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Distribution and Abundance of resting cysts of *Alexandrium tamarense* and/or *A. catenella* (Dinophyceae) in Tokyo Bay, Japan

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Abstract: Sediment samples collected at 33 stations in Tokyo Bay during the autumn of 1999 were examined to determine the distribution and abundance of resting cysts of *Alexandrium tamarense* and/or *A. catenella*. Extremely low concentrations of cysts were observed in Tokyo Bay compared with previously investigated sites in the Seto Inland Sea and Lake Hamana which have almost the same marine environmental conditions as found in Tokyo Bay. Cysts were only found in the upper 2.0–3.0 cm of sediment at three stations along the northwestern coast of the bay, at concentrations ranging from 0.8–2.1 cysts cm^{-3} of wet sediment. All of the observed cysts consisted of the outer thecal integument with no vegetative contents and are therefore concluded to be unable to play a role in the seeding of blooms. Thus, currently the possibility of paralytic shellfish poisoning, caused by a bloom of *A. tamarense* and/or *A. catenella* initiated from benthic cysts, is considered to be low in Tokyo Bay. However, commercial shellfish fisheries and recreational shellfish gathering are expected to remain important activities in this bay. Careful monitoring of toxic dinoflagellates and shellfish toxicity is necessary, because invasion by toxic dinoflagellates into Tokyo Bay via ballast water or oceanic water are potential events in the future.

Key words: *Alexandrium tamarense*, *A. catenella*, cyst, sediment, Tokyo Bay

Introduction

Recently, the occurrence and intensity of toxic dinoflagellates blooms in Japan seems to be on the increase, as well as their geographic distribution becoming more extensive (Yamamoto & Yamasaki 1996, Kotani et al. 2004). However, little attention has been paid to toxic dinoflagellates in Tokyo Bay. According to the annual reports of statistical data on agriculture, forestry and fisheries in the prefectures of Chiba, Kanagawa and Tokyo, the average annual catch of bivalves by industrial fishing in the ten years from 1990 to 1999 has remained at about 17,200 tons in Tokyo Bay, with two main bivalves, the short-necked clam (*Ruditapes philippinarum* Adams et Reeve) and the hen clam (*Mactra chinensis* Philippi), accounting for >98% of this annual catch. These bivalves are not only major commercial resources, but are also the main quarry for recreational shellfish gathering, which is permitted on many beaches facing the bay. Therefore outbreaks of paralytic shellfish poisoning (PSP) could be a major threat to both shellfish fishermen and citizens who enjoy shellfish gathering in Tokyo Bay. Historically, PSP has not broken out in this bay, however, a bloom of *Alexandrium tamarense* (Lebour) Balech has been observed once in June 1984 (Han & Terazaki 1993). Moreover, PSP events caused by *A. catenella* (Whedon et Kofoid) Balech have been confirmed in Lake Hamana (Koizumi & Tanaka 2001) and areas along the Pacific coasts of Chiba Prefecture (Chiba Prefectural Fisheries Experiment Station 1977–1995) neighboring on Tokyo Bay. *A. tamarense* and/or *A. catenella*, which are the most common causative organisms of PSP (Fukuyo et al. 1985) might have intruded into Tokyo Bay and occur at non-bloom, low cell density levels. However, investigations on the toxic dinoflagellates *A. tamarense* and *A. catenella* have been fragmentary and geographically limited within Tokyo Bay.

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Resting cysts of phytoplankton species play an important role in their dispersion, and the initiation and termination of their blooms (Anderson & Wall l978, Matsuoka 1982, Anderson 1998). Therefore, it is important to investigate the distribution and abundance of cysts in the sediments for conducting monitoring programs on the bloom dynamics of harmful phytoplankton (White & Lewis 1982, Kotani et al. 1998). The occurrence of the resting cysts of *A. tamarense* and/or *A. catenella* in Tokyo Bay has been reported based on an investigation carried out during the autumn of 1999 (Matsuoka et al. 2003). The present paper details the distribution and historical abundance of these. Furthermore we discuss in this paper whether the observed cysts are of *A. tamarense* or *A. catenella* based on evidence given in Itakura and Yamaguchi (2005) using statistical methods, it has become possible to distinguish them by measuring the length of the major axis of the cysts.

Materials and Methods

Sediment Sampling

Sediment samples were collected with a gravity core sampler of 40 mm inner diameter at 33 stations in Tokyo Bay (Fig. 1) during the R/V Shirafuji-maru cruise in the autumn of 1999. The samples were sectioned into layers of 0–1 cm, 1–2 cm, 2–3 cm and 3–5 cm, and triplicate cores were mixed before storage in the dark at about 10°C until analysis.

Sample preparation for microscopy

At first, the sediment samples were treated by the chemical method suggested by Matsuoka & Fukuyo (2000). Approximately 2 mg of wet sediment from each sample was placed into a polyethylene beaker, and then sonicated for 15 seconds after suspension in distilled water, and sieved through plankton netting to obtain the size fraction between 20 and 100 μm. The sediment remaining on the 20 μm netting was put into 15 ml polycarbonate centrifuge tubes. Five

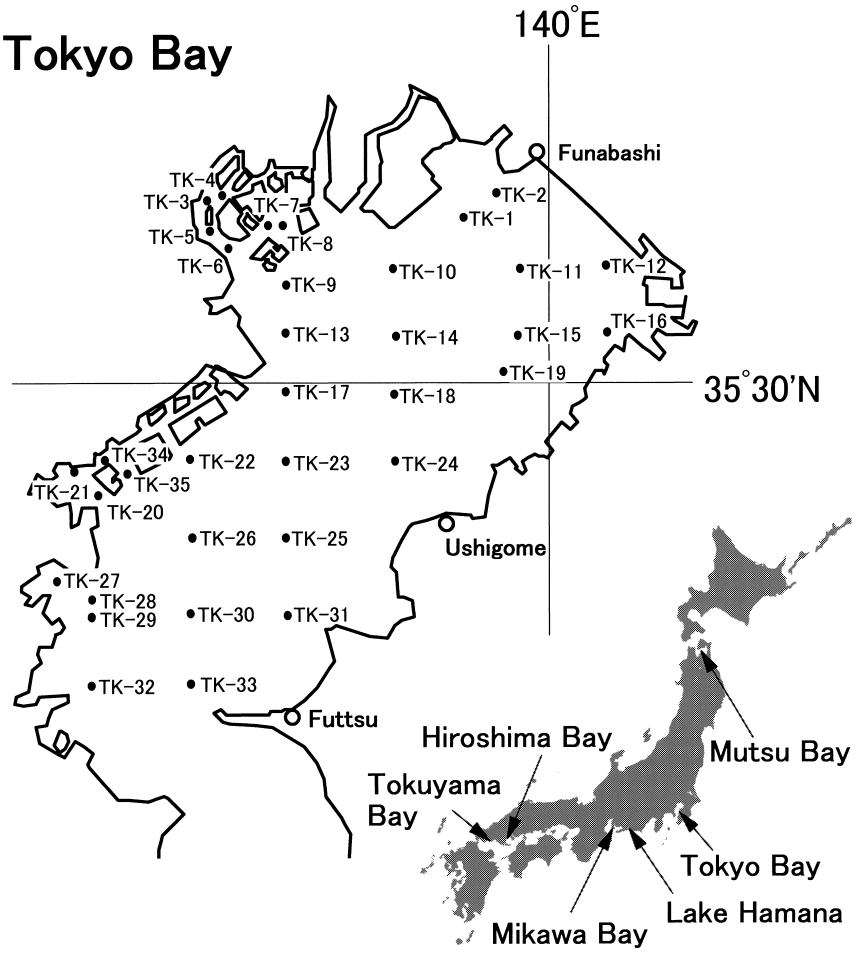

Fig. 1. Map of Tokyo Bay showing the 33 sampling stations during the R/V Shirafuji-maru cruise in the autumn of 1999. In addition, the location of each bay referred to in this paper and Lake Hamana is indicated.

The three investigation points for monitoring toxic phytoplankton and shellfish toxification in Tokyo Bay by the Chiba Prefectural Fisheries Experiment Station, located on the coasts of Funabashi, Ushigome and Futtsu, are indicated with open circles.

ml of cold methanol was added to the tubes for fixation and bleaching. The tubes were placed in a refrigerator for more than two days. The methanol was replaced with 10 ml distilled water using centrifugation. Sediment suspensions were treated to remove calcium carbonate and silicate materials with 10% hydrochloric acid (HCl) and 30% hydrofluoric acid (HF), respectively. After rinsing with distilled water and sieving through 20 μm mesh plankton netting, the sediment remaining on the 20 μm netting was put into the tube again. We confirmed that the cysts were not affected (shrinkage, dissolution or damage to the thecal integument) by the above chemical treatments in a preliminary experiment.

Secondarily, treatment of the cysts for direct counting was carried out using the primuline-staining method (Yamaguchi et al. 1995b). One ml of primuline stock solution (2 mg ml^{-1}) was added to each tube and left for an hour in the dark. After staining and centrifugation, the supernatant containing the remaining fluorochromes was removed by pipetting and the pellet was resuspended in distilled water and centrifuged again. Then the pellets were finally resuspended in 5 ml distilled water for microscopic observations using an inverted epifluorescence microscope (Nikon TE300-EF) under blue light excitation.

Observations and measurements of the cysts

For observing cysts, each milliliter of stained sediment suspension was placed in a 1 ml plate chamber. Observations of cysts in the sediment suspensions were made in triplicate. The minimum detection level of the present method was estimated to be about 0.8 cysts g^{-1} of wet sediment. Cyst concentration was calculated as cysts g^{-1} of wet sediment, and converted to cysts cm^{-3} sediment for comparison to the previous reports from Japanese coasts, by using the specific gravity of each sediment samples obtained according to the method of Kamiyama (1996). At the same time as detecting and observing the cysts by the above-mentioned method, the length of the major axis of cysts was measured using an objective micrometer fixed to the inverted microscope.

Sediment condition by mud content

The sediment samples were also treated for measuring the mud content. Approximately 2 mg wet sediment from each sample was placed into a polyethylene beaker, and then dried at 60°C for about a day in an oven. After weighing, the dried sediment was resuspended in distilled water and sieved through plankton netting to obtain the size fraction above 63 μm. The sediment remaining on the 63 μm netting was put into the same polyethylene beaker, and then dried using the same conditions in the oven and weighed. The mud content of the sediment was calculated as the percentage of the particles less than 63 μm to the dry weight of the total sediment.

Results

Distribution and abundance of the cysts

Table 1 shows the abundance of *A. tamarense* and/or *A. catenella* resting cysts as cysts cm^{-3} sediment at the only stations (TK-9, TK-13 and TK-17) where cysts were found in the sediments in the 2.0–3.0 cm layer. Cysts were distributed in the 2.0–3.0 cm layer of only three stations along the northwestern coast of the bay, at concentrations ranging from 0.8–2.1 cysts cm^{-3} wet sediment. In all of the 0–1 cm, 1–2 cm and 3–5 cm samples cyst concentrations were below the detection limit. Based on the sedimentation rates previously investigated in the vicinity of each station by Matsumoto (1983), the depositional age of each layer could be calculated. Thus, we estimated that the ages of the sediments where the cysts existed in the stations TK-9, TK-13 and TK-17 corresponded to the years 1988–1991, 1992–1995 and 1991–1993 respectively.

Observations and measurements of the cysts

Five cysts obtained from the samples from TK-9, TK-13 and TK-17 were observed to record their shapes, and their major axis was measured under a microscope. All of the cysts were thecal integuments with no vegetative contents, as shown in Fig. 2-1a, b of Matsuoka et al. (2003). Table 2 shows the data on the major axis of the cysts sampled from Tokyo Bay. The average length of the major axis of the cysts in Tokyo Bay was 52.5 μm ($\pm$1.5 μm). It was confirmed beforehand that there was no difference in the variance of the mean length of the cysts among the samples.

Table 1. Historical abundance of *A. tamarense* and/or *A. catenella* resting cysts in the sediment at the stations where cysts were found in Tokyo Bay. Sedimentation rates were estimated from the values in Fig. 2 of Matsumoto (1983).

Station No.	Sampling depth (cm)	Cyst density (cysts cm^{-3})	Sedimentation rate (g cm^{-2} year^{-1})	Gravity of sediment (g cm^{-3})	Sedimentation rate (cm year^{-1})	Estimated deposition age
TK-9	2.0–3.0	0.8	0.50	1.06	0.47	1993–1995
TK-13	2.0–3.0	0.8	0.40	1.11	0.36	1991–1994
TK-17	2.0–3.0	2.1	0.30	1.06	0.28	1989–1992

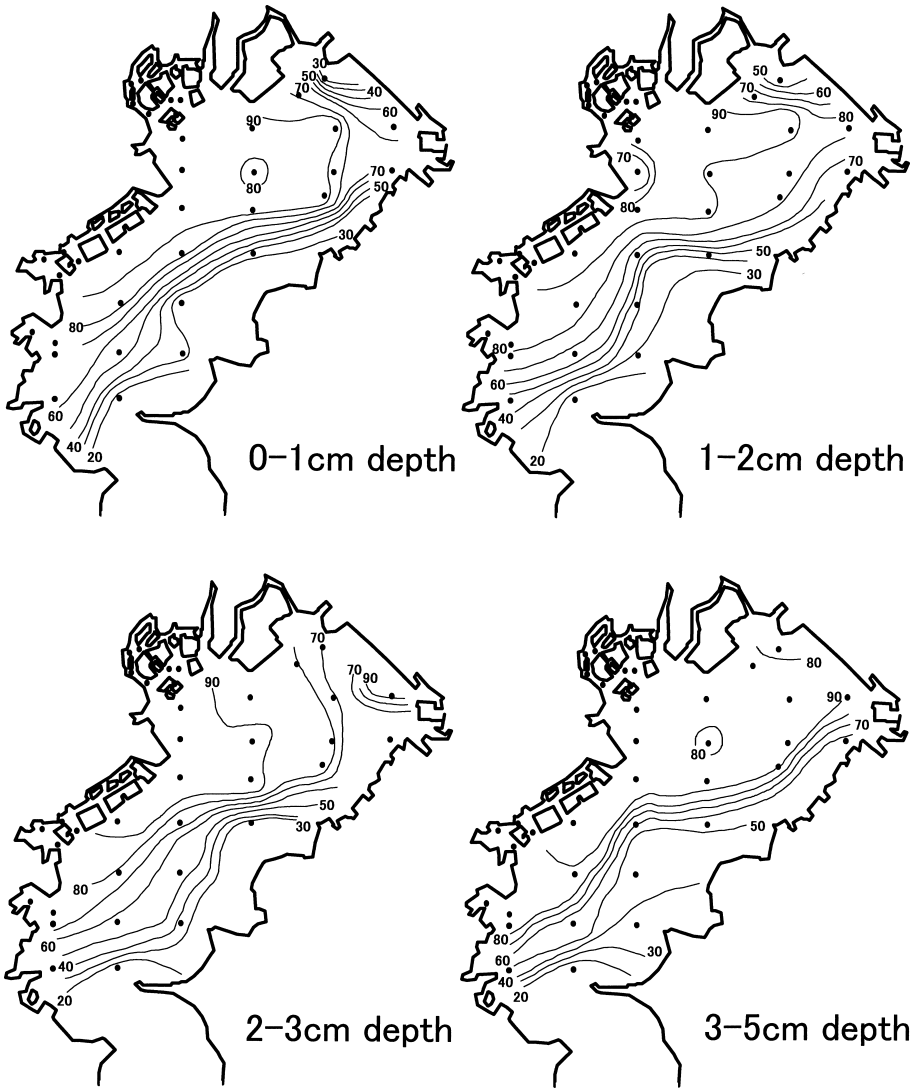

Fig. 2. Distribution of the mud content (percentage of particles less than 63 μm to the dry weight of the total sediment) in Tokyo Bay.

Table 2. Data on the major axis of the cysts of *A. tamarense* and/or *A. catenella* in Tokyo Bay compared with those from Tokuyama Bay and Hiroshima Bay reported by Itakura & Yamaguchi (2005).

	Tokyo Bay	Tokuyama Bay	Hiroshima Bay
Number of samples	5	134	130
Mean length (μm)	52.5	49.1	54.2
SD of mean length	±1.5	±4.6	±4.5
Dominant species	*A. tamarense* ?	*A. catenella*	*A. tamarense*

Discussion

Observations on the vegetative cells of toxic *Alexandrium* species

With regard to marine biotoxin monitoring in Japan, prefectural fisheries experimental stations of local governments regularly monitor the bivalves from fishery grounds for the effects of toxic phytoplankton and characterize the oceanographic conditions in cooperation with prefectural institutes of public health, in spring and summer. However, few of them monitor marine biotoxins in bivalves from public beaches. In Tokyo Bay, only three stations for monitoring toxic phytoplankton and shellfish poisoning have been in existence since 1977 at the coastal stations of Funabashi, Ushigome and Futtsu, indicated by open circles (Fig. 1). However, the Chiba Prefectural Fisheries Experiment Station (1977–1995) has not reported the occurrence of toxic *Alexandrium* species or any PSP events at any of these stations.

Tokyo Bay is a semi-enclosed bay with a narrow central part restricting the exchange of bay waters. Moreover, in the hinterland, Tokyo Bay has a large city with a population of over ten million. Eutrophication in the inner bay, which has deteriorated due to contamination by pollutants from domestic and industrial wastes, has continued since the

1970's (Nomura 1995). Since then, harmful algal blooms have been observed from spring to summer and the main causative species were reported as *Skeletonema costatum*, *Cryptomonas* sp., *Pyramimonas* sp. and *Heterosigma* sp. (Murano 1980, Okaichi 1997). In particular, small diatoms and microflagellates dominate in the spring phytoplankton communities in Tokyo Bay (Han l988). However, toxic *Alexandrium* species were not detected in the previous reports on the phytoplankton in Tokyo Bay, except for by Han & Terazaki (1993), who presented the first record of the occurrence of an *A. tamarense* bloom in Tokyo Bay. In this case, the density of vegetative cells of *A. tamarense* was reported to be 240-33,531 cells L^{-1} at four stations in the central part of Tokyo Bay. Previously, toxic *Alexandrium* species have been mainly distributed in northern Japanese coastal waters (Fukuyo et al. 1985). Over the past decade, *A. tamarense* has expanded its distribution range to southwestern waters and has formed blooms in Hiroshima Bay and Mikawa Bay (Yamaguchi et al. 1995a, Yamamoto & Tarutani 1996). This suggests the possibility that *A. tamarense* populations might already occur in Tokyo Bay. Therefore, we decided to confirm the previous occurrence of *A. tamarense* in Tokyo Bay as reported above, by examining the historical abundance of resting cysts of *A. tamarense* and/or *A. catenella* in this study. However, we concluded that the cysts found in Tokyo Bay would be unable to play a role in initiating future blooms, because all of the observed cysts were assessed to be dead with no vegetative contents. Mishima & Matsuoka (2004) suggested that *Alexandrium* spp. cysts can retain the ability to germinate for more than 8 years, from their observations in Kure Bay. Since 15 years had already passed after cyst formation in 1984, we inferred that the cysts in Tokyo Bay had naturally died and disappeared.

It is important to specify the main causative species of PSP distributed in any marine area. The reason is because the monitoring strategies and the predictive techniques for PSP differ greatly according to the causative species. Fukuyo (1982) reported that cysts of *A. tamarense* and *A. catenella* could not be readily distinguished based on their morphological characters. However, Itakura & Yamaguchi (2005) pointed out that cysts in Tokuyama Bay, where *A. catenella* dominates, are a little smaller and shorter than ones in Hiroshima Bay where *A. tamarense* dominates. It may therefore be possible to determine which species dominates in a sample from statistical data on the major axis of the cysts. Thus the length of the major axis of the cysts collected in Tokyo Bay was measured. The average length of the major axis of the cysts in Tokyo Bay was 52.5 μm ($\pm$1.5 μm). For comparison, those sampled from the sediments of Tokuyama Bay, where *A. catenella* dominates, and Hiroshima Bay, where *A. tamarense* dominates as reported by Itakura & Yamaguchi (2005), are also shown in Table 2. The average length of the cysts from Tokuyama Bay was 49.1 μm ($\pm$4.6 μm), and that of the cysts from Hiroshima Bay was 54.2 μm ($\pm$4.5 μm). There was a significant difference (*t*-test; $p<0.01$) in the mean length of the cyst between Tokuyama Bay and Hiroshima Bay.

The average length of the major axis of cysts in Tokyo Bay was larger than that in Tokuyama Bay, where *A. catenella* dominates, and was almost the same as that in Hiroshima Bay, where *A. tamarense* dominates. This indicates that *A. tamarense* might be the dominant species in Tokyo Bay. However, it is necessary to examine the morphological characters of cysts in more detail, because we think that the size of cysts possibly differs depending on the environmental conditions and the population. Moreover, judging from the precedent that a bloom of *A. tamarense* was observed in June 1984 (Han & Terazaki 1993), there is a high possibility that the cysts in Tokyo Bay are of *A. tamarense*.

Relationships between cyst distribution and oceanic conditions

In l980, cyst surveys were conducted in the western coastal area of Tokyo Bay, for the first time (Fukuyo 1982). At that time, *Alexandrium* cysts were not found at the two stations examined. We planned this investigation because no cyst surveys had been conducted in Tokyo Bay since that study. The distribution of cysts was limited to the northwestern sea area in this investigation, though blooms of *A. tamarense* in June 1984 were widely distributed and had a relatively high biomass in Tokyo Bay (Han & Terazaki 1993). We suggest a potential mechanism for the initiation of *A. tamarense* blooms in 1984 as follows. Along with the southward movement of abnormal cold water (Tominaga 1985), which seemed to be derived from Oyashio water and brought "cold species" of diatoms to the coast of the Kanto district (Suzuki & Nakata 1984), the vegetative cells of *A. tamarense* transported into Tokyo Bay might have grown because the environmental conditions were good for their growth during this period. Intrusion of oceanic water into the bay has sometimes been observed in Tokyo Bay during the summer (Yanagi et al. 1989, Fujiwara et al. 2000). These previous reports support our assumption that vegetative cells of *A. tamarense* were carried into Tokyo Bay from more oceanic area in June 1984. On the other hand, we conclude that the usual sea conditions in Tokyo Bay are not suitable for the proliferation of this species, because the vegetative cells did not grow afterwards and live cysts were not discovered. Hasunuma (1979) reported that anti-clockwise water circulation caused mainly by southwesterly seasonal winds is observed in Tokyo Bay during the summer season within the surface mixed layer. We assume that *A. tamarense*, in 1984, formed cysts that were carried into the northwestern region, where the mud content is high, as shown in Fig. 2. In general, it is known that cysts tend to be carried by currents (Takasugi et al. 1998) and accumulate in regions where the current is weak and the mud content is high (Tyler et al. 1982, Turgeon et al. 1990, Yamaguchi et al. 1996).

Historical abundance of the cysts

Within the northwestern region of Tokyo Bay, we consider the sediments to not be subject to high levels of bioturbation, due to the low density of benthic organisms (Furota 1991). However, estimation of sediment age may be difficult, because of turbation caused by tidal scouring, storms or human activities such as trawl fishing and dredging operations. Nevertheless, the cysts have remained in the 2.0–3.0 cm layer of the sediment, moreover, they were not observed in the shallower 0.0–2.0 cm and deeper 3.0–5.0 cm layers. We assumed that the cysts in the 2.0–3.0 cm layers were about 4–10 years old (i.e., around l989–1995) by calculation from a sedimentation rate of 0.3–0.5 g cm^{-2} yr^{-1} in the northwest stations (Matsumoto 1983). These findings suggest that the cysts in the 2.0–3.0 cm layer might not be derived from the bloom of *A. tamarense* first detected in 1984. To obtain more conclusive results of cyst dynamics in Tokyo Bay, additional studies on the sedimentation rate in the bay are needed.

Considering the previous cyst survey in 1980 (Fukuyo 1982) and recent investigations monitoring toxic phytoplankton, it is clear that *A. tamarense* and/or *A. catenella* have not settled and proliferated in Tokyo Bay within the last two decades. Thus, studies on the reasons why *A. tamarense* has not become a resident species in Tokyo Bay after the bloom in 1984 may yield data on the factors limiting their growth, which in turn may give insights into possible measures to control harmful blooms. Moreover, we are interested in Mutsu Bay (refer to Fig. 1) located in northern Honshu, a sea area that has probably been continuously exposed to invasions by these toxic species (Aomori Prefectural Aquaculture Research Center: personal communication) as well as Tokyo Bay. Because, it has been reported that *A. tamarense* and *A. catenella* have not been observed in Mutsu Bay, neither as vegetative cells (Osaka 1985) nor as cysts (Fukuyo 1982). There may be circumstances or factors that prevent cyst formation and/or the proliferation of the vegetative cells of *A. tamarense* and *A. catenella* in these bays.

Conclusion

The results of this study indicated that *Alexandrium* cysts (considered to be *A. tamarense*) were found at only 3 of the 33 stations examined in Tokyo Bay. However, because all of cysts were of the outer thecal integument only without vegetative contents, it is considered that there is not a significant potential for initiation of *A. tamarense* bloom or associated outbreaks of shellfish poisoning. This study also demonstrates that *A. tamarense* and/or *A. catenella* have not settled and proliferated in Tokyo Bay within the last two decades, in spite of the *A. tamarense* bloom in June 1984 (Han & Terazaki 1993). Occurrence of *A. tamarense* was not observed in monthly investigations of the phytoplankton community in the central part of Tokyo Bay carried out from 1991 to 1993 (Nomura & Yoshida 1997). However, recently blooms of toxic *Alexandrium* species have occurred suddenly after an extended period of no blooms for several years in several locations. For example, bivalves were affected by PSP in Tokuyama Bay, caused by an *A. catenella* bloom in May 1998, after no blooms having been observed since May 1985 (Sakamoto et al. 1999, Baba et al. 2000), in Lake Hamana by *A. catenella* in May 1999 after no blooms having been observed since November 1986 (Koizumi & Tanaka 2001), and in Mikawa Bay by *A. tamarense* in February 2001 after no blooms having been observed since April 1985 (Aichi Prefectural Fisheries Experimental Station 2002). These previous studies suggest that there is a possibility of sudden incidents of *A. tamarense* or *A. catenella* blooms and human intoxication upon the consumption of bivalves affected by PSP in Tokyo Bay, and even in previously unaffected areas. Therefore, we conclude that it is necessary to set up PSP monitoring activities at least in the sea areas where the occurrence of toxic *Alexandrium* species or *Gymnodinium catenatum* have been confirmed, including in Tokyo Bay, to prevent the outbreak of PSP incidents. In the case of Tokyo Bay, the possibility that cysts will accumulate in the northwestern part of the bay is high, judging from the current system in summer (Hasunuma 1979), sedimentation processes (Yanagi & Shimizu 1993) and mud content distribution (Fig. 2). Therefore, investigations of cyst distribution should be conducted focusing on the northwestern part of the bay.

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