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Chemical and Drug Residues in Fish and Fish Products in Southeast Asia
(Biotoxins Monitoring in ASEAN)
Information for Technical Compilation

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1. Introduction

In Cambodia, there are some cases of food poisoning was occasionally occurred and suspected to be associated with biotoxins by consumption of aquatic animals. Most of cases were reported to be associated with the consumption of puffer fish that also known as blow fish. In 2007, it was reported one child was dead and 12 members of two more family were hospitalized after having lunch with puffer fish in Takeo province, and in 2010 it was also reported one person died, and 23 were hospitalized after eating poisonous freshwater puffer fish in Kampong Cham province.

Puffer fish is known as blow fish contains toxins substances that are lethal to human. Some study has revealed that small puffer fish inhabiting brackish water or freshwater in Southeast Asia are also contaminated with toxins that identified as tetrodotoxin (TTX) and saxitoxins (STXs), toxins that belong to the paralytic shellfish poison (PSP) family, were detected as the main toxic principles. In Cambodia, however, PSP contamination in aquatic animals has not been broadly investigated yet, especially toxins in marine bivalves and molluscs that have fed on the toxin-producing dinoflagellates that is potential source of the PSP. On the other hand, PSP is also suggested to be as a potential source of biotoxins contamination in shellfish that is the important indicator for biotoxins monitoring purposes.

Early study conducted by Laymithuna Ngy *et al.*[1] about the toxicity in the horseshoe crab (*Carcinoscopius rotundicauda*) to reveal the presence of tetrodotoxin in this species, but not found paralytic shellfish toxins. The toxicity of two species of wild Cambodia freshwater

[1] L Ngy *et al.*2007. Toxicity assessment for the horseshoe crab *Carcinoscorpius rotundicauda* collected from Cambodia. Toxicon: 49(6):843-7

pufferfish of genus Tetraodon T. Turgudus and Tetraodon sp., was further investigated and found to appear highly in skin and ovary (Ngy L. *et al.*, 2008)[2]. Recent study of toxicity and toxin profiles of Cambodia marine pufferfish (*Takifugu oblongus*) were investigated and revealed that the toxicities ranged from 10-132 mouse units, and mainly concentrate in ovary. The study was also found tetrodotoxin (TTX) as the main component and saxitoxin (STX) as a minor. It was confirmed that T. Oblongus is a hazardous species that unsafe for human consumption (Ngy L. *et al.*, 2009)[3].

Bivalves are considered as one of potential hosts of biotoxins, but these species of aquatic animals have not yet been investigated in Cambodia. Bivalves have been widely distributed and preferably consumed amongst other types of seafood, especially green mussels. This type of bivalves is collected from wild resource and widely marketed in Cambodia. In this study, green mussel (*Perna Viridis*) is identified and investigated for PSP contamination. It is hoped that the result from this study will provide insight for further researches and also provide recommendations for future biotoxins monitoring programme in Cambodia as well as contributing to the biotoxins monitoring programme in Asian region.

2. Objectives and Goals

The main objective of this study is to investigate Paralytic Shellfish Poisoning (SPS) contamination in green mussel *Perna Viridis* in Cambodia marine water using Mouse Bio-assay Method (MBA).

The study is part of biotoxins watch programme in Cambodia marine water contributing to the control of biotoxins contamination in Asian region. This study will monthly monitor for the period started from June 2011 to May 2012.

3. Survey Methodologies

a. Sampling Site:

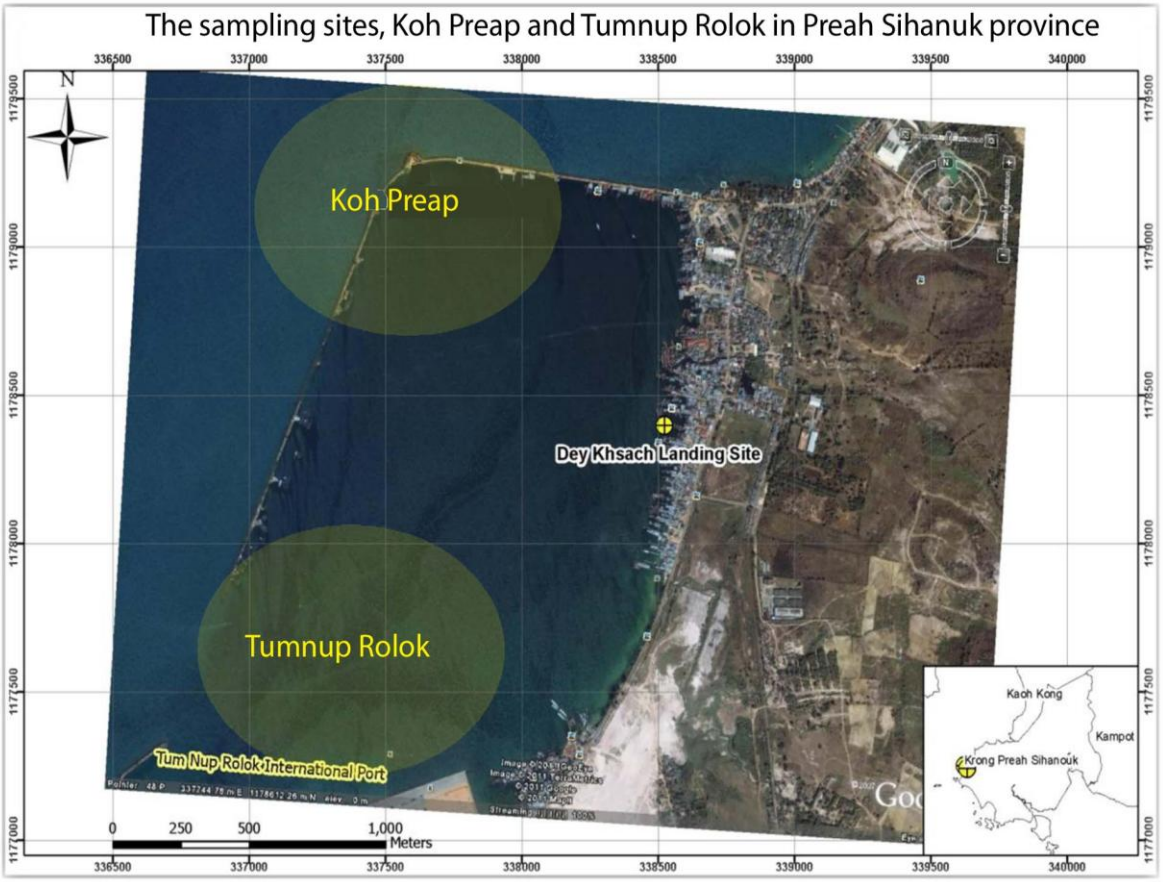
In this study, there are two sampling sites, Koh Preap and Tumnup Rolok, were identified and selected (Figure 1). Koh Preap and Tumnup Rolok are bivalves-inhabiting locations where are the favourite fishing ground for local fishmen. These sampling sites are located in Preah Sihanouk province where is one amongst the four provinces in coastal areas situated in South-western part of the Kingdom of Cambodia. This province is productive biodiversity area in the Cambodia's EEZ where producing all types of seafood include bivalves and molluscs. Most of fisheries products in this area are from wild capture, but some from aquaculture. In contrast, this province is an industrial area that potentially makes risk to the environment in this area. With reference to Asia mussel watch programme conducted during 1997-2001 under implementation of the Centre for Marine Environmental Studies (CMES),

[2] L. Ngy *et al.* 2008. Occurrence of paralytic shellfish toxins in Cambodia Mekong pufferfish Tetraodon turgidus: selective toxin accumulation in the skin. Toxicon: 51(2):2080-8.

[3] L Ngy *et al.* 2009. Co-occurrence of tetrodotoxin and saxitoxin in Cambodian marine pufferfish *Takifugu oblongus*. African Journal of Marine Science: 31(3): 349-354.

Japan revealed some level of the contamination of toxins substances in mussel collected from this area, but PSP contamination in the species has not been investigated yet. Therefore, the selected sampling sites are suitable for this study to investigate the possible PSP contamination in Cambodian bivalves in this coastal province.

Figure 1. Map showing the sampling sites of the study



a. Target Species:

Green mussel *Perna Viridis* are widely distributed and preferably consumed amongst other types of bivalves. This type of bivalves is collected from wild resource and widely marketed in Cambodia. In this study, green mussel *Perna Viridis* is identified for investigation of PSP contamination.

Figure 2. Identification and measurements of the samples of green mussel *Perna Viridis*

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b. Sampling Method:

The samples were collected monthly during the four week of each month started from May 2011 to June 2012. The samples of green mussel (*Perna Viridis*) 4-5 Kg were collected each time at the fishing grounds from each sampling site, and then each piece of identified green mussel was recorded its length and weight (Table 1). The samples were collected in alive form and quickly kept in an icebox to transfer to Phnom Penh city. At the laboratory in Phnom Penh, the samples were cleaned and washed several times by clean water before being cut-opened the shell. The whole tissues of 350-500g were collected and kept in the zip-locked plastic bag with proper labelling and stored in a freezer at -25 degree Celsius until used.

Table 1. Average length and weight of collected samples, green mussel *Perna Viridis*.

Sampling date	Sampling location	Code	Average weight (g)	Average length (cm)
June 2011	Tumnup Rolok	A6	19.60	7.10
	Koh Preap	B6	36.85	8.73
July 2011	Tumnup Rolok	A7	26.39	8.04
	Koh Preap	B7	24.05	7.20
August 2011	Tumnup Rolok	A8	11.58	5.80
	Koh Preap	B8	15.76	6.72
September 2011	Tumnup Rolok	A9	22.41	7.39
	Koh Preap	B9	22.81	7.43
October 2011	Tumnup Rolok	A10	15.40	6.00
	Koh Preap	B10	22.20	7.50
November 2011	Tumnup Rolok	A11	15.79	6.33
	Koh Preap	B11	24.42	7.46
December 2011	Tumnup Rolok	A12	23.01	7.46
	Koh Preap	B12	24.64	7.32
January 2012	Tumnup Rolok	A1	21.87	7.23
	Koh Preap	B1	25.32	7.62
February 2012	Tumnup Rolok	A2	18.35	6.74
	Koh Preap	B2	31.23	8.24
March 2012	Tumnup Rolok	A3	30.84	8.61
	Koh Preap	B3	20.71	7.55
April 2012	Tumnup Rolok	A4	26.21	8.92
	Koh Preap	B4	30.28	8.35
May 2012	Tumnup Rolok	A5	27.50	8.05
	Koh Preap	B5	32.85	8.52
Note: A= Samples from Tumnup Rolok, B= Sample from Koh Preap				

c. Method of Analysis

350-500g whole tissue of green mussel *Perna Viridis* were transferred in icebox to analyse at the laboratory of the Centre of Analytical Service and Experimentation (CASE) in Vietnam. The analytical method was conducted in accordance with the CASE laboratory standard protocol for PSP detection using a Mouse Bio-assay Method (MBA).

In this study, Mouse Bio-assay method (MBA) is used to test PSP level in green mussel *Perna Viridis*. PSP is analyzed qualitatively as amount of toxins (positive or negative). The

internationally recognized method used for analysis of PSP toxins is the standard AOAC mouse bioassay (OAC 1990, AOAC International 1995). This is the only live animal bioassay method that has been fully validated in a collaborative study. The detection level of this method is about 40 microgram STXeq per 100g wet weight of tissue, boiling of 100g of tissue with 100 ml of 0.1 N HCl for 5 minute, adjustment of volume back to 200 ml, of the pH ideally to 3, and intraperitoneal (i.p.) infection of the acidified extract 0.1 N HCL solution injected into white mice (three mice) and observe white mouse carefully in 1 hour. Results are calibrated against a STX standard and expressed in mouse units (MU) which are converted to toxicity units [STX equivalents (STXeq)] using the conversion factor which varies with the sensitivity of the mouse strain used (1 MU = 0.18 to 0.23 microgram STXeq). Standard mouse bioassay an amount of 1 ml of the solution was injected intraperitoneally into three male mice, and they were observed for symptoms and times of death. The median death time (i.e. period between injection and death) was used to calculate the number of mouse units (MU). Toxicity level of the sample (MU g–1) was determined from the dose–death time relationship (Japan Food Hygiene Association 2005), where 1 MU is defined as the amount of toxin required to kill a 20g male mouse within 30 minutes of injection.

d. Limit of Detection and Limit of Quantification

The detection level of this method is about 40 microgram STXeq per 100g wet weight of tissue.

e. National Regulatory Limits

There is no National Regulatory Limits has been set up yet in the Kingdom of Cambodia. Cambodia Fisheries Administration is adopting mainly base on the ASEAN and EU Standards in complying with the international conformity for controlling safety of exported and imported fisheries products.

4. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results (if any)
Not applicable.

b. Survey Results and Discussion

Results of Analysis:

Paralytic Shellfish Poisoning (PSP) in Cambodian green mussel *Perna Viridis* was invested throughout a period from May 2010 to June 2012. All samples were analysed for PSP contamination by using the Standard MBA. The results of this analysis have shown that the level of PSP was not detected (negative) in all analysed samples of green mussels *Perva Viridis* from these two sampling sites throughout the sampling period (Table 2).

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Table 2. Results of the detection of PSP contamination in green mussel *Perna Viridis*

Month and year of sampling	Method	No. of replica analysed	Average sample Weight	Confirmation	
				Koh Preap	Tumnup Rolok
Jun 2011	MBA	3	350-500g	NE	NE
Jul 2011	MBA	3	350-500g	NE	NE
Aug 2011	MBA	3	350-500g	NE	NE
Sep 2011	MBA	3	350-500g	NE	NE
Oct 2011	MBA	3	350-500g	NE	NE
Nov 2011	MBA	3	350-500g	NE	NE
Dec 2011	MBA	3	350-500g	NE	NE
Jan 2012	MBA	3	350-500g	NE	NE
Feb 2012	MBA	3	350-500g	NE	NE
Mar 2012	MBA	3	350-500g	NE	NE
Apr 2012	MBA	3	350-500g	NE	NE
May 2012	MBA	3	350-500g	NE	NE
NOTE: MBA (Mouse Bio Assay), NE (Negative), PO (Positive)					

Discussion

Paralytic shellfish poisoning (PSP) in humans is caused by ingestion of shellfish containing PSP toxins. These PSP toxins are accumulated by shellfish grazing on algae producing these toxins. The PSP toxins are produced mainly by dinoflagellates belonging to the genus *Alexandrium,* which occur marine, but also produced in freshwater cyanobaceria and associated with calcareous red macroalgae have been reported (Deeds, J.R., *et al.*, 2008)[4]. Shellfish grazing on these algae can accumulate the toxins but the shellfish itself is rather resistant to the harmful effects of these toxins. According to FAO's reports during the last 20 years, there seems to have been an increase in intoxications caused by PSP toxins. However, as yet it is unclear whether the increase is real, whether it could be a consequence of improved identification, detection and medical registration, or whether it is due to expanded shellfish culture and consumption.

In this study, we have investigated PSP toxins in green mussel *Perna Viridis* that naturally inhabits in Cambodia marine water. The results of this study showed that PSP toxins in all samples collected from the sampling sites, Koh Preap and Tumnup Rolok, in Preah Shihanuk province were not detected. In principle, PSP toxins are dominant toxins group presented especially in marine shellfish, therefore, our present results can conclude that the Cambodia green mussels *Perna Viridis* inhabiting in this coastal area are non-hazardous and safe for human consumption. On other hand, the results from this study also revealed that the environment where the green mussels inhabiting is still good and not polluted or contaminated with PSP toxins yet, suggesting that probably dinoflagellates that are the marine primary producers of the major causative agents of harmful algal blooms producing chemical biotoxins may not be presented in this area. In contrast, the absence of PSP toxins is probably due to limitation of the detection method used in this study that is not capable to quantify a level of PSP toxins in this species, so it may still have possible risks to human health in future because PSP toxins level detection is probably below the limit detection of 40 microgram/100g.

Therefore, it is suggested that further study should be used quantitative methods in order to clearly elucidate the PSP toxins level in the samples. The results from our present study will

[4] Deeds, J.R., *et al.*, 2008. Non-Traditional Vectors for Paralytic Shellfish Poisoning. Marine Drugs 6(2): 308-348.

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provide insight for further researches about biotoxins contamination in fisheries products as well as contributing to biotoxins monitoring programme in the Asean region.

c. Corrective Actions

PSP toxins contamination in the Cambodia green mussel *Perna Viridis* was not detected in all samples tested during the period from June 2011 to May 2012, so it is confirmed safe for human consumption, and there is no corrective action should be taken.

d. Problems and Challenges Encountered

- The samples were transferred with long distance to analyse at the laboratory in Vietnam due to lacks of laboratory facilities in Cambodia, thus increased the cost of analyses and, moreover, it is sometime difficult to make clearance at check-point.
- Financial support is limited for the study.
- Limitation of background information (references).
- Human resource capacity in this field is still limited.

e. Recommendations and Suggestions for Future Follow Up Action

Cambodia marine water are potentially contaminated with hazardous chemical toxins due to increase industrial activities, therefore it is suggested that further studies or investigations on other potentially toxins-contaminated aquatic species should be continuously conducted and expended widely in order to provide clear background information for controlling biotoxins that are harmful to public health. In addition to this, long term biotoxins monitoring programme should be set up, and human resource capacity building is needed for Cambodia.

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