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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,

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