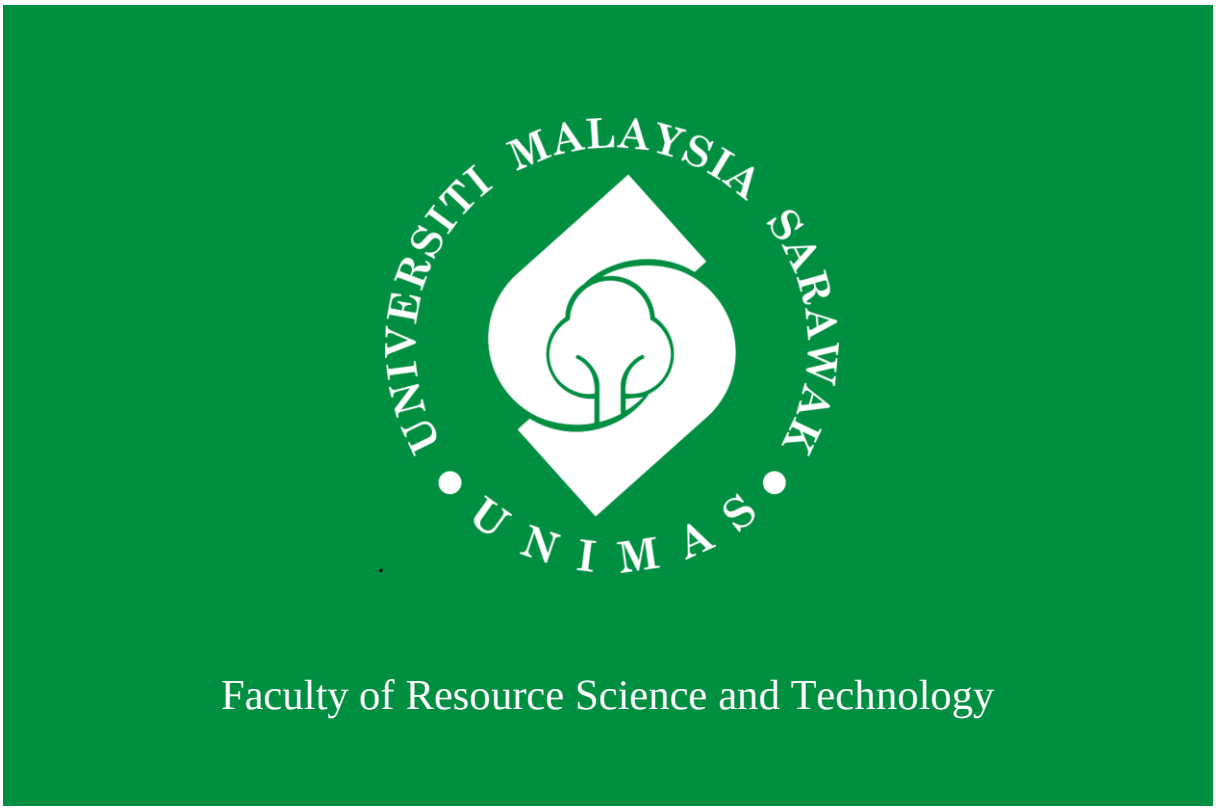

MORPHOMETRIC STUDY AND TOXICITY ASSESSMENT FOR HORSESHOE CRAB COLLECTED FROM KAMPUNG PASIR PUTIH, SARAWAK, MALAYSIA

ER HUEY HUI

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Morphometric Study and Toxicity Assessment for Horseshoe Crab Collected From Kampung Pasir Putih, Sarawak, Malaysia

Er Huey Hui

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

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University Malaysia Sarawak

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DECLARATION

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

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Er Huey Hui

Aquatic Resource Science and Management

Department of Aquatic Science

Faculty of Resource Science and Technology

University Malaysia Sarawak

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List of Abbreviations

HPLC	High Performance Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectrometry
LAL	Limulus Amoebocyte Lysate
MU	Mouse Unit
PSP	Paralytic Shellfish Poisoning
TLC	Thin Layer Chromatography
TTX	Tetrodotoxin

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Er Huey Hui

Aquatic Resource Science and Management
Faculty of Resource Science and Technology
University Malaysia Sarawak

ABSTRACT

In Malaysia, horseshoe crabs encountered the decreases in population, which might due to the anthropogenic factor. Hence, this study was carried out to study the morphometric characteristic and to assess the toxicity of horseshoe crabs collected by local fishermen at 2014 and 2015 from Kampung Pasir Putih, Sarawak, Malaysia. The morphometric and allometric analysis was used to study the morphometric variability between the sexes of horseshoe crabs. All the samples collected were confirmed to be *Tachypleus gigas*. The body weight of both sex had increased at 2015 as compared to 2014. All parameters in females were recorded high especially body weight as compared to the males. The mean body weight of females were 787.22±93.73 g and males were 298.71±29.58 g. Allometric analysis shown that the specimens collected for both sexes have a high degree of correlation between total length and body weight. However, the b values (female=0.1813; male=0.1961) shown a negative allometric growth characteristic. Due to the frequent occurrence of food poisoning after consuming horseshoe crab, toxicity analysis was carried out in this study. For toxin analyses, thin layer chromatography, high performance liquid chromatography, and liquid chromatography mass spectrometer were performed. TLC was used as a screening for TTX, HPLC was to confirm for the presence of TTX and LCMS/MS was used to quantify the amount of TTX presence. TTX was most frequent detected in the eggs with the toxicity range from 3.53 MU/g to 6.5 MU/g. In viscera caecum and soft tissue, the toxic amount range from 1.22 MU/g to 6.81 MU/g and 1.96 MU/g to 6.15 MU/g respectively. This study was inferred that the horseshoe crab *T. gigas* collected are non-toxic (less than 10 MU) and safe to be consumed.

Keywords: Horseshoe crab, *T.* gigas, Morphometric study, Allometric analysis, Tetrodotoxin

ABSTRAK

Di Malaysia, belangkas menghadapi penurunan dalam bilangan populasi yang mungkin disebabkan oleh faktor antropogenik. Kajian ini dijalankan bagi mengkaji ciri-ciri morfometrik dan menilai ketoksikan belangkas yang dikutip oleh nelayan tempatan pada 2014 dan 2015 dari Kampung Pasir Putih, Sarawak, Malaysia. Morfometrik and alometri analisis telah digunakan untuk mengkaji perbezaan ciri-ciri morfometrik antara jantan dan betina belangkas. Semua sampel yang dikutip telah dikenalpasti sebagai Tachypleus gigas*. Berat badan kedua-dua jantina telah meningkat pada tahun 2015 berbanding dengan 2014. Bagi betina, semua parameter direkod tinggi terutamanya badan berat jika berbanding dengan jantan. Purata berat badan betina ialah 787.22 g dan jantan ialah 298.71 g. Analisis alometri menunjukkan bahawa sampel yang dikumpul bagi kedua-dua jantina menpunyai tahap korelasi antara jumlah panjang and berat badan yang tinggi. Walau bagaimanapun, nilai-nilai b (betina=0.1813; jantan= 0.1961) menunjukkan ciri-ciri pertumbuhan negatif alometri. Disebabkan kejadian keracunan makanan kerap berlaku selepas makan belangkas, analisis ketoksikan telah dijalankan dalam kajian ini. Bagi analisis toksin, TLC, HPLC dan LCMS/MS telah diguna untuk mengesan Tetrodotoksin (TTX). TTX adalah paling kerap dikesan dalam telur dengan quantiti antara 3.53 MU hingga 6.5 MU. Dalam viscera caecum dan tisu lembut, toksik tersebut adalah di antara 1.22 MU hingga 6.81 MU dan 1.96 MU hingga 6.15 MU masing-masing. Kajian ini telah menunjukkan bahawa* T. gigas *yang dikumpul adalah tidak bertoksik (kurang daripada 10 MU).*

Kata kunci: *Belangkas,* T. gigas*, morfometrik, analisis allometrik, Tetrodotoksin (TTX)*

1.0 Introduction

Horseshoe crabs are the oldest living marine creature that normally known as "living fossil", it is because after a span of 350 million years this animal has not shown any significant phenotypic change (Chatterji & Pati, 2014). However, there are only four species in this world. They are *Limulus polyphemus* (Linnaeus, 1758), *Tachypleus gigas* (Muller, 1758), *Tachypleus tridentatus* (Leach, 1819) and *Carcinoscorpius rotundicauda* (Latreille, 1802) (Walls *et al*., 2002).

Horseshoe crabs are very important either towards the ecosystem or towards the economy. Ecologically, their eggs are important food source for the migrating shorebird. These birds depend on horseshoe crab eggs to replenish and reserve the fat (Botton, 2009). Hence, the number of the shorebirds is highly influence by the population of the horseshoe crabs (Tsipoura & Burger, 1999; Walls *et al*., 2002). Besides that, the host of invertebrate and some fish species also feed on the eggs and larvae. Economically, horseshoe crabs also act as the food source for human or being used by fisherman as a fish bait to lure conch and eels. In addition, horseshoe crabs' blue blood that contains Limulus Amoebocyte Lysate (LAL) is valuable and can be used for detection of endotoxin in production of biomedical devices (Botton *et al*., 2013). However, the number of horseshoe crabs are now declining because of human disturbances have been attributed to the degradation and destruction of horseshoe crab's habitat (Chatterji & Pati, 2014).

In other aspect, since 1925, several cases of food poisoning reported in Thailand due to consume of horseshoe crab, especially the eggs (Kungsuwan *et al*., 1987). In addition, within year 1994 to 2006, medical service of Chon Buri Hospital receive a total of 280 cases with different severity of tetrodotoxin (TTX) poisoning which cause by the ingestion of the toxic

eggs of horseshoe crab, *C. rotundicauda* (Kanchanapongkul, 2008). TTX is a highly potent toxin when ingested, it inhibits nerve and muscle conduction by selectively binding to sodium channels and impair its to function (Wood *et al*., 2012, Bane *et al*., 2014). In Thailand, Bangladesh, Cambodia and Vietnam, the causative species and the toxins have been characterized as *C. rotundicauda* and TTX is the major toxin in eggs (Kungsuwan *et al*., 1987; Tanu & Noguchi, 1999; Ngy *et al*., 2007; Kanchanapongkul, 2008; Dao *et al*., 2009). The symptoms occur after consume TTX-bearer crab are similar to those paralytic shellfish poisoning (PSP) (Kungsuwan *et al.*, 1987). According to Dao *et al*. (2009), although PSP toxins were also detected in all horseshoe crabs specimen, but the levels were low. In the study, they identified that TTX is the major toxin responsible for the food poisoning after ingestion of *C. rotundicauda* in Vietnam.

In Malaysia, the public is still unaware of the important of horseshoe crab toward ecosystem and economic aspect. On top of that, the consumer are unaware of the potential danger from eating toxic bearer horseshoe crab. There are many local people consumed horseshoe crab especially their eggs and meat, however there were very limited study on the toxicity of horseshoe crab. Hence, this study can create awareness to local people and it also useful for government to prevent extinction of these creatures and prevent food poisoning in our country.

Therefore, the objectives of this study are to: 1) study the morphometric variability and carry out allometric analysis for both sexes of horseshoe crab *T. gigas;* 2) identify the toxic and assess the toxic level of different body part of horseshoe crab *T. gigas* by using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) methods, and Liquid chromatography-mass spectrometry/ mass spectrometry (LC-MS/MS).

2.0 Literature Review

2.1 Horseshoe crab

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Chelicerata

Class: Merostomata

Order: Xiphosura

Family: Limulidae

Genera: *Carcinoscorpius*,

Limulus,

Tachypleus

Phylum Arthropods are animals with an articulated body and limbs. There are 3 major classes under phylum Arthropods which are Arachnids, Crustaceans, and Insects. However, horseshoe crab does not belong to any of the classes mentioned above but they belong to their own class known as Merostomata that means, "The legs attached to the mouth". Although they have the name "crab", but they are marine arthropods which are closely related to spiders and scorpions (Cotner & Moore, 2011).

As stated in Botton (2009) study, horseshoe crabs play an important role in estuarine and coastal communities. They act as predator, prey and hosts for epibionts. Adult horseshoe crabs classified as omnivorous which feed on several species of benthic invertebrates. These benthic invertebrates included bivalves, crustaceans, gastropod and polychaetes (Walls *et al*., 2002). Horseshoe crabs face many challenges throughout their lifecycle. During the embryo stage in the eggs, they are heavily consumed by the migratory shorebirds. This is important because without the sufficient supply of horseshoe crabs' eggs, the shorebirds would not

have enough energy to survive the long distance of migration. Some of the fishes like eels, catfish and crustaceans like sand shrimp will also feed on the eggs and larvae stage horseshoe crab. In addition, adult horseshoe crabs are one of the food sources for loggerhead turtles. Sometime, horseshoe crabs' carapaces will be used by the encrusting invertebrates and algae as a substrate to stay on it. The example of epibionts is barnacles, tube-building polychaetes, oysters and slipper limpets (Fish and Wildlife Research Institute, 2007).

In term of economically importance, horseshoe crabs were commercially harvested during 20th century as fertilizer for crops, livestock feed for chickens, hogs and bait (especially female horseshoe crab) for eel fisheries. Moreover, horseshoe crabs have high demand because of its blue blood that contained LAL. Their compound eyes are also important to the biomedical research. Horseshoe crabs have the largest photoreceptor that can be seen by the naked eyes and this is used to study and understand how human eyes function (Fish and Wildlife Research Institute, 2007). In Malaysia, horseshoe crabs also consumed as a food by local people at Johor, Sabah and Sarawak. They normally consume the horseshoe crab's soft muscle and eggs.

2.2 Horseshoe Crab's Blood

Unlike human, horseshoe crab use hemocyanin to carry oxygen instead of haemoglobin. Hemocyanin that contains copper makes the horseshoe crab's blood appear in blue color (Markl, 1986). According to Walls *et al*., (2002), their blood contains amebocytes that have the same function as white blood cells of vertebrates to defend themselves against pathogen. In biomedical industry, the amebocytes in the blood are separated and lysed in distilled water to produce LAL.

LAL is very useful to detect the endotoxins presence in the biological, pharmaceutical drugs and medical devices that can cause fever. In addition, it is also a crucial tool to control the presence of endotoxin during the process and the equipment used to produce pharmaceuticals (Novitsky, 2009). The level of the endotoxin can be detected within 1 hour. Endotoxins are large molecular weight complex lipopolysaccharides that can be found in the cell wall components of all Gram-negative bacteria (Silveira *et al*., 2004). Walls *et al*. (2002) mentioned that, there is vast number of endotoxin present in the horseshoe crab habitat. Hence, this make horseshoe crab evolved this important system to protect itself from the bacterial. When amebocytes exposed to endotoxin, it will change shape, attach to the sides of the vascular channels and finally form a gel clot (Joiner *et al*., 2002).

According to PBS (2008), in pharmaceutical LAL test used as a substitute to current methods for endotoxin test after identified by Food and Drug Administrations. Today, LAL test has become the standard screening test for the presence of bacterial. Hence, every drug and surgical implants (pacemakers and prosthtic devices) certified by the FDA must go through the LAL test. On the world market, the overall revenues are around U.S. $ 50 million from the LAL industry and around $15000 is needed for a quart of horseshoe crab blood.

2.3 Distribution of Horseshoe Crabs

Horseshoe crab is an invertebrate with a hard-shell that are living on the sea floor in warmer climates. Through geologic record, these creatures are always been shallow water animals (Sekiguchi & Shuster, 2009). They are the benthos that carries out their biogenic activity at the calm sea or estuary and muddy areas (Samsur & Nur Izzatie, 2011). The *L. polyphemus* species can only be found at North America along the Atlantic coastline (Walls *et al*., 2002). Meanwhile the other three species of horseshoe crabs *T.gigas, T. tridentatus*

and *C. rotundicauda* are found in Asia, that are from India to Japan and south of Malaysia and Indonesia (Chatterji & Abidi, 1993; Cartwright-Taylor *et al*., 2011; Botton *et al*., 2013). However, *T. tridentatus* are restricted at East Malaysia (John *et al*., 2012). *T. gigas* that known as coastal horseshoe crab found mainly in coastal habitat including East and West of Peninsular Malaysia. At Peninsular Malaysia, the nesting of horseshoe crab *T. gigas* can be found throughout the year especially in East coast like Balok and Pekan (Zaleha *et al*., 2012). According to Cartwright-Taylor *et al*. (2011), they are two species of horseshoe crab living at sandy to muddy habitats they are Asian horseshoe crab *T. tridentatus* and the coastal horseshoe crab *T. gigas.*

Meanwhile, *C. rotundicauda* are commonly found in muddy area in mangrove at Sarawak, it also known as mangrove horseshoe crab or "belangkas ranggar" or "balangkas padi" by the local people (Samsur & Nur Izzatie, 2011). This species can only be found at Johor, Selangor, Terengganu, Sabah, and Sarawak in Malaysia.

At night-time, horseshoe crabs are commonly found migrate towards the nesting site to spawn during high tide (Zaleha *et al*., 2012). The spawning activity of horseshoe crabs is high during full and new moon season (Jennifer *et al*., 2010; Zaleha *et al*., 2012). According to Brockmann (1990), male horseshoe crabs usually have more number than the female horseshoe crab on spawning beaches, this will create a male-biased sex ratios and intra-competition between male for mates.

2.4 Morphology of Horseshoe Crabs

Horseshoe crabs have an arc shaped carapace or exoskeleton that protect their organ, legs and keeps them upright in rough water. Their body is divided into 3 sections that are cephalothorax (prosoma), abdomen (opisthosoma) and telson as shown in Figure 1.

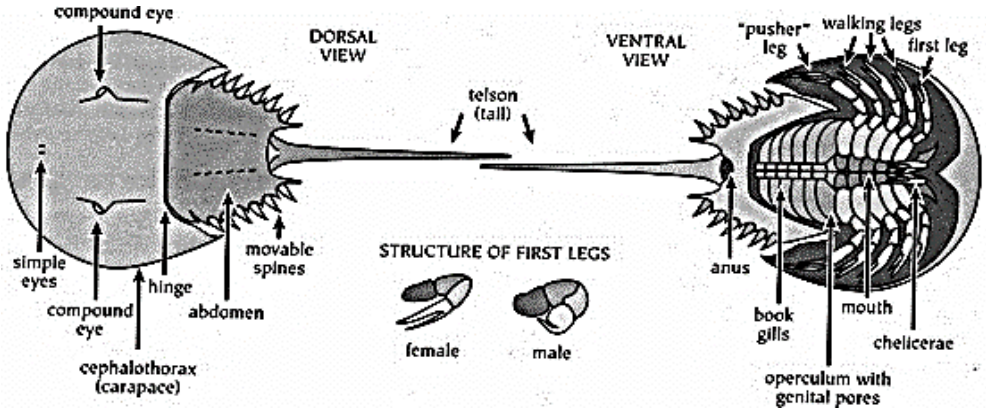

Figure 1. The physical structure of horseshoe crabs.
(Source: Fish and Wildlife Research Institute 2007)

The cephalothorax (prosoma) is the largest section that has several eyes on it. Mouth located at the ventral side of the prosoma with a pair of feeding pincers and five pairs of legs around it. Feeding pincers also known as chelicerae that used to pick up the food and pass to mouth. Abdomen (opisthosoma) found at the second body section looks like a triangle with moveable spines on the sides and a ridge in the centre. It contains five sets of book gills that have a flap-like structure that allow the horseshoe crab to breath under water. The movement of the book gills can keep the flowing of oxygenated water around them and it is used as paddles during swimming. The third body section is a long and pointed shape tail that called telson. A ball and socket joint connected between the abdomen and the telson allows the tail to move in different direction. The function of the telson is to return their body into the right position when it overturned unintentionally on the beaches (Fish and Wildlife Research Institute, 2007).

Horseshoe crabs have 10 eyes that used to detect light and to locate mate. They have the largest photoreceptor in the animal kingdom (Barlow, 2009). According to Fish and Wildlife Research Institute (2007), the two large compound lateral eyes that found on the carapace are used to detect the movement and locate mate. In addition, there are five more eyes located on the top of it prosoma, 2 eyes at the ventral side that near the mouth, and the last eye is the multiple photoreceptors on the telson. Among the three eyes that near the front of the prosoma, two are median eyes and one of it is endoparietal eye. The function of these eyes is to detect the sunlight and moonlight. This is important because this help them to follow the lunar cycle for spawning (Barlow, 2009).

Some of the morphometric characteristic proposed by Walls *et al.* (2002) were used to differentiate between the male and female horseshoe crab. The most obvious feature is that females are about 1/3 bigger than the males. Moreover, the first pair walking legs of the male crab is structurally different from female. Male crab have a modified hook-like structure known as pedipalps (Figure 1) that can be used to grasp onto females' terminal spines from water to spawning beaches (Walls *et al.*, 2002).

Young adult, middle-aged adults, and old aged adults are commonly used to describe the age of horseshoe crab. Young adults normally had a burnish carapace with few or no scratches or epibionts attach on it. The degenerated unmoveable chela of horseshoe crab that will break off after first mate can be used to identify a virgin male. On the same time, the immaculate shell with no mating scars form can make sure that it is a virgin female. Middle-aged adults will have an eroded carapace that started to expose the shell layer that is in black colour. More epibionts can be found on the surface of carapace. Meantime, carapace of female horseshoe crab will have the mating scar with the pressure spot cause by the male horseshoe crab during spawning. For the old-aged adults, the carapace is almost fully

blackened. In some cases, the carapace faces the severe erosion and causes the brownish-coloured layer to be exposed. The carapace become thinner and easier to depress compare to the young horseshoe crab. On the surface of carapace, epibionts are always present and most of the time they are grown to large size (Walls *et al*., 2002).

Table 1 shows the characteristics that used to differentiate the four types of horseshoe crab. The cross section of telson of *C. rotundicauda* is round, while others is triangulate. Hence, this part is usually used to differentiate the *C. rotundicauda* and others. Besides telson, the second and third appendage pair between *C. rotundicauda* and *T. gigas* can also be used to differentiate this two species.

Table 1. Distinguishing morphology characteristics of the four species of extant horseshoe crabs.

(Source: modified Sekiguchi and Nakamura, 1979)

	Characteristics	*Limulus polyphemus*	*Tachypleus tridentatus*	*Tachypleus gigas*	*Carcinoscorpius rotundicauda*
1.	Frontal Margin				
2.	Frontal View				
3.	2nd appendage pair				
4.	3rd appendage pair				
5.	Number of immovable spines on the mid-posterior margin of the opisthosomatic carapace	1	3	1	1
6.	Cross section of telson	Triangulate 	Triangulate 	Triangulate 	Sub-triangulate 
7.	Genital Operculum				
8.	Marginal Spines				

2.5 Morphometric Analysis

Horseshoe crab is classified under IUCN red list which either near threatened or data deficient. Most study shows that the Asian horseshoe crab are declining both locally and regionally. The main reason for the declining of this species is anthropogenic factors that happened in both United State and Asia (Ismail *et al*., 2011; Sahu & Dey, 2013). Although Asian horseshoe crabs are not like Altlantic species that have multiple utilization, but it has high potential usage in pharmaceutical and eco-tourism (Kassim *et al.*, 2008). However, the important of horseshoe crab are still unaware and this species are not listed in the Malaysia Wildlife Conservation Act 2010, hence no conservation practices, legislation or harvest regulations have been enforce to protect and conserve the horseshoe crab in Malaysia. Morphometric study provides both quantitative and qualitative baseline information of the horseshoe crab, which is important for implementation of conservation measures and management planning in future (Tan *et al.*, 2012).

The variation and changes in the morphology like size and shape of living organism can be study by using the morphometric analysis (Webster, 2007). By using this analysis, the quantitative measurement of different body parts can be used for comparing between different living organisms and population. According to Srijiaya *et al.* (2010), variation in the morphometric characteristics are useful in studying the identification and classification of many species. In all species, the main morphological characteristics almost look similar and hard to differentiate. However, when the analysis of data was done statistically among the populations that inhibiting in different area, a significant difference could be observed in their morphometric structures.

As mention by Chatterji and Pati (2014), in a more stable habitat, the marine organisms will show isometric growth in all of their body parts. In contrast, organisms will change their

morphometrical, biological and physiological characteristics if any sudden change in environmental conditions happen. Allometry is described as the study of relationship between differences in one body parameter to the other. This relationship provides a crucial information about the comparative growth of various body parameter (Vijiyakumar *et al*., 2000). The growth of the species can be identify by the understanding of allometry in shell and soft body parts of the crabs (Sahu & Dey, 2013). This concept of allometry was first postulated by Huxley and Tessier in 1936 and then this analysis was widely applied by biologist to estimate the population growth characteristics of multitude organism. The length-weight relationship study is an important prerequisite in fishery biological investigation. In order to identify the fatness, breeding and feeding state and their suitability to the environment, the study of the variations in expected weight from the length groups can be done (Thirunavukkarasu & Shanmugan, 2011).

2.6 Tetrodotoxin (TTX)

TTX is best described following the ingestion of *Fugu* (in Japanese), or puffer fish (Wallace, 2009). It is a small non-proteinaceous toxins which has a low molecular weight with 319.27, and a chemical formula of $C_{11}H_{17}O_8N_3$ (Yu, 2007; Wood, 2012). The unique complex chemical structure of TTX is show in Figure 2.

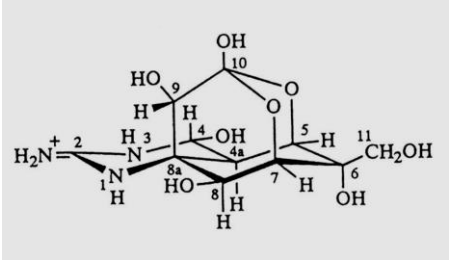

Figure 2. The chemical structure of TTX.
(Source: Tanu & Noguchi, 1999)

TTX are soluble in acidic environment with pKa 8.76, but not in any neutral organic solvent that can limits the extraction of TTX using aqueous solutions. TTX is odourless, colorless, and a heat stable toxin with a change of colouration from colorless to dark with the temperature above 220ºC with decomposition (Yu, 2007; Arakawa *et al*., 2010). In addition, it will not damage by freezing (Wallace, 2009). The fatal capacity is 5000 to 6000 MU/mg. 1 MU (= 0.22 µg) is defined as the quantity of TTX needed to kill a male mouse with 20g in a period of 30 minutes after injection. For human, the minimum lethal dose (MLD) is 10000 MU that corresponding to 2mg of pure TTX crystals (Asakawa *et al*., 2012).

The main origin of toxin in marine organism can be either endogenous or exogenous. Endogenous refer to the organism that produced their own toxin while exogenous refer to the organism that get toxin sources outside their body. According to Tanu *et al*. (1999), horseshoe crabs are exogenous organism this is because the sources of TTX might come

from the prey that the horseshoe crab feed on. Diets of horseshoe crab are mollusc, arthropods and detritus that may contain TTX-bearer bacteria.

TTX is very powerful after ingestion because it will bind to the sodium channels and interfering with muscle and nerve function (Wallace, 2009; Wood, 2012; Bane *et al*., 2014; Itoi *et al.*, 2014). After ingested the causative food, the symptoms will develop shortly and this can lead to fatality at approximately 6 hours (Noguchi & Arakawa, 2008). According to Kanchanapongkul (2008) and Bane *et al*. (2014) study, the initial symptoms after ingested TTX include tingling (paresthesias) of the lips and tongue. For mild poisoning only sensory symptom develop and are come along with nausea, headache and vomiting. However, this may develop to muscle weakness and ataxia. For the most serious cases, TTX may cause death due to paralytic, difficulty in respiratory and / or heart failure. Observation and appropriate supportive care are the only treatment for TTX intoxication. Presently, there is no any antidote available for TTX poisoning (Noguchi & Arakawa, 2008; Bane *et al*., 2014).

2.7 Instrumental Analysis of Tetrodotoxin

TTX detection and determination are not only for investigation and for medication purposes, in addition this is important to raise up the public awareness. Asakawa *et al*. (2012) stated that, there are a few analysis methods used for quantitative and qualitative detection of TTX. This included mouse bioassay, High Performance Liquid Chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LCMS). To quantify TTX, the methods such as Gas Chromatography Mass Spectrometry (GCMS), infrared (IR) spectrometry and nuclear magnetic resonance (H-NMR) spectrometry are frequently adopt. Furthermore, simpler and more practical methods, which are not instrumental analysis, like Thin Layer Chromatography (TLC) and electrophoresis can also use to detect the TTX. These rapid