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Effects of inorganic and organic nitrogen and phosphorus on the growth and toxicity of two *Alexandrium* species from Hong Kong

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ABSTRACT

The effects of three nitrogen (N) and two phosphorus (P) inorganic and organic forms on the growth, toxin content and composition, toxin production, and chemical composition of *Alexandrium catenella* and *Alexandrium tamarense* isolated from coastal waters of Hong Kong were determined. The toxin production rate and cellular toxin content for *A. catenella* were at least 10-fold higher than *A. tamarense*. The highest net production rate (R_{tox}) of the two *Alexandrium* species was generally achieved in the exponential phase. However, the highest cellular toxin content occurred in the stationary phase in all cultures, partly due to the enhancement of cell volume caused by P limitation, except for urea grown cultures where cellular toxin content remained low throughout the growth stage. For *A. catenella*, NH_4 induced the highest growth rate (0.59 d^{-1}), toxin production rate (μ_{tox}, 1.0 μmol L^{-1} d^{-1}; R_{tox}, 2.5 pmol $cell^{-1}$ d^{-1}) and cellular toxin content (2.8 pmol $cell^{-1}$) among the three nitrogen sources regardless of inorganic and organic P. The form of phosphorus had limited effect on *A. catenella*. In contrast, the response of *A. tamarense* to different forms of nitrogen and phosphorus was more complex. NH_4 induced the highest cellular toxin content (445 fmol $cell^{-1}$), while NO_3 yielded the highest toxin production rate (μ_{tox}, 0.71 nmol L^{-1} d^{-1}; R_{tox}, 140 fmol $cell^{-1}$ d^{-1}) and urea produced the highest growth rate (0.57 d^{-1}). For *A. tamarense*, the highest toxin production rate occurred under organic phosphorus. The relationship between toxin accumulation and the form of nitrogen varied with the phosphorus source. *A. catenella* cultures grown on NO_3 and NH_4 have about 80–90% C1/2 toxins and 5–15% GTX 1/4 toxins compared to 65–75% C1/2 toxins and 25–35% GTX 1/4 toxins in cultures grown on urea. Our results suggest that during summer when *Alexandrium* uses NH_4 from local sewage effluent as its preferred nitrogen source, it might become more toxic in combination with episodically occurring P limitation in Hong Kong waters.

1. Introduction

Over the last few decades, numerous studies have shown that toxicity often varies greatly among *Alexandrium* species and strains of the same species that have a different nutritional status (Boyer et al., 1987; Anderson et al., 1990a; Bechemin et al., 1999; John and Flynn, 2000; Hamasaki et al., 2001; Wang and Hsieh, 2002) and environmental conditions (Ogata et al., 1987; Flynn et al., 1994; Hwang and Lu, 2001; Hamasaki et al., 2001). However, little attention has been paid to the effect of the form of the nutrient (e.g. inorganic vs organic) on the level of toxicity of *Alexandrium* species.

Another focus of our toxin study was on the interaction between toxin composition and nutrients. There are two contradictory views on the stability of toxin composition of *Alexandrium* isolates. One view is that toxin composition can be considered as a genetic fingerprint (Cembella et al., 1987; Flynn et al., 1994). The other suggestion is that toxin composition in *Alexandrium* isolates often varies with growth phase and culture conditions (Anderson et al., 1990b, 1994; Hamasaki et al., 2001). Furthermore, Anderson et al. (1990b) reported that nitrogen limitation enhanced the relative production of C1 and C2 for *Alexandrium fundyense*, as well as GTX1 and GTX4, while phosphorus limitation led to an increase in the relative production of GTX2 and GTX3. These two different views likely result from the difference in the experimental design since in the latter studies, cultures were grown for extended intervals with specific degrees of nutrient limitation, allowing the

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cells to modify their toxin composition and achieve a steady state. Clearly, more studies are needed to reconcile this discrepancy and confirm whether this explanation is valid.

Hong Kong waters receive various forms of nitrogen (i.e. NO_3 from the Pearl River discharge in summer and NH_4 and urea from year round local sewage effluent). In summer, the southern and western waters of Hong Kong are potentially phosphorus-limited due to high nitrogen inputs from the Pearl River (Yin et al., 2000; Xu et al., 2009). A routine monitoring program on PSP toxicity in shellfish has been implemented in Hong Kong since 1984 (Lam et al., 1989). Algal blooms of the PSP-producing dinoflagellate *Alexandrium* species have been documented in Hong Kong which have caused fish kills (Chan, 1998). A bloom of *Alexandrium catenella* was recorded in March 2011 in the fish culture zone with high NH_4 concentrations (http://www.afcd.gov.hk/tc_chi/fisheries/hkredtide/update/updata_sub.asp). Little is known about the factors that caused the toxic *Alexandrium* blooms in Hong Kong.

The present study focused on the effect of three nitrogenous (ammonium, nitrate and urea) and two phosphorus sources (inorganic and organic) on the growth, toxin production and toxin composition of two red tide causative *Alexandrium* species from Hong Kong waters during three growth phases, exponential, and early and late senescence. The culture medium had a high N:P ratio (~80:1) since toxin production is higher under P limitation than N limitation and hence the latter two growth phases were P-limited with saturation N concentrations. It is important to determine what forms of inorganic and organic nitrogen or phosphorus can stimulate higher toxin production of *Alexandrium* species to assist in evaluating the factors underlying deleterious effect of toxic *Alexandrium* blooms in the Hong Kong region.

2. Materials and methods

2.1. Dinoflagellate cultures

The two toxic dinoflagellates *Alexandrium tamarense* and *A. catenella* were isolated and identified, based on the morphology of the species, from local HK waters by the Agricultural, Fisheries and Conservation Department (AFCD) of Hong Kong Government in 2002 and by our laboratory in 2005, respectively. The batch cultures were grown in 3 L of natural filter-sterilized seawater in 5 L conical flasks and supplemented with K medium (Keller et al., 1987) without silicate with 320 μM of three nitrogenous nutrients: ammonium (NH_4), nitrate (NO_3), and urea. Each nitrogenous treatment was supplemented with 4 μM of inorganic sodium phosphate (IP) and organic sodium glycerol-phosphate (OP). All experiments were conducted in triplicate at 23.5 °C (similar to in situ water temperatures in Hong Kong), on a 14:10 h L:D cycle at a light intensity of 120 μmol m^{-2} s^{-1} that is optimal for algal growth of these species (Ho, 2007). During the incubation period, pH was measured for each culture in order to assess if carbon limitation occurred. When the pH exceeded ~8.8, Na_2CO_3 was added with the final concentration of 2 mM in the medium. Samples were taken from each culture every 2–3 days for the measurement of cell growth and toxin quantification.

2.2. In vivo fluorescence, cell counts, cell volume, Chl a, nutrients, particulate organic carbon and nitrogen

In vivo fluorescence was measured with a fluorometer (Turner Designs). Cell count samples (1 ml) were fixed in Lugol's solution and counted using an inverted light microscope equipped with a camera. Photos were taken and the diameters of ~50–150 cells from each sample were measured. The cell volume was calculated from the averaged cell diameter assuming all cells were spherical. Chl *a* samples (20 ml) were filtered through a 25 mm GF/F filter, extracted using 90% acetone and determined using in vitro fluorometric methods (Parsons et al., 1984). The filtrate was collected and used to measure inorganic nutrients (NO_3, NH_4, and PO_4) using a Skalar San Nutrient Analyzer following JGOFS protocols (Knap et al., 1996). Urea was measured using the diacetyl monoxime thiosemicarbizide (Price & Harrison, 1987). Samples for particulate organic carbon (POC) and organic nitrogen (PON) (20 ml) were filtered through a 25 mm pre-combusted (450 °C, 4 h) GF/F filter, and POC and PON was determined using a CHNS elemental analyzer (Perkin Elmer PE 2400 Series II) following JGOFS protocols (Knap et al., 1996).

2.3. Toxin analyses

Cells in a 10–15 ml sample were pelleted by centrifugation at 3600 × *g* for 10 min, the supernatant removed, and the pellet was resuspended in 0.5 ml of 50 mM acetic acid. Cells were disrupted by freezing and thawing, followed by sonication on ice. The extracts were then centrifuged at 10,000 × *g* for 5 min at 4 °C and the supernatants were analyzed for toxins using a Hewlett-Packard HP1100 high performance liquid chromatography-fluorescence detection system (HPLC-FLD) with post column derivatization and an intersil C8-5 column, as described by Wang et al. (2006). Three mobile phases were used to separate various toxins at a flow rate of 0.8 ml min^{-1}: (1) 2 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for C toxins; (2) 2 mM l-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for the gonyautoxin group (Wang et al., 2006). (Abbreviations of toxins are as follows: C1, 2 = toxins C1, C2; GTX1/4 = gonyautoxins 1 and 4; GTX2/3 = gonyautoxins 2 and 3; GTX5 = gonyautoxin 5.)

2.4. Calculation of growth rate and toxin production rate

The average growth rates, μ (d^{-1}), of the two *Alexandrium* species in the exponential growth phase were calculated using the following equation:

$$\mu = \ln\left(\frac{N_t/N_0}{t_1 - t_0}\right)$$

where N_t and N_0 were the in vivo fluorescence reading for the initial t_0 and at time t, respectively.

The total toxin (sum of C1/2, GTX1/4, GTX2/3 and GTX5) production rate μ_{tox} (amount of toxin L^{-1} d^{-1}) in the cultures throughout the growth phase was calculated as follows:

$$\mu_{tox} = \frac{\ln(C_2T_2/C_1T_1)}{t_2 - t_1}$$

where the toxin concentration, C_tT_t (amount of toxin L^{-1}), was calculated by multiplying C_t, cell concentration (cells L^{-1}), by T_t, the cellular toxin content (amount of toxin $cell^{-1}$) at time t.

To account for the effect of cell growth rates on toxin production, the net toxin production rate (amount toxin $cell^{-1}$ d^{-1}) was determined over each growth phase in the batch culture following the equation of Anderson et al. (1990a) and Tong et al. (2011).

$$R_{tox} = \frac{C_2T_2 - C_1T_1}{\bar{C}(t_2 - t_1)}$$

where $\bar{C}$ is the ln average of the cell concentration,

$$\bar{C} = \frac{C_2 - C_1}{\ln(C_2/C_1)}$$

2.5. Statistical analyses

A two-way ANOVA followed by Tukey's multiple comparison test (Tukey's HSD) and *t*-test were performed using SPSS 11.0 to compare the differences in growth rate, cell volume and total cellular toxin content among cultures grown on different N and P forms in three growth phases, exponential, early stationary, and mid-late stationary phases.

3. Results

3.1. Growth on inorganic and organic N and P

The cell density and in vivo fluorescence curves for *A. catenella* and *A. tamarense* were similar. All *A. catenella* and *A. tamarense* cultures grew exponentially during days 1–4 (Fig. 1). Both inorganic and organic PO_4 concentrations declined to <0.2 μM P in all cultures on day 4 when nitrogen (NH_4, NO_3 and urea) concentrations remained high (>150 μM N). After a subsequent long stationary phase lasting 18 days, the culture entered senescence phase on days 18–22 (Fig. 1). The maximal cell density was 6–8 × 10^6 cells L^{-1} for all *A. catenella* cultures and 4–6 × 10^6 cells L^{-1} for most *A. tamarense* cultures, except for those grown on NH_4 with OP (Fig. 1).

Table 1
Analysis of variance (ANOVA), Tukey's and *t*-tests of pooled data showing the effect of N and P sources on the growth rate (based on in vivo fluorescence data) of *A. catenella* and *A. tamarense*.

Growth rate	*A. catenella*	*A. tamarense*
Two-way ANOVA		
Tukey's test: effect of N		
Inorganic PO_4	$NH_4 \gg NO_3 > Urea$	$Urea > NO_3 \gg NH_4$
Organic PO_4	$NH_4 \gg Urea > NO_3$	$Urea \gg NO_3 \gg NH_4$
t-test: effect of P		
NH_4	IP > OP	IP ≫ OP
NO_3	IP > OP	IP ≫ OP
Urea	OP > IP	OP ≫ IP

"≫" indicates that the former is significantly ($p < 0.05$) higher than the latter. ">" indicates that the former is higher, but not significantly higher than the later.

For *A. catenella* cultures, irrespective of the phosphorus form, the growth rate (0.59 d^{-1}) for the NH_4 treatments was significantly ($p < 0.05$) higher than those in both NO_3 and urea treatments (0.53–0.56 d^{-1}) in which the growth rates on NO_3 and urea did not

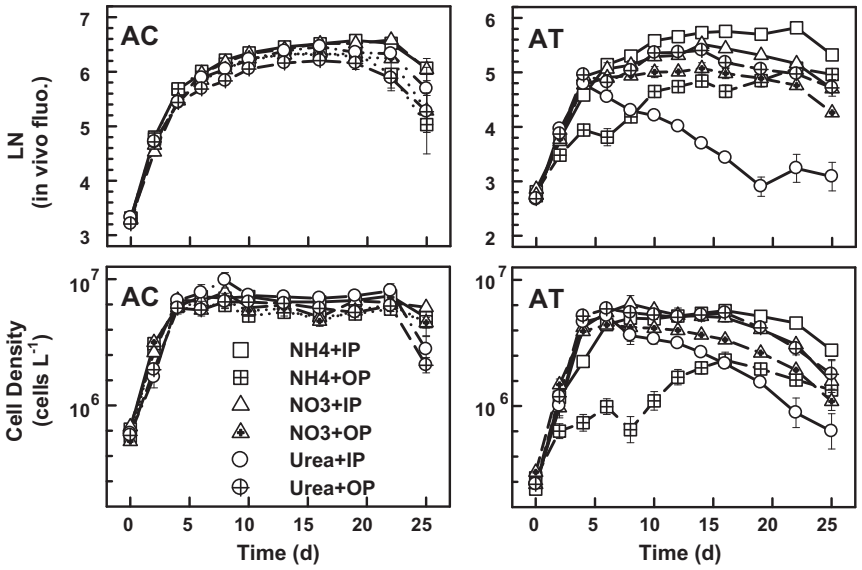

Fig. 1. Changes in in vivo fluorescence and cell density in cultures of *A. catenella* (AC) and *A. tamarense* (AT) grown on three nitrogen sources (NH_4, NO_3 and urea) and two phosphorus sources (inorganic PO_4 (IP) and organic PO_4 (OP)). Data points are means ± 1 SD of 3 replicates. When error bars are not visible, they are smaller than the symbol.

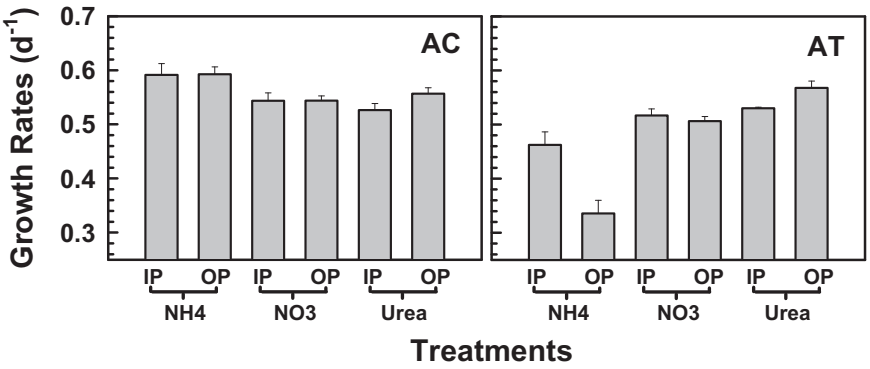

Fig. 2. Growth rates of *A. catenella* (AC) and *A. tamarense* (AT) grown on three nitrogen sources (NH_4, NO_3 and urea) and two phosphorus sources (inorganic PO_4 (IP) and organic PO_4 (OP)) in the exponential growth phase. Data points are means ± 1 SD of 3 replicates.

differ significantly ($p < 0.05$). In contrast, for *A. tamarense* cultures, the growth rate was significantly ($p < 0.01$) different for each of the nutrient sources. The growth rates in the NH_4 treatment was significantly ($p < 0.01$) lower (<0.47 d^{-1}) than those in the NO_3 (0.51 d^{-1}) and urea treatments (0.57 d^{-1}) and the growth rate in the urea treatment (0.57 d^{-1}) was significantly ($p < 0.01$) higher than that in the NO_3 treatment (0.51 d^{-1}) when OP was used. The growth rates of *A. tamarense* grown on inorganic P (0.46–0.51 d^{-1}) were significantly ($p < 0.01$) higher than those on organic P (0.34–0.50 d^{-1}) when inorganic N was used, but was the opposite when organic N was used (Fig. 2 and Table 1).

The cell volume and POC and PON of *A. catenella* and *A. tamarense* increased during growth from exponential to early stationary phase and became a maximum in the mid-late stationary phase, whereas cellular Chl *a* peaked in the early stationary phase and declined in mid-late stationary (Fig. 3). The cell volume of both species was significantly ($p < 0.05$) affected by both N and P nutrient sources (Table 2). Cell volumes of both species were the largest (up to 28,000 μm^3 for *A. catenella* and 25,000 μm^3 for *A. tamarense*) when NH_4 was used and the smallest (<20,000 μm^3 for *A. catenella* and <15,000 μm^3 for *A. tamarense*) when urea was used (Fig. 3). In the mid-late stationary phase, cell

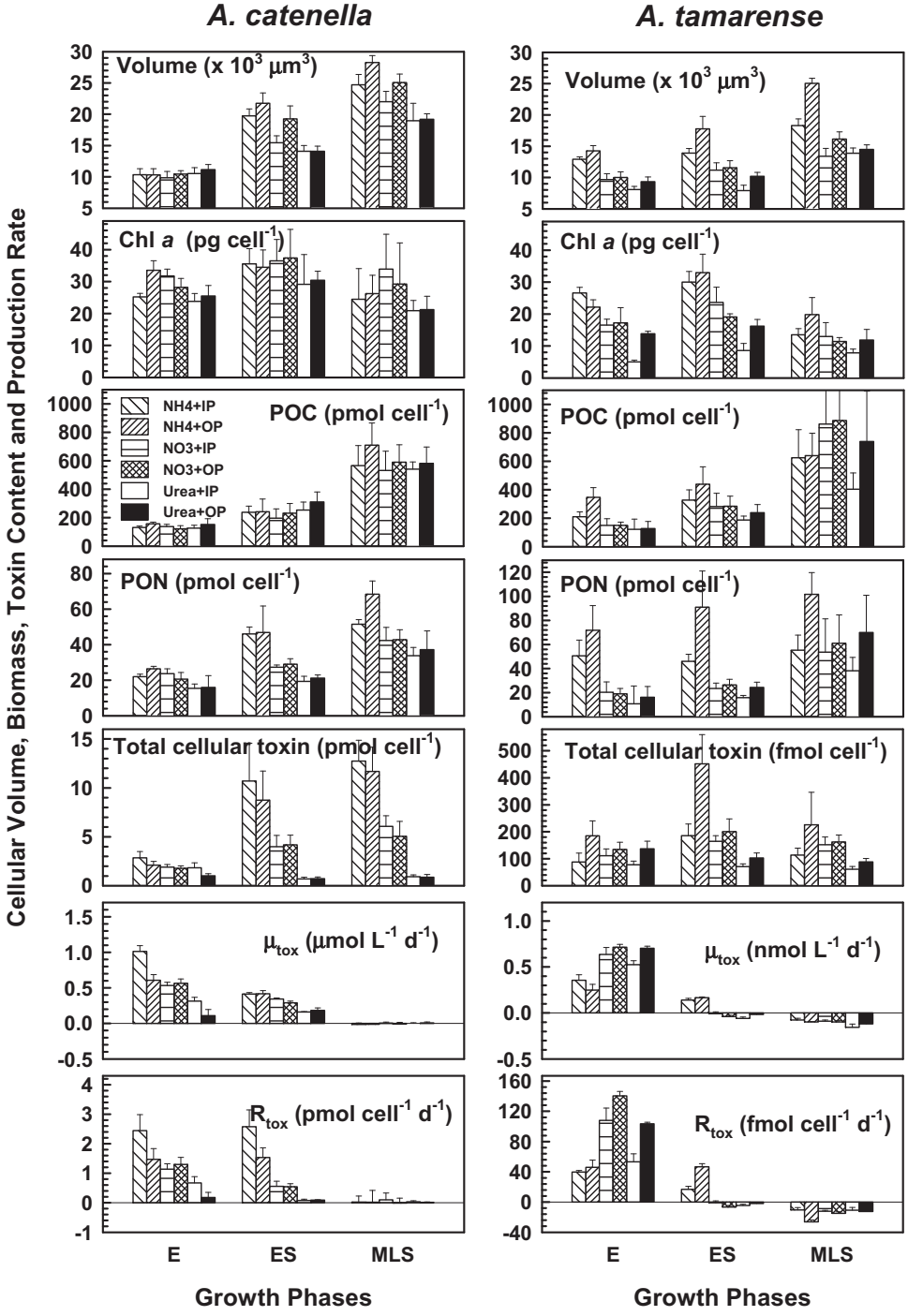

Fig. 3. Changes in cell volume, Chl *a*, particulate organic nitrogen (PON), organic carbon (POC), total cellular toxin content, total toxin production rate (μ_{tox}) and net toxin production rate (R_{tox}) of *A. catenella* (AC) and *A. tamarense* (AT) grown on three nitrogen sources (NH_4, NO_3 and urea) and two phosphorus sources (inorganic PO_4 (IP) and organic PO_4 (OP)) for exponential (E), early stationary (ES) and mid-late stationary (MLS) growth phases. Data points are means ± 1 SD of 3 replicates.

Table 2
Analysis of variance (ANOVA), Tukey's and *t*-tests of pooled data showing the effect of nitrogen (N) and phosphorus (P) on the cellular volume and bulk toxin production rate (μ_{tox}) of *A. catenella* and *A. tamarense*.

	A. catenella			*A. tamarense*		
	Cell volume stage			Cell volume stage		
	E	ES	MLS	E	ES	MLS
Two-way ANOVA						
Tukey's test: N effects						
Inorganic PO_4	Urea > NH_4 > NO_3	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg$ Urea > NO_3
Organic PO_4	Urea > NO_3 > NH_4	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3$ > Urea	$NH_4 \gg NO_3$ > Urea	$NH_4 \gg NO_3 \gg$ Urea
t-test: P effects						
NH_4	OP > IP	OP $\gg$ IP	OP $\gg$ IP	OP $\gg$ IP	OP $\gg$ IP	OP $\gg$ IP
NO_3	OP > IP	OP $\gg$ IP	OP $\gg$ IP	OP > IP	OP > IP	OP $\gg$ IP
Urea	OP > IP	OP > IP	OP > IP	OP $\gg$ IP	OP $\gg$ IP	OP > IP

	A. catenella			*A. tamarense*		
	μ_{tox} stage			μ_{tox} stage		
	E	ES	MLS	E	ES	MLS
Two-way ANOVA						
Tukey's test: N effects						
Inorganic PO_4	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	NA	NO_3 > Urea $\gg NH_4$	NA	NA
Organic PO_4	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	NA	NO_3 > Urea $\gg NH_4$	NA	NA
t-test: P effects						
NH_4	IP $\gg$ OP	OP > IP	NA	IP > OP	NA	NA
NO_3	IP > OP	OP > IP	NA	IP > OP	NA	NA
Urea	OP $\gg$ IP	OP > IP	NA	OP $\gg$ IP	NA	NA

"$\gg$" indicates that the former is significantly ($p < 0.05$) higher than the latter. ">" indicates that the former is higher, but not significantly higher than the later. E: exponential phase; ES: early stationary phase; MLS: mid-late stationary phase.

volumes of both species grown on inorganic PO_4 were significantly ($p < 0.05$) smaller than those on organic P except for urea cultures where there was no significant difference between both phosphorus sources (Table 2).

3.2. Toxin production rate (μ_{tox} and R_{tox}) and cellular toxin content

The effect of nutrients on toxin production rate and cellular toxin content for both species varied with the growth phase and nutrient source (Tables 2 and 3). Total toxin production rate was highest (up to 1.0 μmol L^{-1} d^{-1}) on NH_4 for *A. catenella* and (up to 0.71 nmol L^{-1} d^{-1}) on NO_3 for *A. tamarense* (Fig. 3). For *A. catenella* cultures, the net production rate was generally greatest (up to 248 pmol $cell^{-1}$ d^{-1}) in the exponential phase, while the cellular toxin content or toxin cell quota was lowest (<3 pmol $cell^{-1}$) in the exponential phase and highest (up to 13 pmol $cell^{-1}$ for NH_4 cultures) in the mid-late stationary phase (Fig. 3). Irrespective of the phosphorus source, the net toxin production rate and cellular toxin content differed significantly ($p < 0.05$) among the three nitrogen sources (Table 3), with the lowest toxicity

Table 3
Analysis of variance (ANOVA), Tukey's and *t*-tests of pooled data showing the effect of nitrogen and phosphorus nutrient sources on the total cellular toxin content and net toxin production rate (R_{tox}) of *A. catenella* and *A. tamarense*.

	A. catenella			*A. tamarense*		
	Total cellular toxin content stage			Total cellular toxin content stage		
	E	ES	MLS	E	ES	MLS
Two-way ANOVA						
Tukey's test: N effects						
Inorganic PO_4	$NH_4 \gg NO_3$ > Urea	$NH_4 \gg NO_3 \gg$ Urea	NH_4 > NO_3 > Urea	$NO_3 \gg NH_4 \gg$ Urea	$NH_4 \gg NO_3$ > Urea	NA
Organic PO_4	NH_4 > $NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	NH_4 > NO_3 > Urea	NO_3 > $NH_4 \gg$ Urea	$NH_4 \gg NO_3$ > Urea	NA
t-test: P effects						
NH_4	IP > OP	IP > OP	IP > OP	OP > IP	IP $\gg$ OP	NA
NO_3	IP > OP	IP > OP	IP > OP	OP $\gg$ IP	NA	NA
Urea	IP $\gg$ OP	IP > OP	IP > OP	OP $\gg$ IP	NA	NA

	A. catenella			*A. tamarense*		
	R_{tox} stage			R_{tox} stage		
	E	ES	MLS	E	ES	MLS
Two-way ANOVA						
Tukey's test: N effects						
Inorganic PO_4	$NH_4 \gg NO_3$ > >Urea	$NH_4 \gg NO_3 \gg$ Urea	NH_4 > NO_3 > Urea	$NO_3 \gg$ Urea $\gg NH_4$	$NH_4 \gg NO_3$ > Urea	NA
Organic PO_4	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	NH_4 > NO_3 > Urea	$NO_3 \gg$ Urea $\gg NH_4$	$NH_4 \gg NO_3$ > Urea	NA
t-test: P effects						
NH_4	IP > OP	IP > OP	IP > OP	OP > IP	IP $\gg$ OP	NA
NO_3	IP > OP	IP > OP	IP > OP	OP $\gg$ IP	NA	NA
Urea	IP $\gg$ OP	IP > OP	IP > OP	OP $\gg$ IP	NA	NA

"$\gg$" indicates that the former is significantly ($p < 0.05$) higher than the latter. ">" indicates that the former is higher, but not significantly higher than the later. E: exponential phase; ES: early stationary phase; MLS: mid-late stationary phase.

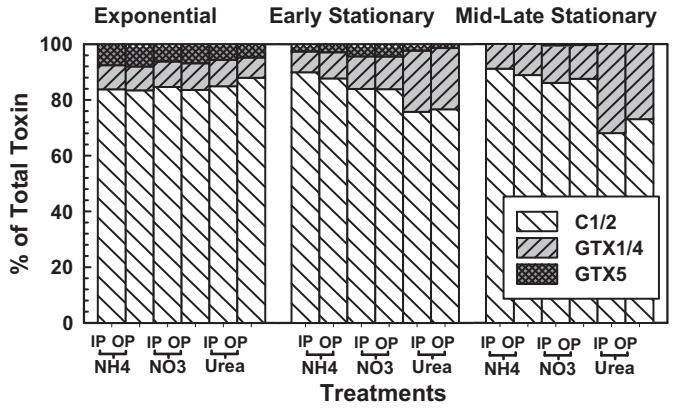

Fig. 4. Change in % of total (sum of C1/2, GTX1/4, GTX2/3 and GTX5) toxin composition for three toxins of *A. catenella* (AC) grown on three N sources (NH_4, NO_3 and urea) and two P sources (inorganic P (IP) and organic P (OP)) at three different growth phases.

(<2 pmol cell^{-1}) on urea, moderate (2–7 pmol cell^{-1}) on NO_3 and the highest toxicity (up to 13 pmol cell^{-1}) on NH_4. When *A. catenella* was grown on IP, it had significantly ($p < 0.05$) higher cellular toxin content (1.8–2.8 pmol cell^{-1}) than on OP (1.0–2.1 pmol cell^{-1}) in the exponential phase (Fig. 3 and Table 3).

The net toxin production rate and cellular toxin content for *A. tamarense* were lower than for *A. catenella*. The net toxin production rate was generally highest (39–140 fmol cell^{-1} d^{-1}) in the exponential phase in all cultures, while cellular toxin content peaked (at 450 and 200 fmol cell^{-1} for NH_4 and NO_3, respectively) in the early-stationary phase in the NH_4 and NO_3 cultures, and at 140 fmol cell^{-1} in the exponential phase in the urea cultures (Fig. 3). When *A. tamarense* was grown on inorganic nitrogen (NH_4 and NO_3), it generally had significantly ($p < 0.05$) higher cellular toxin content than when grown on urea. When *A. tamarense* was grown on OP, generally it had significantly ($p < 0.05$) higher (7–140%) cellular toxin content than on IP throughout the growth stages, regardless of nitrogen source (Table 3).

3.3. Toxin composition

Two PST derivatives, C2 at a high level (>90%) and its epimer derivative C1 at a trace level were detected in all *A. tamarense* cultures throughout all three growth stages. In contrast, *A. catenella* produced five PST derivatives including C1, C2, GTX1, GTX4 and GTX 5. The cellular toxin composition of *A. catenella* changed significantly ($p < 0.05$) in different growth stages and was dominated by C1/C2 (70–90%) (Fig. 4). The cellular mole percentage of C1/2 increased from about 83 to 86% and from 85 to 91% in *A. catenella* cultures grown on NH_4 and NO_3 respectively and decreased from 85–88% to 68–73% when grown on urea (Fig. 4). The cellular mole percentage of GTX1/4 increased from 7–10% to 9–32%, while that of GTX5 decreased from 7–9% to <1% as the cultures of *A. catenella* aged (Fig. 4).

4. Discussion

4.1. Growth of two Alexandrium species

A great deal of attention has been focused on a comparison of nitrogen uptake rates of phytoplankton among various forms of nitrogen during the past decades, indicating that NH_4 is generally a favored N source over NO_3, due to lower energy costs for utilization (Thompson et al., 1989; Dortch, 1990; Levasseur et al., 1993; Twomey et al., 2005). However, few studies have determined if there is a difference in growth rates of toxic dinoflagellates growing on various forms of N and P. Such a study is necessary in order to understand the mechanism of toxin production and the succession of red tides in coastal waters receiving different forms and levels of inorganic and organic nitrogen and phosphorus.

The two *Alexandrium* species in this study differed in their response to various forms of N and P in terms of growth rate and cell volume. The growth rate of *A. catenella* was significantly ($p < 0.05$) stimulated by NH_4, as indicated by higher growth rate on NH_4 relative to NO_3 and urea. In contrast, when *A. tamarense* was grown on NH_4, it had a significantly ($p < 0.05$) lower (20–25%) growth rate, regardless of the phosphorus source, compared to urea and NO_3. Interestingly, *A. tamarense* preferred urea as the N source for its growth, especially when organic phosphorus was used.

The form of phosphorus (IP vs OP) had a significant ($p < 0.05$) impact on the growth rate of *A. tamarense*, but not for *A. catenella*. IP induced significantly ($p < 0.05$) higher growth rates of *A. tamarense* than OP when inorganic nitrogen (i.e. NH_4 and NO_3) was used as the N source, while OP induced higher growth rates of *A. tamarense* when organic nitrogen (i.e. urea) was used as the N source.

Previous studies have also shown that the increase in cell size with P limitation is common among phytoplankton (Latasa and Berdalet, 1994; John and Flynn, 2000), and P limitation stops pigment synthesis as the cells are unable to synthesize RNA (Latasa and Berdalet, 1994; Lippemeier et al., 2003). In this study, cell volume and cellular C and N contents increased in late stationary phase when P was depleted compared with exponential and early stationary phases of all the cultures, while the cellular Chl *a* content did not increase, and even decreased in response to P depletion (Fig. 3). These clearly indicated that P limitation occurred in the stationary phase. NH_4 induced the largest cell volume and also highest PON (Fig. 3 and Table 4), suggesting faster uptake of

Table 4
Analysis of variance (ANOVA), Tukey's and *t*-tests of pooled data showing the effect of nitrogen and phosphorus nutrient sources on the cellular particulate organic nitrogen (PON) of *A. catenella* and *A. tamarense*.

	A. catenella			*A. tamarense*		
	Cellular PON stage			Cellular PON stage		
	E	ES	MLS	E	ES	MLS
Two-way ANOVA						
Tukey's test: N effects						
Inorganic PO_4	$NO_3 > NH_4 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 >$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 > NO_3 >$ Urea
Organic PO_4	$NH_4 > NO_3 >$ Urea	$NH_4 \gg NO_3 \gg$ Urea	$NH_4 \gg NO_3 >$ Urea	$NH_4 \gg NO_3 >$ Urea	$NH_4 \gg NO_3 >$ Urea	$NH_4 \gg$ Urea $> NO_3$
t-test: P effects						
NH_4	IP > OP	IP > OP	IP $\gg$ OP	OP $\gg$ IP	OP $\gg$ IP	OP $\gg$ IP
NO_3	IP > OP	IP > OP	IP > OP	OP > IP	OP > IP	OP $\gg$ IP
Urea	IP > OP	IP > OP	IP > OP	OP $\gg$ IP	OP $\gg$ IP	OP $\gg$ IP

"$\gg$" indicates that the former is significantly ($p < 0.05$) higher than the latter. ">" indicates that the former is higher, but not significantly higher than the later. E: exponential phase; ES: early stationary phase; MLS: mid-late stationary phase.

NH_4 than other N forms under P limitation, possibly due to the lower energetic cost of utilizing NH_4 to synthesize cellular materials. Hence, the form of nitrogen and phosphorus had a significant ($p < 0.05$) effect on the cell volume of the two *Alexandrium* species.

4.2. Toxin production and cellular content

Many studies have been conducted on the relationship between toxin production and nutrient status (Anderson et al., 1990a; Latasa and Berdalet, 1994; Murata et al., 2006). A review of data from both semi-continuous and batch cultures showed that phosphorus limitation or starvation generally increased toxin content, while nitrogen limitation or starvation decreased toxin content (Boyer et al., 1987; Anderson et al., 1990a; Taroncher-Oldenburg et al., 1999; John and Flynn, 2000; Poulton, 2001; Guisande et al., 2002). However, they rarely examined the effect of various forms of nutrients on toxicity (i.e. toxin production rate and cellular toxin content).

In this study, the form of nitrogenous and phosphorus nutrients was found to play an important role in controlling the toxin production rate and toxin content of these two *Alexandrium* species in three growth phases. The greatest net toxin production rate (R_{tox}) occurred in the exponential phase in all cultures. The effect of the form of phosphorus on R_{tox} varied with the form of nitrogen. The form of phosphorus did not have an effect on R_{tox} in NH_4 cultures, while the two *Alexandrium* species demonstrated different patterns in NO_3 and urea cultures, in response to different phosphorus sources.

P deficiency did not stop toxin synthesis completely, while the net toxin production rate significantly ($p < 0.05$) decreased in the early stationary phase when inorganic nitrogen was still present and continuously taken up. As a result, the cellular toxin content peaked in the stationary phase. This is in agreement with previous reports which proposed that P limitation in the presence of excess nitrogen could cause an increase in the availability of intracellular arginine (containing N), a presumed precursor in PST biosynthesis (Anderson et al., 1990a; Flynn et al., 1994). Besides, toxin production rate and cell volume might provide insight into the understanding of a dramatic increase in the cellular toxin content in response to P depletion. In the exponential phase, in NH_4 and NO_3 cultures, toxin production rate (μ_{tox} and R_{tox}) reached a maximum, while cellular toxin content remained low. In contrast, in the stationary phase, toxin production rate began to decrease and cell volume increased due to P depletion. As a result, the cellular toxin content was enhanced dramatically. This observation agrees with Lippemeier et al. (2003) who found that after a P spike to P-limited cells of *Alexandrium minutum*, the cellular toxin decreased due to a reduction in cell volume as a result of enhanced rates of cell division. Hence, an increase in cell volume caused by P depletion might partially contribute to an increase in the cellular toxin content. This suggestion was also supported by the significant ($p < 0.05$) correlation between cellular toxin content and cell volume in inorganic nitrogen treatments for *A. catenella* (Fig. 5). However, cellular toxin content of both species did not increase with cellular N and cell volume in response to P starvation in urea cultures where cells utilized urea to synthesize cellular materials, but not toxins.

Toxin accumulation of these two *Alexandrium* species exhibited different patterns in response to different forms of phosphorus. For *A. catenella*, growth on IP generally led to slightly higher (but not statistically significant) cellular toxin content than OP, irrespective

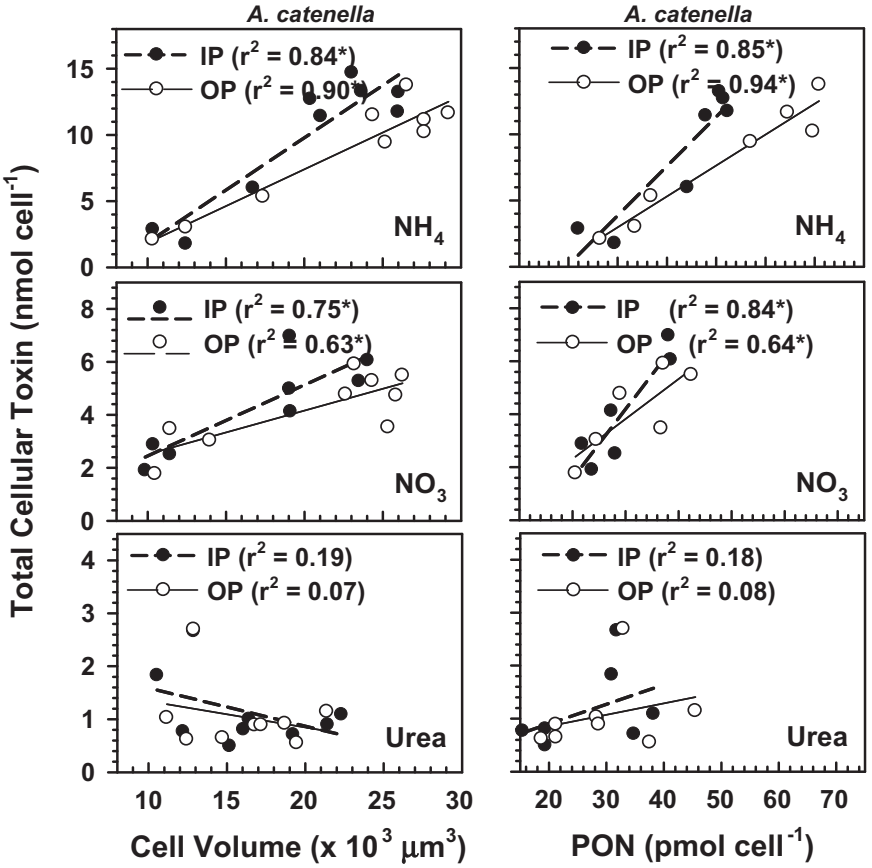

Fig. 5. Total cellular toxin content (sum of C1/2, GTX1/4, GTX2/3 and GTX5) vs. cell volume and particulate organic nitrogen (PON) for *A. catenella* grown on three N sources (NH_4, NO_3 and urea) and inorganic (IP) and organic (OP) sources. * indicates a significant difference.

of the form of nitrogen. In contrast, for *A. tamarense*, OP led to significantly ($p < 0.05$) higher cellular toxin content than IP throughout the growth phase when reduced forms of nitrogen (i.e. NH_4 and urea) were used. This might be partly due to the difference in the net toxin production rate between the two forms of phosphorus. Overall, the form of phosphorus had marked impacts on the toxin production of *A. tamarense*, but not *A. catenella*, while toxin accumulation in both *Alexandrium* species increased in response to P limitation.

In contrast to phosphorus, the form of nitrogen played a more important role in controlling toxin accumulation in *Alexandrium* species. The cellular toxin content in both *Alexandrium* species varied greatly with nitrogen sources in response to P limitation. NH_4 led to the highest cellular toxin content for *A. catenella* under P limitation, which was two-fold higher than NO_3 and 8-fold higher than urea, while P limitation did not lead to an increase in cellular toxin content in the urea grown cultures. This might be attributed to the difference in the net production rate among different nitrogen sources. In the exponential phase when P was not limiting, irrespective of the phosphorus source, R_{tox} in NH_4 cultures was 10–100% higher than NO_3 and 4–8-fold higher than urea for *A. catenella* and these differences in R_{tox} were statistically significant. This suggested that the cells of *A. catenella* grown on NH_4 were able to produce more toxin than on other nitrogen sources, and this pattern continued even in the early stationary phase when R_{tox} began decreasing because of P limitation. This was consistent with previous reports that nitrate has been found to induce more stress on cellular metabolic activities than ammonium (John and Flynn, 2002; Wood and Flynn, 1995). NH_4 induced a higher cellular toxin content than other nitrogen sources (NO_3 and urea) by increasing net toxin production rates and cell volume.

In contrast to *A. catenella*, the relationship between cellular toxin content and nitrogen sources for *A. tamarense* was more complicated. *A. tamarense* grown on NH_4 had higher cellular toxin content than on NO_3 and urea when OP was used, but NO_3 induced the highest toxin production in the exponential phase and mid-late stationary phases and NH_4 in early stationary phase when IP was used. This suggested that the relationship between toxin accumulation and the form of nitrogen varied with the phosphorus source. Cells grown on urea had the lowest cellular toxin content among the three nitrogen sources. Similar results were observed by Dyhrman and Anderson (2003). However, based on N-limited lab cultures, Leong et al. (2004) found that cellular toxin content of *A. tamarense* declined in the following order: $NH_4 >$ urea $> NO_3$, and there was a negative relationship between cellular toxin content and urea concentration. They suggested that the mechanism of utilizing urea to synthesize toxin and cellular materials might differ, compared to NH_4 and NO_3. This discrepancy might also be due to a different experimental design (N limitation vs P limitation). Therefore, further study is necessary to determine the role of urea in toxin production in *Alexandrium* sp. Our study implied that the form of nitrogen, which has been generally less emphasized than phosphorus in previous studies, should not be ignored in toxin studies as it is an important factor regulating toxicity of *Alexandrium*, since our results indicate that the toxicity of *Alexandrium* species is sensitive to the species of nitrogen.

4.3. Toxin composition

In this study, *A. catenella* produced a variety of PSTs, mainly C1/2, GTX1/4, GTX5 and trace amounts of GTX2/3. The form of phosphorus did not affect the toxin composition, while P depletion resulted in changes in the toxin profile of *A. catenella*. GTX5 decreased to a very low level (<1%) when P was exhausted, suggesting P depletion might inhibit GTX5 synthesis. Interestingly, the response of the toxin composition under P depletion varied with the N source. Urea favored GTX1/4 synthesis under P depletion, which increased 2–4-fold in response to P depletion, accompanied by a decline in C1/2 and GTX5. In contrast, in NH_4 and NO_3 cultures, there were only minor changes in C1/2 and GTX1/4. *A. tamarense* produced only C1/2 and a trace amount of dc-GTX2/3, which is consistent with the finding of Wang and Hsieh (2001). Toxin composition of *A. tamarense* remained constant for all of the nitrogen and phosphorus sources that were tested. Our results indicated that the stability of toxin composition varied with species in response to nutrient status.

5. Summary

The growth and toxicity of two *Alexandrium* species varied with the form of nitrogen and phosphorus. NH_4 induced the highest growth rate, toxin production rate (μ_{tox} and R_{tox}) and cellular toxin content for *A. catenella* among the three N sources, followed by NO_3 and urea. The form of phosphorus had little effect on the growth and toxicity of *A. catenella*, while P depletion resulted in an increase in the cellular toxin content and GTX1/4 and a decline in C1/2 and GTX5 in urea cultures, but only minor changes in C1/2 and GTX1/4 in NH_4 and NO_3 cultures. In contrast for *A. tamarense*, the form of N and P had a significant ($p < 0.05$) impact on the growth and toxicity. *A. tamarense* preferred urea as a N source for its growth, but not for toxin production. NO_3 led to the highest production rate (μ_{tox} and R_{tox}) for *A. tamarense*. Toxin composition of *A. tamarense*, with C1/2 being the dominant component, remained constant among various forms of nitrogen and phosphorus. Our findings imply that *Alexandrium* species using NH_4 from local sewage effluent as a nitrogen source might be more toxic in combination with P limitation during summer (Yin et al., 2000; Xu et al., 2009). This information could assist in assessing the harmful effect of *Alexandrium* blooms in Hong Kong waters and the environmental conditions that led to high or low toxicity blooms.

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