Paralytic shellfish toxin production by the dinoflagellate *Alexandrium pacificum* (Chinhae Bay, Korea) in axenic, nutrient-limited chemostat cultures and nutrient-enriched batch cultures

Myungsoo Han¹, Haeok Lee¹, Donald M. Anderson², Baikho Kim^{1*}

¹Department of Life Science, Hanyang University, Seoul 04763, South Korea

² Biology Department, Woods Hole Oceanographic Institution, Woods Hole MA 02543-1049
USA

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*Correspondent: Baikho Kim (tigerk@hanyang.ac.kr)

Department of Life Science

Hanyang University

Seoul 133-791

South Korea

Tel & Fax : +82-2-2290-0960

Abstract

Multiple species within the marine dinoflagellate genus *Alexandrium* produce paralytic shellfish poisoning (PSP) toxins in temperate coastal waters worldwide. Since the 1990s, there have been frequent outbreaks of toxic *A. pacificum* (formerly *A. tamarense*; John et al. 2014) linked to anthropogenic eutrophication in Chinhae Bay (Korea). Such blooms have a detrimental effect on mariculture, more specifically on the oyster, shellfish, and fishing industries. In this study, we examined the production of PSP toxin by axenic *A. pacificum* HYM9704 in chemostat and batch cultures using growth conditions that differed according to dilution rate, nutrient limitations, and enrichments. More specifically, we determined how these variables affected PSP toxin content and profile. We found that phosphate (P)-limited cells in chemostat cultures produced higher levels of PSP toxins with a more diverse toxin profile than nitrogen (N)-limited cells at the highest algal growth rates. In nutrient-limited batch cultures that then received N and P enrichment, the N-enriched *A. pacificum* HYM9704 showed a more diverse toxin profile than the enriched P-limited cells, even though the total concentration of PSP toxins following N enrichment was lower than that in the P-enriched cultures. Based on the observation that nutrient availability strongly influenced PSP toxin profile, we suggest the following order for the biosynthesis of individual toxins: C1, C2 > GTX3 > GTX1 > neoSTX. In summary, although a higher toxicity of axenic *A. pacificum* HYM9704 PSP toxin was found under phosphorus limitation, nitrogen limitation and anthropogenic enrichment could also affect the nature and magnitude of toxicity in *Alexandrium* blooms.

Key words: *Alexandrium pacificum*, batch cultures, chemostat, dinoflagellate, nutrient enrichment and limitation, PSP toxin

Introduction

Many marine dinoflagellates cause problems for the shellfish industry because of their production of paralytic shellfish poison (PSP) toxins (saxitoxins) that accumulate in filter-feeding bivalves and cause illness and death in human consumers (Hallegraeff 1995, Tsujino and Uchida 2004, Persson et al. 2006, Wang et al. 2011). The most widespread of these dinoflagellates are in the genus *Alexandrium*, with multiple species producing saxitoxins (Cembella 1998; Anderson et al. 2012). It is important to have a solid understanding of the factors that influence the biosynthesis and accumulation of PSP toxins because toxic dinoflagellates and their resulting harmful algal blooms (HABs) are an ongoing problem for the seafood industry and continue to threaten human health worldwide (Shumway 1990; Hallegraeff, 1993; Scholin *et al.*, 1994; Scholin and Anderson, 1994; Su, 2004).

It is well known that toxin cell quotas (often termed ‘toxin content’) vary with growth conditions for individual *Alexandrium* isolates, but there is disagreement about whether or not toxin composition profiles (i.e., the suite of different saxitoxin congeners produced by an individual isolate) remain constant under a range of growth conditions. The effects of varying a single environmental parameter, such as light, temperature, salinity, nitrogen (N) or phosphorous (P), have been shown to include alteration of toxin content for several different *Alexandrium* species and strains. For example, N depletion causes the toxin content to decrease, while P limitation often results in higher toxin cell quotas (e.g., Boyer *et al.* 1987; Anderson *et al.* 1990a; Marsot, 1997; Matsuda *et al.* 1996; Béchemin *et al.* 1999; John and Flynn, 2000; Maestrini *et al.* 2000; Etheridge and Roesler, 2005; Wang and Hsieh, 2005). Based on results from previous batch culture studies, toxin composition has been considered a stable feature, i.e., a genetic trait that can be used to distinguish strains or species as a biochemical fingerprint (Boyer et al., 1987; Matsuda et al., 1996; Cembella and Taylor, 1985; Cembella, 1998). However, other studies have shown that, once cells are given time to achieve steady state in semi-continuous, nutrient-limited cultures, the toxin composition varies during progressive N- and P-limitation (Anderson et al., 1990a ; MacIntyre et al., 1997). Using either toxin content or relative proportion (mol% total toxin) of each toxin derivative, N- and P-limitation can be distinguished based on the observed trends in the different STX derivatives. For example, during N- and P-limitations, the absolute toxin content of *A. fundyense* decreased and increased, respectively, with different derivatives being dominant under each of the conditions (Anderson et al., 1990a, b). Other environmental conditions, such as salinity and temperature, have limited effects on toxin composition (Taroncher-Oldenburg et al., 1999; Etheridge and Roesler, 2005).

Marine bacteria directly or indirectly affect the growth and toxin production of *Alexandrium* species (Doucette, 1995; Doucette and Powell, 1998; Dantzer and Levin, 1997; Gallacher et al. 1997; Gallacher and Smith 1999; Mass et al. 2007; Kodama 2010). All of the tested saxitoxin-producing species can produce toxins by themselves (i.e., in a bacteria-free/axenic culture), and antibacterial agents do not have a clear effect on toxin production, nor is there a clear relationship between algal and bacterial abundance (Ho et al. 2006; Hold et al. 2001; Lu et al. 2000; Uribe and Espejo, 2003). Some studies have analyzed toxin production and toxin profile diversity in axenic batch cultures of *A. tamarense* (= *A. pacificum*) (Omura, 1999), while the results of other studies have demonstrated that the low growth rate of axenic *A. tamarense* is due to nutrient deficiencies rather than a lack of bacteria (Singh et al. 1982; Dantzer and Levin, 1997). It has been suggested that the effects of bacteria on PSP toxin production by marine *Alexandrium* species can be explained by altered toxin synthesis pathways, by algae or bacteria alone, or by algal-bacterium interactions (Maas and Brooks 2010). Although the axenic culturing of dinoflagellates poses multiple difficulties, bacteria-free cultures are useful for understanding the characteristics of PSP toxins and the underlying physiology that affects their production by the dinoflagellate, with no complications caused by bacterial interactions.

Chinhae Bay is located in the southeastern part of the Korean peninsula (sampling station: E128°38'; N35°08') and is connected to the open ocean by a narrow delta. Lee and Cho (1990) have noted that this region has active aquacultures of oysters (30% of all seafood production), mussels, and fish; this industrial activity increases the risk of algal blooms. In fact, eutrophication due to domestic and industrial waste has led to frequent and dense blooms involving the dinoflagellates *Ceratium fusus* and *Gymnodinium nagasakiense* (Cho, 1979; Lee et al., 1981; Yang, 1989; Lee et al., 1998). The first bloom of toxic *A. tamarense* (= *A. pacificum*) was reported by Han *et al.* (1992), and many researchers have since described the morphology and physiology of this group (Lee, 1990; Kim et al. 1993a, b; Kim and Lee, 1996; Kim et al., 1996; Kim et al. 2002). Studies have been carried out both in the field and in the laboratory on the toxicity and physiological characteristics of *A. pacificum* and its poisoning of mussels (Kim et al., 1996; Lee et al., 2003; Lee et al., 2006; Jeon et al., 1988; Lee *et al.*, 1992). The PSP toxins in *A. pacificum* and in the mussel *M. edulis* are commonly comprised of two major components: an 11- β -epimer (C2), from which C2, C4, GTX3, and GTX4 are biosynthesized; and an 11- α -epimer, from which C1, C3, GTX1, and GTX2 are biosynthesized. These toxins are formed via spontaneous epimerization (Oshima et al., 1993; Oshima, 1995). Studies have shown that 1) the dinoflagellate *A. pacificum* in Chinhae Bay is toxic; 2) the PSP toxin profiles of the algae and mussels in this bay are very similar; and 3) this toxin represents an ongoing threat to seafood production in mariculture in the southern part of Korea and along its coastline.

The present study examined the biosynthesis of PSP toxins by the axenic dinoflagellate *A. pacificum* HYM9704 under N- and P-limitations and enriched conditions in order to determine how these variables affected PSP toxin levels and the toxin profile.

Materials and Methods

Isolation of *A. pacificum* HYM9704

A. pacificum samples were collected from coastal waters in Chinhae Bay, Korea, in March 2006 using a 25- μ m plankton net. Axenic *A. pacificum* HYM9704 cells were isolated using a method that involved gentle washing and antibiotic treatment (Lee et al., 2006). Seawater T1 medium was used to isolate live cells from the algal bloom and to maintain the *A. pacificum*. The seawater (~30 psu) was filtered using a 0.45- μ m Millipore filter (cellulose nitrate; Whatman). Both isolated and axenic cells were maintained at 100 μ Em⁻²s⁻¹ and 15°C under cool-white fluorescent lighting with a 12L:12D light cycle. The culture conditions for axenic *A. pacificum* HYM9704 were established by Lee (2006), who determined the optimal growth conditions by experimenting with various temperatures, salinity levels, lighting conditions, and culture media.

To prepare the axenic strain prior to treatment with antibiotics, washing and dilution were conducted several times with T1 medium. The stock solution of antibiotics consisted of 100mg (50mg) gentamycin, 40mg (25mg) streptomycin sulfate, and 100mL (50mL) distilled water; the solution was filtered using a membrane filter (0.2 μ m). The fresh algal cells were inoculated directly into flasks that contained six different concentrations of antibiotics (0, 1.5, 2.0, 2.5, 5, and 10%) and were incubated for 48 hours. Only the fastest growing cells were aseptically selected from the antibiotic-treated cultures and re-inoculated into flasks containing fresh T1 medium. To ensure that there was no bacterial contamination, aliquots from each culture were checked regularly using an epifluorescence microscope with DAPI staining (Porter and Feig, 1980) or the bacto-yeast extraction method (Tartakoff, 1989). The bacto-yeast extract was comprised of 10g of yeast extract, 10g of bacto-peptone, and 1L of autoclaved seawater (30 psu), and the presence of bacteria was assessed with a microscope using a stain such as DAPI or propidium iodide. All of the axenic strains were cultured for 48 hours prior to use in the experiments, and the aliquots were always analyzed using a microscope.

Nutrient limitation in chemostat culture

A chemostat system for culturing axenic *A. pacificum* HYM9704 was designed by Lee (2001). Before inoculation of *A. pacificum* into the chemostat, all of the strains were batch cultured in T1 medium (30 psu) at 15°C under cool-white fluorescent lighting with a 12L:12D light cycle. A stock culture (150 mL) was transferred into each 2-L culture vessel with fresh N-limited medium sterilized by filtration through a membrane filter (Advantec K010A047A, 4science). The chemostat system was aerated with sterile air using a flow meter (Omega FS-03) after inoculation with strain HYM9704. The N- and P-limited chemostat (hereafter, NL and PL) cultures of *A. pacificum* HYM9704 were performed using

gentle mixing conditions by bubbling air through the culture medium at different flow rates. The culture was bubbled continuously, with bubbling increasing in intensity each time the cell density increased. To measure algal growth, we sampled 5 mL of each culture daily using a sampling bell and counted the cells under a light microscope (Nikon Eclipse E600). Before the cultures reached maximum cell density, a continuous supply of fresh medium was supplied from a 5-L glass flask reservoir. This medium was fed through a network of Teflon and silicon tubing to the growth units using a peristaltic pump (ATTO; peristaltic mini-pump SJ-1211-L). Following Lee et al. (2006), the dilution rates were established at 0.05, 0.1, 0.15, and 0.2 d⁻¹. The inflow of fresh medium was continued until the culture reached steady-state growth and was sustained thereafter. The cells and spent medium overflow were collected in a 5-L glass flask through a silicon tube. The effluent volume was measured daily in order to confirm the flow (dilution) rate. Steady-state growth was defined as six or more consecutive daily cell density measurements during which the coefficient of variation was less than 10%.

The negative control chemostat system did not contain any *A. pacificum*. The dissolved inorganic nutrient levels in the control system changed very little during the culture experiment. Therefore, changes in dissolved inorganic nutrient levels were considered negligible. The dilution rate ranged from 0.05 to 0.20 d⁻¹ and was confirmed by measuring the daily effluent volume. For the NLC culture, the axenic *A. pacificum* HYM9704 was incubated in modified T1 medium containing 50 µM nitrate plus 50 µM phosphate (N:P=1:1). No efforts were made to remove N or P from the seawater that was used to prepare the culture media. In accordance with the method described by Lee et al. (2006), the cultures were maintained at 15°C in 30-psu seawater under continuous cool-white fluorescent lighting. After analyzing the aliquots of the steady-state cultures, the inflow rate of fresh medium was sometimes changed, and steady-state growth was usually attained by 20 days. In the PL culture, the axenic HYM9704 was incubated in modified T1 medium containing 500 µM nitrate plus 1 µM phosphate (N:P=500:1). The other conditions were the same as for the NL culture.

Nutrient enrichment in batch culture

To investigate the impact of nutrient enrichment on PSP toxin content and composition, samples of algal cells from NL and PL conditions were transferred into batch flask systems that were nitrogen enriched (NEN) and phosphate enriched (PEN), respectively. In the NEN experiments, the strains were cultured for 45 days in fresh chemostat systems in order to select the chemostat in which the algal growth rate was the highest. On the final day, a 600-mL sample of the inoculum was collected from the chemostat system with a dilution rate of 0.1 d⁻¹. Briefly, 1M NaNO₃ was added to the batch culture system and adjusted to a final concentration of 500 µM. Each 600-mL sample that was collected was transferred in 60/mL aliquots to 10 flasks and cultured under the same conditions. The enrichment cultures were grown at 15°C under a light intensity of 150 µEm⁻²s⁻¹.

To measure changes in cell density, toxin level, and nutrients, samples were collected from each flask at the following time points after the cultures were established: 0, 0.5, 1, 3, 6, 12, 24, 48, 96, 120, and 168 hours. For the PEN experiments, 1M phosphate was added to the batch culture system to a final concentration of 50 μ M. After the strains were cultured for 45 days, subsamples of the inoculum were collected from the chemostat system with a dilution rate of 0.05 d⁻¹. The other conditions were the same as in the NEN study. Each 600-mL sample harvested was transferred in aliquots to 10 flasks and cultured under the same conditions. The light intensity and sampling schedules were the same as for the NEN experiments.

To check the changes of nutrients after refeeding of nitrogen and phosphate, the concentrations of nitrite, nitrate, ammonia and phosphate were measured at the same times that samples were taken for cell counts and toxin measurements. After 168 hours, concentrations of nitrate, ammonia and phosphate did not change significantly in NEN cultures, though nitrite gradually increased after 6 hours. In PEN cultures, nitrate and nitrite slightly increased and decreased, respectively, however, the level of was essentially unchanged (data not shown).

Analysis of algal growth and nutrients

To understand the algal growth of *A. pacificum* HYM9704, we directly enumerated the algal cells and measured the chlorophyll-*a* (Chl-*a*) concentrations using an acetone extraction method (APHA, 1995) and/or *in vivo* fluorescence using a fluorometer (Turner Designs, Model 10AU). Acetone extraction was primarily used for chemostat cultures, while the *in vivo* fluorescence method was primarily used for enrichment (batch) cultures. The cell density was enumerated by direct cell counting in a Sedgwick-Rafter chamber (1 mL) using an inverted light microscope (Olympus, Japan) after fixation with Lugol's solution. Although the graphs of these parameters (cell density on the y-axis and *in vivo* fluorescence on the x-axis) are not shown, we obtained the following regression line: $y=434.3x + 46.1$ ($r^2=0.9341$).

During this study, axenic *A. pacificum* HYM9704 NL and PL cultures were controlled in order to determine specific algal growth rates. Based on the relationship between fluorescence and cell density, the growth rate of *A. pacificum* HYM9704 was extrapolated from the *in vivo* fluorescence values and calculated using the following formula: $\mu = \ln [(F_1 - F_0)/(t_1 - t_0)]$, where μ is the specific growth rate (d⁻¹), and F_0 and F_1 represent the *in vivo* fluorescence values at times t_0 and t_1 , respectively. The water samples for the inorganic nutrient analyses were filtered through Whatman GF/F filters and stored frozen (-10°C) until analysis. The concentrations of dissolved inorganic nutrients (PO₄-P, NO₂-N, NO₃-N, and NH₄-N) were determined using ascorbic acid, phenate, colorimetric, and cadmium reduction methods, respectively (APHA 1995). The total phosphate and total nitrate concentrations were determined using the ascorbic acid and cadmium reduction methods, respectively, after digestion with persulfate (APHA 1995). In both the chemostat and batch cultures, the cell quota (Q) of nitrate or

phosphate was calculated using the following equation: $Q = (S_0 - S)/N$, where S_0 and S are the concentrations of nitrate or phosphate before and after their addition, and N is the HYM9704 cell density (cells mL⁻¹).

PSP toxin analysis

Toxin analysis was carried out using a fluorometric HPLC system according to Oshima (1995), with a slight modification (Table 1). The samples (10-mL in a Falcon tube) collected from the flasks were centrifuged at 1,000 × g at 5°C for 10 min. The supernatant was filtered through a 0.45-μm Millex GV4 syringe filter (Millipore, Bedford, MA, USA). The pellets were suspended in 0.5N acetic acid (0.5 mL) and stored in a refrigerator. The HPLC system was comprised of a high-pressure pump (Vintage 2000 LC, Orom Tech Ltd., South Korea), a Varian Rainin Microsorb-MV C18 column (4.6 × 250 mm, Derwood, MD, USA), a double-head reaction pump (TSP millionaire, MN, USA), and a fluorescence detector (Orom FL-300, Orom Tech Ltd., South Korea). Three separate isocratic elutions at a flow rate of 0.8 mL/min were used to isolate the different sets of PSP toxins. The first elution was performed using 1 mM tetrabutyl ammonium phosphate solution adjusted to pH 5.8 with acetic acid to elute the C1–C4 toxins. The second elution was performed using 2 mM sodium 1-heptanesulphonate in 10 mM phosphoric acid adjusted to pH 7.1 with ammonium water to elute the GTX and dcGTX toxins. The third elution was performed with 2 mM sodium 1-heptane sulphonate in 30 mM phosphoric acid adjusted to pH 7.1 with ammonium water plus 30 mM acetonitrile for the STX group. Oxidation was performed at a flow rate of 0.4 mL/min using 7 mL periodic acid in 50 nM potassium phosphate buffer (pH 9.0) in a 65°C water bath using Teflon tubing (10 mm × 0.25 mm i.d.). Acidification was performed at a flow rate of 0.4 mL/min with 0.5N acetic acid. The PSP toxins were detected using wavelengths of 330 nm for excitation and 390 nm for emission. The toxin profiles were calculated from the individual toxin concentrations as percentage of total PSP toxins for each of the samples (mol%). The relative toxicities of PSP saxitoxin (mg STX equiv/kg) were calculated based on the specific toxicities given by Oshima (1995). Toxicity (fmol cell⁻¹) was calculated using the results of HPLC analysis.

Results

PSP toxin production in nitrogen-limiting (NL) conditions

The dinoflagellate *A. pacificum* HYM9704 was cultured for 50 days in axenic, NL conditions at four dilution rates (0.05, 0.1, 0.15, and 0.2 d⁻¹). The time to reach steady-state growth varied for the different dilution rates (Figs. 2A-D). The fastest time to steady state (35 d) and the maximum final

cell density (4.1×10^3 cells ml⁻¹) occurred in the 0.1 d⁻¹ dilution rate culture, and this culture also showed the highest toxicity (17.3 fmol cell⁻¹). As expected, the nitrate cell quota (NQ) was lowest in this culture (12.34 pmol-N cell⁻¹); Fig. 2E). Therefore, there was a significant and positive relationship between maximum steady-state cell density and toxin content ($r^2=0.91$, $p=0.0312$) and a significant, negative relationship between maximum cell density and NQ ($r^2=0.93$, $p=0.0252$). In terms of toxin composition, the PSP toxins produced by HYM9704 included C1, C2, GTX1, and GTX3; but the relative abundance of these congeners varied with dilution rate (Fig. 2F). Toxin C2 comprised approximately 76.9%–100% of the total toxin content; only toxin C2 was found in the 0.2 d⁻¹ culture (Fig. 2f). The other toxins were relatively low in abundance and varied according to dilution rate: C1 (~8.8%–15.5%), GTX1 (~20.7%), and GTX3 (~1.5%).

PSP toxin production in phosphorous-limiting (PL) conditions

The dinoflagellate *A. pacificum* HYM9704 was cultured for 50 days under axenic PL conditions at four dilution rates (0.05, 0.1, 0.15, and 0.2 d⁻¹) (Figs. 3A-D). Time to reach steady-state growth (34 d), maximum final cell density (4.2×10^3 cells ml⁻¹), and lowest phosphate cell quota (PQ) (0.164 pmol-P cell⁻¹) were similar to the values for NL conditions but occurred at a different dilution rate (0.05 vs. 0.1 d⁻¹; Fig. 3E). Regardless of the dilution rate, algal growth or density of HYM9704 in PL conditions increased sharply between days 9 and 15 and then decreased and reached steady-state growth. The final density was <50% of the peak cell densities. Interestingly, the highest toxin content (384.0 fmol cell⁻¹) was recorded at 0.1 d⁻¹ for PL, and this value was 17–80 times higher than that for the NL conditions. Under the PL conditions, no significant relationships were found between final cell density, toxin content, and PQ. During steady-state growth, our isolate of *A. pacificum* contained five kinds of PSP toxins on day 50: C1, C2, GTX1, GTX3, and neoSTX (Fig. 3F). As was the case with the NL conditions, the C2 toxins were predominant (~29.0–89.1%) regardless of dilution rate, while the other toxins (C1, GTX1, GTX3, and neoSTX) were low in abundance and varied with dilution rates. Interestingly, neoSTX (~26.8%) was only found at 0.15 d⁻¹ and 0.2 d⁻¹.

PSP toxin production in NEN and PEN conditions

When *A. pacificum* HYM9704 cultured in NL and PL conditions was transferred to NEN and PEN cultures, respectively, the NEN and PEN cells showed similar growth patterns for seven days or 168 hours (Figs. 4A, B). Cell density did not change appreciably during the first 24 hours after nutrient supplementation but then gradually increased until the end of the study period. There were significant differences in cell density over time between the two treatments: 5.0×10^3 cells ml⁻¹ during the first 24 hours of NEN culture and 8.3×10^3 cells ml⁻¹ between 24–168 hours ($P<0.05$), while there were 4.1×10^3 cells ml⁻¹ during the first 24 hours of PEN culture and 7.9×10^3 cells ml⁻¹ between 24–168 hours ($P<0.05$). Although inoculation density was slightly different in NEN vs. PEN conditions, there

were no significant differences in cell density between NEN and PEN cultures after 24 hours ($P>0.5$), indicating similar patterns of growth. However, there were remarkable differences in PSP toxin production between NEN and PEN cultures (Figs. 4C, D; Table 2). During the first 24 hours of enriched culture, the toxin content in the PEN culture was higher than that in the NEN culture; the former gradually increased, while the latter increased moderately. The average toxin content in the PEN culture was 101.4 ± 9.4 fmole cell⁻¹ within 24 hours and 68.2 ± 21.5 fmole cell⁻¹ after 24 hours, while the level in the NEN culture was 15.4 ± 2.1 fmole cell⁻¹ within 24 hours and 43.4 ± 23.7 fmole cell⁻¹ after 24 hours. In addition, the NEN and PEN cultures showed peaks of 74.9 fmole cell⁻¹ (at 120 h) and 121.0 fmole cell⁻¹ (at 24 h), respectively. After 24 hours, the toxin content in the PEN culture sharply decreased through the end of the experiment, while the rates in the NEN culture increased with an increase in cell density up to 120 hours and then decreased. The toxin content in the NEN culture was higher than that in the PEN culture at 120 hours.

Toxin composition after enrichment

For 168 hours, the NEN and PEN batch cultures of axenic *A. pacificum* HYM9704 cells showed similar growth, and both of these cultures contained five toxins (C1, C2, GTX1, GTX3, and neoSTX). However, the time at which these toxins appeared in the cultures and their relative abundances were clearly different for the two treatments (Figs. 5A-D). In the NEN cultures, three toxins (C1, C2, and GTX1) were detected at the time of enrichment, but two additional toxins, GTX3 and neoSTX, were detected after 1 h and 96 h, respectively. Notably, the appearance of each toxin was closely related to decreases in other toxins: 1) a slight increase in GTX3 was accompanied by a slight decrease in C2 at 0.5 hours; 2) increases in GTX3 and GTX1 were balanced by a gradual decrease in C1 between 6 and 48 hours; and 3) the appearance of neoSTX was accompanied by decreases in GTX3 and C2 between 96 and 168 hours (Figs. 5A, B). In particular, neoSTX was barely detectable in the four NLC cultures but was found in the NEN after 96 hours. On the other hand, the five toxins were found at all of the time points examined in the PEN culture, and their relative abundances did not change, even after a decrease in the overall level of PSP toxin after 24 hours. The exception was an increase in neoSTX accompanied by a slight decrease in C1 and GTX3 between 120 and 168 hours (Figs. 5C, D). In both NEN and PEN cultures, C2 was the most abundant (~56.4%–82.8% in NEN culture and ~70.6%–78.3% in PEN culture); while GTX1, C1, GTX3, and neoSTX were present in minor, variable amounts [GTX1 (~8.2–30.0% for NEN, ~13.9–15.7% for PEN), C1 (~10.7% for NEN, ~7.5% for PEN), GTX3 (~6.1% for NEN, ~10.8% for PEN), and neoSTX (~10.8% for NEN, ~1.8%–4.6% for PEN)].

Discussion

There have been numerous studies conducted on toxin production by various *Alexandrium* species under nutrient-limited conditions in cultures, but few of these were conducted in bacteria-free or continuous cultures to determine toxin content and composition patterns under steady-state conditions with no bacterial interactions. In this paper, we performed studies of a Korean strain of *A. pacificum* grown in axenic, nutrient-limited chemostats and followed those studies by enriching the cultures with pulses of N or P. The results were consistent with those of many previous studies, but they also revealed some significant and important differences.

Toxin content

During the growth of axenic *A. pacificum* under NL conditions, the cellular NQs ranged from 15.2 to 22.6 pmol-N cell⁻¹ (Figs. 2, 3). Of the four dilution rates, the highest cell density and toxin content were observed at 0.1 d⁻¹, an intermediate growth rate, and this culture had the lowest NQ. Some previous studies have reported a positive relationship among nitrogen level, dilution rate, and toxin content of *Alexandrium* species grown under NL conditions (White, 1978; Hall, 1982; Marsot, 1997; Matsuda et al., 1996). Others have reported that toxin production in *Alexandrium* species was inversely proportional to growth rate (Proctor et al., 1975; Ogata et al., 1987). In our cultures, the toxin content of HYM9704 gradually decreased with an increase in dilution rate (i.e., faster growth) except that of 0.05 d⁻¹. For example, the cultures with a 0.2 d⁻¹ dilution rate had a toxin content of 0.9 fmol cell⁻¹, which was lower than the toxin content in the 0.05 d⁻¹ culture. Toxicity peaked at 17.3 fmol cell⁻¹ at a dilution rate of 0.1 d⁻¹. This maximum level was much higher than that of an axenic Japanese strain of *A. tamarens* (= *pacificum*), which had a peak toxicity of 9 fmol cell⁻¹ at 0.1 d⁻¹ and did not decrease at 0.2 d⁻¹ (Omura, 1999). In addition, the relationship between toxin content and growth rate of *A. tamarens* (now *A. fundyense*) in NL continuous culture was not linear and increased at dilutions >0.3 d⁻¹ (Anderson et al., 1990b). Based on these results, we suggest that it would be informative to grow chemostat cultures of *A. pacificum* at dilution rates >0.20 d⁻¹ in order to confirm a relationship between dilution rate and toxicity and to perhaps overcome differences due to the origin of the strain or isolate.

Similar to the NL conditions, under PL conditions, the maximum steady-state cell density of *A. pacificum* tended to decrease with an increase in dilution rate. However, there were some differences between toxin content and the PQ of the treatments. The PQ ranged from 0.16 to 1.04 pmol-P cell⁻¹ between 0.05 and 0.2 d⁻¹ and generally increased with an increase in growth rate. The maximum steady-state cell density was recorded at a dilution rate of 0.05 d⁻¹, but the highest toxicity (384 fmol cell⁻¹) was observed at 0.1 d⁻¹. The toxin content at 0.05 d⁻¹ was approximately 70% of that at 0.1 d⁻¹.

We found that, in both NL and PL conditions, toxin content in axenic *A. pacificum* was strongly related to algal cell density. After switching nutrient-limited cultures to nutrient-enriched conditions, different toxin production patterns were observed after only 24 hours. In the NEN cultures, 24 hours

seemed to be a sufficient period for the N-limited *A. pacificum* to acclimate to the nitrate-rich environment. Twenty-four hours after enrichment, algal cell density in NEN cultures increased gradually, as did cellular toxicity. This result was consistent with the concept that nitrogen enrichment enhances the production of nitrogen-rich compounds such as PSP toxins by dinoflagellates such as *Alexandrium* or *Gymnodinium* (McIntyre et al., 1997; John and Flynn, 2000; Wang and Hsieh, 2002; Wang et al. 2005). It is well known that the *Alexandrium* species have the ability to acclimate to nitrogen level (e.g., Leong et al. 2004). Therefore, when dinoflagellates such as *A. pacificum* are exposed to highly variable or increased concentrations of nitrogen, physiological changes in these cells (e.g., growth rate, toxin content) are expected to occur in order to accommodate the environmental changes.

In general, saxitoxin accumulation is relatively high during exponential growth and low or absent when cultures reach stationary growth. However, factors that inhibit growth, such as low temperature and low phosphate concentration, increase toxicity per cell (Boyer et al., 1987; Hall, 1982; Anderson et al., 1990a,b). In our continuous cultures, although the highest toxin content in the NL and PL conditions was seen at a dilution rate of 0.1 d⁻¹ (the same dilution rate for both), the toxin content per cell in the PL culture was 17 times higher than that in the NL culture, highlighting the higher toxin production when phosphate was limiting. Enhanced toxin production under P limitation has been observed for several *Alexandrium* species (Anderson et al. 1990a,b; Flynn et al., 1994; John and Flynn, 2000; Wang and Hsieh, 2002, 2005; Hu et al. 2006; Guisande et al., 2002; Frangopulos et al., 2004). Anderson et al. (1990b) suggested that, when phosphate is limited, there is an increase in availability of intracellular arginine, which is a precursor of PSP biosynthesis (Shimizu et al., 1984), due to the decreased demands from the competing phosphate-dependent pathways that are involved in cell division. Flynn et al. (1996) reported that toxin synthesis in *A. minutum* is proportional to intracellular arginine concentration. Wang and Hsieh (2005) showed that, in P-limited conditions, cells continue to utilize the available arginine or other cellular constituents for toxin biosynthesis.

The influence of an enhanced phosphate supply on toxin content is a matter of debate. Hu et al. (2006) reported that supplementation of nitrate and phosphate to axenic *A. tamarense* that were cultured in low-level phosphate conditions increase toxin production in comparison to high nutrient levels. In our batch cultures, growth of the dinoflagellate *A. pacificum* was well-established with the one-time enrichment of phosphate, but PSP toxin accumulation (and thus toxin content) decreased. However, our results are consistent with those of Frangopulos et al. (2004), who reported that enrichment with phosphate is unfavorable for the accumulation of toxins, and that temporary loading with anthropogenic phosphate can diminish the natural PSP toxin concentration produced by dinoflagellates under P-limited conditions.

Toxin composition

In the nutrient-limited chemostat cultures in this study, the PSP toxins consisted of toxins biosynthesized from the 11- β -epimer (C2, C4, GTX3, and GTX4) and the 11- α -epimer (C1, C3, GTX1, and GTX2) via spontaneous epimerization (Oshima et al., 1993; Oshima 1995). Similar to the toxin content results, the toxin composition profiles of axenic *A. pacificum* HYM9704 showed different patterns between the NL and PL cultures. The NL cultures contained four toxins (C1, C2, GTX1, and GTX3), while the PL conditions contained the same four plus small amounts of a fifth toxin, neoSTX. At 0.2 d⁻¹, the NL culture contained only the C2 toxin (100%), while the PL culture had 73% C2 and ~27% neoSTX. Taken together, these results demonstrate that there is considerable variation in toxin content and composition in *A. pacificum* as a result of nutrient stress or differences in growth conditions, consistent with results from other *Alexandrium* species (Boczar et al., 1988; Anderson et al., 1990a), in contrast to studies that have claimed that toxin composition is a stable trait that does not change with growth stage or environmental stress; these have generally used batch cultures (e.g., Cembella and Taylor, 1985; Anderson, 1994). Continuous or semi-continuous cultures, as described in this study and in Anderson et al. (1990a,b), allow the cells sufficient time (1-2 months) and generations to alter their composition and adapt to the culture conditions.

In batch cultures of *A. pacificum*, increases in both nitrogen and phosphate levels enhanced growth, but subsequent changes in the levels and profile of PSP toxins were clearer in NEN cultures than in the PEN cultures. Compared to the PEN cultures, supplementation with nitrate (i.e., NEN; batch culture) in axenic *A. pacificum* that was acclimatized to nitrogen limitation more clearly showed the biosynthetic sequence of PSP toxins. Although three toxin congeners, i.e., C2, C1, and GTX1, were always detected in the cultures, two carbamate toxins, GTX3 and neoSTX, were detected later (1 and 96 hours post enrichment, respectively), and their appearance was paralleled by decreases in C2 and C1 toxins. In contrast, the total toxin level in PEN cultures gradually increased during the first 24 hours and then sharply decreased. However, only one change in relative abundance of individual toxins was noted between 0 and 96 hours; an increase in neoSTX at 120 and 168 h, paralleled by decreases in C1 and C2. This result was similar to the NEN results during the same time periods.

Based on the NEN cultures, we suggest the following putative sequence or pathway for toxin production or interconversion by axenic *A. pacificum* HYM9704: C2, C1 > GTX3 > GTX1 > neoSTX (Fig. 6). Taroncher-Oldenburg et al. (1997) reported that increases in the carbamate toxins GTX3 and STX in *A. fundyense* were accompanied by decreases in toxins C2, GTX4 and neoSTX toxins, while the Japanese *Alexandrium* strain showed the following PSP toxin profile pathway: neoSTX > GTX4 > GTX3 > C2 (Omura, 1999). Regardless of nutrient enrichment, we could not determine the full process of toxin synthesis because there were five toxins at all of the time points tested during algal culture. We cannot yet explain the discrepancies in the toxin biosynthesis process between the NEN and PEN cultures.

Summary

For the past two or three decades, many researchers have studied the relationships between algal growth and biotic and abiotic factors that control toxin production variability in toxin content and diversity of individual toxins of *Alexandrium* species (axenic or xenic strains) in cultures. Only a few of these studies have used axenic continuous cultures due to the difficulty of growing these sensitive dinoflagellates in well-mixed, turbulent systems that are free of bacteria. Our results using a Korean strain of *A. pacificum* in both batch and continuous axenic cultures demonstrated that toxin composition changes do occur following extended growth under steady state conditions, and these results confirmed the results of past studies that showed enhanced toxin production under P limitation. We also examined the time course of acclimation following the enrichment of P- and N-limited cultures. The results of our study raised several questions regarding the toxin production mechanisms in the context of cell physiology and toxicology. First, why does the toxin content of cells grown under P-limited conditions gradually decrease with an increase in algal density 24 hours after phosphate enrichment? Cell densities increase, as would be expected, but the toxin per cell decreases. Therefore, how does phosphate supplementation inhibit toxin production? Second, does the addition of nitrate to nitrogen-limited cells really enhance toxin production? Clearly, these results are relevant to the conditions in natural waters where nutrient supplies can fluctuate considerably due to biological uptake and episodic supply from anthropogenic sources.

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Table 1. HPLC analysis of the PSP toxins.

Steps	Conditions
Mobile phases	flow rate of 0.8 ml/min
1) C1-C4 group	1 mM tetrabutyl ammonium phosphate adjusted to pH 5.8 with acetic acid
2) GTX group	2 mM sodium 1-heptane sulfonate in 10 mM phosphoric acid adjusted to pH 7.1 with ammonium water
3) STX group	2 mM sodium 1-heptane sulfonate in 30 mM phosphoric acid adjusted to pH 7.1 with ammonium water + acetonitrile (3:1)
Oxidation	flow rate of 0.4 ml/min 7 mM periodic acid in 50 mM potassium phosphate buffer (pH 9.0)
Reaction	in a 65°C water bath in a Teflon tube (10 m x 0.25 mm. i.d.)
Acidification	flow rate of 0.4 ml/min, 0.5 M acetic acid
Detection	excitation at 330 nm; emission at 390 nm

Table 2. Change in toxin content (fmole cell⁻¹) of axenic *Alexandrium pacificum* after enrichment with nitrate 500 mM at 0.1d⁻¹ (NEN) and with phosphate 0.5mM at 0.05d⁻¹ (PEN).

Experiments	t(h)	C1	C2	GTX1	GTX3	neoSTX
NEN	0	1.52	14.32	1.46		
	0.5	1.49	11.30	1.14		
	1	1.20	10.85	2.30	0.18	
	3	0.92	8.79	1.84	0.15	
	6	1.47	10.97	2.17	0.18	
	12		12.14	2.42	0.33	
	24		15.59	3.99	1.17	
	48		19.92	4.99	1.62	
	96		43.24	11.14	2.48	2.41
	120	1.50	54.93	13.63	1.97	2.92
	168		7.18	3.81	0.35	1.38
PEN	0	3.60	64.33	13.61	3.84	1.56
	0.5	5.01	76.30	14.68	4.05	2.20
	1	4.53	70.47	13.55	3.72	2.43
	3	6.75	63.48	13.29	3.31	3.12
	6	6.10	76.01	14.30	3.66	2.88
	12	2.74	34.55	7.01	1.64	
	24	2.54	94.82	16.89	4.40	2.43
	48	2.99	36.94	7.40	1.53	
	96	2.13	61.44	11.64	2.01	2.07
	120	1.86	35.03	7.20	1.44	2.92
	168		34.71	6.96	1.21	2.06

Figure captions

Fig. 1. Basic structures (upper) of saxitoxin and its congeners (below). Adapted from Franco et al. (1993) and Gallacher et al. (1997).

Fig. 2. Growth of axenic *Alexandrium pacificum* HYM9704 in N-limited chemostats supplied with 50 μM nitrate and 50 μM phosphate at different dilution rates: (a) 0.05 d^{-1} , (b) 0.10 d^{-1} , (c) 0.15 d^{-1} , and (d) 0.21 d^{-1} ; (e) algal cell density (D); nitrogen cell quota (NQ) and toxin content (T); and (f) relative abundances (%) of the five toxin congeners at steady state for each dilution rate.

Fig. 3. Growth of axenic *Alexandrium pacificum* HYM9704 in P-limited chemostats supplied with 500 μM nitrate and 1 μM phosphate at the following dilution rates: (a) 0.05 d^{-1} , (b) 0.10 d^{-1} , (c) 0.15 d^{-1} , and (d) 0.21 d^{-1} ; (e) algal cell density (D); phosphate cell quota (PQ); and toxin content (T); and (f) relative abundances of the five toxin congeners at steady state at each dilution rate.

Fig. 4. Changes in cell density (a) and toxin content (c) of axenic *Alexandrium pacificum* HYM9704 after enrichment with 500 μM nitrate at 0.1 d^{-1} (NEN) and 500 μM phosphate at 0.05 d^{-1} (PEN). The figures on the right indicate maximum mean cell density (b) and toxin content (d) in both the first (0-24h) and second periods (48-168h). The asterisk indicates significance at 0.05 (t-Test); the blue asterisks are comparisons of the two periods, and the red asterisks compare the two enrichments (NEN and PEN).

Fig. 5. Changes in absolute amount (a and c) and relative abundance (b and d) of the five toxin congeners produced by axenic *Alexandrium pacificum* HYM9704 after enrichments with 500 μM nitrate at 0.1 d^{-1} (left; NEN) and with 500 μM phosphate at 0.05 d^{-1} (right; PEN).

Fig. 6. A proposed pathway (red lines) of PSP toxin and congeners adapted from Franco et al. (1993) and Gallacher et al. (1997). The saxitoxins in the yellow boxes were found in this study.

Toxins		R ₁	R ₂	R ₃	R ₄
carbamate	STX	H	H	H	CONH ₂
	NEO	OH	H	H	CONH ₂
	GTX1	H	H	OSO ₃ ⁻	CONH ₂
	GTX2	H	H	OSO ₃ ⁻	CONH ₂
	GTX3	H	OSO ₃ ⁻	H	CONH ₂
	GTX4	OH	OSO ₃ ⁻	H	CONH ₂
decarbamoyl	dc-STX	H	H	H	OH
	dc-NEO	OH	H	H	OH
	dc-GTX2	H	H	OSO ₃ ⁻	OH
	dc-GTX3	H	OSO ₃ ⁻	H	OH
N-Sulfocarbamoyl	C1	H	H	OSO ₃ ⁻	CONHSO ₃ ⁻
	C2	H	OSO ₃ ⁻	H	CONHSO ₃ ⁻
	C3	OH	H	OSO ₃ ⁻	CONHSO ₃ ⁻
	C4	OH	OSO ₃ ⁻	H	CONHSO ₃ ⁻

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.