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# EFFECTS OF LIGHT AND TEMPERATURE ON GROWTH, NITRATE UPTAKE, AND TOXIN PRODUCTION OF TWO TROPICAL DINOFLAGELLATES: *ALEXANDRIUM TAMIYAVANICHII* AND *ALEXANDRIUM MINUTUM* (DINOPHYCEAE)[1]

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**The two tropical estuarine dinoflagellates, *Alexandrium tamiyavanichii* Balech and *A. minutum* Halim, were used to determine the ecophysiological adaptations in relation to their temperate counterparts. These species are the two main causative organisms responsible for the incidence of paralytic shellfish poisoning (PSP) in Southeast Asia. The effects of light (10, 40, 60, and 100 µmol photons $\cdot m^{-2} \cdot s^{-1}$) and temperature (15, 20, and 25° C) on the growth, nitrate assimilation, and PST production of these species were investigated in clonal batch cultures over the growth cycle. The growth rates of *A. tamiyavanichii* and *A. minutum* increased with increasing temperature and irradiance. The growth of *A. tamiyavanichii* was depressed at lower temperature (20° C) and irradiance (40 µmol photons $\cdot m^{-2} \cdot s^{-1}$). Both species showed no net growth at 10 µmol photons $\cdot m^{-2} \cdot s^{-1}$ and a temperature of 15° C, although cells remained alive. Cellular toxin quotas ($Q_t$) of *A. tamiyavanichii* and *A. minutum* varied in the range of 60–180 and 10–42 fmol PST $\cdot$ cell$^{-1}$, respectively. Toxin production rate, $R_{tox}$, increased with elevated light at both 20 and 25° C, with a pronounced effect observed at exponential phase in both species (*A. tamiyavanichii*, $r^2 = 0.95$; *A. minutum*, $r^2 = 0.96$). Toxin production rate also increased significantly with elevated temperature ($P < 0.05$) for both species examined. We suggest that the ecotypic variations in growth adaptations and toxin production of these Malaysian strains may reveal a unique physiological adaptation of tropical *Alexandrium* species.**

***Key index words:*** ***Alexandrium minutum*; *Alexandrium tamiyavanichii*; growth; light; temperature; toxin production; tropical**

***Abbreviations:*** ***E*, irradiance; µ, growth rate; NR, nitrate reductase; PSP, paralytic shellfish poisoning; PPFD, photosynthesis photon flux densities; $Q_t$, cell toxin quota; $R_{tox}$, toxin production rate**

In Southeast Asia, thousands of paralytic shellfish poisoning (PSP) incidents have been reported since the early 1970s. In the Philippines alone, 2107 cases and 117 fatalities have been reported (Gonzales et al. 2002). Initially, PSP in this region was associated solely with the blooms of *Pyrodinium bahamense* Plate var. *compressum* Böhm (Roy 1977, Maclean 1979, 1989). However, since the late 1980s, there have been increasing PSP events due to other species. The first was toxicity due to *Alexandrium tamiyavanichii* Balech in the Strait of Malacca in 1991 (Usup et al. 2002a), which was followed by the events in the Philippines (Montojo et al. 2003). Most recently, *Alexandrium minutum* Halim has emerged as an important PSP-causing species in the regions (Usup et al. 2002a, Bajarias et al. 2003, Lim et al. 2004). Previously, *A. minutum* was reported from Thailand (Matsuoka et al. 1997, Piumsomboon et al. 2001) and Vietnam (Yoshida et al. 2000), although no human toxicity was mentioned.

Many studies on PST-producing dinoflagellates have been focused on the genus *Alexandrium*. This was probably due to the fact that about one third of the 30 currently known *Alexandrium* species are PST producers, in addition to the wide biogeographical

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TABLE 1. Cultures of *Alexandrium tamiyavanichii* and *A. minutum* used in this study.

| Species | Strain | Location | Isolation date | Collector | Morphology description/Genetic characterization |
|---|---|---|---|---|---|
| *A. tamiyavanichii* | AcMS01 | Sebatu, Malacca | October 1997 | P. T. Lim Usup et al. (2002a)/Usup et al. (2002b), Leaw et al. (2005) | |
| *A. minutum* | AmKB02 | Tumpat, Kelantan | September 2001 | P. T. Lim Usup et al. (2002a), Lim et al. (2004)/Leaw et al. (2005) | |

distribution of these species. Studies on *Alexandrium* in batch cultures have shown that cellular toxin content and toxin composition can vary at inter- and intraspecies levels (Oshima et al. 1987, Yoshida et al. 2001), within biogeographical regions (Yoshida et al. 2001), and with growth stage (Prakash 1967, Anderson et al. 1990a, Flynn et al. 1994). Environmental conditions have also been shown to influence the toxicity of the species. Influential factors include salinity (White 1978, Parkhill and Cembella 1999, Hamasaki et al. 2001), light (Ogata et al. 1987, Parkhill and Cembella 1999, Hwang and Lu 2000, Hamasaki et al. 2001), and temperature (Ogata et al. 1987, Anderson et al. 1990a, b, Hamasaki et al. 2001). Most of these studies were conducted on temperate strains. At present, it is not known whether tropical isolates show the same PST-producing characteristics as the toxic temperate counterparts. The objective of the present study was to investigate the effects of temperature and light on growth, nitrate uptake, and PST production of tropical *A. tamiyavanichii* and *A. minutum*. These species have been shown previously to possess distinct salinity tolerances (Lim and Ogata 2005).

## MATERIALS AND METHODS

*Clonal cultures.* Clonal cultures of *A. tamiyavanichii* were established from samples originating from the Strait of Malacca, while *A. minutum* was collected from the northeast coast of Peninsula Malaysia (Table 1). Clonal cultures were maintained in ES-DK medium (Kokinos and Anderson 1995), containing 430 μM nitrate and 13 μM of phosphate and maintained at 25 ± 0.5° C under a 15:9 light:dark cycle at a mean photosynthetic photon flux density (PPFD) of 140 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Seawater (33 psu salinity) from Okkirai Bay was used as the medium base. *A. tamiyavanichii* cultures (strain: AcMS01) were grown at a salinity of 25 psu, while *A. minutum* culture (strain: AmKB02) was grown at 15 psu. Medium salinity was adjusted by dilution with deionized distilled water. The pH of the culture medium was adjusted to 7.8–7.9. Cultures were also kept at Marine Microalgae Cultures Collection, Universiti Kebangsaan Malaysia (UKM-MMCC).

*Light and temperature experiments.* Cultures were grown under light intensities of 10, 40, 60, and 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ PPFD at three different temperatures (15, 20, and 25° C). Cells were acclimated at 20° C for 24 h before transferring to 15° C. Light intensities were adjusted by placing screens over the light sources. Irradiance was measured using a QSL-2100 quantum scalar irradiance meter (Biospherical Instruments Inc., San Diego, CA, USA). The experiments were carried out in duplicate. Cultures were subsampled once every two days for cell counts. Specific growth rate (μ, $day^{-1}$), was calculated over the exponential growth phase using the following equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0} \quad (1)$$

where $N_0$ and $N_1$ are the cell density at time $t_0$ and $t_1$, respectively.

For the light experiments, μ at each light intensity was plotted against irradiance. The data were fitted with an exponential equation according to Webb et al. (1974)

$$\mu = \mu_{max} \times [1 - \exp^{(-\alpha E/\mu_{max})}] \quad (2)$$

where $\mu_{max}$ is the maximum potential growth rate, α is the initial slope of the relationship, $E$ is the irradiance (μmol photons $\cdot m^{-2} \cdot s^{-1}$) expressed as $(E_I - E_C)$, where $E_I$ is the incident irradiance, and $E_C$ is the compensation irradiance. The saturating irradiance, $E_{opt,}$ was also calculated, as the ratio of $\mu_{max}$ to α.

*Measurement of nitrate concentration and determination of nitrate uptake rate.* Nitrate concentrations were determined at 2–3-day intervals by UV-spectrophotometry at 220 nm (Carvalho et al. 1998, Collos et al. 1999). Samples were centrifuged at 3000*g* for 5 min to remove cells and particulate matter, and the supernatant was used to determine nitrate concentrations. Two milliliters of the supernatant was diluted to 10 mL with Milli-Q water, and 0.2 mL 1 N HCl solution was added to the sample and mixed. Absorbance was then read at 220 and 275 nm. The nitrate absorbance was calculated by subtracting two times the absorbance at 275 nm for background correction from the absorbance at 220 nm. A calibration curve was generated with standards in the range of 16–112 μM.

Nitrate uptake rate, $R_{nit}$ (pmol $NO_3^- \cdot cell^{-1} \cdot day^{-1}$), was calculated from the depletion of nitrate in the medium based on the assumption that the depletion was due to uptake by the dinoflagellate cells. Nitrate uptake was calculated from the residual nitrate concentration ($S$) in the medium and the change in cell density ($\overline{N}$) over a period of time ($\Delta t = t_1 - t_0$).

$$R_{nit} = -\frac{(S_0 - S_1)}{\overline{N}\Delta t} \quad (3)$$

$$\overline{N} = \frac{N_1 - N_0}{\ln N_1 - \ln N_0} \quad (4)$$

where $S_0$ and $S_1$ are the residual nitrate concentrations (μM) at time $t_0$ and $t_1$; $N_0$ and $N_1$ were cell densities (cells $\cdot mL^{-1}$) at time $t_0$ and $t_1$, respectively.

*Analysis of PSTs.* Analysis of PSTs by high-performance liquid chromatography (HPLC) was carried out using the isocratic, post-column derivatization method of Oshima (1995b) on a JASCO HPLC system (JASCO, Tokyo, Japan) fitted with a post-column reaction system and fluorescence detector. The samples were run through a Wakosil C18 column (Wako, Osaka, Japan). The chromatographic conditions were as follows: for analysis of STXs, the mobile phase was 2 mM heptanesulfonate in 30 mM ammonium phosphate buffer and 6% acetonitrile, pH 7.1. For GTXs, the mobile phase was 2 mM heptanesulfonate in 30 mM ammonium phosphate buffer, pH 7.1, and for C toxins the mobile phase was 2 mM tetrabutyl ammonium in acetate buffer, pH 5.8. The post-column oxidizing reagent was 7 mM periodic acid in 80 mM sodium phosphate buffer, pH 9.0, while the acidifier was 0.5 M acetic acid. The sample injection volume was 20 μL. Flow rates for the mobile phases were 0.8 and 0.4 mL $min^{-1}$ for each post-column reagent. The

post-column temperature was maintained at 70° C for all runs. Detection wavelengths were set at 330 nm excitation and 390 nm emissions.

The net toxin production rate, $R_{tox}$ (fmol PST · cell$^{-1}$ · day$^{-1}$), was determined using the equations described by Anderson et al. (1990b).

$$R_{tox} = \frac{(T_1 - T_0)}{\overline{N}\Delta t} \quad (5)$$

where $T_t$ is the total toxin concentration at time $t$, by multiplying the toxin content (fmol PST · cell$^{-1}$) with cell density ($N_t$); $\overline{N}$ is the ln average of the cell concentration and $\Delta t$ is the specific time interval of growth.

## RESULTS

*Growth responses to various light and temperature conditions.* In all light and temperature treatments, cell density showed an immediate exponential increase without an observable lag phase (Fig. 1). Neither of the *A. tamiyavanichii* and *A. minutum* clones grew at a PPFD of 10 µmol photons · m$^{-2}$ · s$^{-1}$. Cell density remained constant for several days (~7 days) and declined gradually (data not shown). Generally, µ increased with increasing temperature and irradiance for both *A. tamiyavanichii* and *A. minutum* (Fig. 2). For *A. tamiyavanichii*, the highest µ (0.31 ± 0.03 day$^{-1}$) and maximum cell density (6.07 ± 0.22 × 10$^3$ cells · mL$^{-1}$) were observed at 25° C under 100 µmol photons · m$^{-2}$ · s$^{-1}$. At 20° C, the maximum cell densities decreased around 4- to 6-fold compared with 25° C. Maximum cell density decreased significantly with decreased light ($P<0.01$ at 20° C and $P \ll 0.0001$ at 25° C). For *A. minutum*, µ and maximum cell densities for the three irradiances tested did not vary from each other at 25° C ($P = 0.171$; $P = 0.083$). For the 20° C cultures, the maximum cell densities were 1.4–3.4 times lower than densities reached at 25° C. Both µ and maximum cell densities increased with increasing light, although there was no significant difference between 60 and 100 µmol photons · m$^{-2}$ · s$^{-1}$ ($P = 0.149$). No net growth was observed for the 15° C treatments of either species. Transfer of cultures to higher temperatures such as 20° C and above did promote growth of the cultures (data not shown). Maximum growth rate ($\mu_{max}$) as a function of irradiance increased with increasing temperature (Fig. 3), but the differences were not statistically significant (Table 2). The optimum irradiance for growth for *A. minutum* (>24 µmol photons · m$^{-2}$ · s$^{-1}$) was relatively low compared with *A. tamiyavanchii* (>50 µmol photons · m$^{-2}$ · s$^{-1}$).

*Effect of light and temperature on nitrate uptake.* Nitrate concentrations decreased in the media at 25° C under irradiance of 60 and 100 µmol photons · m$^{-2}$ · s$^{-1}$ (59.8 ± 3.8 and 29.2 ± 2.9 µM) in *A. tamiyavanichii* culture. In the media at 40 µmol photons · m$^{-2}$ · s$^{-1}$, approximately half of the $NO_3^-$ remained at the end of the experiment (227.2 ± 65.4 µM). However, at 20° C, $NO_3^-$ in the media were still high at the end of the experiment, ranging from 334.4 ± 12.4 µM (100 µmol photons · m$^{-2}$ · s$^{-1}$) to 398.4 µM (40 µmol photons · m$^{-2}$ · s$^{-1}$; Fig. 4). For *A. minutum*, $NO_3^-$ was depleted in all media when the cultures entered the stationary phase. Only 7 µM (40 µmol photons · m$^{-2}$ · s$^{-1}$) to 14 µM (60 and 100 µmol photons · m$^{-2}$ · s$^{-1}$) of $NO_3^-$ remained at the end of the experiment (Fig. 4). At the end of the experiment, $NO_3^-$ was exhausted in the media of 100 µmol photons · m$^{-2}$ · s$^{-1}$, but about half of the $NO_3^-$ still remained in the media at 40 µmol photons · m$^{-2}$ · s$^{-1}$ and about one third in media at 60 µmol photons · m$^{-2}$ · s$^{-1}$.

The relationship between nitrate uptake rates and irradiance differed between the two species (Fig. 5). In *A. tamiyavanichii* at 20° C cultures, rates of nitrate uptake showed an increase with elevated PPFD ($P<0.01$). However, the $R_{nit}$ were not significantly different for the 25° C cultures ($P = 0.212$), averaging 17.5 ± 2.32 pmol · cell$^{-1}$ · day$^{-1}$. For *A. tamiyavanichii*, the $R_{nit}$ was 0.5- to 6-fold higher in 25° C cultures compared with 20° C cultures ($P<0.0001$, 2-way ANOVA) over all the light intensities tested (Fig. 5A). In contrast, the maximum $R_{nit}$ for *A. minutum* occurred in low irradiance cultures (40 µmol photons · m$^{-2}$ · s$^{-1}$) at 25° C and 20° C. Two-way ANOVA showed no significant effect of temperature on $R_{nit}$ ($P = 0.982$). The $R_{nit}$ decreased with an increase in irradiance ($P<0.01$) (Fig. 5B), although the $R_{nit}$ at 60 and 100 µmol photons · m$^{-2}$ · s$^{-1}$ did not differ significantly ($P = 0.984$).

*Effect of light and temperature on PSTs production.* In *A. tamiyavanichii*, toxin cell quota, $Q_t$ (fmol PST · cell$^{-1}$) varied markedly with temperature (Fig. 6). At 20° C, $Q_t$ was significantly lower than for cultures grown at 25° C under all light intensities ($P<0.0001$). However, there were no significant variations in $Q_t$ under three different irradiances for either temperature treatment (2-way ANOVA, $P = 0.155$). At 20° C, cultures showed a slight increase in the $Q_t$ during the early stationary phase (day 9), with a maximum $Q_t$ of 60 fmol PST · cell$^{-1}$ under 40 µmol photons · m$^{-2}$ · s$^{-1}$ (Fig. 6a), while under 60 µmol photons · m$^{-2}$ · s$^{-1}$, the $Q_t$ increased slightly at the late stationary phase (day 15) to 65 fmol PST · cell$^{-1}$ (Fig. 6b). However, the $Q_t$ did not differ significantly through the growth cycle under 100 µmol photons · m$^{-2}$ · s$^{-1}$ ($P = 0.261$) (Fig. 6c). At 25° C, cultures grown under different irradiances showed an increase in the $Q_t$ during the early exponential phase (day 8); however, the $Q_t$ decreased markedly at day 13 despite the cell growth (Fig. 6, d–f). Maximum $Q_t$ of cultures grown under higher PPFD (186 ± 18 fmol PST · cell$^{-1}$ at 100 µmol photons · m$^{-2}$ · s$^{-1}$) were prominently higher than the cultures grown under lower PPFD (131 ± 26 fmol PST · cell$^{-1}$ at 40 µmol photons · m$^{-2}$ · s$^{-1}$). However, no significant differences in $Q_t$ of cultures growing under 60 and 100 µmol photons · m$^{-2}$ · s$^{-1}$ were observed (185 ± 18 and 186 ± 18 fmol PST · cell$^{-1}$, respectively) (Fig. 6, e and f).

Carbamate toxins of STX, GTX1–GTX4, N-sulfocarbamoyl group of GTX5, and C2 were detected by

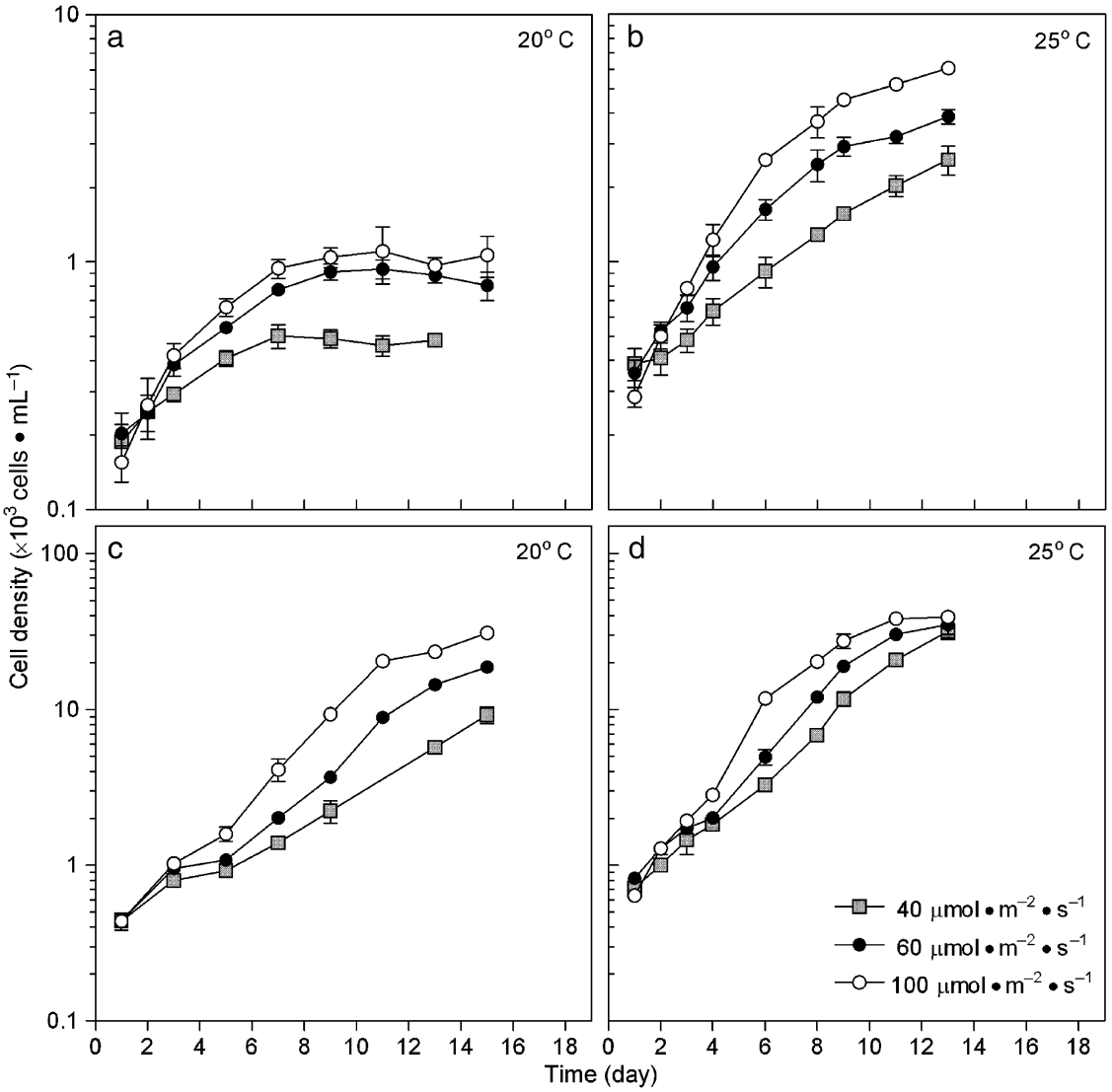


FIG. 1. Growth curves of *Alexandrium tamiyavanichii*, AcMS01 (a and b) and *A. minutum*, AmKB02 (c and d) grown under different temperature and light conditions at salinities of 25 and 15 psu, respectively. The cell densities shown are the averages of duplicate cultures, and the error bars represent the standard deviation (±SD) from the mean values; error bars are absent when the error is smaller than the size of the symbol.

HPLC in *A. tamiyavanichii* cultures at 25° C. Traces of dcSTX, neoSTX, and C1 were also detected (<2%). However, dcSTX, neoSTX, and C1 were below the detection level at 20° C. On a relative molar basis, GTX4 contributed to the largest proportion of total toxin composition (up to 80%) and increased significantly during culture growth under three different irradiances at 25° C treatment ($P<0.0001$). At 20° C, GTX4 and GTX3 were the major toxin components, which contributed to 35%–46% and 23%–42% of the $Q_t$, respectively. The relative proportions of each toxin remained stable over culture time. No significant difference was observed in relative toxin proportions among various PPFDs.

In *A. minutum* cultures, $Q_t$ of cultures differed significantly ($P<0.001$) at two different temperatures, but no significant variation was observed with light intensity ($P = 0.951$). At 20° C, $Q_t$ decreased during the mid-exponential phases and remained within the range of 8–12 fmol PST · cell$^{-1}$ for all three irradiances (Fig. 7). Cultures showed a slight increase in $Q_t$ when entering the early stationary phase (day 15). A similar trend was also observed with 10 μmol photons · m$^{-2}$ · s$^{-1}$ at 25° C (Fig. 8a). In contrast, under higher PPFD (40–100 μmol photons · m$^{-2}$ · s$^{-1}$) at 25° C, $Q_t$ peaked at the mid-exponential phase (day 8–9) to a maximum of 38.4 ± 9.4 (Fig. 8b), 33.3 ± 2.6 (Fig. 8c), and 41.3 ± 2.7 fmol PST · cell$^{-1}$ (Fig. 8d) under elevated irradiances, but decreased abruptly afterward to about half of the maximum $Q_t$.

Toxin composition throughout the experiments remained stable, with the carbamate toxins of epimer pair GTX1 and GTX4 as the major toxin components. However, the relative proportions varied throughout

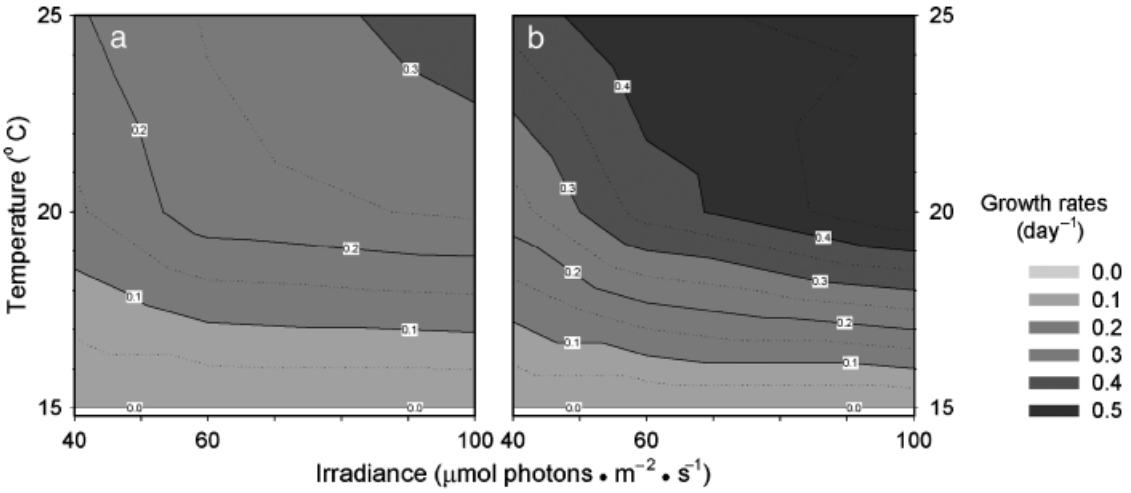


FIG. 2. Specific growth rates of *Alexandrium tamiyavanichii*, AcMS01 (a) and *A. minutum*, AmKB02 (b) as functions of temperature and irradiance.

the culture growth. At the early exponential phase, all cultures in the experiments were dominated by GTX4, which accounted for up to 96% of $Q_t$ (Figs. 7 and 8). A significant decrease of GTX4 to ~30% ($P<0.0001$) and proportional increase of GTX1 were observed during the sudden decline of $Q_t$ (during day 9 for 20° C and day 11 for 25° C). However, GTX4 increased significantly thereafter ($P<0.0001$), reaching similar proportions as previously observed.

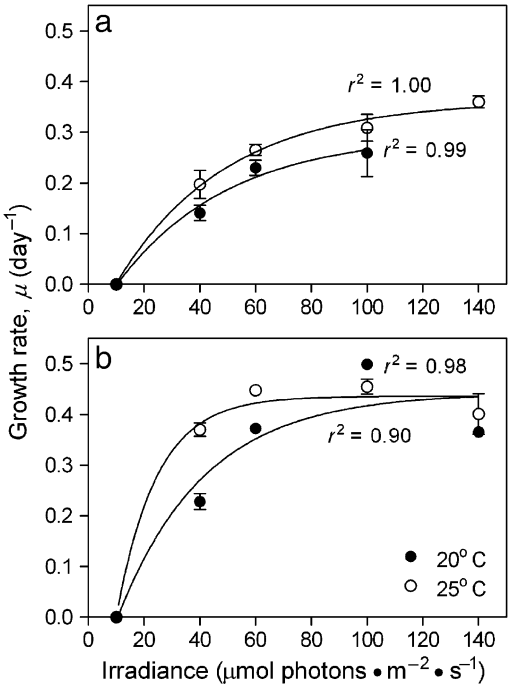


FIG. 3. Specific growth rate (μ, $day^{-1}$), as a function of irradiance, μmol photons $\cdot m^{-2} \cdot s^{-1}$, for *Alexandrium tamiyavanichii*, AcMS01 (a) and *A. minutum*, AmKB02 (b) grown at 20 and 25° C under a 15:9 light:dark cycle.

Toxin production rate increased with elevated light at both temperatures (Table 3), with more pronounced effects observed during exponential growth for both *Alexandrium* species (*A. tamiyavanichii*, $r^2 = 0.95$; *A. minutum*, $r^2 = 0.96$). Toxin production rate also increased significantly with elevated temperature ($P<0.05$) for both species and showed a positive correlation with μ at the exponential phase of *A. tamiyavanichii* culture and the mid-exponential phases of *A. minutum* culture ($0.67<r^2<0.83$) for all temperature and light conditions. In 25° C cultures, significant variations were observed in the $R_{tox}$ at different growth stages (Table 3). The highest rates were observed during the exponential growth and $R_{tox}$ increased significantly with elevated PPFD ($P<0.05$ for *A. tamiyavanichii*; $P<0.01$ for *A. minutum*). In late exponential or early stationary phases, the rates decreased by 50%–100%. However, in 20° C cultures, no significant variation was found in the $R_{tox}$ ($P = 0.101$ for *A. tamiyavanichii*; $P = 0.252$ for *A. minutum*).

## DISCUSSION

*Effects of light and temperature on growth kinetics.* The growth response of Malaysian *A. tamiyavanichii* was temperature and light dependent in the ranges examined. Low temperature and low irradiance depressed the growth of *A. tamiyavanichii*. As expected, the highest growth rates were obtained at 25° C. This was supported by previous findings of the same species from Thailand (previously reported as *A. cohorticula*; Ogata et al. 1989, 1990). However, the growth rate of the Malaysian strain was 2-fold lower in comparison with the Thai strains as reported by Ogata et al. (1990) under comparable culture conditions (25° C, 51 μmol photons $\cdot m^{-2} \cdot s^{-1}$). Neither the Malaysian nor the Thai strains could grow at 15° C. Temperate strains from Japan, however, grew at 15° C (Ogata et al. 1990). This is in agreement with

TABLE 2. Nonlinear curve fits of the specific growth rate versus irradiance for *Alexandrium tamiyavanichii* and *A. minutum*.

| Species | *A. tamiyavanichii* | | | *A. minutum* | | |
|---|---|---|---|---|---|---|
| Treatment | 20° C | 25° C | *P* | 20° C | 25° C | *P* |
| $\mu_{max}$ | 0.297 ± 0.055 | 0.362 ± 0.018 | 0.34 | 0.442 ± 0.081 | 0.436 ± 0.023 | 0.95 |
| *Ec* | 10.30 ± 3.26 | 9.86 ± 1.60 | 0.91 | 10.58 ± 5.63 | 10.02 ± 1.14 | 0.93 |
| $E_{opt}$ | 52.73 ± 30.76 | 50.08 ± 19.60 | 0.95 | 42.15 ± 17.20 | 24.55 ± 3.44 | 0.37 |
| α | 0.007 ± 0.002 | 0.009 ± 0.001 | | 0.014 ± 0.007 | 0.030 ± 0.010 | |

Maximum growth rates ($\mu_{max}$; in $day^{-1}$), initial slope of the growth versus irradiance curve (α), compensation irradiance ($E_c$) and saturating irradiance ($E_{opt}$) in μmol photons $\cdot m^{-2} \cdot s^{-1}$. Mean and standard deviation ( ± SD) were given.

the observation of Ogata et al. (1990) that the tropical strains were less tolerant of low temperature than the temperate strains. In terms of growth, *A. tamiyavanichii* was more tolerant of higher irradiances in the 20–25° C range, and growth rates increased with increasing irradiance. The results of this study are consistent with findings for other species of *Alexandrium* (Anderson et al. 1984, Maranda et al. 1985, Ogata et al. 1987, 1989, Parkhill and Cembella 1999). However, as the temperature increased, the maximum growth rates could be reached at lower irradiances (Smayda 1969, Cloern 1977). This was

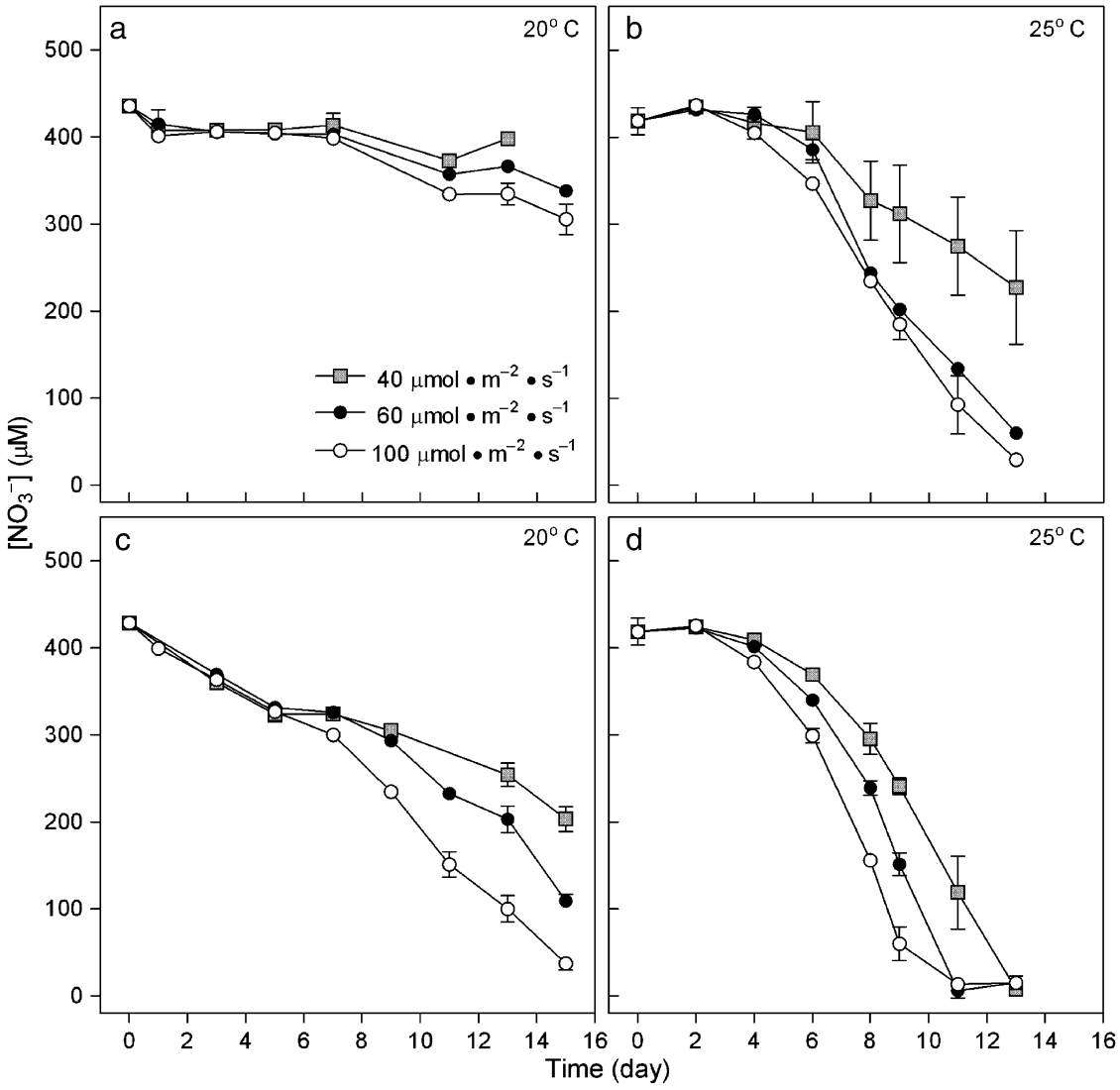


FIG. 4. Changes in nitrate concentration in the culture medium of *Alexandrium tamiyavanichii*, AcMS01 (a and b) and *A. minutum*, AmKB02 (c and d) grown under different temperature and light conditions. Error bars represent ± SD ($n = 4$).

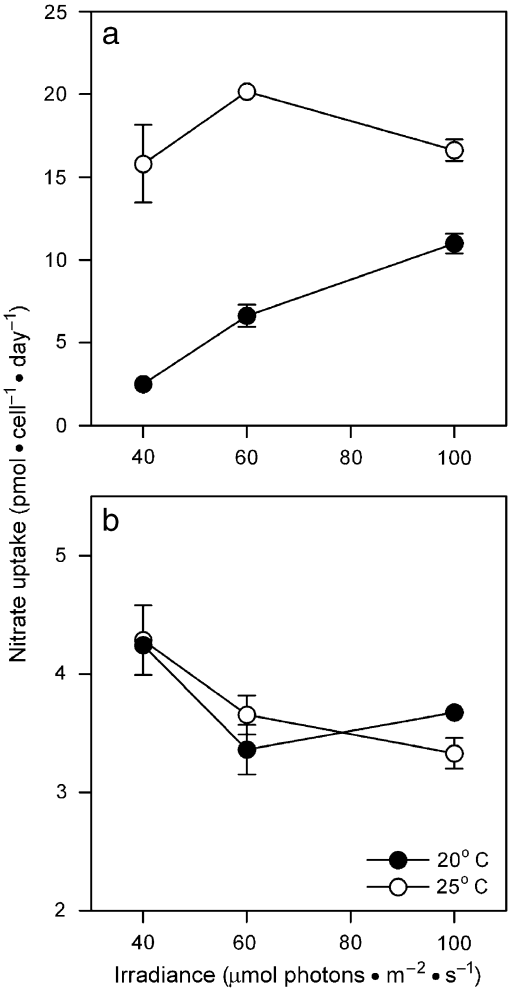


FIG. 5. Rates of nitrate uptake at three different irradiances of *Alexandrium tamiyavanichii*, AcMS01 (a) and *A. minutum*, AmKB02 (b).

observed as a slight decrease of saturating irradiance ($E_{opt}$) with temperature in this study. The Malaysian strain grew sub-optimally at low irradiance (40 μmol photons $\cdot m^{-2} \cdot s^{-1}$).

In contrast, several differences were observed in the growth of *A. minutum* as a function of temperature and light. At optimal temperature (25° C), the strain had a wide optimal irradiance range, ranging from 40 to >100 μmol photons $\cdot m^{-2} \cdot s^{-1}$. The strain could adapt to low irradiance. This was supported by the exceedingly low $E_{opt}$ of 24.55 μmol photons $\cdot m^{-2} \cdot s^{-1}$ at 25° C. The light response of this species indicated that it might be more adapted to changes in irradiance in the water column compared with *A. tamiyavanichii* and other species of *Alexandrium* (Anderson et al. 1984, Parkhill and Cembella 1999). Similar light response characteristics have also been observed in the New Zealand strain (Chang and McClean 1997). At low irradiance levels (<40 μmol photons $\cdot m^{-2} \cdot s^{-1}$), the growth of this species was affected by temperature. However, at irradiances of 60 μmol photons $\cdot m^{-2} \cdot s^{-1}$ and above, growth was not affected by temperature, as there was no difference in growth rates over the range examined. Note also the relatively high $E_{opt}$ at 20° C, suggesting a temperature inhibition.

In Malaysia, blooms of *A. minutum* were found in a shallow semi-enclosed brackish lagoon of less than 5 m depth (Lim et al. 2004). Water clarity in the lagoon was low, due to colored organic matter such as humic acid derived from the surrounding swamp forests that significantly reduced available irradiance. The shade-adaptation strategy (Falkowski and Owen 1980) of *A. minutum* might be a unique characteristic for the species to out-compete other phytoplankton groups in low light environments. Blooms of *A. minutum* in Port River, Australia (Cannon 1990), Bay of Plenty, New Zealand (Chang and Bradford-Grieve 1994), and Tumpat, Malaysia (P. T. Lim et al., unpublished data), where cells were found over the full depth of the water columns, suggest the ability of this species to adapt to a changing irradiance environment. In contrast, blooms of *A. tamiyavanichii* were found to be patchy and close to the clear surface waters in the location where the strain was isolated. This could be explained by the high-irradiance requirement of *A. tamiyavanichii* observed in the laboratory experiments. Other *Alexandrium* species with similar light response characteristics, such as *A. tamarense*, also showed a tendency to occur near the surface under a stratified condition (Cembella and Therriault 1989). The species-specific characteristics of light adaptation may imply different bloom mechanisms and strategies in natural waters.

*Effects of light and temperature on nitrate uptake.* The mechanism of light-stimulated nitrogen uptake and assimilation has been extensively studied in a variety of algae. Several studies have shown that the incorporation of nitrate was strongly dependent on light (MacIsaac 1978, Syrett 1981), while others found contradictory results (Kudela and Cochlan 2000, Fan and Glibert 2005). Paasche et al. (1984), on the other hand, have shown significant variations among species. There exists little information regarding the effects of temperature–irradiance interactions. We have shown that the tropical *Alexandrium*, *A. tamiyavanichii* and *A. minutum*, differ widely in their ability to take up nitrate under various temperature and irradiance conditions.

The uptake kinetics of *A. tamiyavanichii* are similar to those of species that show light-induced nitrate assimilation (Paasche et al. 1984). Analysis of variance demonstrated existence of significant temperature–light effects on the rates of nitrate uptake ($P<0.05$). This suggested that nitrate uptake in this species is light-stimulated as has been observed in *Karenia mikimotoi* (previously reported as *Gyrodinium aureolum*) (Paasche et al. 1984). Many studies have found strong relationships between nitrate uptake rates and nitrate reductase (NR) (Newsholme and Crabtree 1986, Berges and Harrison 1995). The activity of NR is light dependent to some degree in many species of

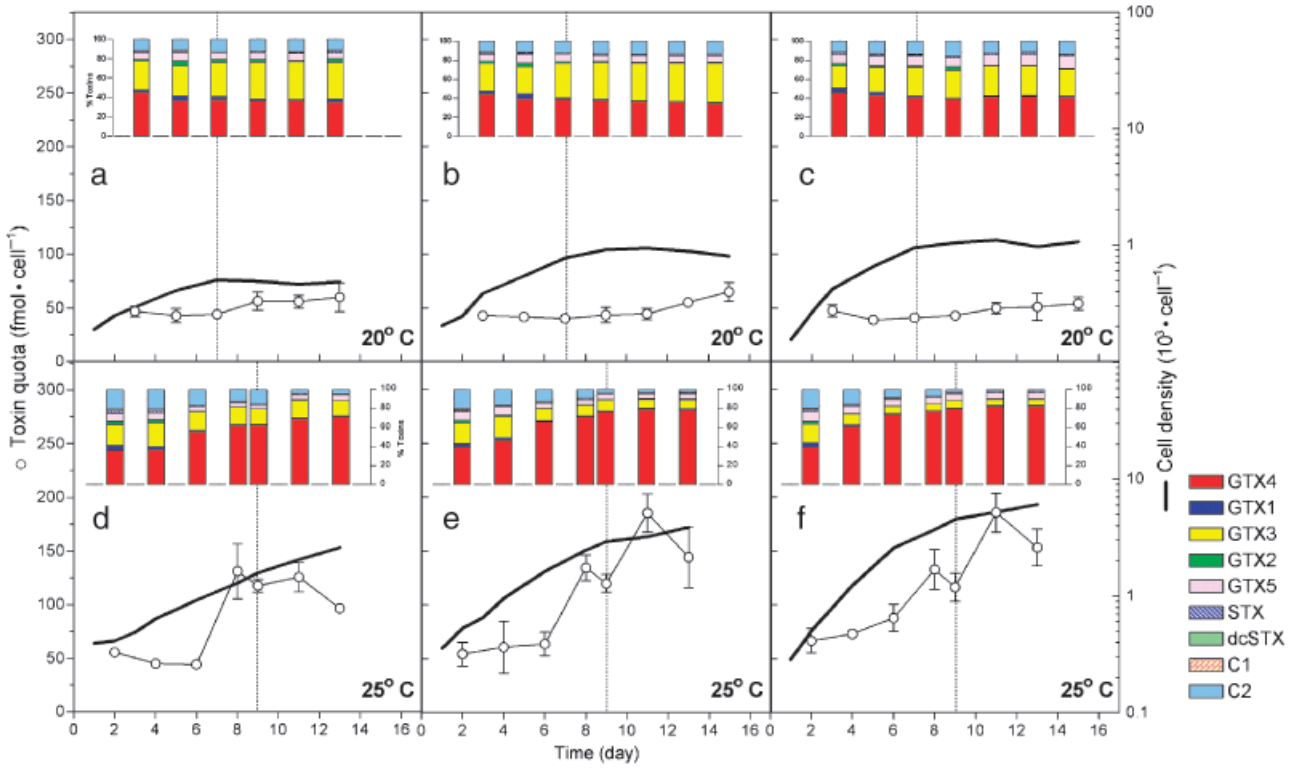


FIG. 6. Toxin quota, $Q_t$ (fmol PST · cell$^{-1}$) of *Alexandrium tamiyavanichii*, AcMS01 grown at 20° C (a–c) and 25° C (d–f) under various irradiances (μmol photons · m$^{-2}$ · s$^{-1}$), 40 (a and d), 60 (b and e), 100 (c and f), with their respective relative proportions of toxins (mol% toxin · cell$^{-1}$, see insets). The $Q_t$ is shown in circles and represent the average of duplicate cultures. Error bars represent ± SD ($n = 4$). The solid lines represent the growth curves shown in Fig. 1. The boundary between exponential and stationary phases was defined by dotted vertical lines.

phytoplankton where nitrate reduction may depend on photosynthetic carbon assimilation or it may involve certain light-activated enzymes (Syrett 1981, Turpin 1991). The rate of nitrate uptake also varied with temperature in the three irradiance treatments; uptake rates measured at optimal growth temperature were greater than those at sub-optimal temperature. This is consistent with the temperature optimum for growth in this species. However, few other studies have shown that the optimum temperature for uptake is different from that for growth (Rhee and Gotham 1981).

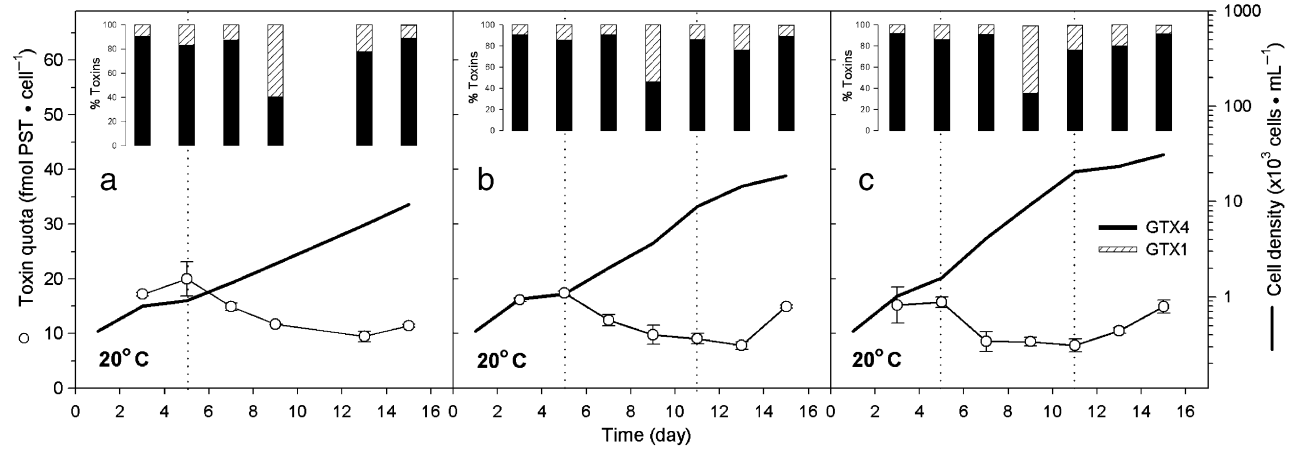


FIG. 7. Toxin quota, $Q_t$ (fmol PST · cell$^{-1}$), of *Alexandrium minutum*, AmKB02 grown at temperature of 20° C under various irradiance (μmol photons · m$^{-2}$ · s$^{-1}$), 40 (a), 60 (b), 100 (c), with their respective relative proportions of toxins (mol% toxin · cell$^{-1}$, insets). The $Q_t$ is shown in circles and represents the average of duplicate cultures. Error bars represent ± SD ($n = 4$). The black solid lines represent the growth curves shown in Fig. 1. The boundaries between early, mid, and late exponential phases are defined by dotted vertical lines.

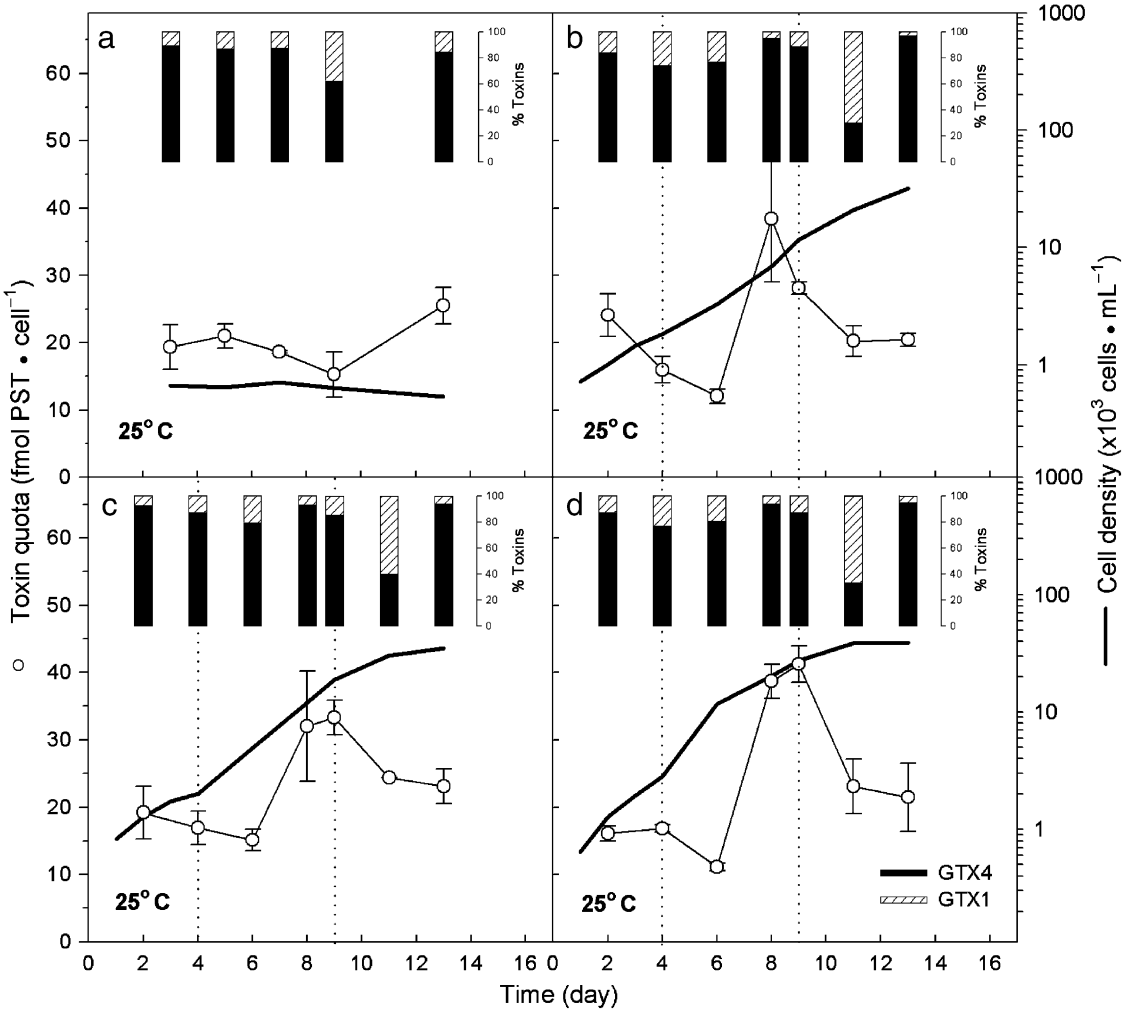


FIG. 8. Toxin quota, $Q_t$ (fmol PST · cell$^{-1}$), of *Alexandrium minutum*, AmKB02 grown at temperature of 25° C under various irradiance (μmol photons · m$^{-2}$ · s$^{-1}$), 10 (a), 40 (b), 60 (c), 100 (d), with their respective relative proportions of toxins (mol% toxin · cell$^{-1}$, insets).

It is noteworthy that the uptake kinetics of *A. minutum* varied from those reported previously. The maximum nitrate uptake rates were found at 40 μmol photons · m$^{-2}$ · s$^{-1}$ for both temperature treatments. At sub-optimal irradiance levels, the increased rate of nitrate uptake may expeditiously increase the nitrogen cell quota that is required for growth. Davis (1976) also showed that cell quotas of the diatom *Skeletonema costatum* were greater at sub-optimal light levels than those at light-saturated growth. It could be an adaptive mechanism at suboptimal irradiance for the species. It is also interesting to note that temperature did not affect the rate of nitrate uptake in this species. This may be explained by the fact that optimum temperature for enzyme reactions is not necessarily the same as those for growth (Innis and Ingraham 1978).

*Effects of light and temperature on toxin production.* Most studies on toxin production of *Alexandrium* in batch cultures have shown that the toxin cell quota, $Q_t$, peaked at the early exponential phase (Boyer et al. 1987, Boczar et al. 1988) when nitrogen sources remain sufficient, and $Q_t$ decreased at the late exponential phase when nitrogen became depleted. However, our results showed that the highest $Q_t$ was observed in late exponential to stationary phase cultures of *A. tamiyavanichii* and *A. minutum*, a pattern that appears inconsistent with the results of other studies. The same phenomenon was observed in the PSP-producing cyanobacterium, *Aphanizomenon* sp. (Dias et al. 2002). The variations indicated that the production or synthesis of PSP toxins in the course of growth cycle may be variable among the PST-producing species.

Increasing the irradiance and temperature of *A. tamiyavanichii* and *A. minutum* cultures led to an increase in $Q_t$, even though the increment was less pronounced for light effects. This is in contrast to other studies of *Alexandrium* species (Table 4). For example, *A. tamarense* showed an increase in cellular toxin content with reduced temperature and irradiance at sub-optimal growth (Ogata et al. 1987, Hamasaki et al. 2001). Proctor et al. (1975) suggested that toxin content was

TABLE 3. Rates of net toxin production, $R_{tox}$ (fmol · cell$^{-1}$ · day$^{-1}$) calculated at early, mid, and late exponential or early stationary growth phases of *Alexandrium tamiyavanichii* and *A. minutum* at various temperature and light conditions (PPFD, μmol photons · m$^{-2}$ · s$^{-1}$).

| Temperature | PPFD | Net toxin production rate at various growth stages | |
|---|---|---|---|
| | | Exponential | Stationary |
| *A. tamiyavanichii* | | | |
| 20° C | 40 | 5.39 ± 0.21 | 2.29 ± 1.80 |
| | 60 | 6.57 ± 1.25 | 5.84 ± 0.21 |
| | 100 | 7.18 ± 0.06 | 3.62 ± 1.78 |
| 25° C | 40 | 26.9 ± 3.87 | 8.03 ± 2.95 |
| | 60 | 32.8 ± 1.52 | 15.2 ± 7.32 |
| | 100 | 38.7 ± 6.50 | 19.2 ± 8.80 |

| Temperature | PPFD | Net toxin production rate at various growth stages | | |
|---|---|---|---|---|
| | | Early exponential | Mid exponential | Late exponential |
| *A. minutum* | | | | |
| 20° C | 40 | 2.65 ± 0.26 | 1.31 ± 0.31 | 2.71 ± 0.66 |
| | 60 | 1.66 ± 0.01 | 2.03 ± 0.60 | 4.40 ± 0.18 |
| | 100 | 3.63 ± 0.76 | 3.14 ± 0.48 | 3.53 ± 0.28 |
| 25° C | 40 | 1.85 ± 0.45 | 11.2 ± 0.21 | 3.97 ± 0.39 |
| | 60 | 2.91 ± 1.69 | 15.8 ± 0.99 | 1.66 ± 0.20 |
| | 100 | 6.90 ± 0.56 | 20.0 ± 0.87 | −2.12 ± 0.79 |

PPFD, photosynthesis photon flux density.

inversely related to growth rate in *A. catenella*. Compilation and re-analysis of previous data by Cembella (1998) also supported a strong correlation between cell toxicity and light- or temperature-dependent growth rate. However, Kodama (1990) found no clear correlation between growth rate and cellular toxin content in different isolates of *A. tamarense*. The results obtained from this study clearly showed that the increase in $Q_t$ was affected by both light intensity and temperature. This increment was observed even during the active cell division when the effect of toxin dilution is high. The growth of *A. minutum* was significantly different between 20 and 15° C. But a similar $Q_t$ was observed over the growth cycle under both conditions. These results clearly showed that the toxin production in these two species was not a function of the growth response to the related exogenous factors (e.g. temperature- and light-dependent growth rate), but indeed a response to temperature and light.

The consensus on temperature effects on *Alexandrium* toxin production is that growth at low temperature favors a high toxin cell quota (Cembella 1998). In contrast, our result showed that $Q_t$ in 25° C grown cultures was 1- to 3-fold greater than 20° C grown cultures in both species. In 20° C cultures, $Q_t$ decreased gradually even though the cells continued to grow. Therefore, low temperature seems to suppress the production of toxins or its related processes. It is interesting to note that similar nitrate uptake rates were observed in *A. minutum* cultures at both temperatures. The reasons why the same amount of nitrate was assimilated but not diverted into toxin constituents are unclear. At optimal temperatures, the maximum $Q_t$ increased with elevated irradiance. Similar observations have been described in another PST-producing dinoflagellate, *Pyrodinium bahamense* var. *compressum* (Usup et al. 1995). Ogata et al. (1996) also demonstrated that both growth and toxin synthesis were suppressed under extremely low light conditions. This may be due to the inhibition of the enzyme RUBISCO and/or nitrate uptake efficiency (Cembella 1998).

Eutrophication is perceived as one of the main triggering factors contributing to the increase of HAB events in the coastal waters throughout the world (Lam et al. 1989, Okaichi 1989). Nitrogen-to-phosphorus ratios (N:P) are typically high in eutrophic waters (Smayda 1990, Conley 1999) and in estuarine waters. In Malaysian estuaries, N:P ratios were found to vary from approximately 25:1 (during neap tide) to approximately 70:1 (during spring tide) and to fluctuate during the rainy and dry seasons (Tanaka and Choo 2000). The high N:P ratio was used in the present study because all of the *Alexandrium* species were isolated from estuarine waters. Moreover, application of this high ratio for assessing the $Q_t$ under culture conditions is relevant for studies of the toxicity of the two species in the natural environment.

Phosphate limitation has been shown to suppress the growth of *A. tamarense* (Boyer et al. 1987), *A. fundyense* (Anderson et al. 1990b, John and Flynn 2000), *A. catenella* (Siu et al. 1997), and *A. minutum* (Hwang and Lu 2000, Lippemeier et al. 2003), but may require N:P ratios significantly higher than the Redfield ratio (>36:1; John and Flynn 2000). In the absence of phosphate, cell growth may continue over several generations (John and Flynn 2000) due to high intracellular storage (Cembella 1998). In this study, phosphate levels were not investigated; therefore, changes of N:P in the cultures over growth cycle could not be determined. However, we observed a continued increase in cell densities up to day 12, and the two species in this study are not likely to be limited by phosphate at least during the exponential phases.

There is no general trend of the effect of phosphorous limitation on PST production. While several studies have reported an increase in cellular PST under P limitation (Boyer et al. 1987, Anderson et al. 1990b, Usup et al. 1994, Lippemeier et al. 2003), others showed that phosphate limitation alone did not result in an increase of PST production (Flynn et al. 1994, 1996, John and Flynn 2000). In the latter cases, an increase in PST was only observed under both P and N limitation. However, the cellular toxin quota of *Alexandrium* was found to be a non-linear function of growth rates under phosphate limitation (Cembella 1998). According to Cembella (1998), the differences in the effect of phosphate limitation were due to the lack of bioenergetic substrates for cell division, and not for toxin biosynthesis. High intracellular storage of phosphate could probably be the mechanism to overcome phosphate starvation when intracellular free

TABLE 4. Irradiance, temperature, and salinity used in this study and others for *Alexandrium* species and their effects on growth and toxin production.

| Species (strain) | Irradiance (μmol photons · $m^{-2} \cdot s^{-1}$) | Temperature (° C) | Salinity (psu) | Growth rate, μ ($day^{-1}$) | Toxicity/cellular toxin quota, $Q_t$ | Location |
|---|---|---|---|---|---|---|
| *A. tamiyavanichii* (AcMS01) | 40–140 | 15–25 | 5–30[a] | μ: 0.1–0.4 $day^{-1}$ (20–30° C, 40–140 μmol photons · $m^{-2} \cdot s^{-1}$); μ: 0.25–0.35 $day^{-1}$ (20–30 psu) | $Q_t$: 50–200 fmol · $cell^{-1}$, $Q_t$ increased with temperature; $Q_t$: 40–>80 fmol · $cell^{-1}$, $Q_t$ decreased with elevated salinity | Malaysia[a,b] |
| *A. tamiyavanichii* (Chula8, Chula18) | 51 | 15–30 | — | μ: 0.1–0.4 $day^{-1}$ (20–30° C) | — | Thailand[c] |
| *A. tamiyavanichii* (Chula5) | 10–80 | 20, 25, and 30 | — | μ: 0.2–0.3 $day^{-1}$ Increased with increasing irradiance and temperature | Toxin content decreased with temperature; toxin content peaked at ~35 μmol photons · $m^{-2} \cdot s^{-1}$ | Thailand[d] |
| *A. tamiyavanichii* (MMBS8811-1, MMBS8811-3) | 51 | 15–30 | — | μ: 0.05–0.3 $day^{-1}$ (20–30° C) | — | Japan[c] |
| *A. tamarense* (Pr18b) | 40–470 | 15 | 10–30 | $\mu_{max}$: 0.41 $day^{-1}$ (230 μmol photons · $m^{-2} \cdot s^{-1}$); 0.5 $day^{-1}$ (25 psu) | 31–179 pg STXeq · $cell^{-1}$ (salinity); 15–95 pg STXeq · $cell^{-1}$ (irradiance) | Canada[e] |
| *A. tamarense* (ATHS-92) | 80–350 | 12, 17, and 22 | 13–38 | $\mu_{max}$: 0.55 $day^{-1}$ (25 psu); $\mu_{max}$: 0.24 $day^{-1}$ (17° C, 350 μmol photons · $m^{-2} \cdot s^{-1}$); μ increased with irradiance at 17 and 22° C | 0.072–0.92 pg STXeq · $cell^{-1}$ (salinity); toxicity increased with decreasing salinity, irradiance at 17 and 22° C | Japan[f] |
| A. *minutum* (AmKB06) | 140 | 25 | 2–30 | μ: 0.3–0.5 $day^{-1}$ (5–30 psu) | $Q_t$: 4–12 fmol · $cell^{-1}$; $Q_t$ decreased with lifted salinity | Malaysia[a] |
| A. *minutum* (AmKB02) | 10–140 | 15–25 | 15 | μ: 0.2–0.5 $day^{-1}$; μ increased with increasing light and temperature | $Q_t$: 10–40 fmol · $cell^{-1}$; $Q_t$ increased with increasing light and temperature | Malaysia[b] |
| *A. minutum* (AM89BM) | 100 | 18 | 12–37 | μ: ≥ 0.5 $day^{-1}$ (20–37 psu) | $Q_t$: 10–50 fmol · $cell^{-1}$; $Q_t$ increased at lower salinities | France[g] |
| *A. minutum* (AMBOP006) | 25–100 | 18 | — | $\mu_{max}$: 0.50 $day^{-1}$; μ increased with elevated irradiance | — | New Zealand[h] |

[a]This study.
[b]Lim and Ogata (2005).
[c]Ogata et al. (1990).
[d]Ogata et al. (1987).
[e]Parkhill and Cembella (1999).
[f]Hamasaki et al. (2001).
[g]Grzerbyk et al. (2003).
[h]Chang and McClean (1997).

amino acid remained sufficient for biosynthesis of toxin (Cembella 1998).

As phosphate levels were not monitored over the growth cycle in our study, the possibility of an increase of cellular PST in some treatments due to phosphate limitation cannot be totally ruled out. On the other hand, the increase of toxin quota, particularly at the exponential phase (when phosphate was sufficient), cannot be explained by the effect of phosphate limitation. In *A. tamiyavanichii*, toxin quota showed differences among the treatments (temperature and irradiance levels) between day 6 and day 8 when phosphate remained sufficient (Fig. 6). In *A. minutum*, phosphate levels reached limiting levels at 25 and 20° C, but an increase of toxin quota was only observed at 25° C and not at 20° C, indicating that it was due to elevated temperature and not phosphate limitation. Nonetheless, the issue of phosphate limitation effect on toxin production of the two *Alexandrium* species should be examined in more detail in future work.

Analysis of PST compositions in both *A. tamiyavanichii* and *A. minutum* demonstrated no significant compositional changes in the toxin profiles. This is in agreement with the results of other studies (Boyer et al. 1987, Cembella et al. 1987, Ogata et al. 1987, Oshima et al. 1990, Cembella and Destombe 1996, Parkhill and Cembella 1999). In addition, the relative abundance of individual toxins (mol%) for both species was stable over the culture growth regardless of the elevated irradiances. This was in partial agreement with the results obtained by Parkhill and Cembella (1999). They found that the GTX3:GTX2 ratios of *A. tamarense* were stable over time under the irradiance range tested (40–470 µmol photons $\cdot m^{-2} \cdot s^{-1}$). However, in *A. tamiyavanichii* cells grown under optimal temperature conditions, the relative abundance of GTX4 increased gradually with culture growth. It was also interesting to note that under a less favorable temperature condition, the toxin composition was relatively constant throughout the course of the experiments, with almost an equal proportion of GTX3 and GTX4. In *A. minutum*, an increase of the relative abundance of GTX1 was observed with the decrease of GTX4 proportionally as the toxin cell quota decreased (day 9 in 20° C cultures; day 11 in 25° C cultures). The lowest epimeric ratio of GTX4 and GTX1 remained for a very short period and reverted to a normal high GTX4:GTX1 ratio thereafter. The changes in the epimeric ratios might be explained by the epimerization of GTX4 to GTX1. Minor variation due to *in situ* transformation (e.g. epimerization) of toxin composition has been reported. In fact, transformation of toxin was not an unusual phenomenon as similar transformation has been observed in both plankton and shellfish (Asakawa et al. 1986, Oshima 1995a). Enzymatic oxidization that converted GTX3 + 2 to GTX4 + 1 has also been documented in *A. tamarense* (Kodama 2000). In the same species, Parkhill and Cembella (1999) suggested that the β-epimer (GTX3) was epimerized to a more thermodynamically favored α-epimer (GTX2) when the culture conditions become less favorable, and thus concluded that the minor variation in toxin composition did not invalidate the concept of genetic stability in toxin profiles. Immediate analysis of the toxin samples to avoid *in vitro* transformation and meticulous analysis of toxin data should be taken into consideration in evaluating the growth and PSP toxin production data.

In this study, we demonstrated remarkable variations of temperature and light adaptations of two tropical *Alexandrium* species from Malaysia. *A. minutum* showed higher temperature and light tolerances compared with *A. tamiyavanichii*. Our previous study on salinity effects showed a distinctive adaptation pattern between the two species (Lim and Ogata 2005). Our results showed that temperature and light were not only affecting growth but also toxin production. It is essential to verify the results obtained in the laboratory with the field determinations.

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