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Variability in toxicity of the dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, western Japan, as a reflection of changing environmental conditions

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The variability of cellular toxin content in the dinoflagellate Alexandrium tamarense isolated from Hiroshima Bay was analyzed under a variety of culture conditions. Growth and toxicity were represented as a function of light (80, 90, 110, 160 and 350 μmol m^{-2} s^{-1}), temperature (12, 17 and 22°C), salinity (13, 16.5, 19.5, 25, 29, 33, 36.5 and 38 PSU) and ammonium concentration (0.11, 0.22 and 0.44 mM). Toxicity was measured by the tissue culture bioassay using mouse neuroblastoma cells, and expressed as saxitoxin concentration equivalents. Cellular toxicity increased with decreasing salinity. At temperatures of 17 and 22°C, maximum toxin content was observed at the lowest light intensity and growth rate. At the lowest temperature of 12°C, maximum toxin content was observed at intermediate light intensities and growth rates. A drastic increase in toxin content with an increase in ammonium concentration from 0.11 to 0.22 mM supported the idea that ammonium utilization for toxin production directly brings about a high toxin content in A. tamarense. Our results ecologically imply that the cells become highly toxic in environments with low salinity and high ammonium concentration, and successive cloudy days. Such environmental conditions may lead to increasing risk of shellfish toxification.

INTRODUCTION

Dinoflagellates in the genus *Alexandrium* produce paralytic shellfish poisoning (PSP) toxins. During blooms of these toxic dinoflagellates, bivalves accumulate the toxins by ingesting the dinoflagellates. In Japan, 11 species of *Alexandrium* inhabit coastal waters and *A. catenella* and *A. tamarense* are major species causing PSP. Hiroshima Bay is the most productive area for oyster culture in Japan. More than 10 000 rafts produce 50–70% of the total amount of oysters consumed in the country. The first infestation of shellfish with PSP toxins in the bay was reported in 1992 (Asakawa *et al.*, 1993). The outbreak of the toxic dinoflagellate *A. tamarense* and PSP toxification of bivalves in the bay is currently a serious problem from an economic and a food hygiene point of view.

The toxicity and growth characteristics of the dinoflagellate can be important information to predict or elucidate its dynamics and toxification events in natural environments. The toxin content of *Alexandrium* species varies with growth stage (Prakash, 1967; Proctor *et al.*, 1975; Oshima and Yasumoto, 1979; Anderson *et al.*, 1990; Flynn *et al.*, 1994) and growth conditions such as nutrient limitation (Boyer *et al.*, 1987; John and Flynn, 2000), light intensity, temperature (Ogata *et al.*, 1987) and salinity (White, 1978). Proctor *et al.* hypothesized that toxin content is inversely proportional to growth rate (Proctor *et al.*, 1975). Although Ogata *et al.* (Ogata *et al.*, 1987) showed that toxin content increases as growth rate decreases due to temperature or light limitation, contradictory results have been obtained so far. Parkhill and Cembella reported a positive correlation between cellular toxicity and salinity-dependent

growth rate (Parkhill and Cembella, 1999). Some environmental conditions affect toxin content or production directly, and others influence it indirectly via an effect on growth. Recent evidence clearly indicates that toxin production is closely coupled to cell cycle events. The toxin content in *A. tamarense* was found by Kim *et al.* to gradually increase from the latter half of the light period to the middle of the dark period, and then decreased rapidly in association with cell division (Kim *et al.*, 1993). Taroncher-Oldenburg *et al.* proved that toxin production of *A. fundyense* occurred within the G_1 phase of the cell cycle (Taroncher-Oldenburg *et al.*, 1997). They suggested that a high toxin content in slow growing cells could be explained by increasing duration of the G_1 phase.

In this study, *A. tamarense* strains isolated from Hiroshima Bay were grown under a variety of conditions in batch culture, and changes in toxicity measured to evaluate the response to environmental factors such as light, temperature, salinity and ammonium concentration. The purpose of this study is to understand which environmental factors enhance dinoflagellate toxicity and predict conditions when high risks of toxification in shellfish in the bay might occur.

METHOD

Culture conditions

Culture experiments were conducted with unialgal cultures of *A. tamarense* isolated from Hiroshima Bay in 1992–1995, when blooming was occurring, which were designated ATHS-92, ATHS-93, ATHS-94 and ATHS-95. The algae were incubated in f/2 medium without silicate on a 12 h light (L):12 h dark (D) cycle under cool white fluorescent lamps. All cultures, except for the ammonium experiments, were maintained in test tubes (13 × 100 mm Pyrex) with 4 ml of medium. For the ammonium experiments, larger test tubes (25 × 200 mm) with 50 ml of medium were used. In the light and temperature experiments, five levels of irradiance were adopted at three levels of temperature with a constant salinity of 36.5 PSU. Temperature was adjusted to 12, 17 and 22°C. Irradiance was adjusted to 80, 90, 110, 160 and 350 μmol m^{-2} s^{-1} with the use of neutral density filters. The effect of salinity was examined at constant light and temperature (350 μmol m^{-2} s^{-1} and 17°C). Salinity was adjusted to 13, 16.5, 19.5, 25, 29, 33, 36.5 and 38 PSU by diluting the sea water evaporated to 38 PSU with distilled water. In the ammonium experiment, NH_4Cl at concentrations of 0.11, 0.22 and 0.44 mM was substituted for 0.88 mM $NaNO_3$, and the culture conditions were set at 350 μmol m^{-2} s^{-1}, 17°C and 36.5 PSU. Prior to the experiments, the cells in maintenance stock cultures were inoculated into each experimental medium and cultured at each experimental condition for 10–20 days to acclimate them. When each culture reached exponential phase, the acclimated cells were inoculated into five replicate tubes with fresh medium at each condition to start measurement. For the ammonium experiment, cells were inoculated into duplicate tubes after acclimation to nitrogen-depleted medium.

Growth was monitored by *in vivo* fluorescence readings using a Turner Designs fluorometer (Model 10-AU) (Brand *et al.*, 1981). Cell number was estimated using the equation between cell number and *in vivo* fluorescence, which was preliminarily obtained for each growth condition. When the cells reached early stationary phase, they were collected by centrifugation at 1500 *g* for 10 min and stored at –80°C until measurement of toxicity. In the experiments for salinity, light and temperature effect, replicate cultures were collected in one bottle for the toxicity measurement. In the ammonium experiment, toxicity was measured independently for each of duplicate cultures. The specific growth rate (μ in day^{-1}) was calculated over the entire exponential phase of growth. The growth rates in the experiments for salinity, light and temperature effect were averaged for the five replicates.

Toxicity measurements

Cells were suspended in 3% acetic acid solution and subsequently ultrasonicated. After centrifugation at 15 000 *g* for 10 min, the supernatant was passed through a SEP-PAK C_{18} cartridge column (Waters Corporation, MA) for partial purification. Freeze–dried eluate was resuspended in a small amount of distilled water. The extracts were analyzed with the tissue culture bioassay (TCBA) using the mouse neuroblastoma cell line Neuro 2A (Kogure *et al.*, 1988). Cell survival was assessed using a tetrazolium salt WST-1 (Dojindo Chemical Inc., Kumamoto, Japan), which produces a water-soluble formazan dye (Ishiyama *et al.*, 1993; Hamasaki *et al.*, 1996). Saxitoxin (STX) was used as the standard for quantification, and thus the toxin concentration was expressed as equivalent to STX.

Compositional change of PSP toxins during growth was analyzed with the post-column derivatization HPLC method described by Nagashima *et al.* (Nagashima *et al.*, 1987). Strain ATHS-95 was grown in 6 l of f/2 medium at 17°C, 35 PSU and 350 μmol m^{-2} s^{-1} in a 12L:12D cycle. Cell number per 1 ml was monitored by direct counting during the incubation. Subsamples of 1000 ml for toxicity measurement were taken three times: at the exponential, early stationary and stationary phase of growth. The incubation time of each subsample was 14, 20 and 44 days, respectively. The toxin composition of gonyautoxins (GTX) 1–4, STX, neoSTX and C1–4 toxins was

analyzed. The mole amount of each compound (fmol cell^{-1}) was multiplied by the specific toxicity and summed to obtain the total toxicity (MU cell^{-1}). The specific toxicities (MU μmol^{-1}) used for the calculation were 2468 for GTX1, 892 for GTX2, 1584 for GTX3, 1803 for GTX4, 15 for C1 toxin, 239 for C2 toxin, 33 for C3 toxin, 143 for C4 toxin, 2483 for STX and 2295 for neoSTX (Oshima, 1995).

RESULTS

Toxin composition

Alexandrium tamarense ATHS-95 produced toxin derivatives of GTX1, GTX3, GTX4, C1, C2, C3, C4, and a trace amount of GTX2 (Table I). Other derivatives of STX and neoSTX were not detected in this culture. GTX1, GTX4 and C2 toxin were major components at the exponential and stationary phase. When the culture entered the early stationary phase, concentrations of GTX1 + GTX4 and C2 toxin remained almost constant. A derivative of C4 toxin, however, increased greatly and became the major component in this phase. The increase in the concentration of C toxins scarcely contributes to the total toxicity of the cell because of their low specific toxicities. Total toxin concentration in terms of mole per cell was highest in the early stationary phase. In contrast, when it was converted to toxicity (MU cell^{-1}), the value in the stationary phase was highest. Toxicity values measured by both HPLC and TCBA were almost comparable for the three samples analyzed.

Effect of environmental factors on toxicity

The two strains ATHS-92 and ATHS-93 were examined for the salinity experiment (Figure 1). Cells grew at all salinity levels examined. A one-way ANOVA indicated that variability of growth rates was significant ($P < 0.001$) among various salinity levels in both cultures. The growth rate of ATHS-92 peaked at 25 PSU ($\mu = 0.55$ day^{-1}) and then decreased with salinity levels higher and lower than 25 PSU, except 38 PSU. In ATHS-93, the growth rate peaked at 29 PSU ($\mu = 0.42$ day^{-1}) and decreased with salinity. Toxicity (toxin cell^{-1}) increased with decreasing salinity in both strains. The toxin concentration of ATHS-92 ranged over 10-fold: the minimum was 0.072 pg cell^{-1} at 25 PSU and the maximum was 0.92 pg cell^{-1} at 33 PSU. In the ATHS-93 cultures, the maximum toxicity (1.4 pg cell^{-1} at 13 PSU) was >20-fold the minimum toxicity (0.059 pg cell^{-1} at 38 PSU). Toxicity was below the detection limit, ~0.03–0.1 pg cell^{-1}, at some salinity levels: 29 and 36.5 PSU for ATHS-92, and 29 and 33 PSU for ATHS-93. The detection limit depends on the final cell yield of each culture.

The two strains ATHS-92 and ATHS-95 were examined for the light and temperature experiments (Figures 2 and 3; Table II). No growth was observed at 12 and 22°C for ATHS-92 and at 17 and 22°C for ATHS-95 under

Table I: Toxin composition and toxicity of A. tamarense ATHS-95 measured by HPLC and TCBA

Sample no.	I Exponential		II Early stationary		III Stationary	
	fmol cell^{-1}	%	fmol cell^{-1}	%	fmol cell^{-1}	%
GTX4	0.32	24	0.29	11	0.45	21
GTX1	0.19	14	0.14	5	0.38	18
GTX3	0.08	6	0.03	1	0.17	8
GTX2	ND		ND		ND	
C4	0.05	4	1.53	57	0.07	3
C3	0.05	4	0.18	7	0.28	13
C2	0.64	47	0.47	17	0.68	32
C1	0.02	2	0.06	2	0.07	3
Total	1.35	100	2.70	100	2.12	100
	Toxicity (× 10^{-6} MU cell^{-1})					
HPLC	1.31		1.25		2.23	
TCBA	1.79		1.54		2.73	

ND, not detected

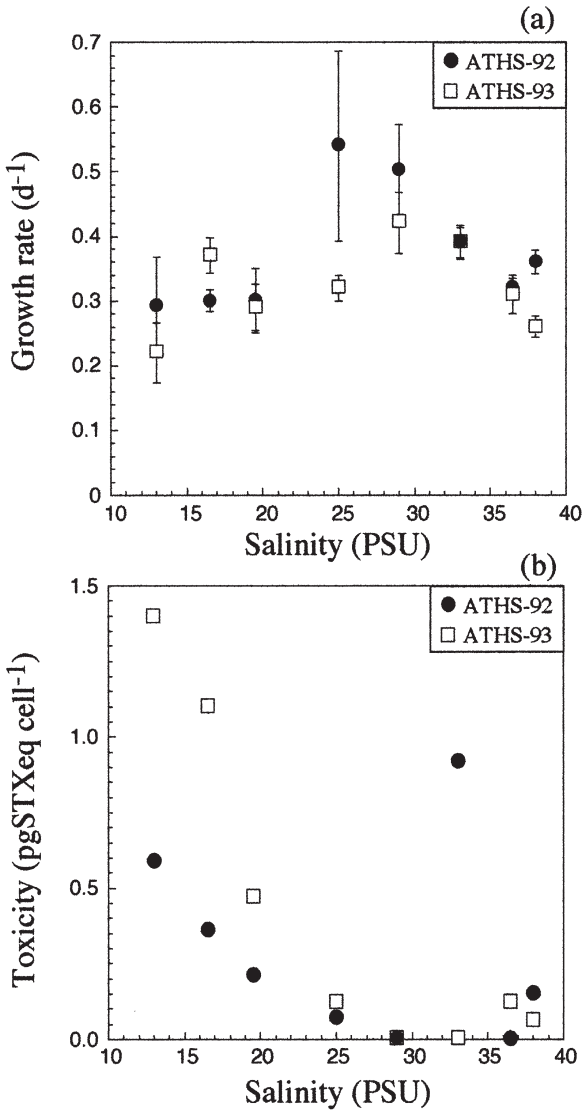

Fig. 1. Growth (**a**) and toxicity (**b**) of *A. tamarense* isolated from Hiroshima Bay as a function of salinity. Toxicity values lower than detection limits are plotted on the *x*-axis.

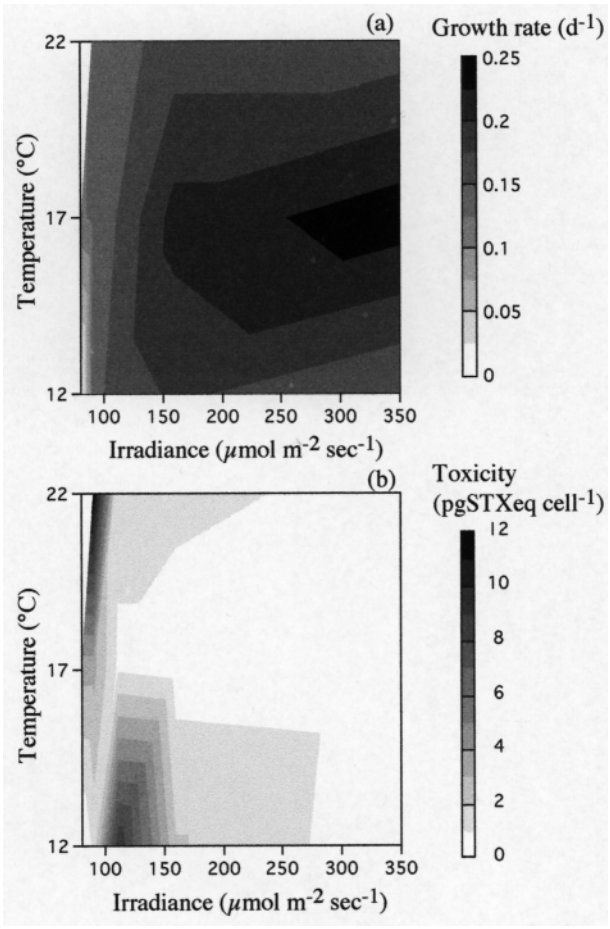

Fig. 2. Growth (**a**) and toxicity (**b**) of *A. tamarense* ATHS-92 isolated from Hiroshima Bay as functions of temperature and light intensity.

80 μmol m^{-2} s^{-1}. Exponential growth continued from 10 to 30 days throughout all cultures. A one-way ANOVA indicated that variability of growth rates was significant ($P < 0.01$) among various light levels at three temperature levels except at 22°C for ATHS-92 ($0.05 < P < 0.10$). Variability of growth rates at a saturated light level, 350 μmol m^{-2} s^{-1}, was also significant ($P < 0.001$) among three temperature levels in both cultures. Growth rates of both strains increased with irradiance at 17 and 22°C. At 12°C, the growth rate peaked at 160 μmol m^{-2} s^{-1} and decreased at the highest irradiance of 350 μmol m^{-2} s^{-1}. Maximum growth rates were observed at 17°C and 350 μmol m^{-2} s^{-1} in both strains: 0.24 day^{-1} for ATHS-92 and 0.33 day^{-1} for ATHS-95. The toxicity showed a peak at 22°C and 90 μmol m^{-2} s^{-1} for ATHS-92, and at 12°C and 110 μmol m^{-2} s^{-1} for ATHS-95. Toxicity increased with decreasing irradiance at 17 and 22°C.

The two strains ATHS-93 and ATHS-94 were examined for the ammonium experiment. Growth was inhibited with increasing ammonium concentrations. Slight growth was observed at 0.44 mM for both strains (Table II). In contrast, the cells grown at 0.22 mM culture were 5- to 15-fold more toxic than those grown at 0.11 mM culture.

DISCUSSION

Cellular toxin content was expressed as STX equivalent toxicity, measured by TCBA, in this study. In the field

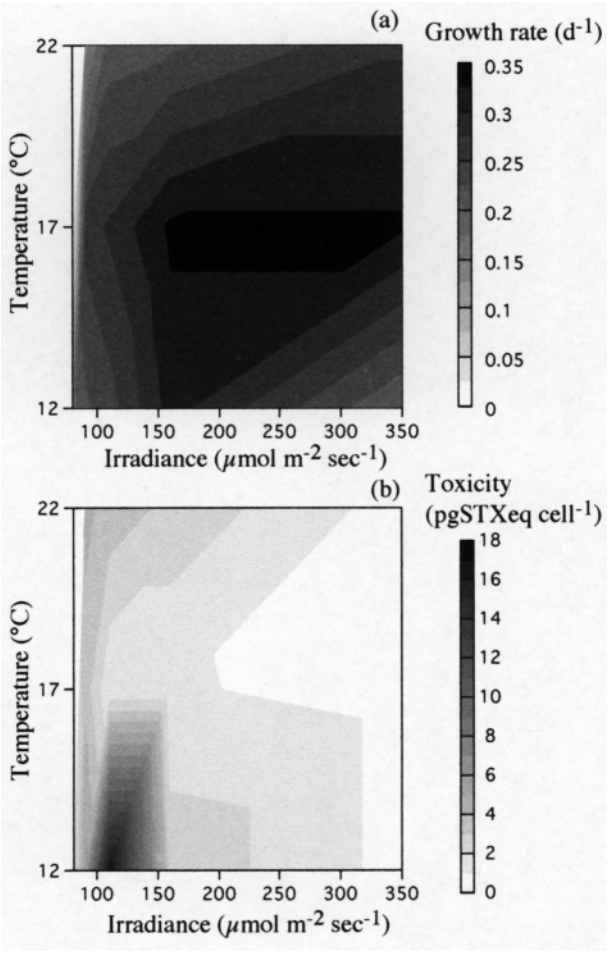

Fig. 3. Growth (**a**) and toxicity (**b**) of *A. tamarense* ATHS-95 isolated from Hiroshima Bay as functions of temperature and light intensity.

monitoring study on *A. tamarense* bloom, toxicity measured by TCBA showed a good correlation to that measured by HPLC when the data were normalized by mouse unit (r = 0.895, n = 43; unpublished data). However, variability of STX equivalent toxicity is not always consistent with that of mole-based toxin concentration, because various PSP toxin analogs have different specific toxicities to each other. STX equivalent toxicity may be applicable as an indicator of toxin concentration when the toxin composition of the cell is constant among various culture conditions. We have no data on the constancy of toxin composition among environmental conditions. Some studies have suggested that the relative toxin composition, so-called 'toxin profile', is a conservative characteristic within an isolate or strain (Cembella and Taylor, 1985; Boyer *et al.*, 1987; Oshima *et al.*, 1993; Anderson *et al.*, 1994; Flynn *et al.*, 1994). Parkhill and Cembella observed no substantial compositional change in the major toxin profiles for all of the environmental factors they tested, such as light (40–470 μmol m^{-2} s^{-1}), salinity (10–30‰) and nitrate concentration (0–0.88 mM), although minor variation (e.g. epimerization) was present (Parkhill and Cembella, 1999). In HPLC analysis of ATHS-95, an increase of C4 toxin changed the toxin profile at the early stationary phase (Table I). However, it does not invalidate the concept of a stable genetic trait in toxin profiles, because C4 toxin is a sulfonated product from GTX4, which is a major component at the other two phases. Although HPLC analysis of toxin composition was only carried out for one of the four strains in this study, previous studies reportedly showed a consistent feature of toxin profile among the strains isolated from Hiroshima Bay. They indicated that GTX1, GTX4 and C2 toxins were dominant constituents of PSP toxins in these strains (Asakawa *et al.*, 1995; Hamasaki *et al.*, 1998). As STX equivalent toxicity may overlook the production of C toxins due to their low potency, it may reflect the oscillation of mainly GTX production in this study.

Table II: Growth and toxicity of A. tamarense at different ammonium concentrations

Ammonium (mM)	ATHS-93		ATHS-94	
	Growth rate (day^{-1})	Toxicity (pg $cell^{-1}$)	Growth rate (day^{-1})	Toxicity (pg $cell^{-1}$)
0.11	0.30	ND	0.32	2.9
0.11	0.32	0.48	0.29	0.56
0.22	0.19	3.8	0.31	8.3
0.22	0.22	4.9	0.29	4.1
0.44	0.06	ND	0.10	ND

ND, not detected.

Effect of environmental factors on toxicity

Salinity is a changeable factor in estuaries or coastal waters where blooms of *A. tamarense* frequently appear, and is an important determinant of population dynamics for many phytoplankton species. An almost constant growth rate within the salinity range examined (13–38 PSU) suggests that isolated strains of *A. tamarense* are well adapted to changeable environments. Toxicity showed a tendency to decrease with increasing salinity within the range of 13–29 PSU, which coincided with the increase in growth rate (Figure 1). However, the overall relationship between toxicity and growth rate was not well defined. The highest toxicity was found at the lowest salinity and the lowest growth rate for ATHS-93. Similar results (enhanced cellular toxin content at the lowest salinity coincided with the lowest growth rate) were obtained for other toxic dinoflagellates, *Pyrodinium bahamense* (Cembella, 1998). However, the contradictory trends have also been reported in previous work. White showed that the toxicity of *Gonyaulax excavata* increased with increasing salinity up to 37 PSU (White, 1978). In a high-toxicity clone of *A. tamarense*, a positive correlation between growth rate and toxicity was reported in the salinity range from 10 to 30 PSU (Parkhill and Cembella, 1999). As was pointed out by previous workers, such discrepancy in the salinity experiments possibly originates from an acclimation state of the cell to various salinity levels. The present study used cells acclimated to each experimental salinity (from 13 to 38 PSU) in advance. White (White, 1978) and Parkhill and Cembella (Parkhill and Cembella, 1999) inoculated cells grown at 30 or 32 PSU into medium of different salinity levels. The stress caused by sudden osmotic change can cause the suppression of toxin production or the leakage of toxins from cells.

The effects of light on toxin production are complex and controversial (Cembella, 1998). Photosynthetic processes could affect toxin biosynthesis at various physiological levels. For example, nitrate reductase levels are often inversely correlated with light-harvesting chlorophyll protein, and photosynthetic electron flow is an important source of reduced ferredoxin for nitrite reduction (Falkowski and Raven, 1997). Ogata *et al.* showed that light retardation during growth interrupted both growth and toxin production (Ogata *et al.*, 1987). They suggested that photoassimilation of the nitrogen source into amino acid precursors is related to such phenomena. The present study showed that the increase in cellular toxin content under reduced light intensity was influenced by temperature (Figures 2 and 3). At temperatures of 17 and 22°C, maximum toxin content was observed at the lowest light intensity. High toxin content with low light intensity coincided with reduced growth rates. These results are supported by previous reports (Proctor *et al.*, 1975; Ogata *et al.*, 1987). However, at the lowest temperature of 12°C, maximum toxin content was observed at intermediate light-dependent growth rates. These results suggested that toxin content or its production was directly affected by light intensity, temperature and their related processes. Inconsistent data at 12°C may be caused by the susceptibility of algal cell metabolism to temperature. Temperature control of toxin productivity has been a possible explanation for high cellular toxicity of *Alexandrium* species inhabiting high latitudes (White, 1986; Cembella *et al.*, 1988; Anderson *et al.*, 1994). In this study, the toxicity values at 12°C were almost comparable to or higher than those at high temperatures. Ogata *et al.* observed that the toxicity of *A. tamarense* at low temperature was higher than that obtained at higher temperatures in a field survey (Ogata *et al.*, 1982). Anderson *et al.* observed that toxin and arginine content at 8°C were higher than that at 15°C (Anderson *et al.*, 1990). They proposed the hypothesis that the effect of low temperature was to reduce protein synthesis, resulting in a surplus of arginine within the cell that could be used for toxin synthesis.

Nitrogen availability would be a critical factor controlling the biosynthesis of PSP toxins because the toxins are nitrogen-rich compounds, accounting for 5–10% of the total cellular nitrogen in highly toxic strains (McIntyre *et al.*, 1997). An intracellular amino acid such as arginine is a precursor of toxin biosynthesis (Shimizu *et al.*, 1984). Nitrate and ammonium ions are actively transported from the environment into the cytoplasm. When amino acid synthesis is required, nitrate should be reduced to ammonium by the sequential action of nitrate reductase (NR) and nitrite reductase (Falkowski and Raven, 1997). These reactions could be limiting to toxin synthesis, because NR is a highly regulated enzyme in aquatic photoautotrophs (Guerrero *et al.*, 1981). Levasseur *et al.* described that the toxin content in *A. excavatum* increased when nitrogen was available in high concentration, but even more so if ammonium was used as a nitrogen source rather than nitrate (Levasseur *et al.*, 1993). Also, John and Flynn showed that a supply of ammonium rather than nitrate is likely to enhance toxin production of *A. fundyense* under phosphorus stress conditions (John and Flynn, 2000). Ammonium ion would be favored as a nitrogen source for toxin synthesis, although a high concentration of ambient ammonium shows an inhibitory effect on the growth of dinoflagellates (Iwasaki, 1973). Our results showed a drastic increase in toxin content with an increase in ammonium concentration from 0.11 to 0.22 mM, while growth was scarcely detected at a concentration of 0.44 mM (Table II). In the culture of ATHS-93, such an increase was possibly caused by a growth rate reduction due to an inhibitory effect of ammonium. However, such

an increase was larger than that observed in the salinity-dependent growth rate reduction of ATHS-93 and appeared without reduction of growth rates in the culture of ATHS-94. These results support the idea that ammonium utilization for toxin synthesis directly induces a high toxin content of *Alexandrium*.

Ecological implications

The toxicity of *A. tamarense* increased with decreasing salinity, decreasing light intensity and increasing ammonium concentration in this study. We need further work to say whether such tendencies are caused directly by environmental factors or by growth rate reduction influenced by environmental factors. However, these results suggest that the cells might become highly toxic in environments with low salinity and high ammonium concentration and successive cloudy days, which could lead to increasing risk of shellfish toxification. Field monitoring of *A. tamarense* bloom in 1998 in Hiroshima Bay revealed that the cellular toxin content of the natural population varied from 0.28 to 224 pg cell^{-1}, which sometimes showed higher values than those in culture conditions. In addition, toxin content data were positively correlated to ambient ammonium concentration regardless of the presence of sufficient other nitrogen sources, such as nitrate and nitrite for growth (unpublished data). The antagonistic effect of ammonium on nitrate metabolism with regard to the synthesis of the enzymes of the nitrate-reducing system is evident in many organisms, including some eukaryotic algae (Guerrero *et al.*, 1981). Furthermore, nutrient uptake kinetics for *A. excavata* (*G. excavata*) was reported to provide higher V_{max} and a lower K_s for ammonium than nitrate (MacIssac *et al.*, 1979). It is speculated that preferential uptake and assimilation of ammonium lead to a higher cellular toxin content of *Alexandrium* species in the natural environment. Wyatt and Jenkinson proposed that PSP toxins are used as chemical signals to promote mating, acting like pheromones in *Alexandrium* species (Wyatt and Jenkinson, 1997). If this hypothesis is true, the observed pattern in this study, in which toxicity in suboptimum environmental conditions with a reduced growth rate is higher than that in optimum environmental conditions, can be explained as an ecophysiological adaptation of the toxic dinoflagellates. A high toxin content and its subsequent release would compensate for the reduced chance of mating due to low cell density in unfavorable conditions for growth, which would contribute to keeping their populations in changeable estuary environments.

Prediction and risk assessment of toxification events require the integration of several biogeochemical models related to toxic phytoplankton growth, their toxin production, and toxin accumulation in shellfish. The physiological changes in toxin content for *Alexandrium* species have been investigated for the last two decades; however, the underlying mechanism is still hard to understand. It seems that toxin variability of *Alexandrium* species differs with strains and regions where they were isolated. Therefore, it is currently important to obtain data on toxin variability in response to environmental factors in each region. Our data are insufficient to clarify the physiological mechanism of cellular toxin production. However, they are useful to understand regional toxification events and predict possible conditions when food hygiene is at risk. The universal mechanism regulating toxin synthesis should be clarified by identifying genes and enzymatic processes in future research.

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