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# Growth and toxin production in batch cultures of a marine dinoflagellate *Alexandrium tamarense* HK9301 isolated from the South China Sea

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**Abstract**

Nutritional and environmental conditions were characterized for a batch culture of the marine dinoflagellate *Alexandrium tamarense* HK9301 isolated from the South China Sea for its growth (cells $ml^{-1}$), cellular toxin content (Qt in fmol $cell^{-1}$) and toxin composition (mol%). Under a nutrient replete condition, Qt increased with cell growth and peaked at the late stationary phase. Toxin content increased with the nitrate concentration in the culture while it reached a maximum at 5 μM phosphate. When nitrate was replaced with ammonia, Qt decreased by 4.5-fold. Salinity and light intensity were important factors affecting Qt. The latter increased two-fold over the range of salinity from 15 to 30‰, while decreased 38% as light intensity increased from 80 to 220 μE $m^{-2}$ $s^{-1}$. Toxin composition varied with growth phase and culture conditions. In nutrient replete cultures, toxin composition varied greatly in the early growth phase (first 3 days) and then C1/C2, C3/C4 and GTX1 remained relatively constant while GTX4 increased from 32 to 46% and GTX5 decreased from 28 to 15%. In general, the composition of GTXs was affected in a much greater extent than C toxins by changes in nutrient conditions, salinity and light intensity. This is especially true with GTX4 and GTX5. These data indicate that the cellular toxin content and toxin composition of *A. tamarense* HK9301 are not constant, but that they vary with growth phase and culture conditions. Use of toxin composition to identify a toxigenic marine dinoflagellate is not always valid. The data also reveal that high salinity and low light intensity, together with high nitrate and low phosphate concentrations, would favor toxin production by this species.


*Keywords:* Paralytic shellfish poison; *Alexandrium tamarense* HK9301; Batch culture; Growth; Toxin production

## 1. Introduction

Paralytic shellfish toxins (PSTs) are a suite of neurotoxic and N-rich compounds that are produced by a number of toxic dinoflagellate species, such as

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*Alexandrium* spp. (Cembella, 1998), *Gymnodinium catenatum* (Oshima et al., 1987, 1993) and *Pyrodinium bahamense* var. *compressum* (Usup et al., 1994). These toxins can accumulate in filter-feeding shellfish that feed on the dinoflagellates, resulting in illness or death to animals at higher trophic levels in the food chain such as marine mammals, birds and humans. In the past few decades, many studies have been focused on the PST-producing dinoflagellates. With batch cultures of these dinoflagellates, it has been shown that the cellular toxin content (fmol $cell^{-1}$) and toxin composition (mol%) vary with growth phase (Prakash, 1967; Proctor et al., 1975; Anderson et al., 1990b; Flynn et al., 1994) and culture conditions, such as salinity (White, 1978; Usup et al., 1994; Hwang and Lu, 2001; Hamasaki et al., 2001), light (Ogata et al., 1987; Usup et al., 1994; Hwang and Lu, 2001; Hamasaki et al., 2001), temperature (Ogata et al., 1987; Anderson et al., 1990b; Hamasaki et al., 2001) 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). However, other studies have indicated that toxin composition is a stable biochemical characteristic of a dinoflagellate and can be used as a 'genetic fingerprint' for chemotaxonomic analysis (Hall, 1982; 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, it is important to understand how PSTs are produced by geographically prevalent dinoflagellates under various environmental conditions to allow an accurate assessment of the environmental health significance of these local dinoflagellates.

The outbreaks of intoxication by PSTs or paralytic shellfish poisoning (PSP) have become a serious food safety problem along the coast of the South China Sea and have caused heavy economic losses of the mariculture industry (Qiu, 1990; Jian and Deng, 1991; Lin et al., 1994; Zhou et al., 1999). A number of studies have indicated that PSP outbreaks along the coast of the South China Sea were mainly caused by *Alexandrium* species (Anderson et al., 1996; Siu et al., 1997; Zhou et al., 1999). Anderson et al. (1996) reported that there were clear similarities between the toxin profiles found in the contaminated shellfish and in the cultured *Alexandrium tamarense* species. However, the effort devoted to the study of PST-producing *Alexandrium* species in this region is still rather limited.

*A. tamarense* HK9301 is a toxic strain isolated from the coastal waters of the South China Sea, which produces carbamoylsaxitoxin, the C1–C4 toxins, and the gonyautoxins 1, 3, 4, 5 and 6, no saxitonxin (STX) and neoSTX were detected in this culture (Wang et al., in press). This species might be a key species responsible for the outbreaks of PSP in this region (Anderson et al., 1996). Despite its potential hazards to mariculture and public health, there has been no detailed study reported on this species. The present study investigated the growth and toxin production of this species over a typical batch culture cycle as they were affected by nutrient status, salinity and light intensity.

## 2. Materials and methods

### 2.1. Culture conditions

*A. tamarense* HK9301 was isolated from the coastal waters of Hong Kong during a red tide in 1993. The original strain was maintained in the Institute of Hydrobiology, Jinan University, Guangzhou, PRC. A culture of this strain was routinely maintained in modified K-medium (Keller et al., 1987) with omission of $Na_2SiO_3$ at 23 °C under 14:10 light:dark cycle.

Growth and toxin production over the growth cycle was studied in 250 ml flask containing 125 ml of K-medium at a regime of 23.5 °C and 80 μE $m^{-2}$ $s^{-1}$. The effects of salinity on growth and toxin production were studied at constant light and temperature (80 nE $m^{-2}$ $s^{-1}$ and 23.5 °C). Salinity was adjusted to 15, 20, 25, 30‰ by diluting with Milli-Q water and to 35‰ by addition of NaCl. Light intensity effects on growth and toxin production were studied at 23.5 °C and a salinity of 31‰ under continuous illumination. The irradiation was adjusted to 80, 140, 180 and 230 μE $m^{-2}$ $s^{-1}$. In nutrient experiments, the effects of nitrate, phosphate and ammonium on growth and toxin production were investigated, no effort was made to remove residual nitrogen and phosphate from the seawater used. In the nitrate experiment, the K-medium was altered by omitting the nitrogen sources ($NaNO_3$ and $NH_4CI$). Nitrate was added

to yield the following concentration gradient: 0, 264 and 880 μmol $l^{-1}$. In the phosphate experiment, the concentration gradient was adjusted to 0, 5 and 10 μmol $l^{-1}$. In the ammonium experiment, $NH_4Cl$ at concentrations of 30, 50 and 70 μmol $l^{-1}$ was substituted for 880 μmol $l^{-1}$ $NaNO_3$. Cultures with 880 μmol $l^{-1}$ nitrate and cultures with 880 μmol $l^{-1}$ nitrate plus 50 μmol $l^{-1}$ $NH_4CI$ were used in a comparative study.

Duplicate 0.5 ml samples from each culture were collected in 1.5 ml Eppendorf tubes and preserved in Lugol's iodine solution for cell counts. Cell counts were done manually under a light microscope.

In all culture experiments, the specific growth rate, $\mu$, for the exponential phase was calculated using the following equation:

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

where $N_t$ is the cell density at time $t$. The subscripts denote values at two time points.

### 2.2. Analysis of toxins

Toxin production over the growth cycle was monitored daily. Algal cells from duplicate samples were collected by centrifugation at 2500 × $g$ for 5 min, suspended in 0.5 ml of 50 mM acetic acid and homogenized with three successive sonications. The supernatant was obtained after centrifugation at 11,000 × $g$ for 30 min and 50 μl each was subjected to analysis. Toxin production under various growth conditions was investigated in the late stationary phase (10 days) using methods described as above.

Toxin analysis was carried out using HPLC with fluorescent detection, as reported previously (Wang and Hsieh, 2001), with slight modifications. Two mobile phases (flow rate 0.8 ml $min^{-1}$) were used for separation of different toxin groups (due to lack of the standard, GTX6 was not studied in this paper): (1) 2 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for C toxins and (2) 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 11) for the gonyautoxin group. An authentic gonyautoxins mixture (GTXs standard, National Research Council, Canada) and C1/C2 mixture standard (provided by Prof. Oshima, Tohoku University, Sendai, Japan) were used as reference standards. C3 and C4 toxins were determined after Proctor enhancement by hydrolyzing samples with 0.1 M HCl for 10 min in boiling water (Hall and Reichardt, 1984). (Abbreviations of toxins are used hereafter: C1, 2, 3, 4 = toxins C1, C2, C3 and C4; GTX1/4 = gonyautoxins 1 and 4; GTX2/3 = gonyautoxins 2 and 3; GTX5 = gonyautoxins 5.)

## 3. Results

### 3.1. Growth and toxin production of strain HK9301

The typical growth curve (in cells $ml^{-1}$), cellular toxin content (Qt in fmol $cell^{-1}$) and toxin composition (mol%) of *A. tamarense* HK9301 in a nutrient-replete batch culture are shown in Fig. 1. The cells grew exponentially between day 1 and 5, with a specific growth rate ($\mu$) of 0.6. A distinct stationary phase was observed after day 5 due to phosphate depletion. During the stationary phase, the cell density remained high and relatively constant. Qt increased with growth and reached a peak of ca. 97 fmol $cell^{-1}$ at day 12. The toxin composition varied greatly in early growth phase (first 3 days), and then the content of C1/C2, C3/C4 and GTX1 remained relatively constant throughout the remaining culture cycle, while GTX4 increased from 32 to 46% and GTX5 decreased from 28 to 15%.

### 3.2. Effects of nitrate

The growth of *A. tamarense* HK9301 required the supplementation of nitrate in the culture (Fig. 2a). However, increase in growth ceased when nitrate exceeded 264 μM ($\mu$ = 0.5). Qt increased when nitrate was further increased. The highest Qt, up to 91 fmol $cell^{-1}$, was found in the late stationary phase in the culture started with 880 μM nitrate (Fig. 2b). Toxin composition in cells varied significantly among cultures with different nitrate concentrations (Fig. 2c). In the culture with no addition of nitrate, only C1/C2 toxins were found. The abundance of C1/C2, C3/C4 and GTX4 remained relatively constant with the increase in nitrate concentration, whereas GTX1 decreased from 19.6 to 6.7% and GTX5 increased from 11.4 to 19.4%.

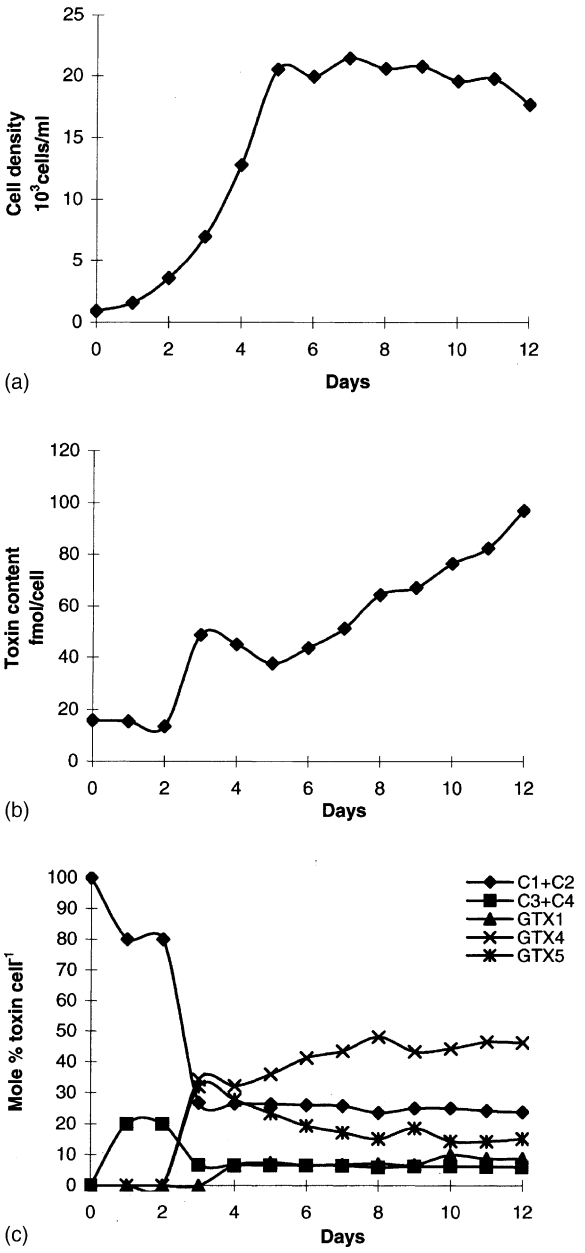


Fig. 1. Growth and toxin production in nutrient-replete batch cultures in K-medium: (a) cell density; (b) toxin content over growth cycle; (c) toxin composition profile over growth cycle.

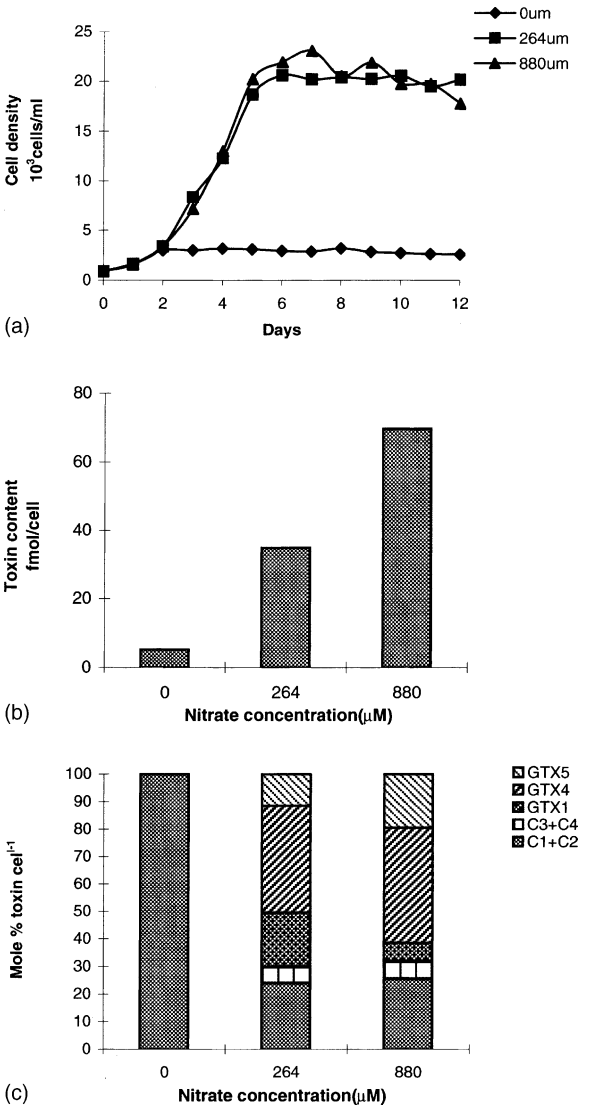


Fig. 2. Growth and toxin production as a function of nitrate concentration: (a) cell density; (b) toxin content; (c) toxin composition.

### 3.3. Effects of phosphate

The growth of *A. tamarense* HK9301 increased with increase in starting phosphate concentration, but the highest toxin content was found at 5 μM; both lower or higher phosphate concentrations caused a reduction of toxin production (Fig. 3). In the culture with no addition of phosphate, C1/C2 were the only PSTs found. When the culture was started with 5 μM of phosphate, other PSTs were found. When the phosphate concentration increased from 5 to 10 μM, the contents of C1/C2, C3/C4 and GTX1 remained relatively constant, while GTX5 decreased from 26.2 to 15.2% and GTX4 increased from 41.6 to 48.2%.

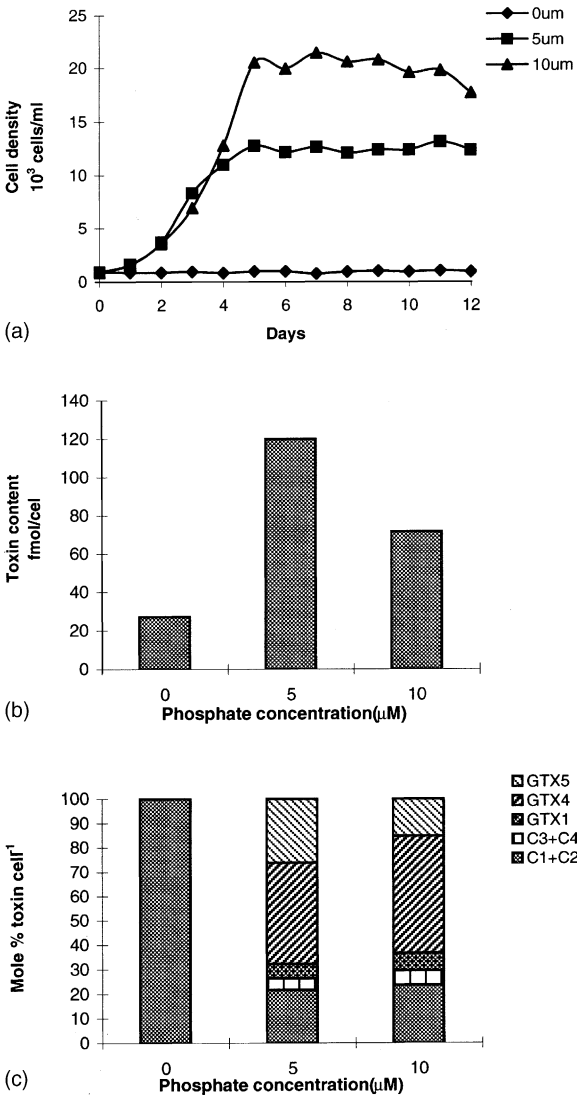


Fig. 3. Growth and toxin production as a function of phosphate concentration: (a) cell density; (b) toxin content; (c) toxin composition.

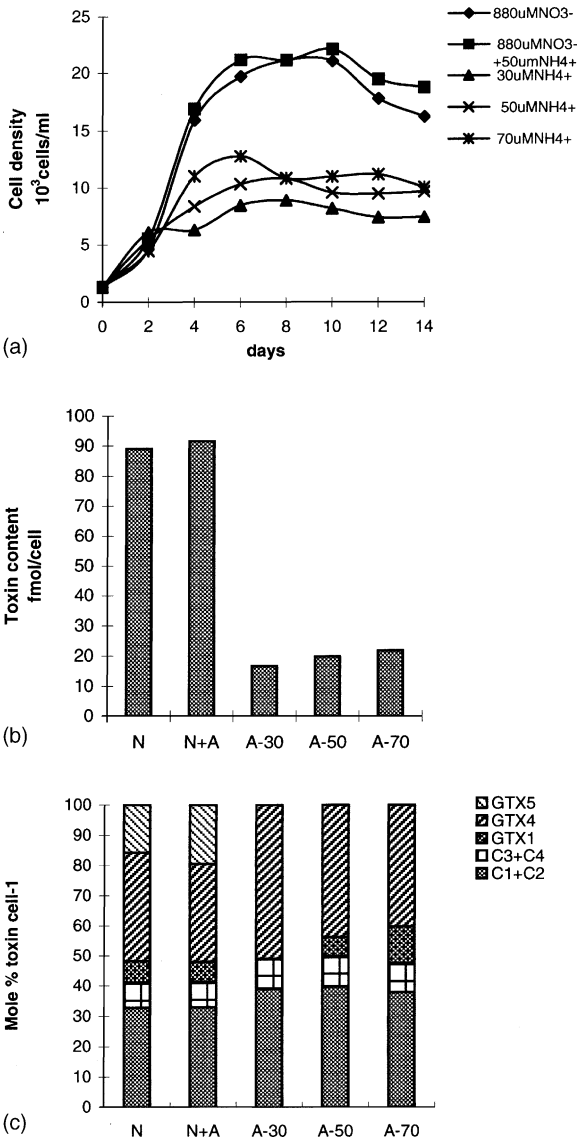


Fig. 4. Growth and toxin production as a function of different nitrogen sources: (a) cell density; (b) toxin content; (c) toxin composition.

### 3.4. Effects of ammonia

Qt and toxin composition of *A. tamarense* HK9301 cultured with different nitrogen sources are shown in Fig. 4. There was no difference in growth and toxin production between the culture with 880 μM nitrate and the culture with 880 μM nitrate plus 50 μM ammonium, but the cell density decreased significantly when ammonium was used as the sole nitrogen source. Qt of the cultures with ammonium alone decreased 4.5-fold compared to those with nitrate or with both nitrate and ammonium. Toxin composition also varied greatly in the cultures with ammonium. GTX1 increased from 0 to 12.4%, and GTX4 decreased from 51.1 to 40.3%. No GTX5 was detected under various ammonium concentrations, however,

the contents of C1/C2 and C3/C4 remained relatively constant among different cultures.

### 3.5. Effects of salinity

This species was able to grow at salinities between 15 and 35‰, with optimal growth between 25 and 35‰ ($\mu$ = 0.6 per day) (Fig. 5). Qt increased over the range of 15–30‰ and peaked at 96 fmol cell$^{-1}$ at a salinity of 30‰, and then decreased with a further increase in salinity. Lower salinity affected toxin composition significantly: GTX4 increased from 44.3 to 53.2% as salinity decreased from 20 to 15‰, while GTX1 decreased from 8.5 to 0%. Toxin composition remained relatively constant when salinity exceeded 20‰ except for GTX5, which decreased from 21.7 to 11.8% with an increase in salinity.

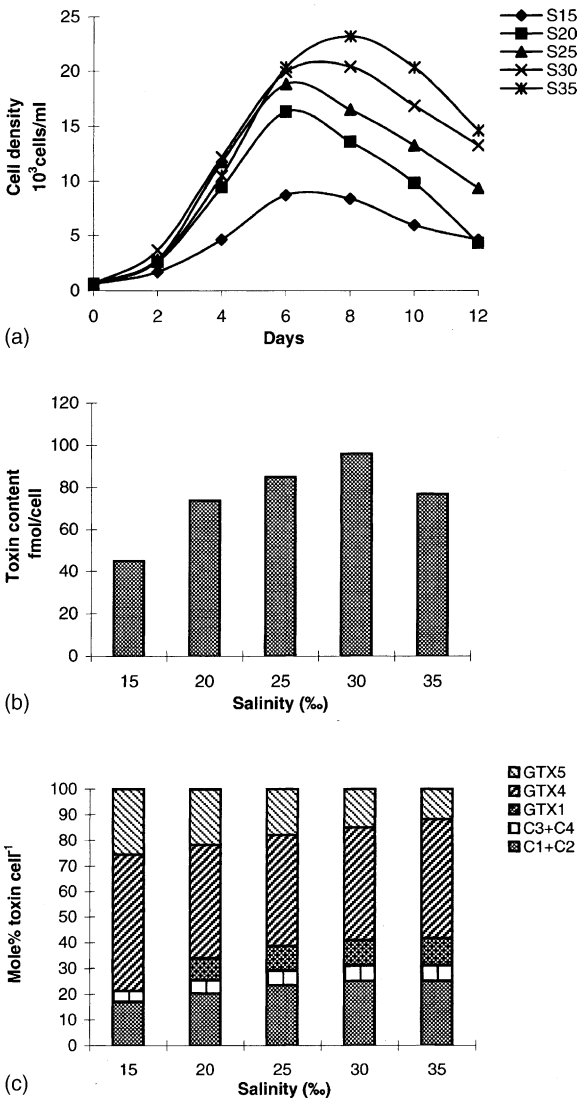


Fig. 5. Growth and toxin production as a function of salinity: (a) cell density; (b) toxin content; (c) toxin composition.

### 3.6. Effects of light

Light intensity caused no adverse effects on the growth rate of *A. tamarense* HK9301 ($\mu$ = 0.5 per day) within the range of 80 to 220 μE m$^{-2}$ s$^{-1}$ (Fig. 6). However, cell density decreased in the stationary phase under higher light intensity. Light intensity variation also affected toxin content and composition. High light intensity inhibited toxin production. Toxin content decreased 38% from 77 to 48 fmol/cell. GTX4 increased by ca. 13% as light intensity increased, while GTX5 decreased by ca. 12%. Other toxin profiles remained relatively constant throughout the growth cycle.

## 4. Discussion

### 4.1. Dynamics of growth and toxin production

There have been many reports on the dynamics of growth and toxin production of *Alexandrium* species (Boyer et al., 1987; Ogata et al., 1987; Anderson et al., 1990b; Flynn et al., 1994, 1996; McIntyre et al., 1997; Parkhill and Cembella, 1999; Hwang and Lu, 2001; Hamasaki et al., 2001). Virtually all studies on toxin production by *Alexandrium* species in batch cultures have shown that Qt reached the highest level in early exponential phase when nitrogen supply was sufficient, and dropped to a low level in the stationary phase when nitrogen became limiting. However, we observed a different pattern of toxin production in cultured *A. tamarense* HK9301, which produced the highest Qt in late stationary phase. We have no clear explanation for the difference seen, except that in this study a different algal species and different nutrient concentrations were used. Previously, we also observed a similar phenomenon in the culture of another species isolated from the South China Sea, *A. tamarense* CI01, which produced the highest

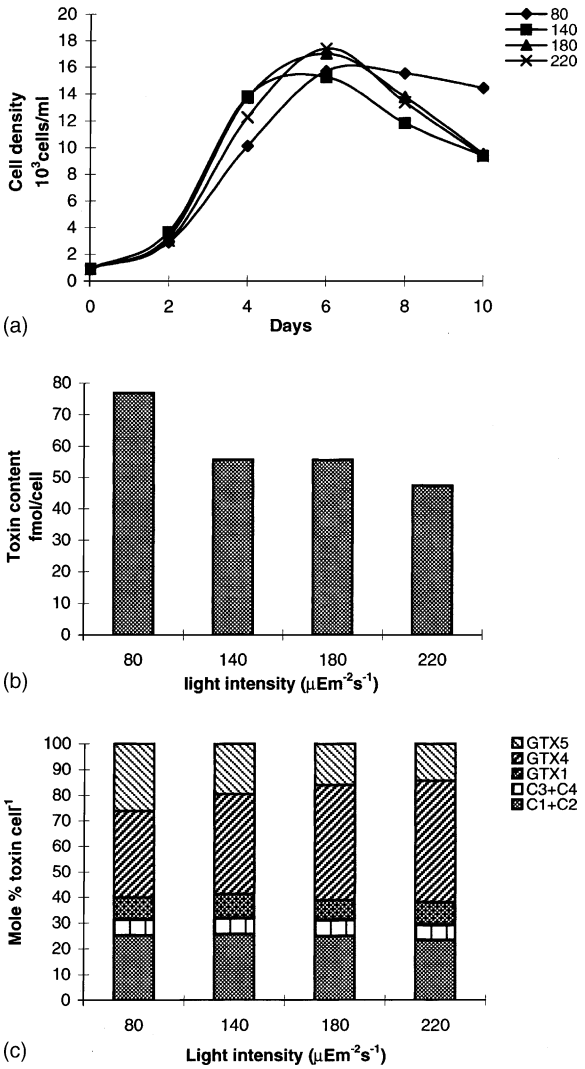


Fig. 6. Growth and toxin production as a function of light intensity ($\mu E\ m^{-2}\ s^{-1}$): (a) cell density; (b) toxin content; (c) toxin composition.

Qt in middle stationary phase (Wang and Hsieh, 2001, 2002). In our studies, K-medium with 880 μM nitrate and 10 μM phosphate was used for algal cultures. Phosphate was exhausted in the exponential phase (day 4), while nitrate maintained in excess (data not shown), which provided sufficient nitrate for toxin biosynthesis under severe phosphate limitation.

Effects of environmental factors on toxin content and composition of *Alexandrium* species have been extensively investigated (Boyer et al., 1987; Ogata et al., 1987; Anderson et al., 1990a, 1990b; Flynn et al., 1994, 1996; McIntyre et al., 1997; Parkhill and Cembella, 1999; Hwang and Lu, 2001; Hamasaki et al., 2001; Wang and Hsieh, 2002). The results reported are complex and inconsistent due to the different experimental designs and different specific species used.

### 4.2. Toxin content variability

PSTs are a family of nitrogen-rich compounds that contain 5–10% of the total cellular nitrogen in *Alexandrium* and *Gymnodinium* sp. (Cembella, 1998). Many investigators (Anderson et al., 1990a; Flynn et al., 1994; McIntyre et al., 1997; John and Flynn, 2000; Wang and Hsieh, 2002) have reported that nitrogen deficiency caused dinoflagellate cultures to decrease in PST yield and cellular toxin content. The present results are consistent with these findings. Qt of *A. tamarense* HK9301 is strongly affected by nitrate supplementation. Although growth topped at a nitrate concentration of 264 μM, Qt continued to increase until the nitrate concentration reached 880 μM, at which Qt was doubled in value indicating that the requirement for nitrate is different between cell growth and toxin biosynthesis. Hence, the supply of ample nitrate is important for high toxin yields.

Ammonium ion was thought to be a favored nitrogen source for toxin biosynthesis, although high concentration of ambient ammonium shows an inhibitory effect on the growth of dinoflagellates (Iwasaki, 1973). Hamasaki et al. (2001) reported a drastic increase in toxin content with an increase in ammonium concentration from 0.11 to 0.22 mM, while growth was scarcely detected at 0.44 mM ammonium. However, our results indicate that ammonium did not enhance toxin production of *A. tamarense* HK9301 and Qt decreased significantly compared to that of cultures containing nitrate, indicating that ammonium is not a suitable nitrogen source for this strain. The results do not support the notion that ammonium utilization directly induces a high toxin content in *Alexandrium.* This observation calls for a reassessment of the role of ammonium in marine environment in terms of the toxicity of *Alexandrium* species.

Although PST molecules do not contain phosphorus, enhancement of bioproduction of PSTs by phosphorus limiting culture conditions has been well

documented (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). In the present study an optimal starting phosphate concentration of 5 μM was observed. Qt decreased when the phosphate condition was either lower or higher than this level. The mechanism of how phosphorus regulates toxin production is not well understood at present. Anderson et al. (1990b) proposed that P-limitation could cause an increase in the availability of intracellular arginine, a presumed precursor in PST biosynthesis (Shimizu et al., 1984), due to reduced demand from competing P-dependent pathways involved in cell division. The role of arginine was corroborated by Flynn et al. (1996) who reported that toxin synthesis in *A. minutum* was proportional to intracellular concentrations of amino acids, arginine in particular. Conceivably, P-limitation may cause cell division to cease, thus allow the cells to continue utilizing available arginine or other cellular constituents for toxin biosynthesis.

Salinity is known to affect nutrient uptake and a variety of active and facilitated transport systems in dinoflagellate cells. There is no fixed optimal salinity for PST production in algal cultures. White (1978) reported that the toxin content of *Gonyaulax excavate* increased two-fold when salinity increased from 31 to 37‰. Similarly, in a highly toxic strain of *A. tamarense*, the highest cell toxicity occurred when the culture was at the highest salinity-dependent exponential phase (Parkhill and Cembella, 1999). Recently, Hwang and Lu (2001) observed that good production of GTX2 and GTX3 in *A. minutum* required a sufficiently high salinity. Our results indicate that toxin content increased with increasing salinity in the range of 15–30‰. However, Usup et al. (1994) found that increasing salinity did not elevate toxin content of *P. bahamense* var. *compressum*, but low salinity enhanced toxicity. Hamasaki et al. (2001) also found that the toxicity of *A. tamarense* showed a tendency to decrease with increasing salinity in the range of 13–29%, while others (Anderson et al., 1990b; Flynn et al., 1996) found no significant effects of salinity on toxin production in some *Alexandrium* species. Discrepancies in the results of salinity experiments were possibly caused by acclimation time, the steepness and range of salinity gradient. In the studies of Hamasaki et al. (2001) and Usup et al. (1994), cells for experiments were acclimated to each experimental salinity in advance, while in the present study and some other studies (White, 1978; Parkhill and Cembella, 1999; Hwang and Lu, 2001), cells grown at high salinity were introduced directly into the medium of different salinity levels. The stress due to a sudden osmotic change might have enhanced toxin production.

Light is of primary importance in the growth and toxin production of dinoflagellates because most of them are photoautotrophic. Ogata et al. (1987) reported that in an *A. tamarense* culture Qt increased with a decrease in light intensity. Hamasaki et al. (2001) found that the increase in Qt under reduced light intensity was influenced by temperature and that high Qt with low light intensity coincided with reduced growth rates. Our results indicated that Qt increased 60% when the light intensity decreased from 220 to 80 $\mu E\ m^{-2}\ s^{-1}$. However, Usup et al. (1994) reported that Qt of *P. bahamense* var. *compressum* decreased when light intensity was reduced. These contrasting results might be related to the different light intensity ranges used in the experiments. In the study of Usup et al. (1994), low light intensity range of 90 to 15 $\mu E\ m^{-2}\ s^{-1}$ was used, while the present study and other studies (Parkhill and Cembella, 1999; Hwang and Lu, 2001) were conducted under a high light intensity range. It is evident that sufficient light energy is required for maximum toxin yield.

### 4.3. Toxin composition

Toxin composition as a characteristic of a particulate dinoflagellate isolate remains unclear. Numerous investigators indicated that the toxin composition in *Alexandrium* isolates 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). Boczar et al. (1988) reported that significant composition changes occurred during prolonged senescence in batch cultures. Anderson et al. (1990a) observed that toxin composition of *A. fundynse* varied under steady-state nutrient limitation. 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. Our data on *A. tamarense*

HK9301 indicate that toxin composition does vary with growth phases and culture conditions. In a culture not supplemented with nitrate and phosphate, only C2 and C2 were found, suggesting that these two toxins might be the parent toxins from which other PSTs were derived when the nutrients were made available. In nutrient-replete cultures, toxin composition varied greatly in early growth phase, and then 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 studies indicate that toxin composition is constant and is independent of culture conditions, although toxin content might change (Boyer et al., 1987; Cembella et al., 1987; Hall, 1982; Ogata et al., 1987; Anderson et al., 1994; Cembella and Taylor, 1985; Oshima et al., 1990; Cembella and Destombe, 1996). Flynn et al. (1994) and Franco et al. (1994) showed that *A. minutum* has a stable toxin profile under various culture conditions. Further support from Parkhill and Cembella (1999) showed that toxin composition of *A. tamarense* remained constant under various salinities, light and inorganic nitrogen conditions. These investigators claimed that toxin composition can be used as a 'genetic fingerprint' of a dinoflagellate isolate. Oshima et al. (1993) also demonstrated that toxin composition of *G. catenatum* is a characteristic of the algae and is stable under various culture conditions. These investigations suggest that toxin composition can be used as a biochemical marker to distinguish between geographical isolates of *Alexandrium* species or *G. catenatum.*

## 5. Conclusions

Our results indicate that toxin content and toxin composition of *A. tamarense* HK9301 in a batch culture vary with growth phases and culture conditions. Sufficient nitrogen and phosphate were essential for growth and toxin production. Ammonium was not a good nitrogen source for growth and toxin biosynthesis. Variations of culture conditions affected compositions of GTXs greatly while C toxins remained relatively constant. Use of toxin composition as a marker of chemical taxonomy may not be always valid. Our results also imply that algal blooms with high PST toxicity might occur in eutrophic marine environments with high salinity and relatively low phosphorus concentrations accompanied by successive cloudy days.

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