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Harmful Algae 4 (2005) 109–121
HARMFUL ALGAE
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Toxin composition variations in cultures of *Alexandrium* species isolated from the coastal waters of southern China

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Received 11 April 2003; received in revised form 23 October 2003; accepted 17 December 2003

Abstract

The composition of the paralytic shellfish toxins (PSTs) of five *Alexandrium tamarense* strains isolated from the coastal waters of southern China and one *Alexandrium minutum* strain from Taiwan Island were investigated. *A. tamarense* CI01 and *A. tamarense* Dapeng predominantly produced C2 toxin (over 90%) with trace amounts of C1 toxin (C1), gonyautoxin-2 (GTX2) and GTX3; two strains of *A. tamarense* HK9301 maintained in different locations produced C1-4 toxins and GTX1, 4, 5 and 6; no PSTs were found in *A. tamarense* NEW, while *A. minutum* TW produced only GTX1-4. The toxin compositions of cultured *A. tamarense* strains did not vary as much during different growth phases as did the toxin composition of *A. minutum* TW. The toxin compositions of *A. tamarense* HK9301-1 did not change significantly under different salinity, light intensity, and nitrate and phosphate levels in the culture medium, although the toxin productivity varied expectably. Another strain HK9301-2 maintained in a different location produced much less toxins with a considerably different toxin composition. Under similar culture maintenance conditions for 3 years, the toxin profiles of *A. tamarense* HK9301-1 did not change as much as did *A. tamarense* CI01. Our results indicate that toxin compositions of the dinoflagellate strains are strain-specific and are subject to influence by nutritional and environmental conditions but not as much by the growth phase. Use of toxin composition in identifying a toxigenic strain requires special caution.

Keywords: Paralytic shellfish toxin; Toxin composition; *Alexandrium*

1. Introduction

Paralytic shellfish toxins (PSTs) are a family of over 20 structurally related imidazoline guanidinium derivatives (Fig. 1) produced by some marine dinoflagellates including the *Alexandrium* species, freshwater cyanobacteria, and dinoflagellate-associated bacteria (Shimizu, 1996; Kodama, 2000). These toxins vary in toxic potency by hundreds of fold due to certain minor structural differences (Oshima, 1995a,b). Some investigators have observed that toxin composition varies among different species and among different strains in a species and even among cultures of a same strain under different nutritional and environmental conditions (Shimizu, 1979; Hall, 1982; Cembella and Taylor, 1985; Cembella et al., 1987; Boczar et al., 1988; Anderson et al., 1990; Flynn et al., 1994; Hamasaki et al., 2001; Hwang and Lu, 2001). Other investigators maintain that the toxin composition of an algal culture is constant and is a biochemical

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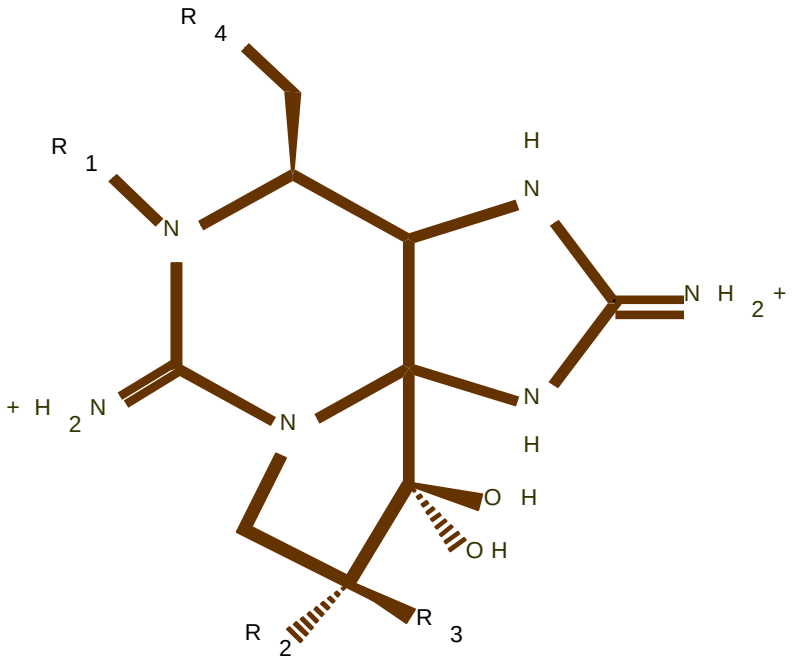
doi:10.1016/j.hal.2003.12.003



R1	R2	R3	R4: OCOHNH2	R4: OCOHNSO3
H	H	H	STX	GTX5
OH	H	H	neoSTX	GTX6
OH	OSO3	H	GTX1	C3
H	OSO3	H	GTX2	C1
H	H	OSO3	GTX3	C2
OH	H	OSO3	GTX4	C4

Fig. 1. Common structure of paralytic shellfish toxins.

characteristic of the organism (Cembella and Taylor, 1985; Boyer et al., 1987; Ogata et al., 1987; Oshima et al., 1990; Anderson et al., 1994; Flynn et al., 1996; Parkhill and Cembella, 1999).

Despite the conflicting views, information on the toxin compositions of geographically prevalent dinoflagellates is important in assessing the environmental health significance of these local dinoflagellates. PSTs are a growing economic and public health concern in coastal areas of southern China, as suggested by the growing number of reports of mariculture losses and threats to public health (Qiu, 1990; Jian and Deng, 1991; Lin et al., 1994; Zhou et al., 1999). A number of studies indicate that the *Alexandrium* species is involved in the production of PSTs in these areas (Anderson et al., 1996; Zhou et al., 1999; Siu et al., 1997).

In the present study, the PST compositions of five strains of *Alexandrium tamarense* isolated from the coastal areas of southern China were investigated and compared under different culture conditions, different growth phases and different lengths of culture maintenance to gain insight into the potential environmental health significance of these dinoflagellates. A species isolated from Taiwan Island, *A. minutum* TW, was also included in the comparison.

2. Materials and methods

2.1. Organisms

Cultures of *A. tamarense* CI01 (ATCI01), *A. tamarense* Dapeng (ATDP) and *A. tamarense* HK9301 were established from germinated cysts isolated from Daya Bay and Dapeng Bay sediments, and *A. tamarense* New was isolated from Hong Kong coastal waters. ATDP, *A. minutum* TW (AMTW) and a strain of *A. tamarense* HK9301 (ATHK9301-2) were maintained by the Institute of Hydrobiology, Jinan University, Guangzhou, PR China. The organisms were cultivated in a modified f/2 medium at 20 °C with 80 μE m^{-2} s^{-1} and a 14/10 h light/dark cycle. The cultures of ATCI01 and another *A. tamarense* HK9301 strain, ATHK9301-1 had been maintained in our laboratory since 1998 in a natural seawater K medium (Keller and Guillard, 1985) at 23 °C. Our stock cultures were sub-cultured every 7–10 days. Cultures used for toxin analysis were cultivated in 125 ml of K medium contained in a 250 ml conical flask, in duplicate, at 23 °C on a 14/10 h light/dark cycle and 80 μE m^{-2} s^{-1} light intensity. Modified K media with lower concentrations of phosphate (5 μM) and nitrate (240 μM) and varied light intensities (80, 120, 180 μE m^{-2} s^{-1}), and salinities (20, 25, 30, 35‰) were used for comparison. Algal growth was monitored by cell count under a light microscope.

2.2. Analysis of toxins

The cultured algal cells were collected by centrifugation at 4000 rpm, suspended in 0.5 ml of 0.05 M acetic acid and homogenized with three successive sonications. The supernatant obtained after centrifugation at 14,000 rpm for 30 min was subjected to analysis.

Toxin analysis was carried out on HPLC with fluorescence detection using reverse phase chromatography with post column derivatization and an intersil C8-5 column (15 cm × 4.6 cm), as reported previously (Wang and Hsieh, 2001), with slight modifications. The following three mobile phases (flow rate 0.8 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 11) for the gonyautoxin (GTX) group; (3) 2 mM 1-heptanesulfonic acid in a mixture of 30 mM ammonium phosphate buffer (pH 7.1) and acetonitrile (100:5) for the saxitoxin (STX) group.

3. Results

3.1. Toxin profiles of the Alexandrium cultures

Five strains of *A. tamarense* isolated from the coastal waters of southern China and one species from Taiwan Island, *A. minutum*, were cultured under the same conditions. The typical HPLC profiles of toxin extracts from individual cultures are shown in Fig. 2. Their cellular toxin content in fmol per cell and the relative abundance of the toxins in mol% in different growth phases are shown in Table 1.

A. tamarense CI01 produced nearly exclusively C2 toxin (C2), with only trace amounts of C1 toxin (C1), GTX2 and GTX3 (Fig. 2A). The other strain, *A. tamarense* Dapen gave the same pattern. In comparison, *A. minutum* TW produced GTX1, GTX2, GTX3 and GTX4, but no C toxins (Fig. 2B). *A. tamarense* HK9301-1 and *A. tamarense* HK9301-2 produced C1-4, GTX1, GTX4, GTX5 and GTX6, but no GTX2/3 (Fig. 2C and D). No PST-like compounds were found to be produced by *A. tamarense* New.

3.2. Variations during growth phases

The toxin compositions of the different isolates of *A. tamarense* did not change significantly during the different phases of growth, compared to the toxin composition of *A. minutum* TW, which varied considerably (Tables 1 and 2). In the latter culture, while the relative abundance (mol%) of GTX1 did not change much during the entire growth cycle, GTX2 and GTX3 decreased but GTX4 increased as the culture moved through the growth phases. Although the relative abundance of toxins produced by the two *A. tamarense* HK9301 strains, which were maintained in different localities, did not change significantly when the cultures moved through the different growth phases, the total toxin yield of strain 2

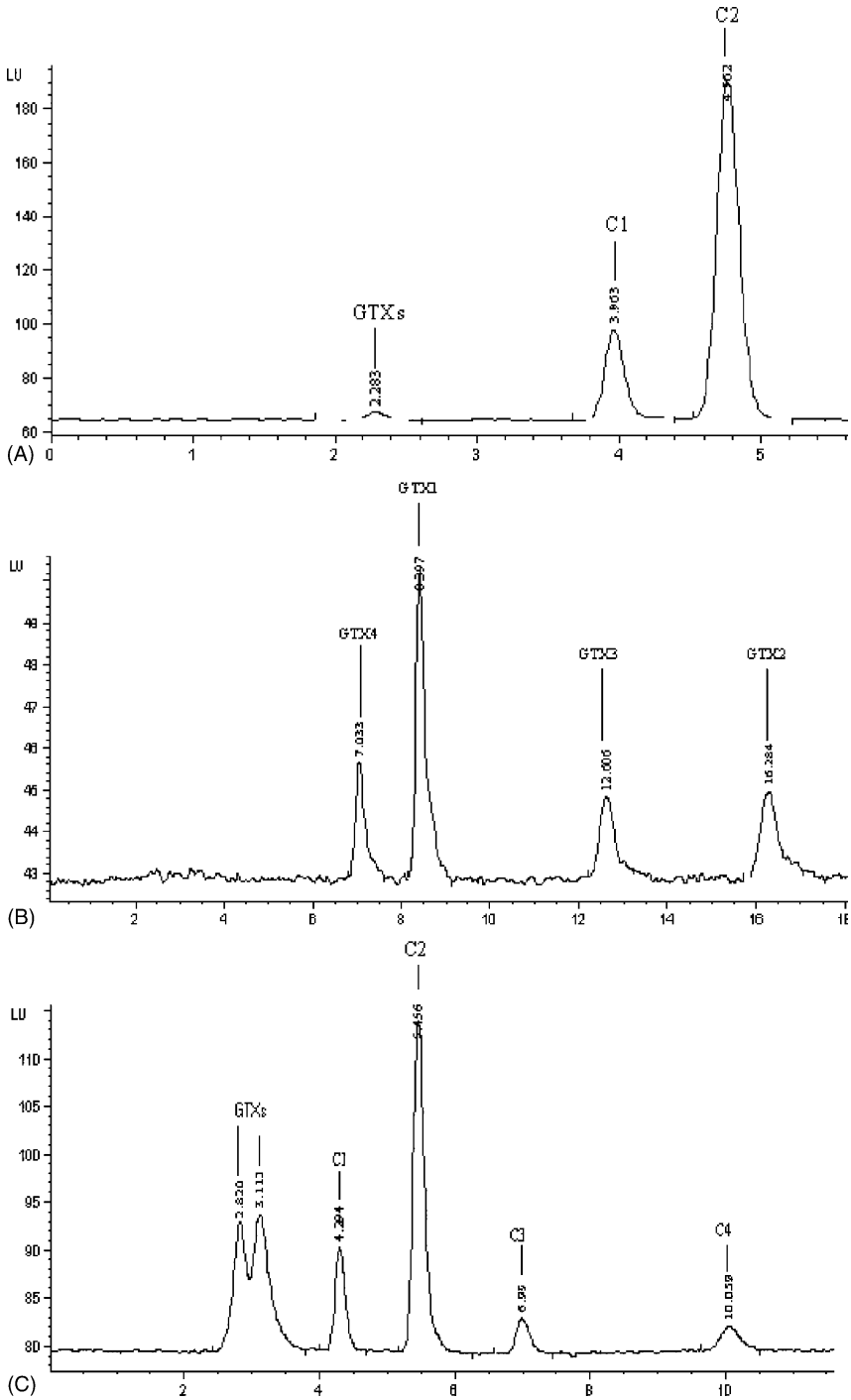


Fig. 2. High performance liquid chromatography of the profiles of paralytic shellfish toxins from cultures of *Alexandrium* species. (A) *A. tamarense* CI01 with C mobile phase; (B) *A. minutum* TW with GTX mobile phase; (C) *A. tamarense* HK9301 with C mobile phase; (D) *A. tamarense* HK9301 with GTX mobile phase.

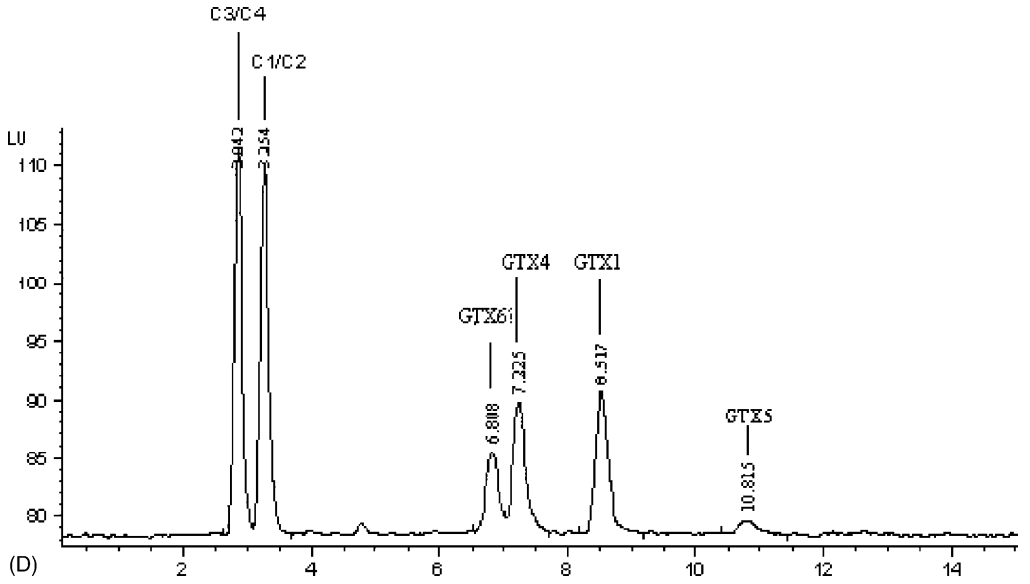


Fig. 2. (*Continued*) .

was considerably lower than that of strain 1 while the yield of GTX1 was higher. Consequently, the relative abundance of GTX 4 and GTX1 differed considerably between the two strains (Table 2). The toxin compositions of *A. tamarense* CI01 and *A. tamarense* Dapen were very simple throughout all the growth phases. C2 was the predominant toxin (over 90%) with highest concentrations found in the late stationary phase.

3.3. Variations under various culture conditions

The toxin compositions of *A. tamarense* HK9301-1 under various culture conditions are shown in Table 3. Based on the data obtained for various cultures harvested in the late stationary phase (day 10), the most significant factor affecting toxin productivity was the phosphate concentration of the culture medium, followed by the received light intensity and then the salinity of the medium. The total toxin productivity in fmol per cell of the culture was highest in the low phosphate medium (LP) and lowest in the low nitrate medium (LN). Within the range of the light intensity and the salinity in the medium used, the toxin productivity varied less than 30%. The relative molar abundance of most toxins in the cells did not differ significantly in various conditions with a few exceptions. GTX1 increased from 6.7 to 19.6% and GTX5 decreased from 19.4 to 11.4% in LN, whereas GTX5 increased from 19.4 to 26.2% in LP. Under different light intensities, the relative abundance of GTX4 increased from 34.6 to 44.6% and that of GTX5 decreased from 23.0 to 15.2%. Under the different salinity used, only the relative abundance of GTX5 decreased from 21.7 to 11.8%.

3.4. Changes during culture maintenance

Changes in the toxin composition of *A. tamarense* HK9301-1 and *A. tamarense* CI01 were investigated by maintaining the cultures under laboratory conditions for more than 2 years. The toxin composition of *A. tamarense* CI01 changed greatly (Fig. 3A) while that of *A. tamarense* HK9301-1 did not (Fig. 3B). In 1999, the CI01 strain was producing C1, C2, GTX1, GTX2, GTX3, GTX4, GTX5 and decarbamoyl GTX (dc GTX) with C2 being the predominant toxin. The same culture in 2000 and 2001 was found to produce only C1 and C2 with a trace amount of GTX3. In comparison, the HK9301-1 strain continued to produce C1, C2, GTX1, GTX3, GTX4, GTX5 and GTX6 with GTX3 being in only a trace amount while GTX4 was the major toxin (the data of C3/4 toxins was not available for 2000).

Table 1
Toxin compositions in different growth phases of *Alexandrium* species

Toxin	ATCI01			ATDP			AMTW			
	EX (fmol per cell (%))	ES (fmol per cell (%))	MS (fmol per cell (%))	EX (fmol per cell (%))	ES (fmol per cell (%))	MS (fmol per cell (%))	EX (fmol per cell (%))	ES (fmol per cell (%))	MS (fmol per cell (%))	LS (fmol per cell (%))
C1/C2	18.2 (100)	20 (100)	28.4 (100)	25.2 (100)	31.4 (100)	51.6 (100)	ud	ud	ud	ud
C3/C4	ud	ud	ud	ud	ud	ud	ud	ud	ud	ud
GTX1	ud	ud	ud	ud	ud	ud	5.5 (44.4)	7.0 (38.5)	7.3 (48.0)	7.3 (54.0)
GTX2	tr	tr	tr	tr	tr	tr	5.2 (41.9)	3.5 (19.2)	1.4 (9.2)	0.0 (0.0)
GTX3	tr	tr	tr	tr	tr	tr	1.7 (13.7)	0.9 (4.9)	0.4 (2.6)	0.4 (3.0)
GTX4	ud	ud	ud	ud	ud	ud	0.0 (0.0)	6.8 (37.4)	6.1 (40.1)	5.8 (43.0)
GTX5	ud	ud	ud	ud	ud	ud	ud	ud	ud	ud
Total	18.2	20	28.4	25.2	31.4	51.6	12.4	18.2	15.2	13.5

ATCI01, *A. tamarense* CI01; ATDP, *A. tamarense* Dapeng; AMTW, *A. tamarense* TW. EX: exponential phase; ES: early stationary phase; MS: middle stationary phase; LS: later stationary phase; tr: trace amount; ud: undetected.

Table 2
Toxin compositions in different growth phases of two *A. tamarense* HK9301 strains maintained in different locations

Toxin	ATHK9301-1				ATHK9301-2			
	EX (fmol per cell (%))	ES (fmol per cell (%))	MS (fmol per cell (%))	LS (fmol per cell (%))	EX (fmol per cell (%))	ES (fmol per cell (%))	MS (fmol per cell (%))	LS (fmol per cell (%))
C1/C2	11.4 (27.7)	10.6 (27.0)	13.9 (26.4)	16.5 (23.2)	5.7 (28.2)	6.8 (23.4)	9.0 (21.4)	10.4 (22.3)
C3/C4	2.8 (6.8)	2.7 (6.9)	3.4 (6.5)	4.1 (5.8)	1.4 (6.9)	1.7 (5.9)	2.3 (5.5)	2.6 (5.6)
GTX1	1.9 (4.6)	2.5 (6.4)	4.3 (8.2)	5.6 (7.9)	5.0 (24.8)	5.9 (20.3)	9.6 (22.8)	9.1 (19.5)
GTX4	19.8 (48.2)	19.1 (48.5)	24.8 (47.1)	33.1 (46.6)	8.1 (40.1)	10.2 (35.2)	14.1 (33.5)	17.2 (36.8)
GTX5	5.2 (12.7)	4.4 (11.2)	6.2 (11.8)	11.7 (16.5)	0.0 (0.0)	4.4 (15.2)	7.1 (16.7)	7.4 (15.8)
Total	41.4	39.3	52.6	71	20.2	29	42.1	46.7

ATHK9301, *A. tamarense* HK9301. EX: exponential phase; ES: early stationary phase; MS: middle stationary phase; LS: late stationary phase.

Table 3
Toxin compositions of *A. tamarense* HK9301-1 under different culture conditions

Toxin	Culture conditions			Light intensity ($\mu E\,m^{-2}\,s^{-1}$)			Salinity (‰)			
	Full (fmol per cell (%))	LN (fmol per cell (%))	LP (fmol per cell (%))	80 (fmol per cell (%))	120 (fmol per cell (%))	180 (fmol per cell (%))	20 (fmol per cell (%))	25 (fmol per cell (%))	30 (fmol per cell (%))	35 (fmol per cell (%))
C1/C2	16.1 (25.5)	8.8 (24.0)	26.1 (21.7)	18.1 (23.5)	11.7 (20.9)	12.0 (21.6)	15.0 (20.3)	20.0 (23.5)	24.1 (25.1)	19.3 (25.1)
C3/C4	4.0 (6.3)	2.2 (6.0)	5.7 (4.7)	4.5 (5.9)	2.9 (5.2)	3.0 (5.4)	3.8 (5.1)	5.0 (5.9)	6.0 (6.2)	4.8 (6.2)
GTX1	4.2 (6.7)	7.2 (19.6)	7.0 (5.8)	10.0 (13.0)	5.3 (9.5)	7.4 (13.2)	6.3 (8.5)	7.8 (9.2)	9.3 (9.7)	7.9 (10.3)
GTX4	26.5 (42.1)	14.3 (39.0)	50.0 (41.6)	26.7 (34.6)	25.2 (45.1)	24.8 (44.6)	32.7 (44.3)	37.0 (43.5)	42.5 (44.1)	35.9 (46.6)
GTX5	12.2 (19.4)	4.2 (11.4)	31.5 (26.2)	17.7 (23.0)	10.8 (19.3)	8.5 (21.7)	16.0 (21.7)	15.3 (17.9)	14.3 (14.9)	9.1 (11.8)
Total	63.9	36.7	120.3	76.9	55.9	55.7	73.8	85.1	96.2	77

Full: Full K medium as control. Modified K medium with lower nitrate (LN) and phosphate (LP).

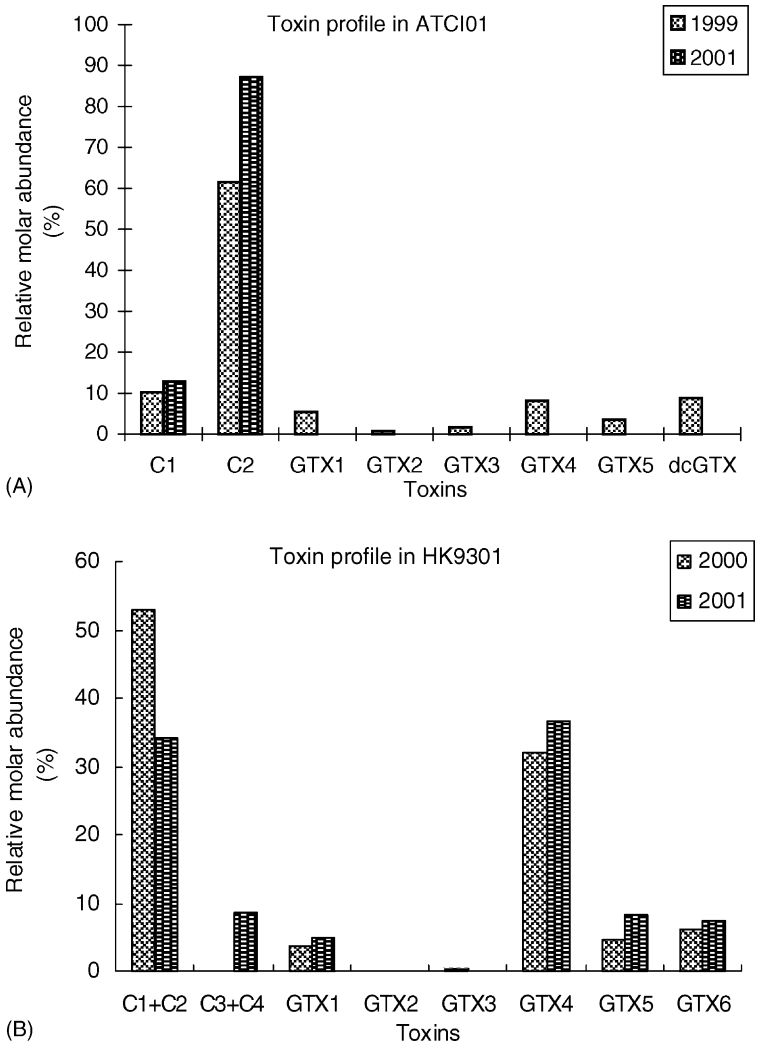


Fig. 3. Changes in toxin composition of *A. tamarense* CI01 (A) and *A. tamarense* HK9301-1 (B) as a result of long culture maintenance.

4. Discussion

4.1. Variations in toxin profiles among Alexandrium species

Studies on the toxin compositions of PST-producing dinoflagellates have led to isolation and identification of over 20 PST analogues. The ability to produce certain combinations of specific toxins by an algal strain has been used in some cases as chemical taxonomy tool for strain and species identification (Boyer et al., 1987; Cembella et al., 1987; Oshima et al., 1990). This practice requires caution as evident from the results of our present study. Our results indicate that the toxin composition of a culture of a toxigenic dinoflagellate can vary among growth phases (Table 2), nutritional and cultivation conditions (Table 3), and length of culture maintenance (Fig. 3), in addition to genetic variations represented by different species and different strains within a species (Tables 1 and 4).

However, there seems to be a consistent toxin composition found in the cultures of strains of *A. tamarense* isolated from the coastal waters of southern China. The cultures of the strains used in our

Table 4
Toxin profiles of PSTs in *Alexandrium* species isolated from the coastal waters of southern China

Algal strains	Toxin													Reference
	C1	C2	C3	C4	GTX1	GTX2	GTX3	GTX4	GTX5 (B1)	GTX6 (B2)	dcGTX3	neoSTX	STX	
A. tamarense CI01-1	+	++	−	−	−	−	+	+	+	−	−	+	+	Anderson et al. (1996)
A. tamarense CI02-1	+	++	−	−	−	−	+	+	+	−	−	+	+	Anderson et al. (1996)
A. tamarense CI03-1	+	++	−	−	−	−	+	+	+	−	+	+	+	Anderson et al. (1996)
A. tamarense CI03	+	++	−	−	−	+	+	−	+	+	−	−	−	Yu et al. (1998)
A. tamarense HK	+	++	−	−	+	+	+	+	+	+	−	+	+	Yu et al. (1998)
A. tamarense CI01	+	++	−	−	+	+	+	+	+	−	+	−	+	Wang and Hsieh (2001)
A. tamarense HK9301-1	+	++	+	+	+	−	−	++	+	+	−	−	−	This study
A. tamarense HK9301-2	+	++	+	+	+	−	−	++	+	+	−	−	−	This study
A. tamarense CI01	+	++	−	−	−	−	−	−	−	−	−	−	−	This study
A. tamarense Dapen	+	++	−	−	−	−	−	−	−	−	−	−	−	This study
A. minutum TW	−	−	−	−	+	+	+	+	−	−	−	−	−	This study
A. minutum TW	−	−	−	−	+	+	+	+	−	−	−	−	−	Hwang and Lu (2001)
A. catenella	+	++	?	?	+	−	+	++	−	+	+	+	+	Siu et al. (1997)

"+" contain; "−" not detected.
"?": peaks not matching with any analytical standards.

study, i.e. *A. tamarense* CI01, Dapeng and HK9301, all predominantly produced the N-sulfocarbamoyl toxin, C2, with trace amounts of C1 and low levels of certain gonyautoxins. Our results compare very well with those of Anderson et al. (1996), who examined toxin production by three *A. tamarense* strains isolated from the coastal waters of southern China and found that these strains predominantly produced C2 (90% of the total) with trace amounts of C1 and certain gonyautoxins. A similar result was also reported by Yu et al. (1998), who worked with two strains isolated from the same region. These studies all point to C2 and its derivatives as the characteristic of the problem associated with toxigenic *A. tamarense* in the coastal areas of the South China Sea, including Hong Kong. From *A. catenella*, an important harmful algal species found in Hong Kong coastal waters, the major toxin produced was GTX4 along with the C toxins and other GTXs (Siu et al., 1997). Trace amounts of STX and/or neoSTX were also found. In the present study, C3 and C4 toxins were, for the first time, detected in the strain of *A. tamarense* HK9301, substantiating the hypothesis of Anderson et al. (1996) that there must exist an algal species capable of producing C3 and C4 toxins in this area.

Variation in toxin compositions of *Alexandrium* isolates at different growth phase under the culture conditions have been suggested by numerous investigators (Boyer et al., 1987; Cembella et al., 1987; Boczar et al., 1988; Anderson et al., 1990, 1994; Flynn et al., 1994; Hamasaki et al., 2001). For example, Anderson et al. (1990) observed that toxin composition varied with growth rate in a semi-continuous culture of *Alexandrium fundyense* under nitrogen- and phosphorus-limited conditions. Nitrogen limitation favored the production of C1 and C2 toxins as well as GTX1 and GTX4, whereas phosphorus limitation favored the production of GTX2 and GTX3.

The effect of culture maintenance on toxin composition and yield may be related to the artificial conditions used in controlled studies. In our study, the inocula of algal cultures were continuously sub-cultured every 7–10 days. The cells were exposed to high concentrations of nutrients (N, 880 μM; P, 10 μM) and optimal temperature and light intensity for long periods of time. Under these conditions, mutations and irreversible physiological changes are likely to occur at elevated rates, resulting in permanent changes in the activities of the algae, including toxin biosynthesis. Oshima et al. (1993) have offered a similar explanation at the biochemical and genetic levels for the change of toxin composition due to culture maintenance. Our result indicates that comparative data should be taken from cultures that have been maintained in the laboratory for as short a time as possible.

4.2. Biosynthesis of PSTs

Our current understanding on the biosynthesis of saxitoxin and its related PSTs is largely derived from studies on a cyanobacterium, *Aphanizomenon flos-aquae* (Shimizu, 1993, 1996). In this species, saxitoxin as the parent compound was thought to be synthesized via a pathway involving arginine, S-adenosylmethionine, and acetate. Subsequently modifications by addition or removal of hydroxyl, carbamyl, and/or hyroxysulfate moieties yield the known 21 derivatives. Relatively little is known about STX biosynthesis in dinoflagellates except about a few enzymes found to be related to modifications of the final toxins. Yoshida et al. (1998) reported on a sulfotransferase in *A. catenella* Acko5, which could produce C1/2 and GTX5 from GTX2/3 and STX, respectively. More recently, Sako et al. (2001) described two sulfotransferases, N-ST and O-ST, found in *Gymnodinium catenatum*, which were involved in sulfation of STX. N-ST could transfer the sulfate group of 3′-phosphoadenisine 5′-phosphosulfate (PAPS) to N-21 of STX and GTX2/3 to give rise to GTX5 and C1/2, respectively. O-ST could convert 11-α, β-hydroxy STX to GTX2/3. The authors hypothesized that STX is converted predominantly to 11-α, β-hydroxy STX by C-11 oxidation and partially to GTX5 by N-21 sulfation. The 11-α, β-hydroxy STX is immediately sulfated and converted to GTX2/3 by O-ST. Finally, GTX2/3 is sulfated at N-21 by N-ST or oxidized at N-1 and converted to C1/2 and GTX1/4. Our results are consistent with this hypothesis. In *A. tamarense* CI01, C1/2 dominated the PST composition with trace amounts of GTX2/3, suggesting that there must exist a fast conversion between GTX2/3 and C1/2 with high activity of N-sulfotransferase (N-ST) in the cells. Recently, the sulfotransferase specific to GTX2/3 was found in *A. tamarense* CI01 in our laboratory. This sulfotransferase can transform GTX2/3 to C2 but not STX

to GTX5 (unpublished data). Our findings and those of others on toxin composition reveal that there are numerous species-specific enzymes involved in the biosynthesis of various PSTs as final products. The expression of these enzymes in cultured algal cells is subject to significant influences of the growth phase as well as nutritional and environmental conditions.

Acknowledgements

The authors thank Garry Chang, Elvina Ong and Pilong Li for their technical assistance. The study was in part supported by the Hong Kong Research Grants Council (HKUST6208/01M) and the Ministry of Education of China Foundation for University Key Teachers.

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