

Marine Biotoxins: Occurrence, Toxicity, and Detection Methods

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Abstract. This review summarizes the role of marine organisms as vectors of marine biotoxins, and discusses the need for surveillance to protect public health and ensure the quality of seafood. I Paralytic shellfish poison (PSP) and PSP-bearing organisms-PSP is produced by toxic dinoflagellates species belonging to the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. Traditionally, PSP monitoring programs have only considered filter-feeding molluscs that concentrate these toxic algae, however, increasing attention is now being paid to higher-order predators that carry PSP, such as carnivorous gastropods and crustaceans. II. Tetrodotoxin (TTX) and TTX-bearing organisms - TTX is the most common natural marine toxin that causes food poisonings in Japan, and poses a serious public health risk. TTX was long believed to be present only in pufferfish. However, TTX was detected in the eggs of California newt *Taricha torosa* in 1964, and since then it has been detected in a wide variety of species belonging to several different phyla. In this study, the main toxic components in the highly toxic ribbon worm *Cephalothrix simula* and the greater blue-ringed octopus *Hapalochlaena lunulata* from Japan were purified and analysed.

Chapter I. Paralytic Shellfish Poison (PSP) and PSP-Bearing Organisms

1. Introduction

PSP is one of the most notorious and hazardous marine biotoxins known. It is mainly produced by toxic marine dinoflagellates species belonging to the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*, and is accumulated up the food chain in many species of marine filter-feeding organisms such as bivalves [1-5]. Ingestion of PSP containing bivalves results in human intoxication. PSP blocks sodium channels distributed on the cell membrane, inhibiting the flow of sodium ions into and out of the cell, which leads to symptoms that include muscle paralysis, and often frequent death (Figure 1).



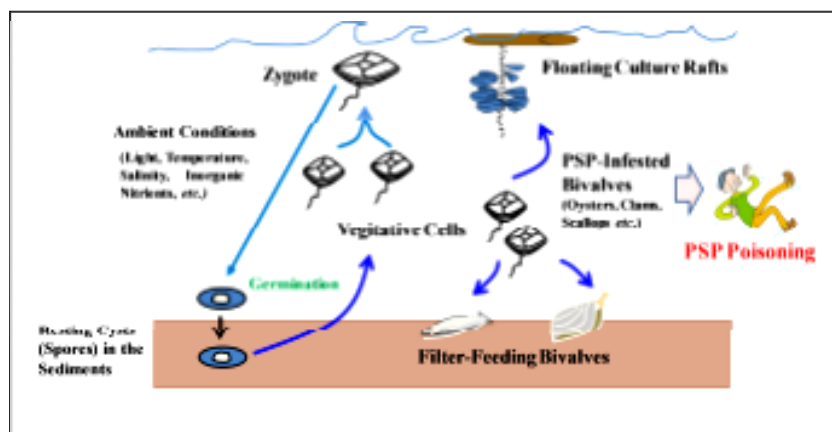
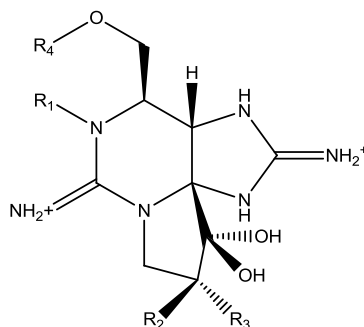


Figure 1. Generalized pathways of human intoxication with PSP via filter feeding bivalves

The PSP-producing dinoflagellates, *Alexandrium tamarense*, *A. catenella*, *A. tamiyavanichii*, and *Gymnodinium catenatum* are found in Japan, where they intoxicate many edible bivalves [6-9]. *Alexandrium* spp. cause problems mainly in cold, temperate waters in the northern hemisphere, while *Pyrodinium bahamense* var. *compressum* is a tropical and subtropical species that occur in Southeast Asia [10,11]. The contamination of bivalves with PSP produced by these species has posed a serious problem to both the shellfish culture industry and public health in various parts of the world. To date, 10 PSP poisoning incidents have been officially recorded in Japan, and PSP food poisoning has also been reported in adjacent countries such as Taiwan and Korea. However, although PSP-infested bivalves are still being reported, food poisonings as a result of the ingestion of these species no longer occurs, except by accident, due to the established monitoring program for toxic shellfish and cell densities of PSP-producing dinoflagellates. In April, 1979, cultured scallops in Funka Bay, Hokkaido, were found to contain high levels of PSP and so were prohibited from being marketed [12]. However, at that time, several people collected and ate the mussels attached to the scallop-culturing rafts, three of whom were poisoned, and one of whom died. PSP is transferred from certain species of toxic dinoflagellates to shellfish by filter feeding with most of the toxins being accumulated in the digestive gland. Consequently, the digestive glands of bivalves are generally much more toxic than other tissues. For example, the adductor muscle has been found to be non-toxic to weakly toxic even in specimens whose digestive glands were extremely toxic (c. 1000 MU/g).

PSP is a group of naturally occurring neurotoxic alkaloids that act as sodium channel-blocking agents in mammals, and causing several symptoms, including paralysis and often death. Saxitoxin (STX) was the first toxin to be isolated from PSP-infested bivalves. However, PSP is composed of at least 20 additional derivatives, all of which contain hydroxyl, carbamyl, and sulfate moieties at four sites (R1 - R4) on the backbone structure [5,13]. These PSP components have been divided into two groups, the low-toxicity group and high-toxicity group. Members of the low toxicity group are known as *N*-sulfocarbamoyl toxins (Figure 2), and are easily converted into other known toxins under acidic conditions.



R1	R2	R3	Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
			R4		
			CONH ₂	CONHSO ₃ ⁻	H
H	H	H	STX (2483)	GTX5 (160)	dcSTX (1274)
OH	H	H	NeoSTX (2295)	GTX6 (180)	dcneoSTX (33)
OH	OSO ₃ ⁻	H	GTX1 (2468)	C3 (33)	dcGTX1 (1500)
H	OSO ₃ ⁻	H	GTX2 (892)	C1 (15)	dcGTX2 (1617)
H	H	OSO ₃ ⁻	GTX3 (1584)	C2 (239)	dcGTX3 (1872)
OH	H	OSO ₃ ⁻	GTX4 (1803)	C4 (143)	DcGTX4 (1080)

C = C toxins; GTX= gonyautoxin; STX= saxitoxin; dc= decarbamoyl

Value in parenthesis: specific toxicity in MU/μmol

The activity was expressed in mouse units (MU); One MU is defined as the dose of toxin required to kill a 20g ddY strain male mouse in 15 min after intraperitoneal injection.

Figure 2. Structure of the PSP analogues and their specific toxicity

For example, gonyautoxin V (GTX5) belongs to this group, and gives rise to STX when heated in dilute hydrochloric acid (HCl), increasing the toxicity sharply (mild acidic hydrolysis) [14]. These low-toxicity components are considered to be the precursors of the high toxicity carbamate toxin group. These substitutions result in analogs that vary by more than three orders of magnitude in toxicity. Some components, such as STX and GTX2, have specific toxicities comparable to tetrodotoxin (TTX). The minimum lethal dose (MLD) of PSP in humans is estimated to be 3,000 MU based mainly on fatal cases induced by this toxin [15]. PSP is roughly three times more toxic than TTX whose MLD in humans is considered to be 10,000 MU. Here, one mouse unit (MU) of PSP and TTX is defined as the amount of each toxin which can kill a 20g ddY strain male mouse in 15 and 30 min, respectively, after intraperitoneal administration.

2. Accumulation of PSP by Filter-Feeding Vectors

2.1 Toxic dinoflagellate and PSP-infested bivalves

Case study in Hiroshima Bay, Hiroshima Prefecture, Japan

Hiroshima Bay is one of the largest oyster farming areas in Japan (Figure 3). Up until 1992, there had been no records of PSP toxification of bivalves in this region. However, in April 1992, levels of PSP that substantially exceeded the quarantine limit of 4 MU/g edible parts as PSP were detected in bivalves with the appearance of *A. tamarensis*, resulting in the prohibition of their harvesting and marketing by the Hiroshima Prefectural Government [1]. Fortunately, no food poisoning resulted from this outbreak. However, since the cultured oysters that are produced in Hiroshima Bay are shipped to many parts of Japan not only as fresh oysters, but also as raw materials for processed foods such as smoked oysters and oyster sauce, this posed a very serious problem to fisher-men and other workers in associated industries as well as to public health. Figure 4 shows a scanning electron microscope image of *A. tamarensis* isolated from Hiroshima Bay in 1992. Subsequent monitoring of the cell density of *A. tamarensis* from 1993 to 2004 showed that oysters, mussels and clams were contaminated with PSP from approximately the end of March until May each year [16].

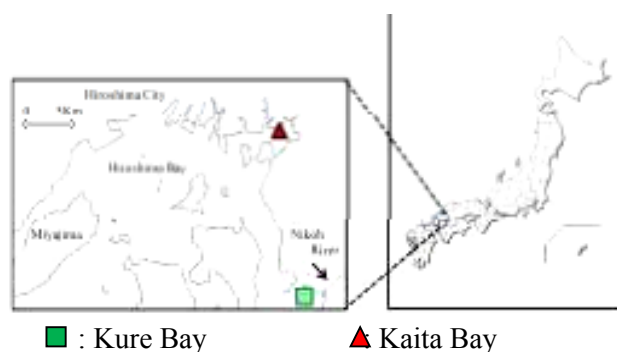


Figure 3. Map showing PSP-producing dinoflagellate of *A. tamarensis* collecting locations in Hiroshima Bay

The location of Hiroshima bay in Japan is shown in the map to the right. The map on the left shows an enlarged image of Hiroshima Bay to pinpoint the sampling locations

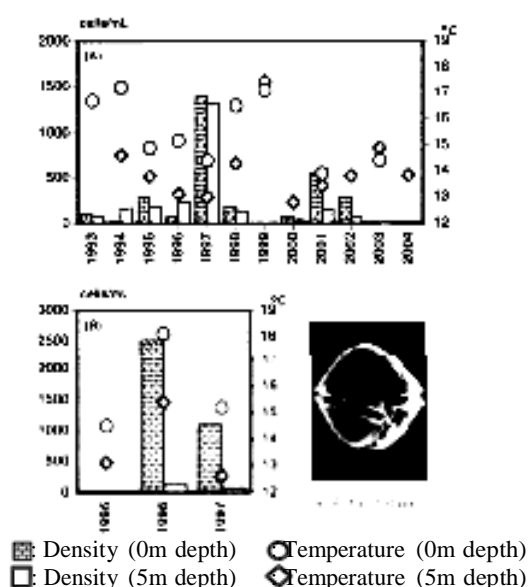


Figure 4. Maximum cells density of *A. tamarensis* in Kure Bay (A) and Kaita Bay (B), Hiroshima Prefecture and scanning electron microscope image showing

For example, in Kure Bay, maximum concentrations of 1,400 and 1,300 cells/ml were observed at 0 and 5 m depths on April 21 and 24, 1997, respectively, while in Kaita Bay, remarkably high concentrations above 1,000 cells/ml were observed in two out of three years investigated. The temperature range at which the natural population of *A. tamarensis* bloomed was generally between from 12°C to 16°C.

Figure 5 shows a high-performance liquid chromatography-fluorescence detection (HPLC-FLD) analysis of the GTX and STX groups of toxins contained in *A. tamarensis* strain AHS-93 isolated from Hiroshima Bay, while Table 1 shows the toxin profiles of *A. tamarensis* isolated from Hiroshima Bay and the toxic bivalves.

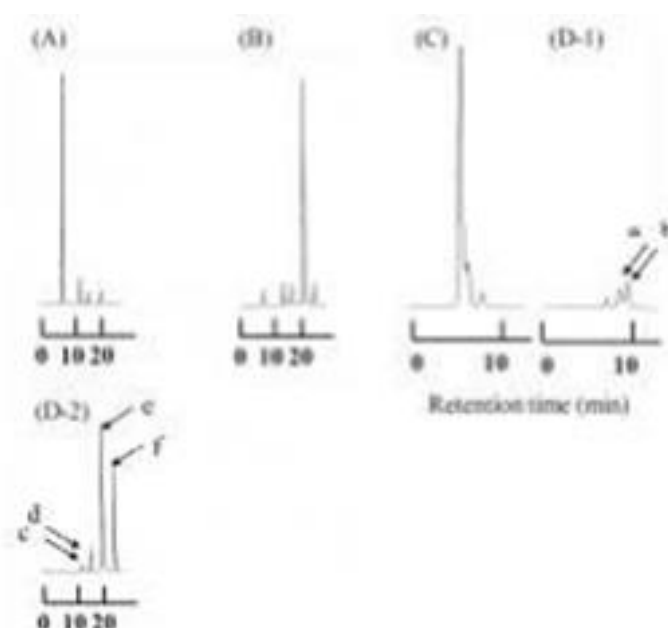


Figure 5. HPLC-FLD analysis of GTX and STX group of toxins containing in *A. tamarens* strain AHS-93 isolated in Hiroshima Bay. (A) Purified toxins from strain AHS-93 (B) hydrolysate of purified toxins with 0.1N HCl for 15 min. (D-1, 2) STX and GTX standards : (a, b) neoSTX, STX, (c,d,e,f) GTX4,1,3,2

Table 1. Toxin profiles of *A. tamarens* isolated in Hiroshima Bay and toxic bivalves

Chemical form	Specimens	Dinoflagellate		Short-necked clam	mussel	oyster
		<i>Alexandrium tamarens</i>				
	Strain	ATHS-92*	ATHS-93			
α -epimer	GTX1	9.1±3.8	11.6	61.8	60.5	42.5
	GTX2	Tr.	Tr.	1.5	1.2	2.9
	C1(epi-GTX8)	0.7±0.4	1.3	0.2	0	0.9
	C3	1.7±1.3	0.2	0	0	0
	subtotal	11.5	13.1	63.5	61.7	46.3
β -epimer	GTX3	1.6±0.2	5.1	7.5	8.8	21.5
	GTX4	33.4±4.8	27.6	23.8	28.4	17
	C2(GTX8)	39.9±8.7	37.0	0.7	0	5.3
	subtotal	74.9	69.7	32	37.2	43.8
	neoSTX	13.5±5.1	17.2	5.1	1.1	9.9

All results are shown in mol%. -: not detected, Tr.: less than 0.1%

*Mean \pm S.D. of five samples

Some fairly large differences were observed in the relative abundance of the toxins in the responsible dinoflagellates and the contaminated shellfish, suggesting that some *in vivo* conversion between the PSP components. The most notable difference was in the amount of C2 toxin, which is considered to be the precursor of GTX3. The two *A. tamarens* strains contained large amounts of C2 toxin (39.9 and 37.0%), whereas the bivalves only contained trace amounts of this low toxicity component, suggesting its *in vivo* conversion following ingestion. It has previously been shown that

toxin composition also varies substantially between shellfish species, suggesting that there is interspecific variation in the metabolism of PSP [17-21].

2.2 PSP-infested marine mossworm

Case study in Funka Bay, Hokkaido, Japan

During the screening for paralytic potency in marine organisms around Funka Bay in Hokkaido, one of the largest scallop farming areas in Japan, a lethal potency was detected in brown seaweed, that was fouled with organisms including mossworm [22]. Figure 6 shows a scanning electron microscope image of marine mossworms that were stripped off a seaweed sample (the dried brown alga *Laminaria japonica*, known as “Kombu” in Japanese) that was harvested from Funka Bay, Hokkaido in summer of 1989. These mossworms had a lethal score of 18 MU/g dry basis, which greatly exceeded the quarantine limit of 4 MU/g. It is generally accepted that mossworms are plankton-feeders, and so, it is assumed that they were contaminated by ingesting PSP-producing toxic dinoflagellates.

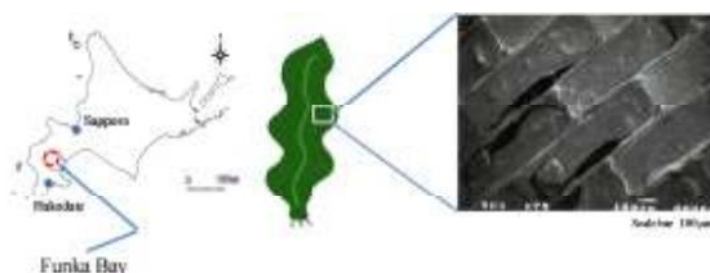


Figure 6. Scanning electron microscope image of mossworm on the brown alga *Laminaria japonica* (“Kombu” in Japanese).

3. Accumulation of PSP by Non Filter-Feeding Vectors

3.1 Echinoderms

Case study of the starfish from Hiroshima Bay, Hiroshima Prefecture, Japan

During surveillance on the toxicity of invertebrates inhabiting the coasts of Hiroshima Bay, the starfish *Asterias amurensis* was found to contain toxins that had strong paralytic action in mice. Figure 7 shows the results from the parallel monitoring on toxicity of this starfish and bivalves. Although this starfish was not found to be toxic in March to April, its toxicity increased unexpectedly in May, concurrent with an increase in the toxicity of the bivalves. This toxicity appeared to be almost exclusively present in the viscera and this toxin was unexpectedly identified as PSP by HPLC-FLD analysis [23]. Ito *et al.* also reported the occurrence of PSP in the starfish *Asterina pectinifera* collected from Kure Bay, Hiroshima Prefecture [24].

3.2 Gastropods

Case study of carnivorous gastropods from Kure Bay, Hiroshima Prefecture, Japan

Figure 8 shows a comparison of the toxicity of a gastropod (the rapa whelk, *Rapana venosa*) and a bivalve (the short-necked clam), along with cell densities of *A. tamarensis*, in the Nikoh River estuary near Hiroshima Bay [25]. The period during which this carnivorous gastropod had paralytic toxicity almost completely coincided with the spring to early summer season when the toxic plankton *A. tamarensis* flourished and plankton-feeding bivalves such as the short-necked clam became infested with PSP, albeit with some time lag. An analysis of the anatomical distribution of toxicity in this gastropod showed that the viscera were significantly toxic, reaching a score of 224 MU/viscera (4.2 MU/g viscera) in one specimen on May 21.

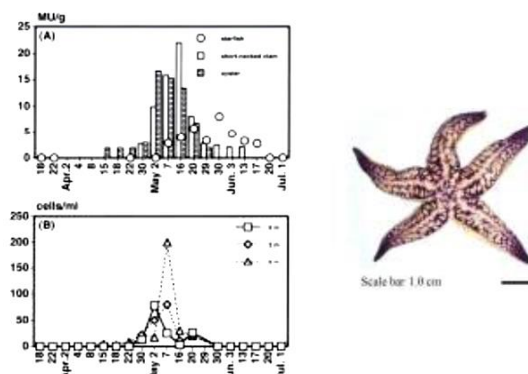


Figure 7. Starfish *Asterias amurensis* (right) and toxicities of starfish, short-necked clams and oysters in Hiroshima Bay in 1996, along with maximum cells density of *A. tamarensis* (B) in each layer.

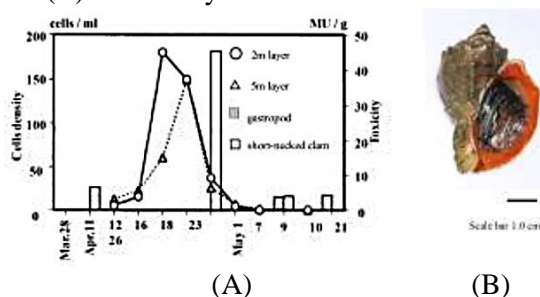


Figure 8. Non-filter feeding (non-traditional) vectors of PSP (1). (A) Carnivorous species (gastropod): rapa whelk *Rapana venosa* PSP was detected in the viscera. (B) Toxicity of *R. venosa* and short-necked clam along with cells density of *A. tamarensis*, in the estuary of Nikoh River in 2001.

Case study of herbivorous gastropods imported from Spain

Ormer are of great commercial value, and widely cultivated in Japan and other countries. Paralytic toxicity in excess of the quarantine limit of 4 MU/g as PSP was detected in ormer, that had been imported from Vigo, Spain, to Japan, between January and April, 1994 (Figure 9) [26, 27]. An analysis of the anatomical distribution of PSP toxicity in 10 samples showed that the muscle was the most toxic tissue in all specimens (31.9MU/g), while the viscera were less toxic (7.1MU/g). HPLCFLD analysis showed that dcSTX was the main component in all tissues, accounting for between 83 mol% (muscle) and 97 mol% (digestive gland) of all components. These results demonstrated that herbivorous marine gastropods had also accumulated PSP, and suggested that ormer possess a unique metabolic pathway for PSP. The exact source of these toxins in ormer remains unknown. Paralytic shellfish poisoning has also been detected in *Haliotis tuberculata* from the Galician coast, Spain, the geographical distribution of which has been investigated, along with the relationship between toxicity and the different lengths and parts of the molluscs [28].

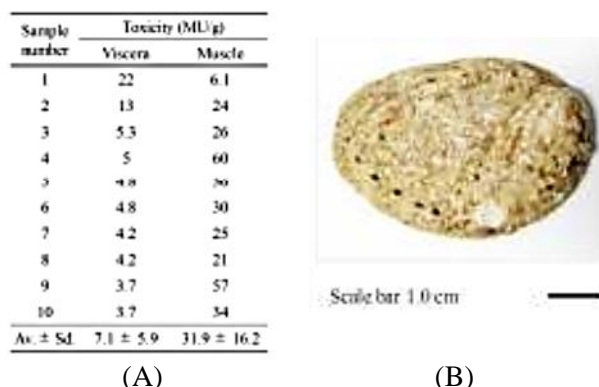


Figure 9. Non-filter feeding (non-traditional) vectors of PSP (2). (A) Anatomical distribution of toxicity (B) Herbivorous species (gastropod): ormer *Haliotis tuberculata* (imported from Spain to Japan) PSP was detected in muscle and viscera.

3.3 Crustaceans

Case study of xanthid crabs from Ishigaki Island, Okinawa Prefecture, Japan, and from Camotes Island, Visayas, the Philippines

Crabs are valued as a popular seafood in many parts of the world, and are widely consumed in many ways such as by boiling, steaming, or in processed foods. While most species of crabs are edible, some are toxic to humans and other mammals.



Figure 10. Highly toxic xanthid crab *Atergatis floridus* (left) from Ishigaki Is. (I), Camotes Is. (II), and *Zosimus aeneus* (right) from Ishigaki Is. Ishigaki specimen contains PSP. (A), (B): locations of sampling in Ishigaki Island. Camotes specimen contains PSP as the major components and a small amount of TTX (tetrodotoxin).

Xanthid crabs inhabiting the tropical and subtropical islands of the Pacific, possess high levels of PSP [29-33], and several cases of human poisoning and fatalities have been reported as a result of the ingesting coral reef crabs. For example, in the Southwestern Islands of Japan, at least 29 people were poisoned and at least 15 died due to the ingestion of xanthid crabs between 1909 and 1988. Figure 10 shows specimens of the highly toxic xanthid crab *Atergatis floridus* from Ishigaki Island, Okinawa Prefecture, Japan and Camotes Island, the Philippines. The muscles of the appendages of these specimens were highly toxic, with a maximum toxicity of $4,641 \pm 972$ MU/g as PSP being recorded. The PSP toxicity scores in each of the tissues of Ishigaki specimens were as follows: carapace, 183 ± 47 to 807 ± 693 MU/g, viscera, 64 ± 41 to 654 ± 137 MU/g, appendages, 88 ± 40 to 1257 ± 607 MU/g and their muscle, $1,408 \pm 404$ to 4641 ± 972 MU/g (mean \pm SD). By contrast, all of the Camotes Island

specimens exhibited high toxicities in all of their tissues as follows: viscera, 105 ± 56 MU/g, appendages 221 ± 189 MU/g and muscle, 719 ± 349 MU/g (mean \pm SD).

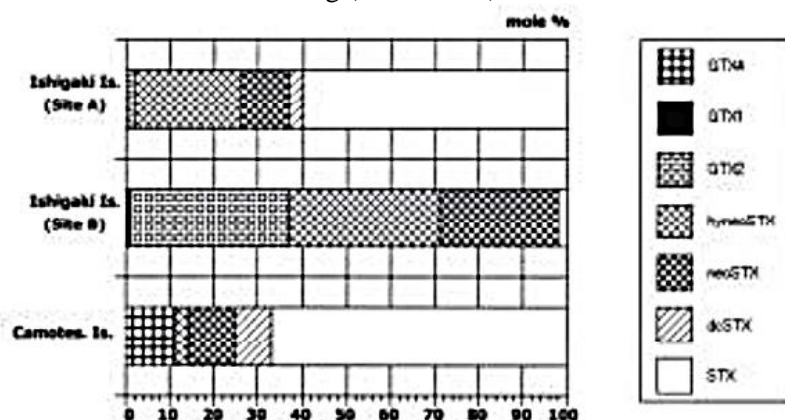


Figure 11. Comparison with the toxin profiles of *A. floridus* collected on reefs of Ishigaki Island and Camotes Island, Cebu Province, Philippines.

The toxin profiles of the viscera of *A. floridus* from sites A and B in June, 2007 are illustrated in Figure 11 [34]. The semi-purified toxins from the viscera of the Camotes Island samples contained rather high relative abundances (mol%) of neoSTX, dcSTX and STX were rather high (89%), along with smaller amounts of GTX4(11%) and hyneoSTX (3%). These PSP compositions were almost identical to those of the Ishigaki specimens, but the HPLC-FLD analysis also detected a trace amount of TTX (data not shown). The highly toxic PSP-bearing xanthid crab *Zosimus aeneus* had the highest toxicity score at 1,777 MU/g in the muscle (Figure 10). The toxicity levels of xanthid crab species are generally high, with previous reports of up to 16,500 MU/g. Since the minimum lethal dose (MLD) of PSP in humans is estimated to be 3,000 MU, as little as one gram of such a toxic crab specimen would be sufficient to kill at least five people.

4. Conclusion

The transfer of PSP to various marine organisms via the food web is shown in Figure 12. PSP is produced by toxic dinoflagellates and then transferred and accumulated throughout aquatic food webs. The occurrence of several new non-filter-feeding vectors of PSP and the secondary intoxication of edible gastropods are huge problems from a food hygiene and fisheries perspective, and so legislation should be adjusted to extend the monitoring of marine biotoxins to a wider range of species besides commercially important edible bivalves. Among non-filter feeding, non-molluscan species, the STX ngroup has been found most commonly in xanthid crabs. The origin of PSP in these toxic crabs remains unclear, as known PSP-producing toxic dinoflagellates species have not been detected in areas that are inhabited by toxic crabs. Consequently, further studies are currently in progress to elucidate the mechanisms involved.

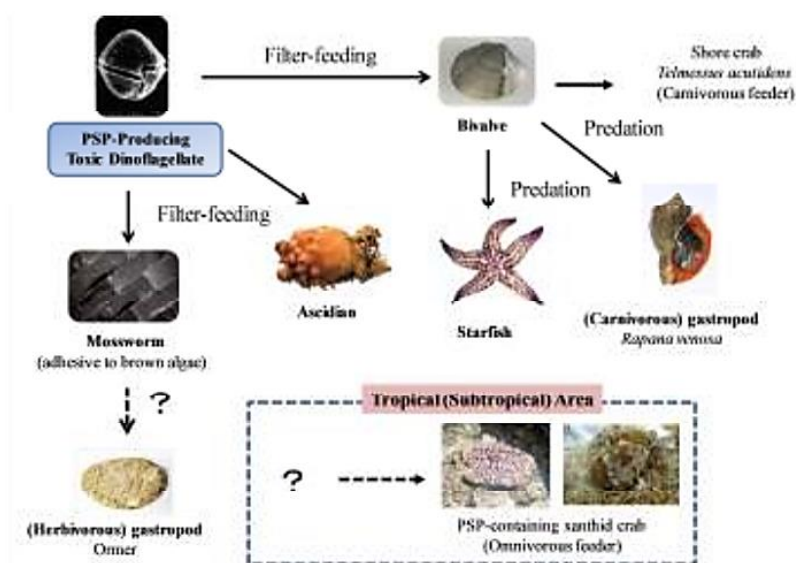


Figure 12. Transfer of PSP to various marine organisms via food web

Chapter II. Tetrodotoxin (TTX) and TTX-Bearing Organisms

1. Introduction

The pufferfish toxin, TTX is one of the most potent neurotoxins. This low molecular weight toxin (319) was first isolated by Yokoo in 1950 as a crystalline prism from a toxic pufferfish [35]. The most characteristic symptom caused by TTX is paralysis with respiratory paralysis occurring in severe cases, often resulting in the victim's death. In Japan, pufferfish have been a traditional food for many years, and so cases of TTX poisoning are frequent occurring on a regular basis not only in Japan but also in other parts of Asia, and sporadically resulting in severe poisoning or even death. Consequently, TTX poses a serious hazard to public health in several Asian countries, with more than 100 deaths per year being recorded until 1960 in Japan.

Figure 13 shows the structure of TTX and its many typical derivatives, as well as their specific toxicities, which vary greatly [36]. TTX is a heterocyclic guanide, and act as a powerful and specific sodium channel blocker. When ingested by humans, it blocks the sodium channels in nerve cells and skeletal muscles, resulting in typical symptoms such as respiratory paralysis and even death in severe cases. The lethal potency of TTX is 5,000 MU/mg, where 1 MU (mouse unit) is defined as the amount of toxin required to kill a 20g male mouse within 30 min after intraperitoneal administration, and the minimum lethal dose (MLD) for humans is estimated to be approximately 10,000 MU, which is equivalent to 2 mg of pure TTX crystals [37]. TTX was long believed to be present only in the pufferfish. Mosher et al. [38] detected TTX in California newt *Taricha torosa*, and since then, it has been detected in several other taxa.

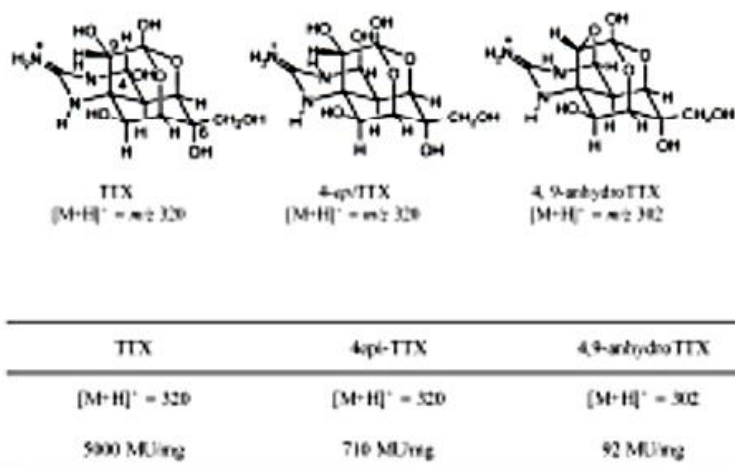


Figure 13. Structure of tetrodotoxin derivatives and their specific

2. Nemertines

2.1. Toxicity of ribbon worm *Cephalothrix simula*

During surveillance of the toxicity of various marine fouling organisms in Hiroshima Bay, specimens of the ribbon worm *Cephalothrix simula* that were adhering to the cultured oyster *Crassostrea gigas* hanging onto floating culture rafts were found to contain toxins that exhibited strong paralytic action in mice [39, 40]. The i.p. injection of a 0.1% acetic acid (AcOH) extract of the worms induced restlessness, paralysis in the hind limbs, and a wobbling gait in the mice, followed by gasping, jumping, and death. Therefore, here, we examined the toxicity of these worms and attempted to identify the toxic component they contain. We carefully collected 615 ribbon worms that were adhering to cultured oysters harvested from floating rafts using tweezers (Figure 14). Based on histological studies, these specimens were identified as *Cephalothrix simula* by Dr.H. Kajihara, Hokkaido University [41]. We then examined the toxicity of each specimen in details.

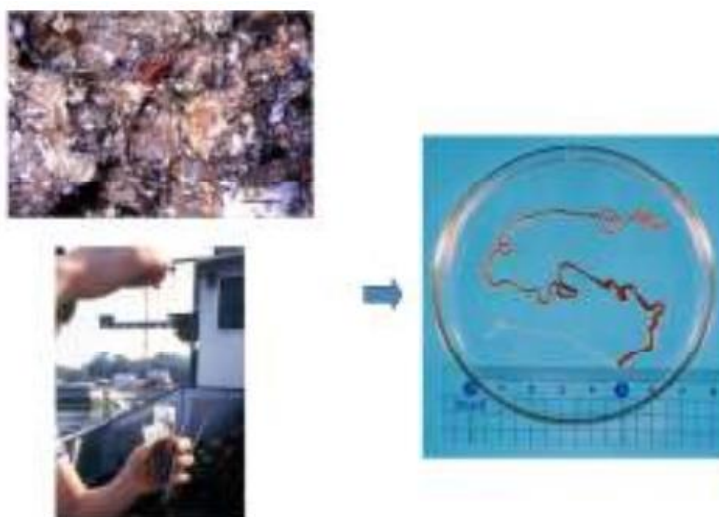


Figure 14. Ribbon worm *Cephalothrix simula* adherent to shells of oysters from Hiroshima Bay.

The toxicity and weight of each ribbon worm are shown in Figure 15. All of the specimens assayed were found to be toxic throughout the season covered, with toxicity scores ranging from 169 to 25,593 MU/g. The maximum to minimum ratio was as high as 151, but the ratio of strongly toxic ($\geq 1,000$ MU/g) to total specimens was 81%. The ratio of extremely toxic ($> 2,000$ MU/g) to total specimens was 53%. By contrast, the ratio of moderately toxic specimens between 100 and 999 MU/g was 100%. There

was no obvious relationship between size and toxicity, as comparable sized specimens that had been collected from the same place at the same time exhibited wide variations in toxicity. The highest toxicity detected was 25,590 MU/g in a specimen collected on June 25(1999). The total toxicity of this specimen was calculated to be approximately 5,631 MU, which is equivalent to about half of the MLD of TTX in humans, 10,000 MU.

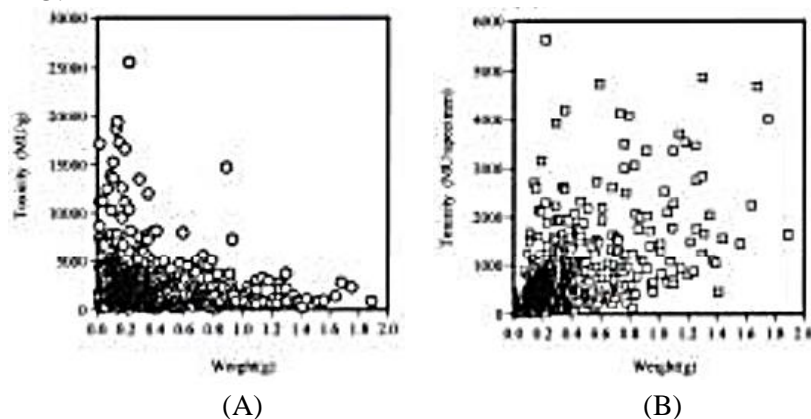


Figure 15. Toxicity of ribbon worm *Cephalothrix simula* from Hiroshima Bay (1998-2005). (A) Relationship between toxicity (MU/g) and body weight (g) (B) Relationship between toxicity (MU/specimen) and body weight (g).

Although these worms were highly toxic, no paralytic toxicity was detected in the shucked meat of the oysters that were fouled with them. However, since the culturing of edible bivalves such as oysters and scallops is a flourishing industry in Japan, surveillance of the distribution of toxic ribbon worms in other areas besides Hiroshima Bay is urgently required.

2.2. Isolation of TTX as the main toxic component from *Cephalothrix simula*

The frozen ribbon worm specimens obtained in the above survey (390g) were semi-defrosted and their contents were extracted with three volumes of 1% AcOH in 80% methanol. The resulting supernatant, which had a toxicity of 2,897,000 MU, was then defatted by chloroform several times. The aqueous layer (2,750,000 MU) was applied to an activated charcoal column and the adsorbed toxin was eluted with 1% acetic acid in 20% ethanol. The eluate was then evaporated to dryness in vacuo, and the residue (total toxicity 2,433,000 MU; specific toxicity 99 MU/mg) was dissolved in a small amount of water and adjusted to pH5.5 with 1N NaOH. This solution was applied to a Bio-Gel P2 column ($\phi 3.5 \times 100$ cm). The column was washed with 3 L of water and then developed with 2 L of 0.03M AcOH. The toxicity was detected exclusively in the 0.03M AcOH fraction. Therefore, this fraction was concentrated to dryness under reduced pressure and the residue (3,300 MU/mg) was dissolved in a small volume of water. The solution was chromatographed on a Bio-Rex 70 column (H+ form, $\phi 1.0 \times 100$ cm) using a linear gradient of 0 to 0.03 M AcOH (Figure 16). The toxic fractions were monitored by mouse bioassay and HPLC-FLD analysis. The main toxic fractions (fr.85-100) were combined, and rechromatographed in the same manner. The toxic fractions thus obtained were then combined, freeze-dried, and then finally dissolved in 0.5 ml of 1% AcOH. Approximately 2.0 ml of methanol and 5.0 ml of diethyl ether were then added to these fractions, and the mixture was left to stand overnight in a refrigerator. During this time, plate-like crystals appeared, which were collected by decantation, and recrystallized. Bio-Gel P-2 column chromatography was found to be very effective for purifying this ribbon worm toxin, increasing the specific toxicity from 99 MU/mg to 3,300 MU/mg. Following recrystallization, the specific toxicity of this toxin was finally increased to 3,520 MU/mg. The combined homogenates had a toxicity of approximately 7,400 MU/g, from which approximately 25 mg of the stratified plate-like crystalline toxin was obtained.

A portion of the toxin was dissolved in a small amount of 1% AcOH, and electrospray ionization mass spectrometry (ESI-MS) was performed using a Hitachi M-1000 mass spectrometer using 50% methanol as the mobile phase with a flow rate of 50 μ l/min. The positive ion mass spectrum was then

measured. The ESI mass spectrum showed an intense ion peak of $(M+H)^+$ at m/z 320 and a weak ion peak of $(M+H - H_2O)^+$ at m/z 302. This mass spectrum agreed well with that of TTX. The molecular weight thus determined was 319, which is in accordance with the reported molecular weight of TTX. In the selected ion-monitored (SIM) mass chromatogram of the trimethylsilyl (TMS) derivatives of alkali-hydrolyzed ribbon worm toxins that were used for gas chromatography-mass spectrometry (GC MS) analysis, mass fragment ion peaks at m/z 376, 392 and 407, appeared at retention times of 8.33 and 8.34 min., which were almost identical to those from the TMS-C9 base derived from authentic TTX.

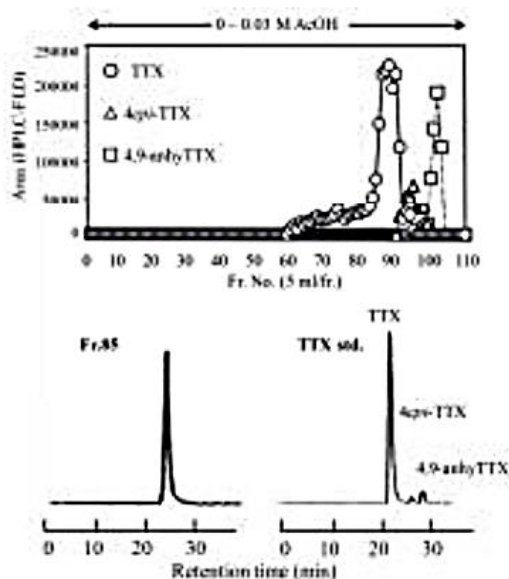


Figure 16. Elution profile of the ribbon worm *C. simula* toxin from a Bio-rex 70 (H^+) column with linear gradient from 0 to 0.03 M AcOH.

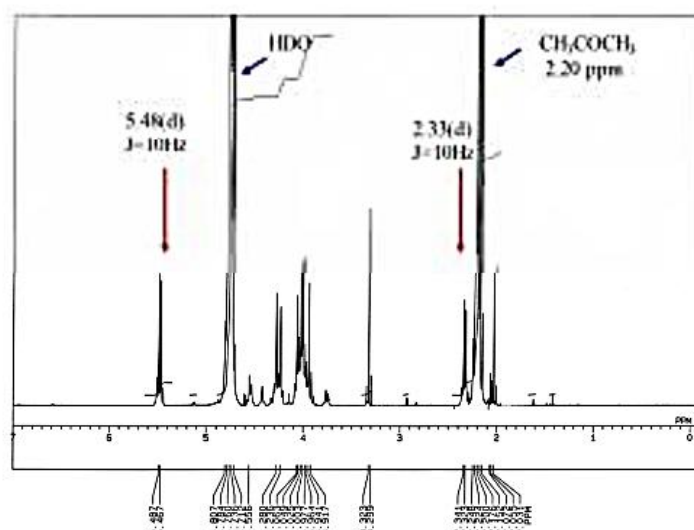


Figure 17. 1H -NMR spectrum of the toxin isolated from the ribbon worm *C. simula*

To examine the 1H -NMR spectrum of the toxin isolated from ribbon worms, 5 mg of the crystals were dissolved in 0.5 ml of 1% CD_3COOD in D_2O , and placed in a test tube. The 1H -NMR spectrum was then obtained with a 500 MHz JEOL JNM-500 spectrometer, using the methyl group proton of

acetone as an internal standard. As can be seen in Figure 17, the ^1H -NMR spectrum of HMT exhibited a singlet at 2.20 ppm (CH_3COCH_3), a doublet centered at 2.33 ppm ($J=10.0$ Hz), a large proton peak at 4.76 ppm (HDO) and a doublet centered at 5.48 ppm ($J=10.0$ Hz). The pair of doublets around 2.33 and 5.48 ppm, which are the hallmarks of TTX and are assigned to H-4a and H-4 respectively, were confirmed to be coupled with each other by double irradiation [42]. In comparison with ^1H -NMR spectral data of the toxin isolated from ribbon worm and the structure of TTX, these results are in agreement with the corresponding data for TTX, with the signals at 4.24, 4.06, 4.28, 3.94, 4.00 and 4.02 ppm are assigned to H-5, H-7, H-8, H-9 and H-11 respectively [43]. These findings demonstrate that TTX is a major component of the paralytic toxins contained in *C. simula*.

What is particularly interesting is how such a large amount of TTX could be concentrated in such a small and simple body with efficiency. Therefore, attempts to clarify this are currently in progress, results from which should be available in near future.

3. Cephalopods

Several species of octopuses secrete a venom from their posterior salivary glands that paralyzes prey organisms.

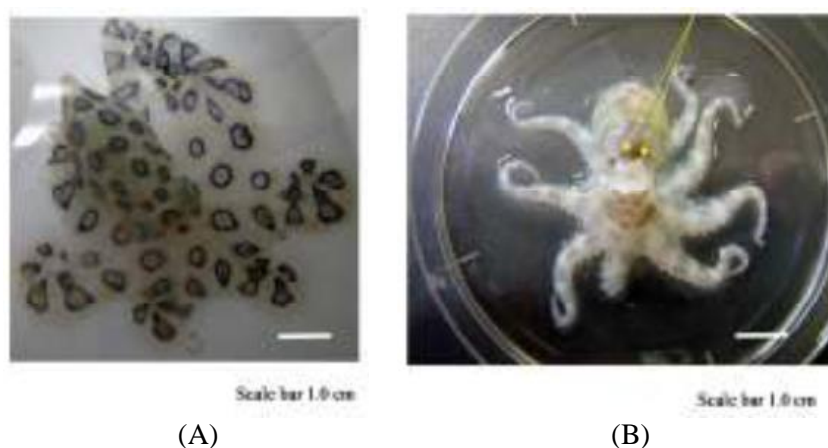


Figure 18. Toxic octopus *Hapalochlaena lunulata* from Ishigaki Island, Okinawa Prefecture, Japan (2015). (A) greater blue-ringed octopus *H. lunulata* (B) posterior salivary gland

Table 2. Toxicity of greater blue-ringed octopus *Hapalochlaena lunulata* from Ishigaki Island, Okinawa Prefecture, Japan

Sample code	N49-43-1		N49-43-1	
Date Collection	Nov, 28, 2015			
Weight of Whole body (g)	9.63		5.24	
Organ	Weight (g)	Toxicity (MU/g)	Weight (g)	Toxicity (MU/g)
Posterior salivary glands	0.37	288.0	0.02	9276.0
Gonad	0.05	52.3	0.03	ND
Hepatopancreas	0.38	58.8	0.17	145.4
Body*	0.52	18.5	0.33	34.5
Arm	3.28	5.3	1.99	9.0

*Posterior salivary glands, gonad, and hepatopancreas are reserved

Bites by these octopuses cause not only pain in the area around the wound, but also occasionally fatal lesions in humans [44]. The blue-lined octopus (*Hapalochaena fasciata*), lesser blue-ringed octopus (*H. maculosa*) and greater blue-ringed octopus (*H. lunulata*) are particularly famous for their toxicity, all of which are distributed in the tropical to subtropical zone. Ishigaki Island, Okinawa

Prefecture, Japan is included in the subtropical zone, and both *H. fasciata* and *H. lunulata* are present in this region. However, information on the toxicity and toxins contained in these two species is limited. A greater blue-ringed octopus collected from Ishigaki Island in November, 2015 is shown in Figure 18. The toxicity of greater blue-ringed octopus *H. lunulata* from this region is provided in Table 2.

Selected ion-monitored liquid chromatography-mass spectrometry (LC-MS) chromatograms of toxin extracted from the posterior salivary glands in greater blue-ringed octopus showed that TTX, 4 ϵ -TTX, 4,9-anhydroTTX and 6 ϵ -TTX were present (data not shown).

4. Conclusion

Based on the findings of the present study and previous studies, the mechanism of TTX accumulation in marine animals is assumed and proposed. It is generally accepted that TTX is produced by some universal organisms such as microbes, based on the observation that it is distributed across a variety of invertebrates and vertebrates, and there are wide individual and regional variations in the content even within the same species.

TTX in toxic pufferfish is not endogenous. Therefore, it must either be derived from the food chain, or, alternatively, may be produced by symbiotic or parasitic bacteria that directly accumulated inside the pufferfish body and not obtained via the food chain (Figure 19).

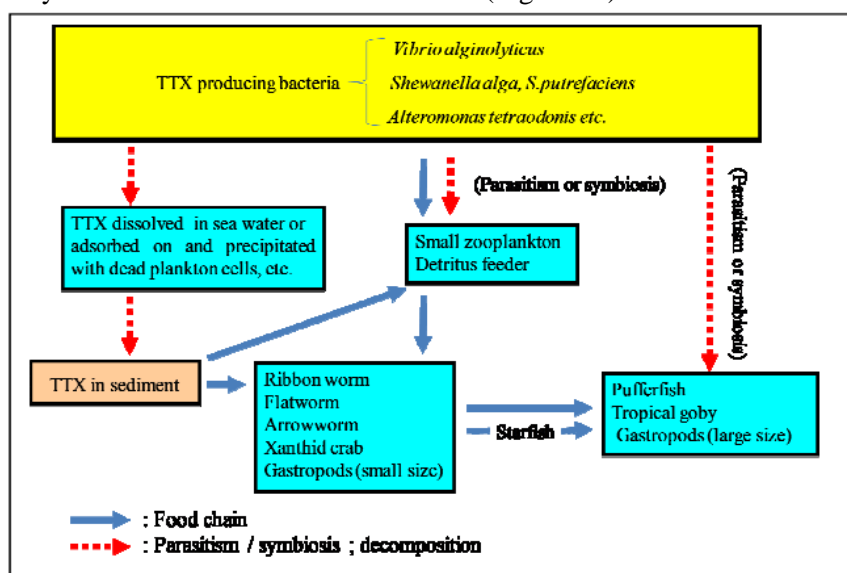


Figure 19. Proposed mechanism of TTX accumulation in marine animals.

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