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Citation	北海道大学水産科学研究彙報, 68(1), 7-16
Issue Date	2018-03-16
DOI	https://doi.org/10.14943/bull.fish.68.1.7
Doc URL	https://hdl.handle.net/2115/68502
Type	departmental bulletin paper
File Information	bull.fish.68.1.7.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Bull. Fish. Sci. Hokkaido Univ.
68(1), 7–16, 2018.
DOI 10.14943/bull.fish.68.1.7

Different Nutrient Availabilities of Surface and Bottom Water under Nutrient-depleted Conditions during Bloom Formation of the Toxic Dinoflagellate *Alexandrium tamarense* in Osaka Bay, Japan

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(Received 17 November 2017, Accepted 11 January 2018)

Abstract

Alexandrium tamarense is a toxic dinoflagellate known to produce neurotoxins cause paralytic shellfish poisoning to human and marine animals. To understand the growth dynamics of *A. tamarense*, the seasonal changes in *A. tamarense* vegetative cells and environmental factors were evaluated using monthly field observations at two fixed stations in Osaka Bay, Japan, from January to May 2008. Additionally, a bioassay with axenic *A. tamarense* clonal cultures was performed to determine the growth potentials and growth-limiting nutrients of seawater samples collected during the field observations. The density of *A. tamarense* increased from February to April, and depletions of dissolved phosphate and silicate were observed in the surface layer during this period. The bioassay showed that phosphorous limitation occurred at the surface water of one station during March and April, while nitrogen limitation occurred in the bottom water. Moreover, at the other station, the growth potentials of the bottom water were higher than those of the surface water during February and April. Thus, the differences of nutrient availabilities between surface and bottom water during spring in Osaka Bay potentially allow *A. tamarense* to grow with nutrients uptake from bottom water by vertical migration.

Key words : *Alexandrium tamarense*, Osaka Bay, Growth potential, Limiting nutrient, Vertical migration

Introduction

Paralytic shellfish poisoning (PSP) is a marine toxin disease, caused primarily by the consumption of poisoned bivalves that afflicts humans and marine mammals. PSP significantly impacts human health and fisheries of cultured and wild bivalves. The toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech, which has a worldwide distribution, is among the most harmful algae that cause PSP (Hallegraeff, 1993 ; Lilly et al., 2007). In Japan, *A. tamarense* shellfish poisoning has mainly occurred in the Tohoku and Hokkaido regions since the 1970s. In addition, the contamination of bivalves by *A. tamarense* toxin has occurred in the Seto Inland Sea, including Osaka Bay, after the 1990s (Imai et al., 2006).

Osaka Bay is a semienclosed and highly eutrophicated embayment, located in the eastern part of the Seto Inland Sea, with an area of approximately 1,450 km^2. The eastern part of the bay has a flat bottom, with a mean water depth of approximately 15 m, and its innermost portion occasionally shows strong stratification due to water runoff from the Yodo River (Fig. 1; Joh, 1986; Fujiwara and Nakata, 1991). Yamamoto (2004) reported that *A. tamarense* has occurred in this area since the 1990s, and the alga has since been monitored by the Osaka Prefectural Fisheries Experimental Station. Yamaguchi et al. (1996) also demonstrated

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that *A. tamarense*/*catenella* (Whedon & Kofoid) cysts were widely distributed in the bottom sediments of the bay. These reports indicate that *A. tamarense* inhabited Osaka Bay prior to the 2000s but did not form the dense blooms that cause shellfish poisoning in excess of the regulatory level (4 MU g^{-1} edible portion ; notification by Ministry of Health, Labour and Welfare could available on http://www.mhlw.go.jp/topics/syokuchu/poison/animal_09.html). *A. tamarense* blooms occurred at 3.7×10^4 cells L^{-1} in the eastern regions of Osaka Bay in spring 2002, and a PSP toxicity of 18.0 MU g^{-1} was detected in Manila clams collected from these regions (Yamamoto, 2004). In spring 2007, *A. tamarense* formed massive blooms of 7.27×10^7 cells L^{-1}, which caused red tides (Yamamoto et al., 2009). Since 2007, high toxin contamination levels of bivalves due to an excess of this species have frequently occurred in the eastern part of the bay during the spring. These facts strongly suggest that environmental conditions in the eastern part of the bay favor the growth of *A. tamarense*.

Several studies have examined the relationships between *A. tamarense* blooms and environmental conditions in Osaka Bay. Yamamoto et al. (2009) reported that low dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorous (DIP) concentrations occurred at the surface layer during the 2007 *A. tamarense* bloom in the eastern part of Osaka Bay. Itakura et al. (2002) also reported the depletion of DIP and dissolved silicate levels during *A. tamarense* bloom periods in Hiroshima Bay of the Seto Inland Sea. These authors proposed that the exhaustion of inorganic nutrients by diatoms in the winter caused the depletion of nutrients preceding the *A. tamarense* blooms during spring. Moreover, Yamamoto et al. (2002b) regarded phosphorous as a liming nutrient for *A. tamarense* growth in Hiroshima Bay. From these prior studies, nutrient availabilities under low nutrient concentrations were thought to be important in understanding favorable environmental conditions for *A. tamarense* growth in Osaka Bay.

Yamamoto et al. (2010) reported that *A. tamarense* practiced diel vertical migration at a fishing port in Osaka Bay and suggested that *A. tamarense* cells might supplement NO_2-N and NO_3-N intake from the bottom water. The importance of vertical migration and nitrate availability in deeper water for the growth of this species has also been suggested by laboratory experiments (MacIntyre et al., 1997) and by field observations in the St. Lawrence estuary, Canada (Fauchot et al., 2005). Moreover, Yamamoto et al. (2002b) simulated competition between *A. tamarense* and the nontoxic diatom *Skeletonema costatum* in Hiroshima Bay using a numerical model that considered DIP uptake by *A. tamarense* from the bottom water after vertical migration ; the results indicated that *A. tamarense* could form blooms even if DIP concentrations were low. Therefore, low nutrient concentrations in surface waters and greater nutrient availability in bottom waters, as accessed by diel vertical migration, most likely play important roles in the succession of *A. tamarense* in Osaka Bay during the spring. Although prior studies in Osaka Bay have reported on the DIN and DIP depletion of the surface water during *A. tamarense* blooms and the diel vertical migration of this species (Yamamoto et al., 2009 and 2010), the nutrients that limit the growth of *A. tamarense* in surface waters and the ability of *A. tamarense* to supplement these growth-limiting nutrients from bottom waters remain unclear.

To clarify the favorable environmental conditions for *A. tamarense* growth, the nutrient dynamics in the surface and bottom waters in the eastern part of Osaka Bay were examined during the *A. tamarense* bloom period. Field observations were performed monthly to assess the environmental conditions in the surface and bottom waters during the *A. tamarense* bloom period, including water temperature, salinity, DIN, DIP, dissolved silicate, chlorophyll *a* (Chl *a*), and other phytoplankton. Moreover, to evaluate the growth potentials and growth-limiting nutrients in the surface and bottom waters, a bioassay with axenic *A. tamarense* clonal cultures was performed using seawater samples collected during the field observations.

Materials and Methods

Field observations

Monthly seawater sampling was performed at two fixed stations (Sts. 11 and 13) in the eastern part of Osaka Bay from January to May 2008 (Fig. 1). The estuarine circulation from the inner part of Osaka Bay primarily spreads to the south along the east coast of the bay (Joh, 1986 ; Fujiwara and Nakata, 1991). The seawater samples were collected with a Kitahara water sampler at depths of 0, 5, and 10 m during the daytime (13 : 00–15 : 30), and the samples were stored in 500-mL acid-rinsed polyethylene bottles on ice. The salinity and water temperature were measured using a CTD (ACL215-DK, JFE Advantech Co. Ltd.). Upon their return to the laboratory, the seawater samples were immediately filtered through a GF/F glass fiber filter (Whatman), and the filtrates were stored at −30°C until analysis. The concentrations of dissolved inorganic nutrients, including DIN (NH_4-N+NO_2-N+NO_3-N), PO_4-P, and SiO_2-Si, were measured using a continuous flow analyzer (Swatt, BL TEC K.K.) according to the methods of Strickland and Parsons (1968). The detection limit for all nutrients was 0.01 μM. All equipment for the nutrient analyses was rinsed with 3 N hydrochloric acid prior to use.

The Chl *a* from the phytoplankton collected on the filters was extracted with 90% acetone, and the concentrations were measured using a fluorometer (10AU005, Turner Designs). For the enumeration of *A. tamarense* and the dominant phytoplankton species, a portion of the seawater samples was fixed with formaldehyde at a final concentration of 0.37% and concentrated 10-fold using the settling method (Utermöhl,

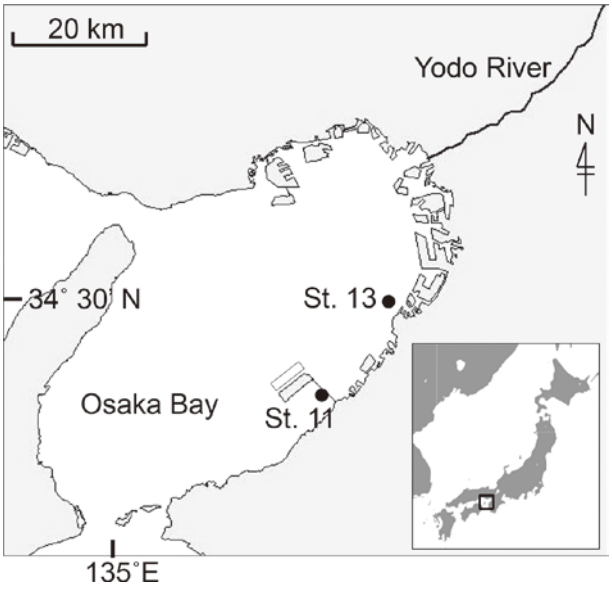

Fig. 1. Locations of the sampling stations in the Osaka Bay (●).

1958). *A. tamarense* cells were counted for the samples from 0, 5 and 10 m in depth, while the dominant phytoplankton species was determined for the 0 m samples. The identification and counts of the dinoflagellates were performed with an epifluorescence inverted microscope (ECLIPSE TE200, Nikon) under UV light (350 nm) excitation after staining the thecal plates with calcofluor-white according to the methods of Fritz and Triemer (1985). The identification and counts of the other dominant phytoplankton were also performed with an inverted microscope (ECLIPSE TE200, Nikon). Species identification was performed according to the methods of Tomas (1997), and the detection limit for *A. tamarense* was 3.3×10^1 cells L^{-1}.

Bioassay experiments with A. tamarense

The strain of *A. tamarense* used for the bioassay was originally isolated from Osaka Bay in 2007, and an axenic clone culture was established following the swimming method of Imai and Yamaguchi (1994). This culture was maintained in modified SWM-3 medium (Chen et al., 1969 ; Imai et al., 1996 ; autoclaved at 121°C for 20 min) at 15°C, with a 12-h light : 12-h dark cycle and a light intensity of 110-130 µmol photons m^{-2} s^{-1}.

Preincubation was performed twice, as described by Nishijima and Hata (1991) and Kimura et al. (1999), to prepare the nutrient-starved culture. During the first preincubation, 1 mL of axenic *A. tamarense* culture was inoculated into 100 mL of 1/20-diluted modified SWM-3 medium. The culture was grown for 17 days until it reached the stationary phase. The second preincubation was performed by inoculating 1 mL of each culture from the first preincubation into 100 mL of 1/100-diluted modified SWM-3 medium. Similarly to the first preincubation, *A. tamarense* was incubated for 14 days until it reached the stationary phase. Acid-rinsed polycarbonate bottles (Nalgene Nunc) were used for the preincubations. The seawater for the preincubations was collected from Osaka Bay in April 2008.

Table 1. Contents and final concentrations of the added nutrients to seawater samples for the bioassay. *P-I metals contain H_3BO_3 (100 µM), $MnCl_2 \cdot 2H_2O$ (3.5 µM), $ZnCl_2$ (0.4 µM), $CoCl_2 \cdot 6H_2O$ and $CuCl_2 \cdot 2H_2O$ (0.1 µM). **S-3 vitamins contain vitamin B_1-HCl (0.05 mg L^{-1}), Ca-pantothenate (0.01 mg L^{-1}), nicotinic acid (0.01 mg L^{-1}), p-aminobenzonic acid (1.0 µg L^{-1}), biotin (0.1 µg L^{-1}), inositol (0.5 mg L^{-1}), folic acid (0.2 µg L^{-1}), thymine (0.3 mg L^{-1}), vitamin B_{12} (0.1 µg L^{-1}).

Abbreviation	Added nutrients	Final conc.
+none	Only Ultrapure-water	
+N	$NaNO_3$	200 µM
+P	NaH_2PO_4	10 µM
+Si	Na_2SiO_3	10 µM
+trace elements	FeEDTA	0.2 µM
	Na_2SeO_3	0.2 nM
	$Na_2MoO_4 \cdot 2H_2O$	10 nM
	P-I metals	*
	Na_2EDTA	3 µM
+vitamins	S-3 vitamins	**

Concentrations of each added nutrients was set for one-tenth of modefied SWM-3 medium

Seawater samples collected from 0 and 10 m in depth at Sts. 11 and 13 from January to April 2008 were used as the experimental media for the bioassay. The seawater samples, having been prefiltered through a GF/F filter after the field samplings, were then sterilized through a sterile 0.2 µm polyethersulfone syringe filter (Minisart, Sartorius). A 3.6-mL aliquot of each sterile seawater sample was dispensed into a polystyrene sterile test tube (13-mm diameter × 100-mm length, Evergreen Scientific). In addition, 0.4-mL aliquots of nutrient solutions (+none, +N, +P, +trace elements, or +vitamins) were added to the media. The contents and final concentrations of the nutrient-added media are shown in Table 1.

After the second preincubation, a 0.1-mL aliquot of *A. tamarense* culture was inoculated into each experimental medium, and the cells were incubated until maximum growth was recorded. The growth was monitored by measuring the *in vivo* Chl *a* fluorescence using a fluorophotometer (10AU005, Turner Designs) every other day. Culturing was performed in triplicate for each medium.

Statistical analysis of the bioassay

The *A. tamarense* cells in the experimental tubes were enu-

merated after the maximum growth measurements, and a significant simple regression line was determined for *in vivo* Chl *a* fluorescence and *A. tamarense* cell density ($r = 0.998$, $p < 0.001$). Using this regression line, units of maximum growth were converted from *in vivo* Chl *a* into cell density. The maximum growths of the +none treatments were regarded as the growth potentials of *A. tamarense* and are shown in Fig. 4. To clarify the differences in the growth potentials of the seawater samples between months and depths, multiple comparisons were conducted using Tukey's test and the t-test, respectively.

To detect significant differences in the maximum growths among the media with added nutrients for each seawater sample, multiple comparisons were performed using Dunnett's test. Differences at $p < 0.05$ were considered significant. When the maximum growth of a nutrient-added medium was significantly higher than that of the comparable +none treatment, the added nutrients were regarded as the growth-limiting nutrients in the seawater sample. The results of the bioassay to identify the growth-limiting nutrients are shown in Fig. 5. The maximum growths are indicated as the relative growth (%) compared to the growth of the blank (+none treatment) as 100% in each seawater sample.

Results

Field observation

Water temperature ranged from 8.7°C to 15.1°C and reached its lowest value between February and March. The salinity varied from 30.0 to 32.8 psu (Fig. 2). The thermocline and halocline clearly developed at both stations in April between 0 and 5 m. The inorganic nutrient concentrations at both stations and for all depths were highest in January (Fig. 2). The total average concentrations were 16 μM for DIN, 0.63 μM for PO_4-P, and 14 μM for SiO_2-Si. In February, all concentrations significantly decreased, with total average concentrations reaching 5.1 μM for DIN, 0.09 μM for PO_4-P, and 0.48 μM for SiO_2-Si (Fig. 2). From March through April, all inorganic nutrient concentrations, particularly those of PO_4-P and SiO_2-Si at 0 and 5 m in depth, were comparatively low (PO_4-P, 0.02-0.05 μM ; SiO_2-Si, <0.01-1.5 μM). However, the concentrations of PO_4-P and SiO_2-Si at 10 m in depth (PO_4-P, 0.07-0.26 μM ; SiO_2-Si, 1.7-7.4 μM) were relatively higher than at 0 and 5 m during March and April. During March and April, the DIN concentrations at 0 and 5 m at St. 11 (1.6-2.1 μM) were much lower than those at St. 13 (4.4-12.8 μM). In May, all nutrient concentrations, except those of PO_4-P, increased at all depths and stations ; the total average concentrations were 5.7 μM for DIN, 0.20 μM for PO_4-P, and 13.1 μM for SiO_2-Si (Fig. 2).

Figure 3 shows the monthly changes in the cell densities of *A. tamarense* and Chl *a* concentrations at 0, 5, and 10 m at Sts. 11 and 13. At both stations, *A. tamarense* was detected from January to April and formed blooms from March to April. The maximum cell densities were 1.6×10^4 cells L^{-1} at St. 11 and 3.9×10^3 cells L^{-1} at St. 13. *A. tamarense* was initially detected in January near the detection limit (appro. 1.0×10^2 cells L^{-1} at both stations) and occurred at relatively low densities (<4.0×10^2 cells L^{-1}) in February. The cell densities at both stations increased to over 1.0×10^3 cells L^{-1} from March to April and then peaked in April (3.9×10^3 cells L^{-1} at St. 13 and 1.6×10^4 cells L^{-1} at St. 11). *A. tamarense* was extensively distributed between 0 and 5 m during the bloom periods. In May, after the bloom, *A. tamarense* disappeared from the water column. The Chl *a* concentrations in January were relatively low, with an average concentration of 1.8 μg L^{-1}, but increased in February at all depths to 8.1-18.4 μg L^{-1} because of the winter diatom bloom, which mainly consisted of *Chaetoceros* spp. (5.4×10^2-6.5×10^2 cells mL^{-1}) and *Eucampia zodiacus* (2.2×10^2-3.2×10^2 cells mL^{-1}). After this diatom bloom, the Chl *a* concentration significantly decreased at both stations in March, averaging 5.6 μg L^{-1}, and then increased because of a dense bloom of the genus *Skeletonema* at 0 m at both stations in April. Chl. *a* concentrations in this month ranged from 11 to 22 μg L^{-1}, and the cell density of *Skeletonema* spp. varied from 2.1×10^3 to 1.4×10^4 cells mL^{-1}. During the sampling period, diatoms were completely dominant, with cell densities reaching 6.0×10^0-1.4×10^4 cells mL^{-1}. The next most-dominant phytoplankton group was dinoflagellates.

Bioassay

The seasonal changes in the growth potentials of the seawater samples collected at 0 and 10 m in depth from Sts. 11 and 13 in Osaka Bay during January to May 2008 are represented in Fig. 4. The growth potentials of *A. tamarense* ranged from 7.3×10^4 cells L^{-1} to 9.6×10^5 cells L^{-1}. This range exceeded the maximum cell density of *A. tamarense* detected during the field observations. The growth potentials in January were much higher than those of the other months at both stations. No significant differences between months were detected at either station during February to May. A significantly higher growth potential at 0 m than at 10 m was only detected in January at St. 13. Conversely, the growth potentials at 10 m in February and April at St. 11, and in May at St. 13, were significantly higher than those at 0 m.

Figure 5 presents the results of the bioassay with *A. tamarense* to identify the growth-limiting nutrients of the seawater samples collected at 0 and 10 m in depth from St. 11 and 13 in Osaka Bay during January to May 2008. In January, the main growth-limiting nutrient for *A. tamarense* was nitrogen in all seawater samples except that from 0 m at St. 13, for which the growth-limiting nutrients were nitrogen and phosphorous. Phosphorous limited *A. tamarense* growths at all

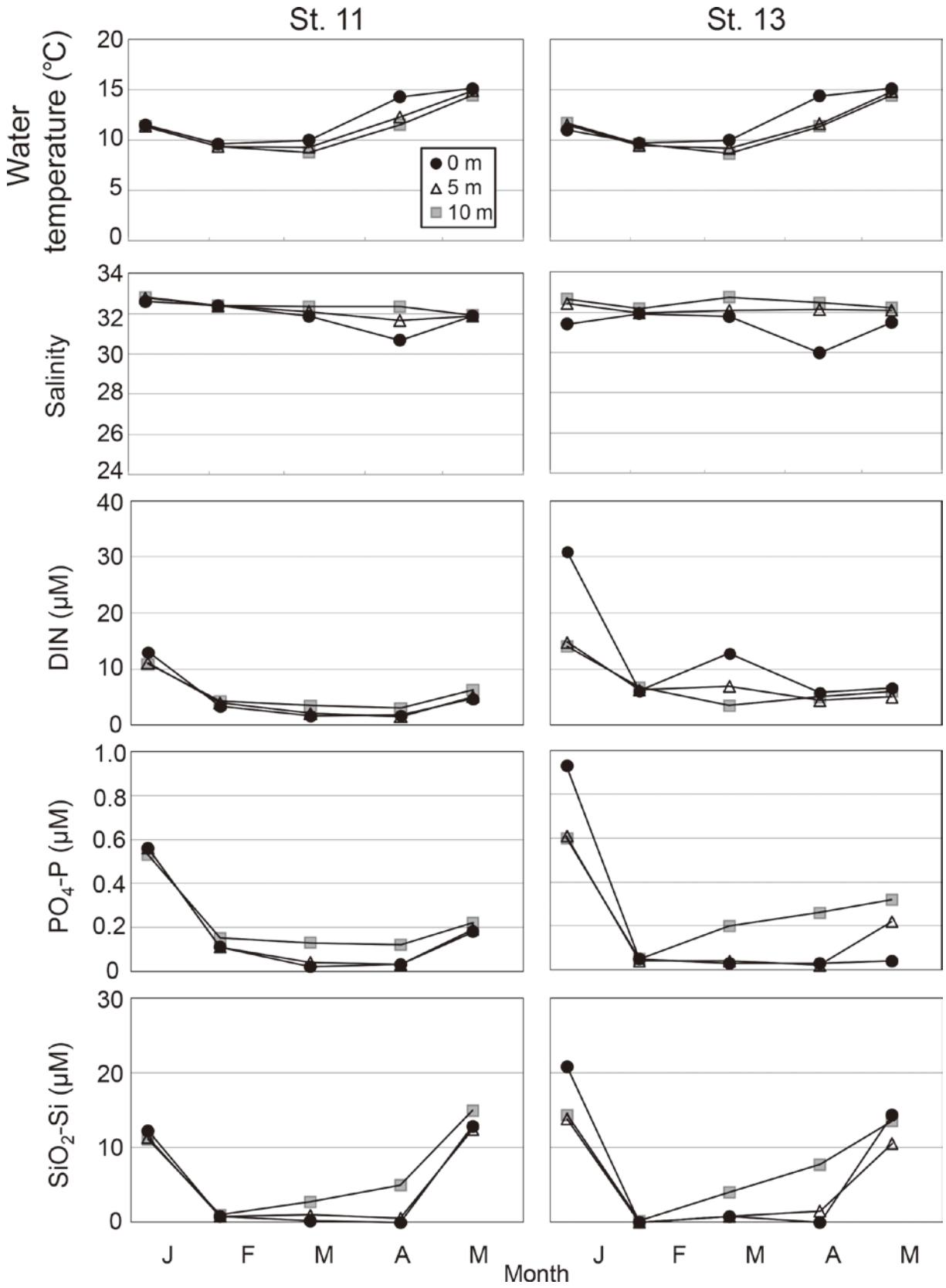

Fig. 2. Monthly changes in the water temperature (°C), salinity (PSU), dissolved inorganic nitrogen concentration (DIN ; μM), phosphate concentration (μM), and silicate concentration (μM) at Sts. 11 and 13 in the eastern part of Osaka Bay from January to May 2008.

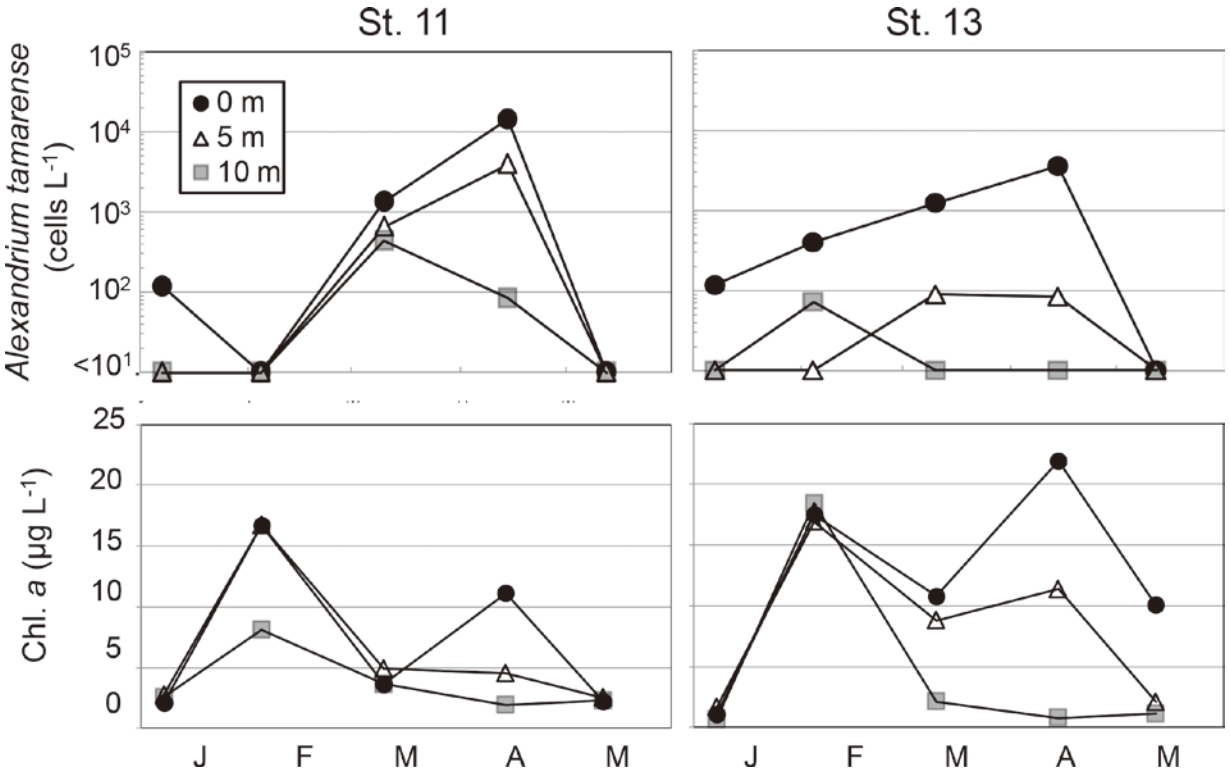

Fig. 3. Seasonal changes in the cell densities of the toxic dinoflagellate *Alexandrium tamarense* (cells L^{-1}) and chlorophyll *a* (Chl *a* ; μg L^{-1}) at Sts. 11 and 13 in the eastern part of Osaka Bay from January to May 2008. N.D.= not detected.

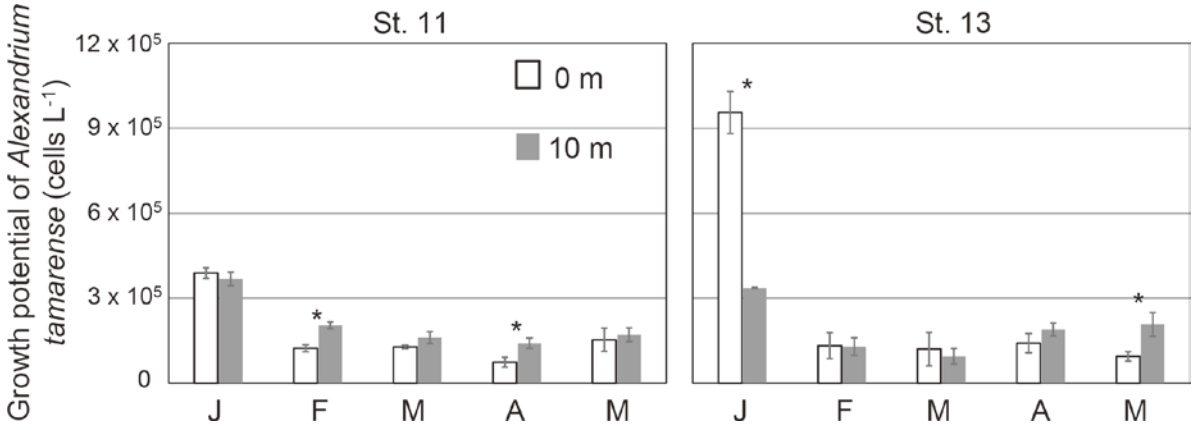

Fig. 4. Results of the bioassay with *Alexandrium tamarense* to determine the growth potentials of seawater samples collected at 0 and 10 m in depth at Sts. 11 and 13 in the eastern part of Osaka Bay from January 2008 to May 2008. An asterisk (*) indicates a significant difference between growth potentials at 0 and 10 m ($P < 0.05$). Error bars indicate standard deviation.

depths and stations in February, except at 10 m at St. 11. The growth of *A. tamarense* in seawater samples from 0 m at St. 13 was limited by phosphorous from March to May. By contrast, nitrogen limitation was observed in the 10 m samples from St. 13 during March to April. No nutrient limitation was observed at St. 11 from March to April or at 0 m at Sts. 11 and 13 in May. The growth-limiting nutrient at 10 m at St. 13 in May was nitrogen.

Discussion

The depletion of inorganic nutrients, particularly DIP and dissolved silicate, and the corresponding occurrences of *A. tamarense* blooms have been widely observed at the surface layer in the eastern part of Osaka Bay from March to April (Figs. 2 and 3). Yamamoto and Tarutani (1999) performed semicontinuous culture experiments and reported that the phosphate concentration required for maintaining the maximum growth of *A. tamarense* isolated from Hiroshima Bay

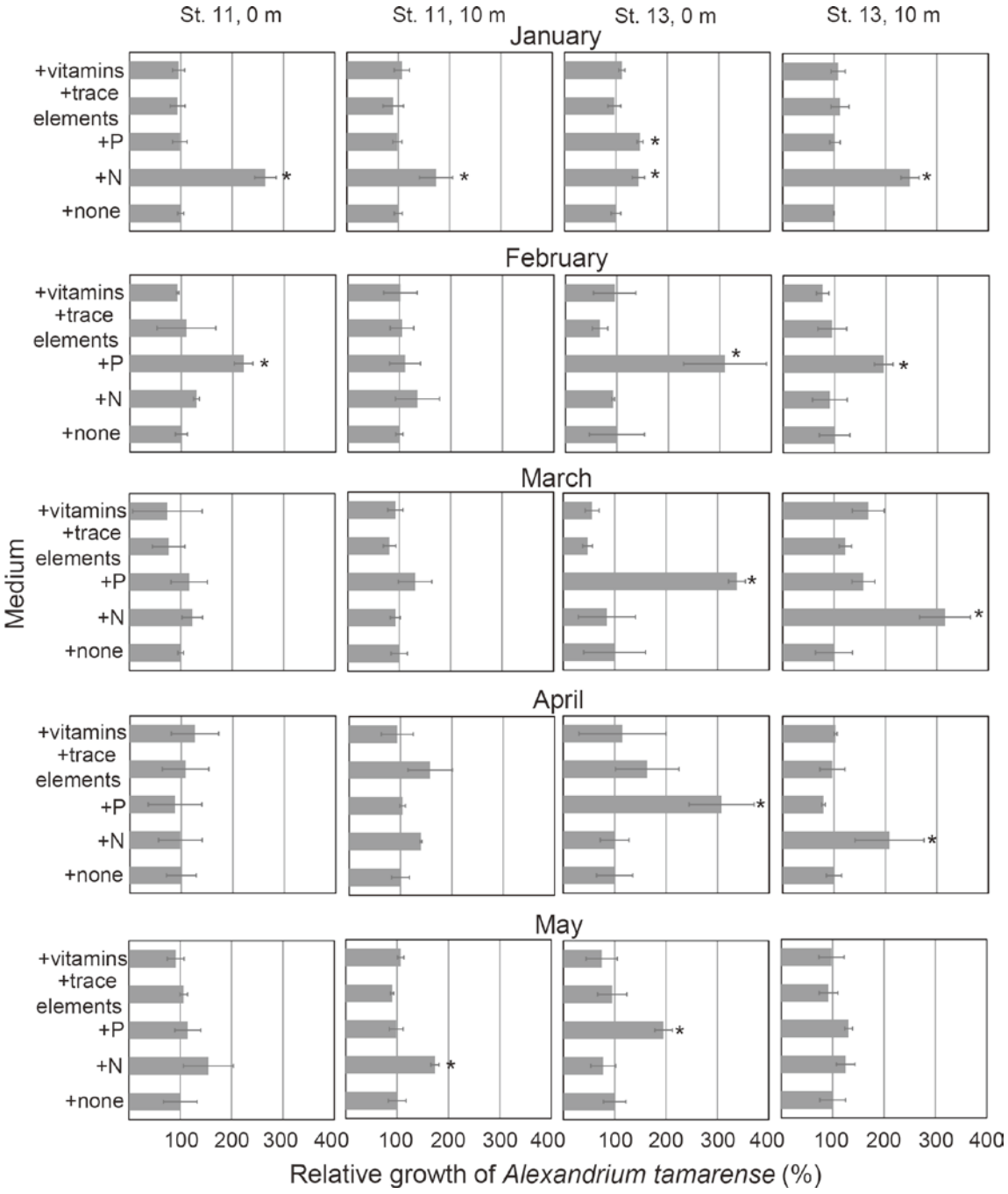

Fig. 5. Results of the bioassay with *Alexandrium tamarense* to determine the growth-limiting nutrients of seawater samples collected from 0 and 10 m in depth at Sts. 11 and 13 in the eastern part of Osaka Bay from January 2008 to May 2008. An asterisk (*) next to a nutrient-added medium indicates a significant increase of the treatment mean over that of the +none treatment. Treatment means were analyzed using Dunnett's multiple comparison test ($P < 0.05$). Error bars indicate standard deviation. Data represent the means of $n = 3$ replicates.

was 0.12 μM. During the *A. tamarense* bloom period in the present study, the observed concentrations of DIP at the surface layer were much lower than this value (Fig. 2). Furthermore, the observed concentrations of dissolved silicate at the surface layer from March to April (Fig. 2) were mostly lower than the reported threshold silicate concentration (2 μM) required to sustain the highest potential growth rate of diatoms (Egge and Aksnes, 1995). Consequently, the lack of inorganic nutrients at the surface layer during the spring was postulated to significantly limit the growth of *A. tamarense* and competitive diatoms in the bay.

The bioassay experiment indicated that phosphorous limitation occurred in the surface water, whereas nitrogen limitation occurred in the bottom water collected from St. 13 during

March and April (Fig. 5). Moreover, the bioassay revealed higher growth potentials in the bottom water than in the surface water from St. 11 in February and April (Fig. 4). These results suggested that the bottom waters in the eastern part of Osaka Bay contained excess nutrients during spring, which *A. tamarense* could gather using diel vertical migration to a deeper layer at night. Fauchot et al. (2005) have reported the diel vertical migration of *A. tamarense* from a depth of 2–4 m during the daytime to below the nutricline, formed at a depth of over 10 m, during the nighttime in the St. Lawrence estuary. The diel vertical migration of *A. tamarense* has also been observed at a station located in a fishing port of Osaka Bay (water depth of 5 m) during an *A. tamarense* red tide (Yamamoto et al., 2010). These reports support that *A. tamarense* is able to vertically migrate below the 10 m layer in Osaka Bay. The maximum yields determined by the bioassay ranged from 7.4×10^4 cells L^{-1} to 1.4×10^5 cells L^{-1} in the surface water during February to April (Fig. 4), when nutrients concentrations were quite low. These yields were approximately 5–10 times higher than the maximum cell density of *A. tamarense* (1.4×10^4 cells L^{-1}) observed in the field (Fig. 3). Therefore, *A. tamarense* could obtain sufficient nutrients to bloom, as was observed during the field observations, but low nutrients concentrations limited its growth and that of the dominant diatoms. In addition, *A. tamarense* growth potentials were expected to increase by approximately threefold at St. 13 during March and April and by 1.7- to 1.9-fold at St. 11 during February and April (Fig. 4), through utilizing the nutrients in the bottom water for growth. These growth potentials were estimated at 2.1×10^5–4.2×10^5 cells L^{-1}, assuming that *A. tamarense* could fully utilize the nutrients in the bottom water by diel vertical migration. These values reached approximately 35% to 70% of the maximum cell densities observed at fixed stations by Yamamoto et al. (2009) in Osaka Bay during the massive *A. tamarense* bloom period in 2007. The results of the bioassay suggested that the nutrient availability in the bottom water in the eastern part of Osaka Bay during spring, accessible by diel vertical migration, was potentially advantageous for the growth of *A. tamarense* and contained part of nutrients necessary for the species to develop a massive bloom.

The present bioassay identified different growth-limiting nutrients and growth potentials between surface and bottom water samples during the *A. tamarense* bloom period in most cases, but no differences were observed among seawater samples collected from St. 11 in March (Figs. 4 and 5). This observation implies that *A. tamarense* obtains the essential nutrients necessary for growth by other mechanisms. For example, Jeong et al. (2010) have reported that *A. tamarense* can ingest heterotrophic bacteria, pico-sized cyanobacteria, or various nanophytoplankton such as haptophytes and cryptophytes. Tada et al. (2003) investigated the seasonal size fractionation of phytoplankton in Osaka Bay and concluded that pico- and nanophytoplankton occurred in all seasons and accounted for 33% and 31%, respectively of the total Chl *a* in the bay. Although the present study did not consider the contribution of particulate matter, including pico- and nanophytoplankton, to the growth of *A. tamarense*, the intake of such matter through phagotrophy by this species in the field should be addressed in the future.

The water temperature and salinity observed during the sampling period were within the ranges at which *A. tamarense* strains can grow (Watras and Chisholm, 1982 ; Yamamoto et al., 1995 ; Yamamoto and Tarutani, 1997) and at which blooms have been observed in Osaka Bay (Yamamoto, 2004 ; Yamamoto et al., 2009). Therefore, water temperature and salinity are not always the only important factors controlling the growth of *A. tamarense*. However, the water stratification observed during the *A. tamarense* bloom period was hypothesized to prevent the constant growth of diatoms because of the lack of DIP and dissolved silicate in the surface layer. Therefore, it is difficult for the species to maintain constant populations. Under the conditions of water stratification formed by a river water plume, *A. tamarense* bloom formations have been reported at the St. Lawrence estuary and the Gulf of Maine (Therriault et al., 1985 ; Franks and Anderson, 1992 ; Fauchot et al., 2008). Water stability also contributed to the formation of *A. tamarense* blooms in Hiroshima Bay (Itakura et al., 2002 ; Yamamoto et al., 2002a). For these reasons, low nutrient concentrations in the surface water and relatively shallow water stratification (10 m or less) are important factors for the formation of *A. tamarense* blooms.

The present study partially identified the nutrient conditions leading to *A. tamarense* bloom formation in Osaka Bay during the spring (Fig. 2). Diatom blooms in late winter usually occur under nutrient-enriched and moderate vertical mixing conditions in the water column. In the present study, high Chl *a* concentrations (Fig. 3) and a bloom of large diatoms, including *Chaetoceros* spp. and *E. zodiacus*, occurred in February. This winter diatom bloom was hypothesized to induce the heavy consumption of nutrients from January to February. Large diatom blooms and the heavy consumption of nutrients in winter have also been reported in Hiroshima Bay before *A. tamarense* blooming (Itakura et al., 2002). Likewise, the decrease of nutrients and increase of *Chaetoceros* spp. cell density in winter were observed before the bloom formation of *A. tamarense* in Osaka Bay in 2007 (Yamamoto et al., 2009). These previous studies also indicated that the depletion of nutrients and water stratification continued during late winter to spring. The continuous depletion of nutrients in the surface water after a winter diatom bloom is thought to be an important factor contributing to the expansion of *A. tamarense* blooms in the eastern part of Osaka Bay, as has been observed in Hiroshima Bay. A lack of nutrients limited diatom growth in the surface water, but

the present study suggested that vertical migration below the nutricline during the night enabled *A. tamarense* to grow through the nocturnal uptake of nutrients in this deeper layer.

Throughout the world, eutrophication has caused numerous environmental problems for coastal marine ecosystems, such as harmful algal blooms and oxygen depletion (reviewed by Rosenberg, 1985 ; Boesch and Rabalais, 1991). The Seto Inland Sea of Japan, including Osaka Bay, is one of the most eutrophicated areas in Japan, and serious damage to fisheries due to harmful red tides of raphidophytes and dinoflagellates has occurred since the 1970s (Imai et al., 2006). The scale and number of these harmful red tides have recently begun to decrease because of the success of water-purity controls, such as regulatory laws and improvements in sewage disposal facilities (Imai et al., 2006). The present study suggested that recent occurrences of toxic *A. tamarense* blooms in Osaka Bay have been highly related to the low conventional (DIN and DIP) nutrient concentrations in the surface water, as has previously been observed in Hiroshima Bay. Moreover, different growth-limiting nutrients and growth potentials were observed between the surface and bottom waters during the *A. tamarense* bloom period, suggesting that the diel vertical migration ability of the species and its uptake of nutrients in bottom waters have been advantageous under low nutrient concentrations in recent years. Thus, recent nutrients decrease potentially lead to the massive occurrences of the toxic species while the decrease of red tide occurrences.

Acknowledgments

The authors are grateful to the captain and crews of the research vessel 'Osaka' of the Marine Fisheries Research Center, Osaka Prefectural Government for assistance of field survey. Prof. M. Yamaguchi (Kitasato University), Prof. I. Yoshinaga (Tottori Environment University) and T. Ajisaka (Kyoto University) provided helpful advice and encouragement. This study was supported by a grant from The Association for the Environmental Conservation of the Seto Inland Sea.

References

Boesch, D.F. and Rabalais, N.N. (1991) Effects of hypoxia on continental shelf benthos : comparison between the New York Bright and the Northern Gulf of Mexico. pp. 27-34, Tyson, R.V. and Pearson, T.H. (eds), *Modern and Ancient Continental Shelf Anoxia*, Geological Society Special Publication, London.

Chen, L.C.M., Edelstein, T. and McLachlan, J. (1969) *Bonnemaisonia hamifera* Hariot in nature and in culture. *J. Phycol.*, **5**, 211-220.

Egge, J.K. and Aksnes, D.L. (1992) Silicate as regulating nutrient in phytoplankton competition. *Mar. Ecol. Prog. Ser.*, **83**, 281-289.

Fauchot, J., Levasseur, M. and Roy, S. (2005) Daytime and nighttime vertical migrations of *Alexandrium tamarense* in the St. Lawrence estuary (Canada). *Mar. Ecol. Prog. Ser.*, **296**, 241-250.

Fauchot, J., Saucier, F.J., Levasseur, M., Roy, S. and Zakardjian, B. (2008) Wind-driven river plume dynamics and toxic *Alexandrium tamarense* blooms in the St. Lawrence Estuary (Canada) : A modeling study. *Harmful algae*, **7**, 214-227.

Franks, P.J.S. and Anderson, D.M. (1992) Alongshore transport of a toxic phytoplankton bloom in a buoyancy current : *Alexandrium tamarense* in the Gulf of Maine. *Mar. Biol.*, **116**, 153-64.

Fritz, L. and Triemer, R.E. (1985) A rapid technique utilizing Calcofluor white M2R for the visualization of dinoflagellate thecal plate. *J. Phycol.*, **21**, 662-664.

Fujiwara, T. and Nakata, H. (1991) Flow characteristics governing the distribution of water and bottom quality in a semi-enclosed sea. *Mar. Pollution Bull.*, **23**, 675-678.

Hallegraeff, G. (1993) A review of harmful algal blooms and their apparent global increase. *Phycologia*, **32**, 79-99.

Imai, I. and Yamaguchi, M. (1994) A simple technique for establishing axenic cultures of phytoflagellates. *Bull. Jpn. Soc. Microb. Ecol.*, **9**, 15-17.

Imai, I., Itakura, S., Matsuyama, Y. and Yamaguchi, M. (1996) Selenium requirement for growth of a novel red tide flagellate *Chattonella verruculosa* (Raphidophyceae) in culture. *Fish. Sci.*, **62**, 834-835.

Imai, I., Yamaguchi, M. and Hori, Y. (2006) Eutrophication and occurrences of harmful algal blooms in the Seto Inland Sea, Japan. *Plankton and Benthos Res.*, **1**, 71-84.

Itakura, S., Yamauchi, M., Yoshida, M. and Fukuyo, Y. (2002) The seasonal occurrence of *Alexandrium tamarense* (Dinophyceae) vegetative cells in Hiroshima Bay, Japan. *Fish Sci.*, **68**, 77-86.

Joh, H. (1986) Studies on the mechanism of eutrophication and the effect of it on fisheries production in Osaka Bay. *Bull. Osaka Pref. Fish. Exp. Stat.*, **7**, 1-174. (in Japanese)

Jeong, H.J., Yoo, Y.D., Kim, J.S., Seong, K.A., Kang, N.S. and Kim, T.H. (2010) Growth, feeding and ecological roles of the mixotrophic and heterotrophic dinoflagellates in marine planktonic food webs. *Ocean Sci. J.*, **45**, 65-91.

Kimura, B., Kamizono, M., Etoh, T., Koizumi, Y., Murakami, M. and Honjo, T. (1999) Population development of the red tide dinoflagellate *Gymnodinium mikimotoi* in inshore waters of Japan. *Plankton Biol. Ecol.*, **46**, 37-47.

Lilly, E.L., Halanych, K.M. and Anderson, D.M. (2007) Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). *J. Phycol.*, **43**, 1329-1338.

MacIntyre, J.G., Cullen, J.J. and Cembella, A.D. (1997) Vertical migration, nutrition and toxicity in the dinoflagellate *Alexandrium tamarense*. *Mar. Ecol. Prog. Ser.*, **148**, 201-216.

Nishijima, T. and Hata, Y. (1991) Growth potentials of red tide phytoplankters in coastal seawater by AGP assay. *Mar. Pollution Bull.*, **23**, 175-179.

Rosenberg, R. (1985) Eutrophication-the future marine coastal nuisance ? *Mar. Pollution. Bull.*, **16**, 227-231.

Strickland, J.D.H. and Parsons, T.R. (1968) A practical handbook of seawater analysis. *Fish. Res. Board of Can. Bull.*, **167**, 1-311.

Tada, K., Ichimi, K. and Hashimoto, T. (2003) Size fractionations of phytoplankton community in Osaka Bay and Kii channel. *Bull. Coastal Oceanogr.*, **40**, 197-204. (in Japanese with English abstract)

Therriault, J.C., Painchaued, J. and Levasseur, M. (1985) Factors controlling the occurrence of *Protogonyaulax tamarensis* and shellfish toxicity in the St. Lawrence Estuary : fresh water runoff and the stability of the water column. pp.141–146, Anderson, D.M., White, A.W. and Banden, D.G. (eds), *Toxic Dinoflagellates*, Elsevier Science, New York.

Tomas, C.R. (1997) Identifying marine phytoplankton. Academic Press, San Diego.

Watras, C.J. and Chisholm, S.W. (1982) Regulation of growth in an estuarine clone of *Gonyaulax tamarensis* lebour : salinity–dependent temperature respons. *J. Exp. Mar. Biol. Ecol.*, **62**, 25–37.

Utermöhl, H. (1958) Zur Vervollkommnung der quantitative Phytoplankton–Methodik. *Mitt Int Ver Theor Angew Limnol*, **9**, 1–38. (In Italian with English abstract)

Yamaguchi, M., Itakura, S., Nagasaki, K. and Imai, I. (1996) Distribution and abundance of resting cysts of the toxic dinoflagellates *Alexandrium tamarense* and *A. catenella* in sediments of the eastern Seto Inland Sea, Japan. pp. 177–180, Yasumoto, T., Oshima, Y. and Fukuyo, Y. (eds), *Harmful and Toxic Algal Blooms*, Inter Governmental Oceanographic Commission of UNESCO, Paris.

Yamamoto, K. (2004) Occurrence of paralytic shellfish toxins in the spring of 2002 in east side of Osaka Bay. *Bull. Osaka Pref. Fish. Exp. Stat.*, **15**, 1–8. (in Japanese with English abstract)

Yamamoto, K., Nakajima, M., Tabuchi, K. and Hamano, Y. (2009) A novel red tide of the toxic dinoflagellate *Alexandrium tamarense* and resultant contamination of paralytic shellfish toxins in bivalves in the spring of 2007 in Osaka Bay. *Bull. Plankton Soc. Jpn.*, **56**, 13–24. (in Japanese with English abstract)

Yamamoto, K., Matsuyama, Y., Ohmi, H. and Ariyama, H. (2010) Diel vertical migration of the toxic dinoflagellate *Alexandrium tamarense*, temporal changes of associated environmental factors and cell toxin content during the course of a large–scale bloom. *Nippon Suisan Gakkaishi*, **76**, 877–885. (in Japanese with English abstract)

Yamamoto, T., Yoshizu, Y. and Tarutani, K. (1995) Effects of temperature, salinity, and irradiance on the growth of toxic dinoflagellate *Alexandrium tamarense* isolated from Mikawa Bay, Japan. *Jpn. J. Phycol.*, **43**, 91–98. (in Japanese with English abstract)

Yamamoto, T. and Tarutani, K. (1997) Effects of temperature, salinity, and irradiance on the growth of toxic dinoflagellate *Alexandrium tamarense* isolated from Hiroshima Bay, Japan. *Jpn. J. Phycol.*, **45**, 95–101. (in Japanese with English abstract)

Yamamoto, T. and Tarutani, K. (1999) Growth and phosphate uptake kinetics of the toxic dinoflagellate *Alexandrium tamarense* from Hiroshima Bay in the Seto Inland Sea, Japan. *Phycol. Res.*, **47**, 27–32.

Yamamoto, T., Hashimoto, T., Tarutani, K. and Kotani, Y. (2002a) Effects of winds, tides and river water runoff on the formation and disappearance of the *Alexandrium tamarense* bloom in the Hiroshima Bay, Japan. *Harmful Algae*, **1**, 301–312.

Yamamoto, T., Seike, T., Hashimoto, T. and Tarutani, K. (2002b) Modelling the population dynamics of the toxic dinoflagellate *Alexandrium tamarense* in Hiroshima Bay, Japan. *J. Plankton Res.*, **24**, 33–47.