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海洋細菌によるフグ毒産生に関する研究
― 特に懸濁粒子を通じての動態 ―

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浜 崎 恒 二

STUDIES ON MICROBIAL PRODUCTION OF TETRODOTOXIN IN MARINE ENVIRONMENT WITH EMPHASIS ON SUSPENDED PARTICLES AS A SITE FOR PRODUCTION AND ACCUMULATION

by
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A dissertation submitted in partial fulfilament of the requirement for the degree of doctor (agriculture)

University of Tokyo
1995

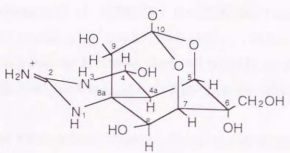
CONTENTS

Historical Perspective and General Introduction

Tetrodotoxin (TTX), which is well known as a pufferfish toxin and as a specific sodium channel blocker in excitable membranes (Narahashi 1974), is one of the potent natural toxins. A poisonous substance in pufferfish has been known since the early Chinese era. In Japan, archaeological investigations around Nara unearthed many bony remnants characteristic of pufferfish (Kao 1966). First description of pufferfish poisoning appeared in poetry, 'Haiku' or 'Senryu', or other literature in the Edo era after A.D.1600. People of those days cooked this fish with avoiding poisonous parts and tasted it as one of the most delicious fishes (Tsuda 1984).

Scientific investigations were started by pharmacologists at 1870's in Meiji era. Pharmacological effect of water extract to dogs or rabbits was investigated (Tani 1945 ; Tsuda 1984). In 1909, Tahara reported the purification procedure of a pufferfish toxin and named this 'tetrodotoxin', although the substance which he could get after "purification" was far from pure in reality. In 1950, Yokoo announced that he succeeded in purification and crystallization of a toxic substance from eggs of *Spheroides rubripes* and named it "spheroidin". Two years later, Tsuda and Kawamura (1952) also successfully purified it from ovaries of *Spheroides rubripes* independently and named "tetrodotoxin". The term "tetrodotoxin" was generally accepted and is widely used nowadays.

Although development of purification procedure accelerated the effort to determine the structure of TTX, it was very difficult because of its unique chemical behavior. In 1964 at the 4th International Symposium on the Chemistry of Natural Products in Kyoto, the structure presented by three independent groups including Hirata, Tsuda and Woodward was agreed with each other. Determination of its unique structure was one of topics in this meeting. TTX was an amino perhydroquinazoline compound with a molecular formula of $C_{11}H_{17}N_3O_8$. It had a unique hemilactal link between two separate rings (position 5 and 10), a guanidium group constituting an integral part of the molecule and many OH groups.



Tetrodotoxin

On the other hand, in the late 1950's, physiological and pharmacological action of TTX started to be investigated using electrophysiological technique. The studies of Narahashi et al. (1960, 1964) revealed that TTX blocks the action potential by a selective inhibition of transient current carried by sodium ions. Unique and highly specific action of TTX stimulated many investigators to utilize it as a tool for a wide variety of physiological and pharmacological studies.

In Kyoto meeting, Mosher et al. reported that tarichatoxin, which was isolated from eggs of the California newt (*Taricha trosa*), was identical to TTX. Toxic substance of this eggs had been discovered and named tarichatoxin by Twitty in the late 1930's (Mosher et al. 1964). This was the first report on the presence of TTX in animals other than a pufferfish. Extracts of adult salamanders from ten different species related to *T. trosa*, collected at northern Pacific perimeter, were injected into mice and produced symptoms resembling those of TTX poisoning (Wakely et al. 1966). In 1973, TTX was isolated from a goby in Ryukyu islands (*Gobius cringer*), which was completely different order from a pufferfish (Noguchi and Hashimoto 1973). A study of toxic frogs led to the isolation of TTX from the skin of the harlequin frog of Costa Rica (*Atelopus varius*) (Kim et al. 1975) and from the skin and eggs of the Costa Rican frog (*A. chiriquiensis*) (Pavelka et al. 1977). Another isolation and identification from the Australian blue-ringed octopus (*Hapalochlaena maculosa*) expanded the diversity of animals containing TTX. This was the first reported case in which TTX had been found to occur in a venom and used for

paralyzing prey (Sheumack et al. 1978). So far TTX has been detected from various species of shellfish (Noguchi et al. 1981, 1984, 1985a, 1985b ; Yasumoto et al. 1981 ; Narita et al. 1981, 1984; Joen et al. 1984), starfish (Noguchi et al. 1982 ; Miyazawa et al. 1985; Maruyama et al. 1985) and xanthid crab (*Atergatis floridus*) (Noguchi et al. 1983a, 1983b).

Distribution of TTX in these diverse animals led to controversies about its origin. Endogenous and exogenous origin have been discussed. If the endogenous origin would be true, the various animals such as fishes, newts, frogs and shellfishes should have genetic properties for TTX production. However, the finding that cultured puffers were non-toxic (Matsui et al. 1982) or the observation of variable toxic levels in puffers with individuals, locations and seasons are hard to explain on the endogenous origin hypothesis. Occurrence of TTX in a shellfish was uncovered by investigations of the incidents of fatal and near-fatal shellfish poisonings in Japan. The toxic starfish "togemomojigai" (*Astropecten polyacanthus*) was shown in the digestive gland of the trumpet shell "boshubora" (*Chalonia sauliae*) which caused food poisoning (Noguchi et al. 1982). They concluded that TTX in the trumpet shell came from food sources. Above or other circumstantial observations supported the idea that TTX came from the exogenous origin. As a result of tracing the exogenous origin along with food chain, Yasumoto et al. (1986) isolated TTX-producing bacteria from a red calcarious algae, and Noguchi et al. (1986) also isolated from intestine of a xanthid crab. The bacterial strains isolated by Yasumoto et al. were reported to be new species and named *Shewanella alga* and *Alteromonas tetraodonis* (Simidu et al. 1990). In addition, TTX-producing bacteria have been isolated from various marine organisms, such as pufferfish (Noguchi et al. 1987 ; Yotsu et al. 1987), starfish (Narita et al. 1987) and blue-ringed octopus (Hwang et al. 1989). Simidu et al.(1987) carried out a screening test with type culture strains of marine bacteria and showed that 12 out of 24 strains tested clearly produced TTX or related substances. Since the finding of TTX-producing bacteria, the works to search TTX-containing organisms have been continued, and flatworm (Miyazawa et al. 1986, 1987), ribbonworm (Miyazawa et al. 1988), horseshoe crab (Kangsuwan et al. 1987) and arrowworm (Thuesen et al. 1988) have been revealed. TTX-containing organisms reported up to now are summarized in Table 1.

Since the findings of TTX-producing bacteria, two hypotheses on the toxification

mechanism of animals have been controversial: (1) TTX is produced by bacteria which live symbiotically with these toxic animals, and (2) TTX is produced in the environment and accumulated in these animals through food webs. The evidences that TTX-producing bacteria were isolated from the intestine of toxic organisms and many of them belonged to *Vibrionaceae* favored the first hypothesis: TTX was produced by the intestinal bacteria and absorbed by the host organisms. However, this story contradicted the facts that cultured puffers were non-toxic and the intestinal microflora of non-toxic and toxic puffers were not different from each other (Sugita et al. 1988). In 1988, Kogure et al. detected high concentrations of sodium channel blocking agents in marine sediment and suggested that a part of these agents was TTX. TTX-producing bacteria were isolated from the same sediment (Do et al. 1990). TTX-producing actinomycetes were also isolated from marine sediment (Do et al. 1991). These reports strongly supported the second hypothesis: TTX was produced in the sediment by marine bacteria, incorporated into benthic meiofauna and transferred to various marine organisms through food webs.

At present TTX-producing bacteria are assumed to have wide distribution in marine environment and to be related to the widespread toxification of marine organisms. The hypothesis that TTX is produced in the environment and accumulated through food webs has been widely accepted although it is not definitive. As for the concrete mechanism, marine sediment was pointed out as a site for TTX production and a primary food source for TTX accumulation (Kogure et al. 1988 ; Do et al. 1990, 1991). However, the works on marine sediment could not explain the fact that a planktonic organism like arrowworm contains TTX. This fact implied the presence of another food source suspended in the upper water. Furthermore, much of organic matter in sediments are supplied from the upper water column by the sedimentation of dead organisms, feacal pellets or others. Consequently in the above speculations, suspended particles would reasonably come to attention as an important source of TTX, and particularly sinking fraction of suspended particles would be important with relation to the supply of TTX in the sediment. These particles are generally colonized by large bacterial populations (Pearl 1973, 1974 ; Fukami et al. 1981, 1983a) and offer a possible site for TTX production to bacteria. It is easily assumed that suspended particles containing TTX are ingested by the detritus feeder at first and TTX is transferred to organisms in higher trophic levels through

detritus food webs. In the context described above, this study aimed to clarify the first step of TTX accumulation mechanism in marine environment.

There was two methodological problems when I started this study. The first one was the way to collect larger amount of suspended particles which was enough for analysis. Filtration of sea water is laborious and time consuming for practical use, and fragile particles would be destroyed and missed. However, otherwise sediment trap system consisting of cylinders settled at the bottom would make easy collection of such particles possible. Suspended particles collected by the sediment trap are particularly called sinking particles. Focusing on sinking particles had the methodological advantage in addition to the importance of themselves as a source of TTX. The second problem was the sensitivity of the methods to detect TTX. The samples analyzed in this study were occasionally expected to contain smaller amount of TTX than the detectable limit of usual methods. In order to achieve more sensitive analysis, the tissue culture bioassay was improved, and immunological methods using anti-TTX monoclonal antibody was uniquely applied. These two points concerned in methodology were included with emphasis in this study.

In Chapter 1, the presence of TTX in sinking particles collected from coastal waters and in animals which feed such particles were investigated. In Chapter 2, improvement of the bioassay method using a mouse neuroblastoma cell line was discussed to measure TTX with higher sensitivity and simpler procedure. In Chapter 3, TTX produced by marine bacteria was examined by using anti-TTX monoclonal antibody which had just been developed recently. This new tool provided us more information about the property of TTX produced by bacteria. In Chapter 4, immunological methods were applied to measure the TTX concentrations in sinking particles and to microscopically visualize the TTX-containing bacteria which were attached to the particles.

Table 1. Animals containing tetrodotoxin and related substances

Animals		References
Platyhelminthes	(Flatworms)	
Tubellaria	Planocera reticulata	Miyazawa et.al. 1987
	P. multitentaculata	Miyazawa et.al. 1986
Nemertinea	(Ribbonworms)	
Anopla	Lineus fuscoviridis	Miyazawa et.al. 1988
	Tubulanus punctatus	
Annelida		
Polychaeta	Pseudopotamilla acelata	Yasumoto et.al. 1986
Mollusca		
Gastropoda	Charonia sauliae	Noguchi et.al. 1985
	Babylonia japonica	Noguchi et.al. 1981
	Tutufa lissostoma	Noguchi et.al. 1984
	Zeuxis siquijorensis	Narita et.al. 1984
	Niotha clathrata	Jeon et.al. 1984
Cephalopoda	Octopus maculosus	Sheumack et.al. 1978
Arthropoda		
Crustacea	Atergatis floridus	Noguchi et.al. 1983
	Zosimus aeneus	Yasumura et.al. 1986
	A. integerrimus	
Xiphosura	Carcinoscorpius rotundicauda	Kangsuwan et.al. 1987
Chaetognatha	(Arrowworms)	
Sagittoidea	Parasagitta elegans	Thuesen et.al. 1988
	Flaccisaggita scrippsae	
Echinodermata	(Starfishes)	
Asteroidea	Astropecten polycanthtus	Noguchi et.al. 1982
	A. latespinosus	Miyazawa et.al. 1985
	A. scoparius	Maruyama et.al. 1985
Vertebrata		
Ostiechthyes	Fugu spp.	
	Gobius criniger	Noguchi et.al. 1973
Amphibia	Taricha spp.	Wakely et.al. 1966
	Triturus spp.	
	Cynops spp.	
	Notophthalmus viridescens	
	Atelopus spp.	Kim et.al. 1975

Chapter 1.

Tetrodotoxin in Sinking Particles from Coastal Waters

INTRODUCTION

In 1979, a paralytic food poisoning occurred through ingestion of trumpet shellfish, *Charonia sauliae* , which had been assumed to be non-toxic. The causative agent was identical to TTX (Narita et al. 1981). Noguchi et al.(1982) reported that the starfish *Astropecten polyacanthus*, often found in the digestive tract of the trumpet shellfish, contained TTX. They concluded that TTX in the trumpet shellfish came from the starfish which was ingested as its food. Feeding experiment by Shiomi et al.(1984) showed that the trumpet shell *C. sauliae* and the ivory shell accumulated TTX when they were fed with muscle of toxic pufferfish as a food. The rate of toxin accumulation was about 20-80 %. Another marine snail *Neptunea arthritica* was also reported to accumulate TTX through a food source containing TTX (Sato et al. 1991). As for the cultured puffers which are almost non-toxic, feeding experiment showed that TTX was accumulated through the diet containing toxic ovary of puffers (Matsui et al. 1981, 1982). Obviously these shellfish and puffers have accumulation mechanism for TTX from their food source. This is supporting the evidence for the toxification mechanism through food webs in marine environment. It should be important to find out the TTX-containing food source of toxic marine organisms.

In 1988, Kogure et al. reported the presence of TTX in sediment samples from 2 stations in coastal waters and 1 station at deep-sea in the western Pacific off the coast of Japan and suggested that bacteria inhabiting the sediment are responsible for the production of the toxin. Do et al. (1990, 1991) demonstrated the productivity of TTX by bacteria isolated from deep-sea sediment and actinomycetes from shallow water and deep-sea sediment. They also showed similar evidence of the presence of TTX in fresh water sediment and TTX production by the isolated bacteria from these (Do et al. 1993). It would be assumed that marine sediment is important as a source of TTX in marine environment.

Particulate organic material (POM) that has settled from upper water column plays a role as the main food source for benthic organisms. Particularly marine snow, the main type of large POM, is heavily colonized by bacteria and serves as a locus for

active metabolism of them (Alldredge and Silver 1988). While sinking through the water column, the particles are altered by microbial degradation in which easily degradable substances such as starch, protein and fat are removed (Karl et al. 1988 ; Smith et al. 1992 ; Hoppe et al. 1991). During declining phase of phytoplankton bloom, some of free-living bacteria become attached and grow on the surface of the particles (Fukami et al. 1983a, 1983b). Attached bacteria are inferior to free-living in biomass but are more metabolically active on a per-cell bases, although the activity is easily affected by the quality of particulate substances (Griffith et al. 1994). High metabolic activity of attached bacteria in POM led to the hypothesis that TTX would be produced by bacteria in not only sediment but also sinking particles, which would be the origin of TTX in toxic organisms as a food source.

A caprellid is an elongated amphipod (crustacea) with clinging feet at the front and hinder ends, and is very abundant in hydroid colonies, eelgrasses, seaweeds, etc (MacGinitie et al. 1968). Keith et al.(1969) reported that floating organic detritus is much of the food for two species of caprellids investigated, *Caprella californica* and*C. equilibra*. Five types of adult feeding mechanisms has been reported: browsing, filter-feeding, scraping, scavenging, and predation. They would consume organic detritus by filter-feeding, scraping and scavenging (Cane 1977).

In this chapter, the occurrence of TTX in sinking particles was investigated, and then caprellids were analyzed as a marine organism which fed these particles.

MATERIALS AND METHODS

Sample collection

Sinking particles were repeatedly collected in Aburatsubo Inlet from May to October in 1991 (sample no. I-VI) using the sediment trap system (Fig. 1-1). Ten sets of traps made of transparent acrylic resin cylinder, 80 cm height and 7.5 cm diameter, were set at the depth of 6-8 m for 1 day (Fig. 1-2). As for sample II and VI, the traps were

moored for 3 and 2 days respectively. Additionally sinking particles were collected from other coastal areas (sample no. VII-XII) ; Tokyo Bay, Sagami Bay and Suruga Bay during KT- 91-12 and KT-92-10 cruise of R/V Tansei-maru Ocean Research Institute Univ. of Tokyo in August 1991 and July 1992 (Fig. 1-3). The depth of settling traps was 10 m. After retrieving the traps, most of the water in the cylinder was discarded using siphon. Obtained particles at the bottom of the cylinder (Fig. 1-4) were kept cool in the case of Aburatsubo Inlet and freezed in the case of Tansei-maru cruises, and brought back to Ocean Research Institute, University of Tokyo.

In July and August of 1991 when sinking particles were collected in Aburatsubo Inlet, more than a thousand of caprellids clinging on the seaweeds were captured (Fig.1-5). Samples were kept cool and brought back.

Toxin extraction

Particles were centrifuged at 16,000 g for 20 min. Obtained particles were suspended in 0.1% acetic acid, subsequently ultrasonicated and boiled for 20 min After centrifugation at 25,000 g for 20 min, the supernatant was purified using SEP-PAK C_{18} cartridge (Waters Associates). The eluate was evaporated to dryness and the residue was dissolved in a small amount of distilled water. Caprellids were homosinized and boiled in 0.1 % acetic acid for 20 min Subsequent treatments were same as sinking particles. The extracts were analyzed by the tissue culture assay method (Kogure et al. 1988b), high performance liquid chromatography (Yasumoto and Michishita 1985) and gas chromatography-mass spectrometry (Suenaga and Kotoku 1980, Narita et al. 1981) to confirm the presence of TTX and related substances.

Bioassay

The tissue culture bioassay (Kogure et al. 1988b) was used for the detection and quantitative measurement of sodium channel blockers (SCBs) in the samples. Briefly, the alkaloid toxin veratridine causes sodium influx in the mouse neuroblastoma cell line Neuro 2A (ATCC, CCL 131), when the function of Na^+-K^+ATPase is inhibited by

ouabain. This results in the cellular swelling and subsequent death. However, in the presence of TTX or similar sodium channel blocking agents, cells continue to grow because TTX counteracts veratridine. The relative abundance of living cells can be used to estimate the concentration of toxin in the sample.

High performance liquid chromatography (HPLC)

Samples were further analyzed by ion-paired reversephase HPLC to identify sodium channel blocking agents according to the methods described previously (Yasumoto and Michishita 1985). A JUSCO Tri Roter-VI with a Senshu Pak column (ODS-3251-D, 8x250 mm, Senshu Scientific Co.) was used as a HPLC system. For detecting fluorescence, the excitation and emission were set at 365 and 510 nm respectively on a Hitachi F1000 fluorescence spectrophotometer.

Gas chromatography-mass spectrometry (GC-MS)

After the analyses by the tissue culture bioassay and HPLC, the extracts were alkali-hydrolyzed to convert TTX or related substances into C9-base, which is detected by GC-MS. The extract mixed with an equal volume of 1N NaOH solution was heated in a boiling water bath for 40 min The alkaline hydrolysate was neutralized with 1N HCl and purified by SEP-PAK C_{18} cartridge (Waters Associates). The eluate was evaporated to dryness and then trimethylsilylated according to the method of Narita et al. (1981). This sample was subjected to GC-MS on a VG AutoSpecE mass spectrometer equipped with a HP 5890 gas chromatograph. GC was performed on a 15 m x 0.25 mm i.d. DB-5 fused silica column, 0.25 μm film thickness, with helium as carrier gas (25 ml/min) (Hewlett-Packard). The oven temperature was raised from 160 to 250 ^{o}C at the rate of 5 ^{o}C/min The electron ionization energy was 70 eV and the ion source temperature was kept at 200 ^{o}C.

RESULTS

The data of sinking particles from Aburatsubo Inlet are summarized in Table 1-1. SCBs were detected from all samples by the tissue culture bioassay. Toxin concentrations are expressed as equivalent to TTX.

All samples except for IV were found to contain TTX or related substances by the HPLC analysis. A peak corresponding to authentic TTX was observed in the HPLC chromatograms of samples I, II, V, VI. Other peaks indicating the occurrence of 4-epiTTX were shown in the samples II, III. Parts of samples II, V, VI, from which a peak corresponding to TTX had been detected, were subjected to ultrafiltration (Amicon Diaflo YM1 ;cut-off limit 1,000 daltons) and charcoal column. These three samples were analyzed by HPLC again. A peak corresponding to TTX was also detected from every sample analyzed. Fig. 1-6 shows the HPLC chromatograms of the extract from sample VI before(a) and after(b) the treatments. However, the peak of 4-epiTTX in sample II could not be found after the treatments. The data of HPLC in Table 1 are based on the analyses before the treatments.

GC-MS analysis proved the presence of C9-base in the alkali-degraded extracts from samples I, II, IV, VI. Fig. 1-7 shows the selected ion-monitored chromatogram (m/z 392,407) and the mass spectrum of sample VI. Selected ion-monitored chromatogram indicated the presence of an identical compound in retention time with authentic TMS-C9-base in the TMS derivative from alkali-degraded extract of sample VI (Fig. 1-7a). The mass spectrum of this compound (Rt 11.11 min) showed ion peaks of m/z 407 (parent peak), 392 (base peak), 376, which are specific to TMS-C9-base derived from TTX and related substances (Fig. 1-7b).

As for the sinking particles from various coastal waters other than Aburatsubo Inlet, SCBs were detected from all samples, and peaks corresponding to TTX and/or anhydroTTX were showed in the chromatograms of samples VII, VIII, IX and XII. The results were summarized in Table 1-2.

From the both extracts of caprellids, peaks corresponding to TTX (Fig. 1-8) and SCBs were detected. Furthermore, C9-base was detected from the sample no.1 (Fig. 1-9a,

1-9b). The results were summarized in Table 1-3.

DISCUSSION

As the result of HPLC analysis of sinking particles, concentrations of TTX in the samples were estimated to be about 200-1,000 ng/g from the peak area of samples compared with authentic TTX. The quantity of the toxin, however, varied from time to time in the inlet. This may be due to the differences in bacterial populations and activity affected by the quality of sinking particles. It seems highly probable that hydrographical conditions and biological components in the inlet lead to the formation of different types of sinking particles from time to time.

Toxin concentration of sample II showed good agreement between the tissue culture bioassay and the HPLC analysis. As for samples I, V, VI, VII, IX, XII, toxin concentrations estimated by the tissue culture bioassay were five to ten times as high as those obtained by the HPLC analysis. The tissue culture bioassay detects any kinds of sodium channel blocking toxins, if the function is similar to TTX. Therefore, it is expected that there are other types of neurotoxins such as saxitoxin or gonyautoxin. These toxins are also reported to occur in various marine organisms and cause paralytic shellfish poisoning (Kao 1966, Shimizu 1978). Two major groups of PSTs, saxitoxin (STX, neoSTX) and gonyautoxin (GTX1, GTX2, GTX3, GTX4), in samples III, IV, V and VI were further analyzed by HPLC following the methods by Nagashima et al. (1987, 1989). As a result, a small peak corresponding to GTX3 was observed in samples IV and VI. From other samples, no peak of PSTs was detected. Thus, concentrations of these two PSTs were too low to explain the difference of concentrations between TTX and sodium channel blocking toxins.

There are possibilities of errors intrinsic to this tissue culture method. Theoretically, any conditions which affect the function of veratridine and/or ouabain could lead to the over or underestimation of TTX. For instance, sea anemone toxin and brevetoxin as well as veratridine cause influx of sodium ions by enhancing persistent

activation of sodium channels (Catterall 1985). Binding of TTX and STX is inhibited by chemical modification of sodium channels (Worley et al. 1986). At present, we have no data of the occurrence of such compounds, although it seems very likely that there are various kinds of unknown compounds which may have certain function to the sodium channel in nature. However, it is noteworthy to point out that Sato et al.(1988) reported that accurate toxicity values were obtained from known amount of STX standards containing <3.5% polypepton, <1.0% meat extract, <1.0% KCl, and <3.5% NaCl respectively by this tissue culture bioassay ; Jellet et al.(1992) noted that co-extracted materials hardly affected the toxicity values after the sample treatment by SPE-C_{18} column. They also reported that the data of the tissue culture bioassay showed good correlations with those of mouse bioassay (correlation coefficients r=0.995,0.992) when they analyzed PSTs of shellfish and dinoflagellate.

On the other hand, although C9-base detected by GC-MS analysis is generally considered to be derived from TTX and related substances, there is a possibility that C9-base itself or any substance sharing partial structure with TTX are also detected as TMS-C9-base. In this investigation, however, three independent analytical methods, two chemical and one biological, indicated the presence of TTX. It is difficult to imagine that compounds other than TTX would be coincidentally detected in three different analyses. Therefore, we concluded that C9-base detected had been derived from TTX and related substances and sinking particles contained these compounds.

Additional experiments in which sinking particles were collected from various coastal waters during R/V Tansei-maru cruise suggested the widespread distribution of TTX in such particles. Their sinking velocity can vary greatly, depending on the origin, composition and size of the particles (Alldredge and Silver 1988). As for sample II, which showed good agreement between the two analyses, we obtained 2.2 g particles in 3 days. These particles contained about 2.0 μg sodium channel blocking agents. As the total base area of the cylinders was 440 cm^2, the sedimentation flux of sodium channel blocking agents was estimated to be 1.5 ng/cm^2/day or 45 ng/cm^2/month. It will be noteworthy that Kogure et al. (1988a) detected 25-90 ng/g sodium channel blocking agents in marine sediment. Although the production and turnover rate of the toxin in sediments are not known, it is assumed that sinking particles may supply a considerable part of

sodium channel blocking agents to sediments. This hypothesis seems to be reasonable because organic matters in sediments are virtually depending on the vertical flux from the upper water column. The toxin may be produced during the bacterial decomposition of easily biodegradable fraction. This is supported by the fact that sinking particles contain one to two orders of magnitudes higher concentration of TTX (200-1,000 ng/g) than in sediments. Do et al. (1990) suggested that TTX in marine sediment was produced by bacteria and that TTX-producing bacteria were quite widespread among various bacterial groups. Although not quantitative, we observed dense bacterial population attached or colonized on sinking particles under the fluorescence microscope. It may be reasonably supposed that the toxins were produced by bacteria in the sinking particles.

The presence of TTX in the extracts of caprellids was confirmed in the same way as sinking particles. The mean concentration of TTX in a caprellid estimated from the data of HPLC was 28 ng (0.14 MU) per individuals or 2300 ng/g, which was higher than sinking particles (200-1000 ng/g). This result proposed that they might absorb TTX from their food source and actively accumulate. As this result was integrated into those of sinking particles, a concrete mechanism of TTX accumulation and important role of sinking particles come up reasonably : TTX is produced by bacteria in sinking particles and these particles are ingested by various detritus feeders, resulting in the toxification of those animals and subsequent transfer to higher trophic levels through food webs.

Research on TTX production by bacteria in sinking particles were described in Chapter 4. This is supposed to be the first report on the occurrence of TTX in particles in aquatic environment. Further investigations are needed to reveal the quantitative significance of the toxin in particles and its contribution to the toxification of marine organisms.

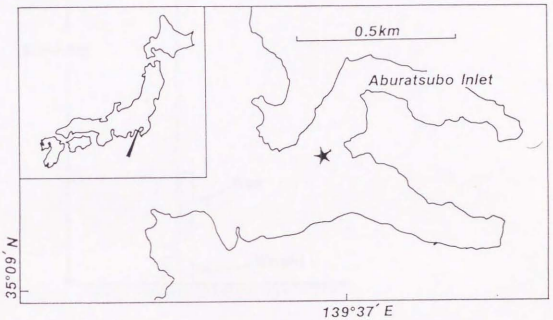


Figure 1-1. Sampling location in Aburatsubo Inlet.

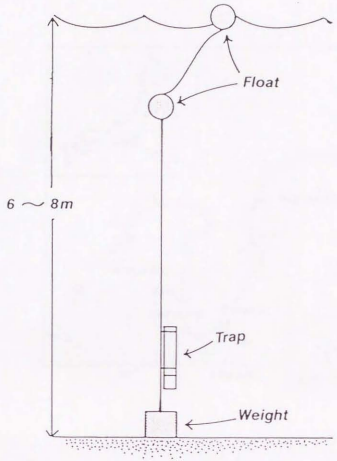


Figure 1-2. Illustration of sediment trap system.

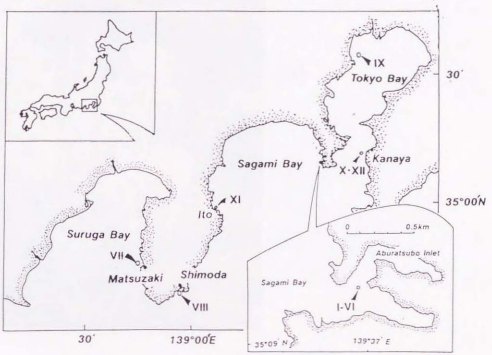


Figure 1-3. Sampling locations in Tokyo Bay, Sagami Bay and Suruga Bay. Roman numerals correspond to the sample no. in Table 1-1 and 1-2.

(a)

(b)

Figure 1-4. Sediment trap cylinder (a) and obtained particles at the bottom of it (b)

(a)

(b)

Figure 1-5. Seaweeds (*Chaetomorpha crassa*) (a) in which large number of caprellids clung (b) .

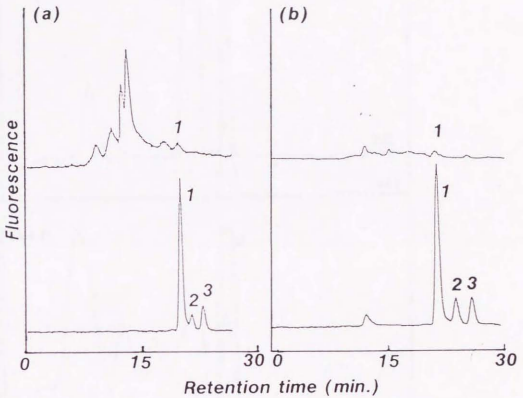


Figure 1-6. HPLC chromatograms of the sinking particles (sample IV) (a) before and (b) after ultrafiltration and charcoal column treatments. Upper plots: sample. Lower plots: standard (1)TTX, (2)4-epiTTX, (3)AnhydroTTX.

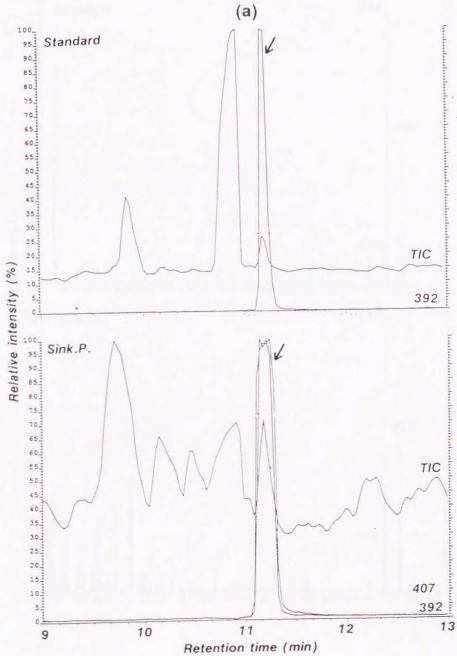


Figure 1-7(a). Total ion chromatogram (TIC) and selected ion-monitored chromatograms (m/z 392, 407) of the trymethylsilyl (TMS) derivative from alkali-degraded extract of sinking particles (Sink.P.)(sample VI, lower plot) and authentic TTX (upper plot). Arrows indicate peaks of TMS-C9-base derived from TTX and/or its analogues.

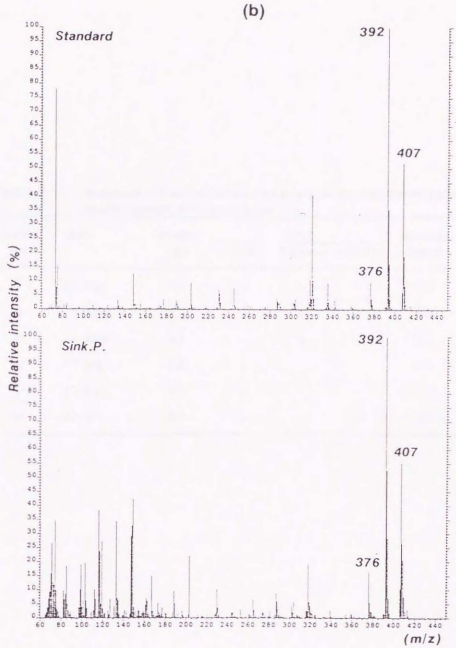


Figure 1-7(b). Mass spectrum of TMS-C-9-base obtained from the authentic TTX (upper plot). Mass spectrum of the peak corresponding with authentic TMS-C9-base (Rt. 11.11 min) in the TMS derivative from sample VI (lower plot).

Table 1-1 Analysis of TTX and related substances in sinking particles collected in Aburatsubo Inlet. NA : not analyzed

Sample No.	Date	Weight (g)	HPLC			Bioassay (ug/g)	GC-MS
			TTX	4-epimer	Anhydro		
I	31 May	1.1	+	-	-	5.6	+
II	3 June	2.2	+	+	-	0.9	+
III	24 June	0.6	-	+	-	3.4	NA
IV	17 July	0.9	-	-	-	0.6	+
V	15 Aug	0.8	+	-	-	12.5	NA
VI	27 Oct	6.1	+	-	-	1.5	+

Table 1-2 Analysis of TTX and related substances in sinking particles collected in the coastal stations of Tokyo bay, Sagami Bay and Suruga Bay

Sample No.	Date	Weight (g)	HPLC			Bioassay (ug/g)
			TTX	4-epimer	Anhydro	
VII	24 Aug.	0.4	+	-	-	0.7
VIII	25 Aug.	0.4	-	-	+	0.4
IX	26 Aug.	0.6	+	-	-	0.9
X	9 July	0.6	-	-	-	1.9
XI	10 July	0.6	-	-	-	6.2
XII	16 July	2.8	+	-	+	0.6

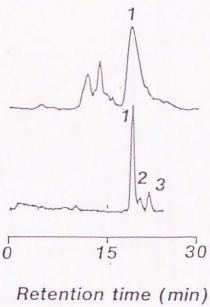


Figure 1-8. HPLC chromatograms of the caprellids captured in August (sample II). Upper plots : sample. Lower plots : standard (1)TTX ; (2)4-epiTTX ; (3)AnhydroTTX.

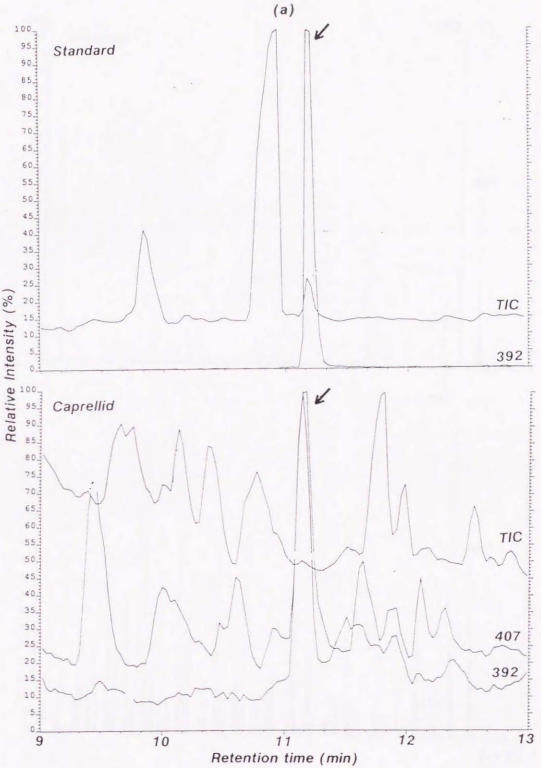


Figure 1-9(a). Total ion chromatogram (TIC) and selected ion-monitored chromatograms (m/z 392, 407) of the trymethylsilyl (TMS) derivative from alkali-degraded extract of caprellids captured in July (sample I, lower plot) and authentic TTX (upper plot). Arrows indicate peaks of TMS-C9-base derived from TTX and/or its analogues.

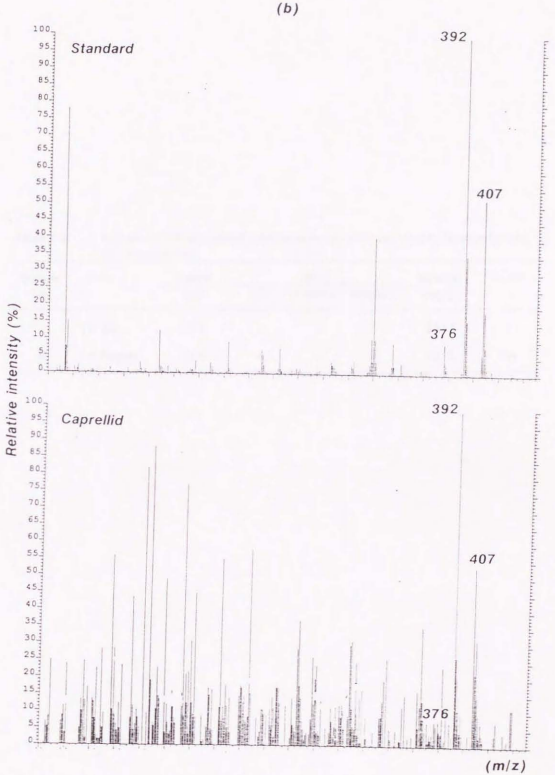


Figure 1-9(b). Mass spectrum of TMS-C-9-base obtained from the authentic TTX (upper plot). Mass spectrum of the peak corresponding with authentic TMS-C9-base (Rt. 11.11 min) in the TMS derivative from sample I of caprellids (lower plot).

Table 1-3 Analysis of TTX and related substances in caprellids captured in Aburatsubo Inlet
NA : not analyzed

Sample No.	Date	Weight (g)	HPLC			Bioassay (ug/g)	GC-MS
			TTX	4-epimer	Anhydro		
I	16 July	15.6	+	-	-	3814	+
II	14 August	41.0	+	-	-	>915	NA

Chapter 2.

Improvement of Tetrodotoxin Bioassay Using a Mouse Neuroblastoma Cell Line

INTRODUCTION

Various methods of chemical (Suenaga and Kotoku 1980 ; Shimada et al. 1983 ; Yasumoto and Michishita 1985), biological (Strong et al. 1973 ; David and Fontelo 1984 ; Kogure et al. 1988a ; Hu and Kao 1991) and immunological (Watabe et al. 1988 ; Huot et al. 1989 ; Matsumura and Fukiya 1992) assays are currently available to identify sodium channel blocking toxins such as TTX and STX (Egmond et al. 1993). The standard method for biological assay is still mouse bioassay (Kawabata 1978), which has been employed in almost all works on these toxins. However, when measuring the accurate toxicity of many samples, it is difficult to supply many mice of same strain with suitable weight and appropriate age. In addition, this method is not sensitive enough for laboratory use : minimum detectable limit is about 0.2 μg of TTX per assay.

The tissue culture bioassay (TCBA) using mouse neuroblastoma cell line, Neuro 2A (ATCC, CCL 131), is a simple and sensitive method for tetrodotoxin (TTX), saxitoxin (STX) and related compounds (Kogure et al. 1988b). TCBA method is specific to sodium channel blocking agents, such as above toxins. In this assay, an alkaloid toxin, veratridine, causes sodium ion influx in this cell, when the function of Na^+-K^+ATPase is inhibited by ouabain. This results in the cellular swelling and subsequent death. In the presence of TTX or similar sodium channel blocking toxins, cells continue to grow as these toxins counteracts veratridine. The relative abundance of living cells has linear relationship with the concentration of a toxin in the samples and therefore, can be used to estimate the toxin. The sensitivity of this method is three order of magnitudes higher than standard mouse assay. Therefore, theoretically TCBA method is useful for detection at low level concentrations of toxins.

As described in Chapter 1 and reported in the previous papers, this method was successfully used for the samples such as sediments and sinking particles for which higher sensitivity was required (Kogure et al. 1988a ; Do et al. 1993). However, this is not always very convenient for routine bioassay, because it solely depends on the cellular morphological change to distinguish dead cells from living cells. It is time consuming and needs some skillness for the distinction. To overcome this problem,

Gallacher and Birkbeck (1992) tried to combine a dye (neutral red) inclusion procedure for counting viable cells. Jellet et al. (1992) tried to stained cells with crystal violet after washing out detached dead cells. In both methods, an absorbance, which can be automatically measured by microplate reader, was used to detect abundance of living cells so that counting procedures changed for rapid and objective ones. However, these methods are still insecure as they require washing steps for removing free dye or dead cells, which will easily affect the data by personal skillness of the investigators. Manger et al. (1993) reported to use a kind of tetrazolium salt 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) as an indicator of living cells in their improved TCBA method for the detection of STX.

In this chapter, two kinds of tetrazolium salts, MTT and [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST-1) were tried to use as an indicator. Tetrazolium salt is cleaved by dehydrogenase enzymes in active mitchondria and produce a dark blue formazan crystal (Mosmann 1983). If absorbance of formazan is measured by a microplate reader, the toxin concentration would be calculated from the absorbance. MTT and WST-1 method would provide us with both omitting a washing process and simplifying a counting process. Formazan crystal generated from MTT is hardly soluble in water so that the solubilization step is required before the measurement of absorbance. Compared with MTT, on the other hand, formazan from WST-1 is water soluble so that the direct measurement would be possible. In this experiment, assay conditions when combining MTT and WST-1 with TCBA method were examined.

When applying this method to bacterial extracts, some compounds included with toxins in the extracts may interfere with accurate measurement. Therefore, it is necessary to check the effect of such compounds on the quantitative analysis. Such effect, which is called matrix interference in this thesis, would be estimated with measuring a sample supplemented with known amount of TTX (spiked sample). To prepare the spiked sample, negative matrix including no TTX should be required. *Escherichia coli* (K-12) would be available, because no production of TTX reported in the previous paper (Simidu et al. 1987) and also shows in the results of Chapter 3. In this experiment, the whole extract (cell and supernatant) and cell-free supernatant of *E. coli* (K-12) were

used to be supplemented and examined for matrix interferences.

MATERIALS AND METHODS

Cell culture and chemicals

Mouse neuroblastoma cells (Neuro2A) were grown in RPMI1640 medium supplemented with 10% fetal bovine serum and 1% Antibiotic Antimycotic Solution (Sigma) in an air atmosphere at 37 °C. Cells were removed from the culture surface by trypsinization and resuspended in the culture medium. Two hundred µl of cell suspension (10^4-10^5 cells/ml) was inoculated into each well of 96 wells microtitre plate. After an incubation of over night at 37 °C, cells were used for the assay.

Tetrodotoxin were purchased from Wako Pure Chemical Industries, Ltd. MTT, ouabain and veratridine were from Sigma. WST-1 included in Cell Counting Kit was purchased from Dojindo.

TCBA assay conditions

Standard TTX was serially diluted with distilled water. Culture medium in each well was pipetted to 80 µl. Ten ul of ouabain, 5 µl of veratridine and 5 µl each of diluted TTX were dispensed into several replicate wells. After the plate was incubated at 37 °C for 20-24 hours, relative abundance of living cells was estimated with MTT assay. To determine optimum concentrations of ouabain and veratridine, mortality of cell depending on the concentration of these chemicals was examined. First, in the presence of 0.5 mM ouabain, veratridine was added to final concentrations of 0.002, 0.005, 0.01, 0.02, 0.03 and 0.05 mM. Second, in the presence of 0.05mM veratridine, ouabain was added to final concentrations of 0.03, 0.05, 0.1, 0.2, 0.5, 1.0 mM. Finally, three combination of ouabain and veratridine concentration (0.5mM ouabain + 0.03mM

veratridine, 0.5mM + 0.05mM, 1.0mM + 0.05mM respectively) were tried to obtain clear dosage response relation with TTX.

MTT assay conditions

Ten μl of filter-sterilized stock MTT solution dissolved in distilled water was added to all wells after the reaction of ouabain, veratridine and TTX. Formazan crystals generated from MTT were solubilized in 100 ul of an appropriate solvent. An absorbance of each wells was measured at 595 nm by the microplate reader (CORONA Co.,Ltd.). To determine an optimum concentration, MTT was added to final concentrations of 0.2, 0.5, 1.0 and 2.0 mM. On the other hand, to confirm the relationship between viable cell number and absorbance, MTT was added to 6 replicate wells including four serial dilutions of cell suspension. At the same time, colorimetric measurement was made after 2, 4, 6 hours respectively in order to find out incubation time enough for reliable detection. Three kinds of solvents, isopropanol containing 0.04N HCl (acid isopropanol), dimethyl sulfoxide (DMSO) and 20% sodium dodecyl sulfate (SDS), for solubilization of formazan were compared.

WST-1 assay

In Cell Counting Kit (Dojindo), WST-1 was dissolved in the solution of 1-methoxy-5-methylphenazinium methylsulfate (1-methoxy PMS), which mediates and accelerates the reduction of WST-1. Mixed solution was filter-sterilized and 10 ul of the solution was added to each well. After the incubation at 37 °C, absorbance was measured at 405 nm by microplate reader, with reference wave length at 595 nm. To determine the incubation time enough for reliable detection, absorbance after 1, 2, 3 and 4 hours of reaction were examined.

Matrix interferences of whole (cell and supernatant) extract

E. coli (K12) was cultured at 20 °C by shaking with modified L medium (ML

medium) containing 0.5% polypepton, 0.1% yeast extract, 0.2% NaCl, 0.2% KCl, 0.4% $MgSO_47H_2O$ and 50mM Tris-HCl in D.W. at pH 7.5-7.6. After cultivation for 48 hours, 1% acetic acid was added to the culture for adjusting pH to 4.0. Whole the culture including both cell and supernatant was ultrasonicated and boiled for 30 min. After centrifugation at 7000 g for 20 min, discarding the precipitates, the supernatant was passed through SEP-PAK C_{18} column and evaporated to dryness. The residue was reconstituted in distilled water and diluted. Two fold dilution was made serially 8 steps. Spiked samples were prepared with supplementing each dilution of the extract by the same amount of TTX. The TTX concentration in spiked samples was prepared to 10 ng/μl, and 5 μl of this sample was added to each well so that each well finally received 50 μl of TTX which would be enough to give 100% relative absorbance in the assay. Percentage recovery of supplemented TTX at each dilution of the extract was estimated in the improved TCBA combining with MTT (MTT-TCBA).

Matrix interferences of bacterial culture supernatant

Cell-free supernatant of *E. coli* cultured in the same conditions described above was directly spiked by serial dilutions of TTX. Dose-response of TTX with or without *E. coli* supernatant was compared in TCBA combining with WST-1 (WST-1-TCBA). The effect of another two concentrations of the supernatant, 1/4 and 1/8, was additionally tested.

RESULTS

TCBA assay condition

In the examination of assay condition, the response of cells treated by chemical reagents were expressed as absorbance of formazan generated from MTT. Dose responses of different concentrations of ouabain and veratridine were presented in

Figures 2-1 and 2-2. Abundance of living cells were shown as relative absorbance to each control well without these chemical reagents. In the presence of 0.2 mM ouabain and 0.03 mM veratridine respectively or higher, relative absorbance of formazan changed lower, which is due to decreasing of dehydrogenase activity of the cells (Fig. 2-1, Fig. 2-2). Combination of 0.5 mM ouabain and 0.05mM veratridine, and of 1.0 mM ouabain and 0.05 mM veratridine showed suitable dosage response to TTX (data not shown). From these results, combination of 1.0 mM ouabain and 0.05 mM veratridine seemed to be suitable.

MTT assay condition

Optimum wave length to obtain maximum absorbance of formazan generated by Nuro2A cells was found to be about 570 nm from the result of scanning analysis (data not shown). However, absorbance value at 595nm which showed 93% of maximum was chosen, because the microplate reader used in this experiment was not equipped with a filter at 570nm. Absorbance of formazan was proportional to the cell number (Fig. 2-3), which indicated that MTT could be used for counting cell numbers of Neuro2A. The absorbance became higher with increase of MTT concentration (Fig. 2-4) and incubation time after addition of MTT (Fig. 2-3). Therefore, the combination of 1.0 mM MTT and 4 hours of its incubation were chosen. Acid isopropanol seemed to be suitable for solubilization of formazan, because mean absorbance of formazan after solubilization was highest (Table 2-1).

WST-1 assay

Longer incubation time gave proportionally higher absorbance in the range from 1 to 4 hours (Fig. 2-5). Therefore, 4 hours of incubation time was employed in this experiment.

Standard dose-response curve

According to the results described above, the procedures of this improved TCBA method was confirmed (Fig. 2-6) and then dose-response curve was obtained. Response of cells was expressed as relative absorbance value which was calculated from following formula ;

$$100 \times (AB-MIN) / (MAX-MIN)$$

AB : absorbance at each concentration of TTX, MAX : maximum absorbance at each concentration of TTX, MIN : minimum absorbance at each concentration of TTX

The dose-response relation fitted to be S-shaped curve in MTT-TCBA (Fig. 2-7). Probit analysis was carried out using probit paper (Sokal and Rohlf 1981). The plotted data on this paper showed a good regression line against TTX concentrations (Fig. 2-8). Also WST-1-TCBA gave almost same regression line as MTT-TCBA (Fig. 2-9). The regression equation was used for quantitative measurement of TTX.

Matrix interferences of whole (cell and supernatant) extract

In the MTT-TCBA, percentage recovery was calculated from dividing the absorbance of wells containing TTX and bacterial extract by that of control wells containing only TTX. *E. coli* extract completely inhibited the reaction of TTX at high concentration, undiluted and 1/2, 1/4 and 1/8 diluted wells (Fig. 2-10). The effect changed between the dilution of 1/8 and 1/16. Percentage recoveries of spiked TTX were 83% and 96% at the dilution of 1/16 and 1/32 respectively and became 100% or more at the dilution of 1/64 and 1/128. Therefore, concentrated bacterial extract would interfere the quantitative measurement of TTX and might cause underestimation in MTT-TCBA.

Matrix interferences of bacterial culture supernatant

Dose-response curves of TTX with and without *E. coli* supernatant were

comparable. However, TTX with supernatant gave 12-39% higher absorbance at all dilution steps (Fig. 2-11). This effect was observed in 1/4 diluted supernatant, but not in 1/8 diluted supernatant (Fig. 2-12). Therefore, matrix interferences of bacterial culture supernatant might cause overestimation in WST-1-TCBA.

DISCUSSION

In TCBA, morphological differences between toxin-treated and untreated cells started to appear 3 hours after the addition of chemical reagents. Kogure et al. (1988b) reported that longer incubation times than 8 hours didn't enhance these differences so much. However, when we applied MTT to these cells, incubation of 16 hours at least was required. Although after the cells swelled and seems to be dead after 8 hours, dehydrogenase activity in the cells remained and formazan was still formed from MTT. Clear relationship of dose-response was obtained after more than 16 hours of incubation.

The MTT method requires solubilization of formazan generated by cellular reduction of MTT. In this experiment, acid isopropanol seemed to be most successful among three reagents tested, acid isopropanol, SDS and DMSO. However, this step is not only laborious, but also may have risk exposing laboratory personal to large quantities of potentially hazardous solutions, particularly DMSO which was used by Manger et al. (1993) for solubilization with his improved method. WST-1, alternative tetrazolium reagents, is reduced to a water soluble formazan product (Ishiyama et al. 1993). This reagent allows direct readings of absorbance, therefore, eliminating a solubilization step and shortening the assay procedure.

Catterall and Nirenberg (1973) showed the effect of TTX concentration on inhibition of veratridine-dependent $^{22}Na^{+}$ uptake in Neuro2A cells. Uptake of $^{22}Na^{+}$ was measured in the presence of 5.0 mM ouabain, 0.1 mM veratridine and serial concentrations of TTX (Fig. 2-13a). In Fig. 2-13b the standard regression line obtained in the MTT-TCBA (Fig. 2-8) is replotted with horizontal axis, TTX molarity, and vertical, relative abundance of living cells. This regression curve is quite similar to the

curve of TTX-dependent Na^+ uptake. It can be a proof that enzymatic activity of formazan formation is affected by Na^+ influx, although the relation between them was not examined here. In another improved TCBA method by Gallacher and Birkbeck (1992), half-maximal inhibition was obtained with 50 nM TTX. As for MTT-TCBA, half-maximal inhibition estimated from the regression equation of Fig. 2-8 was at 12.9 nM TTX, which was very close to that of $^{22}Na^+$ uptake (11 nM) obtained by Catterall and Nirenberg. Eliminating washing steps to remove free dye supposed to lead us to not only simpler procedure but also higher sensitivity.

As a result of experiment for matrix interferences using whole extract of *E. coli*, it was suggested that concentrated bacterial extract would cause underestimation in MTT-TCBA. It was observed that the cells morphologically changed for abnormal, tiny size and aggregation, after addition of the concentrated extract (1-1/8 dilution). Therefore, the extract was considered to be cytotoxic, which caused the interferences in the assay. Gallascher and Birkbeck (1992) applied their method directly to bacterial culture supernatant, and reported that undiluted marine broth or culture supernatant of *Alteromonas tetraodomis* were cytotoxic in the absence of veratridine and ouabain, and that direct cytotoxicity was not detectable when they were diluted 2 fold or more. They suggested that high concentration of NaCl suppressed SCB activity, however, 1 % (w/v) of NaCl did not significantly affect. It is conceivable that high concentration of culture supernatant or NaCl in a sample would give us underestimation of SCB toxin in MTT-TCBA. Theoretically, similar effect would be caused in WST-1-TCBA. More purification steps might be required when highly concentrated bacterial extract should be analyzed. In the next chapter, it will be shown that 200 times concentrated bacterial culture supernatants analyzed by MTT-TCBA method did not shows cytotoxicity when purified by activated charcoal column, ion exchange column (Amberlite CG-50) and SEP-PAK C_{18} column. These purification procedures might be available to obtain accurate SCB activity in MTT-TCBA, although such procedures have the disadvantage of losing large amount of initial activity.

On the other hand, matrix interferences of the supernatant of *E. coli* suggested that undiluted supernatant of bacterial culture might cause the overestimation in WST-1-TCBA. As for the ML medium used in this experiment, NaCl concentration is low (0.2 %)

enough to avoid suppression of SCB activity, and the culture supernatant of *E. coli* is expected to show no cytotoxicity and little suppression at undiluted concentration (see the arrow in Fig. 2-10). Otherwise, *E. coli* supernatant seems to accelerate formazan formation. In general, WST-1 is metabolized less efficiently than MTT so that electron-coupling agents like metoxy PMS is necessary to enhance cellular reduction of WST-1. Addition of electron-coupling agent makes the assay system more complex and possibly easy to be affected by matrix constituents. However, we can avoid overestimation by diluting a sample since accelerating effect did not appeared at 1/8 dilution of *E. coli* supernatant (Fig. 2-12). Alternatively, extra reduction originating from bacterial supernatant can be estimated from control experiment, in which the absorbances of sample wells are compared with those of wells containing only cells in the absence of ouabain and veratridine.

When the supernatants of *Alteromonas tetraodonis*, *Vibrio alginolyticus* and *Bacillus subtilis* cultured by ML medium were directly analyzed by WST-1-TCBA method (Table 3-9 in Chapter3), accelerating effect was observed in the supernatants of *A. tetraodonis* and *V. alginolyticus*, and not in *B. subtilis*. It may vary with bacterial species or culture conditions. Net SCB activities of *A. tetraodonis*, *V. alginolyticus* and *B. subtilis* estimated by correction with the control experiment were 19, 24 and 48 ng/ml respectively, which were expressed in terms of TTX concentration. Therefore, WST-1-TCBA method can be well applied for the direct detection of SCB activity in supernatant of bacterial culture, if nonspecific WST-1 reduction by a matrix should be cared.

When applying these improved method to other natural samples such as animals, sinking particles and sediment, matrix interferences may be the problem. At present time, there is no information about the degree of interferences which may vary with samples. However, as indicated in this experiment of matrix interferences, the problem will be overcome by doing appropriate purification or including control experiments. We should choose most suitable way with each particular case.

In conclusion, MTT-TCBA and WST-1-TCBA methods were successfully developed in this investigation. The outline for utilization of these methods are summarized in Table 2-2. They have two advantages : First, this method is so simple and convenient that it is available for laboratory quantification of SCB toxin, and then this

method enables us to analyze large number of samples rapidly and sensitively. Therefore, this is useful for the first screening of SCB toxins from natural samples. We should, however, keep in mind that some compounds in the samples may interfere with accurate quantitative measurement. As for replacing mouse bioassay with TCBA method, which has no need to kill many mice, some more works should be aimed at quantitatively and qualitatively comparing these two methods.

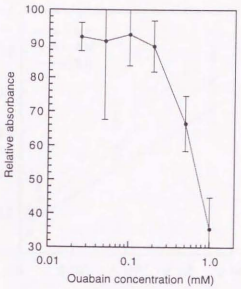


Figure 2-1. Effect of ouabain concentration on the viability of Neuro2A cells in the presence of 0.05 mM veratridine

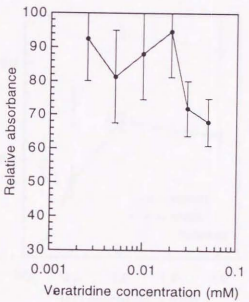


Figure 2-2. Effect of veratridine concentration on the viability of Neuro2A cells in the presence of 0.5 mM ouabain

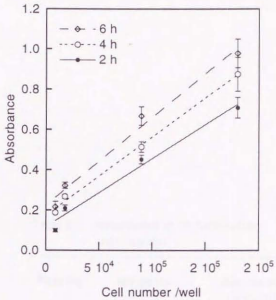


Figure 2-3. Relationship between viable cell number and absorbance of formazan in different incubation times (2, 4 and 6 hours) after addition of MTT

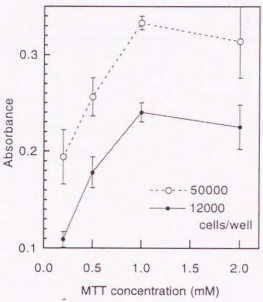


Figure 2-4. Relationship between MTT concentration and absorbance of formazan in different cell numbers (1.2×10^4 and 5.0×10^4) per well

Table 2-1 Absorbance of formazan after solubilization

Flask No.	solubents	absorbance (595nm)
1	Acid isopropanol	0.814
2		0.696
3	DMSO	0.532
4		0.623
5	20%SDS	0.509
6		0.481
7		0.518

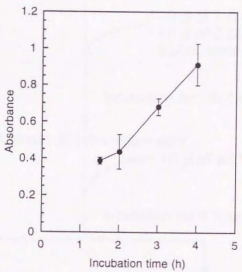


Figure 2-5. Relationship between absorbance of formazan and incubation time after addition of WST-1

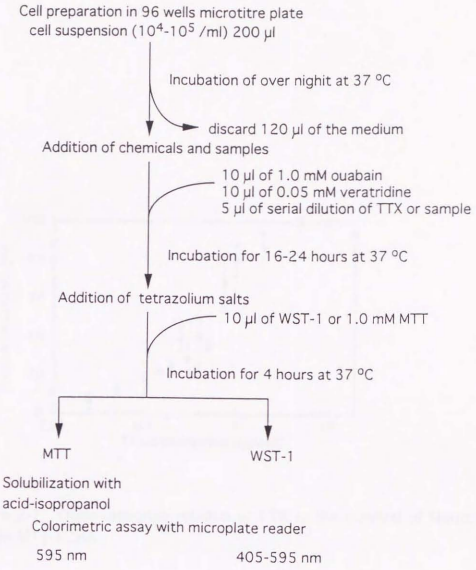


Figure 2-6 Procedure of improved TCBA methods, MTT-TCBA and WST-1-TCBA

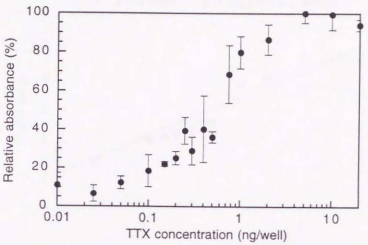


Figure 2-7. Dose-response relation of TTX to the survival of Neuro2A cells in MTT-TCBA

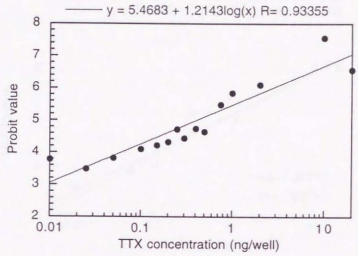


Figure 2-8. Regression line and equation for quantitative measurement of TTX in MTT-TCBA

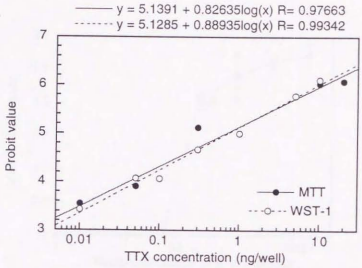


Figure 2-9. Comparison of regression lines between MTT-TCBA and WST-1-TCBA

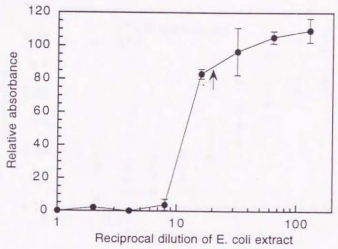


Figure 2-10. Interference of *E. coli* whole (cell and supernatant) extract in MTT-TCBA. The extract concentrated by 20 times was serially diluted, and the arrow indicates the possible relative absorbance at the dilution of 1/20

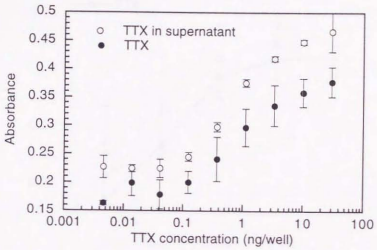


Figure 2-11. Effect of undiluted*E. coli* supernatant on formazan formation in WST-1-TCBA. Dose-response curves were obtained in the presence and absence of the supernatant

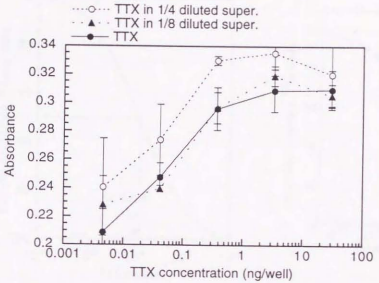


Figure 2-12. Effect of 1/4 and 1/8 diluted *E. coli* supernatant on formazan formation in WST-1-TCBA. Dose-response curves were obtained in the presence and absence of the supernatant

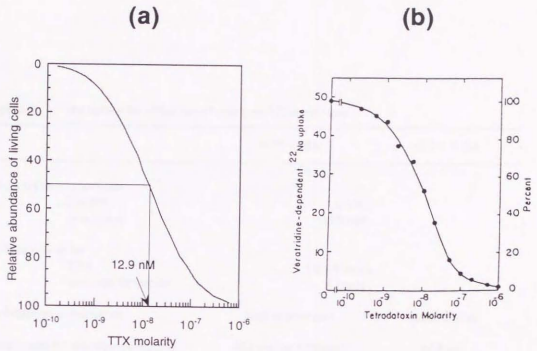


Figure 2-13. Comparison between the effect of TTX concentration on veratridine-dependent sodium ion uptake and that on enzymatic activity of the cells. (a): Dose-response curves in MTT-TCBA (b): Dose-response of Neuro2A cells on veratridine-dependent sodium ion uptake reported by Catterall and Nirenberg (1973)

Table 2-2 The outline for utilization of improved TCBA methods

	MTT-TCBA	WST-1-TCBA
Concentratin of chemicals		
Ouabain	1.0 mM	
Veratridine	0.05 mM	
Incubation time for		
TCBA	16-24 hours	
Formazan formation	4 hours	
Solubilization of formazan	Acid-isopropanol	water soluble
Wave length for absorbance reading	595 nm (or 570 nm)	405 nm reference : 595 nm
Possible interferences of		
Bacterial extract	underestimation	underestimation
Bacterial supernatant	not examined	overestimation

Chapter 3.

Immunological Detection of Tetrodotoxin Produced by Bacteria under the Culture Conditions

INTRODUCTION

TTX-producing bacteria have been isolated from various sources since the reports by Yasumoto et al. (1986) and Noguchi et al. (1986). Various kinds of media and conditions were so far used, when productivities of TTX were examined (Table 3-0). However, there are few works on the culture conditions for TTX production by bacteria. Simidu et al.(1987) showed that anhydroTTX was mainly detected in their screening test for TTX production by type strains of marine bacteria when ORI medium was used for cultivation. Kobayashi et al.(1992) reported that composition of culture medium, growth phase or temperature affected 4-epiTTX production of *Vibrio* sp. which they isolated from the intestine of puffer fish. They could hardly detect TTX in any conditions tested, although 4-epiTTX could easily convert to TTX. Gallacher and Birkbeck (1993) reported that SCB activity of *A. tetraodonis* increased in phosphate-limited cultures and was repressed when phosphate was added at the onset of stationary phase.

Limitation of works on the effect of culture conditions to TTX production by bacteria is mainly caused from technical problems for detection and quantification of TTX. In the HPLC method, a peak identical to TTX should be defined by combination with other methods, because the identification is based on the retention time. The GC-MS method is very sensitive and specific to C9-base, however, the presence of C9-base does not always indicates the presence of TTX because it would be derived from all compounds related to TTX. Mouse bioassay was not sensitive and not suitable for analyzing many samples. Biorecepter assay using crude brain membranes (Davio et al. 1984) was highly sensitive but not available for large scale screening. MTT-TCBA and WST-1-TCBA methods was simple and sensitive but not specific to TTX.

Immunoassays using anti-TTX antibodies would provide a rapid, sensitive and highly specific detection and quantification of TTX. If TTX produced by bacteria can be easily detected in immunological assay systems, isolation and laboratoly analyses of TTX-producing bacteria in sinking particles would be facilitated. Huo et al.(1989) reported the production of two anti-TTX monoclonal antibodies (mAbs), but they could not develop highly sensitive immunoassay system because the affinity of their mAbs was not so high

(IC_{50}, 50 nM and 200 nM). Raybould et al. (1992) reported production of a high-affinity mAb against TTX and its use in developing sensitive and specific competitive inhibition enzyme immunoassays (CIEIAs) for detecting TTX in biological matrices (IC_{50}, 21 nM). Another indirect competitive enzyme immunoassay for TTX using anti-TTX antiserum was developed by Matsumura and Fukuya (1992), and subsequently anti-TTX mAb and highly sensitive mAb based immunoassay was produced (IC_{50}, 15.7 nM) (Matsumura 1995).

In this chapter, toxin fractions of TTX-producing bacteria which had been confirmed in the previous works were analyzed by mouse neutralization assay system using anti-TTX mAb developed by Matsumura (1995). Toxin fractions were further analyzed by MTT-TCBA, HPLC and GC-MS. In the additional works, TTX-producing bacteria were cultured again, and the presence of TTX in the extracts was analyzed by CIEIA using another anti-TTX mAb.

MATERIALS AND METHODS

1. Neutralization test using anti-TTX mAb

Bacterial strains and culture conditions

Bacterial strains used in this study were listed in Table 3-1. *Shewanella alga* (OK-1) was isolated from red calcalious algae and confirmed as TTX producer for the first time by Yasumoto et al. (1986). *Alteromonas tetraodonis* was isolated from puffer fish and confirmed as TTX producer by Yotstu et al.(1987). These two bacteria were taxonomically characterized and reported as new species by Simidu et al. (1990). *Streptmyces* sp.(No.21) was isolated from marine sediment and confirmed as a TTX producing actinomycetes by Do et al.(1991). Each strain was cultivated in a 12 liter liquid medium for 7 days at 25 °C. Compositions of media were showed in Table 3-2.

Extraction

Bacterial cultures were centrifuged at 11,000 g and separated into cells and supernatants. Cells were destroyed by ultrasonication in 0.1% acetic acid and boiled for 20 min. After centrifugation at 23,000 g for 15 min., supernatant was evaporated to dryness. The residue was dissolved in distilled water and treated with Amberlite CG50 (NH^{4+} form) column. The column was washed with D.W. and eluted with 10% acetic acid. The eluate was evaporated, dissolved in D.W. and treated with SEP-PAK C_{18} column. The solution coming through the column was concentrated by evaporation. The final volume of cell extracts was 6 ml.

At first, the pH of the supernatant was adjusted to 3.5 with acetic acid. It was boiled for 20 min and evaporated to dryness. The residue was treated 2 times with methanol containing 1% acetic acid to eliminate salts. After evaporation, the residue was dissolved in D.W. and put into the charcoal column. The column was washed with D.W. and eluted with 20% ethanol containing 1% acetic acid. The eluate was evaporated and dissolved in D.W. The solution was further purified by Amberlite CG50 and SEP-PAK C_{18} column with same procedure as described above. The final volume of an extract from supernatants was also 6 ml.

The extracts from both cells and supernatants were analyzed by mouse bioassay, neutralization test using mAb, HPLC, TCBA and GC-MS.

Mouse bioassay and Neutralization test using mAb

According to the official method of Kawabata (1978), 1 ml of each extract was intraperitoneally injected to male ddY mouse. The extracts which killed mice were mixed with equal volumes of mAb solutions (250-500 μg/ml). Concentrations of mAb in mixed solutions were enough to neutralize 3-6 MU standard TTX. After waiting for 30 min. at room temperature, 1 ml of the mixed solution was injected to a mouse.

TCBA

An improved method using MTT described in Chapter 2 was used to analyze sodium channel blocking agents.

HPLC

The method was already described in Chapter 1.

GC-MS

A preparation procedure for C9-base was same as described in Chapter 1. The sample was subjected to GC-MS on 5791 series mass spectrometer equipped with series II 5890 gas chromatograph (Hewlett Packard). GC was performed on a 30m x 0.25mm i.d. HP-5 capillary column with helium as carrier gas. The oven temperature was raised from 120 °C to 200 °C at the rate of 5 °C/min. and from 200 to 280 °C at the rate of 10 °C/min. The ion source temperature was kept at 250 °C.

2. CIEIA using T20G10 anti-TTX mAb

Bacterial strains and culture conditions

Bacterial strains used here are listed in Table 3-1. All strains except *B. subtilis* and *E. coli* (K-12), negative control, were previously confirmed as TTX-producing bacteria. *V. alginolyticus* (NCMB1903) was reported to produce anhydroTTX in ORI medium (Simidu et al. 1987). Another *V. alginolyticus* isolated from starfish was reported to produce highest amount of TTX (213 MU in the 500 ml culture) (Narita et al. 1987). Although productivity of TTX by *B. subtilis* (IFO3134) has not been examined yet, a half of 10 isolates identified in genus *Bacillus* from marine sediment produced sodium channel blocker when cultured in L medium (Do et al. 1990). ML medium and PSY medium were used for cultivation in this experiment (Table 3-4). Each bacterial strain was cultured in a 200 ml liquid medium for 2 days at 20 °C.

Extraction

Bacterial cultures were centrifuged at 11,000 g for 15 min and separated into cells and supernatants. Supernatants were directly analyzed by WST-1-TCBA method without extraction procedures. Extraction from cells was same as described above. Cell extracts were partially purified by SEP-PAK C_{18} column, concentrated by evaporation and finally lyophilized.

CIEIA

TTX concentrations in extracts were measured by CIEIA repeatedly. For the positive samples, spike and recovery experiment was carried out. Details of CIEIA and "spike and recovery experiment" are described in MATERIALS AND METHODS in Chapter 4.

TCBA

A portion of culture supernatant was centrifuged at 11,000 g for 5 min to eliminate precipitate, sterilized by filtration (0.22 μm pore size membrane filter) and analyzed by WST-1-TCBA method. As for cell extracts, the assay system did not work well because the samples showed toxic effect to the culture cells.

GC-MS

After the analysis by CIEIA, extracts in which TTX was detected were further confirmed by GC-MS. Samples were prepared and analyzed following the method described in Chapter 1.

RESULTS

1. Neutralization test using anti-TTX mAb

Mouse bioassay and neutralization test using mAb

The mice were killed by the injections of all the supernatant extracts tested. On the other hand, the cell extracts did not kill the mice although *Streptomyces* cell extract showed weak toxicity but was not fatal. The supernatant extracts from *S. alga* and *A. tetraodonis* showed lethal toxicity within 30 min, and that from *Streptomyces* sp. showed it after 4 hours. The neutralization test was tried on these three supernatant extracts. However, the lethal effects of them were not neutralized by the mAb.

TCBA

Sodium channel blockers were detected from all extracts. Toxin concentrations are expressed as equivalent to TTX. The SCB concentrations of supernatant extracts were higher than cell extracts. Those were ranged from 0.3 to 6.8 MU per ml of extracts (Table 3-3). Control experiments described in DISCUSSION of Chapter 2 indicated that every extracts did not have cytotoxicity which could cause underestimation.

HPLC

Peaks corresponding to TTX appeared in the HPLC chromatograms of the supernatant extract from *S. alga* and both cell and supernatant extracts from *Streptomyces* sp. (Fig. 3-1).

GC-MS

Alkaline degradation products of all the samples except *A. tetraodonis* were proved clearly to contain C_9-base which could be derived from TTX or related substances.

Fig. 3-2 shows the selected ion-monitored chromatogram (m/z 392, 407 and 376) of *Streptomyces* sp. samples. The typical peaks corresponding to authentic C9-base appeared at about Rt. 25.5 min in the chromatograms. The results of all analyses were summarized in Table 3-3.

2. CIEIA using T20G10 anti-TTX mAb

CIEIA

The results of CIEIA are shown in Table 3-4. In the first CIEIA, inhibitory effect against TTX specific reaction of T20G10 antibody was detected from *B. subtilis* cell extract. TTX concentration in the sample calculated from the inhibitory effect was 7.5 ng per ml of extract. In the second CIEIA, *A. tetraodonis* cell extract became positive in addition to *B. subtilis*. Calculated TTX concentrations were 8.0 and 16.0 ng/ml respectively. Lyophilized sample were dissolved in a buffer solution just before the first CIEIA, and stored in the refrigerator until the second. More dissolution during the storage might be the reason why quantitative data of the second were higher than the first. Inhibitory effect from other strains were undetectable. In CIEIA, 2-3 ng/ml (IC_{20}) was considered to be a minimum detectable concentration. As for these two extracts, spike and recovery experiment was conducted to know the matrix interferences (Table 3-5). Measured TTX concentrations in spiked samples were higher than expected. Mean percentage recovery were 241 and 130 % as showed in Table 3-5.

TCBA

Sodium channel blockers were detected from the supernatants of *A. tetraodonis*, *B. subtilis* and *V. alginolyticus* (Table 3-6). The concentrations showed as equivalent to TTX were estimated to be 19, 48 and 24 MU per 200 ml culture. All the others were undetectable, minimum detectable limit of 9 MU/200ml which showed 20 % protection in the assay.

GC-MS

Cell extracts of *A. tetraodonis* and *B. subtilis* were further analyzed by GC-MS. Alkaline degraded extracts of *B. subtilis* showed a small peak corresponding to authentic C9-base in the selected ion-monitored chromatogram (m/z 392). The results of all analyses were summarized in Table 3-7.

DISCUSSION

Cross-reactivities of the monoclonal antibody used in the neutralization test were 0.001 % against GTX group, <0.0001 % against STX, 0.7 % against tetrodonic acid and 16.7 % against anhydroTTX. The net cross-reactive value against anhydroTTX must be lower than 16.7 % because partial amount of anhydroTTX was considered to be converted to TTX in PBS solution (Matsumura 1995). Although we don't know now about cross-reactivity against 4-epiTTX, less cross-reactivity against anhydroTTX indicated that this mAb recognize the C-4 and C-9 structures of TTX. From this reason, this mAb is not expected to cross-react against 4-epiTTX.

Interpretation of the result was described below about each bacterial strains ; *Shewanella alga* (OK-1), *Alteromonas tetraodonis* (GFC), *Streptomyces* sp. (no.21) and *Bacillus subtilis* (IFO 3134).

Shewanella alga (OK-1) : Mouse toxicity of the supernatant extract was not neutralized by anti-TTX mAb, so that this toxicity would come from compounds other than TTX (Table 3-3). At least TTX should not be major toxic compound which showed mouse lethality. If the peak corresponding to TTX in HPLC would actually originate from TTX, it is estimated that the supernatant extract contained TTX at the concentration of 12 MU/ml (Table 3-3, Fig. 3-1). This assumption would be rejected by the result of neutralization test. It is reasonably assumed that the peak originating from other compounds overlapped. As for the cell extract, the presence of TTX was not confirmed

even by CIEIA, in which 2 ng (0.01 MU) TTX per 200 ml culture could be detectable (Table 3-7). Neverthless, the presence of C9-base in both extracts suggested that they contained TTX related substances. The SCB activities supported this suggestion (Table 3-3).

Alteromonas tetraodonis (GFC) : It was the same as*S. alga* that mouse toxicity of the supernatant extract would come from compounds other than TTX from the result of neutralization test (Table 3-3). Although CIEIA analysis indicated the presence of TTX in the cells of *A. tetraodonis*, it was suspicious because much higher percentage recovery of *A. tetraodonis* spiked sample (241 %) suggested matrix interference to the assay system, and C9-base was not detected by GC-MS analysis (Table 3-7). Therefore, TTX productivity of this strain could not be revealed here. However, C9-base was not detected from the cell extract when 200 ml cultivation with ML medium, but was detected when 12 liter cultivation with ORI medium (Table 3-3). This might come from the difference in the total amount of cells. The presence of C9-base and SCB activities suggested that this strain also produced TTX related substances. SCB activity was detected from both culture supernatants of ORI medium and ML medium (Tables 3-3 and 3-6). When cultured in ORI medium, the supernatant was treated for extraction and partial purification, whereas in ML medium, the supernatant was directly analyzed. The measured SCB value was 4.8 and 40.1 MU/g (wet weight) respectively. Initial amount of SCB must be lost during extraction and partial purification. Therefore, initial SCB concentrations in the cells and supernatants were expected to be higher than what is shown in Table 3-3. If SCB productivities of this bacteria would be same in both, percentage recovery of SCB after the treatment for purification (methanol treatment, Amberlight CG50, charcoal, SEP-PAK C_{18}) is estimated at 12.0 %.

Streptomyces sp. (no. 21) : As for the supernatant, same pattern of results as *S. alga* was obtained from the neutralization test, HPLC, GC-MS and TCBA (Table 3-3). The results also suggested the presence of TTX related substances. The cell extract uniquely showed a peak corresponding to TTX (Fig. 3-1), which did not appear in other two cell extracts. Possible TTX concentration estimated from the peak area, 53 MU per ml of extract, would be enough to kill a mouse. Neverthless, the toxicity showen in the mouse assay was weak and not fatal. This fact indicated the possibility that a peak of some other

compound was overlapping that of TTX in the HPLC chromatogram. It was not definitive whether the mouse toxicity was caused by TTX or not, because the neutralization test was not done. Even in the CIEIA, which is much more sensitive, TTX was not detected from the cell extract (Table 3-7). Therefore, although the presence of TTX was not clarified, the presence of TTX related substances should be certain.

Bacillus subtilis (IFO 3134) : In the two times CIEIA, TTX was repeatedly detected from only the cell extract of this strain (Table 3-4). The cell extract was considered to contain TTX from following three reasons. First, mean percentage recovery of this spiked sample (130 %) was higher than 100 % but not so much. Second, TTX concentrations estimated from undiluted (9.9 ng/ml) and a half diluted (4.6 x2 = 9.2 ng/ml) samples were comparable (Table 3-5). Third, GC-MS analysis suggested the presence of C_9-base which would be derived from TTX (Table 3-7). The presence of SCB in the supernatant also suggested the TTX production by *B. subtilis* (Table 3-6). This may be the first finding of TTX production by *B. subtilis*. Members of the genus Bacillus are among the most common organisms in soil. Many bacilli produce antibiotics, such as bacitracin, polymyxin, tyrocidin, gramicidin, and circulin. In most cases, antibiotic production seems to be related to the sporulation process, where the antibiotic is released when the culture enters the stationary phase of growth and after it is committed to sporulation (Brock et al. 1994). Certain bacilli, most notably *B. huringiensis*, are insect pathogens due to a toxic crystalline protein produced during sporulation. This toxin called deltaendotoxin causes the swelling of cultured insect cells, TN-368, by the stimulation of Na^+ influx and K^+ efflux in the isotonic NaCl solution, and this effect is inhibited by TTX or ouabain (Himeno et al. 1985). The finding of TTX production by *B. subtilis* bring up various interests : Bacteria in genus Bacillus could be an important candidate of TTX-producers in marine sediment, there could be some interaction between TTX and its antagonists produced by bacilli in natural environment, and *B. subtilis* could be useful for works on biosynthetic mechanism of TTX in bacteria.

Many TTX analogues have been reported so far in puffer fish, newts and other organisms (Fig. 3-3) (Yasumoto et al. 1988 ; Endo et al. 1988 ; Yotsu et al. 1990 and 1992). Chiriquitoxin (CqTX) from the Costa Rican frog, *Atelopus chiriquiensis* , differs from TTX only in that a glycine residue is replaced by a methylene hydrogen of the C-11

hydroxymetyl function. On the voltage-clamped frog skeletal muscle fiber, CqTX is as potent as TTX in blocking the sodium channel, with an ED_{50} of 3.8 nM (Yang and Kao 1992). Yasumoto and his co-workers have reported 11-deoxyTTX in puffer fish and newts and 11-norTTX-6-ols and 11-oxoTTX in puffer fish (Endo et al. 1988 ; Yasumoto et al. 1988 ; Wu et al. 1991). These derivatives were considered to be compounds at different stages of progressive oxidation. Chiriquitoxin was speculated to be derived from glycine and aldehyde group at C11 position of 11-oxoTTX as a result of condensation. It was noteworthy that Kotaki and Shimizu (1993) found unique N-hydroxy and ring-deoxy derivative of TTX (1-hydroxy-5,11-dideoxytetrodotoxin) in the newt, *Taricha granulosa*. From the lack of oxygen function on the ring, this unique derivative was considered to be a precursor of 11-deoxyTTX. As another important aspect, N-hydroxy derivatives play an significant role in the toxigenesis of other guanidine-containing toxins because these compounds are easy to lose the hydroxy group reductively and occasionally change to highly toxic derivatives in biological systems (Shimizu and Yoshioka 1981 ; Kotaki et al. 1985). Fig. 3-3 showed the speculated pathway of TTX derivatization mainly at the branching portion, C6,11-position. As for other derivatives, isomers at C-4 or C-6 position and anhydro compounds at C-4 and C-9 were isolated from newts in Okinawa (*Cynops ensicauda*) : 4-epiTTX, 6-epiTTX, 11-deoxy-4-epiTTX, 4,9-anhydroTTX, 4,9-anhydro-6-epiTTX and 4,9-anhydro-11-deoxyTTX (Yasumoto et al. 1988). TTX analogues studied have various degree of reduced potency except CqTTX and 11-oxoTTX. When actions of 11-norTTX-6-ols, 6-epiTTX and 11-deoxyTTX were measured on the frog skeletal muscle fiber, relative potencies to TTX were 10-40 % for 11-norTTX-6-ols, 4.2 % for 6-epiTTX and 0.9 % for 11-deoxyTTX (Kao 1982 ; Yang et al. 1992). Relative potencies of 4-epiTTX and anhydroTTX on the squid axon were 39 % and 1.8 % respectively in comparison with TTX (Kao and Yasumoto 1985). CqTTX and 11-oxoTTX is unusual among TTX analogues in possessing a high degree of potency. Particularly, 11-oxoTTX was about 4 times as potent as TTX (Wu et al. 1991).

In this experiment, TTX was not detected from bacterial strains except *B. subtilis* under the culture conditions and assay sensitivity used. The only rational interpretation for the contradictory results is that these bacteria produced TTX related compounds which would have high toxicity. Above reports of various TTX derivatives including highly toxic

compounds strengthen the rationality of this interpretation. The mAbs used in this experiment was so specific to TTX that it would not react with other derivatives. As HPLC system used here could only identify three compounds (TTX, 4-epiTTX, anhydroTTX), other TTX analogues could give peaks corresponding to these three compounds in this system although retention times are not known. All derivatives related to TTX including unknown ones were detectable by GC-MS, and also by TCBA if they had a sodium channel blocking activities. Although TTX analogues except 4-epiTTX and anhydroTTX have never been isolated from bacteria, the results of this experiment seems to promise their presence.

In conclusion, TTX was only detected from the cell extract of *B. subtilis* in this study. The amount of TTX produced by *B. subtilis* was estimated to be 0.04 MU per 200 ml of culture, which was very low productivity even if there would be lost during the extraction. It is supposed that final metabolites in the microbial biosynthetic pathway related to the production of TTX such as *S. alga* (OK-1), *A. tetraodonis* (GFC) and *Streptomyces* sp. (no.21) would not be TTX but other analogues under the conditions used in this investigation.

In the future, the work to isolate unknown TTX derivatives from bacteria would be very hopeful, and further works to find out suitable conditions or strains for higher TTX production should be required. Immunological method using anti-TTX mAb like CIEIA might be one of useful tools for such future works.

Table 3-0 Compositions of culture media when productivities of TTX were examined in the previous papers

	Shewanella alga	*Vibrio* sp.	*Alteromonas tetraodonis*	*Vibrionaceae* (family)	*Streptomyces* sp.
	(%)	(%)	(%)	ORI medium (%)	L medium (%)
Polypepton	1.0	1.0	0.5		0.5
Proteose pepton				0.2	
Pyton				0.1	
Yeast extract			0.01	0.2	0.1
NaCl	3.0	1.0			1.8
K_2HPO_4					0.1
$MgSO_4$					0.25
$FePO_4$			0.01		
Tris			0.6		
	D.W.	D.W.	S.W.	S.W.	D.W.
	Yasumoto et al. 1986	Noguchi et al. 1986	Yotsu et al. 1987	Simidu et al. 1987	Do et al. 1990

Table 3-1 List of bacterial strains and culture media

Bacterial Strains		Culture Media	
		1. Neutralization (12 liter)	2. CIEIA (0.2 liter)
Shewanella alga Yasumoto et al. 1986	(OK-1)	L	ML
Alteromonas tetraodonis Yotsu et al. 1987	(GFC)	ORI	ML
Streptomyces sp. Do et al. 1991	(no.21)	PSY	PSY
Vibrio alginolyticus	(NCMB 1903)		ML
Bacillus subtilis	(IFO 3134)		ML
Escherichia coli	(K-12, IAM 1264)		ML

*NCMB: National Collection of Marine Bacteria
*IFO: Institute of Fermentation, Osaka
*IAM: Institute of Applied Microbiology

Table 3-2 Compositions of culture media

	L (%)	ML (%)	ORI (%)	PSY (%)
Polypepton	0.5	0.5		0.6
Proteose pepton			0.2	
Phyton			0.1	
Yeast extract	0.1	0.1	0.2	0.1
Soluble starch				1.0
NaCl	2.0	0.2		
KCl	0.2	0.2		
MgSO	0.4	0.4		
pH	7.5-7.6	7.5-7.6 (Tris-HCl)	7.5-7.6	7.0
	D.W.	D.W.	S.W.	S.W.

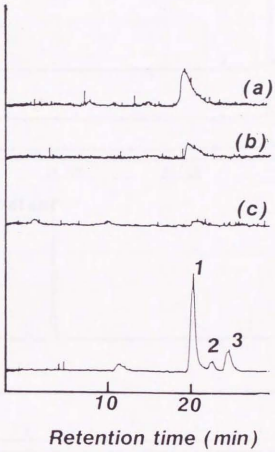


Figure 3-1. HPLC chromatograms of bacterial extracts. (a): cell extract of *Streptomyces* sp., (b): supernatant extract of *Streptomyces* sp., and (c): supernatant extract of *Shewanella alga* . Bottom plot : standard (1)TTX , (2)4-epiTTX , (3)AnhydroTTX.

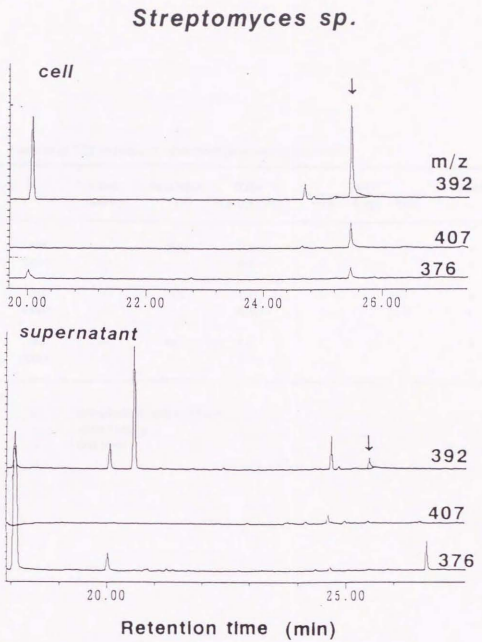


Figure 3-2. Selected ion monitored chromatograms (m/z 392, 407, 376) of the trymethylsilyl (TMS) derivative from alkali-degraded cell (upper) and supernatant (lower) extract of *Streptomyces* sp. Arrows indicate peaks identical to authentic TMS-C9-base derived from standard TTX

Table 3-3 Analyses of TTX and related substances produced by bacteria

Bacterial spiecies		Mouse toxicity	Neutralize (TTX)	TCBA SCB (MU/ml)	HPLC TTX	HPLC 4-epi	HPLC Anh.	GC-MS
Shewanalla	cell	-	NA	0.3	-	-	-	+
alga	super.	++	-	5.2	+	-	-	+
Alteromonas	cell	-	NA	1.6	-	-	-	+
tetraodonis	super.	++	-	6.8	-	-	-	+-
Streptomyces	cell	+-	NA	1.1	+	-	-	+
sp.	super.	+	-	1.7	+	-	-	+

NA: not analyzed

Mouse toxicity ++ : lethal toxicity within 30 min.
+- : weak toxicity
- : non-toxic

Table 3-4 CIEIA analysis of bacterial cell extracts

	Sample No.	D. weight (g)	CIEIA-1 TTX(ng/ml)	(ng/g)	CIEIA-2 TTX(ng/ml)	(ng/g)
A.tetraodonis	11	0.107			8.0	75
B.subtilis	13	0.096	7.5	78	16.0	167

Table 3-5 Spike and recovery experiment of bacterial cell extracts

	TTX in diluted sample (ng/ml)	spiked TTX (ng/ml)		Expected TTX in spiked sample (ng/ml)	Measured TTX in spiked sample (ng/ml)	Percent recovery (%)
A.tetraodonis	2.5	5.0	undiluted	7.5	22.3	297
			1/2	3.8	7.0	184
						mean=241
B.subtilis	2.3	5.0	undiluted	7.3	9.9	136
			1/2	3.7	4.6	124
						mean=130

Table 3-6 Analysis of sodium channel blockers in bacterial culture supernatant

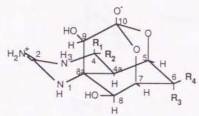
	TCBA (MU)
A. tetraodonis	19
S. alga	-
Streptomyces	-
V. alginolyticus	24
B. subtilis	48
E. coli	-

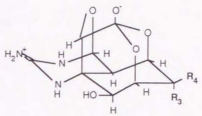
*TTX concentrations were expressed as toxin amounts included in 200 ml of the culture

Table 3-7 Analyses of TTX produced by bacteria

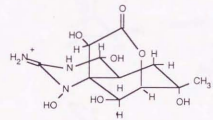
	TTX		spike exp.	
	CIEIA-1 (ng)	CIEIA-2 (ng)	recovery (%)	GC-MS
A. tetraodonis	-	4.0	241	-
S. alga	-	-	NA	NA
Streptomyces	-	-	NA	NA
V. alginolyticus	-	-	NA	NA
B. subtilis	3.25	8.0	130	+-
E. coli	-	-	NA	NA

NA: not analyzed
*TTX concentrations were expressed as toxin amounts included in 200 ml of the culture





	R1	R2	R3	R4		R3	R4
TTX	H	OH	OH	CH_2OH	4,9-anhydroTTX	OH	CH_2OH
4-epiTTX	OH	H	OH	CH_2OH			
6-epiTTX	H	OH	CH_2OH	OH	4,9-anhydro-6-epiTTX	CH_2OH	OH
11-deoxyTTX	H	OH	OH	CH_3	4,9-anhydro-11-deoxyTTX	OH	CH_3
11-deoxy-4-epiTTX	OH	H	OH	CH_3			
11-oxoTTX	H	OH	OH	CHO			
11-norTTX-6(R)-ol	H	OH	H	OH			
11-norTTX-6(S)-ol	H	OH	OH	H			
Chiriquitoxin	H	OH	OH	$CH(OH)CH(NH_2)COOH$			



1-hydroxy-5,11-dideoxyTTX

Figure 3-3 Structure of TTX derivatives

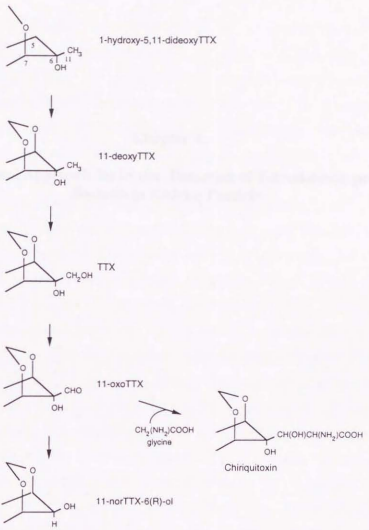


Figure 3-4 Speculated pathway in derivatization of TTX

Chapter 4.

Immunological Approach for *in situ* Detection of Tetrodotoxin-producing Bacteria in Sinking Particles

INTRODUCTION

Presence of TTX was clalified in sinking particles collected from coastal waters in Chapter 1. The evidence that such particles were colonized by a large population of marine bacteria and various strains of TTX-producing bacteria were isolated from marine environment leads us to feasible explanation that TTX would be produced by bacteria attaching to sinking particles. This is, however, difficult to prove by checking the isolated strains from sinking particles, because, first, only a part of them can be isolated by enrichment culture method, and second, suitable conditions for TTX production are still not clear. Therefore, we should take another more direct approach.

Immunostaining technique is very useful in defining the location of antigen in biological systems. The immunofluorescence method particularly has been the most preferable technique because of the possibility of labeling living cells, the convenience of double labeling and the relative ease of identifying even a few fluorescent cells in the dark nonfluorescent tissue sections (Choe and Cho 1994). This method is also very useful in microbial ecology as one of the few methods which can directly identify microbial cells in the natural environments. The great specificity of antibodies prepared against cell surface constituents of a particular organisms can be exploited to identify the organisms in a complex habitat such as soil (Brock et al. 1994).

As described in Chapter 3, several anti-TTX antibodies have been developed (Watabe et al. 1989 ; Huot et al. 1989 ; Kauf man et al. 1991 ; Matsumura and Fukiya 1992 ; Matsumura 1995). The mAb used in this chapter was developed by Raybould et al.(1992). They established enzyme immunoassay system which was called CIEIA to specifically and sensitively measure TTX concentration. In this chapter, immunofluorescent technique using anti-TTX mAb was applied to marine sinking particles to prove the bacterial origin of TTX in them. First, TTX concentration in sinking particles was measured by CIEIA, and then, fixed sinking particles were stained with fluorescent labeled antibody to microscopically visualize *in situ* TTX-producing bacteria.

MATERIALS AND METHODS

Sample collection

Four samples of sinking particle and three sediment samples were collected in Tokyo Bay and Shimizu harbor during the KT-93-13 cruise by the R/V Tansei-maru, Ocean Research Institute, University of Tokyo, in September 1993 (Fig.4-1). Other three samples of sinking particle were collected in Aburatsubo Inlet in August 1994 (Fig. 1-1). Sediments were taken using an Ekman-Birge sampler and sinking particles using a sediment trap system. The trap was equipped with 6 transparent acrylic cylinder, 10 cm in diameter and 50 cm in height (Fig. 4-2). Two sets of the trap were moored at the depth of around 10m for 12-48 hours. After the traps were retrieved, most of the water in the cylinders was discarded using a siphon. Obtained sinking particle suspensions and sediments were kept frozen until extraction. The list of samples is shown in Table 4-1.

Toxin extraction

Particle suspensions were centrifuged at 16000 g for 20 min. Particles obtained were suspended in 0.1% acetic acid, subsequently ultrasonicated and boiled for 20 min. After centrifugation at 25000 g for 20 min, supernatant was purified using SEP-PAK C_{18} cartridge (Waters Associates) and charcoal column. The eluate was finally lyophilized. Sediment was boiled for 20 min in distilled water at acidic condition (pH 3-5) prepared with acetic acid. After the filtration, supernatant was evaporated to dryness and resuspended in methanol containing 1% acetic acid. Insoluble residue was eliminated by filtration. Methanol treatment was repeated 3 times to eliminate salts from sea water. After this procedure, an extract was purified using SEP-PAK C_{18} cartridge and charcoal column, and finally lyophilized. Extracted samples were analyzed by immunoassay using an anti-TTX monoclonal antibody and gas-chromatography mass-spectrometry (GC-MS) following the method previously described.

CIEIA

The lyophilized extracts were solubilized in 0.5ml PBS containing 0.25% BSA, 0.5% Tween20 and 0.02% NaN_3 (BPT buffer) at pH 6.5. Microtiter plates were coated with 100 µl/well of BSA-TTX formaldehyde conjugate (BSA-TTXF). Plates were washed three times with PBS containing 0.05% (v/v) Tween20 (PBS-T). After blocking with 200 µl/well of 1% BSA in PBS for 1h at room temperature, or 18h at 4 °C, the plates were washed four times with PBS-T. Fifty µl/well of standard TTX serially diluted with BPT was put into the wells to draw a standard curve for quantitative measurement. In the case of samples, 50 µl/well of each sample, diluted in three concentration steps with two or three fold, was added. In addition to the standard TTX or a sample, 50 µl/well of T20G10 anti-TTX monoclonal antibody conjugated to alkaline-phosphatase (AP) and diluted in PBS containing 1% BSA was put into the wells. After 1h of incubation at room temperature, plates were washed 4 times with PBS-T. Then, 200 µl of 1 mg/ml p-nitrophenyl-phosphate (pNPP) (SIGMA 5mg tablets), diluted in alkaline phosphatase substrate buffer (pH9.5, 0.15 M NaCl, 5mM $MgCl_2$, 0.02% (w/v) NaN_3) was added to each well. The plates were incubated for another 1h at room temperature, and the absorbance of each well was read on a Titertek Multiskan MC using a sample wavelength of 414 nm and a reference wavelength of 690 nm. CIEIA was repeated two times for all samples.

CIEIA spike and recovery experiment

As for the positive samples in the first and second CIEIA, spike and recovery experiment was carried out. Based on the TTX concentration measured by CIEIA, samples were diluted with BPT buffer (pH6.5) at the optimum concentration. To arrange spiked samples, known amount of TTX (5 ng/ml) was added to each sample dilution. TTX concentrations in an non-spiked sample, a spiked sample and half diluted spiked sample were simultaneously measured by CIEIA for each sample.

GC-MS analysis

After the quantitative measurement of TTX by CIEIA, positive samples were analyzed with GC-MS to confirm the presence of C9-base which should be derived from TTX after alkaline degradation. Samples were prepared and analyzed following the method described in the previous chapter.

Data analysis

All samples tested by CIEIA were run in triplicate, and the mean result of each set of replicates was calculated. BSA-coated wells treated with AP-conjugate and substrate were included on each plate to measure background color development. The mean OD at 414 nm of these wells was subtracted from the mean OD (414 nm) of each set of standard and sample replicates prior to data analysis. For CIEIAs, standard curves were constructed for each experiment using a set of TTX standard dilutions. B/Bo values for each standard curve was calculated by dividing the mean OD414 of a given set of replicates containing TTX inhibitor by the mean OD414 of all the wells containing no inhibitor. Unknown TTX concentrations in the samples under test were calculated from the OD414 of the sample dilutions that fell within the linear portion of standard curve.

Immunofluorescence

A portion of sinking particle was fixed with 4% formaldehyde in a microtube. After washing three times with PBS, 250 µl of PBS containing 1% BSA and 10 mM glycine was added to each tube to block nonspecific staining. After waiting for 1h at room temperature, a sample in each tube was washed three times with PBS. The samples were then incubated with 100 µl T20G10 anti-TTX murine mAb (3 mg/ml) diluted 1/100 and 1/500 in BPT buffer for 1h at room temperature. After washing three times with PBS, the antigenic sites were visualized using 200 µl fluorescein-isothiocyanate (FITC)-conjugated anti-mouse goat IgG (H+L) (1 mg/ml, CalTag Laboratories) diluted 1/500 in PBS. Following 1h of incubation, the samples were washed three times with PBS and

resuspended in small amount of PBS. Small aliquot of each suspension was dropped on a slide and observed under the fluorescent microscope. Control experiment was included to verify the specificity of the primary and secondary antibody reactions. It was carried out in a same procedure except following manners. Two aliquot of each BSA-coated sample were used to incubate with mouse nonimmune ascites fluid diluted 1/100 in BPT buffer instead of T20G10 anti-TTX murine mAb (primary antibody). Another one aliquot of each BSA-coated sample was used to incubate with FITC conjugated goat anti-mouse IgG (secondary antibody) omitting the step of primary antibody reaction.

RESULTS

CIEIA

The data of CIEIA were summarized in Table 4-2. At the first CIEIA experiment, inhibitors against TTX specific reaction of T20G10 antibody were detected from all the extracts of sinking particle and one extract of sediment (no. 9). As for samples no.1 and no.9, these effects were detected from undiluted and half diluted samples. The effects of other samples were detected from only undiluted wells because of their low concentrations. At the second CIEIA experiment, one more sediment extract (no. 10) became positive in addition to the positive samples at the first experiment. In this time, all samples exhibited higher amounts of inhibitory effects than the first, so that these effects were detected from undiluted, 1/2 and 1/4 diluted wells.

Spike and recovery experiment

Nine positive samples at the first and second CIEIA experiments were tested to see the presence of matrix interferences in these samples. The results were summarized in Table 4-3. Except no. 4 and 5, percentage recoveries were almost 100%. So no matrix interferrnce was found in these samples. As for samples no.4 and 5, measured TTX

concentrations in spiked samples were higher than expected. Mean percentage recoveries of no.4 and 5 were 155 and 144%, respectively.

GC-MS

Remaining portion (20-120 µl) of each positive sample was alkaline-hydrolyzed and analyzed with GC-MS. The results including above two experiments are summarized in Table 4-4. It is clear that hydrolyzed samples no. 1, 2, 3, 4 and 6 contained C9-base which could be derived from TTX and/or its analogues. No. 7, 10 and 13 were suspicious, and No. 5, 9 and 11 were undetect able or negative. Fig. 4-3 shows selected ion-monitored chromatograms grams of no.1 and standard samples. The peaks corresponding to standard C9-base appeared in the sample's chromatogram monitored by m/z 392, 407 and 376.

Immunofluorescence

Immunostaining with FITC labeled mAb was carried out for all samples of sinking particles. FITC should give green fluorescence when observed with blue light excitation (495 nm) under the fluorescent microscope. Bright and small green spots were found in all samples with the sizes of bacteria (Fig. 4-4). Such spots were not observed in the samples of control staining. As for the sample no.1, additional experiment of double staining with 4',6-diamino-2-phenylindole (DAPI) was tried to identify the small green spots. DAPI is DNA specific dye and is generally used for identifying and counting bacteria in the soil and water samples. After the procedure of immunofluorescence , the sample was incubated in 200 µl DAPI solution (0.2 µg/ml) for 1 hour at room temperature, and was washed 3 times in PBS and observed in two kinds of excitation light (blue and UV). DAPI should give bluish-white fluorescence in the ultraviolet (UV) light excitation. The result shows that, some of the small green spots observed in blue excitation were also stained by DAPI with exactly similar sizes and shapes under UV light. In other fields, green spots could not be confirmed as bacteria because they were not indistinguishable from particles even in DAPI preparation. Fig. 4-5(a) showed the photomicrograph of the mAb treated

sample (no.1) in blue excitation. Three green spots were found in the photograph, and one of them could be taken simultaneously as a bluish spot in UV excitation in the same field (Fig. 4-5(b)). Although the bluish spot in the photograph looked indistinguishable because of quick fading of fluorescence during exposure to UV light, it was clearly distinguishable when observed by eyes.

DISCUSSION

Lyophilized samples were dissolved in a buffer solution just before the first CIEIA. After the assay, the sample solutions were kept in a refrigerator over night until the second CIEIA. TTX in the samples was expected to be dissolved more during the storage. This might be the reason why quantitative data of the second CIEIA were higher than the first one.

In the CIEIA system, TTX concentration was calculated from the inhibitory effect against an antigen-antibody specific reaction. Calculated TTX concentrations from undiluted, half diluted and 1/4 diluted wells were almost same with samples no. 2, 3, 5 and 6. In the similar way, undiluted and half diluted wells of samples no. 1, 4, 7 and 9, gave reasonable values (Table 4-2). This means that inhibitory effect of these samples represented similar dose response as standard TTX. The inhibitors detected from these samples were very likely to be TTX. In the spike and recovery experiment, all samples except no. 4 and 5 provided almost 100 % percentage recovery so that the quantitative data of them was considered to be reliable (Table 4-3). As the samples no.4 and no. 5 showed high matrix interferences (155 and 144 %), the quantitative data might be suspicious. For further confirmation, another data from biological or chemical analyses should be compared. HPLC system was not available because the sensitivity was not enough in this case. Although TCBA was sensitive enough, SCB toxin other than TTX, which was occasionally contained in sinking particles as described in Chapter 1, could interfere the precise quantification of TTX concentrations. GC-MS system was neither specific to TTX nor quantitative. However, this system was sensitive enough and specific

to C9-base which was so far considered to be derived only from TTX and/or its analogues. The combination with GC-MS system is thought to be currently the best. From the results of GC-MS analysis, 5 out of 7 samples of sinking particles (no.1, 2, 3, 4, 6) were found to surely contain C9-base which could be derived from TTX (Table 4-4). No.5 and 9 were negative, and no.7 and 10 were suspicious. No.7 could be considered positive because of its CIEIA results. Theoretically, C9-base can be derived from not only TTX but also other TTX analogues. As for samples no.1, 2, 3, and 6, the CIEIA and spike recovery experiment indicated the presence of TTX and little matrix interferences. It is quite reasonable that C9-base detected from these samples was originated from TTX. Although sample no.4 was qualitatively confirmed to contain TTX and/or its analogues by GC-MS analysis, presence or a precise concentration of TTX was not clear because of high matrix interferences.

Some TTX concentrations measured by CIEIA (0.3-260 ng/g) were lower than by HPLC (200-1000 ng/g) in the previous experiment (Chapter 1). This is supposed to come from the higher sensitivity and specificity of CIEIA. Lower limit for the detection of HPLC was 100 ng/g in this experiment. In addition, peaks which is originated from other substances and corresponding to TTX might overlap and give us overestimation.

Immunofluorescent technique worked very well and showed the fluorescent spot which was considered to be TTX in bacterial cells. The reasons for the choice of direct staining without sectioning and fluorescent labeling are ; (1) Making thin sections have no advantage to the samples such as marine particles and sediments because it can not be expected to observe their structure. (2)If TTX detected from these samples originates from attached bacteria, the antibody must react against TTX in bacterial cells. A size of bacteria may be too small to be sectioned. (3)A fluorescent label is more suitable for detecting bacterial cells as small spots when stained. Another successful technical point of this work might be formaline fixation. Formaldehyde was used for conjugating TTX to protein to make imunogens and ELIZA coating antigen. This might also link TTX through the guanidyl group to protein amino groups, and, therefore, the sample could avoid the loss of TTX during processing.

Absence of the small green spots in the control experiments strongly suggests that the spots observed in the mAb treated samples would be generated from the mAb specific

reaction against TTX. This fact was supported by the extra control experiment for one sample, in which absorbed mAb was prepared by mixing 100 ng TTX with 100 µl of 1/100 mAb solution for 1 h at room temperature, subsequently used for primary antibody in the same immunofluorescent procedure as normal mAb. No fluorescent spots appeared in this case. Furthermore, double staining with DAPI revealed that such spots would originated from bacterial cells.

In conclusion, the presence of TTX in sinking particles was confirmed by the immunological method using anti-TTX mAb in addition to the previous confirmation by the chemical and biological methods. TTX-producing bacteria were further detected *in situ* in the same samples. These evidences clearly demonstrate that TTX in sinking particles would be originated from the attached bacteria.

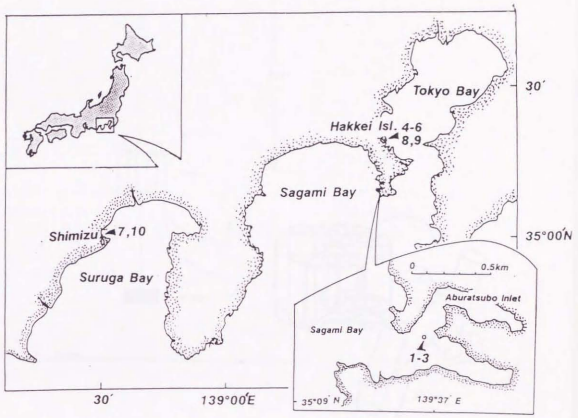


Figure 4-1. Sampling locations in Aburatsubo Inlet, Tokyo Bay and Shimizu Harbor. The Arabic numerals mean the sample no. listed in Table 4-1

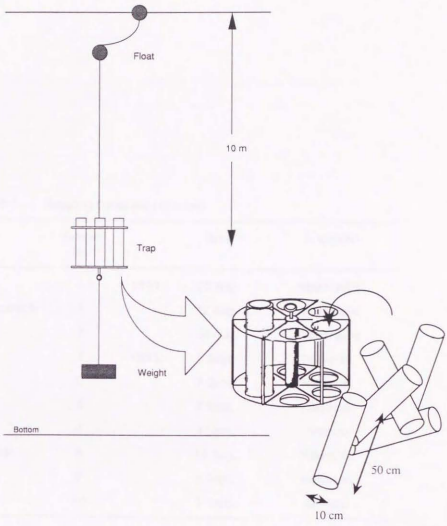


Figure 4-2. Illustration of improved sediment trap system

Table 4-1 Sampling Dates and Locations

	Sample No.		Date	Location
Sinkig	1	1994	20 Aug.	Aburatsubo
particle	2		22 Aug.	Aburatsubo
	3		24 Aug.	Aburatsubo
	4	1993	1 Sept.	Hakkei Isl.
	5		7 Sept.	Hakkei Isl.
	6		7 Sept.	Hakkei Isl.
	7		4 Sept.	Shimizu
Sediment	8		31 Aug.	Hakkei Isl.
	9		6 Sept.	Hakkei Isl.
	10		3 Sept.	Shimizu

Table 4-2 CIEIA analysis of TTX in sinking particles and sediments collected in Aburatsubo Inlet, Tokyo Bay and Shimizu Harbor

	Sample No.	D. weight (g)	CIEIA-1 TTX(ng/ml)		(ng/g)	CIEIA-2 TTX(ng/ml)		(ng/g)
				mean			mean	
Sinkig particles	1	0.10	undiluted	23.4	26.1	261	undiluted	38.6	35.8	358
			1/3	28.8			1/2	33.0		
							1/4			
	2	4.38		15.6		3.6		19.3	21.9	5.0
								22.2		
								24.2		
	3	7.31		14.1		1.9		20.4	25.0	3.4
								23.9		
								30.6		
	4	0.05		*5.1		102		13.8	14.2	284
								14.6		
	5	0.03		9.6		320		21.9	28.6	954
								34.9		
								29.1		
	6	30.8		10.2		0.3		22.0	26.9	0.9
								26.8		
								31.8		
	7	14.0		13.5		1.0		20.4	17.6	1.3
								14.8		
Sediment	9	2180		18.0	23.0	0.011		24.8	24.3	0.011
				27.9				23.7		
	10	490						6.6		0.013

Table 4-3 Spike and recovery experiment of sinking particles and sediments collected from Aburastubo Inlet, Tokyo Bay and Shimizu Harbor

	Sample No.	TTX in diluted sample (ng/ml)	spiked TTX (ng/ml)		Expected TTX in spiked sample (ng/ml)	Measured TTX in spiked sample (ng/ml)	Percent recovery
Sinkig particles	1	3.0	5.0	undiluted	8.0	10.0	125
				1/2	4.0	4.4	110
			15.0	undiluted	18.0	28.6	159*
				1/2	9.0	11.1	123
				1/4	4.5	4.5	100
							mean=115
	2	3.1	5.0	undiluted	8.1	9.2	114
				1/2	4.0	4.8	120
							mean=116
	3	6.0	5.0		11.0	10.2	93
					5.5	5.9	107
							mean=100
	4	2.1	5.0		7.1	9.4	132
					3.5	6.2	177
							mean=155
	5	3.1	5.0		8.1	9.6	119
					4.0	6.8	170
							mean=144
	6	4.1	5.0		9.1	9.9	109
					4.6	3.9	85
							mean=97
	7	2.4	5.0		7.4	8.4	114
					3.7	3.6	97
							mean=105
Sediment	9	2.9	5.0		7.9	9.1	115
					3.9	4.0	103
							mean=109
	10	0.5	5.0		5.5	6.5	118
					2.8	2.0	71*
							mean=118

"+" These data should be omitted, because they are calculated from the edge of the log-linear portion of the standard curve

"Percent recovery"=100*measured TTX/expected TTX

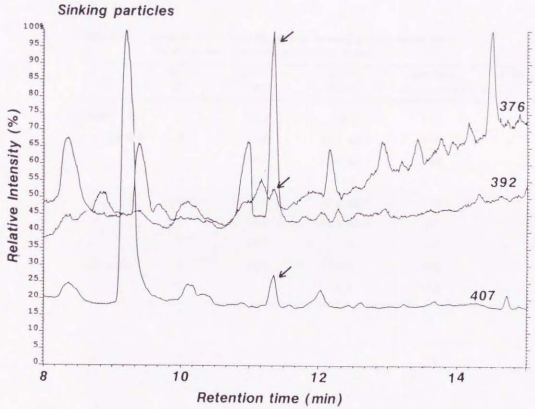


Figure 4-3. Selected ion monitored chromatograms (m/z 392, 407, 376) of the trymethylsilyl (TMS) derivative from alkali-degraded extracts from sinking particles collected in Aburatsubo Inlet (sample no.1). Arrows indicate peaks identical to authentic TMS-C9-base derived from standard TTX

Table 4-4 Analyses of TTX in sinking particles and sediments collected from Aburatsubo Inlet, Tokyo Bay and Shimizu Harbor

	Sample No.	CIEIA-1 TTX(ng/ml)	CIEIA-2 TTX(ng/ml) ±SD	spike exp. percent recovery	GC-MS
Sinkig	1	26.1	35.8	115	+
particle	2	15.6	21.9 ±2.5	116	+
	3	14.1	25.0 ±5.2	100	+
	4	5.1	14.2	155	+
	5	9.6	28.6 ±6.5	144	-
	6	10.2	26.9 ±4.9	97	+
	7	13.5	17.6	105	+
Sediment	9	23.0	24.3	109	-
	10		6.6	118	+

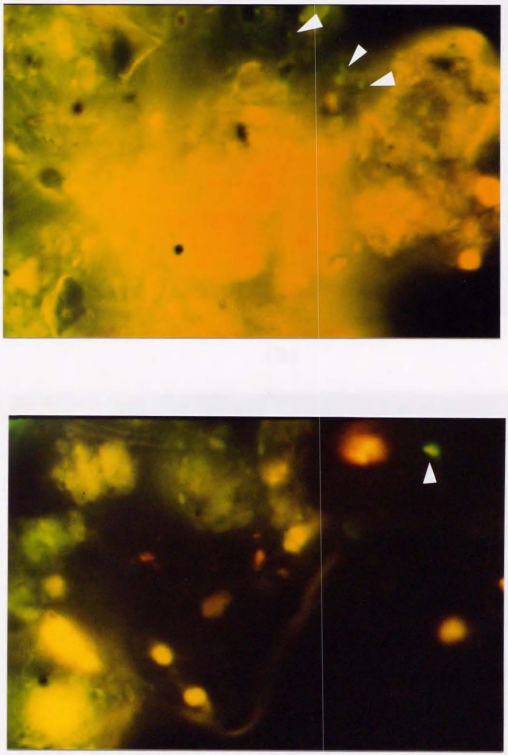
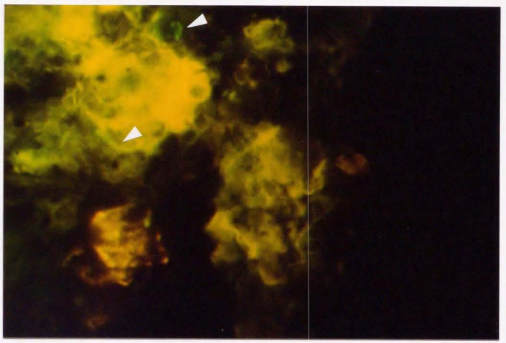


Figure 4-4. Photomicrographs of sinking particles immunologically stained with FITC labeled anti-TTX monoclonal antibody. Blue light excitation (495 nm) of fluorescent microscope (x1000). The arrows indicate the "small green spots"

(a)



(b)

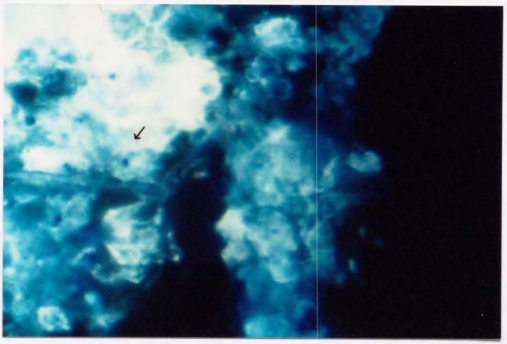


Figure 4-5. Photomicrographs of sinking particles with double staining. (a): Immunostaining with FITC labeled anti-TTX monoclonal antibody (mAb). Blue light excitation of fluorescent microscope (x1000). The arrows indicate the "small green spots". (b): staining with DAPI. Ultra violet excitation of fluorescent microscope (x1000). The arrows indicate the bacterial cells stained with both mAb and DAPI.

Summary and General Discussion

In Chapter 1, the occurrence of TTX in marine sinking particles was investigated. Sinking particles were collected in 1991 using sediment trap system in the coastal area of Aburatsubo Inlet. TTX and related substances were analyzed by TCBA, HPLC and GC-MS detecting from six samples. The TTX concentrations were estimated to be about 200 to 1000 ng/g by the HPLC analyses. Similar results were obtained from additional works in the coastal areas in Tokyo Bay, Sagami Bay and Suruga Bay. This is the first report on the occurrence of TTX in sinking particles in an aquatic environment. When sinking particles were collected in Aburatsubo Inlet, an additional experiment to detect TTX from marine organisms which feed such particles was included. More than a thousand of caprellids clinging on the seaweeds were captured, treated for TTX extraction and analyzed by HPLC, GC-MS and TCBA. As a result, the presence of TTX in caprellids were demonstrated. TTX concentration estimated from the data of HPLC was 16 ng (0.08 MU) per individual or 1300 ng per gram wet weight, which was higher than sinking particles. It was proposed that they might absorb TTX from their food source and actively accumulate.

In Chapter 2, the TCBA was improved to establish a simple and sensitive assay for TTX. The problem of the original method was cell counting procedure, in which living cells were morphorogically distinguished from dead ones and counted under the microscope. This process required some experience and was very time consuming. To solve this problem, two kinds of tetrazolium salts, MTT and WST-1 were applied to stain living cells so that the cell counting procedure was successfully replaced with automatic measurement of absorbance using a microplate reader. TTX concentrations can be measured in the range of 2-70 nM. The sensitivities of these methods were higher than the original TCBA with half-maximal inhibition of MTT-TCBA to be 12.9 nM TTX. Applicability of these methods for the detection of TTX produced by bacteria was also investigated. It was indicated that TTX in a supernatant of bacterial culture could be directly measured. MTT-TCBA and WST-1-TCBA are so simple and sensitive that they may be useful for routine assay or screening test of TTX, in which smaller volume and rapid analysis of each sample are required.

In Chapter 3, toxin fractions of *Shwanella alga*, *Alteromonas tetraodonis* and *Streptomyces* sp. which had been isolated as TTX producing bacteria were analyzed by

mouse neutralization test using anti-TTX mAb. Mouse toxicities of all supernatant extracts were not neutralized. In another highly sensitive immunological assay, CIEIA, TTX was not detected from cell extracts of these strains. However, other chemical and biological analyses showed the contradictory results: (1) Peaks corresponding to TTX appeared in the HPLC chromatograms from the supernatant of *S. alga* and the cell and supernatant extracts of *Streptomyces* sp., and (2) C9-base and sodium channel blockers were detected from all the extracts. Most rational interpretation of all the results would be that detected substances from these strains were not TTX but its derivatives which had high bioactivity. Results of CIEIA indicate that the presence of TTX in the cell extract of *B. subtilis*. It was supported by the results of GC-MS and TCBA. It is very interesting that many members of the genus *Bacillus* produce various antibiotics, and its production seems to be related to the sporulation in most case. *B. subtilis* could be useful for studies on biosynthetic mechanism of TTX.

In Chapter 4, the occurrence of TTX in sinking particles and sediments was proved by the sensitive immunoassay system, CIEIA, and TTX-producing bacteria attaching to the particles were detected in situ by using immunofluorescent technique. Sinking particles and sediments were collected at Tokyo Bay and Shimizu harbor in 1993, and Aburatsubo Inlet in 1994. TTX was detected from 10 samples by CIEIA. From the results of recovery experiment and GC-MS analysis, 6 out of 10 samples were confirmed to contain TTX. Measured concentrations by CIEIA were from 0.9 to 300 ng/g, which were lower than those by HPLC described in Chapter 1. It might be come from higher sensitivity and specificity of CIEIA. In the experiment of immunofluorescence, a portion of fixed sinking particles was stained by the indirect staining method. Small green spots of bacterial size were observed under the fluorescent microscope in every samples. Control experiments indicated that these spots were generated in the specific reaction of anti-TTX mAb. Since the spots were also stained by DAPI in double staining, it was strongly suggested that they were originated from TTX in bacterial cells.

As the results in Chapter 1 and 4 are integrated overall, a concrete mechanism of TTX accumulation and important role of sinking particles come up reasonably: TTX is produced by bacteria in sinking particles, these particles are ingested by detritus feeders like caprellids and cause the toxification of them, and subsequently TTX is transferred

to higher trophic levels through food webs. In addition, sinking particles may be a supplier of TTX to the bottom because of its property of sedimentation and also play an important role for the toxification of benthic animals.

The observations that former reported bacterial strains to be TTX producers did not show enough production of TTX after second transfer in the laboratory conditions imply the difficulties of this kind of works. Since the result from immunofluorescent work, in which TTX-producing bacteria could be directly detected without incubation, indicated that TTX would be produced by bacteria in natural environment, there should be some conditions to be required for TTX production. As appropriate conditions might vary with bacterial strains, we should try screening to find out highly productive strains or conditions. Such works, which require simple and rapid assay of large amount of samples, may become possible by using TCBA method improved in Chapter 2 or CIEIA system applied in Chapter 3 and 4.

Immunostaining work in Chapter 4 would be the first report of *in situ* detection of TTX in natural environment. This technique is very promising for future studies: (1) The number of TTX producing bacteria could be directly counted by using image analyzer or flow cytometer, (2) immunoelectron microscopy could reveal the localization of TTX in a bacterial cell, and also (3) the localization of TTX in other TTX-bearing organisms could be revealed.

In conclusion, this investigation emphasized the importance of sinking particles as a site for TTX production by marine bacteria and as a source of TTX in it accumulation system through food webs, and represented new methodological aspects for future works on TTX production and accumulation mechanism.

Acknowledgements

I am grateful to my mentor Professor Kouichi Ohwada, whose great insight and appropriate advices led me to the successful completion of this degree. He introduced me the way of work at the sea, which was very exciting, attractive and captivating. The experience at the sea will be the base of work all in my life.

I am also grateful to Dr. Kazuhiro Kogure for his suitable suggestions and continuous supports. His two reports in 1988, the development of TCBA and TTX in marine sediment, gave me the cue for starting this work. I was influenced by his enthusiasm and accomplishments in the laboratory works.

Two persons, who had been participants in the laboratory, guided me to the work on TTX-producing bacteria at the beginning of my graduate course. I thank Dr. Usio Simidu, whose book, "Pursuit of the mystery on pufferfish toxins", was impressed, for providing me with the opportunity of doing this work, and Dr. Hyun-Ki Do for kindly teaching me the basic techniques.

I thank Dr. Tamao Noguchi at the University of Tokyo, Dr. Yasuo Shida at the Tokyo College of Pharmacy, Dr. Ichiro Takeuchi at the Otsuchi Marine Research Center, Dr. Kendo Matsumura at the Yamaguchi Prefectural Institute of Health, Dr. Toyoji Kaneko at the Ocean Research Institute and Dr. Gary S. Bignami at the Hawaii Biotechnology Group Inc. for their technical supports, advice and information. The data of GC-MS analysis were obtained with the help of Dr. Noguchi and Dr. Shida, who gave me a lot of suggestions about the chemical analyses of TTX. Dr. Takeuchi helped me with getting various information about caprellids. Dr. Matsumura kindly assisted me in the collaborative studies using the monoclonal antibody that he developed. His energy, drive and enthusiasm inspired me to doing the work. Dr. Bignami sincerely accepted my temporal stay in his laboratory, where I fortunately had a chance to learn the immunological techniques. Furthermore, he supplied the monoclonal antibody that was required for the work of immunofluorescence.

Some samples such as sinking particles and sediments were collected in Aburatsubo Inlet, using facilities of Misaki Marine Biological Station, University of Tokyo. I thank the stuffs of the station for their help with collecting samples and also Miss Yoko Ueda for her assistance of making and applying the sediment trap system. Other sinking particles and sediments were collected during the cruises of R/V Tansei-maru.

Thanks should be extended to officers and crew members of the vessel.

I am grateful to the stuffs and fellow students in the laboratory, Dr. Kumiko-Kita Tsukamoto, Dr. Masahiko Nishimura, Dr. Minoru Wada, Mr. Yong-Te Park, Mr. Tokuki Sakiyama, Mr. Michinari Sunamura, Mr. Akira Shibata and Mr. Hidetoshi Urakawa, for their help, advice and comradeship. Good friendship including daily frivolities we all shared made me feel brave.

Finally, I am deeply indebted to my parents. I have been able to only bring my degree to completion, when their love and support has been involved.

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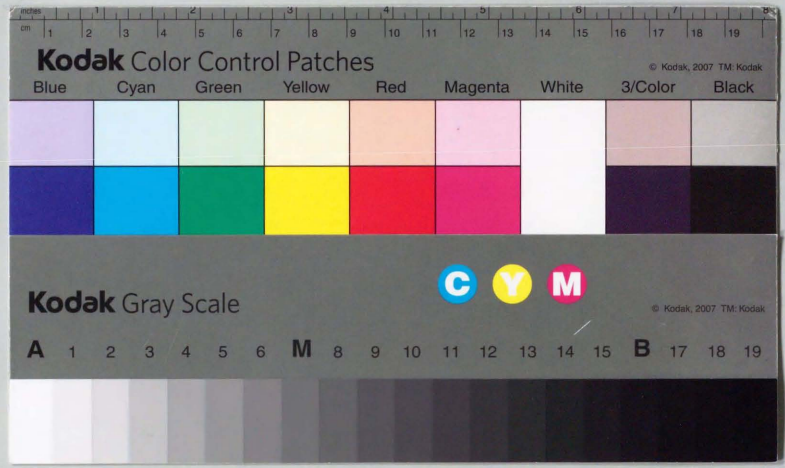
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