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## Comparative Analysis of Paralytic Shellfish Toxin Content and Profile Produced by Dinoflagellate *Gymnodinium Catenatum* Isolated from Inokushi Bay, Japan

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Paralytic shellfish toxin contents and profiles were investigated using shellfish toxin-producing dinoflagellate *Gymnodinium catenatum* strains isolated from Inokushi Bay during the March and July 2002 outbreak. The March strains, which were 72 strains isolated from bloom water, exhibited average growth rates of 0.25 divisions day<sup>-1</sup> due to sub-optimal temperature. The average toxin content was 321 f mole cell<sup>-1</sup>. The principal toxins were C1+2 (79.8 mole%) and GTX 5+6 (18.0 mole%), and minor components were characterized as GTX 2+3, dcGTX2+3, and neoSTX+STX. Interestingly, 7 strains were clearly distinguished from those of other strains by the complete presence of GTX1+4. In the July strains, which were 33 strains isolated from bloom water, the average growth rate and toxin content was 0.34 divisions day<sup>-1</sup> and 160 f mole cell<sup>-1</sup>, respectively. C1+2 (83.3 mole%) and GTX 5+6 (10.6 mole%) were the principal toxins, as in the March strains. However, carbamoyl toxins in the July strains were increasing with decreasing N-sulfocarbamoyl toxins in the March strains. We also observed major changes in toxin profile, evident by the increase of C1+2 and the decrease of GTX 5+6 in response to increasing temperature, using a single March strain. Minor components of July strains were characterized by GTX2+3, neoSTX, STX and dcGTX2+3. Thus, if we were to consider the toxin profile percentage based on temperature variation, *G. catenatum* outbreaks in March and July may have originated from one and the same population.

**Keywords:** *Gymnodinium catenatum*; paralytic shellfish toxin; toxin content; toxin profile; Inokushi Bay

### INTRODUCTION

*Gymnodinium catenatum* Graham is a toxic dinoflagellate that causes paralytic shellfish poisoning (PSP) in coastal waters (Hallegraeff *et al.*, 1989). The first *G. catenatum* bloom associated with PSP was reported from Spain in 1977 (Estrada *et al.*, 1984), followed by cases of human fatalities from the Gulf of California in 1979 (Morey–Gaines, 1982). Reports that this species had spread across many parts of the world began to appear (Hallegraeff *et al.*, 1989). In Japan, *G. catenatum* was first suspected to be involved with PSP outbreaks in Senzaki Bay of western Japan, 1986. Since then, contaminations have occurred almost every year in western Japan (Ikeda *et al.*, 1989; Nishioka *et al.*, 1993; Baba *et al.*, 2001). The introduction (e.g. through ballast water) or migration of *G. catenatum* to central and northern Japan is possible where the maximum surface water temperature of 20 °C is favorable for this species

(Matsuoka and Fukuyo, 1994).

It has been proven that the capability to produce certain toxin profile is inherited (Anderson, 1990; Oshima *et al.*, 1993a, b). Given that differences in toxin profiles have a genetic basis, specific toxin profile might be used as biochemical markers for a number of PSP-producing dinoflagellate species. However, toxin profiles can vary due to differing environmental conditions (Ogata *et al.*, 1987; Maranda *et al.*, 1985; Cembella *et al.*, 1987; Boyer *et al.*, 1987; Boczar *et al.*, 1988; Anderson, 1990; Ishida *et al.*, 1993; Taroncher–Oldenburg *et al.*, 1997). Given this fact, a number of studies investigated species population dynamics based on toxin profiles. For example, Chang *et al.* (1997) reported on the toxin profile of *Alexandrium minutum* isolated from coastal areas of New Zealand, showing that it is quite different from that of species isolated from other parts of the world. It is quite possible that *A. minutum* may have been introduced to New Zealand via ballast water given its isolated location and high maritime traffic from all around the world. In Australia, *G. catenatum* is regarded as an exotic species since only being noticed in coastal waters from the 1980's, and supported by the fact that its cysts were not observed in sediment prior to 1980s. Hallegraeff and Bolch (1992) suggested that cysts of *G. catenatum* were brought to Tasmania by ballast water in ships from Japan and Korea. However, toxic profiles of the Tasmanian strains are clearly distinguishable from those isolated from Japan (Oshima *et al.*, 1993a). This might indicate whether they are autochthonal species or intro-

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duced from countries other than Japan and Korea. Recently, Park *et al.* (2004) observed strains produced C3 and C4 toxins around Korean coastal waters, similar to the Tasmanian strains.

A major causative organism of PSP in Inokushi Bay, which has the highest production of cultured short-necked clam in Japan, was reported to be *G. catenatum* (Miyamura *et al.*, 2007). This species has been observed in the bay throughout the year, which is of interest as water temperatures between winter and summer are significantly different. Moreover, *G. catenatum* attributed PSP events in summer did not occur in conjunction with *G. catenatum* outbreak in the bay, in spite of increasing cell density. This may have been due to specific physiological conditions related to environmental parameters between summer and winter. The aim of this study was to compare the population dynamics of *G. catenatum* between the May and July 2002 blooms, based on toxin profiles in the bay.

## MATERIALS AND METHODS

### Culture isolates of *Gymnodinium catenatum*

*G. catenatum* was isolated from Inokushi Bay, Oita Prefecture, Japan (Fig. 1) in March and July 2002 on a light microscope using a micropipette (ca.  $\phi$  50–100  $\mu$ m), coinciding with high cell numbers in the water column (Fig. 2). Temperature and salinity were observed using a conductivity–temperature–depth system (Model 58, YSI/Nanotech Inc., Tokyo, Japan). The isolated cells were rinsed at least four times by filtered seawater (0.22  $\mu$ m pore size; GSWP, Millipore, MA, USA), and then placed in culture tubes with 5 ml modified f/2 medium (Guillard and Ryther, 1962) sterilized at 121  $^{\circ}$ C for 1 hr. From sea-

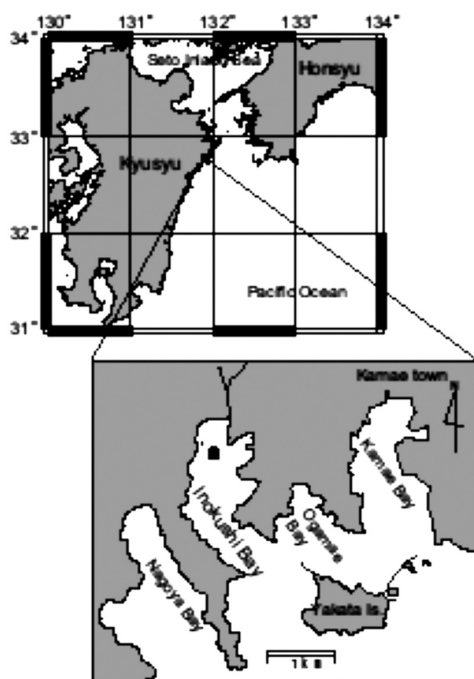


Fig. 1. Study area in Inokushi Bay, Japan (●: sampling station).

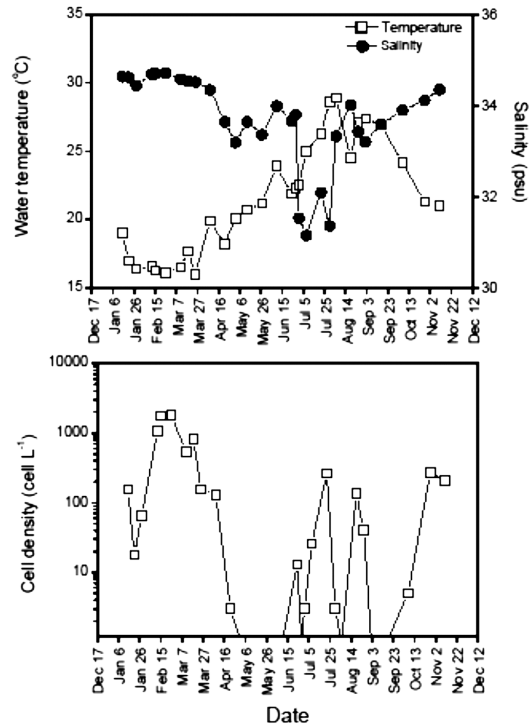


Fig. 2. Temporal changes of temperature, salinity and cell density of *Gymnodinium catenatum* in Inokushi Bay, 2002.

water sampled in March and July, 72 and 33 clonal strains of *G. catenatum* were isolated, respectively (hereafter referred to as the March and July strains). Isolated cells were cultured at 30 psu, with an illumination of 100  $\mu$ mol  $m^{-2} s^{-1}$  using cool-white fluorescent lamps (12 h light: 12 h dark cycle). Incubation temperature was set at the *in situ* temperature (18  $^{\circ}$ C for March strains and 25  $^{\circ}$ C for July strains). Culturable cells (i.e. viable strains after isolation) were 55 of the 73 March strains (76.4%) and 28 of the 33 July strains (84.8%). To remove substances such as ammonia and metals, all equipment and glassware were washed with approximately 30% v/v HCl and thoroughly rinsed with distilled water before being autoclaved (202 kPa, 20 min).

Growth rate (division  $day^{-1}$ ) estimates of each strain were calculated using a least square regression on log-transformed cell density data during exponential growth phase:

$$\mu = \frac{1}{\Delta t} \ln \frac{N_t}{N_0},$$

where

$N_0$ ,  $N_t$ : initial and final *in vivo* fluorescence of the exponential period (cells  $ml^{-1}$ ),

$\Delta t$ : period of exponential growth phase (day).

### Cell harvest and toxin analysis

Cultured cells were harvested by a plankton net (10  $\mu$ m pore size) in late exponential phase at 6:00 p.m. Pellets were extracted with 0.03 N acetic acid by sonication, and cell debris removed by centrifugation (10,000  $\times$  10 min). Analysis of PSP toxins using high-performance

liquid chromatography with fluorescence detection (Model-700, Hitachi, Tokyo, Japan) was performed following the post-column oxidative fluorescence method of Oshima (1995). Toxins were resolved by reverse-phase chromatography using a silica-base column (Inertsil C8; 0.46×15 cm, GL Science, Tokyo, Japan). Three separate isocratic elutions were employed to separate the spectrum of PSP-toxins at a flow of 0.8 ml min<sup>-1</sup>. The gonyautoxins (GTX1–4) and the N-sulfocarbamoyl toxin were resolved using 10 mM aqueous ammonium phosphate at pH 7.1, with 2 mM heptane sulfonate (Na salt) serving as the ion-pairing reagent. To separate neosaxitoxin (neoSTX) and saxitoxin (STX), 2 mM heptane sulfonate (Na salt) was substituted as the ion-pairing reagent in 30 mM aqueous ammonium phosphate buffer (pH 7.1) with 4% acetonitrile. The N-sulfocarbamoyl (C-) toxins were resolved using 2 mM tetrabutyl ammonium phosphate adjusted to pH 6 as the mobile phase. The eluate from the column was continuously mixed with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 9.0) at 0.4 ml min<sup>-1</sup>, heated at 65 °C by passing through Teflon tubing and then mixed with 0.5 N acetic acid at 0.4 ml min<sup>-1</sup> just before entering the monitor. The fluoromonitor was set at an excitation wavelength of 330 nm and emission of 390 nm.

Gonyautoxins 5 and 6 (GTX 5+6) were determined in accordance with the increased yield of neosaxitoxin (neoSTX) and saxitoxin (STX) formed after heating (1 hr, 100 °C) with addition of 0.1 N HCl (Nagashima *et al.*, 1987), as the standard toxins for GTX 5+6 were not available. Meanwhile, STX, neoSTX, gonyautoxins 1–4 (GTX1–4), decarbamoylgonyautoxins 2+3 (dcGTX2+3) and C1–4 were estimated from standard toxins obtained

from Japan Fisheries Resource Conservation Association, and/or from Prof. Yasukatsu Oshima of Tohoku University, Japan. The results were expressed in relative amounts of each toxin on a molar basis (mol%) and toxic content (f mole cell<sup>-1</sup>).

### Toxin contents and profiles of *Gymnodinium catenatum* in response to changing temperature

To examine the toxin contents and profiles of *G. catenatum* in response to changing temperatures, we used a single strain (GCINO2002M14) isolated from Inokushi Bay in March 2002. The effect of temperature was determined at 18 °C and 25 °C, which corresponded to the in situ temperature of each sampling period. Salinity and irradiance was constant during incubation period (30 psu and 100 μmol m<sup>-2</sup> s<sup>-1</sup>). To avoid culture shock due to change in incubated temperature, temperatures of cultures were raised about 1 °C a day, and were finally maintained at 25 °C. The acclimated cells were inoculated into five replicate flasks with fresh modified f/2 medium at 18 °C and 25 °C. Toxin content was estimated using cells in late exponential phase as mentioned above.

## RESULTS

### Relationship between hydrographic condition and cell density of *Gymnodinium catenatum*

Temperature and salinity during the sampling period ranged from 16.0–28.9 °C and 31.15–34.72 psu, respectively (Fig. 2). *G. catenatum* exhibited increasing cell density from January to March (16–21 °C and 34.0–35.0 psu), and from July to August (25–27 °C and 31–32 psu). Maximum cell density was obtained at

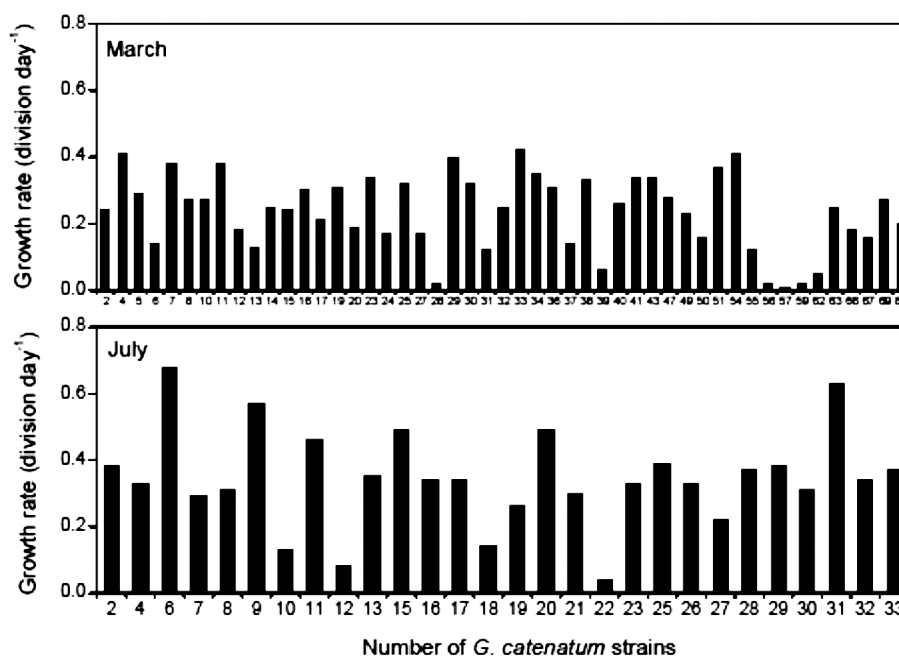


Fig. 3. Growth rates of *Gymnodinium catenatum* isolated from Inokushi Bay in March and July, 2002. Experimental conditions; 18 °C for strains isolated from the March strain and 22 °C for the July strains. Salinity 30, pH 8.0, 300 μmol m<sup>-2</sup> s<sup>-1</sup> (cool-white fluorescent lamps, 12:12 h LD cycle).

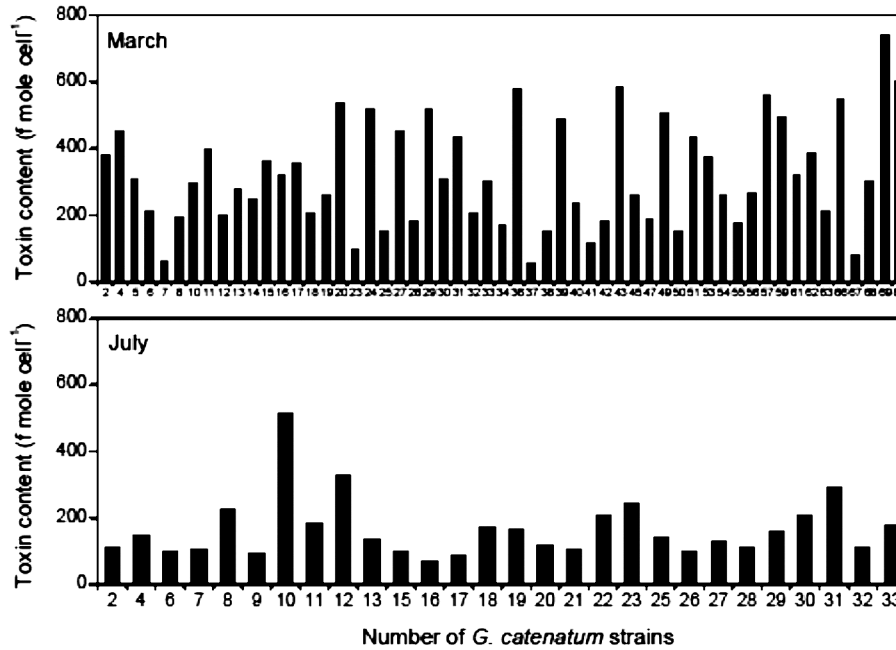


Fig. 4. Toxin contents of *Gymnodinium catenatum* isolated from Inokushi Bay in March and July, 2002. Cultured cells were harvested by plankton net (20  $\mu\text{m}$  pore size) in late exponential phase (See Fig. 3 for incubation conditions).

16.1  $^{\circ}\text{C}$  and 34.72 psu (1,808 cells  $\text{L}^{-1}$ ). However, cell densities from July to August remained at 265 cells  $\text{L}^{-1}$ , which was lower than that of February (temperature 26.3  $^{\circ}\text{C}$  and salinity 32.09 psu).

#### Toxin contents and profiles of *Gymnodinium catenatum* strains isolated from Inokushi Bay

Growth rates of individual strains are shown in Fig. 3. The average growth rates were significantly different at 0.25 division  $\text{day}^{-1}$  for the March strains and 0.34 division  $\text{day}^{-1}$  for the July strains (one-way ANOVA;  $P < 0.001$ ). The average toxin content was  $321 \pm 160$  f mole  $\text{cell}^{-1}$  in the March strains and  $160 \pm 92.8$  f mole  $\text{cell}^{-1}$  in July strains. Non-toxin strains were not observed. Toxin

content of the strains varied greatly between the minimum (56.6 f mole  $\text{cell}^{-1}$ ) and maximum values (740 f mole  $\text{cell}^{-1}$ ; Fig. 4). Moreover, a one-way ANOVA indicated that variability of toxin contents were significant among individual strains ( $p < 0.001$ ). When toxin contents were plotted against individual growth rates, the relationship showed an inverse linear trend, despite the low correlation coefficient ( $r = -0.38$ ;  $P < 0.001$ ; Fig. 5).

In the toxin profile of the March strains, C1+2 toxin was the dominant toxin (ca.  $79.8 \pm 10.0$  mole% among whole PSP toxin; Fig. 6). However, C3+4 toxin was completely absent in all strains. GTX5+6 toxin was occupied by proportions of  $18.0 \pm 8.42$  mole%. Minor components of the March strains were characterized by GTX2+3 ( $0.21 \pm 0.37$  mole%), dcGTX2+3 ( $0.32 \pm 0.25$  mole%) and neoSTX+STX ( $1.65 \pm 3.11$  mole%) (Fig. 6). In the July strains, proportions of C1+2 and GTX 5+6 toxins were  $83.3 \pm 6.63$  mole% and  $10.6 \pm 5.78$  mole%, respectively. In contrast, minor components were characterized by GTX2+3 ( $2.26 \pm 2.78$  mole%), dcGTX2+3 ( $0.72 \pm 0.79$  mole%) and neoSTX+STX ( $3.14 \pm 3.37$  mole%; Fig. 7).

#### Toxin content and profiles of *Gymnodinium catenatum* under different water temperatures

Table 1 shows the toxin content of *G. catenatum* strain (GCINO2002M14) incubated at 18  $^{\circ}\text{C}$  and 22  $^{\circ}\text{C}$  with in situ temperature. The toxin content of strain incubated at 18  $^{\circ}\text{C}$  and 25  $^{\circ}\text{C}$  were  $136 \pm 21.6$  f mole  $\text{cell}^{-1}$  and  $58.0 \pm 20.3$  f mole  $\text{cell}^{-1}$ , respectively. The toxin contents in 18  $^{\circ}\text{C}$  were ca. 2 times higher than those of 25  $^{\circ}\text{C}$ . This corresponded well with results which showed that average toxin contents of the March strains were 2 times higher. The toxin profile also varied with that of C1+2

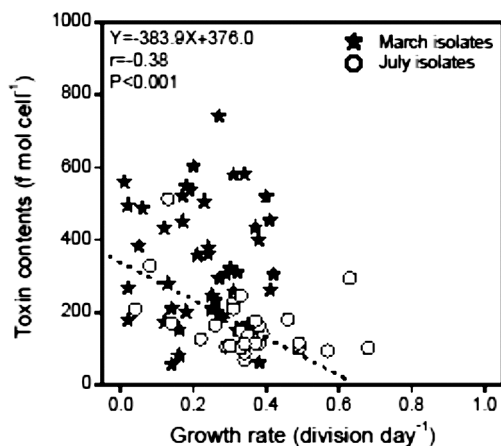


Fig. 5. Correlation between growth rate and toxin contents of *Gymnodinium catenatum* isolated from Inokushi Bay in March and July, 2002.



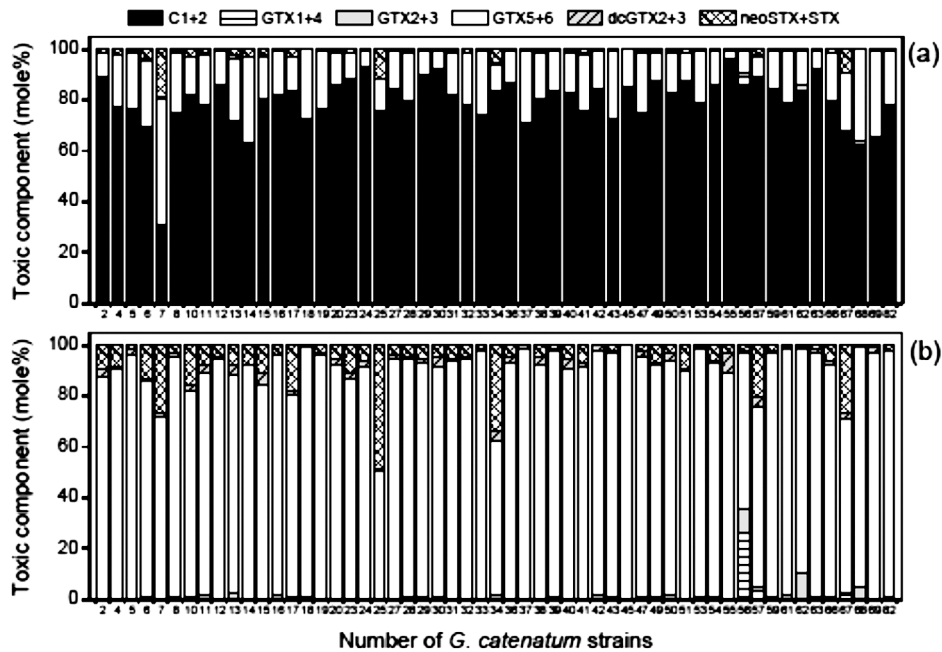


Fig. 6. (a) Toxin profiles in *Gymnodinium catenatum* isolated from Inokushi Bay in March, 2002. (b) Toxin profiles of *G. catenatum* strains, with the exception of C1+2 (See Fig. 3 for incubation conditions).

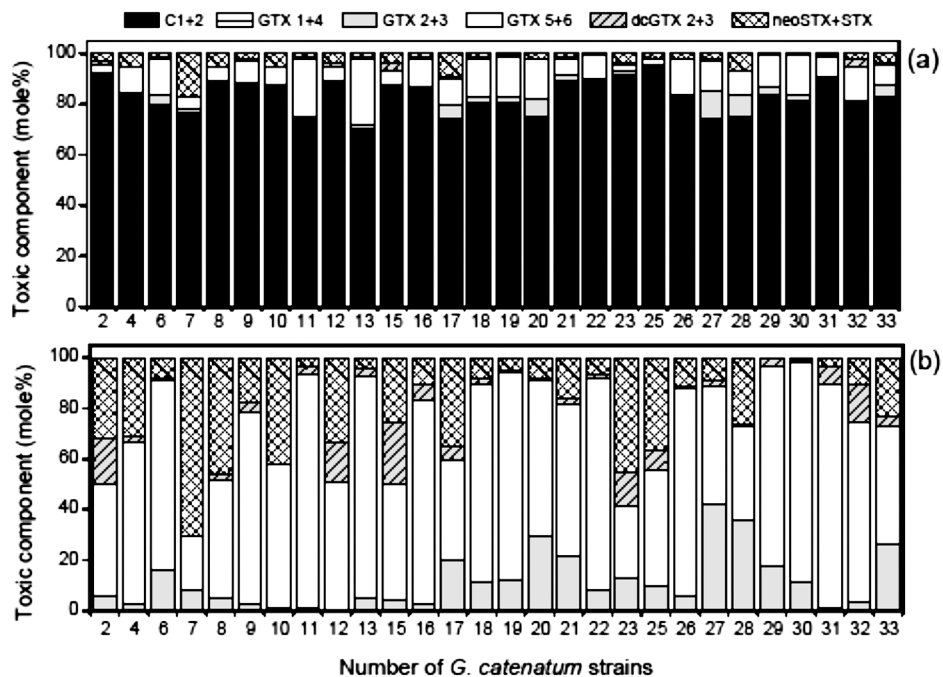
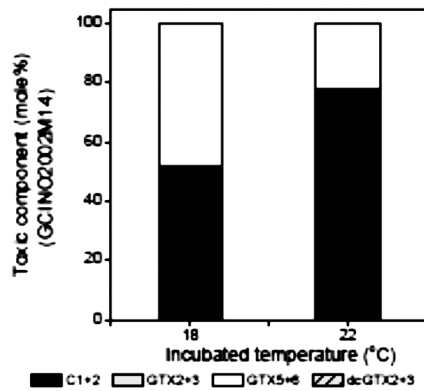


Fig. 7. Toxin profiles in *Gymnodinium catenatum* isolated from Inokushi Bay in July, 2002. (b) Toxin profiles of *G. catenatum* strains, with the exception of C1+2 (See Fig. 3 for incubation conditions).

increasing from  $50.6 \pm 1.30$  mole% to  $76.9 \pm 1.68$  mole%, while GTX 5+6 decreased from  $48.5 \pm 1.31$  mole% to  $22.0 \pm 1.71$  mole% with increasing temperature, although GTX2+3 did not vary between the two temperature ranges (neoSTX+STX was not detected; Fig. 8).

## DISCUSSION

*G. catenatum* blooms occur from January to March and from July to August in Inokushi Bay. Water temperature during these outbreaks ranged from 16 °C to 21 °C



**Fig. 8.** Changes in toxin profile of *Gymnodinium catenatum* with increasing temperature from 18 °C to 25 °C.

and from 25 °C to 27 °C, respectively. Bravo and Anderson (1994) reported that the optimum temperature of the Spanish strain was between 22–28 °C. In tropical regions, such as Palau, Venezuela and Philippines, optimum growth temperature is between 23–29 °C (Hallegraeff and Fraga, 1998). In laboratory experiments using strains isolated from Hiroshima Bay of Japan and Yosuea Bay of Korea, optimum temperatures ranged from 20–30 °C (Yamamoto *et al.*, 2002; Oh and Yoon, 2004). These temperatures are consistent with temperatures observed in this study from July to August. However, the Tasmanian strain was able to better grow in lower temperatures (Hallegraeff *et al.*, 1989). Blooms in Tasmanian waters occur from December to June when water temperature ranges from 12 °C to 18 °C, which were replicated in laboratory experiments by Blackburn *et al.* (1989). These temperatures are consistent with temperature from January to March of this study.

*G. catenatum* outbreaks in Inokushi Bay during different temperature ranges indicate the presence of various populations with different physiological characteristics. Sakamoto and Kotani (1998) reported that *Alexandrium tamarense* isolated from Hiroshima Bay have plural populations due to wide variation of toxin profiles among strains isolated from the same seawater. Ichimi *et al.*, (2002) also suggested the presence of various planktonic populations around the same area. Based on these informations, we cannot rule out the possibility that the same population is in fact present during both sampling periods, compared to the notion that two different populations were present.

Average of growth rate was 0.25 division day<sup>-1</sup> in the March strains and 0.34 division day<sup>-1</sup> in the July strains (Fig. 3). Different studies have reported maximum growth rates of *G. catenatum* at 0.4 divisions day<sup>-1</sup> from the Denmark strain (Ellegaard *et al.*, 1993), 0.53 divisions day<sup>-1</sup> from the Spanish strain (Bravo and Anderson 1994) and 0.44 divisions day<sup>-1</sup> (0.31 day<sup>-1</sup> in the original paper) from the Japan and Korea strains (Yamamoto *et al.*, 2002; Oh *et al.*, 2004) These maximum growth rates were similar to the growth rate of the July strains in this study, which was incubated near its optimum temperature. On the other hand, growth rates of the March

strains were similar to that of laboratory experiments conducted at the same temperature (18 °C) and salinity (30 psu) (i.e. ca. 0.22 divisions day<sup>-1</sup>; Yamamoto *et al.*, 2002). However, we can not explain the contrasting growth rates despite being isolated from the same season and area. Further studies are required to investigate if the difference is related to hereditary characteristics or other physiologically related factors.

Average toxin content of the March strains was 2 times higher than that of the July strains. This discrepancy was similar to that of the laboratory experiment. Causative factors may be related to the production of intracellular amino acid, which is a precursor in toxin biosynthesis. In amino acid synthesis nitrate should be reduced to ammonium by the sequential action of nitrate reductase (Lomas and Glibert, 1999). Enzyme activity increases with decreased temperature (Kristiansen, 1983; Gao *et al.*, 1993; 2000). Thus, increasing of toxin content might have been caused by increasing of intercellular amino acid at relatively low temperature. Anderson *et al.* (1990) also proposed the hypothesis that the effect of low temperature was to reduce protein synthesis, and surplus of arginine within the cell could be used for toxin synthesis. The other reason for increase of toxin content with decreasing temperature may be related to growth rate. High growth rate with optimum temperature conditions and no nutrient limitation may decrease toxin content (Fig. 5). This may be because of transfer of toxic content to daughter cells with faster division (Anderson *et al.* 1990). These results may be a possible explanation for the *G. catenatum* related PSP event in Inokushi Bay inn summer.

Our study confirmed that the major toxins in the March and July strains were C1+2 and GTX5+6. In previous studies, major *G. catenatum* toxins isolated from Australia, Japan, Philippine, Portugal and Spain were shown to be C1–4 and GTX 5+6 (Franca *et al.*, 1993; Fukuyo *et al.*, 1993; Ikeda *et al.*, 1989; Ohsima *et al.*, 1993a, b; Takatani *et al.*, 1998). However, Japan strains have been reported to be devoid of C3+4 (Ohsima *et al.*, 1993a, b). Singapore strains were also clearly distinguished from those of other countries by the complete absence of C3+4 (Holmes *et al.*, 2002).

One-way ANOVA indicated that the C1+2 and GTX5+6 proportions of the March strains were significantly different from those of the July strains ( $P < 0.001$ ), attributed to carbamoyl toxins in the July strains increasing with decreasing N-sulfocarbamoyl toxins in the March strains. Oshima *et al.* (1993a) and Flynn *et al.* (1996) reported that toxin profile in *G. catenatum* is not significantly affected by temperature, light or nutrient status. If toxin profile is stable as reported by Oshima *et al.* (1993a) and Flynn *et al.* (1996), the March population might be different to the July population.

However, we observed major changes in toxin profile with increased temperature (Fig. 9). Some authors have reported changes in the composition of PSP affected by physical and chemical environmental parameters (Anderson, 1990; Sakamoto and Kotani, 1998; Hwang and Lu, 2000; Ichimi *et al.*, 2002). Thus, by considering

the percentage of toxin profile based on temperature variation, the March and July strains could probably be the same population. However, some strains are significantly different from those of other strains. Our study observed GTX1+4 from 7 strains (GCINO2002M17, 27, 29, 56, 57 and 67) among the March strains. This toxin has not been previously reported, except from Singapore strains (Holmes *et al.*, 2002). Furthermore, in Neo-STX+STX, 3 strains (GCINO2002M17, 25 and 56) from the March strains reached more than 10 mol%. These results suggested that some strains could be distinguished from populations of *G. catenatum* species in Inokushi Bay, raising the probability that these strains were introduced from other regions (Ichimi *et al.*, 2002). Based on toxicological analysis, we showed that *G. catenatum* blooms in March and July may have originated from one and the same population. Future research initiatives need to investigate the population dynamics of strains based on molecular biological methods, such as gene analysis, in order to validate the results of this study.

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