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Salinity effect on growth and toxin production of four tropical *Alexandrium* species (Dinophyceae)

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Abstract

Four tropical PSP toxins-producing dinoflagellates, *Alexandrium minutum*, *Alexandrium tamiyavanichii*, *Alexandrium tamarense* and *Alexandrium peruvianum* from Malaysian waters were studied to investigate the influences of salinity on growth and toxin production. Experiments were conducted on constant temperature 25 °C, 140 μE mol m^{-2} s^{-1} and under 14:10 light:dark photo-cycle with salinity ranged from 2 to 30 psu. The PSP-toxin congeners, GTX 1–6, STX, dcSTX, NEO and C1–C2 were analysed by high performance liquid chromatography. Salinity tolerance of the four species in decreasing order is *A. minutum* > *A. peruvianum* > *A. tamarense* > *A. tamiyavanichii*. Specific growth rates and maximum densities varied among these species with *A. minutum* recorded as the highest, 0.5 day^{-1} and 6×10^4 cells mL^{-1}. Toxin content decreased with elevated salinities in *A. minutum*, the highest toxin content was about 12 fmole $cell^{-1}$ at 5 psu. In *A. tamiyavanichii*, toxin content peaked at optimal growth salinity (20 and 25 psu). Toxin content of *A. tamarense*, somehow peaked at sub-optimal growth salinity (15 and 30 psu). Results of this study implied that salinity fluctuation not only influenced the growth physiology but also toxin production of these species.

Keywords: *Alexandrium minutum*; *A. tamiyavanichii*; *A. tamarense*; *A. peruvianum*; Paralytic shellfish poisoning toxins; Salinity tolerance

1. Introduction

Dinoflagellates from the genus *Alexandrium* have been well known as producers for the potent neurotoxins that cause paralytic shellfish poisoning (PSP) in many coastal countries throughout the world (Anderson et al., 1994; Halegraeff et al., 1995). Blooms of *Alexandrium* and outbreak of poisoning cases have caused serious economic losses in the fishery industries of the affected countries. Public health concern arose with increasing poisoning cases due to the consumption of contaminated bivalves. This has been particularly pronounced in countries where seafood is the main source of protein. Malaysia is not an exception from this menace. Before 1990s, *Pyrodinium bahamense* is the only PSP causative organism in Malaysian waters with blooms and PSP outbreaks confined to Sabah. The perception changed with the increased in number of toxic *Alexandrium* species found as well as the PSP cases reported in Peninsula Malaysia (Usup et al., 2002; Lim et al., 2002). Five species of *Alexandrium* have been identified. Out of these species, *Alexandrium tamiyavanichii* and *Alexandrium minutum* were reported as toxic (Usup et al., 2002). Two additional *Alexandrium* species, namely *Alexandrium taylori* and *Alexandrium peruvianum* were later found in

Abbreviations: psu, pratical salinity unit; PSP toxins, paralytic shellfish poisoning toxins; GTXs, gonyautoxins; STXs, saxitoxins; dcSTX, decarbomoyl-saxitoxin; NEO, neosaxitoxin; C toxins, *N*-sulfo-carbamoyl-toxins.

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Sarawak and successfully established into cultures. Both species have been confirmed as for the first time as saxitoxin producers (Lim et al., 2005).

Interestingly, all these *Alexandrium* species were found in or close to estuarine regions. *A. minutum* was found in estuarine semi-enclosed lagoon where bloom of this species caused one casualty and six persons were hospitalized (Lim et al., 2002). *Alexandrium tamarense* was found near to the blood cockle (*Anadara granosa*) farming site in Prai River estuarine in the northern part of Malacca Strait while *A. tamiyavanichii* was found not far from Muar River estuary in southern region. *A. taylori* and *A. peruvianum* were found in a few kilometers upstream of Samariang estuary, Sarawak, which might be advected by the tidal current.

Blooms of toxic *Alexandrium* have been always associated with the estuarine coastal regions (Cembella and Therriault, 1989; Larocque and Cembella, 1990). Giacobbe and co-workers (1996) showed that the spring blooms of *A. minutum* in Mediterranean Sea coincided with the increase in rainfall and freshwater runoff that increased the stratification of the water column. Cembella et al. (1988) in his study documented that blooms of *A. tamarense* in St Lawrence estuary was initiated by the near shore cyst bed. Study on the same species in Gulf of Maine found that low salinity coastal current promoted the increase in cell density dramatically in Casco Bay regions (Anderson, 1998). These works showed that salinity is an important exogenous factor in the bloom dynamics of *Alexandrium* species.

Effects of environmental factors including salinity on cellular growth and toxin production have been studied for several species of *Alexandrium* (Anderson et al., 1990; Chang et al., 1997; Cembella, 1998; Grzebyk et al., 2003). Unfortunately, these studies mainly worked on a single species, e.g. *Alexandrium catenella* (Anderson et al., 1990), *A. tamarense* (Parkhill and Cembella, 1999), and *A. minutum* (Grzebyk et al., 2003). The incongruence in methodological settings among the studies has hampered the effort to have valid physiological comparisons between species. Furthermore, these studies conducted mainly on *Alexandrium* originated from temperate or subtropical water that might possess different physiological adaptation compared to the tropical counterpart. On the other hand, information on the growth and toxin production of tropical *Alexandrium* is still very limited. Present study was carried out as part of the effort to compare the growth physiology of tropical and temperate *Alexandrium*. The main purpose was to determine the variability of growth and toxin production at different salinity regimes on four tropical *Alexandrium* species.

2. Materials and methods

2.1. Culture establishment and salinity tolerance experiment

Clonal cultures of *Alexandrium* were established from live plankton specimens collected from different locations throughout Malaysia waters (Table 1). Cultures were maintained in ES medium, with addition of 380 μM nitrate $[NO_3]^-$ and 12 μM of phosphate $[PO_4]^-$. The pH was adjusted to pH 7.8–7.9 for all the natural seawater used in the experiment. Clonal cultures were kept in 25 °C, 140 μE m^{-2} s^{-1} and 14:10 h light:dark photoperiod.

Table 1
Alexandrium species used in this study

Species, strain	Location of isolation	Date of collection
Alexandrium minutum, AmKB06	Geting River, Tumpat, Kelantan	September 2001
Alexandrium tamiyavanichii, AcMS01	Sebatu, Malacca	October 1997
Alexandrium tamarense, AtPA01	Aman Island, Penang	June 2002
Alexandrium peruvianum, ApKS01	Samariang River, Kuching, Sarawak	August 2003

Seven salinity treatments with salinity of 2, 5, 10, 15, 20, 25, and 30 psu were prepared by diluting seawater with distilled water. Cells were pre-acclimatized at different salinities prior to the salinity experiments. The exponential phase cells were then inoculated into tubes containing medium at desired salinity. Growth was monitored daily by cell density counting and in vivo fluorescence measurement. Specific growth rate (μ, unit: day^{-1}) was calculated for both cell density and in vivo fluorescence over the exponential growth phase using the following equation

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_0 and N_1 are the cell density or in vivo fluorescence reading at time t_0 and t_1.

2.2. Cell diameter and mean cell volume calculation

Sub-samples for cell diameter measurement at different salinity treatments were fixed in Lugol's solution. Measurement was made using an Olympus BX40 microscope (Olympus, Melville, NY) under 200× magnifications. Measurement was calibrated with micrometer. The mean cell volume (v) were calculated from a total of 30–80 measurements of cell diameter and presented as mean. Calculation of mean cell volume (μm^3) was made with the assumption of spherical shape of dinoflagellate cell (Hillebrand et al., 1999) using the following equation

$$v = \frac{\pi}{6} d^3$$

where d is the diameter of cells.

2.3. Toxins analysis

Analysis of high performance liquid chromatography (HPLC) was carried out using the isocratic, post-column

derivatization method of Oshima (1995a) on JASCO HPLC system fitted with post-column system and fluorescence detector. The post-column temperature was kept at 70 °C for all runs. Twenty microliter of sample was injected in each run. GTXs (GTX 1–6), STXs (STX, dcSTX, NEO) and C toxins (C1–C2) were analysed separately. The toxins were quantified with authentic toxin standards.

The net toxins production rate, R_{tox} (fmole toxin cell^{-1} day^{-1}) was determined using the equations as described by Anderson et al. (1990)

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

$$\bar{N} = \frac{N_1 - N_0}{\ln N_1 - \ln N_0}$$

where $\bar{N}$ is the cell density at each stage while T is the total toxin concentration at time, by multiplying the toxin content (fmole cell^{-1}) with cell density (N).

3. Results

Four clonal cultures of tropical *Alexandrium* species, to wit *A. minutum* (strain AmKB06), *A. tamiyavanichii* (strain AcMS01), *A. tamarense* (strain AtPA01), and *A. peruvianum* (strain ApKS01) were used in this salinity study. Growth of the *Alexandrium* strains at different salinity regimes were presented in Fig. 1. Strong correlation was observed between in vivo fluorescence and cell counts in all four species in this study (Fig. 2). Highest correlation was obtained in *A. minutum* ($r^2 = 0.94$) and good correlation for all the other species, 0.77, 0.90, and 0.79 for

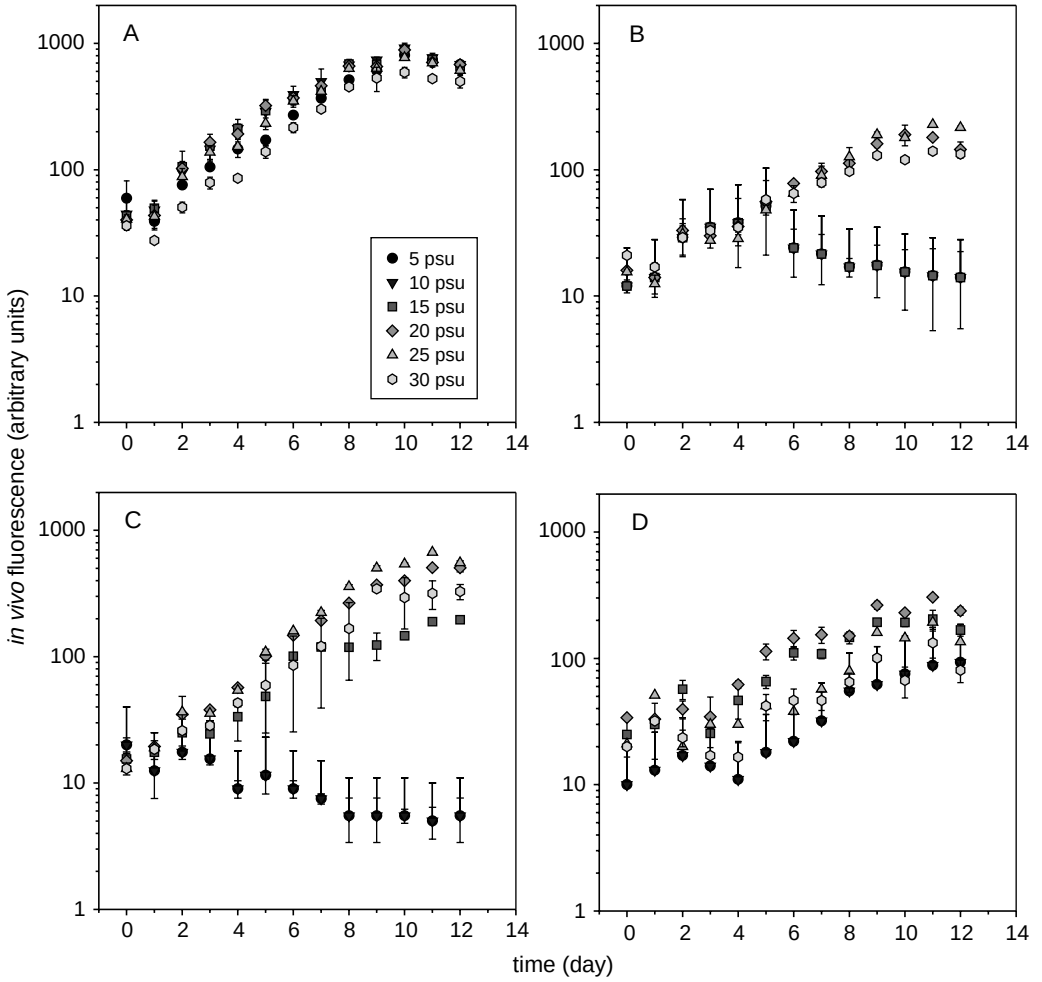

Fig. 1. Growth of *Alexandrium* species monitored using in vivo fluorescence. (A) *A. minutum*, (B) *A. tamiyavanichii*, (C) *A. tamarense*, (D) *A. peruvianum*.

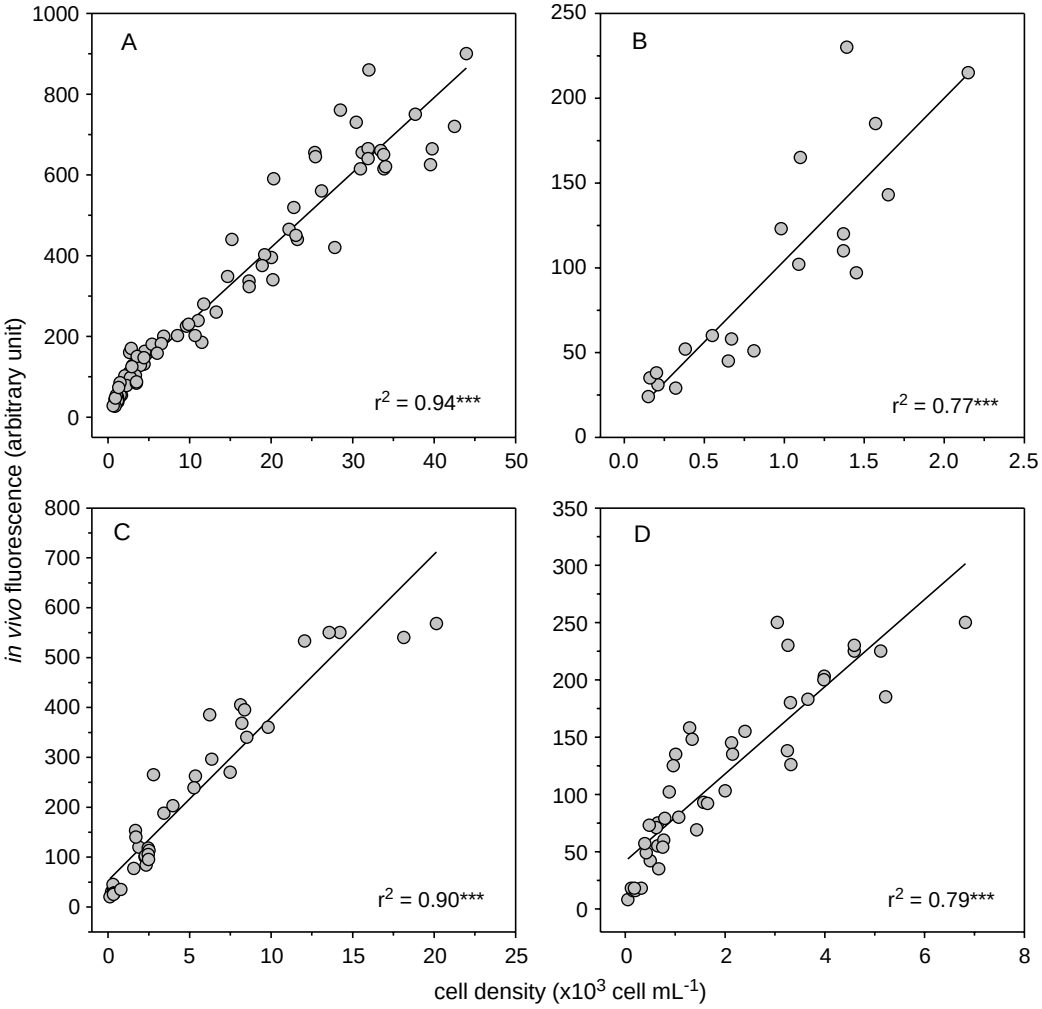

Fig. 2. Correlation between data points of cell density vs. in vivo fluorescence for all salinity treatments. (A) *A. minutum*, (B) *A. tamiyavanichii*, (C) *A. tamarense*, (D) *A. peruvianum*.

A. tamiyavanichii, *A. tamarense* and *A. peruvianum*, respectively. However, no strong correlation ($r^2 = 0.50$) was obtained for the specific growth rate, μ calculated based on cell count and in vivo fluorescence (Fig. 3).

3.1. Growth at different salinity regimes

A. minutum grew at 0.3–0.50 day^{-1} in the salinity ranged from 5 to 30 psu but died off at 2 psu (Fig. 4A). Cells exhibited wide salinity tolerance and no significant differences in growth rate within the salinity range tested except 2 psu and below. However, the highest cell density was obtained at 15 psu with 6×10^4 cells mL^{-1}. Despite the consistent growth rates, cell mean volume was significantly increased with the increase in salinity ($P < 0.01$) (Table 2). Cells in salinity of 5 and 10 psu were nearly 40% smaller in term of cell volume compared to higher salinities. *A. tamiyavanichii* grew at salinity 20–30 psu (μ of 0.25–0.35 day^{-1}) with optimum growth at 25 psu (Fig. 4B). Cells survived without undergoing any division at salinity 15 psu but did not survive at 10 psu and below. Higher mean cell volume was observed in lower salinity (20 psu) coincided with the low growth rate. *A. tamarense* grew in salinity range of 20–30 psu with 0.30–0.45 day^{-1} but the growth rate decreased dramatically to 0.1 day^{-1} at 15 psu (Fig. 4C). Cells did not survive at salinity 10 psu and below. Similar to *A. tamiyavanichii*, the mean cell diameter and volume were antithetical to the salinity where cell volume tended to be higher at low salinity (15 psu). In addition to that when growth rates were high, average cell volumes became smaller. *A. peruvianum* can tolerate wider ranges of salinity from 10 to 30 psu with growth rates of 0.20–0.35 day^{-1} (Fig. 4D). Cells died at 5 psu and below. Mean cell diameter and volume throughout the salinity treatments did not show any significant changes with the range of 24.0–28.0 μm and 0.8–1.2 $\times 10^4$ μm^3, respectively.

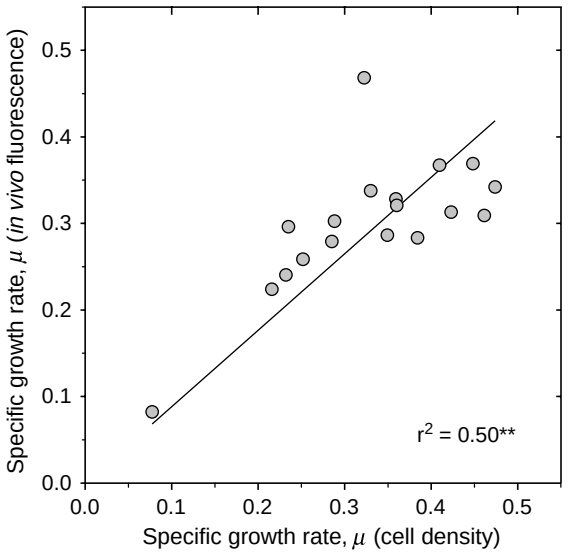

Fig. 3. Correlation between specific growth rates (μ) calculated from in vivo fluorescence vs. μ from cell counts.

3.2. Toxin content, composition and net toxin production rate

A. tamiyavanichii was the most potent species among the four species with toxin content up to 80 fmol cell^{-1}, followed by *A. minutum*, and *A. peruvianum*. *A. tamarense* was only weakly toxic with less than 1 fmole cell^{-1}. All *Alexandrium* species in this study possessed high proportion of gonyautoxins with GTX4 and GTX1 as the major toxin congeners. However, the proportion of congeners varies between species. GTX5 and GTX6 were also found in *A. tamiyavanichii* and *A. peruvianum*, respectively. In *A. minutum*, GTX1+4 made up to 95 mole% of the toxin composition with GTX2+3 as the minor propotion, STX and NEO were only found as traces.

In the salinity experiment, toxin content of *A. minutum* strain tended to be higher in lower salinity regimes, up to 12 fmole cell^{-1}, while the lowest toxin content was observed at salinity of 25 psu (Fig. 5A). Calculation of toxin content based on mean cell volume showed that toxin content per cell volume ranged from 0.6 to

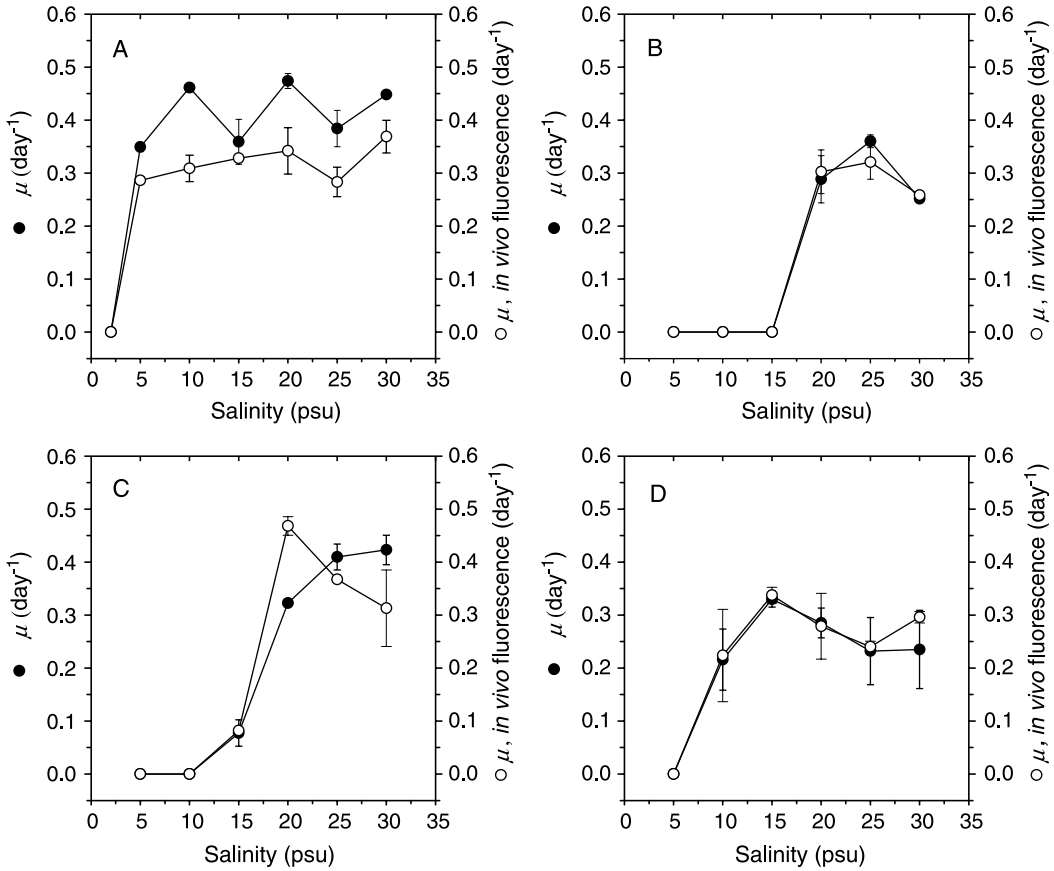

Fig. 4. Specific growth rates, μ of *Alexandrium* species calculated from cell density and in vivo fluorescence at different salinity regimes. (A) *A. minutum*, (B) *A. tamiyavanichii*, (C) *A. tamarense*, (D) *A. peruvianum*.

Table 2
Mean cell diameter (μm) and cell volume (μm^3) of *Alexandrium* species at different salinity regimes

Salinity (psu)	*A. minutum* (AmKB06)	*A. tamiyavanichii* (AcMS01)	*A. tamarense* (AtPA01)	*A. peruvianum* (ApKS01)
5	20.5±2.33 (4482±7)	NA	NA	NA
10	20.7±2.55 (4628±9)	NA	NA	28.3±4.56 (11811±50)
15	21.5±3.09 (5172±15)	NA	39.1±4.91 (31307±62)	25.7±2.88 (8842±12)
20	22.4±3.43 (6268±21)	44.2±4.55 (45313±49)	31.5±5.6 (16295±92)	24.8±3.16 (7943±16)
25	22.9±3.42 (6268±21)	38.6±5.14 (30121±71)	29.2±4.43 (12989±45)	26.1±3.04 (9272±15)
30	24.5±2.81 (7706±12)	38.7±5.62 (30356±93)	31.8±5.28 (16845±77)	25.7±3.58 (8832±24)

NA, not applicable.

2.5 fmole 10^{-3} μm^{-3}. As similar to *A. minutum*, GTX1+4 remained as the major toxins (up to 45 mole%) in *A. tamiyavanichii*; however, the proportion of GTX2+3 (19 mole%) and C2 (up to 24 mole%) were significantly higher compared to *A. minutum* (Fig. 5B). Toxin content per cell decreased with elevated salinity. The highest toxin content was 80 fmole per cell at 20 psu. The toxin content decreased almost 1-fold at 30 psu.

Both *A. tamarense* and *A. peruvianum* were weakly toxic. Toxin content of *A. tamarense* ranged from 0.1 to 0.80 fmole $cell^{-1}$ with highest toxin content obtained in 15 psu (Fig. 5C). Toxin content was slightly higher at sub-optimal salinity, 15 and 30 psu. GTX1+4 contributed to 25–40 mole% of total toxin content. Interestingly, NEO was considerably higher compared to other *Alexandrium* strains with almost equal proportion to GTX1+4

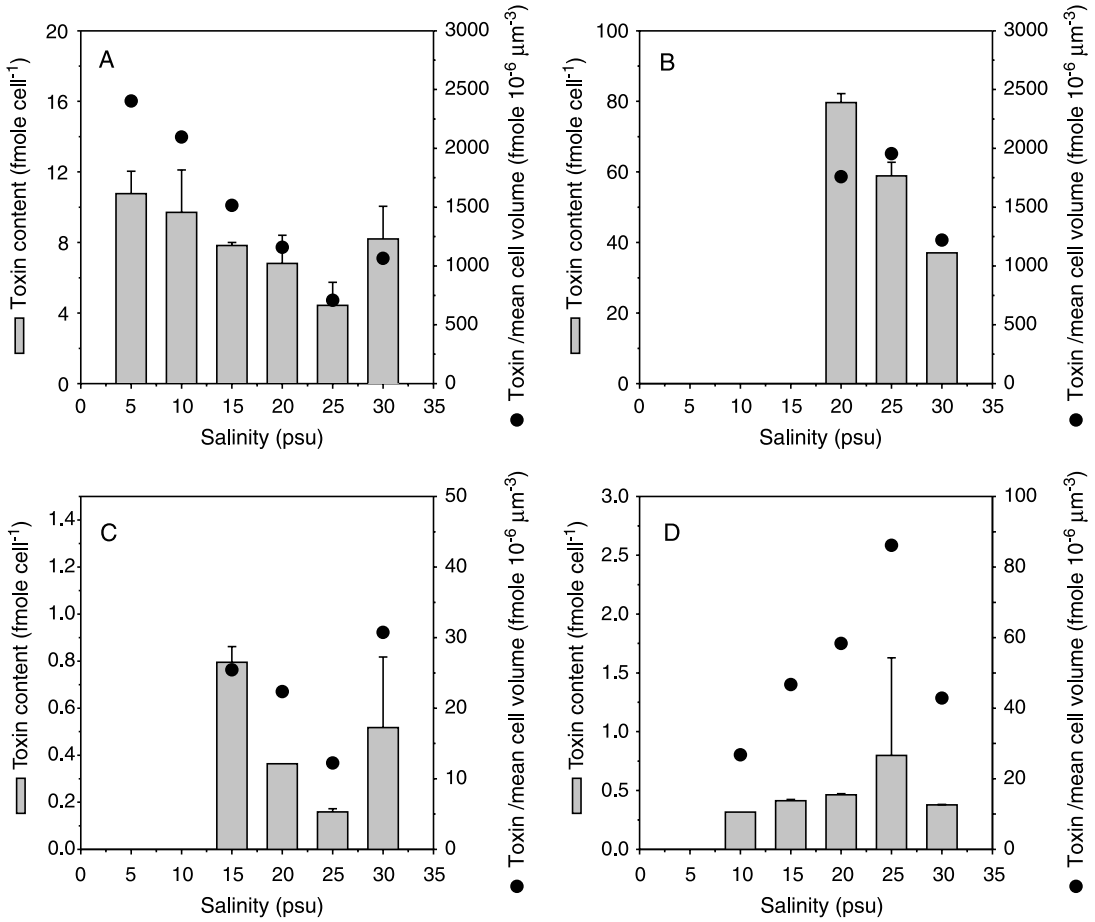

Fig. 5. Toxin content at late exponential phase of *Alexandrium* species at different salinity regimes. (A) *A. minutum*, (B) *A. tamiyavanichii*, (C) *A. tamarense*, (D) *A. peruvianum*.

(ie. 30–40 mole%). Toxin content of *A. peruvianum* was in the range of 0.30–0.80 fmole cell^{-1}. GTX6 and GTX4 make up to 70 mole% of the total toxin followed by NEO and dcSTX. Toxin content of *A. peruvianum* was almost constant throughout the salinity experiment (Fig. 5D).

In *A. minutum*, toxin compositions of both α/β epimer pairs GTX1+4 and GTX2+3 were varied with salinity at different growth stages (Fig. 6). In early exponential phase, toxin content per cell basis for both GTX1+4 and GTX2+3 was constant over the range of salinity regimes. The effect

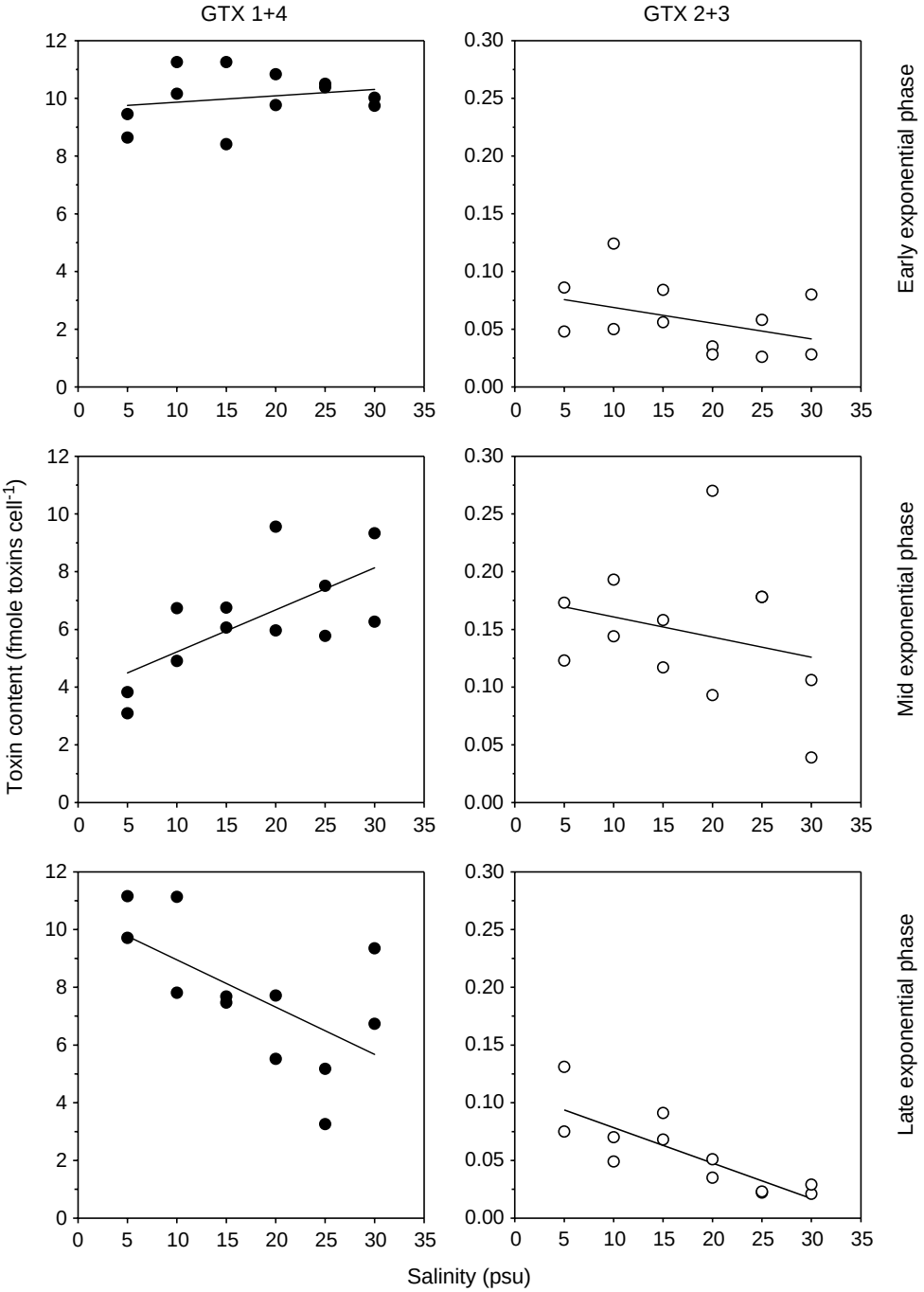

Fig. 6. Toxin content of GTX1+4 and GTX2+3 of *A. minutum* at different growth phases for all salinity treatments.

of salinity became more pronounced at mid exponential phase with GTX2+3 increased gradually while toxin content of GTX1+4 descended. The abrupt descending in GTX1+4 was observed particularly at lower salinities. In late exponential phase, toxin content of both GTX1+4 and GTX2+3 reverted to as in early exponential phase. Total toxins were produced at rates between 0.27 and 2.08 fmole cell^{-1} day^{-1} at 10–30 psu.

4. Discussion

Four tropical *Alexandrium* species in this study showed various pattern of growth responses in the salinity ranges tested. *A. minutum* exhibited exceptionally strong tolerance towards salinity changes hence it was a euryhaline species while *Alexandrium tamiyanvanchii* was more stenohaline. Salinity tolerance of the four species in decreasing order was *A. minutum*, *A. peruvianum*, *A. tamarense*, and *A. tamiyavanichii*.

A. minutum exhibited strong salinity tolerance, with a range from 5 to 30 psu. This is not surprising as the species was isolated from an estuarine lagoon with salinity fluctuation between approximately 10 and 25 psu. As is often the case, species that tolerated low salinity can also tolerate very high salinity (Taylor and Pollingher, 1987). However, the species cannot survive at salinity 2 psu and below that can be explained by osmotic acclimation of the species. A salinity of nearly 5 psu is a lethal barrier for most estuarine planktonic algae, and the species suffered severe osmotic stresses at this salinity level (Kies, 1997). The optimal growths in batch cultures were observed in salinities of about 10–20 psu. This is coincided with the salinity ranges recorded during the bloom of the species in September 2001. The average exponential growth rates (0.35–0.45 day^{-1}) were consistent throughout the salinity optimum range examined (5–30 psu). The growth rates, however, were slightly lower than those reported for the same species (Cannon, 1993; Chang and McClean, 1997; Grzyberk et al., 2003).

Almost all *A. minutum* from different geographical areas have high tolerance towards salinity changes (Zaghloul and Halim, 1992; Cannon, 1993; Chang et al., 1995; Hwang and Lu, 2000; Grzebyk et al., 2003). However, similar salinity optimum range has only been observed in strains from Taiwan (Hwang and Lu, 2000). The France isolates, however, have a higher salinity optimum range of 20–37 psu (Grzebyk et al., 2003). Hence, two physiologically distinctive groups of *A. minutum* were suggested based on the salinity tolerance, i.e. high-euryhaline with optimum salinity at 20–30 psu and low-euryhaline with optimum salinity at 15 or lower.

The Malaysian isolates of *A. minutum* were dominated by GTX4+1 which was similar to those reported from Taiwan (Hwang and Lu, 2000), Vietnam (Yoshida et al., 2000), Australia (Oshima et al., 1990), Spain (Franco et al., 1994), but differed from New Zealand strain which was dominated by NEO (Chang et al., 1997). At salinity range of 5–25 psu, the toxin content in both cell and cell volume basis decreased with increasing salinity. However, the toxin content was slightly increased at 30 psu. It is noteworthy that growth rates were almost consistent throughout the salinity range tested. Thus, the toxicity was not related to the optimum growth salinity. This is somehow different from that reported by Grzebyk et al. (2003) for the same species showing that toxin content was increased below the optimum growth salinity. However, our results showed some congruence with the previous studies indicating that toxicity was low at higher salinity (Hamasaki et al., 2001; Grzebyk et al., 2003).

Toxin composition of *A. minutum* remained constant in early exponential phase in the entire salinity regimes but in contrast, became more diverse at late exponential phase. The results showed that the toxin composition of *A. minutum* indeed changed over the growth stages. Comparison of toxin composition at three different growth stages showed that the proportion of GTX2+3 was relatively higher at mid exponential phase. This was only explainable with increase of specific enzymatic activity that phased with the growth stage. The transformation might be due to an increase of reductant activity that is responsible for the reduction of N1–OH group (GTX1+4) to N1–H group (GTX2+3). However, the percentage increase of GTX2+3 in mid exponential phase does not correspond to the reduction of GTX1+4 in this stage. With the considering of no C toxins was detected in this strain, transformation of C toxins to GTX2+3 has been ruled out. The change in proportion of transformation might as well due to high dilution effect of the toxins in active growth at mid exponential phase. Toxin transformation was not an unusual phenomenon as similar transformation has also been observed in clams, *Polymesoda similis* fed with *A. minutum*. GTX2+3 increased while GTX1+4 decreased over the depuration period (unpublished data). Chemical transformation of GTX1+4 to GTX2+3 was also observed in scallop, *Patinopecten yessoensis* (Oshima, 1995b). This phenomenon occurred mainly due to the reductants glutathione and cysteine, which were commonly found in shellfish that involved in the reduction process (Asakawa et al., 1987; Oshima, 1995b). Similar transformation was also reported in bacterial cultures (Kotaki et al., 1985). On the other hand, enzymatic oxidization that converts GTX3+2 to GTX1+4 has also been documented in *A. tamarense* (Kodama, 2000).

A. tamiyavanichii was the most widely distributed toxic *Alexandrium* in the Asia Pacific regions. The occurrence of this species has been reported in Thailand (Kodama et al., 1988), Japan (Ogata et al., 1990), Philippine (Furio and Gonzales, 2002) and Malaysia (Usup et al., 2002). Toxin profile of this strain was similar to those reported for Japanese and Thailand (Ogata et al., 1990). Both highly and less toxic strains were also reported from Thai and Japanese waters (Ogata et al., 1990). This showed that

biogeographical variation in toxicity also occurred in *A. tamiyavanichii*. The AcMS01 strain was stenohaline with optimal growth observed between 20 and 25 psu. The highest toxin contents were also observed in these salinities, corresponding to the optimum growth condition. In the survival range of salinity, the toxin content of AcMS01 strain was decreasing with lifted salinity. However, Anderson et al. (1990) reported a different pattern of toxicity changes with salinity in *Alexandrium fundyense*. They found no significant difference in toxin content throughout the salinity experiments. However, White (1978) reported an increase in toxicity with increasing salinity. The divarications may be due to differences in experimental setup and strain specific or species specific physiological responses.

Growth physiology and toxin production of *A. tamarense* have been well studied. However, most studied strains were originated from the temperate waters (White, 1978; Anderson et al., 1990; Parkhill and Cembella, 1999; Hamasaki et al., 2001). The toxicity of Malaysian strain of *A. tamarense* was previously not detected (Usup et al., 2002); however, found to be one of the PSP toxins producer in our recent effort to reexamine the toxicity of the species. Present study of tropical *A. tamarense* strain showed that the strain tolerated to similar salinity ranges as reported in other studies (e.g. Parkhill and Cembella, 1999; Hamasaki et al., 2001). However, the growth rates were relatively low at low salinities (10 and 15 psu) and ascending with the increase of salinity. This was somewhat different from the temperate Japanese strains reported by Hamasaki et al. (2001) showing almost constant growth rates within the salinity range examined (13–38 psu). Result of this study also showed that cell volumes tended to be smaller at high growth rates. Similar variation has been documented in *A. tamarense* (*Gonyaulax tamarensis*) (Prakash et al. 1973) and *Alexandrium ostenfeldii* (Jensen and Moestrup, 1997). Although White (1978) reported that no change in cell size in *A. tamarense* with growth rate.

The AtPA01 strain showed low toxin content per cell compared to other temperate *A. tamarense* strains, such as North American strains (Anderson et al., 1990; MacIntyre et al., 1997; Parkhill and Cembella, 1999) and some of the Japanese strains (Ogata et al., 1987) with toxins more than 100 fmole cell^{-1}. Although nontoxic strains of *A. tamarense* were reported from some localities, such as Australia (Negri et al., 2003) and Gulf of Thailand (Pholpunthin et al., 1990) while weakly toxic strains have been reported in Japanese water (Hamasaki et al., 2001; Leong et al., 2004). Variation of toxicity in *Alexandrium* species have been observed among *A. tamarense* from different biogeographical origins (Anderson et al., 1994). Two trends of relationship between toxicity and salinity were observed in *A. tamarense*. First, toxicity per cell was inversely proportional to salinity and growth rates. This was observed in both weakly toxic Malaysian AtPA01 strain and the Japanese strains. While in highly toxic clone of *A. tamarense*, the relationship of toxicity per cell and salinity was the other way round, in which the toxicity was directly proportional to salinity with increasing growth rates (Parkhill and Cembella, 1999).

Very limited physiological study was carried out on *A. peruvianum*. This mainly due to the limited distribution and unavailability of culture materials. The ApKS01 strain can tolerate a broad range of salinity (10–30 psu). However, low division rate (<0.1 day^{-1}) was observed at 10 psu. The species survived in medium of 5 psu but without any cell division. This is well agreed with the natural habitat of this strain which was found in a few kilometers upstream from the Samariang River where the salinity fluctuation is common. The toxin content per cell was almost constant throughout the salinity regimes with constant growth rates, indicating that salinity factor did not affect the growth and toxin production of this species.

Salinity has been well acknowledged as a pivotal physical factor in estuaries or coastal waters where blooms of toxic *Alexandrium* were commonly occurred. Salinity fluctuation in these regions was mainly due to seasonal or diurnal tidal changes. These phenomena were especially pronounced in tropical with high precipitation during wet monsoon season. Usup et al. (1989) reported the blooms of *P. bahamense* were coincided with the calm weather condition after rainy season. Rainfall was also proposed as a trigger factor on blooms of *Gymnodinium catenatum* in Australia (Hallegraeff et al., 1995). At cellular level, salinity was thought to play a significant regulatory role in toxin biosynthesis. Salinity might interfere the cell osmoregulation through the amino acid biosynthesis, cell membrane receptors and changed the homeostasis and growth of cells (Cembella, 1998).

Based on our results of salinity effects on growth and toxin production, the species were separated into three group, which are low-eurhyhaline, high-euryhaline and stenohaline species. Low-euryhaline species originated from low salinity environments have low optimum growth salinity (<15 psu). Cell toxin content was dependent of salinity but not salinity-dependent growth rate. *A. minutum* was a typical member of this group. In contrast, high-euryhaline species have optimum growth at higher salinities (20–30 psu). Toxicity was affected by salinity-dependent growth. *A. tamarense* in this study was probably belongs to this group. While high-stenohaline species did not survive at lower salinities and salinity-dependent growth inversely affected the toxicity. The species were mostly originated from higher salinity environments with less influenced by river plumes. *A. tamiyavanichii* showed typical characteristic in this group.

Several explanations have been proposed on the salinity vs. toxin production relationship. One of the well accepted explanation was salinity-dependent growth inversely affecting cell toxin quota in PSP-producing species (Hamasaki et al., 2001). In rapid growth culture condition, cell toxin quota reduced in the daughter cells. While at lower growth

rate, toxin was presumably 'accumulated' in the parental cells thus contributed to higher cell toxin quota. This explained the inverse relationship of cell toxin quota and growth rate (Hamasaki et al., 2001; this study).

Variation of toxin content was also thought to be related to sexual reproduction. Role of PSP toxin has been proposed as chemical signal in promoting mating in *Alexandrium* species (Wyatt and Jenkinson, 1997). Hamasaki et al. (2001) hypothesized that if the hypothesis of PSP toxins as pheromones is true, the higher toxin content in sub-optimum condition might be a strategy of PSP-toxin producing species to increase the chance of mating and to compensate the low cell density in an unfavorable growth condition. This can be seen as survival strategy of these estuarine species to maintain the population at unpredictable estuary environments. However, this explaination does not apply in some species that showed positive correlation between cell toxin quota and growth (White, 1978; Parkhill and Cembella, 1999). Many have pointed out the discrepancy that might be due to the difference in experimental settings. Acclimation of cells in the test salinity conditions prior to experiments has been suggested since sudden osmotic stress might possibly suppress in toxin production and induce cellular toxin leakage (Parkhill and Cembella, 1999).

A. minutum in this study which showed inverse relationship of toxicity and salinity but growth rate was independent from salinity changes. This suggested that there are other mechanisms underlaid that might control the toxin production of the species. The physiological adaptation of a particular species was very unique and the presence of ecotypes in *Alexandrium* have been well accepted. Species originated from low salinity environments (such as *A. minutum*) might possess different strategy towards salinity fluctuation. In the present study, mean cell volume of *A. minutum* appeared to be inversely correlated to the toxin content. There was a significant increase in cell volume while toxicity decreased in lifted salinities. Mean cell volumes were about 50% higher at 30 psu than at 5 psu. Increase in cell sizes will reduce the surface area/volume ratio that might enhance the osmoregulation capability of the cell. On the other hand, more nutrients might be allocated for growth to grow in size while reduced the portion of nutrients (such as arginine) available for toxin production. This might explain lower toxicity in higher salinity of *A. minutum* cells. Arginine was widely known as a precursor in the biosysnthesis of saxitoxins (Shimizu et al., 1984). Anderson et al. (1990) showed that intracellular arginine in cell of *A. fundyense* was inversely correlated with the cellular toxin level. In addition, intracellular free amino acid, FAA (including arginine) was proven to play an important role in the cell osmoregulation. Comparatively, lower cell surface area/volume ratio also reduced the efficiency of nutrient uptake through diffusion and active transport. Hence, a speculation was made for salinity effects on low- and high-euryhaline species. In low-euryhaline species, cells tended to be larger while toxin content per cell decreased at higher salinities. In these species, intracellular FAA might allocate for osmoregulation rather than toxin production. Since growth rate did not vary significantly in the range of salinity examined. This assured the toxin production in *A. minutum* was affected directly by salinity but not growth. For high-euryhaline species, cells conduced to be larger while toxin content per cell tended to be higher with sub-optimum growth condition (low growth rates) at lower salinities. Combination of cell physiological adaptation by changing cell size, utilization of nutrient (FAA) for growth and toxin production might explain the variation of cell toxin quota in different species in this studies.

Ecophysiology and toxin production of *Alexandrium* species have been investigated for decades. Unfortunately, these works were mainly on the species originated from temperate waters. The effect of environmental changes on toxin production in tropical *Alexandrium* species remains poorly understood. Our results though limited and scattered, it provided a brief starting point of the physiology of these tropical *Alexandrium* species. Effects of salinity on the organisms were not only regional-dependent but also species-dependent. A better view of salinity–toxicity relationship can only be foreseen with further examination of natural population of these species. Study on other environmental factors is essential to give a holistic interpretation on growth and toxin production of these tropical species.

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