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Response of the dinoflagellate *Alexandrium tamarense* to a range of nitrogen sources and concentrations: growth rate, chemical carbon and nitrogen, and pigments

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Abstract

The responses of cellular C and N, pigments and growth rates of *Alexandrium tamarense* to different sources of N at high concentrations (6, 12, 25, 50 and 100 μM-N) were examined. Nitrate induced the highest concentration of cellular C (an indicator of biovolume) and cellular N, followed by ammonium and then urea. Cellular C to N ratio (an indicator of physiological status) also varied between N sources. Nitrate grown cells had lower range of C:N ratios and ammonium grown cells had highly variable range. Urea cultures had the highest range of cellular C:N ratio. Pigment composition remained unchanged with all N substrates. The pigments decreased with increasing nitrate concentrations, but with ammonium pigments increased. On the other hand, urea concentrations had no clear relationship with cellular pigments. Variability in the growth of cells was due to both the physiological condition and pigments. When the cells are exposed to different N environments and concentration, they exhibit a physiological acclimation by regulating their cellular materials which is associated with growth.

Introduction

Anthropogenic input of macronutrients, such as nitrogen (N), to coastal waters is one of the stimuli for phytoplankton blooms including blooms of harmful taxa to develop (Smayda, 1990; Hallegraeff, 1995; Paerl, 1997). N in both inorganic and organic forms is generally available for phytoplankton from either autochthonous or allochthonous sources. Primary production supported by allochthonous N is known as "new" production (Dugdale & Goering, 1967; Paerl, 1997), while autochthonous production is referred as "regenerated" production. In coastal areas, new N is continuously supplied by terrestrial runoff (Middelburg & Nieuwenhuize, 2000), through atmospheric deposition (Howarth et al., 1996), vertical supplies or by upwelling (Richard, 1981). Anthropogenic enrichment is considered as one of the most pervasive changes altering coastal environments world wide. Regenerated N such as ammonium and urea derived from bacteria, zooplankton and higher heterotrophs is also recycled directly back to the phytoplankton.

High concentrations of N are usually found in discharge areas such as bays (Matsuoka, 1999), estuaries and also at the bottom layer of the water column (McCarthy & Kamykowski, 1972; Eppley et al., 1978). When dinoflagellates are exposed by either horizontal dispersion or diel vertical migration to this high concentration of N, some physiological changes such as growth and cellular chemical composition, can be predicted to occur in the cells particularly at the higher range of the nitrogen concentrations.

In the present study, *Alexandrium tamarense* was chosen because it appears as blooms in many parts of the world, and has been linked to paralytic shellfish poisoning (PSP) detected in shellfish (e.g. Hallegraeff, 1995; Anderson, 1997). Like most phytoplankton, dinoflagellates are able to utilize and assimilate vari-

ous forms of nitrogen (Chang & McClean, 1997; Dortch et al., 1984). Dinoflagellates can utilize the oxidized forms such as nitrate as well as the reduced forms, chiefly ammonium and urea. But these various forms of N must be converted to ammonium before incorporation into amino acids and the synthesis of macromolecular N such as protein and nucleic acids (Collos & Slawyk, 1980; Syrett, 1981; Wheeler, 1983). Nitrate uptake is known to be light dependent (MacIsaac & Dugdale, 1969), but in recent years, dark nitrate uptake is also known to occur in some natural phytoplankton communities (Cochlan et al., 1991; Présing et al., 1999) and harmful dinoflagellate *Lingulodinium polyedrum* (Kudela & Cochlan, 2000). In contrast, ammonium and urea can be taken up in the light as well as in the dark (Goldman & Glibert, 1983; Cochlan et al., 1991; Kudela & Cochlan, 2000). Although *Alexandrium* often encounters high nutrient concentrations in temperate coastal waters (e.g. Paerl, 1997; Matsuoka, 1999; Seitzinger et al., 2002), their growth rates, cellular composition and pigments in response to high concentration N sources are not well documented.

Here we report the effect of N sources (nitrate, ammonium and urea) and concentrations (6, 12, 25, 50 and 100μM-N) on the growth rate, cellular carbon (C) and N, and pigments of *Alexandrium tamarense*.

Materials and methods

Alexandrium tamarense Balech (strain ATHS-95) was isolated during the 1995 spring bloom in Hiroshima Bay, Japan. The axenic stock cultures were maintained at 17°C, 35 PSU salinity, and 350μmol m^{-2} s^{-1} of cool fluorescent light on a 12h light:12 h dark cycle (Hamasaki et al., 2001). A modified enriched f/2 medium without silicate was employed throughout the present study (Guillard & Ryther, 1962) so that the medium contained 6μM-N of one N source (nitrate, ammonium or urea) in aged seawater collected from Manazuru Bay, Japan. Cells were preconditioned in semi-continuous culture by transferring into new medium at least 10 times and acclimatized to this 6μM-N concentration of nitrogenous nutrients for about 8 weeks before the actual experiments. Occasional microscopic inspection revealed no contamination by bacteria throughout the present study.

Prior to the incubation experiments all aged seawater was checked for the concentration of ambient nitrogen. Concentrations of ambient nitrate, ammonium and urea were <2.0, <1.0 or undetectable and <0.5μM-N or undetectable, respectively.

Exponentially growing cells were inoculated into enriched seawater medium containing one N source—nitrate, ammonium or urea, at 5 different concentrations: 6, 12, 25, 50 and 100μM-N. The initial cell concentration for all experiments was <100 cells ml^{-1}. All treatments were conducted independently in triplicate runs in 1L sterilized screw-top polycarbonate bottles and incubated for a period of 14 days under the same conditions of constant temperature and light as the stock cultures.

Growth was monitored by direct microscopic counts of cells from subsamples taken every second day. Specific growth rates (k, day^{-1}) were calculated over the exponential phase of growth using the following exponential growth equation,

$$k = \frac{\ln N_t - \ln N_0}{t_1 - t_0}, \quad (1)$$

where N_t and N_0 is the cell density in cells ml^{-1} at the start; t_0 and end; t_2 of the exponential phase, respectively.

At the late exponential phase of growth (day 8), triplicate subsamples were taken for the analyses of cellular carbon, nitrogen and pigments. Subsamples for the analyses of particulate organic carbon and nitrogen were filtered onto GF/A glass fiber filter, which was precombusted for 2 h at 500 °C. The cell samples on filters were oven-dried overnight, and stored at room temperature in a desiccator until analysis. Particulate organic carbon and nitrogen were analyzed using an elemental analyzer (Fisons Instrument, NA 1500 series 2). Cellular carbon and nitrogen contents were determined with reference to acetanilide as the standard (Nagao et al., 2001).

For pigment analysis, subsamples collected were centrifuged for 10 min at 3500 rpm and the cells were washed into a 50 ml centrifuge tube. The cells were filtered onto GF/A filters and stored at −60 °C until analysis. The cells collected on filters were ground in 90% acetone (2 ml) using a glass homogenizer fitted with a Teflon pestle. The ground samples were then transferred into a 15 ml centrifuge tube and sonicated for 10 min on ice and extracted in the dark at −20 °C for 24 hours. Finally, the extracts were run on a high performance liquid chromatographic (HPLC) Beckman (168 Diode Array Detector) C18 reversed-phase Ultrasphere 3 μm column, using a solvent gradient system, as described by Head & Horne (1993). Integrated HPLC peak areas were quantified with standards

for chlorophyll-(Chl) *a*, Chl-*c*, peridinin, diadinoxanthin, diatoxanthin, and *beta* carotene obtained from the International Agency for ^{14}C determination.

For each nitrogen substrate, Kruskal–Wallis nonparametric tests were performed to determine a possible variation of each parameter in relation to N concentration. Growth rate versus N concentration was fit to a Michaelis–Menten function. Curve-fit was iteratively performed to investigate possible relationships between N concentration and variation of each parameter. Differences between N treatments were analyzed using a Student's *t* test.

Results

Growth in the three independent batch cultures was similar for all N sources and at all concentrations (Fig. 1) (data for experimental runs 2 and 3 not shown). All the N sources were not depleted at sampling day 8. With nitrate as the sole N source, growth rates of *A. tamarense* increased significantly with increasing concentrations ($p < 0.001$) and the maximum mean growth rate (k_{max}) of 0.31 day^{-1} was found at concentration of $\geq$ 50 μM with half saturation constant (K_s) at 1.31 μM-N (Fig. 2A). In ammonium-enriched cultures, growth rates were not significant with a mean value of 0.34 day^{-1} ranging from 6 to 50 μM, but growth rate was greatly reduced to 0.16 day^{-1} at 100 μM (Fig. 2B). Omitting the growth rate at 100 μM, the k_{max} was 0.34 day^{-1} and K_s was 0.12 μM-N. Cells exposed to increasing urea concentrations were characterized by an increase in growth rates similar to those observed in nitrate-enriched cultures ($p < 0.001$), with k_{max} of 0.31 day^{-1} and K_s of 1.69 μM-N (Fig. 2C). At concentrations <50 μM-N, cells grown with ammonium had higher growth rates than those cultured with either nitrate or urea ($p < 0.05$).

When grown with 6 μM of nitrate, cells contained a maximum mean of 3.7 ng carbon (C) cell^{-1} and the cellular C contents decreased significantly to 2.9 ng C cell^{-1} at 100 μM-N by 23% ($p < 0.001$) (Fig. 3A). On the other hand, cellular N contents showed no clear relationship with nitrate concentrations (Fig. 3B). The mean C:N ratio decreased from 7.1 to 6.3 at the same range of concentrations ($p < 0.05$) (Fig. 3C).

For ammonium-enriched cultures, cellular mean C contents were relatively conservative ranging from 2.8 to 3.1 ng C cell^{-1} with increasing concentrations from 6 to 50 μM-N and at 100 μM-N a marked increase in cellular C contents by almost 2 times was observed ($p < 0.001$) (Fig. 4A). Cellular mean N contents ranged from 0.31 to 0.47 ng N cell^{-1} between 6 and 50 μM-N and increased to a maximum mean of 1.5 ng N cell^{-1} at 100 μM-N by 5 times when compared to the cellular N content at 6 μM-N ($p < 0.001$) (Fig. 4B). The increase in cellular N resulted in a de-

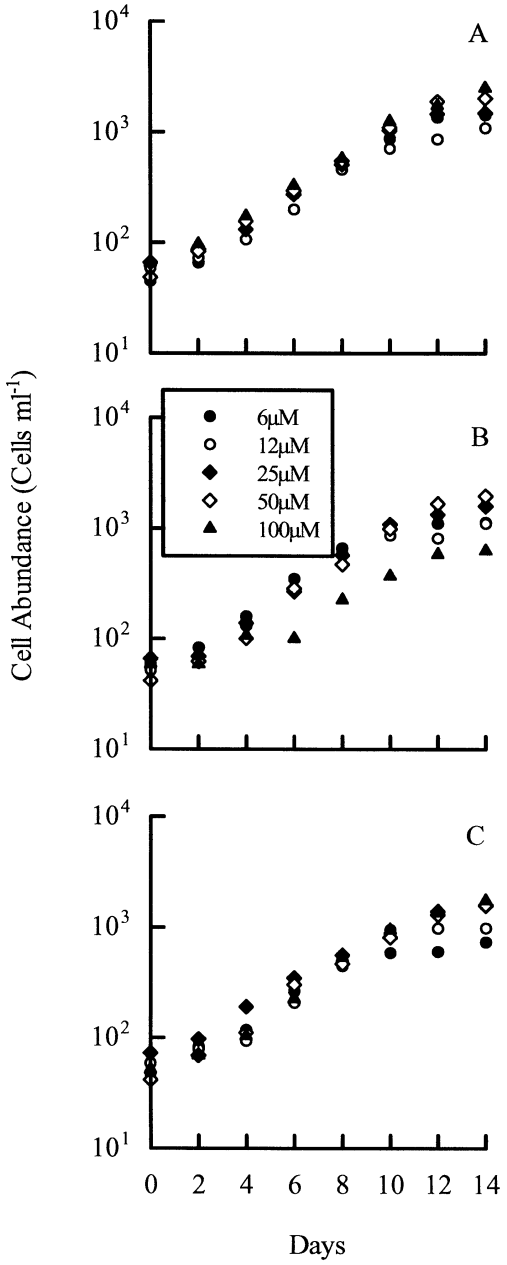

Figure 1. Cell abundances of *Alexandrium tamarense* during experimental run 1 for nitrate (A), ammonium (B) and urea (C) at 6, 12, 25, 50 and 100 μM.

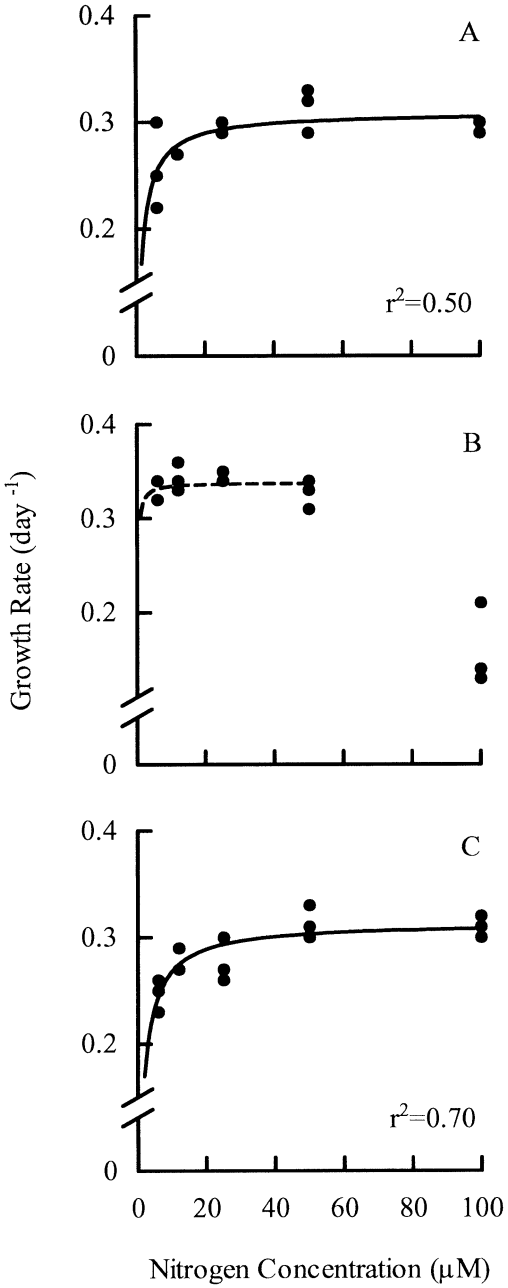

Figure 2. Growth rates of *Alexandrium tamarense* as a function of nitrogen concentrations. (A) Nitrate. (B) Ammonium. (C) Urea.

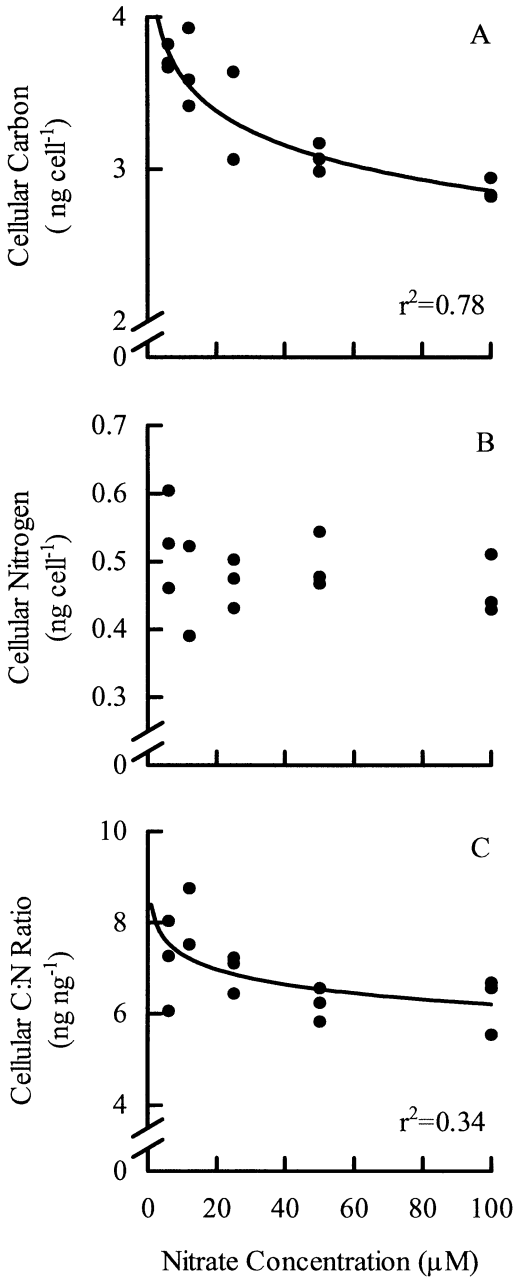

Figure 3. Variations in cellular carbon contents (A), nitrogen contents (B) and cellular C:N ratios (C) of *Alexandrium tamarense* due to the effect of nitrate.

crease in cellular C:N ratio from 10.2 at 6 μM-N to 3.8 at 100 μM-N ($p < 0.001$) (Fig. 4C).

Variations in cellular C, N and C:N ratio of the urea grown cells were similar to those observed in nitrate cultures. Cellular mean C contents showed a significant decrease from 3.1 to 2.2 ng C cell^{-1} over increasing urea concentrations ($p < 0.001$) (Fig. 5A). While cellular N contents showed no significant changes with a mean value of 0.29 ng N cell^{-1} over similar range of concentrations (Fig. 5B). Cellular C:N ratio decreased significantly with increasing urea concentrations from 10.7 at 6 μM-N to 7.5 at 100 μM-N ($p < 0.001$) (Fig. 5C).

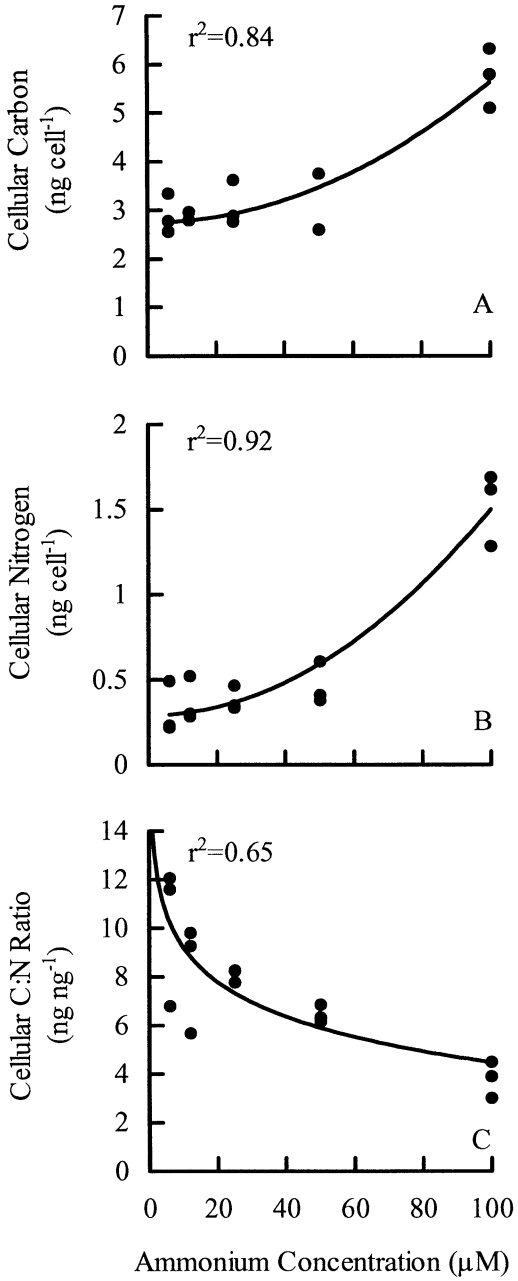

Figure 4. Variations in cellular carbon contents (A), nitrogen contents (B) and cellular C:N ratios (C) of *Alexandrium tamarense* due to the effect of ammonium.

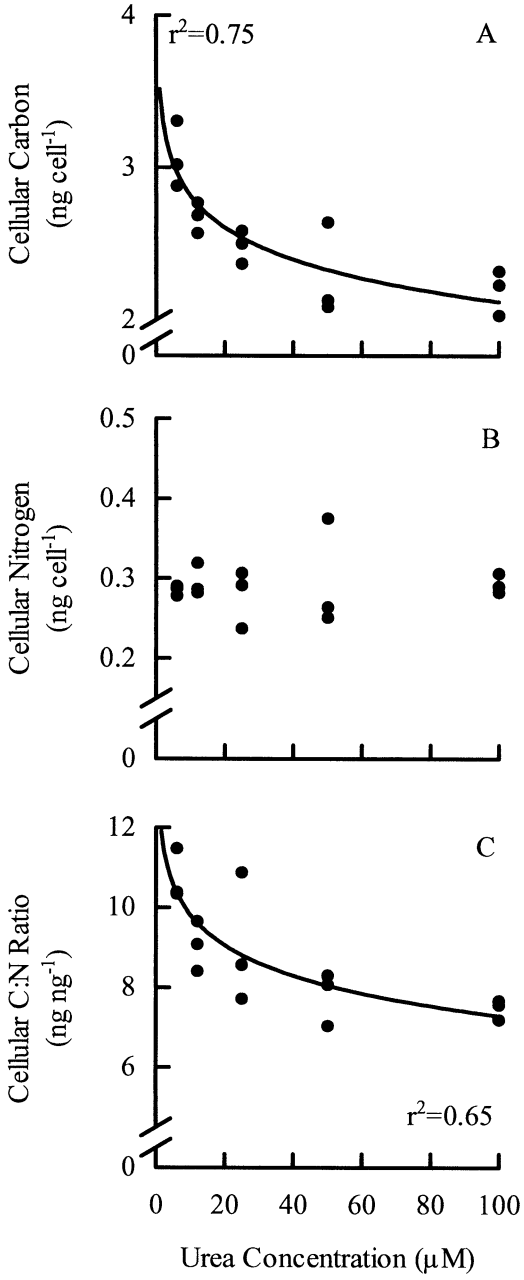

Figure 5. Variations in cellular carbon contents (A), nitrogen contents (B) and cellular C:N ratios (C) of *Alexandrium tamarense* due to the effect of urea.

Except for the apparent inhibition of ammonium at 100 µM-N, nitrate induced the highest cellular C and N contents, followed by ammonium and then urea ($p < 0.001$). Differences in cellular C and N due to the effect of increasing concentrations of each N substrate, resulted in different ranges of cellular C:N ratio. Urea cultures had the highest range of cellular C:N ratio, while nitrate grown cells had lower range and ammonium grown cells had highly variable range, overlapping the values of nitrate cultures.

The pigment composition of cells was dominated by Chl-*a* (49–53%) and peridinin (Perid) (27–

Table 1. Relative pigment composition of nitrate, ammonium and urea enriched cultures with average and one standard deviation. DD + DT indicates diadinoxanthin plus diatoxanthin.

Culture	Pigment	Pigment composition (%)						
		6 μM	12 μM	25 μM	50 μM	50 μM	Average	SD
Nitrate	Chlorophyll-*a*	51.07	52.64	53.65	54.99	53.88	53.25	1.48
	Peridinin	30.88	27.94	30.32	25.00	27.13	28.25	2.40
	Chlorophyll-*c*	10.32	10.32	9.74	10.85	10.99	10.44	0.50
	DD + DT	7.13	8.29	5.73	8.47	7.35	7.39	1.10
	beta Carotene	0.60	0.82	0.55	0.69	0.66	0.66	0.10
Ammonium	Chlorophyll-*a*	52.54	46.58	51.79	50.24	53.05	50.84	2.61
	Peridinin	24.72	31.52	28.57	27.95	28.31	28.21	2.42
	Chlorophyll-*c*	12.59	12.94	11.91	11.74	10.34	11.90	1.00
	DD + DT	9.93	8.72	7.18	9.31	7.94	8.62	1.09
	beta Carotene	0.22	0.25	0.55	0.77	0.36	0.43	0.23
Urea	Chlorophyll-*a*	51.13	44.83	46.58	51.68	52.72	49.39	3.47
	Peridinin	27.28	24.59	29.40	25.79	27.12	26.84	1.80
	Chlorophyll-*c*	12.79	19.63	14.91	14.01	12.90	14.85	2.81
	DD + DT	8.18	10.59	8.59	7.98	7.10	8.49	1.30
	beta Carotene	0.62	0.37	0.51	0.54	0.17	0.44	0.18

28%) with smaller amounts of Chl-*c* (10–15%) and diadinoxanthin plus diatoxanthin (Diadino+Diato) (7–9%) regardless of the N sources utilized. A trace amount of *beta* carotene (*bb*-car) was <1% (Table 1). There were no marked changes in pigment composition for all N sources and concentrations.

All cellular pigment contents per cell decreased with increasing nitrate concentrations. The cellular Chl-*a* contents per cell decreased significantly with increasing nitrate concentrations ($p < 0.001$) (Fig. 6A). Cells contained a mean cellular Chl-*a* per cell of 24.9 at 6 μM-N and 13.3 (pg Chl-*a* cell^{-1}) at 100 μM-N of nitrate. Cellular Perid per cell decreased significantly from 15.1 to 6.58 (pg Perid cell^{-1}) at the same range ($p < 0.001$) (Fig. 6B). Other pigments like Chl-*c*, Diadino+Diato and *bb*-car per cell decreased in a similar manner ($p < 0.001$) (data not shown). Total pigments per cell decreased significantly with increasing nitrate concentrations in the same manner as each individual pigment ($p < 0.001$) (Fig. 6C).

In ammonium-enriched cultures all pigments per cell increased with increasing ammonium concentrations. Cellular Chl-*a* per cell ranged from 8.34 to 13.7 (pg Chl-*a* cell^{-1}) with increasing concentrations from 6 to 50 μM-N and increased sharply to 43.0 (pg Chl-*a* cell^{-1}) at 100 μM-N ($p < 0.001$) (Fig. 7A). Cellular Perid per cell increased by 6.9 times, from 3.35 to 23.0 (pg Perid cell^{-1}) ($p < 0.001$) (Fig. 7B). All minor pigments per cell showed similar increases with increasing ammonium concentrations ($p < 0.001$) (data not shown). Total pigments per cell also demonstrated a significant relationship with ammonium concentrations ($p < 0.001$) (Fig. 7C).

In the case of urea-enriched experiments, all individual pigment and total pigments showed no clear relationship with increasing urea concentrations. Chl-*a* and Perid per cell had a mean value of 11.0 pg Chl-*a* cell^{-1} and 6.0 pg Perid cell^{-1} over the entire range of concentration, respectively (Figs 8A and B). All minor pigments and total pigments per cell also showed similar trend with urea concentrations (Fig. 8C).

Discussion

A. tamarense is able to utilize the three major forms of N sources supplied, showing that nitrate, ammonium and urea can serve as a direct N source for *A. tamarense* in the coastal environment. The high growth rates and k_{max} obtained in ammonium-enriched cultures in this study showed that ammonium is the preferred N source. The K_s estimated from the Michaelis–Menten curve fit for urea-enriched cultures was higher than those of nitrate and ammonium suggest that nitrate and especially ammonium could be utilized rapidly in lower concentrations when compared to urea. The

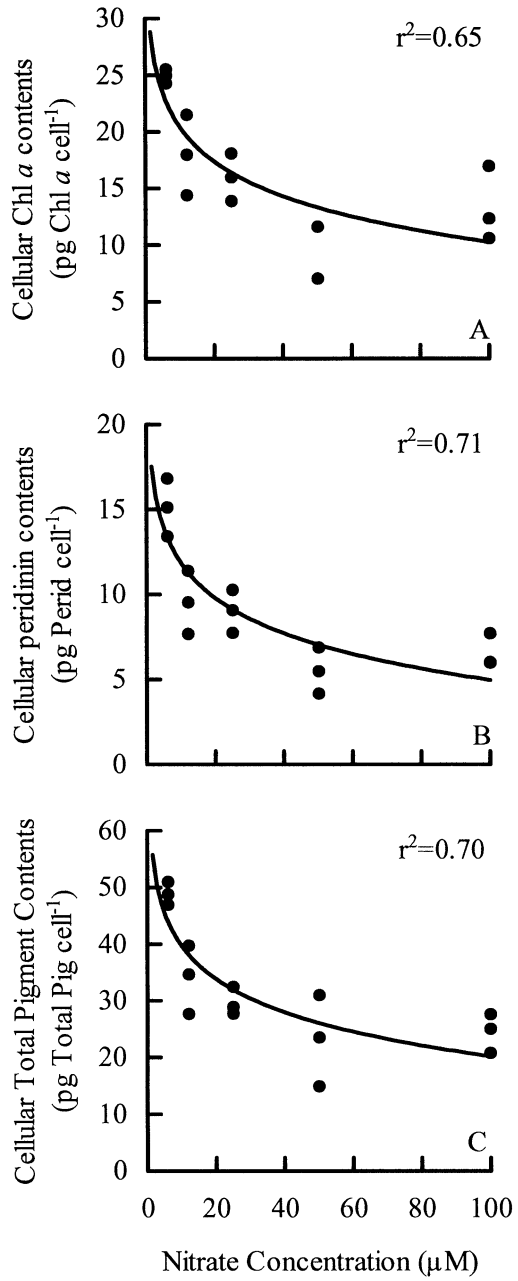

Figure 6. Variations in cellular chlorophyll-*a* (Chl-*a*) (A), peridinin (Perid) (B) and total pigments (Total Pig) (C) of *Alexandrium tamarense* to increasing nitrate concentrations.

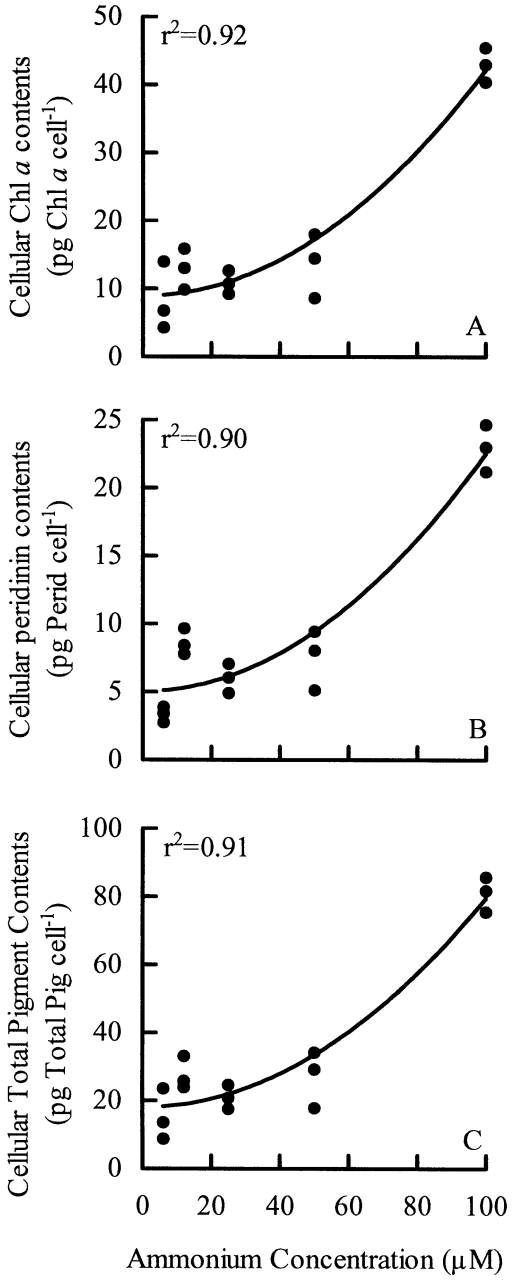

Figure 7. Variations in cellular chlorophyll-*a* (Chl-*a*) (A), peridinin (Perid) (B) and total pigments (Total Pig) (C) of *Alexandrium tamarense* to increasing ammonium concentrations.

results presented in this study are consistent with the results of other dinoflagellate *L. polyedrum* described by Kudela & Cochlan (2000). Other dinoflagellates such as *A. minutum* (e.g., Chang & McClean, 1997) and other phytoplankton such as *Heterosigma carterae* (e.g., Chang & Page, 1995) also showed similar changes in growth rates due to increasing nitrate, ammonium and urea concentrations. Dinoflagellates can normally grow well with increasing nitrate concentrations as observed in the present study, even at very high concentration (ca. >800 μM) (e.g., Parkhill & Cembella, 1999). However, reduced N sources such as

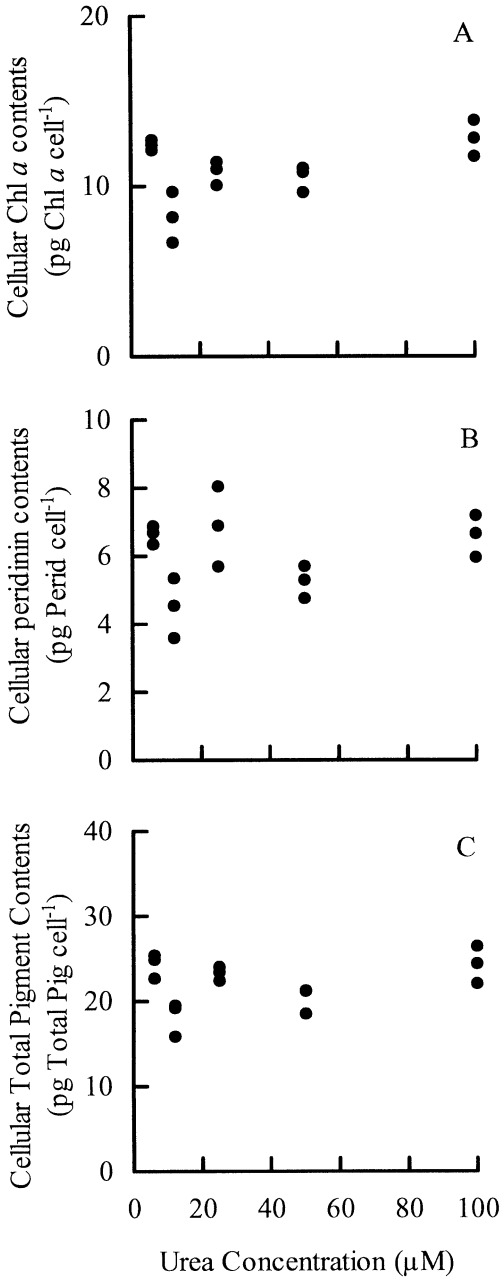

Figure 8. Variations in cellular chlorophyll-*a* (Chl-*a*) (A), peridinin (Perid) (B) and total pigments (Total Pig) (C) of *Alexandrium tamarense* to increasing urea concentrations.

ammonium or urea at high concentration can lead to inhibition of growth rates in some species. Chang & McClean (1997) observed that the growth of *A. minutum* was inhibited at concentrations >25 μM-N of ammonium and urea. While the present study showed that ammonium >50 μM-N inhibited the growth of *A. tamarense*, there was no inhibition of growth with urea. This difference illustrates that the mechanism of N utilization and ammonium toxicity maybe different between species, even if they belong to the same genus. The tolerance level for each N substrate is also species specific.

In general, cellular C is known as an indicative index for phytoplankton biomass. Many studies had shown that cellular C was significantly related to cell volumes (e.g., Montagnes & Berges, 1994; Menden-Deuer & Lessard, 2000). Variations in cellular C contents under various N conditions indicate variability in biovolume (Figs 3–5). These results suggest that cell volume is dependent on the type of N utilized and its concentration. These results also show the differences in the C accumulation due to different N sources (e.g., John & Flynn, 2000). The extremely high cellular C observed at 100 μM-N of ammonium grown cells indicated large cells and presumably the consequent inhibition of low growth or cell division. In addition, cellular C can be divided into three main groups – light-harvesting proteins, biosynthesis apparatus and energy storage reserve as described by Geider et al. (1996). Although the present study did not show the distribution of cellular C among these three groups, difference in C contents due to various N sources suggests that each N form may affect the size of each individual pool differently.

Each N source influenced the cellular N content differently; cellular N per cell showed no clear relationship with nitrate and urea, and increased with ammonium concentrations (Figs 3–5). Protein and RNA are the dominant forms, accounting for 70–90% and 10–15% of cellular N, respectively (Wheeler, 1983). Storage of macromolecules such as protein and RNA requires less energy, than maintaining large concentration of small metabolites (Wheeler, 1983). The ability to store N provides a means of maintaining a high growth rate (Wheeler, 1983; Dortch et al., 1984). The differences in N content of cells observed in this study imply that the abundance of N stored in the cells to maintain growth is dependent on the form of N utilized and its concentrations.

C:N ratio can be used as an indicative index for nutritional status and physiological behavior (Cullen & Horrigan, 1981). Differences in the range of C:N ratio among N sources suggest that N metabolism may play a role in regulating growth rate as the growth of dinoflagellates are related to their physiological status (Flynn & Flynn, 1995; Geider et al., 1998), but the accumulation and utilization of N for maintaining

growth remain to be explored. The increases in C:N ratios of the nitrate and urea grown cells at lower N concentrations in association with lower growth rates in this study may be a consequence of enhanced energy reserve polymers as suggested by Geider et al. (1998). While lower C:N ratios at higher concentrations suggests that more energy reserve is used up in order to maintain high growth rate. On the other hand, it is unclear what is the causes in the variation of C:N ratios of the ammonium grown cells with relatively higher growth rates than those cells grown with other N sources when the toxic effect at 100 μM-N is omitted. This difference is probably due to the sudden exposure of different ammonium concentrations. In addition, it may be the consequence of changes in N:P ratio which was resulted from the adjustment of N concentrations in the present study. Differences in N:P ratio may affect the physiological status of cells (e.g., John & Flynn, 2000). Although, the present study cannot clearly evaluate the metabolic pathways of N utilized and the assimilation of each individual N substrate, it shows the physiological response of *A. tamarense* to variable N and its nutritional status. Variations in cellular C:N ratio also suggest that a physiological acclimation takes place under each N condition, which is associated with growth rates.

Although the pigment composition did not change with concentration of the three N substrates, cellular Chl-*a* per cell decreased with increasing nitrate concentrations and increased with ammonium. On the other hand, urea did not induce any changes. Variations in pigments such as Chl-*a* and Perid in relation to the increase in N concentration can be viewed as a physiological acclimation as described by Geider & MacIntyre (2002). The decrease in cellular Chl *a* with increasing nitrate concentrations may not necessary indicate the energy demand but a redistribution of N from light harvesting proteins to other catalysts such as enzymes (Geider & Osborne, 1992). On the other hand, high cellular Chl-*a* per cell observed at 100 μM-N of ammonium is mostly the consequence of low growth and larger cell due to the detrimental effect of ammonium. The lack of a relationship between cellular pigments and urea concentrations in this study suggests that the mechanism of utilizing urea may be different from those of nitrate and ammonium. It may also be a time consuming process because of slow diffusive property although urea is highly soluble in seawater (Pandian, 1975). Geider et al. (1998) stated that phytoplankton cells required coupling of light harvesting with carbon dioxide fixation and nutrient assimilation, which was associated with cell growth and therefore suggesting that the components of light harvesting apparatus change in abundance with increasing N concentrations in order to optimize growth. Furthermore, the change in abundance of pigments is associated with the balance between light harvesting and photosynthetic carbon assimilation (Geider et al., 1998). In short, this physiological acclimation serves to optimize growth rate under each N condition and it is dependent on the form of the N source utilized and its concentration.

The response of *Alexandrium* upon exposure to high N concentrations for a period of eight days was studied to evaluate the physiological response to these high concentrations as nutrient supply particularly N is always changing in coastal areas. The cells were likely to divide at least two times on the day of harvest. Cochlan et al. (1991) suggested that the nutritional history of phytoplankton cells might determine their ability to take up N and this controlling factor might differ for different forms of N. The present study showed that when the cells were exposed to different N environments and concentrations, they exhibited physiological acclimation by regulating their cellular materials which was associated with growth. The ability of *Alexandrium* to utilize various forms of N sources is an advantage for *Alexandrium* to survive and maintain high growth in the competitive coastal ecosystems. The ability to take up, adapt and regulate cellular contents depending on the N source suggests that N sources may affect the dynamic of dinoflagellate bloom in the coastal ecosystem. In short, *Alexandrium* species exhibit physiological acclimation and other behavioral mechanisms in response to highly variable nutrient condition are a beneficial strategy for ensuring uninterrupted growth and proliferation.

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