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Carbon and nitrogen acquisition by the toxic dinoflagellate *Alexandrium tamarense* in response to different nitrogen sources and supply modes

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ABSTRACT

Alexandrium tamarense was exposed to increasing nitrogen (N) concentrations from three sources; nitrate, ammonium and urea and two supply modes—pulsed and continuous, and its carbon (C) and N uptake kinetics were examined. Cellular properties and nutrient status of cells were found to be dependent on the type of supply, *viz*. pulsed *vs*. continuous. The type of N source and the supply mode induced variability in the C and N assimilation and their interaction. Both the supply mode and concentration of N were important in explaining the observed variability in cellular C and N uptake. Moreover, it was found that *A. tamarense* had the ability to take up substantial amounts of nitrate, ammonium and urea in the darkness. The present study showed that the changes in the C:N uptake ratios were dependent on the N supply mode rather than the nutrient status of cells. Variability in the C:N uptake ratios indicated unbalanced growth and this kind of uptake–growth strategy could be viewed as an ecological advantage in maintaining uninterrupted growth when nutrients are not supplied continuously. Evaluation of the uptake dynamics of *A. tamarense* in response to N conditions and the supply mode, enhance our understanding of how anthropogenic activities in global coastal regions can play an important role in controlling the nutrient uptake of this toxic dinoflagellate, and provide insights into the duration of a bloom.

1. Introduction

Harmful blooms of the dinoflagellate *Alexandrium* are an increasing problem in coastal and estuarine environments. *Alexandrium* species are a causative organism for paralytic shellfish poisoning (PSP) and can cause significant damages in coastal areas. The increases in frequencies and geographical distribution of harmful algal blooms (HABs), such as *Alexandrium tamarense* in global coastal waters, have led to the necessity in predicting such blooms particularly, their initial occurrence in order to ameliorate their detrimental impacts on coastal ecosystems (Leong and Taguchi, 2005). Moreover, the magnitude and the duration of a bloom may also determine the degree of impact on a coastal ecosystem.

A. tamarense appears to have a very wide niche and it is also widely distributed in different geographic areas (Anderson, 1997; Leong et al., 2004). Based on ribosomal RNA sequence, Scholin et al. (1994, 1995) have shown that isolates from each area have their own or unique genotype. Generalizing the environmental factors, particularly the nutrient requirement of *Alexandrium* species is difficult as it has the ability to grow in a variety of habitats. However, blooms of *Alexandrium* species generally occur in nutrient-rich coastal waters (Townsend et al., 2001, 2005; Spatharis et al., 2007).

Globally, both nitrogen (N) and phosphorus are commonly limiting nutrients in aquatic systems. For instance, phosphorus is mostly limiting in freshwater systems while N is the limiting nutrient in marine systems (e.g., Schindler, 1977; Howarth, 1988). One of the serious problems threatening our aquatic ecosystems is the overabundance of these two nutrients due to anthropogenic enrichment (Leong and Taguchi, 2004). Eutrophication from excess of these two nutrients can lead to numerous changes in aquatic systems such as noxious algal blooms. Nutrient enrichment in the form of N is one of the most pervasive impacts affecting coastal environments worldwide (Leong and Taguchi, 2004) and the input of N to coastal waters is an essential factor for the production of phytoplankton blooms including blooms of harmful taxa (Smayda, 1990; Paerl, 1997). Dinoflagellate blooms appear to be associated with elevated N concentration (e.g., Lomas and Glibert, 2000; Glibert et al., 2001, 2006), particularly the reduced forms of N

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sources such as ammonium and urea (e.g., Glibert et al., 2001). In some estuaries, the concentration of the N sources (nitrate, ammonium and urea) can be very high, reaching as high as 100 μM-N (e.g., Yin et al., 2001; Glibert et al., 2001; Turner et al., 2004; Yin and Harrison, 2008). This over-enriched N can cause changes in the bloom dynamics of harmful algae. Moreover, *A. tamarense* has the ability to adapt quickly to changes in the N concentration in its environment (e.g., Leong et al., 2004; Leong and Taguchi, 2006), making it more likely to proliferate and survive in a constantly changing environment. Therefore, the nature of the N source (i.e., the type and the amount of N and/or the supply mode: constant *vs.* pulsed supply) is believed to have consequences on the uptake kinetic parameters of bloom-forming dinoflagellate. Knowledge of the variability in the nutrient uptake kinetic parameters and nutrient requirement of dinoflagellates such as *Alexandrium* species can aid in the development of models to predict its blooms. However, the mechanism by which N is being taken up and assimilated in response to its variations is still a subject of considerable debate.

In the present study, *A. tamarense* cells were exposed to three different N sources for a range of concentrations, in pulses and continuously to determine the carbon (C) and N uptake kinetic parameters.

2. Materials and methods

2.1. Culturing conditions

Dinoflagellate *A. tamarense* Balech (strain ATKR-020415) was isolated during the 2002 spring bloom in Kure Bay, Japan. The axenic stock cultures were maintained at 17 °C, 35 PSU salinity, and an irradiance of 350 μmol m^{-2} s^{-1} provided by cool fluorescent light on a 12 h light:12 h dark cycle. A modified f/2 medium without silicate was employed (Guillard and Ryther, 1962) throughout the present study and N was provided as nitrate, ammonium or urea in aged seawater. Except for the N sources, all other nutrients were added at f/2 concentrations.

All aged seawater was checked for concentration of ambient N before being used for preparation of media. Nitrate concentrations were found to be <0.5 μM-N or undetectable while ammonium and urea were not detected.

2.2. Experimental designs

Uptake experiments with pulsed supply or continuous supply of N were conducted (Fig. 1) as follows: in the pulsed N supply experiments, exponentially growing cells were maintained at 6 μM-N of a single N source (nitrate, ammonium or urea) and a cell density of approximately 400 cells ml^{-1} as a semi-continuous culture by transferring to fresh culture medium every second day for at least 8 days. Cells acclimatized to this concentration (6 μM-N) of the three nitrogenous nutrients were inoculated into seawater medium spiked with labeled ^{13}C ($NaH^{13}CO_3$) (atom% = 98; Isotec, USA) and ^{15}N; $Na^{15}NO_3$ (atom% = 99.1; Shoko Co., Japan), $^{15}NH_4Cl$ (atom% = 98; Cambridge Isotope Laboratories, Inc., USA) or $(^{15}NH_2)_2CO$ (atom% = 98; Cambridge Isotope Laboratories, Inc., USA) of the corresponding N source. The final N concentration was adjusted to produce five concentrations of 6, 12, 25, 50 and 100 μM-N and incubated for 1 h. Dark experimental bottles were also prepared in the same manner but without any dark acclimation prior to the uptake experiment.

In the continuous N supply experiments, exponentially growing cells were acclimatized to three different N sources; nitrate, ammonium or urea, at five different concentrations: 6, 12, 25, 50 and 100 μM-N in semi-continuous culture for a minimum period of 8 days and the cell density was maintained at around

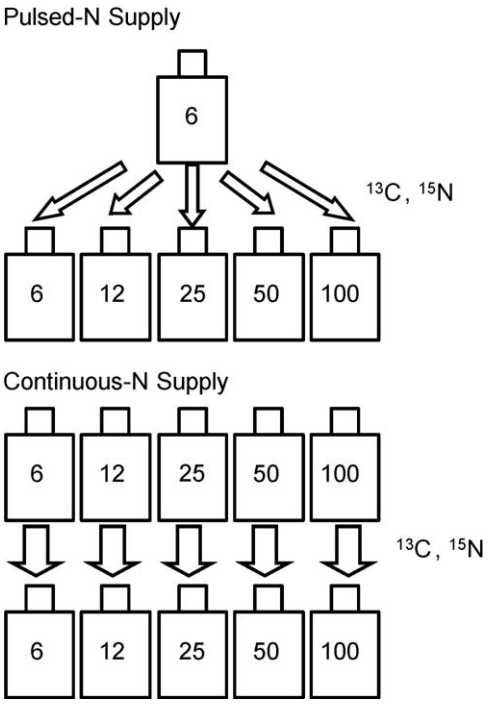

Fig. 1. Schematic diagram of the nitrogen uptake experimental design. Numbers are nitrogen concentration in μM-N. Top panel: Pulsed-N supply mode—*Alexandrium tamarense* cells acclimatized to 6 μM-N of a single nitrogen source; nitrate, ammonium or urea were inoculated into seawater medium spiked with labeled ^{13}C and ^{15}N of the corresponding nitrogen source to produce five concentrations of 6, 12, 25, 50 and 100 μM-N and incubated for 1 h in ambient light and in the dark. Bottom panel: Continuous N supply mode—cells acclimatized to three different nitrogen sources; nitrate, ammonium or urea at five different concentrations of 6, 12, 25, 50 and 100 μM-N were transferred to fresh seawater medium spiked with labeled ^{13}C and ^{15}N of the corresponding nitrogen source and incubated for 1 h under ambient light and dark condition.

400 cells ml^{-1}. Then, the acclimatized cells were transferred to fresh seawater medium spiked with labeled ^{13}C and ^{15}N of the corresponding nitrogenous source and incubated for 1 h under ambient light and dark conditions. No dark acclimation was undertaken prior to the uptake experiment.

The amount of labeled ^{13}C and ^{15}N added was around 5 and, 10–50% of the final concentration, respectively. All the experiments were conducted in triplicate in 4 l sterilized screw-top polycarbonate bottles and at 6 h after the light phase to avoid discrepancy from diel rhythm effects. Duplicate samples from each experimental bottle were collected prior to the uptake experiment (T_0) and after 1 h incubation (T_1) for the measurements of cell abundance, N concentration, particulate organic carbon (POC), particulate organic nitrogen (PON), ^{13}C and ^{15}N atom%.

2.3. Nutrients analyses

Subsamples for N analyses were filtered through 0.45 μm filter unit (Millipore) and nitrate concentrations were measured using a BRAN + LUEBBE nutrient auto-analyzer (AACS-II Compact system). Ammonium was determined using the phenolhypochlorite method (Solorzano, 1969). Urea was assayed using the diacetylmonoxime reagent method (Mulvenna and Savidge, 1992).

2.4. Cell count

Cell density was determined by direct microscopic counts of cells from subsamples that were centrifuged for 10 min at

3500 rpm and re-suspended in 250 μl filtered seawater (0.22 μm). A minimum of 400 cells were counted from each subsample (Lund et al., 1958).

2.5. Cellular carbon and nitrogen

Subsamples for analyses of POC and PON were filtered onto GF/F glass fiber filters previously combusted at 500 °C for 2 h. Cell material on filters was oven-dried at 60 °C for 24 h and stored in a desiccator for analysis. POC and PON were measured using an elemental analyzer (Fisons Instruments, NA 1500 series 2) with acetanilide (C_8H_9NO) as the working standard.

2.6. Cellular ^{13}C and ^{15}N uptake

For ^{13}C and ^{15}N atom% analyses, subsamples were filtered onto pre-combusted GF/F glass fiber filters and cell material on filters was oven-dried for 24 h at 60 °C and stored in a desiccator. Analyses of ^{13}C atom% and ^{15}N atom% were undertaken using an elemental analyzer (Fisions Instrument, NA 1500) coupled to an isotope ratio mass spectrometer (Tracer mat, Finnigan). Glycine (^{13}C atom% = 1.09) and urea (^{15}N atom% = 0.366) were used as the standards. Uptake rate of ^{13}C was calculated using the equation described by Hama et al. (1983) while uptake rate of ^{15}N was calculated using the model described in Dugdale and Goering (1967) and the equation cited in Owen et al. (1986).

2.7. Statistical analyses

The normality of the data set was tested using the Shapiro–Wilk Test. One-way analysis of variance (ANOVA) was then performed to determine if there was a significant difference in the variables for each parameter as a function of N concentrations. In the case where the data did not pass the normality test, a Kruskal–Wallis Test was done to determine a significant difference in the variables as a function of N concentrations. Two-way ANOVA was used to assess the combination effects of N sources and concentrations, and also the supply mode and concentrations effects in each parameter. Bonferroni multiple comparison tests were used to test the differences among treatments and N sources.

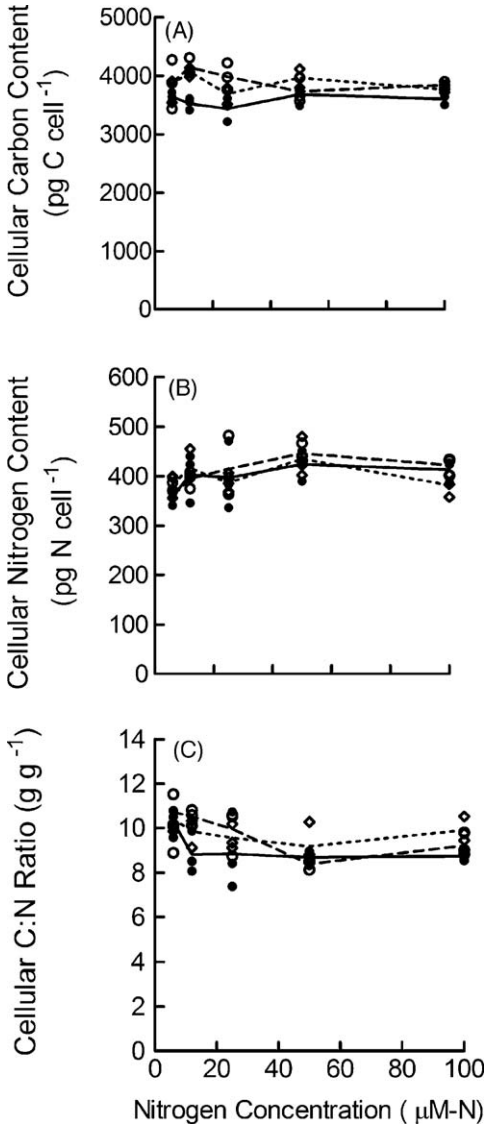

Fig. 2. Cellular carbon, nitrogen content and cellular C:N ratios of *Alexandrium tamarense* grown on nitrate (closed circle; solid line) (A), ammonium (open circle; dashed line) (B) and urea (open diamond; dotted line) (C) supplied in pulses. Lines indicate average values.

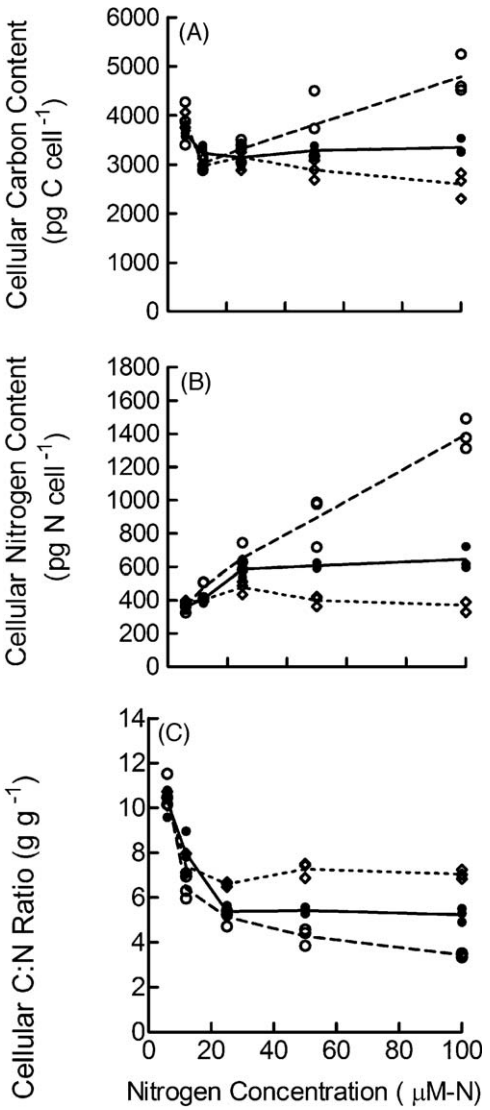

Fig. 3. Cellular carbon, nitrogen content and cellular C:N ratios of *Alexandrium tamarense* grown on nitrate (closed circle; solid line) (A), ammonium (open circle; dashed line) (B) and urea (open diamond; dotted line) (C) supplied continuously. Lines indicate average values.

3. Results

A. tamarense cells acclimatized to the two modes of N supply displayed differences in the cellular C, N and C:N ratio (Fig. 2). When cells were grown and acclimatized to 6 μM-N of nitrate, ammonium and urea in the pulsed mode of nutrient delivery, cells contained an initial average cellular C content of 3579 ± 144, 3914 ± 252 and 3865 ± 197 pg C cell^{-1}, respectively, prior to the uptake experiments (Fig. 2A). The initial average cellular N content ranged from 399 ± 43 to 408 ± 41 pg N cell^{-1} with no dramatic difference among the N sources (Fig. 2B). Cellular C:N ratio ranged from 9.1 ± 1.0 to 9.8 ± 0.7 among the three N sources (Fig. 2C).

On the other hand, when cells were supplied with N constantly and acclimated to a range of concentrations in the continuous mode of nutrient delivery, obvious differences in cellular content were observed (Fig. 3). When grown with 6 μM-N of nitrate, cells contained a maximum cellular C content of 3646 ± 70 pg C cell^{-1} decreasing significantly to 3353 ± 223 pg C cell^{-1} at 100 μM-N (one-way ANOVA, $p < 0.05$) (Fig. 3A). In the case of ammonium acclimatized cells, there was an initial cellular C drop from 3852 ± 438 to 3020 ± 257 pg C cell^{-1} for cells grown in 6–12 μM-N, followed by an increase to 4786 ± 402 pg C cell^{-1} at 100 μM-N ($p < 0.01$) (Fig. 3A). Urea acclimatized cells showed significant decreases in the initial cellular C with increasing concentrations ($p < 0.001$) (Fig. 3A). Two-way ANOVA analysis revealed that the variance of cellular C under continuous-N supply mode was mainly due to the combination effects of both N sources and concentrations (ca. 39%) ($p < 0.0001$) (Table 1). In contrast, cellular N contents increased by almost 80% (358 ± 17 to 643 ± 68 pg N cell^{-1}) for cells grown at concentrations ranging from 6 to 100 μM-N nitrate ($p < 0.001$) (Fig. 3B). In the case of cells acclimatized to ammonium, cellular N increased from 361 ± 32 to 1394 ± 91 pg N cell^{-1} (almost 290%) for cell grown at a range from 6 to 100 μM-N ($p < 0.001$) (Fig. 3B). For the urea acclimatized cells, the cellular N ranged from 369 ± 36 to 478 ± 38 pg N cell^{-1} ($p < 0.05$) (Fig. 3B). Similar to cellular C, the variations in cellular N were controlled by the interaction of both the N sources and concentrations ($p < 0.0001$) (Table 1). Cellular C:N ratios decreased with increasing N concentrations, and ranged from 10.3 ± 0.6 to 5.2 ± 0.3, 10.7 ± 0.7 to 3.4 ± 0.1, and 10.4 ± 0.3 to 7.0 ± 0.2 for nitrate, ammonium and urea acclimated cells, respectively ($p < 0.05$) (Fig. 3B). Concentration of N (ca. 78%) was an important factor in explaining the observed variability in cellular C:N ratio ($p < 0.0001$) (Table 1). In addition, the type of supply or the supply mode (ca. 30–55%) was observed to contribute largely to the variability in the initial cellular properties of cells ($p < 0.01$) (Table 2).

Nitrate, ammonium and urea supplied in different concentrations and via the two modes were found to influence the cellular uptake kinetics parameters of *A. tamarense* differently. When cells were pulsed with nitrate, cellular C uptakes did not differ significantly at all nitrate concentrations and an average value of 120 ± 14.4 pg C cell^{-1} h^{-1} was estimated (one-way ANOVA, $p > 0.05$) (Fig. 4A). In the case of pulsed ammonium supply, the cellular C uptake rates increased from 104 ± 5.6 to 151 ± 5.5 pg C cell^{-1} h^{-1} with increasing concentrations from 6 to 50 μM-N and decreased to 130 ± 14.7 pg C cell^{-1} h^{-1} at 100 μM-N ($p < 0.01$) (Fig. 4B). With urea as the N source, C uptake rates were found to increase significantly from 110 ± 0.5 to 139 ± 2.5 (26% increase) with increasing concentrations of the nitrogenous nutrient ($p < 0.001$) (Fig. 4C). Two-way ANOVA analysis showed that the variance of cellular C uptake rates under pulsed-N supply mode was due to the combination effects of both the N sources and concentrations, (ca. 41%), with 23% of the variance attributable to N concentrations alone ($p < 0.001$) (Table 3).

In the case of continuous supply, increasing nitrate concentrations induced a significant increase in cellular C uptake rates of almost 90% (116 ± 1.8 to 220 ± 5.6 pg C cell^{-1} h^{-1}) for nitrate concentrations from 6 to 100 μM-N (one-way ANOVA, $p < 0.001$)

Table 1
Two-way ANOVA of the effects of nitrogen concentrations and nitrogen source on cellular carbon content (pg C cell^{-1}), nitrogen content (pg N cell^{-1}) and C:N ratio, df: degrees of freedom.

Source	Cellular carbon content			Cellular nitrogen content			Cellular C:N ratio		
	df	%Variance	*p*-Value	df	%Variance	*p*-Value	df	%Variance	*p*-Value
Continuous supply									
Concentrations	4	21.3	0.0001	4	32.3	0.0001	4	78.2	0.0001
Nitrogen sources	2	22.6	0.0001	2	28.9	0.0001	2	10.7	0.0001
Interaction	8	39.0	0.0001	8	35.5	0.0001	8	8.3	0.0001
Residual	30			30			30		

Table 2
Two-way ANOVA of the effects of nitrogen concentrations and supply mode on cellular carbon content (pg C cell^{-1}), nitrogen content (pg N cell^{-1}) and C:N ratio, df: degrees of freedom.

Source	Cellular carbon content			Cellular nitrogen content			Cellular C:N ratio		
	df	%Variance	*p*-Value	df	%Variance	*p*-Value	df	%Variance	*p*-Value
Nitrate									
Concentrations	4	28.2	0.0111	4	37.7	0.0001	4	42.7	0.0001
Supply mode	1	30.5	0.0003	1	31.6	0.0001	1	32.4	0.0001
Interaction	4	8.7	ns	4	20.8	0.0001	4	14.6	0.001
Residual	20			20			20		
Ammonium									
Concentrations	4	24.0	0.0123	4	37.0	0.0001	4	36.1	0.0001
Supply mode	1	2.3	ns	1	30.0	0.0001	1	47.9	0.0001
Interaction	4	45.2	0.0005	4	29.9	0.0001	4	13.2	0.0001
Residual	20			20			20		
Urea									
Concentrations	4	20.5	0.0001	4	29.1	0.0189	4	32.6	0.0001
Supply mode	1	54.5	0.0001	1	0.6	ns	1	46.5	0.0001
Interaction	4	18.2	0.0001	4	32.0	0.0128	4	13.2	0.0004
Residual	20			20			20		

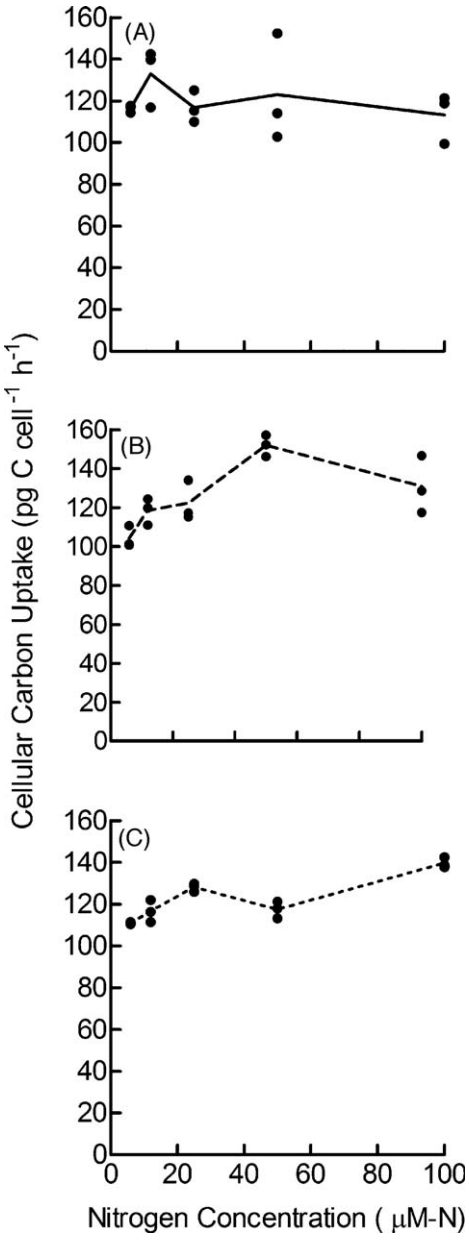

Fig. 4. Variation in the cellular carbon uptake of *Alexandrium tamarense* under nitrate (A), ammonium (B) and urea (C) supplied in pulses. Lines indicate average values.

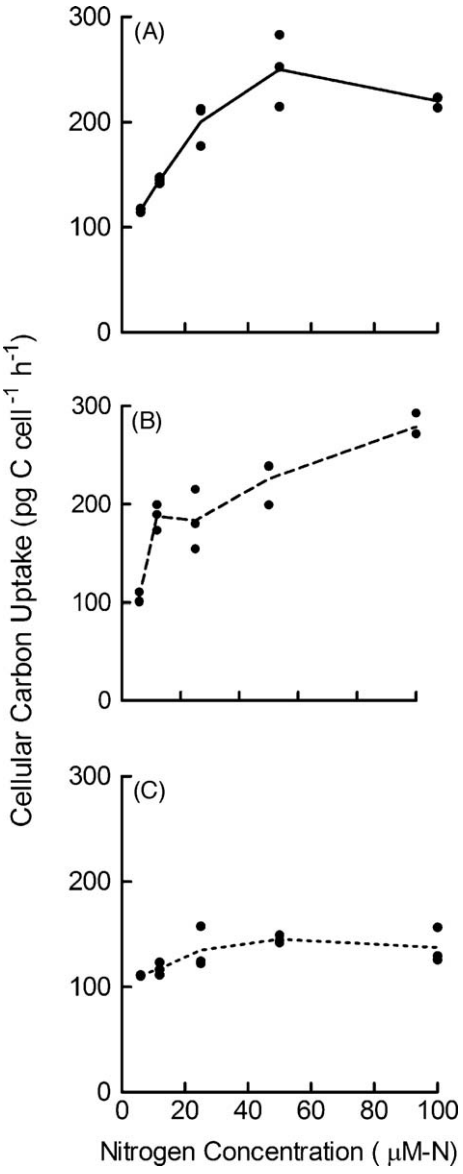

Fig. 5. Variation in the cellular carbon uptake of *Alexandrium tamarense* under nitrate (A), ammonium (B) and urea (C) supplied continuously. Lines indicate average values.

(Fig. 5A) as opposed to those observed with pulsed-nitrate supply. Ammonium and urea uptake rates ranged from 104 ± 5.6 to 279 ± 12.2 pg C cell^{-1} h^{-1} (167%) ($p < 0.001$), and 111 ± 0.5 to 137 ± 16.7 pg C cell^{-1} h^{-1} (24%) ($p < 0.05$), respectively, for the

Table 3
Two-way ANOVA of the effects of concentrations and nitrogen source on carbon uptake rate (pg C cell^{-1} h^{-1}), nitrogen uptake rate (pg N cell^{-1} h^{-1}) and C:N uptake ratio, df: degrees of freedom.

Source	Carbon uptake rate			Nitrogen uptake rate			C:N uptake ratio		
	df	%Variance	*p*-Value	df	%Variance	*p*-Value	df	%Variance	*p*-Value
Pulse supply									
Concentrations	4	23.3	0.0025	4	9.0	0.0001	4	5.5	0.0001
Nitrogen sources	2	2.1	ns	2	82.5	0.0001	2	89.4	0.0001
Interaction	8	41.1	0.001	8	7.3	0.0001	8	1.9	0.0463
Residual	30			30			30		
Continuous supply									
Concentrations	4	47.2	0.0001	4	6.5	0.0001	4	32.3	0.0001
Nitrogen sources	2	29.0	0.0001	2	83.2	0.0001	2	33.2	0.0001
Interaction	8	17.7	0.0001	8	8.5	0.0001	8	32.9	0.0001
Residual	30			30			30		

Table 4
Two-way ANOVA of the effects of concentrations and supply mode on carbon uptake rate (pg C cell^{-1} h^{-1}), nitrogen uptake rate (pg N cell^{-1} h^{-1}) and C:N uptake ratio, df: degrees of freedom.

Source	Carbon uptake rate			Nitrogen uptake rate			C:N uptake ratio		
	df	%Variance	*p*-Value	df	%Variance	*p*-Value	df	%Variance	*p*-Value
Nitrate									
Concentrations	4	23.2	0.0001	4	15.5	0.0001	4	28.6	0.0001
Supply mode	1	43.5	0.0001	1	47.0	0.0001	1	33.8	0.0001
Interaction	4	26.1	0.0001	4	33.5	0.0001	4	36.9	0.0001
Residual	20			20			20		
Ammonium									
Concentrations	4	38.4	0.0001	4	15.3	0.0001	4	8.3	0.0001
Supply mode	1	39.3	0.0001	1	55.1	0.0001	1	66.5	0.0001
Interaction	4	17.6	0.0001	4	26.3	0.0001	4	21.8	0.0001
Residual	20			20			20		
Urea									
Concentrations	4	53.2	0.0001	4	34.8	0.0003	4	26.8	0.003
Supply mode	1	5.4	ns	1	0.1	ns	1	3.4	ns
Interaction	4	15.3	0.0468	4	45.3	0.0001	4	46.5	0.0001
Residual	20			20			20		

entire range of concentrations (6–100 μM-N) (Fig. 5B and C). Under a continuous supply of N, variance in cellular C uptake rates (Table 3) could not be explained solely by either concentrations or N sources but by combination effect of both. Furthermore, the different nutrient regimes (pulse *vs*. continuous) also resulted in highly significant differences in cellular C uptake rates ($p < 0.0001$), except for urea grown cultures ($p > 0.05$) (Table 4). For instance, supply mode alone could explain much of the variance in cellular C uptake rates (ca. 40%) when cells were acclimated to nitrate and ammonium (Table 4). In general, cellular uptake of C under constant N supply was significantly higher compared that when N was supplied in a pulsed mode ($p < 0.01$) (Bonferroni Test). Moreover, both the supply mode and concentration of N were important in explaining the observed variability in cellular C uptake (Table 4).

A. tamarense was able to readily take up the three major forms of N sources supplied. When supplied in pulses, N uptake rates of *Alexandrium* cells were found to increase from 16.6 ± 0.2 to 42.9 ± 6.4 pg N cell^{-1} h^{-1} and 8.1 ± 1.2 to 13.7 ± 2.1 pg N cell^{-1} h^{-1} an increase of 158 and 69% respectively, with increasing nitrate and urea concentrations from 6 to 100 μM-N ($p < 0.001$) (Fig. 6A and C). On the other hand, a minimum mean uptake rate of 55 ± 3.3 pg N cell^{-1} h^{-1} was observed at 6 μM-N in the ammonium acclimated cells and the uptake rates increased significantly to a maximal value of 143 ± 14.4 pg N cell^{-1} h^{-1} at 50 μM-N (158%), then dropped to 103 ± 8.5 pg N cell^{-1} h^{-1} at 100 μM-N ($p < 0.001$) (Fig. 6B). Of the three N sources, ammonium induced the highest N uptake rates, followed by nitrate and then urea. The effects of N sources supplied in pulses on the variability in cellular N uptake rates were larger (ca. >80%) than those due to N concentrations and interaction (Table 3). All the three factors affected the cellular N uptake rates significantly ($p < 0.0001$) (Table 3).

On the other hand, when *Alexandrium* cells were supplied with nitrate continuously, an initial increase in cellular N uptake rate was observed at concentrations from 6 to 12 μM-N with a maximum rate of 31 ± 1.5 pg N cell^{-1} h^{-1} at 12 μM-N. However, the N uptake rates decreased markedly from 12 to 50 μM-N (Kruskal–Wallis Test) ($p < 0.05$) (Fig. 7A). At concentrations ≥50 μM-N, the cellular N uptake rates were relatively conservative with no significant difference (Fig. 7A). Ammonium and urea acclimated cells showed significant variation in the uptake rates (one-way ANOVA, $p < 0.001$), similar to those observed for nitrate uptake with values of 60 ± 5.78 and 17 ± 3.42 pg N cell^{-1} h^{-1} at 12 μM-N, respectively (Fig. 7B and C). However, at ammonium concentrations of >50 μM-N high uptake rates were also observed. Ammonium induced the highest uptake rates of N, followed by nitrate and then urea, similar to those observed under pulsed N supply. N sources supplied via continuous mode were also found to induce more variability in cellular N uptake rates (ca. >80%) than solely due to N concentrations, similar to those observed under pulsed-supply mode (Table 3). In general, cellular uptake rates of N under pulsed-N supply were significantly higher than when N was supplied continuously ($p < 0.05$) (Bonferroni Test). With the exception of urea grown cells, both supply modes and concentrations of N were essential in explaining the >60% of observed variability in cellular N uptakes (Table 4).

Substantial amount of dark N uptake was observed in *A. tamarense* regardless of the supply mode. When provided in pulses, dark nitrate uptake was around 67% relative of nitrate uptake in the light. In the case of reduced N sources, dark ammonium uptake accounted for up to 70% of the ammonium uptake in light, and the ratio of dark to light urea uptake was >0.9. When nitrate, ammonium and urea were provided continuously, a significant amount of dark N uptake was also observed. Dark nitrate uptake could attribute to as high as 67% relative to the light uptake, however, at high supply of nitrate, dark uptake decreased dramatically to <10% relative to light uptake. Dark ammonium and urea uptakes accounted for up to 88 and 98% of light uptake, respectively. In addition, at lower N concentrations (6–12 μM-N), ammonium induced the highest dark N uptake rates, followed by nitrate and then urea, similar to those observed under pulsed N supply. However, from 25 to 100 μM-N, dark N uptake rates were lowest for nitrate acclimated cells, with intermediate values for urea acclimated cells, followed by ammonium acclimated cells with the highest values.

Cellular C:N uptake ratios under light conditions (dark uptake ratio not included) with the exception of urea acclimated cells, decreased with increasing concentrations of N when supplied in pulses ($p < 0.05$) (Fig. 8A and B). Nitrate grown cultures had intermediate range of cellular C:N uptake ratios ranging from 7.0 ± 0.2 to 2.7 ± 0.6, while cells grown under ammonium exhibited the lowest range of cellular C:N uptake ratios (1.9 ± 0.2 to 1.3 ± 0.1) with increasing concentrations. On the other hand, the cellular C:N uptake ratios of urea acclimated cells did not vary significantly with increasing concentrations ($p > 0.05$), and averaged 11.5 ± 2.0 over the entire range of concentrations (Fig. 8C). Of the three N sources, urea acclimated cells exhibited the highest cellular C:N uptake ratio. Both the type of N sources and their concentrations had significant effects on the variance of cellular C:N uptake ratios, and N alone could explain about 89% of the variance (Table 3). Significant interaction between N and concentrations was also observed, but its contribution

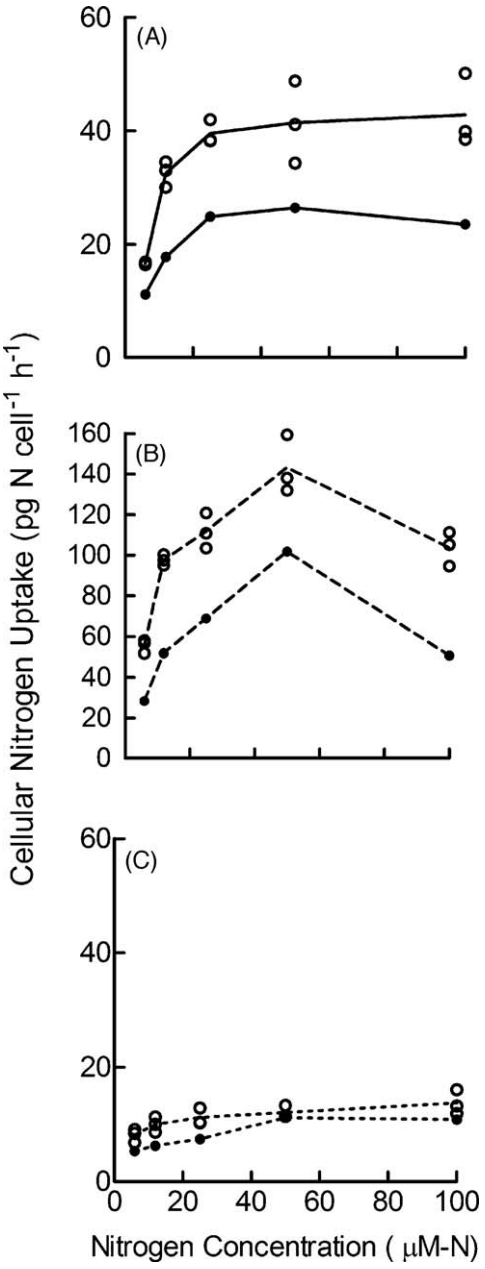

Fig. 6. Cellular nitrogen uptake in darkness (closed) and under ambient light condition (open) as a function of nitrate (A), ammonium (B) and urea (C) concentrations supplied in pulses. Lines indicate average values.

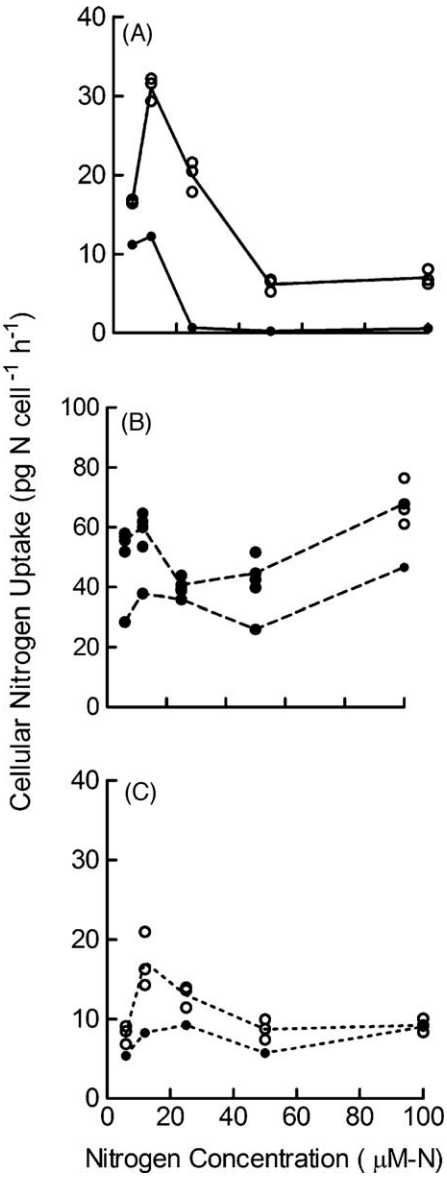

Fig. 7. Cellular nitrogen uptake in darkness (closed) and under ambient light condition (open) as a function of nitrate (A), ammonium (B) and urea (C) concentrations supplied continuously. Lines indicate average values.

to the total percent of variance was low (ca. <2%) (Table 3). When the cultures were supplied with N continuously, the cellular C:N uptake ratios showed different patterns compared to those under a pulsed supply. C:N uptake ratios did not differ significantly under continuous supply of nitrate for concentrations ranging from 6 to 25 μM-N and averaged 7.2 ± 2.3 ($p > 0.05$), and then it increased dramatically at concentration >25 μM-N (Fig. 9A). Ammonium on the other hand, induced a significant increase in C:N uptake ratios from 1.9 ± 0.2 to 4.2 ± 0.6 with increasing concentrations ($p < 0.001$) (Fig. 9B). With urea as the N source, a decrease in cellular C:N uptake ratios was observed from 6 to 12 μM-N, followed by an increase at 12–100 μM-N ($p < 0.01$). Under continuous supply of N, all the factors -N sources, concentrations and interaction of both factors, were equally important in explaining the variability in cellular C:N uptake ratios (Table 3). In addition to the difference in magnitude observed in C:N uptake ratios among N species, the supply mode was also essential in explaining the observed variability in cellular C:N uptake ratios, except for the urea acclimatized cells (Table 4).

4. Discussion

Pulsed *vs.* continuous supply modes of N sources have been shown to induce differences in cellular properties and cellular nutrient status. Variations in cellular C contents under various N conditions indicate differences in the C accumulation due to growth in different N sources (e.g., John and Flynn, 2000), especially when cells were exposed to a continuous supply of N. The extremely high cellular C content observed for *A. tamarense* at 100 μM-N of ammonium supplied continuously was presumably the consequent decrease in growth rate or cell division as observed by Leong et al. (2004). The differences in N content of cells, particularly those observed under continuously N supply, imply that the amount of N stored in the cells for the maintenance of growth is dependent on the form of N, its concentrations and also the type of supply (pulses or continuous). In short, the metabolic

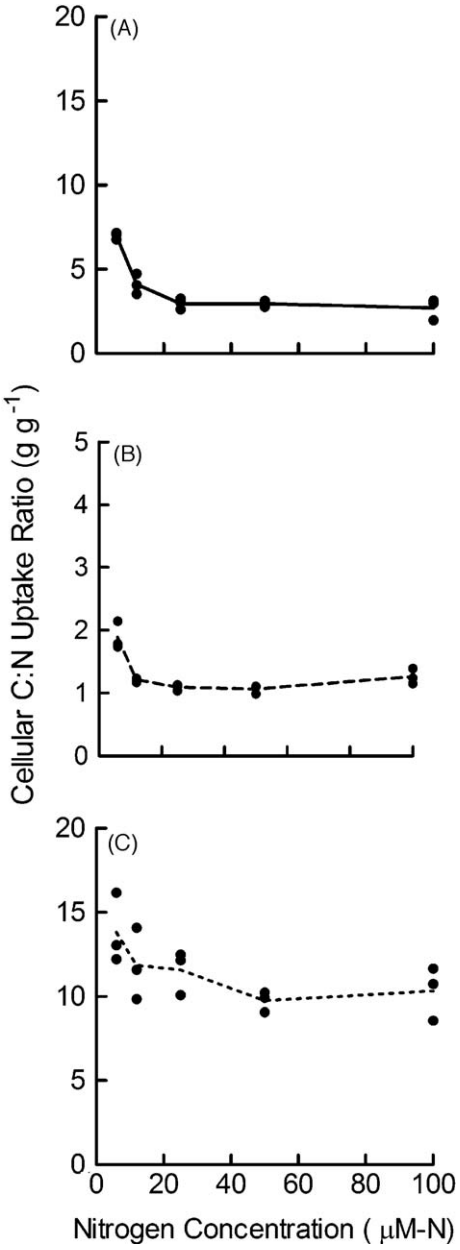

Fig. 8. Variation in the cellular C:N uptake ratio of *Alexandrium tamarense* under nitrate (A), ammonium (B) and urea (C) supplied in pulses. Lines indicate average values.

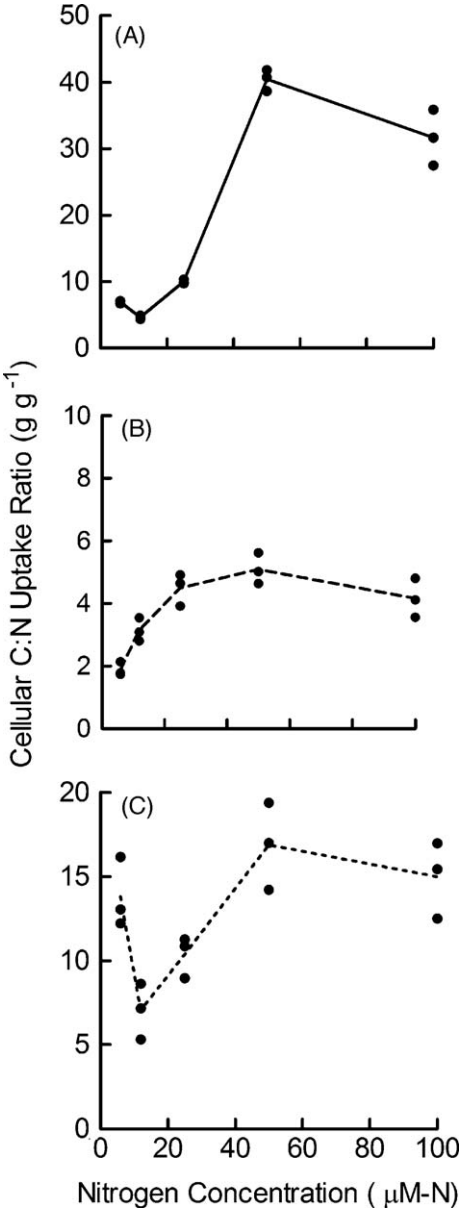

Fig. 9. Variation in the cellular C:N uptake ratio of *Alexandrium tamarense* under nitrate (A), ammonium (B) and urea (C) supplied continuously. Lines indicate average values.

pathways and assimilation are different for each N substrate. Cellular C:N ratio is known as an index for nutritional status and physiological behavior. The nutritional and physiological state of cells is determined by the balance between cellular C and N as photosynthesis and N metabolism are known to interact integrally with each other (Turpin et al., 1988). Varying the cellular C:N ratio within the cells under different N conditions and supply mode were largely due to the carbon requirement for the assimilation of N into protein as described by Vanlerberghe et al. (1990) and Turpin (1991).

The C uptake kinetics of another dinoflagellate *A. catenella* acclimated to several N sources were reported by Collos et al. (2004, 2007). They showed that the C uptakes (specific-C uptake rate: V_{DIC}; units h^{-1}) generally decreased with increasing N concentrations, with nitrate having the greatest influence on the C uptake rates. However, in the present study, the C uptake rates (cell-specific C uptake rate; units pg C $cell^{-1}$ h^{-1}) either increased or remained unchanged with increasing N concentrations. In spite of the different unit used in both the studies, the trend of uptake rate remained unchanged regardless of the unit of the uptake rate (i.e., h^{-1} *vs.* pg C $cell^{-1}$ h^{-1}). This discrepancy in trend observed in both the studies, despite the similar range of N concentrations and the type of supply (i.e., pulses) could be due to several reasons such as differences in the acclimation method or the acclimated concentration. The acclimated N concentration or conditions of the study of Collos et al. (2004) were not reported in detail, therefore, it is difficult to know whether this discrepancy was elicited by a difference in the physiological characteristics caused by different N condition or otherwise. Moreover, different species might have different nutritional needs and nutrient strategy.

Since the photosynthesis–light relationship was first described by Ryther (1956) five decades ago, the photosynthetic rate or C uptake kinetic in response to irradiance has been well documented by many workers (e.g., Ryther and Menzel, 1959; Platt and Jassby,

1976; Taguchi et al., 1988; Behrenfeld and Falkowski, 1997; Kudela and Cochlan, 2000; Behrenfeld et al., 2004). However, the C uptake kinetic in response to N sources and their availability and, in particular the influence of the N supply mode has not been adequately described. The present study showed that most of the variations in C uptake rate could be explained by the combination effects of both N source and concentrations when there is a sudden change in N concentration, i.e., pulsed-supply. On the other hand, under stable condition such as continuous supply of N, the concentrations of N source play an important role in controlling C uptake and thus photosynthesis of phytoplankton. For example, cells might be taking up N from the surroundings and enhancing or restricting photosynthesis at the same time depending on the N conditions. Furthermore, of the three N sources, variability in the cellular C could be explained mostly by supply mode for nitrate and ammonium cultures, but for urea, concentration was the most essential factor. In short, the N availability including the form of N source and supply mode other than light could be viewed as a predominant factor controlling C fixation or photosynthesis. These complex relationships should be considered and applied to coastal management for the mitigating of dinoflagellate blooms.

In addition, the concern regarding the use of urea as a C source is still unclear for this particular species and is still subject to debate. Carlsson et al. (1998) described that only a minor amount or an undetectable amount of the dissolved organic C would turn into particulate C. Moreover, in the present study, the C uptake rates were not enhanced dramatically compared to the other two N sources. This implies that *Alexandrium* species are not capable of utilizing urea as a C source readily, and urea would not contribute significantly to the variability in C uptake as observed in the present study.

The cellular N uptake rates of *A. tamarense* generally, displayed an opposing trend for the two supply modes at >12 μM-N. The maximum N uptake rate was different for each nitrogen source, and it was dependent on the N concentration as well as the mode of supply. Of the three N sources, ammonium is the source taken up most rapidly, but at high concentration ammonium could be toxic to dinoflagellates (Leong and Taguchi, 2004). Higher cellular ammonium uptake rates of >2 times was observed, compared to those of nitrate. This difference could be as high as 10 times depending on the type of supply and concentration. Moreover, the relative cellular N uptake rate of ammonium acclimated cells to those acclimated to urea ranged from 3 to 12 times. Of the three N sources, ammonium was also found to induce highest growth rate for the same *A. tamarense* strain (Leong et al., 2004). Different bloom-forming dinoflagellates such as *A. minutum* and *Prorocentrum minimum* were also shown to exhibit high ammonium uptake rates relative to other N sources; amino acids, nitrate, and urea (e.g., Fan et al., 2003; Maguer et al., 2007). However, *P. minimum* preferred urea to nitrate in contrast to the dinoflagellate *A. tamarense* in the present study. Another bloom-forming dinoflagellate *Lingulodinium polyedrum* was shown to prefer urea over ammonium and nitrate (Kudela and Cochlan, 2000). This suggests that the preference of N source by dinoflagellates could be viewed as species-specific. Specific reason for the preference of one N source over others is unclear.

To date, most studies on N uptake kinetics were based on nutrient pulsing of N sources (e.g., Kudela and Cochlan, 2000; Fan et al., 2003; Maguer et al., 2007), and most of the studies showed that by increasing the strength of the N pulse (i.e., N concentration), enhanced N uptake rates were observed. The present study as well as several previous studies (e.g., Fan et al., 2003; Maguer et al., 2007) have demonstrated that increase in cellular N uptake rates as a function of the strength of the N pulse could be described by the Michaelis–Menten function. However, the present study has showed that this might not be the case when N was provided continuously. A decrease in the cellular N uptake may not necessarily indicate a lack in N demand or extreme inhibition but possibly a strategy in regulating the input of N into the cells or even N distribution within the cells in order to maintain uninterrupted growth. The contradicting trend of cellular N uptake kinetics observed at concentration >12 μM-N between the two supply modes indicates that cells might have different N demand or requirement when cultured. The possible explanation is that cells exposed to high concentrations and sufficient amount of N continuously do not require to take up surplus N for growth maintenance. Although, the effects of N sources and concentrations on the N uptake and/or growth kinetics were examined by several workers (e.g., Dugdale and Goering, 1967; Eppley et al., 1971; Bury et al., 1995; Leong and Taguchi, 2004; Collos et al., 2007), the understanding of N distribution within the cells for cellular maintenance and growth, and also the N distribution due to nutrient or energy demand is still far from complete. The present study shows that supply mode could be a main factor for most of the variability in the cellular N uptake kinetics among the N sources except for urea. Moreover, both supply modes showed that N sources could explain >80% of the variability in the cellular uptake kinetics. Therefore, the nature of the N sources (i.e., the strength and type of supply) is an important factor in regulating the N uptake kinetics of phytoplankton especially in bloom-forming dinoflagellates such as *A. tamarense*.

The present study showed that *A. tamarense* could take up substantial amounts of N in the dark (without dark acclimation), particularly the ammonium and urea acclimated cells, even under surplus conditions regardless of the supply mode. Having the ability to take up N upon a sudden change in the light environment could be an advantage because when cells migrate to depth with low/no light, cells could probably take up and assimilate N to satisfy their daily nutrient requirements (Harrison, 1976). However, upon a long period of dark exposure, the ability to take up N would likely diminish as the cellular energy would be used up. Paasche et al. (1984) showed that the dark N uptake ability of marine dinoflagellates differs greatly. Their study showed that each dinoflagellate species had a different ability to take up different forms of N. Some dinoflagellates could take up a particular form of N in the dark (e.g., nitrate) more efficiently and effectively than others. Moreover, most dinoflagellates could take up more reduced than oxidized N (i.e., ammonium *vs.* nitrate) in the dark (e.g., Dugdale and Goering, 1967; Paasche et al., 1984). In the present study, *A. tamarense* also showed similar uptake kinetics. Discrepancies in the dark N uptake kinetic among marine dinoflagellates suggest that the ability in taking up different forms of N in the dark is species-specific. The present study also showed that the supply mode of N could affect the dark N uptake take significantly, particularly in the case of nitrate. The low dark nitrate uptake under continuous supply could be due to the bio-energy budget for maintaining high growth. Dark N uptake ability has been shown as a competitive advantage over other species such as the diatoms (Harrison, 1976). In short, the ability to take up significant amount of N in darkness could be viewed as an important role in maintaining uninterrupted growth. Clearly, this dark N uptake strategy and its variability need to be investigated in great detail in order to further understand the dark uptake mechanism in response to variable environmental conditions such as nutrients.

The present study has shown for the first time the variations in C:N uptake ratio for the dinoflagellate *A. tamarense* in response to three N sources at a range of concentrations under two supply modes. There is a vast amount of literature on the C or N uptake kinetics of phytoplankton including harmful dinoflagellates in response to environmental conditions particularly irradiance, however, they have generally been examined separately (e.g.,

Cochlan et al., 1991; Gilstad et al., 1993; Lomas and Glibert, 2000). A few studies have examined the simultaneous uptake of C and N in phytoplankton under laboratory and/or field conditions (e.g., Dugdale and Goering, 1967; Bury et al., 1995; Dauchez et al., 1995). Reported values of C relative to N uptake ranged from as low as 0.1–>1000 (mol/mol) (e.g., Bury et al., 1995; Dauchez et al., 1995; Collos et al., 2004, 2007). The range of the C:N uptake values (1.07–40.4 g/g or 1.25–47.2 mol/mol) of *A. tamarense* observed in the present study is consistent with previous studies and fall within the reported range. Even though, the inter-relationship between the assimilation of C and N is not uncommon, the factors contributing to the variability in this uptake ratio are still far from being understood. The present study has shown that the C:N uptake ratio of *A. tamarense* is not completely dependent on nutrient status of cells as indicated by the cellular C:N ratio (Fig. 10). Apart from the C:N uptake ratio of cells exposed to ammonium constantly, no clear trend was observed between cellular C:N ratio and C:N uptake ratio. However, the present study showed that nitrate exposed cells could be divided into two groups. Cells with C:N ratio <6 tend to take up more C relative to N, than cells with C:N ratio of >6 (Fig. 10). On the other, ammonium grown cells exhibited a clear relationship between cellular C:N ratio and C:N uptake ratio under continuous supply ($p < 0.01$). Cells with higher cellular C:N ratio of >8 are likely to take up more N relative to C and vice versa. As in the case of urea grown cells, all data seems to cluster around a C:N ratio of 8, with no clear trend. Although, the relationship between cellular C:N ratio and C:N uptake ratio could not be clearly established, variability in the C:N uptake ratio indicates major interactions between C and N metabolism. This interaction may allow *A. tamarense* to maintain high growth under different N environments. It might also be the energetic competition between C and N assimilations. The widely variable C:N uptake ratios could be due to a sudden change in environmental conditions or dynamic acclimation to varying environmental conditions, particularly those observed in pulsed-supply experiments. In general, C:N uptake ratio tend to be low at high N concentrations (Dauchez et al., 1995). Similar observation was found in the pulsed-supply N experiment in the present study as well as in the study of Collos et al. (2004). However, when cells were supplied with N at a constant rate, a contradicting trend was observed. Clearly, the type of supply mode could account for the discrepancy in trends of the C:N uptake ratio. Moreover, the low C:N uptake ratio observed at high N concentration could be due to unbalanced growth from the addition of high N concentrations (Collos et al., 2004). Low C:N uptake ratio could be an indication of the accumulation of a significant amount of N in dinoflagellate cells before cell division—a lag period between N uptake and growth (Collos, 1986). The lag period between cellular N uptake and growth indicated that N was stored in cells for further usage rather than a growth response to a pulse nutrient addition (Collos, 1986). This kind of uptake–growth strategy could be viewed as an advantage to maintain uninterrupted growth when nutrients are not supplied constantly, thus prolonging the life span of a bloom.

The nature of the N supply has been demonstrated to affect the C and N uptake kinetics of the bloom-forming dinoflagellate. The supply–uptake relationships that we have shown could provide a better understanding of the bloom dynamics of *Alexandrium* species. This information could assist in refining models to better predict the development of a bloom, its magnitude and duration, and thus serve as a means in the mitigation of harmful dinoflagellates. Moreover, nutrient requirement of bloom-forming dinoflagellates is a key factor in determining their growth dynamics. The knowledge and information of the nutrient requirements from uptake kinetic studies could enhance our ability in controlling and preventing a harmful or toxic bloom from being fully developed, and could also provide early warning to the public to reduce the impact of a bloom.

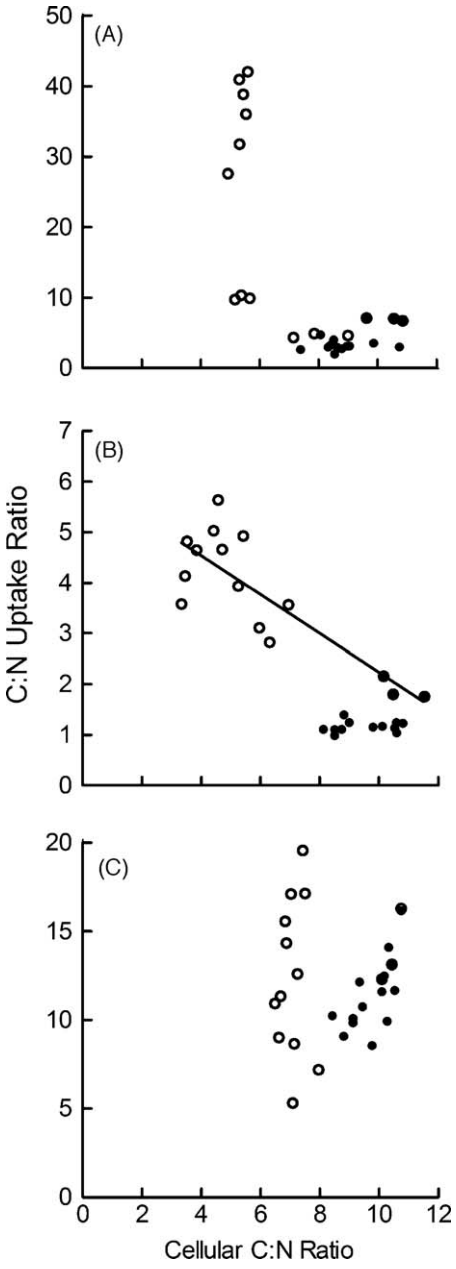

Fig. 10. Relationship between cellular C:N ratio and cellular C:N uptake ratio of *Alexandrium tamarense* grown on nitrate (A), ammonium (B) and urea (C) supplied in pulses (closed circle) and continuously (open circle). A line indicates significant relationship between cellular C:N ratio and cellular C:N uptake ratio ($r^2 = 0{:}70$; $p < 0.01$).

The present study showed that nitrate, ammonium and urea all serve as good N sources for *A. tamarense*. However, differences in the C and N uptakes were observed among the three N sources and for the two supply modes. Phytoplankton cells are bound to be exposed to changes in environmental conditions such as N concentrations and form of supply. This change in N environments might be a factor determining the balance between C and N uptake. This interaction may allow *A. tamarense* to maintain high and uninterrupted growth under varying N environments. Therefore, the form of N supply in coastal environments could play an important role in controlling the nutrient uptake of toxic dinoflagellate, and thus the duration of a bloom.

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