TOXIN COMPOSITIONS of GREEN MUSSEL, *Perna viridis* FED WITH TOXIC DINOFLAGELLATE, *Pyrodinium bahamense* var. *compressum*

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ABSTRACT

Harmful algal blooms, especially *Pyrodinium bahamense* var. *compressum* (*Pbc*) has been the focus of research in Sabah due to its negative impacts toward aquaculture industry and human health. This type of algae can cause Paralytic Shellfish Poisoning (PSP). However, studies on acclimatization and depression periods of PSP toxin in mussels has not been done in the affair, although the mussels are highly consumed by the people of Sabah. Paralytic Shellfish Poison (PSP) toxins for this study were investigated by means of high-performance liquid chromatography with post column fluorescence derivatization (HPLC-FLD). Shellfish samples were collected from places that historically affected by this toxin; Kuala Penyu, Kota Merudu, Kota Belud and Mantanani Island. Sample was collected from June 2015 until April 2016. Three species of shellfish (*Perna viridis*, *Geloina* sp. And *Anadara* sp.) was collected from all sampling locations. *Perna viridis* collected from Kuala Penyu, shown the presence of PSP toxin analogue (decarbamoyl derivatives) which is Gonyautoxin (GTX 4), with toxin content of 30µgeq/100g tissue. However, this GTX 4 need to be reconfirmed in future analysis by using Liquid Chromatography – Mass Spectrometry (LC-MS) in order to differentiate either the peak formed was imposter or not. Then, for accumulations and depuration study of PSP toxin inside the mussel, toxic dinoflagellate, *Pbc* was cultured in f/2 media and fed to the green mussels, *Perna viridis*. Initial toxin content detected from cultured *Pbc* was 963 fmol/cell and toxin component detected were STX, Neo STX and GTX 6. The mussel ingested more than 99% of the *Pbc* cells (3×10^5 cells) once at the beginning of the experiment and accumulated a maximal amount of toxin (201fmol/100 g) at 96 hours and started to decrease after 120 hours of rearing periods. Composition of the PSP toxin accumulated in the mussels obviously different from *Pbc* at 3 hours after the cell supplied. The variation in toxin composition derived presumably from the transformation of toxin analogues in mussels was observed after 144 hours of the rearing period. Whereas, PSP toxin component (STX, Neo STX and GTX 6) in residue water was almost same as its origin dinoflagellate. From this study, it was clearly demonstrated that toxins from *Pbc* take a short period of time to accumulate and depurate toxin in the mussel's tissues after the digestion process. These findings also can be used as a basic guideline for PSP cases in the future.

ABSTRAK

KOMPOSISI TOKSIN DI DAALM KUPANG, Perna viridis YANG DIBERI MAKAN DINOFLAGELAT BERTOKSIN, Pyrodinium bahamense var. compressum

Fenomena ledakan populasi alga berbahaya terutamanya Pyrodinium bahamense var. *compressum (Pbc) menjadi fokus utama penyelidikan di Sabah kerana ianya mampu memberi kesan negatif kepada perusahaan akukultur dan juga ancaman kepada kesihatan manusia. Alga jenis ini boleh menyebabkan keracunan kerangan-kerangan (PSP). Namun begitu, kajian mengenai tempoh masa pengumpulan dan penyingkiran toksin dari jenis kupang masih belum pernah dilakukan di Sabah, walaupun kupang antara jenis kerangan yang banyak dimakan oleh penduduk di negeri Sabah. Untuk kajian ini, toksin PSP dianalisa mengunakan HPLC. Kerangan yang dikutip dari tempat yang pernah direkodkan mengalami kejadian ledakan alga bahaya (Kuala Penyu) berpunca dari Pbc menunjukan kewujudan salah satu kumpulan toksin PSP iaini Gonyautoxin (GTX 4), dengan kandungan toksin 30µgeq/100g tisu. Walaubagaimanapun, kewujudan GTX 4 ini perlu disahkan semula menggunakan LC – MS untuk membezakan samada puncak yang terhasil dari analisa HPLC adalah palsu atau pun tidak. Manakala, untuck kajian pengumpulan dan pengenyahan toksin di dalam kupang, alga bertoksin, Pbc telah dikultur menggunakan media f/2 dan diberi makan kepada kupang dalam tempoh 168 Jam. Kandungan awal toksin Pbc yang dikultur dikesan ialah 963 fmol/sell dan komposisi toksin dikesan ialah STX, Neo STX dan GTX 6. Kupang mula memakan lebih 99% sell PBc (3 x 10^5 sell) pada awal eksperimen dan mengumpulkan paling tinggi kandungan toksin (201 fmol/100 g) pada tempoh 96 jam kandungan toksin mulai menyusut selepas tempoh masa 120 jam pendedahan. Komposisi toksin PSP yang terkumpul didalam kupang ternyata berbeza mulai tempoh 3 jam pertama didedahkan dengan alga beracun tersebut. Kewujudan variasai komposisi toksin diantara toksin alga dan juga kupang selepas tempoh 144 jam menunjukan berlakunya proses transformasi analog toksin melalui proses bio-transformasi. Manakala analisa dari sisa air menunjukan tiada perubahan variasi toksin* (STX, Neo STX and GTX 6) *jika dibandingkan dengan alga penyebab toksin. Kajian ini menunjukkan bahawa toksin daripada alga berbahaya (Pbc) mengambil masa yang singkat untuk akumulasi dan disingkirkan di dalam tisu kupang selepas tempoh penghadaman sel Pbc berlaku. Dapatan kajian ini juga boleh digunakan sebagai garis panduan asas bagi mengelakan keracunan kerangan (PSP) di masa hadapan.*

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LIST OF ABBREVIATIONS

AcOH	Acetic acid
AOAC	Association of Official Analytical Chemist
ASP	Amnesic shellfish poisoning
AZP	Azaspiracid shellfish poisoning
CO_2	Carbon dioxide
ddH_2O	Double distilled water
DSP	Diarrhetic shellfish poisoning
ESI	Electro spray ionization
fmol	fentamole
GTX 1	Gonyautoxin 1
GTX 2	Gonyautoxin 2
GTX 3	Gonyautoxin 3
GTX 4	Gonyautoxin 4
GTX 5	Gonyautoxin 5
GTX 6	Gonyautoxin 6
H	Hydrogen
HABs	Harmful algal blooms
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
HPLC-FLD	High Performance Liquid Chromatography with Fluoresce Detector
LC-MS	Liquid chromatography Mass Spectrometry

LD	Light day
MU	Mouse units
nd	Not detected
Neo-STX	Neo Saxitoxin
NSP	Neurotoxic shellfish poisoning
OH	Hydroxide
OSO_{3-}	Organosulfate
P	Phosphorus
Pbc	*Pyrodinium bahamense* var. *compressum*
PHEA	PolyHydroxyEthyl Aspartamide
PSP	Paralytic shellfish Poisoning
PST	Paralytic shellfish toxin
STX	Saxitoxin
TLC	Thin layer chromatography

LIST OF SYMBOLS

%	Percentage
>	Greater than
≤	Less and equal than
^{0}C	Degree Celsius
µg	Microgram
fmol	fentamole
g	Gram
L	Liter
m	Meter
M	Molar
mL	Milliliter
mM	miliMolar
MU	Mouse units
N	Nitrate
Na^+	Sodium
nmol	nanomole
psu	Practical salinity unit
Rt	Retention time

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CHAPTER 1

INTRODUCTION

Pyrodinium bahamense var. *compressum* (*Pbc*) is a very important member of paralytic shellfish toxin (PST) producing marine dinoflagellates especially in tropical waters. This species have caused more human illnesses and fatalities than any other PSP producing dinoflagellates such as *Alexandrium* spp. and *Gymnodinium* spp (Hallegraeff, 1993; Holmes *et al.*, 2002; Krock *et al.*, 2007). First incidences of toxic blooms of the species occurred in Brunei and Sabah, Malaysia in 1976, where 202 victims were reported to be suffering from PSP and seven deaths and the most recent cases of PSP outbreak in Sabah was recorded in 2013 that caused about two death and more than 40 victims hospitalized (Daily Express, 2013).

At present, *Pbc* continues to be a significant cause for Paralytic Shellfish Poisoning (PSP). PSP is the most potent toxin compared to others shellfish poisons (diarrhetic, neurotoxic and amnesic) (Yasumoto & Murata, 1993) . In general PSP toxin typically comprised of Saxitoxin (STX) and STX-related derivatives that grouped into three main groups: carbamate toxin (saxitoxin (STX), neoSTX, gonyautoxin (GTX) 1,2,3,4), decarbomyl toxins (dcSTX, dcneoSTX, dcGTX1,2,3,4) and N-sulfocarbomyl toxins (GTX5,6 and C-toxins (C) 1,2,3,4) (Oshima 1995; Smith *et al.,* 2001; Donovan *et al.*, 2009; Wiese *et al.*, 2010). Peak toxin content for *Pbc* species is normally about 300 – 400 fmol/cell in laboratory cultured condition (Usup *et al.,* 1994; Gedaria *et al.*, 2007). Culturing method using variety of media also proposed including usage of f/2 media, ES-I media, and ES-DK media.

These toxins responsible for acute and often fatal poisonings caused by the consumption of certain contaminated shellfish that have ingested huge quantities of toxic and planktonic microalgae by filter feeding (Lehane, 2001). The toxicity of STX is attributable to the reversible blockage of voltage-activated sodium channels on excitable cells, which stops the flow of nerve impulses

Bivalve shellfish such as mussels, oysters and clams serve as a vector that concentrate high level of PSP in the digestive glands as a result of the continuous filtration of toxic dinoflagellates (White *et al.*, 1993). However, rate of the accumulation and depuration periods of PSP among the bivalves differ significantly according to different species of causative algal and shellfish species. (Hurst & Gilfillan, 1977; Oshima, *et al.*, 1982; Takatani *et al.,* 1998). Mussels were known as a faster species to accumulate and to eliminate the toxins than most other species of shellfish. Rate of accumulation and depuration is also vary depend on environmental conditions (Cembella *et al.,* 1994) and low environment temperatures evidently slowdown detoxification (Aalvik & Framstad, 1981). Several method also have been developed in order to detect PSP toxin including mouse bioassay, Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Liquid chromatography Mass spectrometry (LC-MS).

This study was the first attempted in Sabah. *Pbc* was fed to the green mussel (*Perna viridis*), and accumulation and depuration of PSP toxin component and composition were investigated in details.

1.1 Significance of Study

Pbc blooms in tropical water have caused major public health and economic impact in the Indo-Pacific, tropical Atlantic and Southeast Asian region (Ting & Joseph, 1989). In Sabah itself, *Pbc* blooms have already caused numerous cases of paralytic shellfish poisoning (PSP), death and economic loss. Still very few studies have been done on this species compared to other "red tides" forming species.

Therefore, these study was essential to understand more about the toxin content of *Pbc* cells as well as to identify the acclimatization and depuration period of PSP toxin in the mussels. So this study can form a prompt result for early warning and create early awareness and preventions regarding PSP toxin.

1.2 Research Objectives

This study aimed on the Paralytic Shellfish Poisoning (PSP) toxin that caused by *Pbc* in green mussels in Sabah. Hence, the specific objectives of this study are:

a. To determine the concentration of PSP toxin in bivalves collected from locations with a history of *Pbc* bloom.
b. To assess the PSP toxicity level and toxin component of cultured *Pbc.*
c. To characterize the accumulation profiles of PSP toxins in the green mussel, *Perna viridis* fed with cultured *Pbc*

CHAPTER 2

LITERATURE REVIEW

2.1 Harmful Algae Blooms

Phytoplankton play important role in supporting the productivity of aquatic ecosystem. They harvest light, carbon dioxide and a range of organic and inorganic compound. Light and carbon dioxide serves as the energy sources while nutrients play role concerning cell structure and metabolism (Hecky & Kilham, 1988). Under certain conditions, it can grow massively and form harmful blooms (Paerl *et al.*, 2001). A bloom is defined as a `significant population increase' (Smayda, 1997b), which leads to a peak. Whereas, harmful alga blooms defined as the phenomena where the density of phytoplankton drastically increase in the water column beyond normal until causes the physical, chemical and biological changes in term of water colors, and odors. (Smayda, 1997b). Even have been generally viewed as periodically occurs, this phenomena are still unpredictable and difficult to control (Smayda, 1997a).

The content of harmful alga blooms (HABs) is a mixture of various microscopic species of phytoplankton in the water column. However, the phytoplankton, a photosynthetic dinoflagellate, is commonly counted as the occurrences of harmful alga blooms. Whereas, macro algal and benthic microalgae preferable dominate at shallow water level with poor water movement, lagoons, stagnant water, coral reef, and rocky intertidal or subtidal habitats (McManus & Woodson, 2012). Under normal environment, HAB cell in water column's presence in small concentration, therefore no significant impact towards aquatic environments and human safety (Deeds *et al.*, 2008). Consideration of blooms concentration also varies depends on alga species. For example, *Alexandrium spp.* considered blooms when the cell number reached more than 10^5 cells /L whereas for *Aureococcus anophagefferans* considered blooms when cell numbers more than 10^9 cells /L (Cosper *et al.*, 1987). On certain study,

blooms also characterized based on concentration of chlorophyll pigment. Where, >100 mg Chl/M^3 considered as blooms (Smayda, 1997b).

Although widely known as red tides (due to color imparted by dominant concentration of the photopigment peridinin algae suspended in water in the water column), certain alga species also can undergo blooms not in red colors, either forming green, yellow or brown phenomena depend on dominant species alga that suspended in the water column (Glibert *et al.*, 2005). Examples of harmful alga blooms that are readily associated with water discoloration are blooms of many species of cyanobacteria, generally visible as floating green colonies in coastal environments; two alga species (*Aureococcus* and *Aureoumbra)* that turn coastal lagoons water column become dark chocolate brown and the dinoflagellates such as *Alexandrium* spp., *Gymnodinium breve, Pyrodinium* bahamense var. *compressum* (after this written as *Pbc*) , *Noctiluca* spp. and *Cochlodinium* spp., that cause changed in red color of water column (normally known as red tides) (Hallegraeff, 1993). The most interesting study found that, certain alga species that blooms, may not be visible when it occurred, due to free-chlorophyll pigmented (Cosper *et al.*, 1987). These algae, which are phytoplankton specifically, contains photosynthetic pigment with various colors to capture different wavelength of sunlight. Harmful algal blooms may be either contain toxin or not. But, both of these blooms will cause high damage either toward human healthy or other aquatic organism or/and environment. Fish killer blooms such as *Cochlodinium* spp. will cause depletion of oxygen level (anoxia) due to excessive respiration or decomposition rate in water column. Whereas, toxic blooms such as *Pbc* will produce toxin compounds that can alter cellular proses of other aquatic organism from plankton to humans. In serious cases, it can lead to death within a few hours after contaminated with this toxin blooms algae (Sellner *et al.*, 2003). Until year 2000, 80 toxic species and about 200 nontoxic species out of about 4000 marine planktonic were potentially recorded involved in harmful algal blooms all around the world (Zingone & Oksfeldt, 2000).

2.2 Factors That Influence Harmful Alga Blooms

To date, no significant factor can be concluded as the main factor that triggers or contributes directly in harmful algal blooms phenomena. Environmental conditions play an important role that can lead to this phenomenon, since different environmental conditions, will have a different triggering factor. A numbers of factors thought to trigger algal blooms likely came from a combination of natural and anthropogenic factors, including available nutrients, temperature, sunlight, ecosystem disturbance (stable/mixing conditions, turbidity), hydrology (river flow and water storage levels), meteorological conditions and the water chemistry (pH, conductivity, salinity, carbon availability etc.) (Paerl, 1988).

It has also has been proved that there is a strong correlation between the number of red tide cases reported the presence of coastal pollutions that mainly came from sewage, urban development and some form of industrial waste (Jingzhong *et al.*, 1985; Howarth *et al.*, 2002). Waste water and raw sewage discharging into the water column without treatment often cause eutrophication (nutrient overloading) which then easily triggers bloom outbreaks. Two macronutrients in human activity, phosphorus (P) and nitrate (N), are of most concern in eutrophication (Schindler, 1977). Agriculture and urban development activities are major sources of phosphorus and Nitrate that washed away to aquatic ecosystems during climatic changes and atmospheric deposition (Carpenter *et al.*, 1998; Hallegraeff, 2010).

Besides, the development of aquaculture and maricultural also playing roles in harmful algal blooms phenomena. This included open fish culture and caged cultural system. Cultured shellfish and finfish populations secrete large amounts of feces, pseudo feces, and other excretory products rich in Nitrate and Phosphorus that important to algal growth (Smayda, 2006). Intensive aquaculture system causes self-pollution as a result of excess feeding practical, fish feces and aging-water. Long term aquaculture in the same area (normally cage culture), over-crowded cage arrangement and intensive fish culture result in eutrophication of the marine aquaculture site, and thus environment preferable for algae to grow and form harmful blooms (Qi *et al.*, 2004).

Non-anthropogenic factors such as climate changes and atmospheric pressure also tense to trigger blooms phenomena. The distributions of blooms likely depend on wind direction or flow of ocean currents (Qi *et al.*, 2004). Climate change altered marine ecosystems with multifactorial stressors, such as increased temperature, enhanced surface stratification, alteration of ocean currents, intensification of weakening of nutrient upwelling, stimulation of photosynthesis by elevated CO_2, reduced calcification from ocean acidification, and changes in land runoff and micronutrient availability (Hallegraeff, 2010). Oceanic and estuarine circulation and river flow greatly influence the abundance and distribution of plankton and the combined physical (e.g., currents, upwelling, etc.) -chemical (e.g., salinity, nutrients, etc.) factors of these systems, combined with unique life cycles and behaviors of some harmful algal species, result in blooms that impact coastal ecosystems and populations.

There is also evidence that harmful algal blooms accidentally introduced to the new uncontaminated area via ship ballast tank or infected shellfish (Hallegraeff, 1998). The probability of ballast water intake of harmful algal taxa is significantly dependent upon shipping patterns, seasonality of plankton blooms in where water was pumped into ballast tank and the presence of local sediment cysts. Cyst that introduce either from discharged water from ballast tank or a result from depurated process from infected shellfish, then will blooms in future, under favorable conditions (Bolch & Hallegraeff, 1993) .

The seasonal variation of temperature and salinity reflects the combined effects of convection and water column stability, which can control vertical movement of plankton and other parameters essential to its growth. Yñiguez *et al.*, (2012) on their study at Sorgoson Island, Philippines found that *Pbc* blooms develop a few months after the beginning of rains, relatively lower temperature, and stratification of the water column. Pronounced blooms are observed at a particular time of the year and in certain areas of the Bay.

Besides, presence of wind blowing are closely related to the potential to re-suspend cysts that settled down at sea floor (Dyble *et al.*, 2008). In the low force of wind, tidal currents in the inner part of the water column, unable induce resuspension