

Effect of temperature on production of okadaic acid, dinophysistoxin-1, and pectenotoxin-2 by *Dinophysis acuminata* in culture experiments

Takashi Kamiyama^{1,*}, Satoshi Nagai¹, Toshiyuki Suzuki², Kazuyoshi Miyamura³

¹National Research Institute of Fisheries and Environment of Inland Sea, Maruishi 2-17-5, Hatsukaichi, Hiroshima 739-0452, Japan

²National Research Institute of Fisheries Science, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan

³Fisheries Research Institute, Oita Prefectural Agriculture Forestry and Fisheries Research Center, 194-6 Tsuiura, Kamiura, Saeki, Oita 879-2602, Japan

ABSTRACT: We cultivated *Dinophysis acuminata*, a mixotrophic dinoflagellate causing diarrhetic shellfish poisoning worldwide, at different temperatures by providing a mixotrophic ciliate *Myrionecta rubra* as a food source. We examined the effects of temperature on growth rates and monitored production of the toxins okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) using liquid chromatography-tandem mass spectrometry. Cell densities of *D. acuminata* increased at temperatures of 10, 14, 18, and 22°C, and the mean specific growth rates during the exponential growth phase were higher at higher temperatures (0.14 to 0.28 d⁻¹). The concentration of all toxins increased as cell densities increased. The mean cellular PTX2 content during the exponential growth phase was greater at lower temperatures, but clear differences in the other toxin concentrations in relation to temperature were not observed. The cellular content of OA and DTX1 significantly increased during the exponential growth phase at all temperatures except at 10°C for OA, whereas that of PTX2 did not significantly increase at temperatures greater than 10°C. Cellular toxin production rates temporarily increased at or just before the end of the exponential growth phase at 14, 18, and 22°C, and the mean rates for OA production over the entire incubation period were higher at higher temperatures. Our results indicate that increasing temperature generally stimulates toxin production in *D. acuminata* populations due to an increase in cell density, but cellular content and production rates of OA and PTX2 in response to temperature differ and are influenced by growth phase.

KEY WORDS: *Dinophysis acuminata* · Growth rate · Temperature · Diarrhetic shellfish poisoning · *Myrionecta rubra* · Okadaic acid · Dinophysistoxin-1 · Pectenotoxin-2

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Several species within the dinoflagellate genus *Dinophysis* are the cause of bivalve contamination with toxins causing diarrhetic shellfish poisoning (DSP) and with lipophilic toxins (Hallegraeff & Lucas 1988, Hallegraeff 1993, Quilliam et al. 1993); this results in serious economic losses to shellfish culture industries worldwide (Yasumoto et al. 1985, Della Loggia et al. 1993).

The population dynamics of the causative species and their cellular toxin content are important factors in

the nature and magnitude of bivalve contamination. Information on abundance, seasonal distribution, and toxicity of several *Prorocentrum* species (potential DSP toxin producers) has been reported (Ten-Hage et al. 2000, Foden et al. 2005, Cruz et al. 2006, Maranda et al. 2007a,b). In addition, field observations on the population dynamics of *Dinophysis* species have been carried out in various areas (Koike et al. 2001, Nishitani et al. 2002, 2005, Lindahl et al. 2007).

The toxin content of *Dinophysis acuta* and *D. acuminata* in natural populations varies spatially and tempo-

*Email: kamiyama@affrc.go.jp

rally (MacKenzie et al. 2005, Lindahl et al. 2007). The toxin content of *Dinophysis* spp. can change markedly over an investigation period (Suzuki et al. 1997, Miyazono et al. 2008), and nontoxic *D. acuminata* has been observed (Hoshiai et al. 1997). Little is known, however, about the cellular dynamics of *Dinophysis* toxin production, and factors influencing toxin variations have not been examined due to the inability to culture *Dinophysis* spp.

Since the first successful cultivation of *Dinophysis acuminata* by Park et al. (2006), physiological characteristics of other *Dinophysis* species from laboratory culture experiments have been reported (Kim et al. 2008, Nagai et al. 2008, Nishitani et al. 2008a,b, Riisgaard & Hansen 2009). As a result, potential growth rates of *D. acuminata*, *D. caudata*, *D. infundibulus*, and *D. fortii* have been clarified, and the effects of prey concentration and light condition on the growth of *D. acuminata* have been demonstrated (Kim et al. 2008, Riisgaard & Hansen 2009), although other environmental factors influencing the growth of *Dinophysis* species have not been identified. Furthermore, toxin production by *D. acuminata* has been confirmed in laboratory experiments (Hackett et al. 2009, Kamiyama & Suzuki 2009), indicating a need to examine the effects of environmental factors on toxin production in order to clarify the dynamics of DSP and lipophilic toxins within marine ecosystems.

Temperature may be one of the more important environmental factors influencing population dynamics and cell toxin content of *Dinophysis* species because growth rates of plankton generally increase with increasing temperature within a certain range. This leads us to assume that the production of cellular DSP and lipophilic toxins by *Dinophysis* species also depends on environmental temperature.

Our present study was conducted to clarify the effects of temperature on the production of the main toxins causing DSP (okadaic acid [OA] and dinophysistoxin-1 [DTX1]) and a lipophilic toxin (pectenotoxin-2 [PTX2]), as well as on the growth rates of *Dinophysis acuminata* in culture experiments.

MATERIALS AND METHODS

Isolation of clonal strains. The marine ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax amphioxeia* were isolated from Inokushi Bay, Oita Prefecture, Japan (32° 47' N, 131° 53' E) at the end of February 2007 (Nishitani et al. 2008a). The *M. rubra* culture was maintained by mixing 0.7 ml of the culture (5.0 to 7.0 × 10³ cells) with 2.3 ml of a modified f/2 medium and 10 to 15 µl of *T. amphioxeia* culture (containing 0.5 to 1.0 × 10² cells) as food source into each well of 12-well

microplates (Iwaki). This culture medium was made up with 1/3 nitrate, phosphate, and metals and 1/10 vitamins of the f/2 medium (Guillard 1975, Nagai et al. 2004) based on the enrichment of Japanese coastal seawater (salinity adjusted to 30). A part of the ciliate culture was transferred into fresh culture medium containing the food source once a week, and the culture were maintained at a temperature of 18°C under an irradiance of 100 to 150 µmol photons m⁻² s⁻¹ provided by cool-white fluorescent lamps with a 12 h light:12 h dark cycle. The *T. amphioxeia* culture was also maintained in the modified f/2 medium in a 24-well microplate (Iwaki) under the same conditions as *M. rubra* (Nagai et al. 2008).

Dinophysis acuminata cells were isolated by micropipetting from a seawater sample collected from Inokushi Bay, Oita Prefecture, Japan (32° 80' N, 131° 90' E) in November 2007 and established as clonal cultures. The established cultures of *D. acuminata* were maintained in 12-well microplates by repeating inoculation of 300 µl of the *D. acuminata* culture (containing 150 to 250 cells) into 2.7 ml of the *Myrionecta rubra* culture (containing 3000 to 3500 cells ml⁻¹ diluted by one-third with fresh culture medium) once a month under the same conditions as the *Teleaulax amphioxeia* and *M. rubra* cultures. All cultures were nonaxenic but were all clonal.

Growth experiment at 4 temperatures. Effect of temperature on toxin production was investigated at 4 temperatures (10, 14, 18, and 22°C) under the same conditions as specified for the maintenance cultures except for temperature. *Dinophysis acuminata* in northern Japan and northern Europe mostly appear within this temperature range (e.g. Hoshiai et al. 2003, Lindahl et al. 2007). When full consumption of *Myrionecta rubra* cells by a clonal strain of *D. acuminata* (DA0711INO26) was observed (2 wk after re-inoculation of the culture), the *D. acuminata* cultures in the 12-well microplates were placed at each temperature and pre-incubated for 3 wk.

After maintenance re-inoculation of the *Myrionecta rubra* culture, the *Dinophysis acuminata* cultures were pre-incubated at each temperature for 2 wk. Re-inoculation of the cultures (except at 10°C), as described for culture maintenance, took place once after 1 wk pre-incubation. The *M. rubra* cultures were grown at each temperature until the late exponential growth (EG) phase (ca. 8.5 × 10³ cells ml⁻¹) and then put together in a plastic vessel. They were diluted with the culture medium to give initial concentrations of ca. 2.3 to 2.4 × 10³ cells ml⁻¹. *D. acuminata* culture (2000 cells ml⁻¹) was added to the *M. rubra* cultures to give an initial concentration of ca. 50 cells ml⁻¹, and 1.0 ml aliquots of the mixed cultures were pipetted into >50 wells of 48-well microplates (Iwaki) for each temperature treat-

ment. The incubation experiment was conducted for 24 d at 14, 18 and 22°C and for 30 d at 10°C under the same light conditions as specified for the maintenance culture. Every 3 d, cultures in 5 wells were mixed in a tube and used for cell counts (0.5 ml, in triplicate) and for DSP and lipophilic toxin analysis (1.0 ml, in triplicate). Cell densities of *D. acuminata* and *M. rubra* were determined using an inverted microscope. Samples for toxin analysis were kept in a freezer (<−20°C).

Treatment of plankton samples for toxin analysis.

For toxin analysis, we used the solid phase extraction procedure, as described by Kamiyama & Suzuki (2009; originally reported by Suzuki et al. 1997, 1998). The cell suspensions were thawed, sonicated in an ultrasonic cleaner for 2 min, and loaded onto a Sep-Pak C18 cartridge column (Waters), which had been previously conditioned with 5 ml methanol and 10 ml distilled water. The cartridge was washed with 5 ml distilled water to remove the seawater salt, and the toxins were eluted with 5 ml methanol, which was then evaporated. The residue was dissolved in 200 µl methanol and a part of the solution was used for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Toxin analysis. LC-MS/MS analysis of toxins was essentially carried out as described by Kamiyama & Suzuki (2009). A model 1100 liquid chromatograph (Agilent) was coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTrap 3200; PE-SCIEX). Separations were performed on Quicksilver cartridge columns (50 × 2.1 mm internal diameter) packed with 3 mm Hypersil-BDS-C8 (Keystone Scientific) and maintained at 20°C. Eluent A was water and eluent B was acetonitrile–water (95:5, vol:vol), both containing 2 mM ammonium formate and 50 mM formic acid. Toxins were eluted from the column with 50% eluent B at a flow rate of 0.2 ml min^{−1}. The toxin concentration of the sample was determined by comparing the peak areas of each toxin with those of the standard toxins for OA, DTX1, and PTX2, which were provided by Japan Food Research Laboratories (Tokyo, Japan). The lowest detection limits for OA, DTX1 and PTX2 were 0.6, 0.6 and 1.6 ng ml^{−1}, respectively. These levels are equivalent to 1.2 pg cell^{−1} of OA/DTX1 and 3.2 pg cell^{−1} of PTX2, when 100 cells of the toxic plankton were analyzed using our LC-MS/MS method.

Data analysis. Specific growth rates (µ; d^{−1}) of *Dinophysis acuminata* between sampling intervals (*t*; 3 d) was calculated from the EG equation:

$$\mu = \frac{\ln(N_2 / N_1)}{t} \quad (1)$$

where N_1 and N_2 are the cell densities of *D. acuminata* at the first and subsequent sampling, respectively. The

mean specific growth rates of *D. acuminata* were determined from the slope of the linear regression of the natural logarithm of cell density versus incubation time during the EG period.

Cellular content of OA, DTX1, and PTX2 of *Dinophysis acuminata* was determined by dividing the toxin concentration by the cell density at each time point of incubation. Effects of temperature on cellular toxin content and toxin production rates of *Dinophysis acuminata* were examined by 1-way ANOVA, or by the Kruskal-Wallis test if the variance of data at each temperature was not homogeneous, and the differences in these values and growth rates between 2 temperatures were examined by the Student's *t*-test. The trends of cellular toxin content and production rate during the incubation period were examined by Spearman's rank correlation coefficient (Spearman's RCC).

RESULTS

Growth at different temperatures

Cell density of *Dinophysis acuminata* increased at all temperatures from the initiation of incubation and reached maximum values of 1.69×10^3 to 4.17×10^3 cells ml^{−1} (Fig. 1). Analysis of the growth curves showed the EG phase to be shorter at higher temperatures: 24 d at 10°C, 18 d at 14 and 18°C, and 15 d at 22°C. In contrast, cell density of *Myrionecta rubra* exponentially decreased after 6 d of incubation, with this decrease occurring more rapidly at higher temperatures (Fig. 1), and cells disappeared after 9 d of incubation at 22°C, 12 d at 18°C, 18 d at 14°C, and 27 d at 10°C. The mean specific growth rates of *D. acuminata* during the EG phase ranged from 0.14 to 0.28 d^{−1} (equivalent to 0.20 to 0.40 divisions d^{−1}) and was greater at higher temperatures (Table 1). The differences in the growth rates between 10°C and higher temperatures and between 14°C and 22°C were significant (Student's *t*-test, $p < 0.05$). The maximum specific growth rates per 3 d sampling interval ranged from 0.29 to 0.50 d^{−1} (0.41 to 0.73 divisions d^{−1}; Table 1).

Toxin production at different temperatures

Toxin concentrations in *Dinophysis acuminata* cultures increased exponentially during the EG phase (Fig. 2). Toxin yields reached a maximum of 16.0 to 120.2 ng ml^{−1} for OA, 1.2 to 3.9 ng ml^{−1} for DTX1, and 203.9 to 447.3 ng ml^{−1} for PTX2 and were greater at higher temperatures for all toxins.

During the EG phase, the cellular toxin content increased significantly with increasing incubation

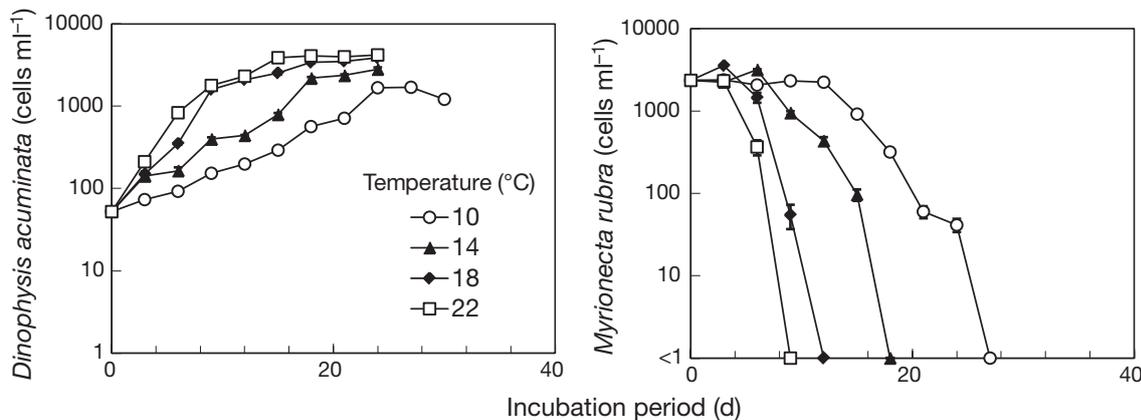


Fig. 1. *Dinophysis acuminata* and *Myrionecta rubra*. Growth of *D. acuminata* and *M. rubra*. Cell densities at 4 different temperatures. Vertical bars denote SE of the mean (n = 3)

periods at temperatures of 14, 18, and 22°C for OA and at all temperatures for DTX1 (Spearman's RCC, $p < 0.05$) (Fig. 3). If the cellular contents of all components after the EG phase are excluded, as mentioned in the Discussion, the cellular toxin contents reached a maximum of 11.9 to 16.8 pg cell^{-1} at 14, 18, and 22°C for OA and 0.5 to 0.7 pg cell^{-1} at all temperatures for DTX1, which is 2.8 to 5.1 and 2.5 to 4.2 times higher than initial values, respectively. This relationship with the incubation period was not observed at 10°C for OA. Mean cellular toxin content at each temperature during the EG period was 8.06 to 8.66 pg cell^{-1} for OA and 0.24 to 0.34 pg cell^{-1} for DTX1 (Table 2). Differences among temperatures were not significant for either OA or DTX1 (Kruskal-Wallis test, $H = 0.69$, $p > 0.05$ for OA; 1-way ANOVA, $F = 0.56$, $p > 0.05$ for DTX1). For PTX2, cellular content of the toxin as a function of incubation period did not show a significant trend at 14 to 22°C (Spearman's RCC, $p > 0.05$), whereas at 10°C, the value significantly increased with incubation period. Mean cellular toxin content at each temperature during the EG period ranged from 57.10 to 107.05 pg cell^{-1} and was significantly greater at lower temperatures (1-way ANOVA, $F = 8.49$, $p < 0.01$).

Table 1. *Dinophysis acuminata*. Maximum and mean (SE) growth rates during the exponential growth (EG) phase at 4 temperatures in culture experiments

Temp. (°C)	Duration of EG phase (d)	Specific growth rate (μ , d^{-1})		n
		Maximum	Mean (SE)	
10	24	0.29	0.14 (0.01)	9
14	18	0.34	0.19 (0.02)	7
18	18	0.50	0.24 (0.03)	7
22	15	0.47	0.28 (0.04)	6

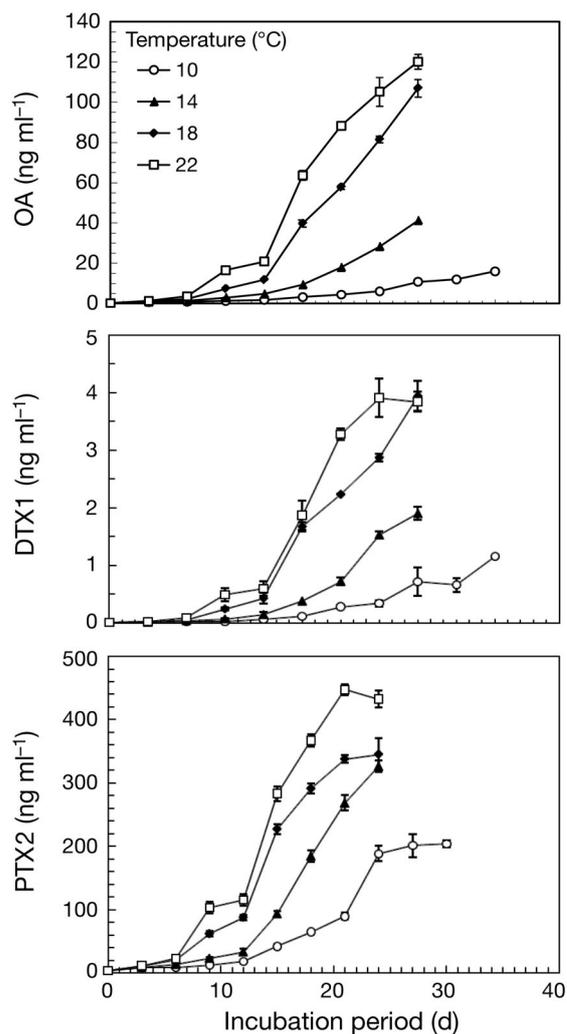


Fig. 2. *Dinophysis acuminata*. Changes in concentrations of okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) in cultures at 4 different temperatures. Vertical bars denote SE of the mean (n = 3)

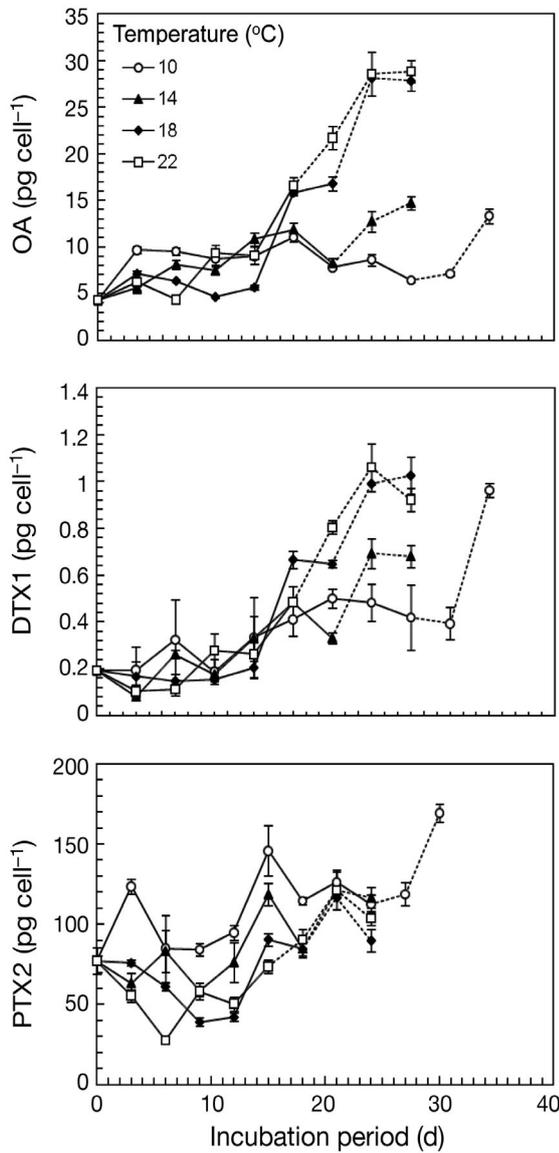


Fig. 3. *Dinophysis acuminata*. Changes in cellular content of okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) at 4 different temperatures. Data on dashed lines denote the values during the stationary growth phase. Vertical bars denote SE of the mean (n = 3)

DISCUSSION

Effects of temperature on growth rates

In the present study the mean specific growth rates of *Dinophysis acuminata* at temperatures from 10 to 22°C ranged from 0.14 to 0.28 d⁻¹ (maximum 0.29 to 0.50 d⁻¹) during the EG period. *In situ* growth rates of *D. acuminata* have previously been reported as ranging from 0.09 to 0.67 d⁻¹ (Table 1 in Nagai et al. 2008), and maximum values based on previous culture experiments range from 0.45 to 0.95 d⁻¹ at 20°C (Park et al. 2006, Kim et al. 2008, Riisgaard & Hansen 2009) and 0.67 d⁻¹ at 15°C (Kamiyama & Suzuki 2009). Compared to values from previous culture experiments, the maximum growth rates of *D. acuminata* in the present study are somewhat low. The growth rates could be influenced by other experimental conditions, such as quality of prey organisms, experimental scale, and genetic characteristics of *D. acuminata*.

Temperature is an important factor influencing growth of plankton. The specific growth rate (μ) of protists is expected to increase exponentially with increases in temperature (e.g. Eppley 1972), and the Q₁₀ model is often used to fit the relationships (e.g. Hansen et al. 1997). However, Montagnes et al. (2003) proposed that the growth rate of aquatic protists usually responds linearly to temperature. Hence, the relationship between the growth rate of *Dinophysis acuminata* and temperature in this study was fitted to a linear regression, with the slope of the regression line calculated to be 0.012 d⁻¹ °C⁻¹ (n = 4, SE = 0.000, r² = 0.999). This is lower than the values reported for other dinoflagellates (0.014 to 0.077 d⁻¹ °C⁻¹) (Montagnes et al. 2003), suggesting that the growth rates of *D. acuminata* are not as sensitive to temperature as those of other heterotrophic and autotrophic dinoflagellates. Alternatively the relationship of temperature to growth rates of *D. acuminata* presented here could have been influenced by factors contributing to the lower observed growth rates.

Dinophysis acuminata is a cosmopolitan species that is distributed in coastal waters worldwide and is a

Table 2. *Dinophysis acuminata*. Cellular toxin contents during the exponential growth (EG) phase at 4 temperatures in culture experiments

Temperature (°C)	Duration of EG phase (d)	Cellular toxin content (pg cell ⁻¹)						n
		OA		DTX1		PTX2		
		Mean	SE	Mean	SE	Mean	SE	
10	24	8.34	0.66	0.34	0.04	107.05	7.72	9
14	18	8.06	1.01	0.26	0.05	80.19	7.39	7
18	18	8.66	2.01	0.31	0.09	67.24	7.68	7
22	15	8.31	1.88	0.24	0.06	57.10	7.29	6

causative species of DSP and lipophilic toxin contamination of bivalves in New Zealand, Chile, Japan, and along the Atlantic coasts of Europe (van Egmond et al. 1993, Reguera & Pizarro 2009). In coastal waters of the western Pacific Ocean, *D. acuminata* appears abundantly in embayments in contrast to the other *Dinophysis* species, *D. fortii* (Hoshiai et al. 2003). The variations in distribution and seasonal occurrence reported in previous studies (Hoshiai et al. 2003, Miyazono et al. 2008) can be explained by the ability of *D. acuminata* to grow over a wide temperature range, as demonstrated in the present study.

Toxin contents and production

The results from the present study show that cultured *Dinophysis acuminata* actively produces OA and PTX2, supporting the findings of Kamiyama & Suzuki (2009) and Hackett et al. (2009). In Japan, accumulated data based on extensive monitoring of shellfish toxin using mouse bioassays indicate that DSP events (here, contamination of shellfish toxin due to okadaic acid analogues and other lipophilic toxins) exceeding a quarantine level have only infrequently occurred in northern and western regions (Yamamoto and Yamasaki 1996), although *Dinophysis* species commonly appear in coastal waters of western as well as northern Japan (Imai et al. 2006). The strain of *D. acuminata* used in the present study was isolated from seawater off western Japan. High concentrations of PTX2 and detection of OA and DTX1 at all temperatures suggest a risk of DSP despite having never been reported.

Although some toxins of *Dinophysis* spp. are released into surrounding seawater (MacKenzie et al. 2004), we assumed that the 3 toxins measured in the present study were components of the *D. acuminata* cells during the EG phase because of the results of a previous study (Kamiyama & Suzuki 2009). In our previous culture experiments, we did not detect toxins (PTX2 and DTX1) released into seawater from *D. acuminata* cells at the late EG phase or early stationary growth phase. However, 4 d after these phases, the released toxins accounted for 13% (PTX2, $n = 4$) and 24% (DTX1, $n = 4$) of total toxin content, on average, in the suspension (Kamiyama & Suzuki 2009, raw data not shown). This suggests that the amount of toxin released into seawater from cells relative to the total toxin concentration rapidly increases with incubation period during the stationary growth phase, even if it is negligible in the initial phase of cultivation. In our present study, cellular toxin content calculated by dividing toxin concentration by cell density often increased rapidly after the EG phase. However, overestimation of the cellular toxin content during the stationary growth

phase due to an increase in dissolved toxin fractions cannot be ruled out.

Cellular toxin content in natural *Dinophysis acuminata* cells ranges from 0 to 40 pg cell⁻¹ for OA in coastal waters of Europe, Japan, Canada, and Sweden, 3 to 50 pg cell⁻¹ for OA in the Bay of Seine (France), and from 0 to 0.02 pg cell⁻¹ and 0.1 to 2.4 pg cell⁻¹ for DTX1 in coastal waters of Sweden and New Zealand, respectively (Marcaillou et al. 2001, MacKenzie et al. 2005, summary in Lindahl et al. 2007). Recent work using LC-MS/MS analysis also indicates that PTX2 is a dominant toxin of *D. acuminata* around the coast of the South Island of New Zealand (cellular toxin content 2.4 to 25.8 pg cell⁻¹; MacKenzie et al. 2005) and off Hokkaido, northern Japan (cellular toxin content 0 to 50.2 pg cell⁻¹; Miyazono et al. 2008, Suzuki et al. 2009). Kamiyama & Suzuki (2009) showed that the cellular toxin content of cultured *D. acuminata* was 4.8 to 14.7 pg cell⁻¹ for PTX2 and 2.5 to 4.8 pg cell⁻¹ for DTX1. PTX2 levels (57 to 107 pg cell⁻¹) of *D. acuminata* in the present study were considerably higher than those from previous field samples and culture samples reported by Kamiyama & Suzuki (2009). In contrast, DTX1 (0.24 to 0.34 pg cell⁻¹) and OA (8.06 to 8.65 pg cell⁻¹) levels in the present study were within the range of previous data, although the DTX1 values were low compared to the data of Kamiyama & Suzuki (2009).

The toxin content of toxic dinoflagellates reflects the initial amount of toxin in a cell and gross toxin production. The gross production reflects the balance between net toxin synthesis and toxin losses due to catabolism and leakage or cell division (toxin partly transferred to new daughter cells) (Anderson et al. 1990). The loss of toxin due to cell division is probably important in culture experiments. Hence, to clarify the dynamics of toxin production, cellular toxin production rates (CTP; pg cell⁻¹ d⁻¹) were determined from the following equation for each time point:

$$\text{CTP} = \frac{(T_2 - T_1)}{N_m \times t}, \quad N_m = \frac{(N_2 - N_1)}{\ln(N_2 / N_1)} \quad (2)$$

where N_1 and N_2 are the cell densities of *D. acuminata* and T_1 and T_2 are the toxin concentrations, both parameters at the first and the subsequent sampling times, respectively, and N_m is the geometric mean density of *D. acuminata* during the sampling period.

As a result, the mean cellular toxin production rates for the entire incubation period were 1.18 to 2.31 pg cell⁻¹ d⁻¹ for OA, 0.06 to 0.08 pg cell⁻¹ d⁻¹ for DTX1, and 10.7 to 15.6 pg cell⁻¹ d⁻¹ for PTX2. The CTP are available after the EG phase as well because the toxins released into the surrounding waters were also produced by *Dinophysis acuminata* cells. The mean cellular production rate of OA increased with increasing temperature (Kruskal-Wallis test, $H = 8.39$, $p < 0.05$).

The mean cellular production rate of DTX1 was fairly similar at all temperatures (1-way ANOVA, $F = 0.16$, $p > 0.05$) and that of PTX2 did not significantly differ between temperatures (1-way ANOVA, $F = 0.42$, $p > 0.05$). The cellular production rates of PTX2 decreased with increasing incubation time at 18°C and 22°C (Spearman's RCC, $p < 0.05$). The temporary peaks of the cellular toxin production rates occurred at or just before the end of the EG phase, except for OA and DTX1 at 10°C (Fig. 4). This can be explained by the temporary decrease in the loss of toxin from cell division when the growth mode of *D. acuminata* shifts from the active to inactive state. Anderson et al. (1990) found that specific toxin production rates of *Alexandrium* spp. mostly corresponded to between 2 and 0.5

times the specific growth rates. The results of our study also indicate a significant linear correlation between toxin production rates and specific growth rates in *D. acuminata* for all 3 toxins (Fig. 5). However, 44 to 53% of the specific toxin production rates were outside the range denoted by 2 and 0.5 times the specific growth rates. This suggests that toxin production rates in *D. acuminata* may vary in response to factors other than growth rate.

Previous studies have reported that the content and composition of paralytic shellfish poisoning toxins of *Alexandrium* spp. are strongly influenced by temperature (Etheridge & Roesler 2005, Navarro et al. 2006). Based on those studies and the results from our present study, we expected that temperature would be an

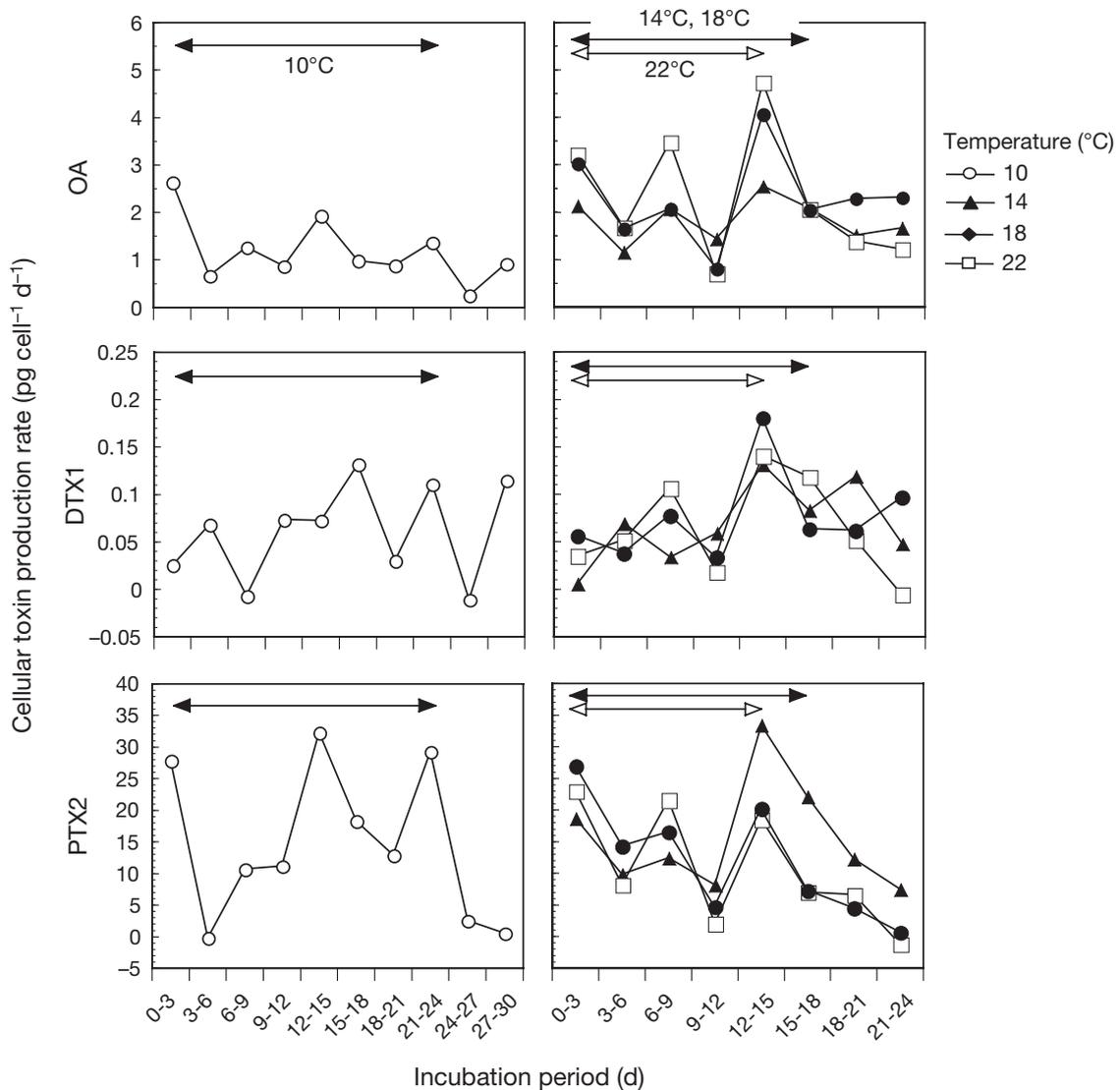


Fig. 4. *Dinophysis acuminata*. Changes in cellular toxin production rate (CTP) of okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxin-2 (PTX2) at 4 different temperatures. Arrows indicate the period of exponential growth at 10°C (left column) and at the other temperatures (right column)

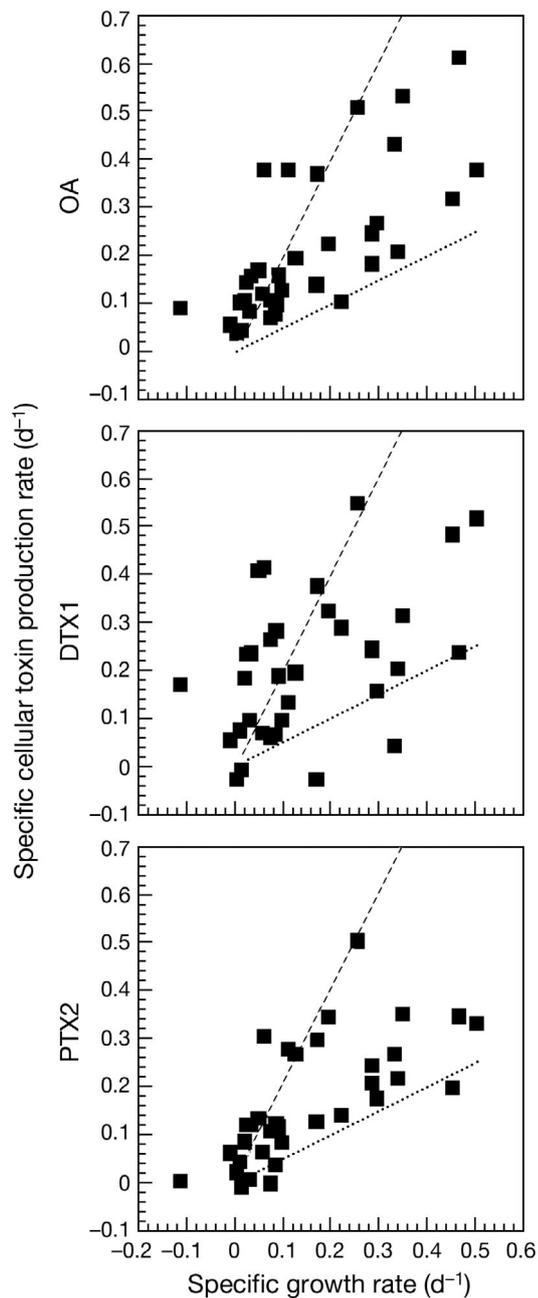


Fig. 5. *Dinophysis acuminata*. Relationship between specific toxin production rate and specific growth rate during each 3 h sampling interval at all temperatures. Dashed and dotted lines denote toxin production rates 2× and 0.5× that of specific growth rates, respectively

important factor in the production of the 3 toxins by *Dinophysis acuminata*. Interestingly, OA/DTX1 and PTX2 production clearly differed in response to temperature. The mean cellular content of OA and DTX1 during the EG phase was independent of the temperature, although cellular content of the toxins increased with increasing incubation time during the EG phase

at higher temperatures. This suggests that temperature does not directly influence toxin production but that population growth in response to temperature influences cellular content and production of OA and DTX1. In particular, a rapid increase in cellular OA and DTX1 occurred after consumption of *Myrionecta rubra*, suggesting an association with plastids originating from prey and their function, as discussed below. On the other hand, the mean cellular content of PTX2 during the EG phase was higher at lower temperatures, and during the first 21 d, was higher at 10°C than at other temperatures (Fig. 3). A significant variation in response to incubation period only occurred at 10°C. These results suggest that temperature may directly influence cellular production of PTX2. The different responses of OA/DTX1 and PTX2 production to temperature may be caused by a difference in the toxin production mechanism.

Uptake of prey (*Myrionecta rubra*) is not only important for growth (Park et al. 2006, Kim et al. 2008, Riisgaard & Hansen 2009) but may also be an important factor in the variations observed in cellular toxin content and production during incubation. Uptake of *M. rubra* by *Dinophysis acuminata* influences dinoflagellate photosynthetic activity (Riisgaard & Hansen 2009). Toxin synthesis by the DSP toxin producer *Prorocentrum lima* is considered to be a light-mediated metabolic process (Pan et al. 1999), implying that photosynthesis influences cellular toxin production by toxic phytoplankton. *Dinophysis* spp. depend on plastids from prey for photosynthesis (kleptoplastidy) (Park et al. 2006, Nagai et al. 2008). Hence, uptake of plastids from the food source through the feeding process possibly induced the variations in cellular toxin production that we observed. Also, the heterotrophic energy source obtained by feeding on *M. rubra* may be directly associated with toxin production. A more rapid increase in toxin concentration with incubation periods at higher temperature corresponded to a more rapid decrease in *M. rubra* due to active feeding. However, an experiment to estimate feeding rates of *D. acuminata* on *M. rubra* was not conducted in this study. Further studies are needed to clarify the effects of feeding on prey organisms on toxin production and the complex relationship between photosynthesis and toxin production.

Toxin concentration increased with increasing *Dinophysis acuminata* cell density at all temperatures, showing that *D. acuminata* growth rate is clearly an important factor promoting the production of toxins. Additionally, the combined effects of temperature and growth phase on cellular toxin content and production rates may explain the large variation in cellular toxin content of *Dinophysis* spp. in seawater. Since *D. acuminata* occurs in a wide range of temperature condi-

tions, the cellular content of PTX2 and OA in the *D. acuminata* population possibly varies seasonally, with PTX2 increasing during cooler seasons and OA increasing during warmer seasons when *D. acuminata* is actively growing. However, in the present situation, we cannot further discuss the differences in cellular toxin contents reported in previous studies on the basis of the effects of temperature on the cellular toxin contents observed in the present study, because the range of cellular toxin contents of field *D. acuminata* is very broad even if samples are collected in the same season (Andersen et al. 1996). Cell toxicity in field *D. acuminata* is probably influenced not only by temperature but also by other factors such as genetic characteristics. Further studies are necessary to document temperature-dependent variations in toxicity for each *Dinophysis* species in natural seawaters.

CONCLUSIONS

This is the first culture experiment addressing the effect of temperature on the growth and toxicity of *Dinophysis acuminata*. Our results showed that temperature affects growth rates and toxin production of *D. acuminata* and that cell toxicity varies during the EG phase. These findings partly suggest that there is a wide variation in cell toxicity in natural populations of *D. acuminata*. However, toxin production by *Dinophysis* spp. may depend on genetic characteristics or the fact that populations from different strains may show different growth responses to temperature. Further investigations are needed on the toxicity of various strains of *Dinophysis* spp. isolated from seawater from different areas having a range of environmental conditions.

Acknowledgements. This work was supported by a Grant-In-Aid for Scientific Research (Kiban-B) from the Japan Society for the Promotion of Science (No. 20380116). We thank the Japan Food Research Laboratories for providing OA, DTX1, and PTX2 standards.

LITERATURE CITED

- Andersen P, Benedicte H, Emsholm H (1996) Toxicity of *Dinophysis acuminata* in Danish coastal waters. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission of UNESCO, Paris, p 281–284
- Anderson DM, Kulis DM, Sullivan JJ, Hall S, Lee C (1990) Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. *Mar Biol* 104:511–524
- Cruz PG, Daranas AH, Fernández JJ, Souto ML, Norte M (2006) DTX5c, a new OA sulphate ester derivative from cultures of *Prorocentrum belizeanum*. *Toxicon* 47:920–924
- Della Loggia R, Cabrini M, Del Negro P, Honsell G, Tubaro A (1993) Relationships between *Dinophysis* spp. in seawater and DSP toxins in mussels in the Northern Adriatic Sea. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, p 483–488
- Eppley RW (1972) Temperature and phytoplankton growth in the sea. *Fish Bull* 70:1063–1085
- Etheridge SM, Roesler CS (2005) Effects of temperature, irradiance, and salinity on photosynthesis, growth rates, total toxicity, and toxin composition for *Alexandrium fundyense* isolates from the Gulf of Maine and Bay of Fundy. *Deep-Sea Res II* 52:2491–2500
- Foden J, Purdie DA, Morris S, Nascimento S (2005) Epiphytic abundance and toxicity of *Prorocentrum lima* populations in the Fleet Lagoon, UK. *Harmful Algae* 4:1063–1074
- Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, NY, p 26–60
- Hackett JD, Tong M, Kulis DM, Fux E, Hess P, Bire R, Anderson DM (2009) DSP toxin production *de novo* in cultures of *Dinophysis acuminata* (Dinophyceae) from North America. *Harmful Algae* 8:873–879
- Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. *Phycologia* 32:79–99
- Hallegraeff GM, Lucas IAN (1988) The marine dinoflagellate genus *Dinophysis* (Dinophyceae): photosynthetic, neritic and non-photosynthetic, oceanic species. *Phycologia* 27: 25–42
- Hansen PJ, Bjørnsen PK, Hansen BW (1997) Zooplankton grazing and growth: scaling within the 2–2000- μ m body size range. *Limnol Oceanogr* 42:687–704
- Hoshiai G, Suzuki T, Onodera T, Yamasaki M, Taguchi S (1997) A case of non-toxic mussels under the presence of high concentrations of toxic dinoflagellate *Dinophysis acuminata* that occurred in Kesennuma Bay, Northern Japan. *Fish Sci* 63:317–318
- Hoshiai G, Suzuki T, Kamiyama T, Yamasaki M, Ichimi K (2003) Water temperature and salinity during the occurrence of *Dinophysis fortii* and *D. acuminata* in Kesennuma Bay, northern Japan. *Fish Sci* 69:1303–1305
- Imai I, Yamaguchi M, Hori Y (2006) Eutrophication and occurrences of harmful algal blooms in the Seto Inland Sea, Japan. *Plankton Benthos Res* 1:71–84
- Kamiyama T, Suzuki T (2009) Production of dinophysistoxin-1 and pectenotoxin-2 by a culture of *Dinophysis acuminata* (Dinophyceae). *Harmful Algae* 8:312–317
- Kim S, Kang YG, Kim HS, Yih W, Coats DW, Park MG (2008) Growth and grazing responses of the mixotrophic dinoflagellate *Dinophysis acuminata* as functions of light intensity and prey concentration. *Aquat Microb Ecol* 51: 301–310
- Koike K, Otake H, Takagi M, Yoshida T, Ogata T, Ishimaru T (2001) Recent occurrences of *Dinophysis fortii* (Dinophyceae) in the Okkirai Bay, Sanriku, Northern Japan, and related environmental factors. *J Oceanogr* 57: 165–175
- Lindahl O, Lundve B, Johansen M (2007) Toxicity of *Dinophysis* spp. in relation to population density and environmental conditions on the Swedish west coast. *Harmful Algae* 6:218–231
- MacKenzie L, Beuzenberg V, Holland P, McNabb P, Selwood A (2004) Solid phase adsorption toxin tracking (SPATT): a new monitoring tool that simulates the biotoxin contamination of filter feeding bivalves. *Toxicon* 44:901–918
- MacKenzie L, Beuzenberg V, Holland P, McNabb P, Suzuki T, Selwood A (2005) Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. *Harmful Algae* 4:75–85

- Maranda L, Corwin S, Dover S, Morton SL (2007a) *Prorocentrum lima* (Dinophyceae) in northeastern USA coastal waters. II Toxin load in the epibiota and in shellfish. *Harmful Algae* 6:632–641
- Maranda L, Corwin S, Hargraves PE (2007b) *Prorocentrum lima* (Dinophyceae) in northeastern USA coastal waters. I. Abundance and distribution. *Harmful Algae* 6:623–631
- Marcaillou C, Gentien P, Lunven M, Le Grand J and others (2001) *Dinophysis acuminata* distribution and specific toxin content in relation to mussel contamination. In: Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ (eds). *Harmful algal blooms 2000*. Intergovernmental Oceanographic Commission of UNESCO, Paris, p 356–59
- Miyazono A, Suzuki T, Baba K, Sugawara R (2008) Occurrence of *Dinophysis* spp. and temporal changes in diarrhetic shellfish poison and lipophilic toxins in *Dinophysis* spp. in Funka Bay, Hokkaido. *Bull Plankton Soc Japan* 55:25–28 (Japanese with English abstract)
- Montagnes DJS, Kimmance SA, Atkinson D (2003) Using Q_{10} : Can growth rates increase linearly with temperature? *Aquat Microb Ecol* 32:307–313
- Nagai S, Matsuyama Y, Oh SJ, Itakura S (2004) Effect of nutrients and temperature on encystment of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) isolated from Hiroshima Bay, Japan. *Plankton Biol Ecol* 51: 103–109
- Nagai S, Nishitani G, Tomaru Y, Sakiyama S, Kamiyama T (2008) Predation by the toxic dinoflagellate *Dinophysis fortii* on the ciliate *Myrionecta rubra* and observation of sequestration of ciliate chloroplasts. *J Phycol* 44:909–922
- Navarro JM, Munoz MG, Contreras AM (2006) Temperature as a factor regulating growth and toxin content in the dinoflagellate *Alexandrium catenella*. *Harmful Algae* 5: 762–769
- Nishitani G, Sugioka H, Imai I (2002) Seasonal distribution of species of the toxic dinoflagellate genus *Dinophysis* in Maizuru Bay (Japan) with comments on their autofluorescence and attachment of picophytoplankton. *Harmful Algae* 1:253–264
- Nishitani G, Yamaguchi M, Ishikawa A, Yanagiya S, Mitsuya T, Imai I (2005) Relationships between occurrences of toxic *Dinophysis* species (Dinophyceae) and small phytoplankton in Japanese coastal waters. *Harmful Algae* 4:755–762
- Nishitani G, Nagai S, Sakiyama S, Kamiyama T (2008a) Successful cultivation of the toxic dinoflagellate *Dinophysis caudata* (Dinophyceae). *Plankton Benthos Res* 3:78–85
- Nishitani G, Nagai S, Takano Y, Sakiyama S, Baba K, Kamiyama T (2008b) Growth characteristics and phylogenetic analysis of the marine dinoflagellate *Dinophysis infundibulus* (Dinophyceae). *Aquat Microb Ecol* 52: 209–221
- Pan Y, Cembella AD, Quilliam MA (1999) Cell cycle and toxin production in the benthic dinoflagellate *Prorocentrum lima*. *Mar Biol* 134:541–549
- Park MG, Kim S, Kim HS, Myung G, Kang YG, Yih W (2006) First successful culture of the marine dinoflagellate *Dinophysis acuminata*. *Aquat Microb Ecol* 45:101–106
- Quilliam MA, Gilgan MW, Pleasance S, Defreitas ASW and others (1993) Confirmation of an incident of diarrhetic shellfish poisoning in Eastern Canada. In: Smayda TJ, Shimizu Y (eds) *Toxic phytoplankton blooms in the sea: Proc 5th Int Conf Toxic Marine Phytoplankton*, Newport, RI, USA. Elsevier Science Publication, New York, NY, p 547–552
- Reguera B, Pizarro G (2009) Planktonic dinoflagellates that contain polyether toxins of the old 'DSP Complex'. In: Botana LM (ed) *Seafood and freshwater toxins: pharmacology, physiology, and detection*, 2nd edn. CRC press, Boca Raton, FL, p 257–284
- Riisgaard K, Hansen PJ (2009) Role of food uptake for photosynthesis, growth and survival of the mixotrophic dinoflagellate *Dinophysis acuminata*. *Mar Ecol Prog Ser* 381: 51–62
- Suzuki T, Mitsuya T, Imai M, Yamasaki M (1997) DSP toxin contents in *Dinophysis fortii* and scallops collected at Mutsu Bay, Japan. *J Appl Phycol* 8:509–515
- Suzuki T, Mitsuya T, Matsubara H, Yamasaki M (1998) Determination of pectenotoxin-2 after solid-phase extraction from seawater and from the dinoflagellate *Dinophysis fortii* by liquid chromatography with electrospray mass spectrometry and ultraviolet detection: evidence of oxidation of pectenotoxin-2 to pectenotoxin-6 in scallops. *J Chromatogr A* 815:155–160
- Suzuki T, Miyazono A, Baba K, Sugawara R, Kamiyama T (2009) LC-MS/MS analysis of okadaic acid analogues and other lipophilic toxins in single-cell isolates of several *Dinophysis* species collected in Hokkaido, Japan. *Harmful Algae* 8:233–238
- Ten-Hage L, Delaunay N, Pichon V, Couté A, Puisseux-Dao S, Turquet J (2000) Okadaic acid production from the marine benthic dinoflagellate *Prorocentrum arenarium* Faust (Dinophyceae) isolated from Europa Island coral reef ecosystem (SW Indian Ocean). *Toxicon* 38:1043–1054
- van Egmond HP, Anne T, Lasso P, Speijers GJA, Waldock M (1993) Paralytic and diarrhetic shellfish poisons: occurrence in Europe, toxicity, analysis and regulation. *J Nat Toxins* 2:41–64
- Yamamoto M, Yamasaki M (1996) Japanese monitoring system on shellfish toxins. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) *Harmful and toxic algal blooms*. Intergovernmental Oceanographic Commissions of UNESCO, Paris, p 19–22
- Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J (1985) Diarrhetic shellfish toxins. *Tetrahedron* 41: 1019–1025

Editorial responsibility: Patricia Glibert,
Cambridge, Maryland, USA

Submitted: November 12, 2009; Accepted: March 15, 2010
Proofs received from author(s): May 31, 2010