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Growth and toxin production of tropical *Alexandrium minutum* Halim (Dinophyceae) under various nitrogen to phosphorus ratios

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Abstract Effects of nitrogen to phosphorous (N/P) ratios of two nitrogen sources (nitrate and ammonium) on growth and toxin production of a tropical estuarine dinoflagellate, *Alexandrium minutum* Halim, were examined using a strain isolated from a bloom at Tumpat Estuary, Malaysia in September 2001. Experiments were carried out in batch cultures, using either nitrate ($N\text{-}NO_3$) or ammonium ($N\text{-}NH_4$) as the nitrogen source at a constant amount, and with initial N/P ratios ranging from 5 to 500. Cell density, residual N and P in the medium, cellular toxin quota (Q_t), and toxin composition were analyzed throughout the growths. Our results showed that cell densities and growth rates of *A. minutum* were severely suppressed under high N/P ratios (>100) in both $N\text{-}NO_3$ and $N\text{-}NH_4$ treatments. Cells tended to be larger at lower growth rate and P-limited cultures. Toxin profile was relatively constant throughout the experiments, with GTX4/GTX1 as the dominant toxin congeners. Cellular toxin quota (Q_t) increased with elevated N/P ratios in both $N\text{-}NO_3$ and $N\text{-}NH_4$ treatments. Toxin production rate, R_{tox}, however was enhanced in $N\text{-}NH_4$-grown cultures when P was limited, but showed no difference between $N\text{-}NO_3$- and $N\text{-}NH_4$-grown cultures when P was replete. Our results clearly showed that N/P ratios as well as the nitrogen compounds not only affected the growth of *A. minutum*, but also the cellular toxin quota and its toxin production rate.

Keywords *Alexandrium minutum* · N/P ratio · Growth · PST production · Tropical

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Introduction

Coastal eutrophication has been increasingly reported not only in developed but also in developing countries, mainly due to the increasing of coastal inhabitants, maricultures, terrestrial origin runoff, as well as other human activities. Dissolved organic nitrogen (N) and phosphorus (P) and nutrients from allochthanous sources in eutrophic waters are the main source of eutrophication (Glibert et al. 2001). Increases of nitrogenous and phosphorus nutrients in estuarine and coastal waters have resulted in changes in nutritional status which favored the proliferation of a selected group of phytoplankton (Balode et al. 1998), and decreases in N/P ratios due to phosphorus loading have been related to harmful algal bloom events (Hodgkiss and Ho 1997). This includes toxic dinoflagellates of the genus *Alexandrium* that are frequently associated with paralytic shellfish poisoning intoxications.

In tropical estuarine coastal regions, seasonal and intratidal fluctuation in nitrogen to phosphorus (N/P) ratios is common over the annual cycle. For example, these ratios vary from 25:1 to ca. 70:1 during monsoonal rainy and dry seasons as well as neap and spring tides in Malaysian mangrove estuaries (Tanaka and Choo 2000). Changes of the nutrient pool affect

not only the growth of the organisms but also their biochemical composition. In laboratory controlled setting, cellular toxin (Q_t) of some paralytic shellfish toxins (PSTs) producing species are induced under P-limited or high N/P ratio conditions (Boyer et al. 1987; Anderson et al. 1990b; Bechemin et al. 1999), while other studies showed that an increase of Q_t is due to simultaneous N and P limitations (Flynn et al. 1994; John and Flynn 2000). The difference might be due to ecotypic variation in ecophysiological adaptation in the environment from where they originated. Thus, studies of species/strains of *Alexandrium minutum* from different environments or geographical regions are necessary to obtain a clear picture of the growth physiology of the species.

Toxic *A. minutum* was found for the first time in Tumpat estuarine coastal lagoon, northeastern of Peninsula Malaysia in September 2001 (Lim et al. 2004). Blooms of the species occurred in a semi-enclosed lagoon, with salinity ranging from 15 psu near the mouth of the lagoon to 11 psu in the inner part of the lagoon, which is a meeting point between the Golok River and the South China Sea that bordered Malaysia and Thailand. In our previous study, we found that this species adapts to wide ranges of salinity (Lim and Ogata 2005) and has a unique light adaptation strategy (Lim et al. 2006) compared to other strains and species reported elsewhere. Therefore, it is important to determine the growth characteristic of this tropical species under different nutritional status. In this study, the effect of nitrogen to phosphorus ratios on growth and toxin production of *A. minutum* from Tumpat Estuary was studied.

Materials and methods

Clonal cultures of *A. minutum* were established from Tumpat, the northeast coast of Peninsula Malaysia in September 2001. The cultures were grown in ES-DK medium (Kokinos and Anderson 1995) and maintained at 25±0.5°C under a 15:9 L/D cycle at a mean photosynthetic photon flux density of 140 μmol photons m^{-2} s^{-1}. The AmKB06 strain was used in this study. Seawater (33 psu salinity) from Okkirai Bay was used as the medium base. Medium salinity was adjusted to 15 psu by dilution with deionized distilled water. The pH of culture medium was adjusted to 7.8–7.9.

Experiment was carried out with nitrate ($N\text{-}NO_3$) or ammonium ($N\text{-}NH_4$) as the sole nitrogen source. Cultures were grown in 200 mL of ES-DK medium (Kokinos and Anderson 1995) enriched with initial constant concentrations of $N\text{-}NO_3$ at 430 μM or $N\text{-}NH_4$ at 200 μM. The high concentration of NO_3 was supplied to ensure sufficient N to sustain growth in batch culture throughout the growth cycle. The concentration of NH_4 was applied at lower concentration compared to NO_3 in this experiment due to growth inhibition at a concentration higher than 200 μM. Different molar concentrations of phosphate ($P\text{-}PO_4$) were added to yield five N/P regimes for each nitrogen source (Table 1). Orto-phosphate (PO_4^{3-}) was used as the phosphorus source. All treatments were conducted in duplicate. Cell abundance, the concentration of NO_3^-, NH_4^+, PO_4^{3-}, and toxin content per cell were analyzed at 2–3-day intervals throughout the experiment.

Culture growth was monitored by subsampling for cell counts in 2-day intervals. Cells were fixed in Lugol's solution and counted microscopically using Sedgewick-Rafter chamber. Specific growth rate, μ (day^{-1}), was calculated over the exponential growth phase using the following equation (Guillard 1973):

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_0 and N_1 are the cell density at time t_0 and t_1, respectively.

Samples for cell diameter measurement were taken at day 12 and fixed in Lugol's solution. Measurement was made using an Olympus BX40 microscope under ×200 magnification and calibrated with a micrometer as described earlier. Mean cell volume was calculated with assumption of the spherical shape of the dinoflagellate cell as described by Hillebrand et al. (1999) using the following equation:

$$v = \frac{\pi}{6} d^3$$

Table 1 Nitrogen to phosphorus (N/P) ratios at the initiation and during the exponential phase of each N/P treatment with nitrate (N) and ammonium (A) as the nitrogen source

N/P treatments		Initial N/P	Average N/P at the exponential phase
Nitrate as N source			
NLLP	P-limited	500	496.4 ± 11.5
NLP		100	143.2 ± 4.0
NP		33	31.1 ± 13.6
NHP		16	13.3 ± 1.9
NHHP	P-replete	6	4.6 ± 0.6
Ammonium as N source			
ALLP	P-limited	230	230.1 ± 41.4
ALP		70	135.3 ± 73.9
AP		27	63.2 ± 50.6
AHP		15	16.7 ± 6.9
HAHP		16	13.1 ± 2.4
AHHP	P-replete	6	3.0 ± 2.3

N nitrate, *A* ammonium, *LP* low phosphate, *HP* high phosphate, *LLP* extremely low phosphate, *HHP* extremely high phosphate

where d is the diameter of cells. Cell volume (v) was calculated from a total of 30–80 cells and presented as the mean.

The relationship between specific growth rates (μ) and phosphate concentrations was described by modeling μ as a function of [P-PO_4] according to the Michaelis–Menten equation (Turpin 1988):

$$\mu = \mu_{\max} \frac{S}{S + K_s}$$

where $\mu_{\max}$ is the maximum growth rate, S is the concentration phosphate, and K_s is the half saturation constant.

Nutrient concentrations were measured immediately after sampling using the following protocols. Nitrate (N-NO_3) concentrations were determined by UV spectrophotometry at 220 nm (Carvalho et al. 1998; Collos et al. 1999). Samples were centrifuged at 3,000×g for 5 min to remove cells and particulate matter, and the supernatant was used to determine nitrate concentrations. Two milliliters of the supernatant was diluted to 10 mL with Milli-Q water, and 0.2 mL 1 N HCl solution was added. Absorbance was then read at 220 and 275 nm. N-NO_3 absorbance was calculated by subtracting two times the absorbance at 275 nm for background correction from the absorbance at 220 nm. A calibration curve was generated with standards in the range of 16–112 μM.

Measurement of ammonium (N-NH_4) concentrations was carried out using indophenol blue method according to Koroleff (1970). Concentrations of N-NH_4 were determined spectrophotometrically at 630 nm. A calibration curve was generated using standards in the range of 0–40 μM.

Measurement of phosphate (P) concentrations was carried out according to the modified method of Strickland and Parsons (1968). The absorbance was measured spectrophotometrically at 885 nm. A calibration curve was generated with known concentration of phosphate in the range of 0–30 μM.

Analysis of PSTs by high performance liquid chromatography (HPLC) was carried out using the isocratic, post-column derivatization method of Oshima (1995) on a JASCO HPLC system as described earlier (Lim et al. 2006). The net toxin production rate, R_{tox} (femtomole PST per cell per day) was determined using the equations described by Anderson et al. (1990b).

Results

Growths

In nitrate-grown (N-NO_3) cultures, the highest cell density of *A. minutum* was observed in nitrate high-phosphate (NHP) culture (with average N/P ratio of 13 during the exponential phase, Table 1) with a mean value of 68,000 cells mL^{-1} (Fig. 1a). Increase in cell densities among NHP, NP (average N/P ratio of 30), and nitrate extremely high-phosphate (NHHP; average N/P ratio of 5) cultures did not vary significantly (ANOVA $p>0.05$). However, cell densities decreased markedly (ANOVA $p<0.01$) in P-limited nitrate low-phosphate (NLP; average N/P ratio of 140) and nitrate extremely low-phosphate (NLLP; average N/P ratio of 500) cultures, with the lowest cell density of 2,200 cells mL^{-1} in NLLP cultures.

In ammonium-grown (N-NH_4) cultures, again growth was impeded in P-limited cultures (ammonium low-phosphate (ALP) and ammonium extremely low-phosphate (ALLP)) with the lowest cell density of 1,300 cells mL^{-1} in ALLP cultures (Fig. 1b). The highest cell density of 18,000 cells mL^{-1} was achieved at P-balanced ammonium high-phosphate (AHP) cultures (average N/P ratio of 17), even though growths of AHP and ammonium extremely high-

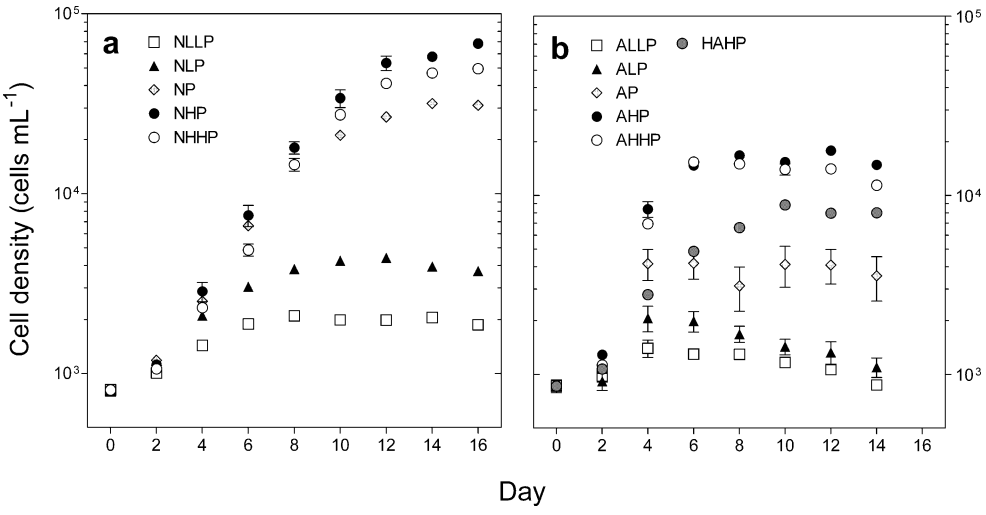

Fig. 1 Cell densities of *Alexandrium minutum*, AmKB06 under various N/P treatments in nitrate-grown (**a**) or ammonium-grown (**b**) cultures

phosphate (AHHP) cultures did not differ significantly over the growth cycle (ANOVA $p>0.05$). A sharp decrease (close to fourfold) in cell yields was found in AP cultures compared to AHP and AHHP cultures. P was taken up in both AP and ALP cultures causing drastic increases in N/P ratios during the exponential phases (Table 1). Interestingly, this tropical strain of *A. minutum* grew in medium with excess ammonium concentration (400 μM) when P was replete (HAHP culture). However, the maximum cell density (9,000 cells mL^{-1}) was twofold lower than cultures grown in medium with 200 μM N-NH_4 (AHP).

The specific growth rates (μ) were elevated with increasing phosphate concentration in both nitrate and ammonium media (Fig. 2), with the highest μ (0.65 day^{-1}) observed in N-NH_4-grown cultures. The half saturation constant, K_s, for uptake of phosphate was 3.64 and 2.51 μM for N-NO_3- and N-NH_4-grown cultures, respectively. Cells tended to be larger in cultures with lower concentrations of phosphate for both nitrate and ammonium regimes (Fig. 3).

Toxins

In the exponentially growing N-NO_3 cultures, high toxin cell quota, Q_t, was observed when N/P ratios >100 (NLP and NLLP cultures), with Q_t around 20 fmol PST $cell^{-1}$ (Fig. 4a). In N-NH_4-grown cultures, changes of Q_t with different N/P treatments were more drastic. The highest Q_t was observed in P-limited cultures (ALLP), about fourfold (80 fmol PST $cell^{-1}$) higher than N-NO_3-grown cultures (Fig. 4). No significant changes in Q_t over the exponential phases in AHP and AHHP cultures, as well as cultures with two times higher ammonium concentration (HAHP; Fig. 5). Toxin composition throughout the experiments remained relatively stable, with GTX4 and GTX1 as the major toxin components, accounting for 70–95 mol% of total toxins. No C1/2 toxins were found in detectable amounts over the growth stages.

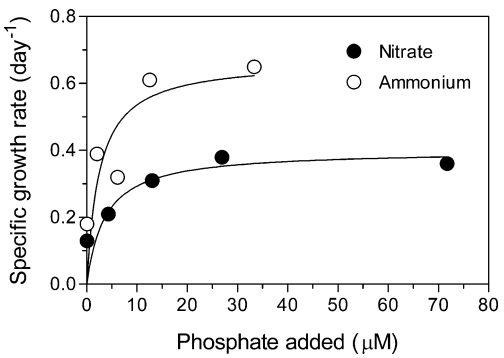

Fig. 2 Specific growth rate of *Alexandrium minutum*, AmKB06 in nitrate-grown (*filled circle*) or ammonium-grown (*empty circle*) cultures

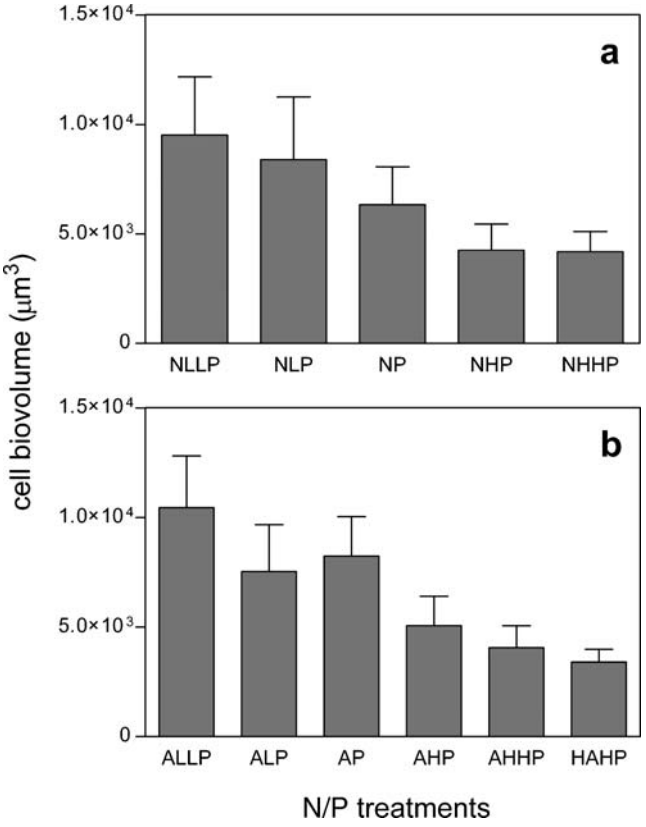

Fig. 3 Changes of cell biovolume of *Alexandrium minutum*, AmKB06 under various N/P treatments in nitrate-grown (**a**) or ammonium-grown (**b**) cultures

Toxin production rate, R_{tox}, varied among different N/P treatments and also with different sources of nitrogen. In N-NO_3-grown cultures, an increase of R_{tox} was observed in cultures when P was replete (Fig. 6). The highest R_{tox} was found in P-balanced NP cultures (N/P ratio ~30). R_{tox} decreased slightly with elevated P concentrations and remained constant. On the other hand, in N-NH_4-grown cultures, R_{tox} decreased dramatically with elevated P concentrations (Fig. 6). The highest R_{tox} was observed at P-limited ALLP cultures (N/P ratio ~230).

Discussion

Blooms of *Alexandrium minutum* Halim have been frequently associated with nutrient-enriched coastal waters, including estuaries, lagoons, ports, and semi-enclosed bays (Delgado et al. 1990; Lim et al. 2004). Several studies have been carried out to investigate the effect of N/P supply ratios on growth physiology of PSTs producing species, including the species *A. minutum*. However, most of the studies only focused on temperate or subtropical species. Thus, it is important to examine the growth physiology of tropical *A. minutum*. Different levels

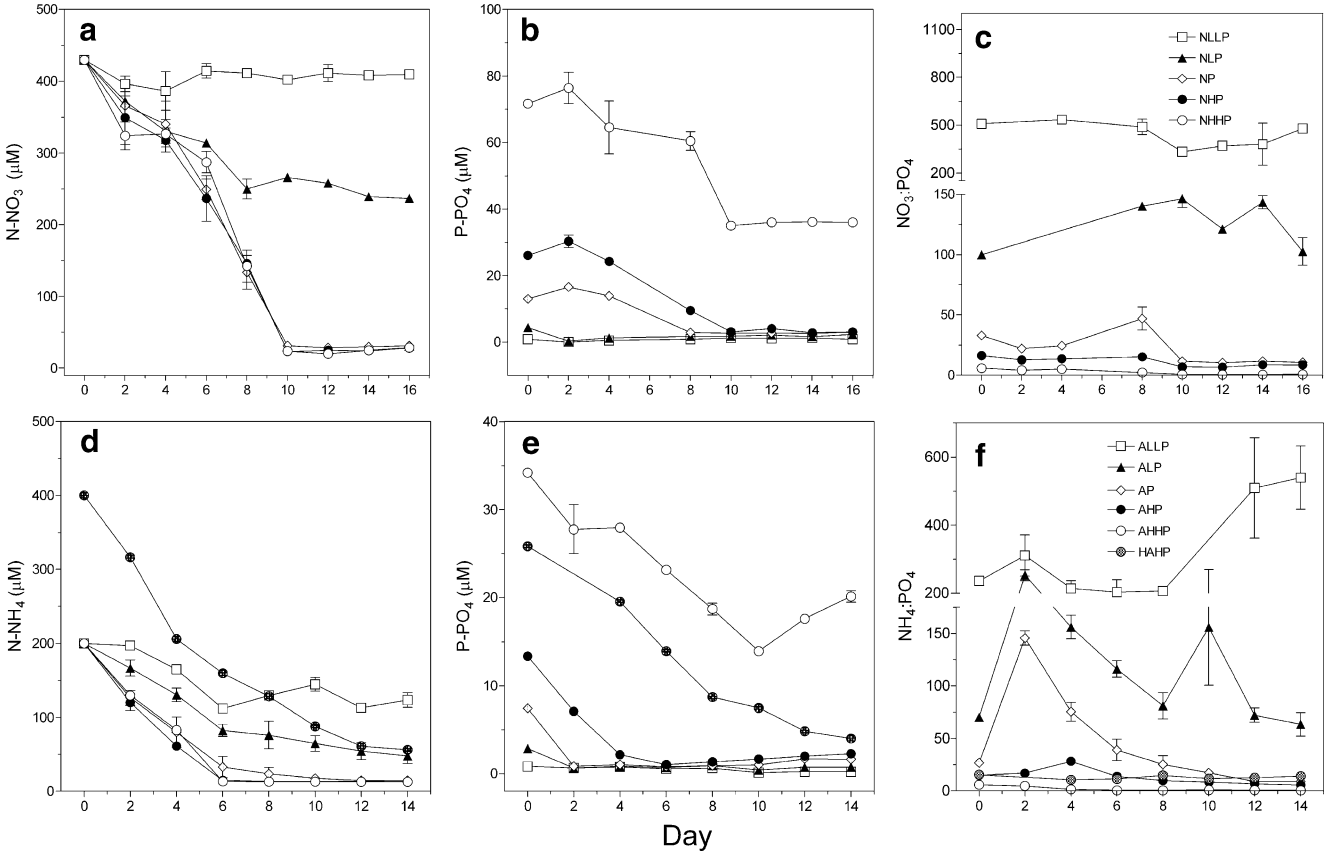

Fig. 4 Extracellular N, P, and N/P ratios of *Alexandrium minutum*, AmKB06 in nitrate-grown (**a**–**c**) and ammonium-grown (**d**–**f**) cultures throughout the growth cycle for all the N/P treatments

of N have been applied in N/P studies with some previous studies using high concentration of N (100–500 μM) in their experimental setup (Boyer et al. 1987; Lim et al. 2006; Anderson et al. 1990a), while others used low concentration of N (Flynn et al. 1994; John and Flynn 2000). These variations may yield differences in cell physiology. Cells of *A. minutum* originating from different trophic waters might represent distinct ecotypes in adaptation to nutrient uptake. Our results from this study may imply growth and toxin production of *A. minutum*

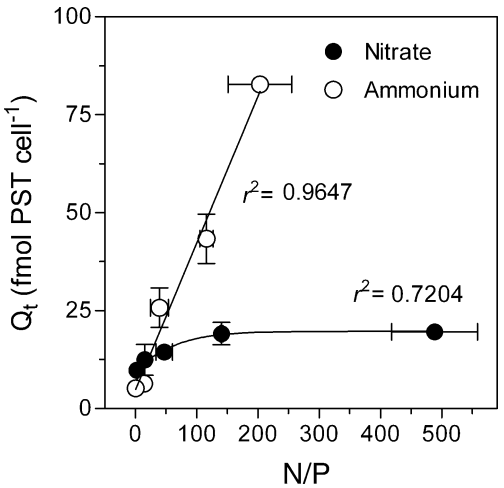

Fig. 5 Cellular toxin quota (Q_t) of *Alexandrium minutum*, AmKB06 under various N/P treatments in nitrate-grown (*filled circle*) or ammonium-grown (*empty circle*) cultures

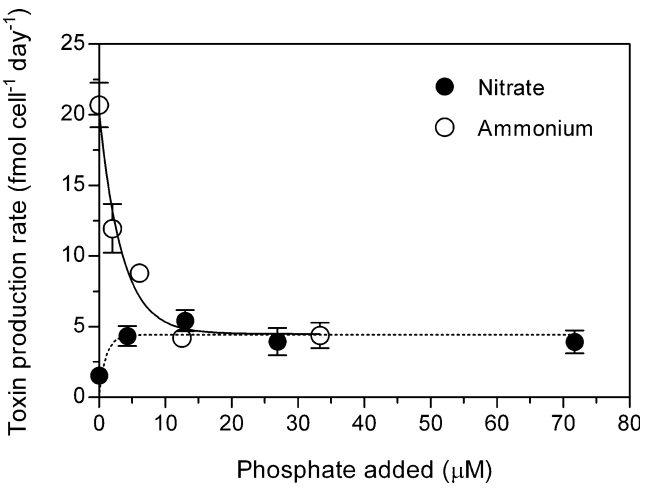

Fig. 6 Toxin production rate (R_{tox}) of *Alexandrium minutum*, AmKB06 under different N/P treatments in nitrate-grown (*filled circle*) or ammonium-grown (*empty circle*) cultures

from highly eutrophic waters. The other studies might represent ecotype/strain from oligotrophic waters (e.g., John and Flynn 2000).

In the growth response to N/P supply ratios of *A. minutum*, AmKB06 was generally similar to those reported in some other *Alexandrium* species, such as *A. tamarense* (Boyer et al. 1987) and *A. fundyense* (Anderson et al. 1990a). Growths were suppressed under P limitation. Because no P was added in LLP cultures, *A. minutum* could only obtain limited external phosphate that was available in the natural seawater (<1 μM) or by intracellular phosphate storage. The culture inoculums were originated from cultures at late exponential phase. Thus, the amount of macronutrients carried over from the parent culture stock was insignificant. This was further verified by the level of N and P detected in the initial phase and throughout the culture experiment (Fig. 4). High N-NO_3 concentration was supplied in the experiments to ensure no nitrogen limitation during the exponential phases.

In the absence of phosphate, the growth and carbon fixation of *Alexandrium* species were shown to continue for several generations, and phosphate may only be limited under N/P ratios which are significantly higher than the Redfield ratio (John and Flynn 2000). John and Flynn (2000) also suggested that N/P ratios higher than 36 (mass ratio) is required for the culture to reach a P-limited condition. In our nitrate-grown culture experiments, P was only seemed to be limited when the ambient N/P ratios were higher than 100 (Fig. 4). However, it is noteworthy that in the ammonium-grown cultures, P was taken up in a relatively high rate at the early growth stage (day2) for all the N/P treatments and become limited before entering the exponential phase, regardless of the N/P ratio treatments. This was probably due to the luxury uptake of cells to store an excess amount of phosphate when it was available in the culture medium. Luxury uptake is an adaptation for uptake in excess of that required for immediate growth.

Our experiments showed that the optimum growth rates of the strain were observed between N/P ratios of 5:13 and 3:16 in both nitrate- and ammonium-grown cultures, respectively. The optimal N/P ratios for growth slightly differed from previous studies on other strains of *A. minutum* (Giacobbe et al. 1996; Bechemin et al. 1999; Ignatiades et al. 2007). However, variation in optimal N/P ratios has been reported among different species. Some bloom-forming dinoflagellates showed optimum growth at N/P ratios lower than the Redfield ratio (N/P=16). Hogkiss and Ho (1997), for example, demonstrated that three *Prorocentrum* species, *P. micans*, *P. sigmoides*, and *P. triestinum* grew optimally between N/P ratios of 5 to 15.

N/P ratios also affected the cell size and biovolume. Our results showed that the increase of N/P ratios (which increased P stress) resulted in an increase of the cell size of *A. minutum*. This was also observed in other species (Latasa and Berdalet 1994; John and Flynn 2000). John and Flynn (2000) suggested that this increase in cell biovolume is due to the arrest of cells in the G1 phase (Vaulot et al. 1996) without undergoing cell division, while other non-P compounds continued to be synthesized.

Ammonium at high concentration (>400 μM) also showed a growth inhibitory effect. Our results showed that at high N-NH_4 cultures (HAHP, average N/P ratio of around 13) both growth and yield were lower compared to cultures with 200 μM of N-NH_4 (AHP, average N/P ratio of around 16). Dixon and Syrett (1988) suggested that high-N systems may cause a cell-density-dependent cessation of growth. Even though ammonium is commonly found in the natural water, the role of ammonium in the bloom development is still unclear. Maguer et al. (2004) found that ammonium regeneration rates during the blooms of *A. minutum* were only slightly higher than before the blooms. Thus, ammonium in the water body might not be the main N source to sustain the blooms (Maguer et al. 2004).

The half saturation constants, K_s, for uptake of phosphate in this study were found to be much higher than those observed in some other studies of *Alexandrium* species (Table 2). However, the value is much lower than those observed in *P. minimum* (Cembella et al. 1984). The difference might due to ecotypic variation among different species. The high level of phosphate applied in this study in relation to other studies might also contribute to the difference in K_s.

Table 2 Summary of maximum growth rate (μ_{max}) and half saturation coefficient (K_s) for phosphate uptake of various *Alexandrium* species

Species (strain)	μ_{max}	K_s (μM)	Reference
A. minutum (AmKB06)	0.40	3.64	This study, in N-NO_3-grown culture
A. minutum (AmKB06)	0.67	2.51	This study, in N-NH_4-grown culture
A. minutum (AL1V)	0.298	1.16	Frangópulos et al. (2004)
A. minutum (L1)	0.24	0.12	Ignatiades et al. (2007)
A. tamarense (MDQ1096)	0.276	1.68	Frangópulos et al. (2004)
A. tamarense (EF04)	0.253	1.00	Frangópulos et al. (2004)
A. tamarense (ATHS92)	0.54	2.6	Yamamoto and Tarutani (1999)

Phosphate is required as one of the key elements in biosynthesis or regulatory enzymes in PSTs biosynthesis (Cembella 1998). Previous studies on PSTs producing dinoflagellates have shown that P limitation induced toxin production and increased intracellular toxin concentrations (Boyer et al. 1987; Anderson et al. 1990a; Siu et al. 1997; Hwang and Lu 2000; Lippemeier et al. 2003). In contrast, some other studies showed that only P limitation did not affect the intracellular toxin concentrations; indeed, toxin content increased with simultaneous N and P limitations (Flynn et al. 1994; John and Flynn 2000). At P-limited conditions, our results consistently showed the increase of toxin content in *A. minutum*. However, when ammonium was supplied as the nitrogen source in the P-limited culture, higher Q_t was observed compared to nitrate-grown cultures. This was strongly supported by the studies of Wood and Flynn (1995) and John and Flynn (2000). Wood and Flynn (1995) proposed that cultures with nitrate as the nitrogen source caused a level of N stress in the cells' metabolic activity that would be likely to decrease the availability of N for toxin synthesis, while John and Flynn (2000) suggested that P stress might affect the intracellular N cycling, thus a supply of ammonium rather than nitrate is likely to enhance toxin synthesis.

The toxin composition of *A. minutum* is relatively stable over the different N/P ratio treatments as well as under both nitrate- and ammonium-grown cultures. However, variation was observed in toxin composition in the late exponential phase of nitrate-grown cultures. The increases in the mole percent of STX and GTX2 might be due to suppression of toxin production of β-form (GTX4/1), which is the predominant toxin in this species. Lower GTX4/1 resulted to higher proportion of STX and GTX2 as observed in this study. The question arose on why this phenomenon was only observed in nitrate-grown cultures but not in ammonium-grown cultures. One of the explanations for this phenomenon is that nitrate resulted in cell metabolic stress, thus the supply of ammonium rather than nitrate will enhance toxin synthesis as suggested by Wood and Flynn (1995).

In conclusion, our results clearly showed that N/P supply ratios affected not only the growth of *A. minutum*, but also its cellular toxin quota and toxin production rate. P limitation (high N/P) led to an increase of cellular toxin quota. Cellular toxin quota was enhanced in ammonium-grown relative to those grown in nitrate. Further study on the occurrence of this species in Tumpat Estuary and its bloom conditions will be essential to understand the bloom dynamics of this tropical estuarine *Alexandrium* species.

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