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Paralytic shellfish toxin profiles and toxin variability of the genus *Alexandrium* (Dinophyceae) isolated from the Southeast China Sea

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Abstract

Paralytic shellfish toxin (PST) profiles of 16 *Alexandrium* isolates from the Southeast China Sea were analyzed by high-pressure liquid chromatography. Toxin content and composition of three *A. tamarense* isolates, ATDH01, ATGX02 and ATMJ02, were also investigated at different growth phases and under various culture conditions. Our results showed that six strains of *A. affine* were non-toxic, while 10 strains of *A. tamarense* and *A. catenella* were toxic. These toxic isolates grown in the same culture conditions consistently produced an unusually high proportion of the *N*-sulfocarbamoyl toxin C1/2 (around 60–80% of total toxins) and medium amounts of gonyautoxin GTX5 (around 15–30% of total) with only trace quantities (<5% of total) of other saxitoxin derivatives (i.e. GTX1, GTX3, GTX4 and neoSTX). The toxin composition of three *A. tamarense* isolates did not vary with the growth phases, although higher toxin contents (Qt, fmol cell^{-1}) were found in the exponential phase. Variations in temperature, salinity and nutrient levels affected toxin content of three *A. tamarense* isolates but they did not have pronounced effects on the toxin composition (mole %). These results indicate that toxin composition remained relatively constant under various culture conditions, suggesting that toxin composition could be used as a stable biomarker for the *Alexandrium* species in this region. However, comparison of toxin profiles between isolates from different localities require special caution since isolates even from the same region can have distinct toxin profiles.

Keywords: *Alexandrium* spp.; Paralytic shellfish toxin; Toxin variability; Southeast China Sea

1. Introduction

Alexandrium is a widely spread genus throughout many regions of the world (Anderson et al., 1994; Hallegraeff et al., 1995). It is well known that many species within this genus are able to produce

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paralytic shellfish toxins (PSTs), a family of potent neurotoxins, which are the causative agents of paralytic shellfish poisoning (PSP) (Cembella, 1998). These toxins can be accumulated in filter-feeding shellfish which have fed on the dinoflagellates, resulting in illness or death to animals at higher trophic levels, such as marine mammals, birds and humans. The incidence of *Alexandrium* blooms has increased markedly in the past few decades (Anderson et al., 1994; Hallegraeff et al., 1995), and this has attracted significant attention to the toxin profiles and absolute toxicity of different *Alexandrium* strains isolated from different geographic areas. As a result, there have been extensive studies on PSP toxin profiles of *Alexandrium* from different geographic areas (Hall, 1982; Oshima et al., 1982; Maranda et al., 1985; Kim et al., 1993, 2005; Anderson et al., 1994; Kim, 1995; Chang et al., 1997; Yoshida et al., 2001; Wang and Hsieh, 2005). These results indicate that the toxin profiles produced by this genus varied among species as well as different strains of the same species. Moreover, some studies showed that toxicity (fmol cell^{-1}) and toxin composition (mole %) even among cultures of the same strain varied under different nutritional and culture conditions, such as salinity (White, 1978; Usup et al., 1994; Hwang and Lu, 2001; Hamasaki et al., 2001; Lim and Ogata, 2005; Etheridge and Roesler, 2005), light intensity (Ogata et al., 1987; Usup et al., 1994; Hwang and Lu, 2001; Hamasaki et al., 2001; Etheridge and Roesler, 2005), temperature (Ogata et al., 1987; Anderson et al., 1990b; Hamasaki et al., 2001; Etheridge and Roesler, 2005) and nutrients (Boyer et al., 1987; Anderson et al., 1990a; Bechemin et al., 1999; John and Flynn, 2000; Hamasaki et al., 2001; Wang and Hsieh, 2002, 2005; Poulton et al., 2005). However, other investigators have indicated that toxin composition is a stable biochemical characteristic of a dinoflagellate and can be regarded as a 'genetic fingerprint' for chemotaxonomic analysis (Hall, 1982; Cembella and Taylor, 1985; Boyer et al., 1987; Ogata et al., 1987; Oshima et al., 1993; Anderson et al., 1994; Flynn et al., 1996; Parkhill and Cembella, 1999).

Despite the conflicting views concerning the toxin composition of *Alexandrium*, information on the toxin profiles of geographically prevalent dinoflagellates is still important in assessing the environmental health significance of these local dinoflagellates. In China, PSP toxins are becoming economic and' public health concerns, and losses in mariculture and threats to human life due to exposure to these toxins have been documented increasingly (Qiu, 1990; Jian and Deng, 1991; Lin et al., 1994; Zhou et al., 1999). Although a few studies indicated the possible involvement of *Alexandrium* species in the production of PSTs in these areas (Anderson et al., 1996; Zhou et al., 1999; Siu et al., 1997; Wang et al., 2005), the toxigenic potential of these species in most geographic areas of the China Sea is still unknown. During an investigation of dinoflagellate cyst production along the China Sea coast, *Alexandrium* cysts were found to exist widely in the sediments, which suggested the potential for toxic *Alexandrium* blooms in this area (Gu et al., 2003).

First, details of the PSP toxin composition profiles of 16 *Alexandrium* isolates collected from the Southeast China Sea are presented. The toxin content and composition variations of three *A. tamarense* isolates (ATDH01, ATGX02 and ATMJ02) at different growth phases and under various culture conditions (nutrients, temperature, and salinity) are then investigated.

2. Materials and methods

2.1. Dinoflagellate cultures

The cultures were established from germinated cysts isolated from the sediments of the Southeast China Sea. The isolation information is shown in Table 1. Upon collection, the cysts were germinated and clonal strains were established from vegetative cells of these original cultures. Species were identified by light and scanning electron microscopy together with phylogenetic analysis based on ribosomal RNA gene sequences. Details of the methods used for identifying are to be published elsewhere (Gu et al., 2006). All cultures were routinely maintained in natural seawater supplemented with f/2-Si nutrients at 20 °C, 60 μE m^{-2} s^{-1} light intensity and 12 h:12 h light:dark cycle.

Toxin content and composition of *Alexandrium* isolates over the growth cycle were studied in 1000 ml conical flask containing 500 ml of f/2-Si medium under same regime. The effects of salinity on toxin content and composition of three *A. tamarense* isolates were studied at constant light and temperature (60 μE m^{-2} s^{-1}and 20 °C). Salinity was adjusted to 15‰, 20‰, 25‰ and 30‰ by diluting seawater with Milli-Q water and 35‰ by addition of NaCl. Temperature effects on toxin

Table 1
List of *Alexandrium* isolates and isolation information

Culture code	Location	Isolation
ATDH01	East China Sea (29°N, 122.5°E)	November, 2002
ATDH03	East China Sea (29°N, 122.5°E)	January, 2003
ATDH04	East China Sea (29°N, 122.5°E)	April, 2003
ATDH05	East China Sea (30° N, 122.8°E)	December, 2003
ACDH01	East China Sea (29°N, 122.5°E)	November, 2002
ACDH02	East China Sea (29°N, 122.5°E)	November, 2002
ACDH03	East China Sea (29°N, 122.5°E)	November, 2002
ATMJ01	Minjiang Estuary (26°N, 120°E)	January, 2003
ATMJ02	Minjiang Estuary (26°N, 120°E)	January, 2003
ATGX02	South China Sea (21°N, 109°E)	May, 2003
AAGX01	South China Sea (21°N, 109°E)	May, 2003
AATS01	Taiwan Strait (27.6°N, 121.2°E)	March, 2005
AATS02	Taiwan Strait (27.6°N, 121.2°E)	March, 2005
AA01	Junk Bay, Hong Kong (22.3°N, 114.3E)	November, 1997
AA02	Junk Bay, Hong Kong (22.3°N, 114.3E)	December, 1997
AA06	Junk Bay, Hong Kong (22.3°N, 114.3E)	August, 1998

Note: AT—*Alexandrium tamarense*; AC—*Alexandrium catenella*; AA—*Alexandrium affine*; DH—Donghai (East China Sea); MJ—Minjiang; GX—Guangxi; TS—Taiwan Strait.

content and composition of three *A. tamarense* isolates were studied at constant light and salinity ($60\,\mu E\,m^{-2}\,s^{-1}$ and a salinity of 30‰). The temperature was adjusted to 12, 16, 20 and 24 °C. In the nutrient experiments, f/2-Si medium and two modified f/2-Si media—without nitrate and without phosphate were used for comparison. No effort was made to remove phosphate and nitrate from the natural seawater used due to the low concentrations found.

Algal growth was monitored by cell counts under a light microscope (Olympus, CX31). At set time intervals (every two days), duplicate 0.5 ml samples from each culture were collected in 1.5 ml Eppendorf tubes and fixed in Lugol's iodine solution. Cell counts were done in a phytoplankton counting chamber under the microscope. At least 400 cells per sample were counted.

2.2. Analysis of toxins

Toxin content and composition over the growth cycle and under various growth conditions were monitored at the exponential and stationary phase. Algal cells from duplicate samples were collected by centrifugation at $2500 \times g$ for 5 min, followed by subsequent re-suspension in 0.5 ml of 50 mM acetic acid, and then followed by homogenization with three successive sonication cycles. Twenty microliter of the supernatant obtained after centrifugation at $10{,}000 \times g$ for 30 min was subjected to toxin analysis, which was carried out by HPLC with fluorescent detection using reverse phase chromatography with post column derivatization and an intersil C8-5 column (15 cm × 4.6 cm), as described previously (Oshima, 1995 as modified by Wang and Hsieh, 2001). Toxin standards, GTXs, STX and neo-STX bought from the National Research Council, Canada, and C1/C2 kindly provided by Prof. Hsieh (Hong Kong University of Science and Technology, HKSAR, China), were used for identification and quantification purpose. Three mobile phases (flow rate $0.8\,ml\,min^{-1}$) were used for separation of different toxin groups: (1) 2 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for C toxins; (2) 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for the gonyautoxin group; and (3) 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1): acetonitrile (100:5) for the saxitoxin group. (Abbreviations of toxins are used hereafter: C1, 2 = toxins C1, C2; GTX1/4 = gonyautoxins 1 and 4; GTX2/3 = gonyautoxins 2 and 3; GTX5 = gonyautoxin 5; STX = saxitoxin; neoSTX = neosaxitoxin).

3. Results

3.1. Toxin profiles of the Alexandrium cultures

Sixteen isolates of *Alexandrium* from the Southeast China Sea were cultured under identical

conditions. Typical HPLC profiles of toxin extracts from individual cultures are shown in Fig. 1. Their cellular toxin content in fmol cell^{-1} and the relative abundance of the toxins in mole % in the exponential phase are shown in Fig. 2. Apart from C1, C2 (*N*-sulfocarbomoyl toxin), GTX1, 3, 4, 5, and neoSTX, no other PSP toxin derivatives were detected in these cultures. The total toxin contents of the isolates ranged between 11.9 and 64.0 fmol cell^{-1}, the highest was found in isolate ATDH03 from the East China Sea.

All these toxic isolates grown in the same culture conditions produced unusually high proportions of the low toxicity *N*-sulfocarbamoyl toxins C2 (around 60–80% of the total) and medium amounts of gonyautoxin GTX5 (around 15–30% of the total), with trace or non-detectable levels of other STX derivatives, such as C1, GTX1, GTX4, GTX3 and neoSTX.

3.2. Variations during growth phases

The total toxin content (Qt) and toxin composition (mole %) at different growth phases were investigated in three *A. tamarense* isolates, ATDH01, ATGX02 and ATMJ02 (Table 2). Higher Qt values were found at the exponential phase of all isolates, and the total toxin content decreased rapidly when cells entered the stationary phase. Nonetheless, the toxin composition of these isolates varied slightly during different growth phases: C2 and GTX5 were consistently the dominant toxins at both exponential phase and stationary phase, and they contributed 60–80% and 15–30% to total toxin content, respectively.

3.3. Effects of nutrients on toxin content and composition

Toxin content and toxin composition of three *A. tamarense* isolates, ATDH01, ATGX02 and ATMJ02, under different nutrient conditions were investigated (Fig. 3). Toxin content (Qt) decreased slightly in ATMJ02 and ATGX02 isolates grown under nitrate deficiency when compared to those grown under replete-nutrients. However, toxin content was enhanced in the cultures grown under phosphate deficiency, especially in ATDH01 and ATMJ02, their Qt increased three times and twice, respectively. Nutrient deficiency had no effects on toxin composition of these three isolates, all toxin composition values remained relatively constant in each isolate, with C2 and GTX5 as the dominant toxins in all cases.

3.4. Effects of temperature on toxin content and composition

The toxin content and composition of three *A. tamarense* isolates, ATDH01, ATGX02 and ATMJ02 under various temperature conditions (12, 16, 20 and 24 °C) are shown in Fig. 4. Variations of temperature significantly affected toxin content of the three isolates, and toxin content decreased as temperature increased, so that the highest Qt was found in the lowest culture temperature (12 °C) in all three isolates. However, the toxin composition of the isolates remained relatively constant (the variation was $< 6\%$) under various temperatures, although a minor variation was observed in the composition of the trace toxin components. C2 and GTX5 were the dominant toxins at all culture temperatures.

3.5. Effects of salinity on toxin content and composition

Effects of salinity on toxin content and composition of three *A. tamarense* isolates, ATDH01, ATGX02 and ATMJ02 were investigated (Fig. 5). Variations of salinity significantly affected toxin content of ATGX02 and ATMJ02, Qt decreased with the elevated salinity, and the highest toxin contents were found at the lowest salinity. The Qt of ATMJ02 and ATGX02 decreased nine and three times, respectively, over the range of 15–35‰. Qt values of ATDH01 were more variable as salinity increased, the lowest Qt was observed in the culture with salinity of 30‰. The toxin composition of the three isolates did not change significantly, and C2 and GTX5 were the dominant toxins in all cases. However, a slight variation was found in ATMJ02, where C2 decreased from 72% to 64% and GTX5 increased from 16% to 23% as salinity increased from 15‰ to 20‰.

4. Discussion

4.1. Toxin composition profiles of regional *Alexandrium* isolates

The diversity of toxin profiles produced by *Alexandrium* species from different geographic areas has been well documented in many regions of the

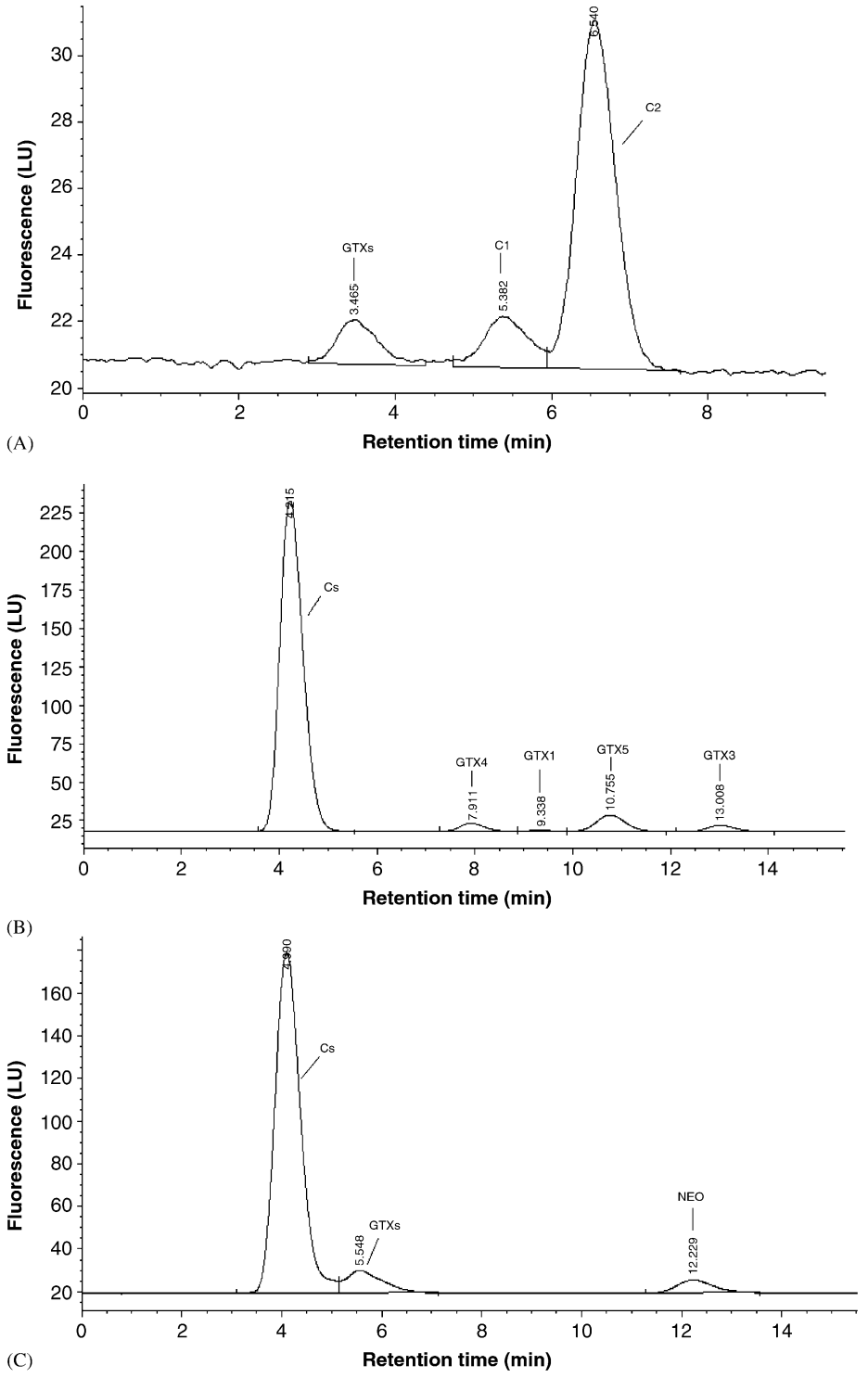

Fig. 1. High performance liquid chromatography paralytic shellfish toxin profiles of *Alexandrium* species: (A) ATDH03 with C mobile phase; (B) ATDH03 with GTX mobile phase; and (C) ATDH03 with STX mobile phase.

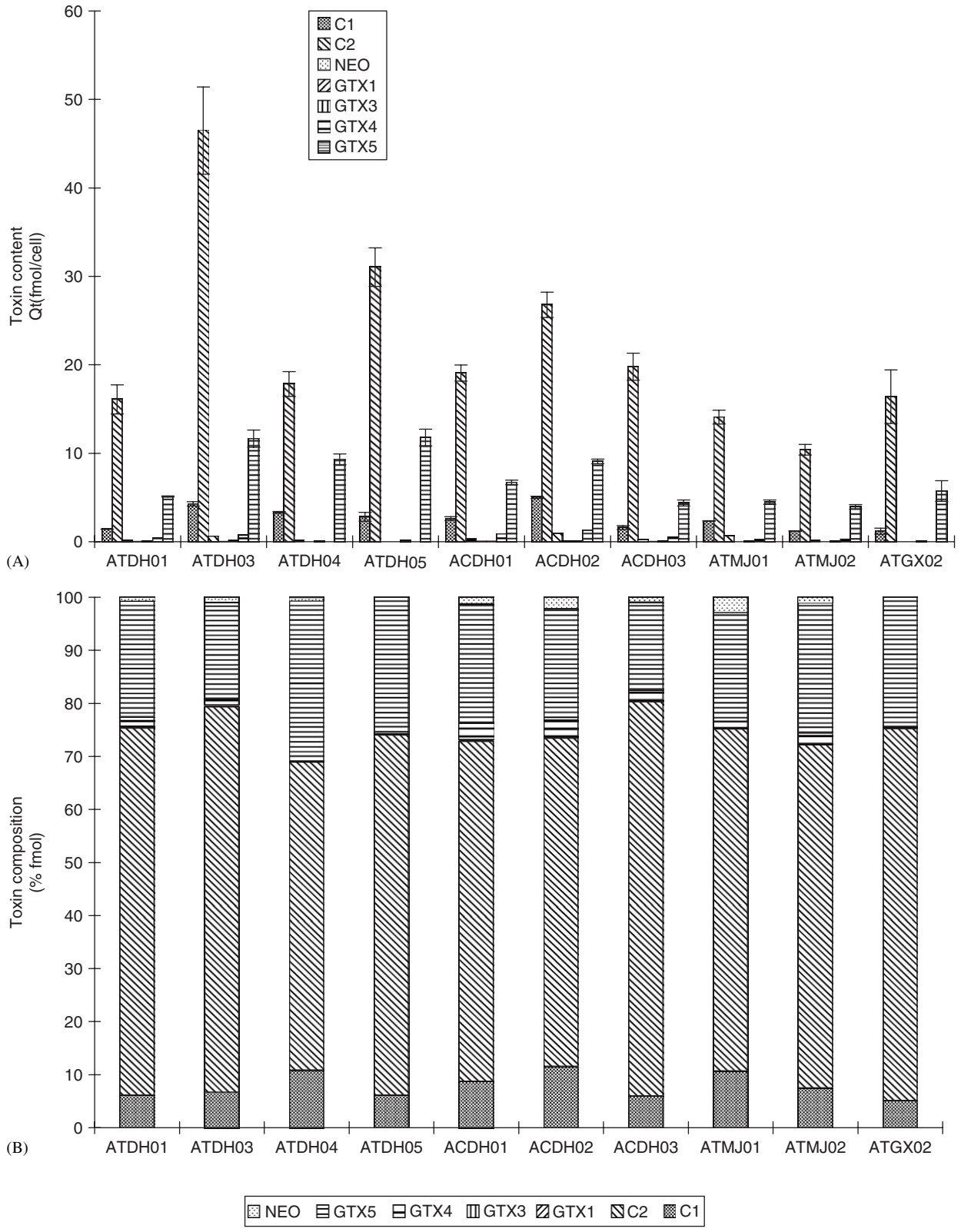

Fig. 2. Toxin content (Qt, fmol cell^{-1}) and composition (fmol %) of 10 toxic *Alexandrium* isolates from the Southeast China Sea: (A) toxin content; (B) toxin composition.

Table 2
Variations of toxin content (fmol cell^{-1}) and composition (in brackets: fmol %) of three *A. tamarense* isolates at different growth phases

	ATDH01		ATGX02		ATMJ02	
	EX	ST	EX	ST	EX	ST
C1	2.19(5.3)	1.05(4.5)	3.19(7.1)	1.8(5.9)	3(7.3)	1.65(6.9)
C2	28.17(68.3)	16.60(71)	27.58(61.7)	19.64(64.0)	24.48(59.7)	
14.93(62.9)						
GTX1	T	T	–	–	0.08(0.2)	0.07(0.3)
GTX3	0.07(0.2)	0.04(0.2)	0.07(0.2)	0.06(0.2)	0.11(0.3)	0.07(0.3)
GTX4	0.83(2.0)	0.56(2.4)	–	–	1.34(3.3)	0.78(3.3)
GTX5	9.5(23.1)	4.83(20.7)	13.81(29.9)	9.17(30.9)	11.11(27.1)	5.71(24.0)
NEO	0.45(1.1)	0.29(1.2)	0.03(0.1)	T	0.85(2.1)	0.55(2.3)
Total	41.21	23.37	44.68	30.67	40.97	23.76

Note: EX—exponential phase; ST—stationary phase; T—trace; "–"—nondetectable.

world. However, information on PSP toxin profiles of *Alexandrium* in China's coastal waters is still lacking and this study represents the first detailed investigation of PSP toxin profiles in these waters. Sixteen *Alexandrium* isolates were established, each from a single cyst collected from the Southeast China Sea. All toxic isolates produced C1, C2, GTX1, GTX3, GTX4, GTX5 and neoSTX. These toxin profile patterns do not seem to be in agreement with previous studies conducted in the same geographic region. Previously, Anderson et al. (1996) investigated three strains of *A. tamarense* isolated from the South China Sea, which produced C1, C2, GTX2, 3, 4, 5, neoSTX and STX. On the other hand, Wang and Hsieh (2005) found that the *A. tamarense* CI01 strain produced C1, C2, GTX1, GTX2, GTX3, GTX4, GTX5 and dcGTX, while another two *A. tamarense* HK9301 strains produced C1, C2, C3, C4, GTX1, GTX4, GTX5 and GTX6 from the same geographic region. In another study of an *A. catanella* strain isolated from Hong Kong coastal water, C1, C2, GTX1, GTX3, GTX4, GTX6, dcGTX3, neoSTX and STX were detected (Siu et al., 1997). These results are not surprising, for toxin composition is known to vary widely among different *Alexandrium* isolates, even if they conform to the same morphotype. Kim (1995) compared toxin composition and productivity of several strains of aseptic *A. tamarense* and *A. catenella* from Ofunato and Tanabe Bay, Japan, and showed that three strains of *A. tamarense* contained C1, 2, GTX1, 2, 3, 4, and neoSTX, whereas the *A. catenella* strains contained C1, 2, GTX1, 2, 3, 4, 5, and STX. Ichimi et al. (2002) investigated toxin profiles of 20 isolates of *A. tamarense* from Sendai Bay and Nagatsuraura, Japan, and C1, C2, GTX1, GTX2, GTX3, GTX4, neoSTX and STX were detected in these isolates. Recently, Kim et al. (2005) analyzed toxin composition profiles of *Alexandrium* isolates from Korean coastal waters, and divided regional isolates into two groups according to toxin profiles: two isolates of *A. catenella* produced C1, 2, GTX1, 2, 3, 4, 5, dcGTX2, 3, neoSTX and STX with trace or non-detectable levels of C3 and C4; while six isolates of *A. tamarense* produced C1, 2, GTX1, 2, 3, 4, dcGTX3, neoSTX with trace or non-detectable levels of C3, 4, dcSTX and STX, and no GTX5 and dcGTX2. These studies implied that PSP toxins might be differentially characterized by *Alexandrium* populations and/or species. Further studies on more *Alexandrium* regional isolates by toxin analysis together with molecular species designation are required to confirm this assertion.

Toxin profiles have been used as a biochemical marker in some cases to distinguish strains within species of the *Alexandrium* genus as complementary or alternative means of discrimination in addition to the morphological fine description. Yoshida et al. (2001) divided Japanese regional isolates of toxic *A. tamarense* and *A. catenella* into distinguishable regional populations based on toxin composition profiles. They also suggested that toxin composition could be a population-specific marker. Anderson et al. (1994) investigated toxin profiles of 28 toxic *Alexandrium* strains from the northeastern United States and

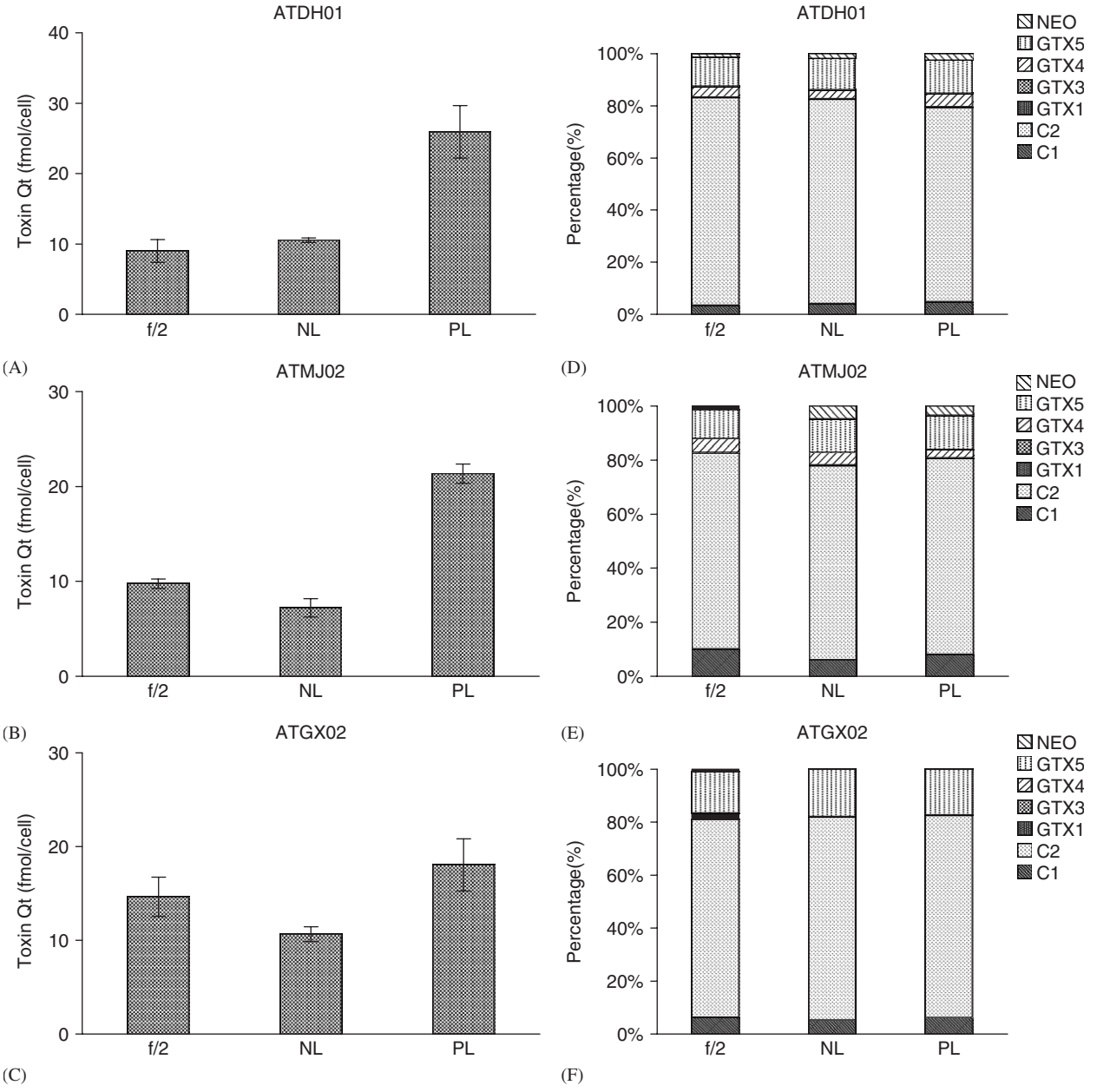

Fig. 3. Effects of nutrients on toxin production (A, B, C) and composition (D, E, F) of three *A. tamarense* isolates. Media: f/2; nitrate limiting (NL) and phosphate limiting (PL).

Canada and clustered two distinct groups of these isolates from toxin profiles: northern isolates, *A. fundyense* with strong signature of neoSTX, STX and GTX2/3; and southern isolates, (a mixture of *A. tamarense* and *A. fundyense*) with a strong signature of C1, C2, dcGTX and GTX1/4. Ciminiello et al. (2000) reported that the toxin composition of *A. andersoni*, which has been known as non-toxic from the Gulf of Naples, Italy, was distinct from those of other *Alexandrium* species, which were dominated with STX and neoSTX. Lilly et al. (2002) proved, using toxin composition profiles together with molecular methods, that a PSP-producing *A. catenella* was introduced into Thau Lagon, France from the Western Pacific using toxin composition profiles together with molecular methods.

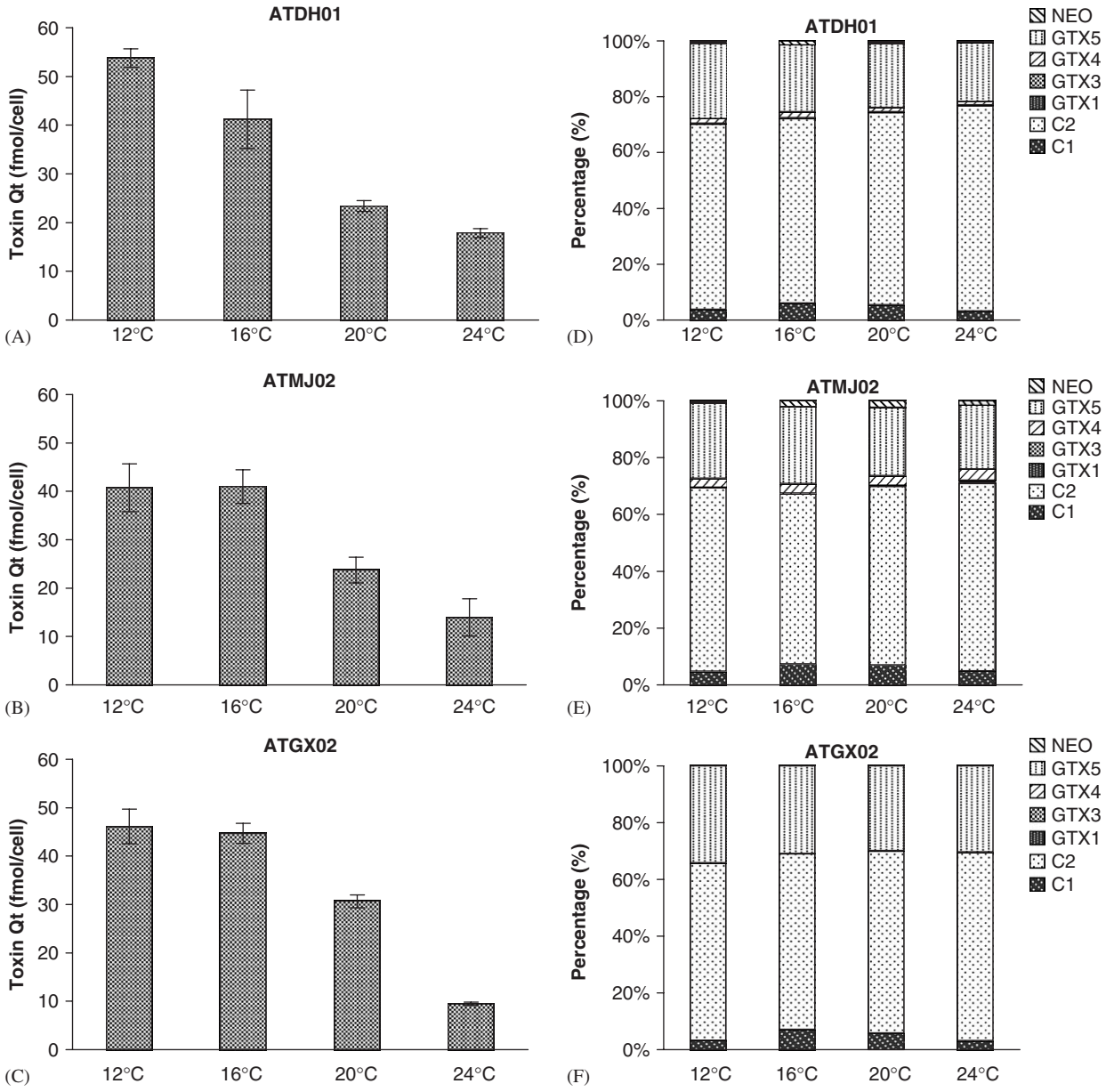

Fig. 4. Effects of temperature on toxin production (A, B, C) and composition (D, E, F) of three *A. tamarense* isolates.

Comparing the toxin profiles with other regional *Alexandrium* strains, the toxin profile of *Alexandrium* strains along the Southeast China Sea is highly unusual. The predominance of C2 and GTX5 in combination with the complete absence of GTX2 and STX has not been observed in many other laboratory strains or from field populations. This profile clearly distinguishes China strains from the relatively consistent toxin profiles of Asia–Pacific strains of *Alexandrium* from Japan, Korea, and North America.

4.2. Toxin content variability

Varying content of *Alexandrium* species with growth phase, nutrient availability and environmental factors has been extensively investigated (Boyer et al., 1987, 1996; Ogata et al., 1987, 1996; Anderson et al.,

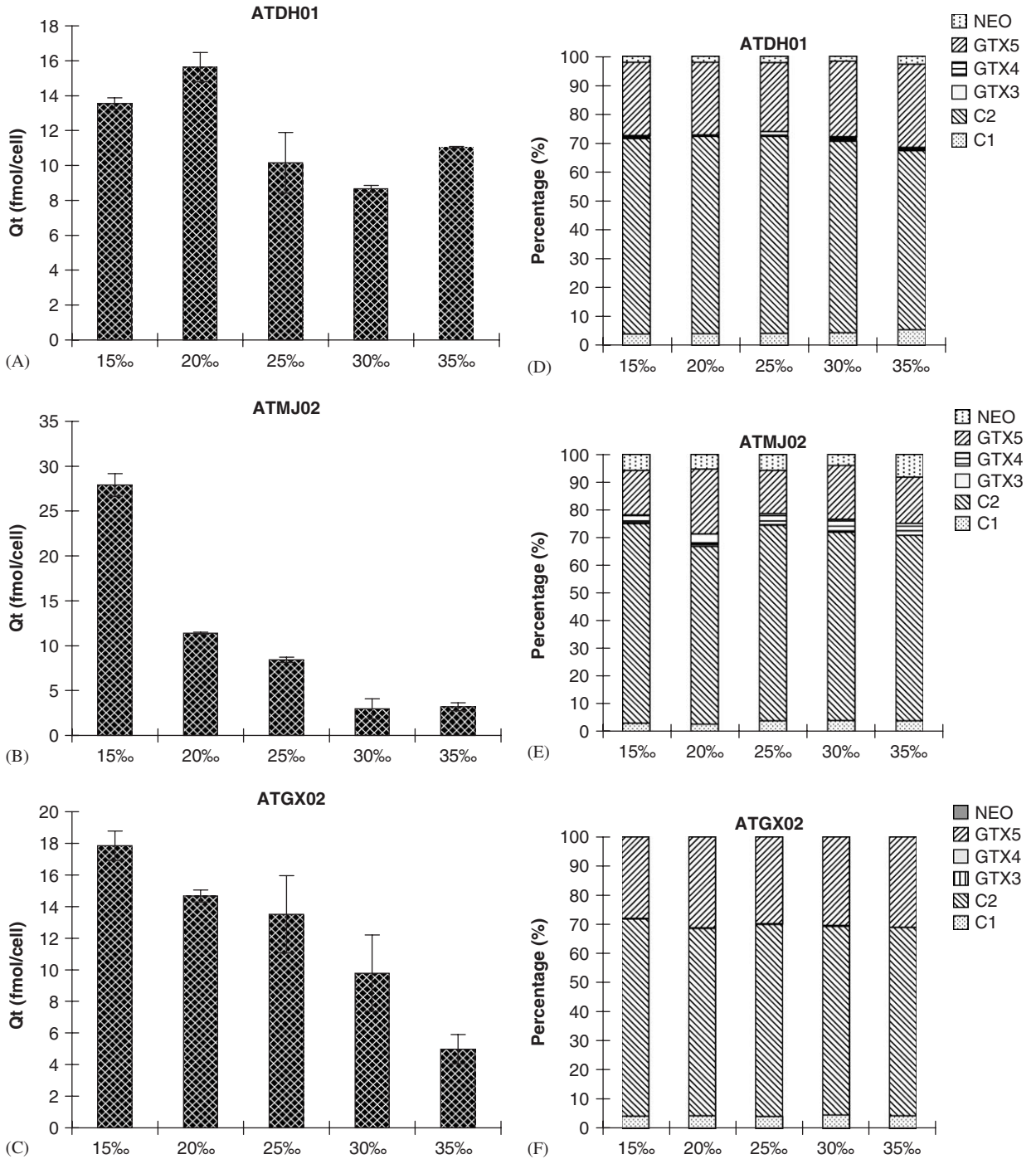

Fig. 5. Effects of salinity on toxin production (A, B, C) and composition (D, E, F) of three *A. tamarense* isolates.

1990a, b; Flynn et al., 1994, 1996; MacIntyre et al., 1997; Parkhill and Cembella, 1999; Hwang and Lu, 2001; Hamasaki et al., 2001; Wang and Hsieh, 2002, 2005; Wang et al. 2005; Lim and Ogata, 2005). Virtually all studies indicated that toxin content peaked in the early exponential phase and was reduced in the stationary phase. Our results present a similar pattern in three toxic *A. tamarense*

isolates grown in nutrient-replete batch culture; toxin contents were higher in the exponential phase than in the stationary phase. However, Wang and Hsieh (2001, 2005) reported a different toxin production pattern in the *A. tamarense* CI01 strain: toxin content peaked in the mid-stationary phase. The reason for this difference is not clear at the moment. One speculation is that the different initial phosphorous (P) concentration in the culture medium might account for such a difference. In most previous studies, and in the present study, the initial P concentration was 30 μM which might not cause P limitation for cells during the entire growth phase. However, whereas in Wang and Hsieh's studies, the initial P concentration was 10 μM, and this was exhausted during the exponential phase (day 4), while nitrate, which was maintained in excess, was sufficient for toxin biosynthesis under severe phosphate limitation.

It is well documented that nitrogen deficiency decreased toxin production and cellular toxin content (Qt) in *Alexandrium*, while phosphorus limitation enhanced toxin production and cellular toxin content (Boyer et al., 1987; Anderson et al., 1990b; Flynn et al., 1994; Bechemin et al., 1999; John and Flynn, 2000; Wang and Hsieh, 2001, 2002). The present results are in agreement with these findings. Nitrate deficiency reduced the toxin contents of three *A. tamarense* isolates while phosphorus deficiency enhanced toxin production. PSTs are a suite of nitrogen-rich compounds, with nitrogen accounting for 17–35% of the molecular weight of a PST and about 5–10% of the total cellular nitrogen in *Alexandrium* and *Gymnodinium* sp. (Cembella, 1998). Thus, a supply of sufficient nitrogen is essential for PST biosynthesis. The mechanism by which phosphorus regulates toxin production is not well understood at present. P-limitation might cause cell division to cease, thus allowing the cells to continue utilizing available arginine, a presumed precursor in PST biosynthesis, or other cellular constituents for toxin biosynthesis (Shimizu et al., 1984; Anderson et al., 1990b).

There have been consistent observations that high temperature reduced PST production in *Alexandrium* (Ogata et al., 1987; Anderson et al., 1990b; Usup et al., 1994; Hwang and Lu, 2001; Etheridge and Roesler, 2005). The present results further supported these findings. Toxin contents of three *A. tamarense* isolates decreased with the elevation of temperature, the highest Qt was found in the lowest culture temperature. The direct effect of temperature on PST biosynthesis is not clear. Anderson et al. (1990a, b) observed that arginine and toxin content of *A. fundyense* at 8 °C were higher than that at 15 °C. Usup et al. (1994) reported that the rate of increase of toxin content due to a decrease in culture temperature was higher than the rate at which growth rate decreased over the same temperature range. They postulated that the elevation in toxin content at low temperature is not simply caused by low division rate, but is related to other factors such as turnover rates of cellular components. Cells might allocate more cellular nitrogen to toxin synthesis and less to protein synthesis at low temperature. This needs further study at the biochemical and molecular level.

The effects of salinity on toxin content are controversial. Some studies indicated that high salinity enhanced toxin production, and that toxin content increased with the salinity of the culture medium (White, 1978; Parkhill and Cembella, 1999; Hwang and Lu, 2001; Wang and Hsieh, 2005), while other investigators reported contrary results. Usup et al. (1994) found that low salinity enhanced toxin production of *P. bahamense* var. *compressum*. Hamasaki et al. (2001) also found that the toxin content of *A. tamarense* decreased with increasing salinity in the range 13–29‰. Recently, Lim and Ogata (2005) investigated the effects of salinity on toxin production of four tropical *Alexandrium* species, and found that the toxin content of *A. minutum, A. tamiyavanichiii* and *A. tamarense* decreased with elevated salinities, while it increased with elevated salinities in *A. peruvianum*. Our results indicated that toxin content of three *Alexandrium* species decreased with increasing salinity, and the Qt of ATMJ02 and ATGX02 decreased nine and three-fold, respectively, over the range 15–35‰. Such discrepancies in the results of salinity experiments have no clear explanation. Lim and Ogata (2005) postulated that the effects of salinity on *Alexandrium* species were not only region-dependent but also species-dependent. Moreover, differences in acclimation time, and in the steepness and range of salinity gradient may be other explanations. In the studies of Hamasaki et al. (2001) and Usup et al. (1994), cells were acclimated to each experimental salinity in advance, while in the present study and other studies (White, 1978; Parkhill and Cembella, 1999; Hwang and Lu, 2001), cells were introduced directly into the media with different salinity levels, such that the stress due

to a sudden osmotic change might alter the metabolism related to toxin biosynthesis.

4.3. Toxin composition

Many studies have been devoted to toxin composition in different *Alexandrium* species and strains. Some investigators have indicated that toxin composition varied with growth phase and culture conditions (Boyer et al., 1987; Cembella et al., 1987; Boczar et al., 1988; Anderson et al., 1990a, 1994; Flynn et al., 1994; Hamasaki et al., 2001; Wang and Hsieh, 2005). Anderson et al. (1990a, b) observed that toxin composition varied with growth rate in a semi-continuous culture of *A. fundyense* under nitrogen- and phosphorus-limited conditions: nitrogen limitation enhanced the production of C1 and C2 toxins as well as GTX1 and GTX4, whereas phosphorus limitation enhanced the production of GTX2 and GTX3. Wang and Hsieh (2005) reported that toxin composition of *A. tamarense* HK9301varied with growth phases and culture conditions: in nutrient-replete cultures, toxin composition of *A. tamarense* HK9301varied greatly in the early growth phase. GTX4 increased ca. 14% and GTX5 decreased ca. 13% throughout the rest of the culture cycle. C toxins remained relatively constant under various culture conditions, while GTXs varied greatly.

However, other investigators thought that toxin composition was stable and did not vary with growth phase or culture conditions, and this has been used in some cases as a chemical taxonomic tool for strain and species identification (Boyer et al., 1987; Cembella et al., 1987; Oshima et al., 1993; Kim et al., 2005). Our present results further supported proved this conclusion. In our batch cultures of *Alexandrium*, compositional changes in toxins were subtle, even under nutrient-deficient conditions, and were negligible when compared to variations between the isolates from different geographic regions.

The above conflicting views on toxin composition might be due to differences in experimental design and long maintenance of cultures in artificial conditions. For example, in some studies of the effects of salinity on toxin production, the cells for the experiment were acclimated to each experimental salinity in advance (Hamasaki et al., 2001; Usup et al., 1994), while in other studies, the cells were introduced directly into the experimental salinity (White, 1978; Parkhill and Cembella, 1999; Hwang and Lu, 2001). Artificial culture conditions were also thought to cause toxin composition variations. Wang and Hsieh (2005) found that toxin composition of *A. tamarense* CI01 strain varied significantly and some toxin components were not detected after several years' maintenance in artificial conditions. Claudia et al. (2004) reported that one formerly toxic *A. lusitanicum* strain lost the ability to produce toxin after prolonged treatment with antibiotics, and they concluded that genetic mutation had occurred in *A. lusitanicum*. Oshima et al. (1993) postulated that the toxin profile is an expression of enzymes involved in toxin biosynthesis, the presence or absence of certain toxins reflecting genetic differences in the enzymes involved between and within populations. Artificial conditions might cause mutations and irreversible physiological changes at elevated rates, resulting in permanent changes in the activities of the algae, including toxin biosynthesis.

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