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Preliminary Study on Larvae Production of Green Spotted Puffer, *Tetraodon nigroviridis*

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Preliminary Study on Larvae Production of Green Spotted Puffer, *Tetraodon nigroviridis*

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This dissertation is submitted in partial fulfillment of the requirement for the degree of Bachelor Science with Honours in Aquatic Resource Science and Management

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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List of Abbreviations

dah: Day after hatch

g: Gram

ml: Milimeter

mm: micrometer

min: Minute

nm: Nanometer

NaOH: Sodium hydroxide

cm: Centimetre

GPS: Global Positioning System

GSP: Green spotted puffer fish

HAS: Heptanesulfonic acid

HPLC: High Performance Liquid Chromatography

TLC: Thin Layer Chromatography

TTX: Tetrodotoxin

SXT: Saxitoxin

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Preliminary Study on Larvae Production of *Tetraodon nigroviridis*

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Abstract

Scientific name for green-spotted puffer is *Tetraodon nigroviridis* and this species belongs to the family of Tetraodontidae. *T. nigroviridis* is distributed along the brackish water, mangrove area, river and flood plains. *T. nigroviridis* is importance in genomic study and also famous as ornamental fish. The morphology of *T. nigroviridis* was examined according to their body weight, standard length and total length. Range of body weight for male *T. nigroviridis* is 74.92±30.1 g and for female is 106.00±36.96 g. In larvae production, *T. nigroviridis* was cultured in the laboratory and the artificial breeding was induced by Ovaprim hormone. Ovaprim hormone was used in ornamental finfish brood stock only and it was injected according to the fish body weight. Chromatography analyses (TLC, HPLC and LC-MS) were carried out to analyse the toxic level in the different tissues of brood stock of *T. nigroviridis.* Among the tissues, egg was found to have the highest concentration of TTX with the result 1330.91 MU/g, followed by intestine and muscle. The baseline data for culturing *T. nigroviridis* in captivity can be used as a guideline for larvae production in the future. On the other side, the TTX data in *T. nigroviridis* tissues could be important information and can be used in order to mitigate puffer fish poisoning cases especially in East Malaysia water.

Keywords: *Tetraodon nigroviridis*, Ovaprim hormone, Tetrodotoxin (TTX)

Abstrak

Nama saintifik bagi ikan buntal hijau ialah Tetraodon nigroviridis dan ikan ini tergolong dalam keluarga Tetraodontidae. T. nigroviridis boleh didapati sepanjang kawasan paya bakau, sungai dan limpahan hujan. T. nigroviridis penting dalam kajian genetik dan juga terkenal sebagai ikan hiasan. Ciri-ciri T. nigroviridis telah dikaji mengikut berat badan, dan panjang keseluruhan badan. Berat badan T. nigroviridis jantan dalam lingkungan 74.92±30.1g dan bagi betina adalah 106.00±36.96 g. Dalam penghasilan larva, T. nigroviridis telah dikultur di makmal dan pesenyawaan artifikal telah dihasilkan dari hormon Ovaprim. Hormon Ovaprim selalu digunakan di kalangan ikan hiasan dan kuantiti hormon yang disuntik berdasarkan berat badan ikan tersebut. Analisis kromatografi (TLC, HPLC and LC-MS) telah dijalankan untuk mengkaji paras toksin di dalam tisu T. nigroviridis yang berbeza. Antara tisu-tisu yang dikaji, telur T. nigroviridis menunjukkan kepekatan TTX yang paling tinggi iaitu 1330.91 MU/g dan di ikuti oleh tisu usus dan tisu badan. Data asas untuk kultur T. nigroviridis boleh di gunakan sebagai panduan untuk menghasilkan lebih banyak larva di masa hadapan. Selain itu, data TTX dalam T. nigroviridis tisu boleh dijadikan sebagai data yang penting dalam usaha untuk mengurangkan kes keracunan ikan buntal di perairan Malaysia Timur.

Kata kunci: Tetraodon nigroviridis, hormon Ovaprim, Toksin(TTX).

1.0 Introduction

Puffer fish are often called as puffers, balloonfish, blowfish, bubblefish, globefish, and swellfish (Torda *et al.,* 1973 cited in Aydin, 2011). Puffer fish come from Tetraodontidae family which their name comes from the Greek word, 'tetra' meaning four and 'odontos' meaning teeth. There are three major types of puffer fish that can be identified around the world which are freshwater, marine and brackish puffer fishes. Puffer fish can be serves as an exotic meal and also as an ornamental fish. Puffer fish is very famous in Sarawak for its meat, egg and also its skin. In Sarawak, there is a festival of puffer fish that has become a tourist attraction. The festival was called 'yellow puffer fish festival' and was celebrated in every year (Azman & Norhana, 2013).

T. nigroviridis are important in the ornamental industry and also in genetic study (Watson *et al.,* 2009; Zauker *et al*., 2014). To produce high amount of *T. nigroviridis* stock for ornamental and genomic analysis, culturing of the fishes were tried in the laboratory and the artificial breeding were induced by the Ovaprim hormone. Watson *et al.,* (2009), demonstrated a simple and reproducible breeding protocol for tertraodon. The suitable water quality measurements are needed to induce the spawning in fish culture. Larvae stages of the teleost life cycle is considerate to be the most sensitive toward environmental impact (Berlinsky *et al*., 2004)

In *T. nigroviridis*, toxicity level is highest under the skin (Noguchi & Arakawa, 2008). Puffer fishes are commonly known of all type of fish poisoning and has been recognized from ancient time (Aydin, 2011). Most of puffer fish species contains tetradotoxin (TTX) and saxitoxin (STX) (Hajeb *et al.,* 2012). The main implication from consume raw puffer fish are having difficulties in breathing, numbness in hand and will lead to death. TTX is very dangerous and until now the antidote still cannot be found (Hajeb *et al.,* 2012). To examine

and identify the level of toxicity, High Performance Liquid Chromathography (HPLC) and Thin Layer Chromatography (TLC) were used. But before that the samples must be extracted to continue with the HPLC and TLC. HPLC and LC-MS are among the most powerful and sensitive tool for determination of TTX (Azman *et al.*, 2014)

However, there are very limited research reports about larvae production in species of *T. nigroviridis*. Baseline data in culturing *T. nigroviridis* in captivity can give some important information to produce a large stock of the fish. A proper way of artificial breeding can be a guideline for many culturists in the future. Although lots of report in puffer fish poisonous, local people still not aware of the dangerous toxin and still consume on the puffer fish. The report about toxicity level of *T. nigroviridis* can give benefit to local people, government, Ministry of Health and also to the public.

Therefore, the objectives for this study were:

1) to examine the suitable condition for culturing brood stock of *T. nigroviridis* for larvae production.

2) to document the embryonic and larvae development of *T. nigroviridis.*

3) to screen toxin properties using TLC, HPLC and LC-MS

2.0 Literature Review

2.1 Green-spotted Puffer, *Tetraodon Nigroviridis*

2.1.1 Taxonomy, Morphology and Distribution

Kingdom: Animalia

Phylum: Chordata

Class: Actinopterygii

Order: Tetraodontiformes

Family: Tetraodontidae

Genus: *Tetraodon*

Species: *Tetraodon nigroviridis*

Puffer fish (Family Tetraodontidae) comprise approximately 120 species, many of which produce the potent neurotoxin tetrodotoxin (Zakon *et al*., 2009). The morphological characteristics of puffer fish are, they have a variety of colour, have bulging eyes and also has an elongated snout. *T. nigroviridis* also known as 'Buntal Hijau' or 'Buntal Bako' (Atack, 2006) because of their green body colour. They also distribute abundantly in mangrove area at Kampung Bako, Sarawak. In adult GSP fish, the top of their body is rich in green colour with dark spotted while under the body (belly) the colour is whitish. For the juvenile, the green colour is not darkens as adult. Male of *T. nigroviridis* have brighter colour than female while female is less green and more brownish.

T. nigroviridis is a very sensitive fish and they were influence by their surrounding environment. They will change their body colour to black or dark green if there were under stress condition. They have no desire to eat, and only swim at the bottom of the river bed. The result from this stress condition are they will not growth, breed, and will lead to death if not

consume food for one week. *T. nigroviridis* is an omnivorous which means they eat plant and also animal. Example, they feed on algae on rock or on coral. In addition, they also ingest small invertebrate and shrimps. They need to be feed snails/ shells to prevent their teeth grow too large.

The unique characteristic of puffer fish is they can expand their body quickly when they facing danger. They are named after their habit of inflating themselves with water or air when threatened, making it difficult for a predator to swallow them (Aydin, 2011). The spikes will cover their body for protection from the prey. The spines were covered around the bottom body and it gives difficulty to the predator from eating them. Their body will inflate bigger than their usual size when they inhale the air. When they inhale air, they will produce some sound to scare the predator.

Puffer fish is a widespread species in the world. They can be found in South and Southeast Asia from Sri Lanka to Indonesia to China and also in the Phillippines (Rainboth 1996; Froese and Pauly 2008 cited in Watson *et al.*, 2009). The habitat for green spotted puffer fish is at brackish water which is near to coastal region and mangrove ecosystem.

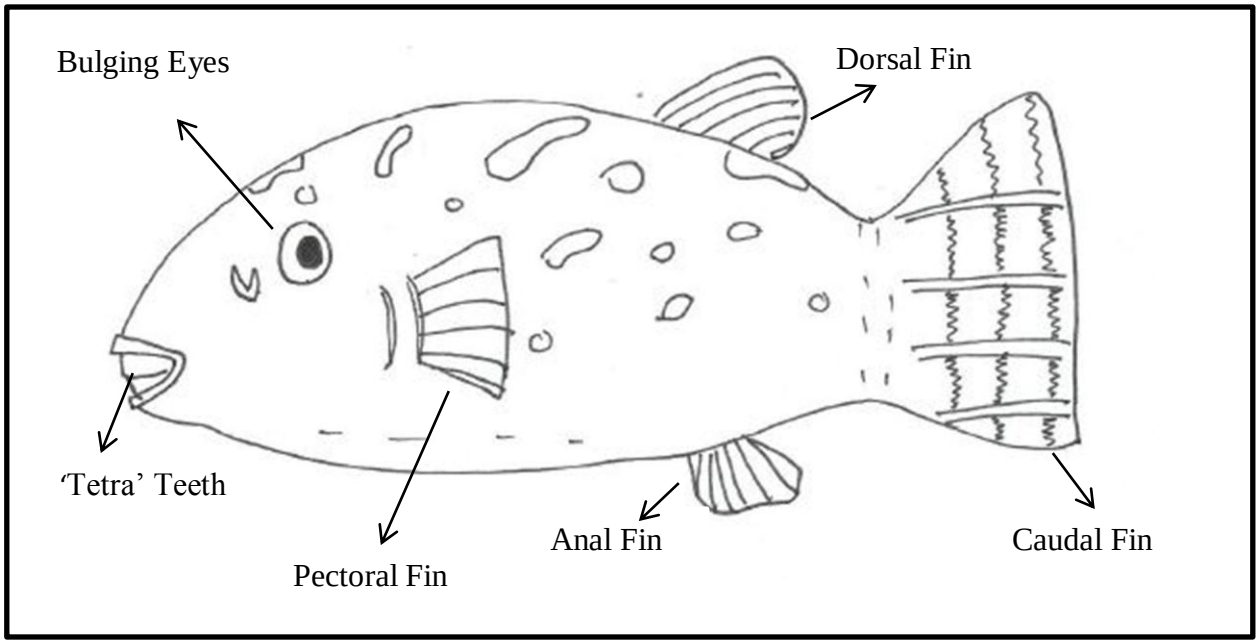

Figure 1: Puffer Fish Morphology

T. nigroviridis has unique reproduction method. Female *T. nigroviridis* will lay eggs at the shore after the male have pushed them to a certain place. Spawning will occur in brackish water (Watson *et al.*, 2009). The larvae were covered with shell and within 3-4 days it will hatch. After a few days the shell will break and the larvae will develops fins, teeth and all necessary part. Puffers are missing the two pelvic fins that on ordinary fish would be located on their belly directly underneath their pectoral fins.

2.1.2 Ecological Importance and Commercial Potential of Green- spotted Puffer

Puffer fish is important as food sources in Japan, Korea, Bangladesh and United State. Local people in Sarawak also consume *X. naritus* and famous through the 'yellow puffer fish festival'. According to Chulanetra *et al.* (2011), puffer meat is relatively cheap therefore it was mixed with fish flesh for making fish balls. In addition, some open grill restaurant called 'Moo-Kata' which is recently become popular in Thailand for selling puffer flesh. There are lots of food poisoning due to the mainly ingestion of the puffer fish meat, liver, and the eggs

(Noguchi & Arakawa, 2008). In Taiwan, to keep the seafood (puffer fish) safe to eat is by examined the puffer fish properly and removed the toxin by expert chef (Ha & Sato, 2011).

Beside, puffer fish is important in food web and food chain. *T. nigroviridis* is an omnivorous animals and its will maintain the estuarine ecosystem by control the population of snail, gastropods, crustaceans, brine shrimp and also small fishes. Because of *T. nigroviridis* is cheap, easily can be obtained and small in size, they widely being used for research purpose. Many research about genome analysis, toxin contains (TTX) and osmoregulation behavior studies have been done (Watson *et al.,* 2009; Zaucker *et al.,* 2014). Mention by Tang and Lee (2011), *T. nigroviridis* are widely used in studies of fish osmoregulation. *T. nigroviridis* is chosen as the experimental model for osmoregulation because it can tolerate wide variation in water (Pinto *et al.,* 2010) Tetrodotoxin (TTX) and saxitoxin (STX) were detected in the most puffer fish species (Azman and Norhana, 2013).

2.1.3 Pufferfish Aquaculture

2.1.3.1 Environmental Condition for Culturing

Fish were being cultured because of their protein sources, research development and also for ornamental purpose. For culturing a fish, an excellent condition must be prepared. To have a good result in culturing, the artificial habitat must be prepared as their real habitat. All the water parameter like salinity, pH, temperature, dissolved oxygen and hardness of water must be exactly same as their former habitat. These factors will influence the growth, reproduction and survival rate of the fish in culturing system.

Temperature is one of the most important environmental variables for all aquatic organisms. It influences the oxygen content of the water, the primary product which is the source of food in the water body and the reproduction and growth of all species (Barnabe, 1990). Range suitable

temperature in culturing a fish is 25-29°C. Temperature increase in water body can cause the metabolic rate of aquatic organisms increase thus they need a double energy requirement. Temperature, in association with other physical and chemical parameters such as salinity, plays a role in the progress of the reproductive cycle from gametogenesis to the survival of the larval stages (Barnabe, 1990). Too high of temperature will hinder the fish to reproduce and growth to a maximum size.

Tetraodon nigroviridis is a brackish fish and can survive in freshwater and also in saltwater (low concentration of salt) but to breed the fish, saltwater (brackish water) with 17-20 ppt is needed (Watson *et al.*, 2009). *T. nigroviridis* is a euryhaline which means can tolerate with wide differences of salinity. However they only breed and reproduce in saltwater. In general, salinity has an effect on reproduction (gametogenesis and tolerance of larvae to euryhaline conditions), nutrition and growth of aquatic organisms (Barnabe, 1990). pH level that suitable for culturing *T. nigroviridis* is in range of 6-8. Too acidic and too basic water body will result in death. Most species can tolerate a variation between pH 6 and pH 9 which is a far wider range than that encountered in their normal environment.

According to Barnabe (1990), dissolved oxygen is essential for respiration, helps in breakdown of organic detritus, and enables the completion of biochemical pathways. Dissolved oxygen present in the water result from the exchange between atmosphere gas with water surface. To accelerate the production of dissolved oxygen is by using aeration system in the culturing aquarium or pond. Aeration is easily the most widely used water treatment employed in aquaculture (Barnabe,1990). Decreasing in dissolved oxygen in water can lead to hypoxia and lead to the death of the fish.

2.1.3.2 Feeding

In culturing a fish, the other most important things are their feeding behavior during their different stages in life-cycle. Different species of puffer will have different type of feeding. Some of them are omnivores while others are carnivores. *Tetraodon* puffer have some unique characteristic which their teeth will growth fast and it will give difficulties to them to eat. They need some hard food like gatropodes, bivalves and other shell food to inhibit their teeth growth fast.

For larvae puffer, they mostly consume small organism like *Artemia* sp and microalgae. The reason they feed on small organisms because of their teeth and mouth not fully develops. *Artemia* sp can be produced easily, cheap, long lasting and suitable to most animals. According to Barnabe (1990) *Artemia* sp are an excellent food for fry in the development of aquaculture. Other than *Artemia* sp, puffer larvae also can be feed on microalgae. Microalgae also easy to been produce like *Artemia* sp.

For adult puffer they were preferred worm, snail, shrimp and other types of gastropode. For example, *T. nigroviridis* mostly consume shrimp and worm in culture pond or in aquarium. The feed no need to mince into small pieces because the adult puffer can chew the large food by themselves. For juvenile puffer, the food need to mince into small portion for them easier to eat and prevent them from chocked when eating.

2.1.3.3 Reproduction and Artificial Breeding in Captivity

T. nigroviridis is importance as genetics model animal due to its small, well-mapped genome and because of their beautiful body pattern make them a popular aquarium fish (Watson *et al*., 2009). Because of information about reproduction *T. nigroviridis* in nature habitat is lacking, some of researchers try to manipulate artificial breeding in aquarium. According to Riehl and Baensh (1991) in Watson *et al*, (2009) said that spawning in aquaria reportedly occurred in brackish water, the eggs were laid on the substrate and the male guarded the larvae. Unfortunately, if reproduce of *T. nigroviridis* in a nature way it will consume a lot of time. To accelerate the spawning and breeding of *T. nigroviridis*, artificial breeding (hormone injection) can be done.

Many types of hormone can be induced the artificial breeding in fish culture like example is Chorulan (human chorionic gonadotropin), ovaprim and LHRH-a (analogues of luteinizing hormone-releasing hormone). According to Watson *et al.* (2009) and Zaucker *et al*, (2014), *T. nigroviridis* was injected with Chorulan hormone in their induced spawning technique. Amount of hormone injected into fish body were determine by their body size (weight). The heavier the sample, the higher the dosage of hormone injected. Around 36 hours later the female and male fish were stripped their stomach for eggs and milt. The eggs and milt (sperm) of fish will breed outside female body by mixed them together in a brackish water body. All the water parameters need to be control for the successful artificial breeding of *T. nigroviridis.*

2.1.4 Gonadosomatic Index (GSI)

Gonadosomatic index(GSI) is the calculation of the gonad mass as a proportion of the total body mass. GSI always been used to measure the condition of fishes in term of maturity and stress. GSI value can determined and identify the right time for spawning season in the fish. Apart from that, GSI gives an indication of the percentage of the fish weight that was used in egg production at the maturation stage (when the eggs were shed) (Nzeh & Lawal, 2012). The value of GSI affected by many factor such as food availability, physical factors and the physiology of fish including its gonad maturity stage (Kreiner *et al.*, 2001).

According to the Nzeh and Lawal (2012), in fish, females had higher GSI than males indicating that more percentage of the weight of the females was used in ovary maturation. Female were seen to be more sensitive than males when it comes to the GSI index (Sadekarpawar & Parikh, 2013). However in male tilapia species, the GSI was very low during immature stages but reached a maximum high value during mature stage (Bhatta *et al.*, 2012). For family Mormyridae, female had higher GSI than male indicating that more percentages of the weight of the females were used in ovary maturation (Nzeh & Lawal, 2012). The value of GSI index is different depends on the species.

2.2 Tetrodotoxin (TTX)

2.2.1 Chemical Structure of TTX

TTX is a potent neurotoxin of low molecular weight that usually distributed in puffer fish of the family Tetraodontidae and is believed to be originated from bacteria of the genera *Vibrio*, *Pseudoalteromonas* and others (Yasumoto, 2005). The structure of TTX is shown in Figure 2 (Noguchi & Arakawa, 2008).

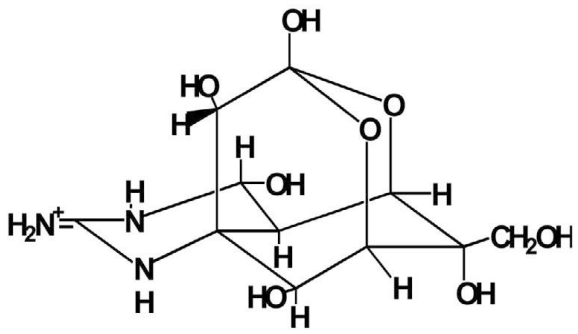

Figure 2: Chemical structure of TTX. Adopted from Noguchi & Arakawa (2008).

At this time, more than ten variants of TTX have been identified from a wide range of organisms including xanthid crabs, horseshoe crabs, frogs, and newts in addition to puffer (Miyazawa & Noguchi, 2001). Not all types of puffer fish contain TTX, example freshwater puffer fish contained saxitoxins(STX) rather than tetrodotoxins. Distribution of TTX in puffer fish bodies appears to be species-specific (Noguchi *et al.*, 2006). Zakon *et al.* (2009) mentioned, TTX is not sequestered within a gland but is accumulated in various body tissues. For example mentioned by Noguchi *et al*. (2006), Thai/Bangladeshi brackish water puffers exhibit the highest toxicity in the skin. Female puffer fish are more toxic compare to the male puffer fish as they accumulated the TTX in the ovaries and eggs during the spawning period (Bane *et al.*, 2014).

Puffer fish were thought to accumulate TTX through the food chain, which start from marine bacteria *Vibrio alginolyticus*, *Shewanella sp*., *S. putrefaciens*, *Alteromonas tetraodonis* and others (Matsui *et al*., 1990; Yasumoto *et al*., 1986 in Azman *et al.*, 2014). Proposed mechanism of TTX accumulate in marine animal is shown as Figure 4 below. TTX is selectively binds to voltage-gated sodium channel (NA_v). Binding of TTX to NA_v channels in muscle and nerve tissues causes paralysis and result from respiratory failure (Soong & Vankatesh, 2006). TTX is very stable structure and it can stand high temperature and also weak acid environment. Arakawa *et al*. (2010) mentioned that the toxin is stable in neutral to weakly acidic solution and does not decompose by cooking (i.e., the application of heat).

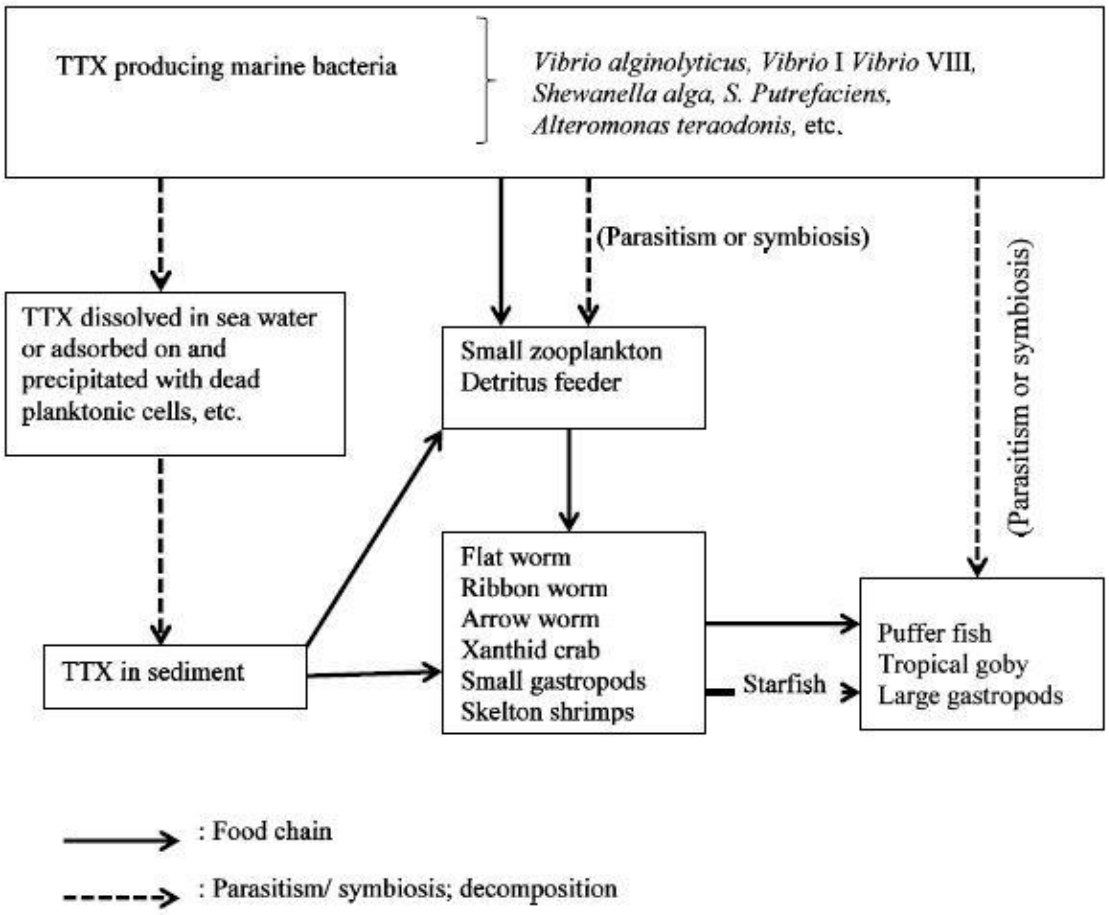

Figure 3: Proposed mechanism of TTX accumulation in marine animal (Bane *et al*., 2014)

2.2.2 TTX Poisoning Cases Related to Pufferfish

In Japan, over the past 13 years (1995-2007), mortality due to puffer fish poisoning has been 6.4% (Table 1).

Table 1: Puffer fish poisoning incidents in Japan (Taken from Noguchi & Arakawa, 2008)

Year	Number of incidents	Number of patients	Number of deaths	Mortality (%)
1965	106	152	88	57.9
1970	46	73	33	45.2
1975	52	75	30	40.0
1980	46	90	15	16.7
1985	30	41	9	22.0
1990	33	55	1	1.8
1995	30	42	2	4.8
1996	21	34	3	8.8
1997	28	44	6	13.6
1998	27	39	4	10.3
1999	20	34	2	5.9
2000	29	40	0	0.0
2001	31	52	3	5.8
2002	32	49	5	10.2
2003	28	35	3	8.6
2004	43	58	2	3.4
2005	40	49	2	4.1
2006	25	32	1	3.1
2007	24	38	2	5.3

As mention in Noguchi and Arakawa (2008) paper research, a 48-year-old man in Nagasaki, Nagasaki Prefecture, caught a wild marine puffer fish *Takifugu poecilonotus*, and ate more than four slices of slightly cooked ‘kimo’ with some flesh in the evening and after 30 to 60 minutes after ingestion, he began to suffer from numbness in his hand and limbs, followed by cyanosis and respiratory failure during the next 60 minutes. Although immediately hospitalized, he died during the following hour.

58 cases for TTX poisoning were reported in Taiwan from 1988 to 2011, and resulted in 192 people intoxicated and 22 deaths (Table 2) (Lin & Hwang, 2012).