Plankton Biol. Ecol. 52 (2): 76–84, 2005

plankton
biology & ecology

Horizontal distribution of toxic *Alexandrium* spp. (Dinophyceae) resting cysts around Hokkaido, Japan

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Received 14 February 2005; Accepted 26 June 2005

Abstract: To clarify the distribution of toxic *Alexandrium* spp. resting cysts throughout the coastal waters around Hokkaido, sediment samples from 152 stations were examined using the primuline-staining direct count method. Cysts were found to be distributed especially in the cold current areas, from the coast of the Pacific Ocean to the Sea of Okhotsk. Large concentrations of cysts were found in Funka Bay (max. 2,568 cysts g^{-1} sediment) and the Sea of Okhotsk (max. 1,022 cysts g^{-1} sediment). On the other hand, there were no cysts in the warm current areas, from the coast of the Sea of Japan to the Tsugaru Strait. There was a significant correlation between the cyst abundance and the frequency of past PSP occurrences in each area around Hokkaido. Therefore, the cyst abundance was concluded to be a useful parameter for predictions of the frequency of PSP occurrence.

Key words: *Alexandrium tamarense*, cyst, paralytic shellfish poisoning, prediction, Hokkaido

Introduction

The toxic dinoflagellate *Alexandrium tamarense* is known as one of the typical species that cause paralytic shellfish poisoning (PSP) in Japan (Fukuyo 1985). Since 1978, PSP has often occurred because of *A. tamarense*, and has had a harmful effect on the shellfish fishery, especially for the scallop (*Mizuhopecten yessoensis*) fishery from the coast of the Pacific Ocean to the Sea of Okhotsk around Hokkaido (Nishihama 1985). Since *A. tamarense* has a resting cyst (hypnozygote) stage as well as the planktonic vegetative cell stage in the life cycle (Anderson & Wall 1978; Turpin et al. 1978; Yoshimatsu 1992), the cyst distribution is important information when considering the dynamics of blooms and the potential to predict *A. tamarense* appearances. It has been observed that the vegetative cells of *A. tamarense* show an annual regular pattern of increase from spring to summer (Shimada et al. 1996; Shimada 2000) where high concentrations of cysts (approx. 200-1,100 cysts g^{-1}) occur in Funka Bay, south western Hokkaido (Miyazono & Shimada 2000). However, surveys on the cyst distribution have only been carried out in limited areas such as Funka Bay (Fukuyo 1982; Miyazono 2000; Miyazono 2002), Lake Saroma (Fukuyo 1982), Akkeshi Bay (Fukuyo 1982) and the coast of Tokachi subprefecture (Hokkaido 1987), because cyst counting using a normal light microscope is highly labor intensive work. However, it has been possible to survey cyst distribution rapidly and extensively, after Yamaguchi et al. (1995) reported the "primuline–staining direct count method" which uses an epi-fluorescence microscope. Thus we attempted to elucidate the horizontal distribution of toxic *Alexandrium* spp. cysts all around Hokkaido as a fundamental database for the prediction of PSP occurrence, to determine if there is a relationship between the cyst abundance and the frequency of past PSP occurrences in each area.

Abbreviations: PSP, paralytic shellfish poison; FB, Funka Bay; SP, Pacific coast of southern Hokkaido; EP, Pacific coast of eastern Hokkaido; NS, Nemuro Strait; OS, Sea of Okhotsk & Soya Strait; JP, Sea of Japan; TS, Tsugaru Strait.

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Materials and Methods

Sampling *in situ*

Surveys were carried out from 1999 to 2000 at 152 sta-

Table 1. Sampling stations, periods, sampling gear and research vessels used in each area in the survey.

Area		Number of Stations	Period	Sampling Gear*	Research Vessel**
Funka Bay (FB)		60	Jul./1999	1,4	Kinsei Maru
Pacific coast of southern Hokkaido (SP)		9	Aug./2000	1,3	Kinsei Maru
Pacific coast of eastern Hokkaido (EP)	Pacific Ocean	14	Jul.–Aug./2000	1,3	Hokushin Maru
	Akkeshi Bay & Lake Akkeshi	8	Aug./2000	2,3	Boat
Nemuro Strait (NS)	Southern Nemuro Strait	15	Jun./1999	2,3	Boat
	Northern Nemuro Strait	8	Jul./1999	1	Boat
Sea of Okhotsk & Soya Strait (OS)	Sea of Okhotsk & Soya Strait	16	Aug./1999	1	Hokuyou Maru
	Lake Notoro	4	Jun./1999	1,3	Boat
	Abashiri Bay	12	Aug./1999	1	Boat
Sea of Japan (JP)		5	May–Jul./2000	1,3	Oyashio Maru
Tsugaru Strait (TS)	Hakodate Bay	1	Aug./2000	1,3	Kinsei Maru
	Total	152	Jun./1999–Aug./2000		

* 1: Smith–McIntyre grab sampler, 2: Ekman–Birge grab sampler, 3: TFO gravity corer, 4: HR type gravity corer

** Hokushin Maru: 214 ton, R/V of Hokkaido Kushiro Fisheries Experimental Station
Kinsei Maru: 69 ton, R/V of Hokkaido Hakodate Fisheries Experimental Station
Hokuyou Maru: 237 ton, R/V of Hokkaido Wakkanai Fisheries Experimental Station
Oyashio Maru: 178 ton, R/V of Hokkaido Central Fisheries Experimental Station
Boat: Light fishing boat <20 ton

tions in 7 areas around Hokkaido (Table 1, Fig. 1). High intensity sampling stations occurred in Funka Bay, near Akkeshi area, Nemuro Strait and near Abashiri area. We used a Smith–McIntyre, an Ekman–Birge grab sampler, a TFO gravity core-sampler (inner diameter 10 mm), or an HR type gravity corer (inner diameter 110 mm) as the sampling gear. Surface sediments from the 0–3 cm layer were collected as the samples for cyst counting, and were preserved in the dark at 3°C.

Sample treatment and cyst counting

We used the primuline-staining direct count method (Yamaguchi et al. 1995). Aliquots of 0.3–0.5 g of wet sediment were suspended in distilled water, and were sieved through a plankton net to obtain the size fraction between 10 and 100 μm after ultrasonic vibration treatment for 2 min. Size fractionated samples were kept in 10 ml centrifuge tubes. The samples were preserved in 90% methanol for 1 day to remove all fluorescence of algal pigments after fixation with 1% glutaraldehyde for 30 min. The samples were stained with 0.2 mg ml^{-1} primuline solution for 1 h in the dark after being placed in distilled water. The 5 ml samples were then resuspended in distilled water for cyst counting after removing the primuline solution. Centrifugation (700 rpm, 15 min) was used to remove the waste solutions and for transfer to other tubes. Subsamples (0.2 ml) of the 5 ml samples were used for each count under a normal microscope (Nikon OPTIPHOT-2) fitted with an epi-fluorescence illuminator (Nikon EFD-2). The countings were repeated 3–5 times to obtain the number of cysts in each sample, and the obtained numbers were converted into cysts per g–wet sediment. We counted only the long–ellipse–shaped *Alexandrium* cysts as "toxic *Alexandrium* spp. cysts", since it is not possible to differentiate the cysts of *A. tamarense* or *A. catenella* from the morphological characters. The cysts of Funka Bay were identified to species level by microscope observation of the thecal plates of the germinated vegetative cells after culturing in GeO_2 added filtered seawater at 10°C under a 14 : 10 h L : D cycle. The condition of each sediment sample was recorded as "mud"; rarely containing particles size of >100 μm or "sand/gravel"; obviously contained particles size of >100 μm, by visual identification when sieving.

Data analysis

We analyzed the relationships between the cyst abundances and the frequencies of past PSP occurrences in the 7 areas around Hokkaido in 1980–2000 and the mean of the maximum PSP toxicity in each year, using the database of PSP occurrences in each area since 1980 (Data source: Hokkaido Prefecture). The frequency of past PSP occurrences and the mean value of the maximum PSP toxicity in 1980–2000 were calculated in the following way.

The frequency of past PSP occurrences:

number of PSP occurrences detected in the year over the critical value (4 mouse units g^{-1} whole meat of scallop)
÷21 (number of all years)

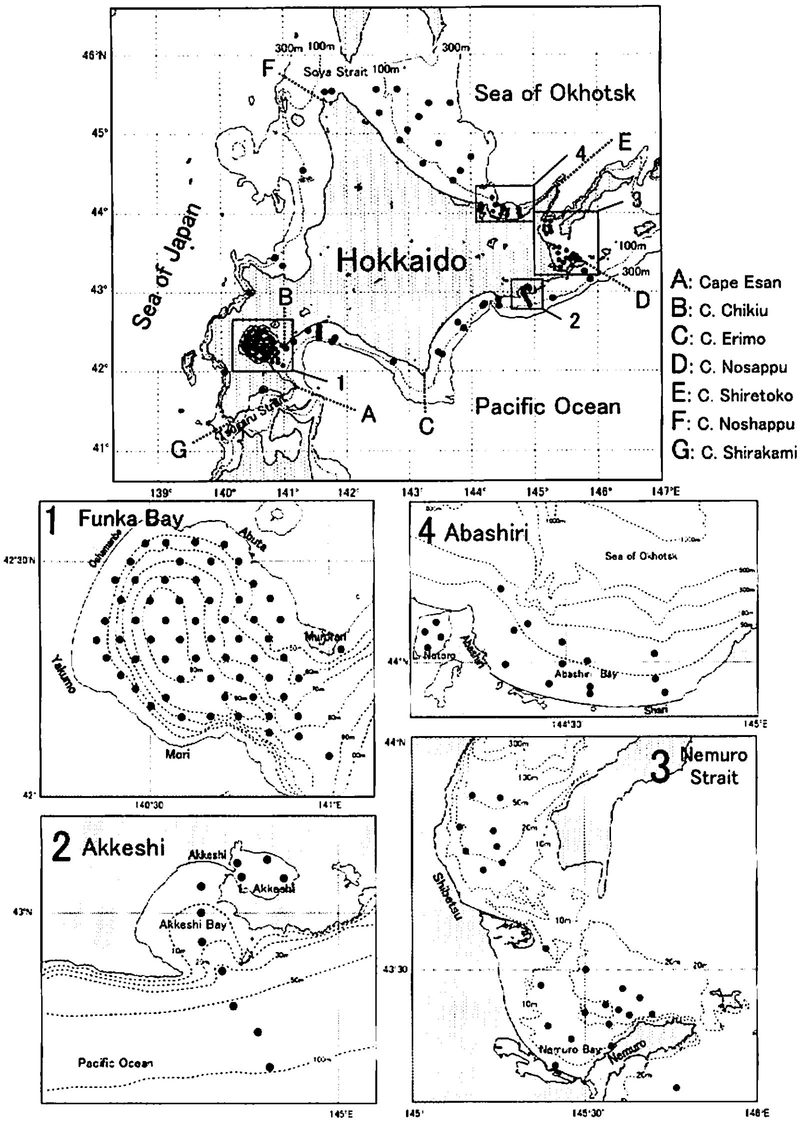

Fig. 1. Maps showing sampling stations around Hokkaido in the present survey. The area of A–B: Funka Bay (FB), B–C: Pacific coast of southern Hokkaido (SP), C–D: Pacific coast of eastern Hokkaido (EP), D–E: Nemuro Strait (NS), E–F: Sea of Okhotsk & Soya Strait (OS), F–G: Sea of Japan (JP), A–G: Tsugaru Strait (TS) Maps 1–4 are enlargements of the squares 1–4 on the main map.

The mean value of the maximum PSP toxicity in each year:

Sum of the maximum PSP toxicity in each year
$\div 21$ (number of all years)

The 7 areas investigated are as follows: Funka Bay (FB, Cape Esan to C. Chikiu), Pacific coast of southern Hokkaido (SP, C. Chikiu to C. Erimo), Pacific coast of eastern Hokkaido (EP, C. Erimo to C. Nosappu), Nemuro Strait (NS, C. Nosappu to C. Shiretoko), Sea of Okhotsk & Soya Strait (OS, C. Shiretoko to C. Noshappu), Sea of Japan (JP, C. Noshappu to C. Shirakami) and Tsugaru Strait (TS, C. Shirakami to C. Esan). Then, correlations between the cyst abundance and the frequency of past PSP occurrences in each area were tested.

Results

Toxic *Alexandrium* resting cysts were found in FB, SP, EP and OS, though no cysts were found in JP and TS (Fig. 2). The cysts of Funka Bay were identified as *A. tamarense* by microscope observations of vegetative cells in a culture established after the germination of cysts. It can thus be supposed that most of the cysts were *A. tamarense*, but a few cysts might possibly be *A. catenella*. Several high concentrations of cysts ($>10^3$ cysts g^{-1}) were found in FB, and one high concentration was also found in OS. High concentrations of cysts (10^2–10^3 cysts g^{-1}) were observed widely around FB and in certain areas of SP, EP, NS and OS. Some cysts ($<10^2$ cysts g^{-1}) also existed in salt lakes such as L. Notoro of OS and L. Akkeshi of EP. Cyst abundances in each area were FB>SP>OS>NS>EP, ordered by the

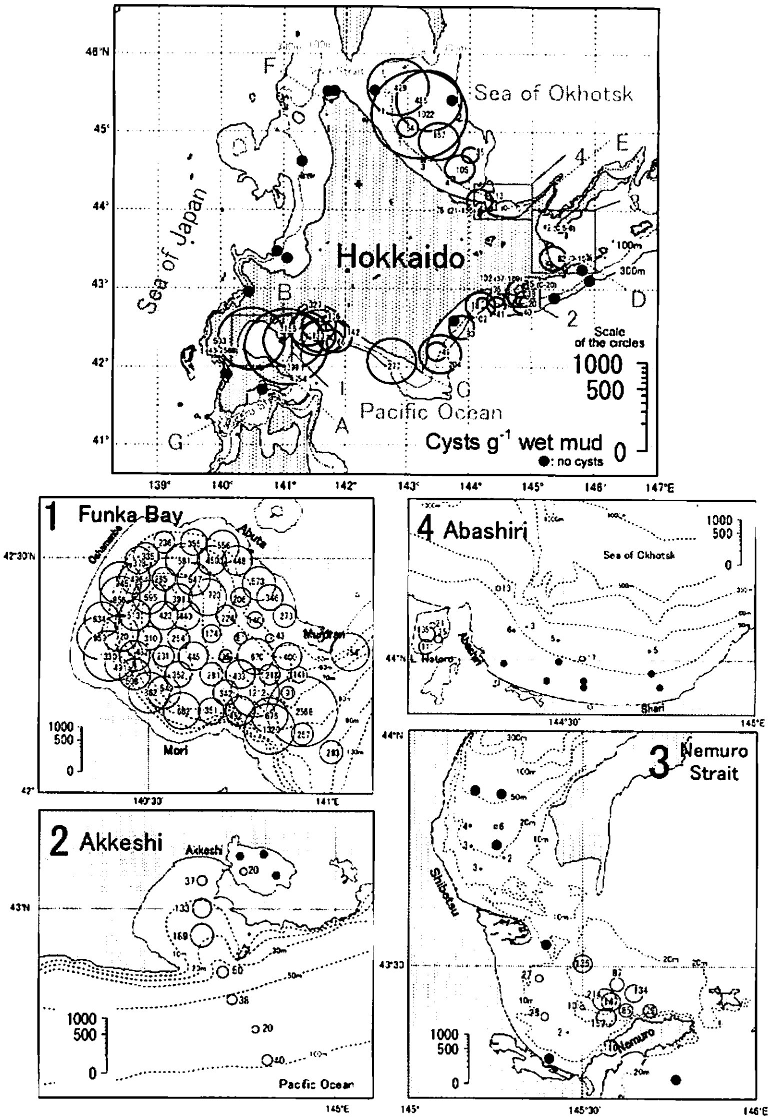

Fig. 2. Maps showing the horizontal distribution of toxic *Alexanadrium* spp.resting cysts around Hokkaido in the present study. Black spots "●" indicate no cysts. Numbers in parentheses are the range of cyst concentrations at the plural sampling stations.

mean cyst abundance (Table 2).

In FB, dense concentrations (1,212–2,568 cysts g^{-1}) were found at the mouth and the southwestern area of the bay, while relatively high concentrations occurred near the coastal region of FB. Near Akkeshi of EP, a high concentrations of cysts (189 cysts g^{-1}) was found at the mouth area of Akkeshi Bay, while the cyst abundance in Lake Akkeshi was relatively low (0–20 cysts g^{-1}). In NS, high concentrations of cysts (116–214 cysts g^{-1}) were found in the southern area, while the cyst abundance in the northern area was relatively low (0–6 cysts g^{-1}). Near Abashiri of OS, high concentrations of cysts (111–135 cysts g^{-1}) were found in Lake Notoro while the cyst abundance in Abashiri Bay was relatively low (0–13 cysts g^{-1}) and a low concentrations of cysts were found at the offshore stations.

As concerns the relationship between cyst abundance and sediment type, excluding JP and TS where no cysts were

Table 2. Cyst abundance around Hokkaido in the present study in 1999–2000.

Area	Number of stations	Cyst abundance (cysts g^{-1} wet sediment) Range	Mean±SD
Funka Bay (FB)	60	43–2568	503±392
Pacific coast of southern Hokkaido (SP)	9	66–754	265±212
Pacific coast of eastern Hokkaido (EP)	22	ND–204	48±60
Akkeshi Bay	4	37–189	102±72
Nemuro Strait (NS)	23	ND–214	54±67
Sea of Okhotsk & Soya Strait (OS)	32	ND–1022	84±207
Sea of Japan (JP)	5	ND	ND
Tsugaru Strait (TS)	1	ND	ND

found, the cyst abundance was relatively higher in "mud", and lower in "sand/gravel" (Fig. 3). Relative frequency of "mud" stations were in the order SP>FB>EP>OS>NS>JP&TS (Fig. 4). The cyst abundance tended to be relatively lower in the areas where the frequency of "mud" stations was less than 50% (NS and JP&TS).

Table 3 shows the frequency of PSP occurrence years and the mean of the maximum PSP toxicity in each year with the range (mean±SD) for each area during 1980–2000. The frequency of PSP occurrence years were in the order FB>EP>SP>NS>OS=TS>JP (Fig. 5). There was a significant correlation between the frequency of PSP occurrence years and the mean cyst abundance in each area. However, there were two exceptions, such as EP where the cyst abundance was low despite the high PSP frequency, and TS where no cysts were found despite the considerable PSP frequency. From records from EP of PSP, confirmed outbreaks have occurred only in Akkeshi Bay except for an outbreak in 1985 outside of the Bay. Substituting the frequency of PSP occurrence years and the mean cyst abundance of Akkeshi Bay for those of EP, led to a more significant correlation (Fig. 5). The means of the maximum PSP toxicities in each year were in the order FB>SP>TS>EP>NS>OS>JP. The other significant correlation was found between the mean of the maximum PSP toxicity in each year and the mean cyst abundance in each area (Fig. 6).

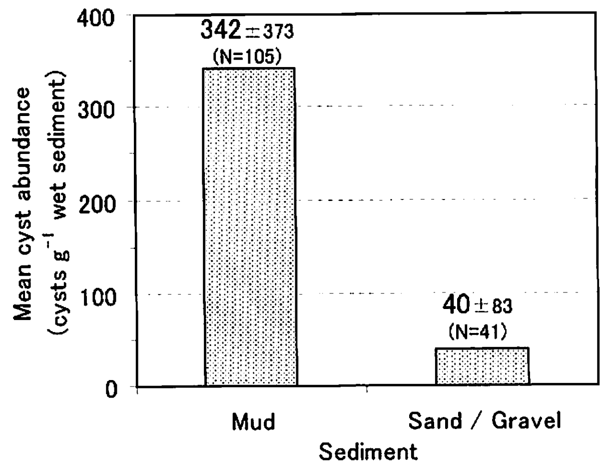

Fig. 3. Comparison of the mean cyst abundance (cysts g^{-1} wet sediment, ±SD) between the two sediment groups, "Mud" and "Sand/Gravel" in the present study, excluding the data for JP and TS where no cysts were found. There was a significant difference between the two groups (*t*-test, $p<0.01$).

Discussion

The cysts were distributed only in the areas under the influence of cold currents, and there were no cysts in the warm current areas (see Fig. 2), referring to the schematic paths of ocean currents around Hokkaido (Isoda & Kishi 2003, Fig. 7). Fig. 8 shows the annual variation of PSP oc-

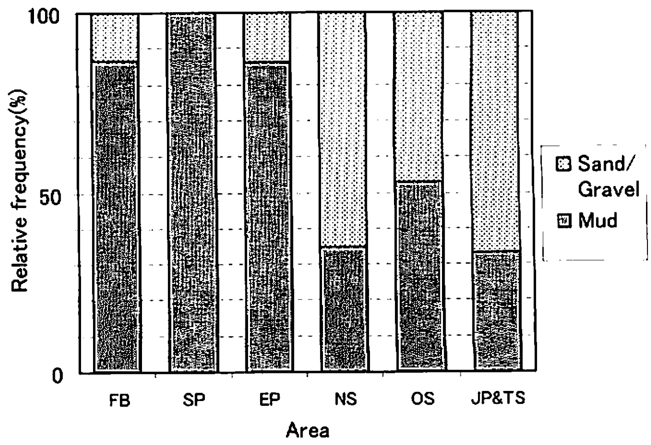

Fig. 4. Sediment composition (relative frequency of "Mud" or "Sand/Gravel") in each area around Hokkaido.

Table 3. Summary of PSP occurrences around Hokkaido in 1980–2000. (Data source: Hokkaido Prefecture)

Area	Frequency of PSP occurrence years (%)	Maximum PSP toxicity in each Year (mouse units g^{-1} digestive diverticula of scallop) Range	Mean±SD
Funka Bay (FB)	76.2	10.4–2812.2	559.0±699.1
Pacific coast of southern Hokkaido (SP)	57.1	3.5–1742.3	326.0±511.1
Pacific coast of eastern Hokkaido (EP)	61.9	2.2–182.7	55.0±49.8
Akkeshi Bay	57.1	2.2–122.4	48.7±41.3
Nemuro Strait (NS)	33.3	2.0–171.8	42.0±49.6
Sea of Okhotsk & Soya Strait (OS)	23.8	2.0–189.1	26.8±43.4
Sea of Japan (JP)	0	—	—
Tsugaru Strait (TS)	23.8	2.0–329.1	69.7±116.0

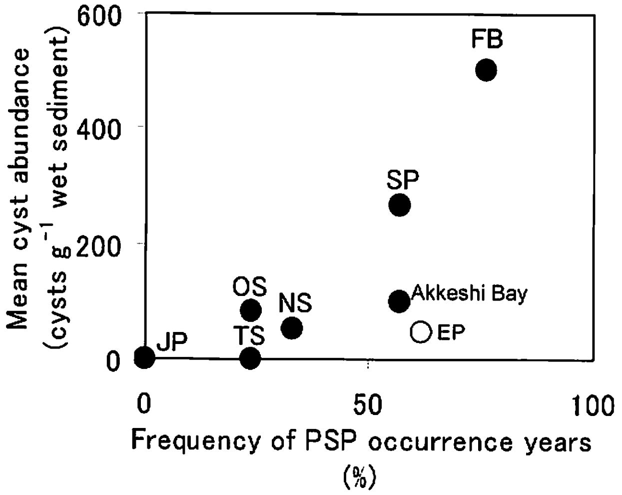

Fig. 5. Relationship between frequency of PSP occurrence years (%) in 1980–2000 and mean cyst abundance (cysts g^{-1} wet sediment) in the present study in each area around Hokkaido. There was a significant correlation which was more significant in the case of using the data of Akkeshi Bay ($n=7$, $r^2=0.717$, $p<0.05$) than EP ($n=7$, $r^2=0.586$, $p<0.05$).

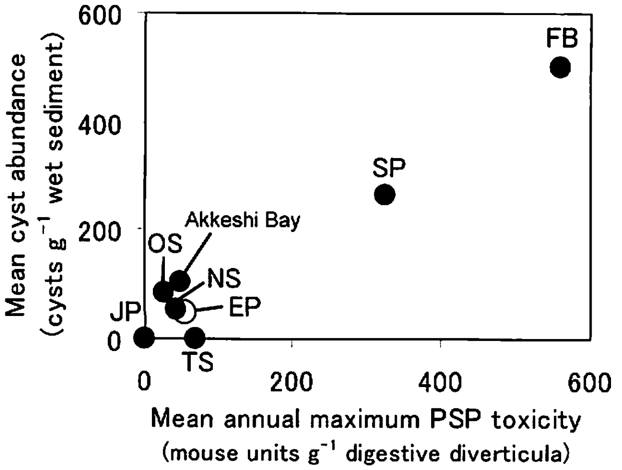

Fig. 6. Relationship between the mean annual maximum PSP toxicity (mouse units g^{-1} digestive diverticula of scallop) in 1980–2000 and the mean cyst abundance (cysts g^{-1} wet sediment) in the present study for each area around Hokkaido. There was a significant correlation in both cases using the data of Akkeshi Bay ($n=7$, $r^2=0.945$, $p<0.01$) and EP ($n=7$, $r^2=0.960$, $p<0.01$).

currences since 1980 around Hokkaido. It is clear that PSP has occurred only in areas under the influence of cold currents (see Figs. 7 and 8). *Alexandrium tamarense* is recognized to be the major PSP causative species in Hokkaido (Nishihama 1985; Shimada unpublished data), except for a PSP occurrence caused by *A. catenella* which was recorded in FB in autumn 1988 (Hayashi 1989; Noguchi et al. 1990). Since the cysts from Funka Bay were identified as *A. tamarense* by the observation of culture cells, the cysts of other areas are considered to be *A. tamarense*. Cyst distribution has been found to be greatly influenced by the past

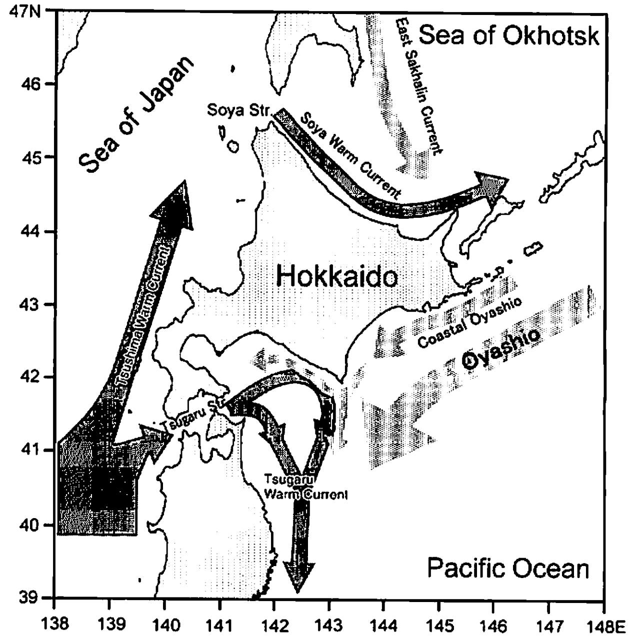

Fig. 7. Schematic paths of the warm currents (Tsushima, Soya and Tsugaru Warm Currents) and cold currents (East Sakhalin Current, Coastal Oyashio and Oyashio) around Hokkaido (Isoda & Kishi 2003).

occurrence of vegetative cells (White & Lewis 1982; Anderson & Keafer 1985; Turgeon et al. 1990; Yamaguchi et al. 1996; Yamaguchi et al. 2002). We suppose that *A. tamarense* is widely distributed and increases in number especially in the cold current areas, so that consequently the cysts exist in these area.

As to the relationship between the cyst abundance and the sediment type, cyst abundance was found to be relatively higher in "mud", and lower in "sand/gravel" areas (Figs. 4 and 5). Cyst abundance also tended to be higher in the higher mud content sediment in the Seto Inland Sea (Yamaguchi et al. 1996; Yamaguchi et al. 2002). These results suggest that cysts that have been deposited are accumulated with the mud, because the particle sizes of the cysts closely approximate those of mud (<63 μm). On the other hand, no records of PSP exist of JP, a warm current area, and no cysts were found in the area. In TS, a considerable PSP frequency was recorded, even though cysts were not found at the station that was investigated. The Tsugaru Warm Current is usually dominant in TS. However, a temporary flow to the west along the coastline of Hokkaido sometimes occurs when the Tsugaru warm current temporarily becomes weak (Ohtani 1987; Nishida 2003). It can be supposed that the temporary flow might transport *A. tamarense* vegetative cells from the Pacific Ocean to the culturing ground of scallops on the coast of TS and bring about the PSP occurrence. So it is important to investigate several areas in TS to determine conclusively whether cysts occur there or not.

In FB, it was reported that *A. tamarense* vegetative cells primarily increased in the southwestern area of the bay

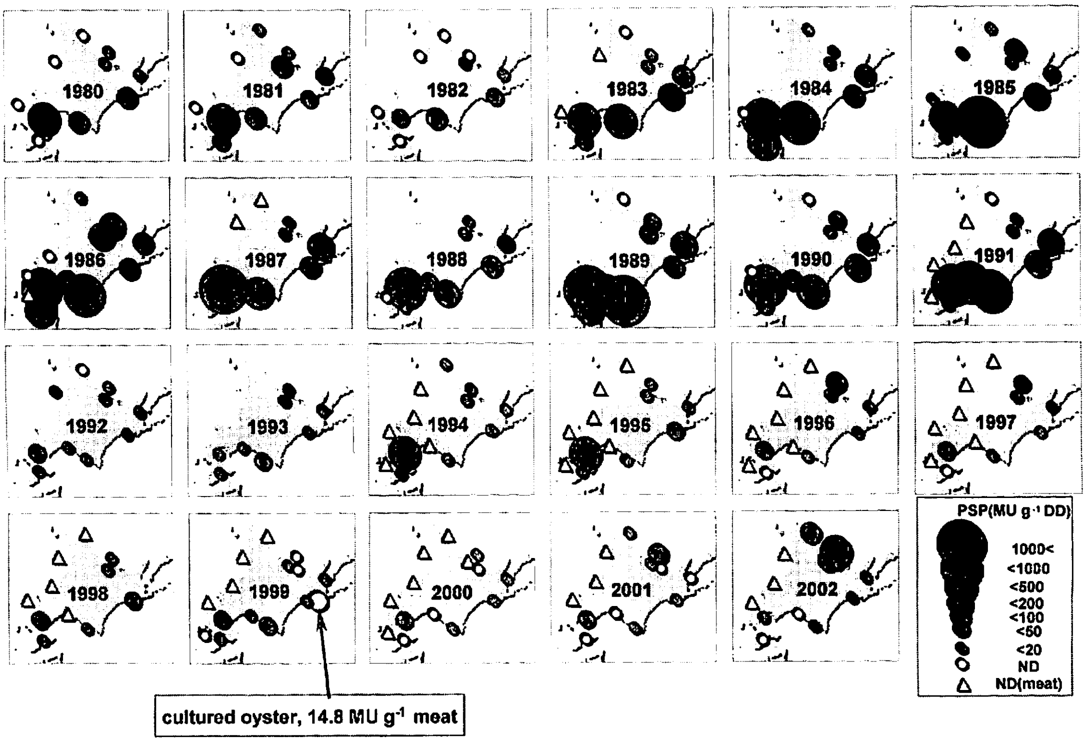

Fig. 8. Diagram showing the annual variation of PSP occurrences since 1980 around Hokkaido (Data source: Hokkaido Prefecture). Sizes of circles indicate annual maximum PSP toxicity (mouse units g^{-1} digestive diverticula or whole meat of scallops).

from April, and became widespread throughout the bay, then disappear in late July, when the cell density tended to be higher near the coast (Shimada et al. 1996). In the present study, the cyst abundance was relatively higher near the coast and dense distributions existed in the southwestern and the mouth area of the bay. It can be supposed that the distribution characteristics of the cysts resemble that of the vegetative cells (Fig. 2). Since vegetative cells may produce resting cysts during growth periods (Yoshimatsu 1985), it was supposed that the cyst distribution closely reflects the horizontal distribution of vegetative cells. Ohtani & Kido (1980) reported that a thermohaline front appeared near the mouth of FB in May–June, when the vegetative cells optimally increase, because warm and low-salinity surface waters formed inside the bay. This suggests that such dense cyst concentrations occur at the mouth of FB because phytoplankton tend to form blooms generally around such thermohaline front areas (Seliger et al. 1979; Holligan 1979; Franks 1997). A similar mechanism can be considered for the high concentrations of cysts observed at the mouth of Akkeshi Bay of EP.

Dense concentrations of cysts ($>10^3$ cysts g^{-1}) were found in OS as well as FB. According to past PSP occurrences, it is likely that some cysts exist in the bottom sediments in OS. However, it is surprising that such a large concentration of cysts was found in the area. High cyst concentrations existed on the continental shelf from northern Hokkaido to Sakhalin Island, and near the thermohaline front between the East Sakhalin cold current and the Soya Warm Current in the present study. Since dense blooms ($>10^3$ cells l^{-1}) of *A. tamarense* were observed near the thermohaline front (Nishihama 1994; Shimada et al. unpublished data), dense cyst concentrations would also be expected to occur in the frontal area. It has been reported that large numbers of *Alexandrium* cysts were found in the coastal area of Sakhalin (Orlova 2003), on the continental shelf area that extends from Hokkaido. We therefore suggest that the seed ground of *A. tamarense* may presumably occur on the continental shelf.

In the present study a significant correlation between the frequency of PSP occurrence years and the mean cyst abundance was found in each area with the two exceptions of EP and TS (Fig. 5). Namely, the higher the frequency of PSP, the higher the concentration of *Alexandrium* cysts distributed in the area. One of the reasons why a high PSP frequency but low cyst abundance may have occurred in EP may be that most of the scallop production is concentrated in Akkeshi Bay (Kushiro Fisheries Technical Guidance Office, personal communication) and the monitoring of PSP toxicity has also been concentrated inside the bay, so that recorded dynamics of PSP toxicity might not reflect the real dynamics of the whole of EP. The other reason may be that Akkeshi Bay is in fact the area of highest PSP frequency and cyst abundance in EP. Substituting the data of Akkeshi Bay for those of EP, the correlation became more significant (Fig. 5). On the other hand, the reason for the exception in TS cannot be sufficiently explained without further investigation of several areas. The other significant correlation was found between the mean of the maximum PSP toxicity and the mean cyst abundance (Fig. 6), namely, the higher the maximum PSP toxicity, the more the *Alexandrium* cysts tend to occur in the area. Thus, it can be supposed that the mean cyst abundance in each area can be a useful parameter for the prediction of the frequency of PSP occurrence and toxicity level, if the cysts maintain their ability to germinate. Miyazono (2002) reported that the cysts not only in the surface layer (0–3 cm) but also in the deeper layer (3–12 cm) have a high germination ability when investigated with culture experiments using core samples from Funka Bay. Therefore, it can be supposed that the cysts in the surface layer in the present study may also have sufficient germination ability. We can therefore conclude that the cyst abundance in each area can be a useful parameter for the prediction of the frequency of PSP occurrence in most cases. Suga et al. (1997) reported that the sedimentation rate could be estimated at 1.8 mm $year^{-1}$ off Mori in Funka Bay. Applying this result to the present study, our samples (0–3 cm) may be equivalent to the sediment from the 17 years after 1982, approximately. Since most years of high PSP toxicity are recorded in this period (Fig. 8), it can be supposed that the estimation on the frequency of PSP occurrence from the cyst abundance may be appropriate in the present study. However it may be necessary to estimate the period of sedimentation in each area around Hokkaido.

PSP occurrences suddenly became less common in 1992 and thereafter (Fig. 8). However, the present study suggests that cysts are widely distributed except for in the warm current areas, so that rarity of PSP occurrences may not necessarily be the case in the future. We recommend that monitoring of the toxicity and distribution of *A. tamarense* vegetative cells should be continued, especially in the areas of high cyst concentrations. The coastal areas around Hokkaido, however, are too vast to monitor vegetative cells, because it is troublesome and expensive work. This is why monitoring of the vegetative cells has been carried out only in the major production areas of scallops, and only toxicity monitoring has been conducted in other areas. Surveys on cyst distribution may be an alternative method for the prediction of the frequency of PSP occurrence in areas other than those that are major aquaculture areas for shellfish. In conclusion, we suggest that similar studies to the present one will be essential at least every 10 years in order to obtain data on the current cyst abundance, since the cyst abundance may fluctuate owing to the occurrence of the vegetative cells.

Acknowledgments

The authors wish to thank Dr. Mineo Yamaguchi of the National Research Institute of Fisheries and Environment of Inland Sea for helpful comments during the preparation of this manuscript. We are also grateful to all of the following, Mr. Ryuji Fukuyama and the staff of the Hokkaido Institute of Environmental Sciences, Mr. Kenji Nishina of the Geological Survey of Hokkaido, and the many staff of local fishery cooperatives, and the captains and crews of the R/V Hokuyo Maru, Hokushin Maru, Kinsei Maru and Oyashio Maru of Hokkaido Fisheries Experimental Stations for kindly helping us with sampling *in situ*.

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