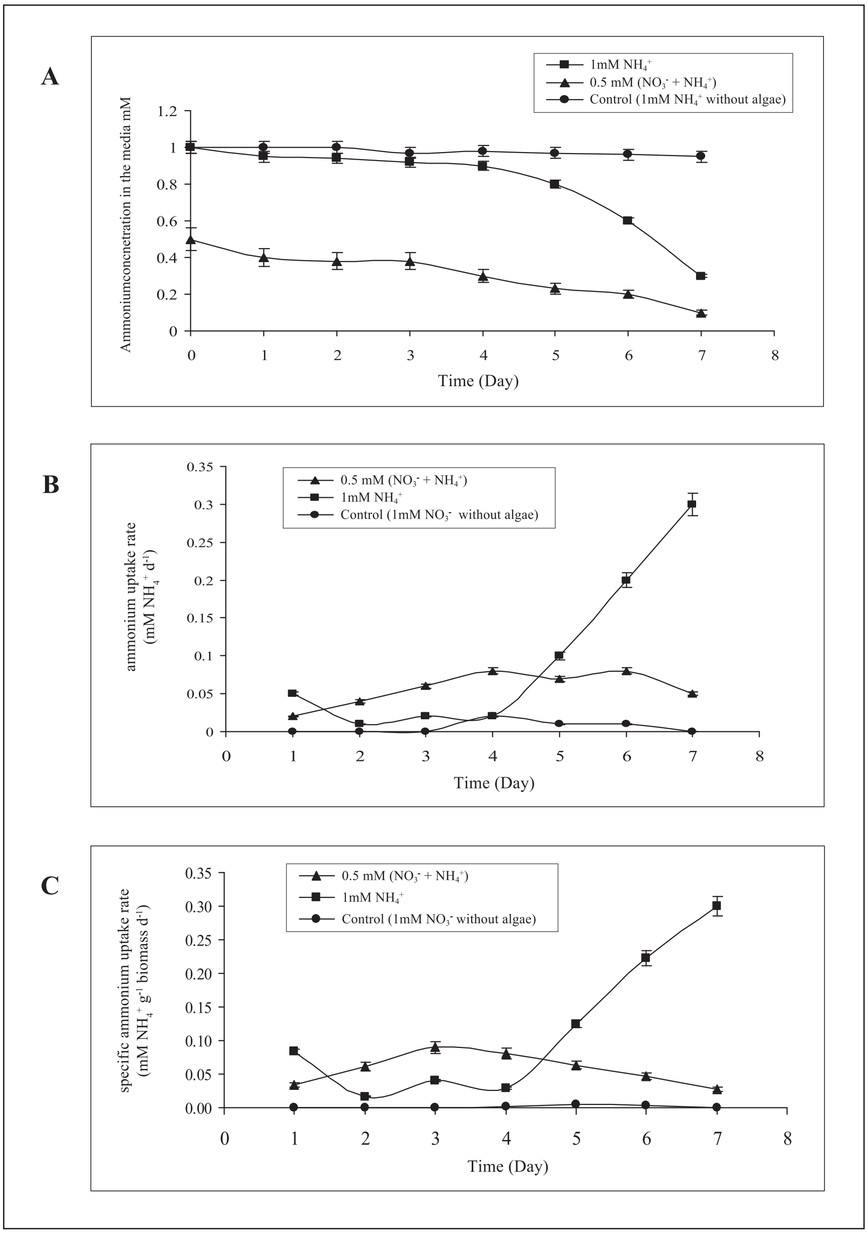

Fig. 4. A) Change in ammonium concentration over time by *Chlorella vulgaris* in fall weather condition. B) Removal rate of ammonium over time by *Chlorella vulgaris* in fall weather condition. C) Specific removal rate of ammonium over time by *Chlorella vulgaris* in fall weather condition.

A, B, and C) fall weather condition 10 h lights (15ºC)/14h dark (8ºC). Artificial wastewater with different nitrogen source was used as the media. The values are mean of 3 replications. A) The control contained 1mM ammonium without microalgae inoculation.

The summer/fall ratio for nitrate uptake (290%) was significantly greater than for ammonium (120%) ($p < 0.01$).

The effect of environmental condition and nitrogen source on biomass production is shown in Fig. 5. In all experiments, biomass production in the summer condition was significantly higher than in the fall condition ($p < 0.01$). In all experiments, the biomass on the first day was 0.5 g/l (f wt). Regardless of the nitrogen source, biomass production in the summer condition was, as expected, greater than for the fall condition. The amount of biomass in the media containing NO_3^- and ($NH_4^+ + NO_3^-$) showed no significant differences; both had greater biomass than the medium containing NH_4^+. The lowest (1 g/L (f wt)) and the highest (5 g/L (f wt)) biomass was obtained for the [NH_4^+ medium in fall condition] and [NO_3^- medium in summer condition], respectively.

In the NO_3^- medium, the maximum biomass in fall and summer was 2 g/L and 4.7 g/L (f wt), respectively. In the NH_4^+ medium, the maximum

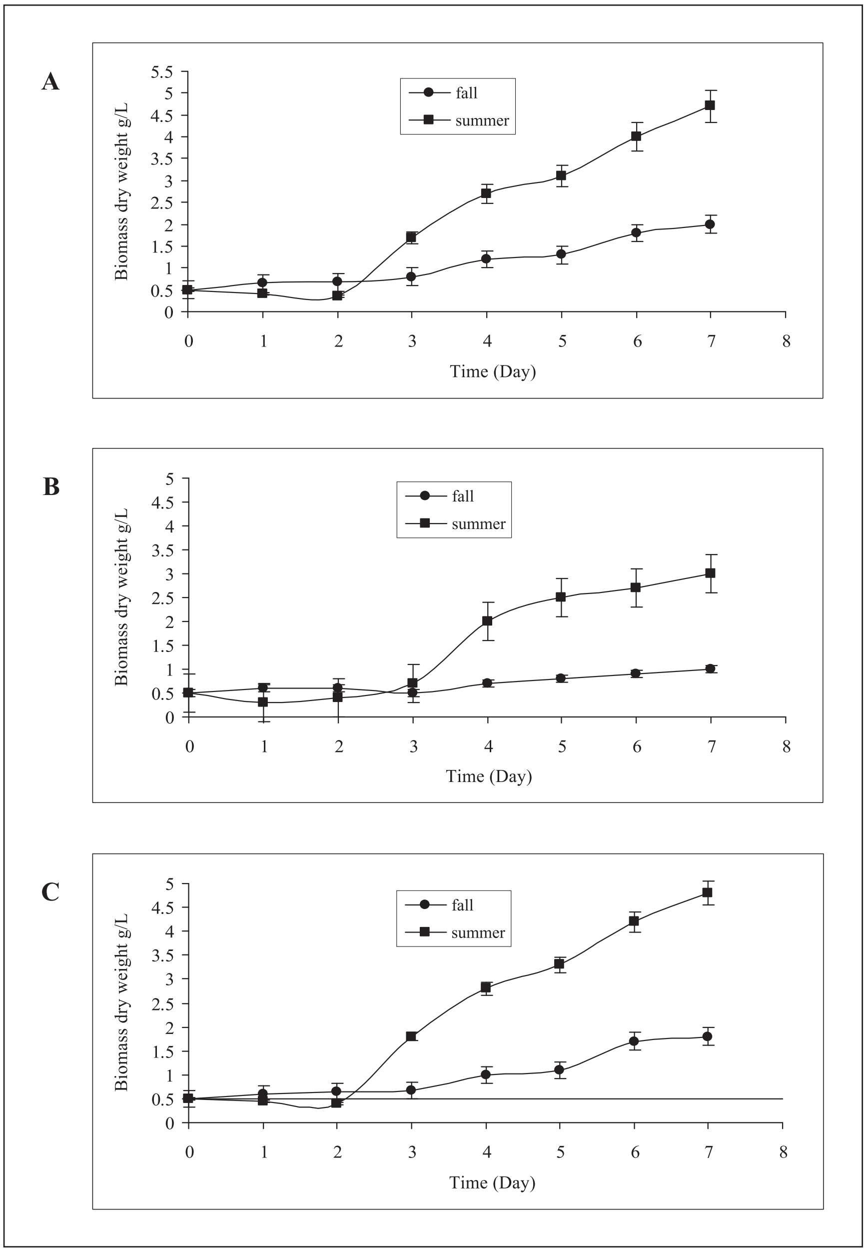

Fig. 5. Biomass production by *Chlorella vulgaris* in fall and summer condition with different nitrogen source. A) *NO_3^- medium* that containing 1mM KNO_3 as nitrogen source. B) *NH_4^+ medium* that containing 1mM NH_4Cl as nitrogen source. C) *(NH_4^+ + NO_3^-) medium* that containing 0.05mM NH_4Cl + 0.05 mM KNO_3 as nitrogen source.

The values are mean of 3 replications.
Fall condition: 10h light (15ºC)/14h dark (8ºC)
Summer condition: 14h light (35ºC)/10h dark (25ºC)

biomass was 3 g/L and 1 g/L (f wt) in summer and fall conditions, respectively. The summer/fall ratio for biomass production was 235%, 300%, and 265% for NO_3^-, NH_4^+, and (NH_4^+ + NO_3^-), respectively.

The interaction of nitrogen source and environmental condition was not significant. The summer/fall ratio of biomass on day 7 [(biomass on day 7 in summer condition)/(biomass on day 7 in summer condition)] for all media was 2.5±0.2.

DISCUSSION

The quantity and quality of nutrient uptake by algae is affected by internal demand, external concentration, and type of the cell transport system (Taiz and Zeiger 2006). When there is no restriction on external nutrient concentration, internal demand is the most important factor affecting uptake quantity. In microalgae, the internal demand is related to growth (Giangrande *et al.,* 2007) and strongly related to the environmental condition. This creates differences in the pattern of nutrient uptake during fall and summer. The results showed that uptake and growth in the summer condition were higher than in the fall condition, as expected, because the photoperiod and thermoperiod in the summer condition is more suitable for growth.

The results showed that, in the present study, uptake rate was controlled by internal demand. *Chlorella vulgaris* preferred ammonium over nitrate as a nitrogen source when both were available. This is in agreement with the results of previous studies (Bauer and Robinson 2002; Giangrande *et al.,* 2007; Thakur and Kumar 1999). This preference may be a response to the lower energy cost of metabolism of ammonium by the algal cell. In addition, the presence of ammonium as an end product of nitrate reduction which it can inhibit nitrate uptake from the feedback inhibition effect (Becker 1993).

It is generally believed that little or no nitrate uptake occurs at ammonium concentrations above 1 μM, although there are reports of low concentrations of ammonium stimulating nitrate uptake (Dortch 1990). Other reasons include the negative charge in the plasma membrane that enhances cation uptake over anion uptake (Taiz and Zeiger 2006). The preference for ammonium uptake can be a consideration in aquaculture in the initial steps of wastewater treatment where ammonium concentration is greater than nitrate concentration.

It can be concluded that *Chlorella vulgaris* removes ammonium before nitrate. The specific uptake rate was more initially than toward the end of the test period; this is probably because the algae absorbed enough nitrogen to fill the internal pool (Kim *et al.,* 2010). Results show that, in the presence of adequate nitrate for maximum biomass production, the existence of ammonium is not essential. The highest biomass was obtained in NO_3^- medium, but there was no significant difference between these results and those for ($NH_4^+ + NO_3^-$) medium. The lowest biomass (3 g/L (f wt)) was obtained in NH_4^+ medium.

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