Technical Compilation of Biotoxins Monitoring in ASEAN Region

2009 - 2012

Marine Fisheries Research Department

Technical Compilation of Biotoxins Monitoring in ASEAN Region

Japanese Trust Fund II:

Chemical and Drug Residues in Fish and Fish Products in Southeast Asia

2009 – 2012

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Organised by: Marine Fisheries Research Department (MFRD)

Southeast Asian Fisheries Development Center (SEAFDEC)

In collaboration with: The Government of Japan (Japanese Trust Fund II Project)

SOUTHEAST ASIAN FISHERIES DEVELOPMENT CENTER

The Southeast Asian Fisheries Development Center (SEAFDEC) is a technical organization devoted to the accelerated development of fisheries in the region. The member countries of SEAFDEC are Japan, Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam. SEAFDEC has four departments, namely, the Marine Fisheries Research Department (MFRD) in Singapore, the Training Department (TD) in Thailand, the Aquaculture Department (AQD) in Philippines and the Marine Fishery Resources Development and Management Development (MFRDMD) in Malaysia.

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Foreword

The wholesomeness of fish for food safety and food quality has always been a primary concern for many in the fisheries sector in the region. Fish has always been a critical source of protein for the people in the Southeast Asia region. Sadly, we have seen an increasing number of human intoxications from the consumption of contaminated shellfish and fish in the region and around the world.

In view of the fact that marine biotoxins pose a significant and increasing threat to human health in the region, monitoring of seafood for toxicity is henceforth, an important step in managing marine biotoxins risk.

For this reason, the Marine Fisheries Research Department (MFRD) of the Southeast Asian Fisheries Development Center (SEAFDEC) in Singapore co-ordinated the Japanese Trust Fund II Project on "Chemical and Drug Residues in Fish and Fish Products in Southeast Asia – Biotoxins Monitoring in ASEAN", and worked in partnership with the ASEAN member countries. It is with the hope that this project would help to develop methodologies on biotoxins analyses through human resource training, obtain an understanding of levels of biotoxins occurrences and incidences in fish and fishery products in ASEAN and enhance the regional capability in marine biotoxins testing.

This Technical Compilation, which is the final output of the project, would be beneficial to policy makers, technologists and scientists as well as regulatory personnel in the fisheries sector. I would like to express my sincere gratitude and appreciation to the fisheries officers in the Member Countries and the officers of MFRD for their hard work and great effort in making this Technical Compilation possible.

In addition, I would like to thank the Government of Japan for funding this project under the Japanese Trust Fund Program. I am sure this compilation would be a useful guide for the fisheries trade and industry in ensuring the success of providing safe and quality seafood for the Southeast Asian region.

Mr. Yeap Soon Eong
Chief, MFRD Programmes

Table of Content

Introduction

The consumption of a variety of shellfish and fish causes an increasing number of human intoxications around the world. There are approximately 400 poisonous fish species existing in the world and, the substances responsible for their toxicity are mainly attributed to biotoxins. Marine biotoxins represent a significant and expanding threat to human health in many parts of the world, with visible impacts ranging from human poisoning or in severe cases, death from the consumption of contaminated shellfish or fish, and at times, the mass killings of fish and shellfish.

The Codex Alimentarius Code of Practice for Fish and Fishery Products (CAC/RCP 52-2003) defined biotoxins as poisonous substances that are naturally present in fish and fishery products or accumulated by the animals feeding on toxin producing algae, or in water containing toxins produced by such organisms. Therefore, biotoxins monitoring is essential to manage food risks and ensure food security.

In view of this, MFRD worked together with the ASEAN member countries to implement a project on biotoxins monitoring in order to increase the attention in expanding and improving initiatives to monitor, detect and share information on marine biotoxins so as to reduce the public health risks associated with the consumption of contaminated shellfish and fish.

The purpose of this project aims to:

- Develop methodologies on biotoxins analyses through human resource training
- Obtain an understanding of levels of biotoxins occurrences and incidences in fish and fishery products in ASEAN
- Establish protocols for harmonization
- Encourage member countries without monitoring system to establish their own system
- Establish a directory of reference of experts and responsible persons
- Enhance analysis capability to an acceptable confidence level with the 1-year survey

This project comprised of 5 activities as follows:

Activity 1: Regional Technical Consultation

A Regional Technical Consultation Meeting was held in Singapore from 26 – 28 August 2009 to initiate the project and to plan for all the project activities. A total of 19 participants from ASEAN SEAFDEC member countries and 8 MFRD officers attended the meeting. Dr Toshiyuki Suzuki, a Japanese expert from the National Research Institute of Fisheries Science also attended the meeting as a resource person to provide technical advice to the meeting. The meeting came to a consensus of testing on Paralytic Shellfish Poisoning (PSP) in Green Mussel or Baby Clam, as the focus of the one year survey. The Administrative Report for the meeting is attached in Annex 1.

Activity 2: Regional Training Course in Singapore

The Regional Training Course was conducted in Singapore at the Veterinary Public Health Centre, Agri-Food & Veterinary Authority of Singapore (AVA) from 28 June – 7 July 2010, with 22 participants from ASEAN SEAFDEC member countries.

Dr Toshiyuki Suzuki and Dr Ryuichi Watanabe from the National Research Institute of Fisheries Science, Japan, Dr Yasukatsu Oshima from Kitasato University were the lead trainers for the course. In addition to these three Japanese experts, two trainers each from Oasis Solutions Pte Ltd and Tropical Technology Centre, Japan, was also at the course to conduct trainings on

biotoxins analysis rapid methods.

In total, five analysis methods for various biotoxins were taught:

1. Multi-component testing of Diarrhetic Shellfish Poisoning (DSP) and lipophilic toxins (Yessotoxin (YTX), Pectenotoxins (PTX) by High Performance Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) method (*Annex 1*)
2. DSP rapid method (*Annex 2*)
3. Paralytic Shellfish Poisoning (PSP) by High Performance Liquid Chromatography (HPLC) (*Annex 3*)
4. PSP ELISA rapid method (*Annex 4*)
5. Tetrodotoxin (TTX) by LC/MS/MS (*Annex 5*)

These methods were selected based on the feedback from member countries on the methods they wish to build up capability in during the Regional Technical Consultation held for the project in 2009.

Activity 3: Biotoxins Survey

The Biotoxins Survey was conducted for one year, from 2011 to 2012. Nine countries, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam, participated in the one year survey.

This survey targeted at PSP monitoring in Green Mussel (*Perna viridis*), with the exception of Indonesia and Vietnam who targeted PSP monitoring in Baby Clam (*Meretrix spp*). Meanwhile, Myanmar and Singapore had also expanded their survey scope to include the monitoring of Amnesic Shellfish Poisoning (ASP) and DSP in Green Mussel (*Perna viridis*). For the purpose of this survey, majority of the countries used mouse bioassay as the screening method followed by HPLC as the confirmation method.

Participating countries submitted their survey results on a half-yearly basis during the one year period and it was found that the species monitored by the countries gave mostly negative returns for their PSP content.

Activity 4: Publication of Technical Report

The results from the one year Biotoxins Survey was collated and published into a Technical Report for dissemination to all ASEAN SEAFDEC member countries in 2012.

Activity 5: End-of-Project (EOP) Seminar

An End-of-Project (EOP) seminar was conducted in 2012 to conclude the project, to present and disseminate the technical compilation on Biotoxins Monitoring in ASEAN, as well as to deliberate on the desirable scope for new projects.

Upon completion of all five activities, participating member countries had upgraded their regional laboratory capabilities and credibility for testing of DSP, PSP and TTX biotoxins as well as established their own monitoring programmes for routine surveillance testing of fish and fisheries products, which was particularly beneficial to countries that do not have such programmes in place. Meanwhile, member countries had also deepened their knowledge and understanding on the levels of biotoxins occurrences and incidences in fish and fish products in the ASEAN region, which in turn, facilitated the exchange of information among Member Countries through the establishment of a directory of biotoxins experts and responsible persons/national authorities in each Member Country.

Survey of Biotoxins in ASEAN Region

- Cambodia
- Indonesia
- Lao PDR
- Malaysia
- Myanmar
- Philippines
- Singapore
- Thailand
- Vietnam

CAMBODIA

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I. Introduction

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

Puffer fish contains toxic substances that are lethal to human. Some studies have revealed that small puffer fish inhabiting in brackish water or freshwater areas in Southeast Asia are also contaminated with toxins that are identified as tetrodotoxin (TTX) and saxitoxin (STX), toxins that belong to the paralytic shellfish poisoning (PSP) family, and are detected as the main toxic principles. However, in Cambodia, PSP contamination in aquatic animals has not been broadly investigated yet, especially toxins in marine bivalves and molluscs that feed on the toxin-producing dinoflagellate which is the potential source of PSP. On the other hand, PSP is also suggested to be a potential source of biotoxins contamination in shellfish which makes it an important indicator for biotoxins monitoring purposes.

Early studies conducted by Laymithuna Ngy *et al*[1]. about the toxicity in the horseshoe crab (*Carcinoscopius rotundicauda*) revealed the presence of TTX in this species, but nil presence of paralytic shellfish toxins. The toxicity of two species of wild Cambodia freshwater puffer fish of genus *Tetraodon T. Turgudus* and *Tetraodon* sp., was further investigated and found to have high toxicity in their skin and ovary (Ngy L. *et al*., 2008)[2]. Recent studies on toxicity and toxin profiles of Cambodia's marine puffer fish (*Takifugu oblongus*) were investigated and revealed that the toxicities ranged from 10 - 132 mouse units, with the main concentration found in ovaries. The study also found that TTX is the main component while STX is a minor component. It was confirmed that T. *Oblongus* is a hazardous species that is unsafe for human consumption (Ngy L. *et al*., 2009)[3].

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

[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.

Bivalves are considered as one of the potential hosts for 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 Mussel. This species of bivalves is collected from the wild and widely marketed in Cambodia.

In this study, Green Mussel (*Perna viridis*) is identified and investigated for PSP contamination. It is envisaged that the results from this study would provide insights for further researches to be conducted, provide recommendations for future biotoxins monitoring programme in Cambodia, as well as contribute to the biotoxins monitoring programme in the Asian region.

II. Objectives and Goals

The main objective of this study is to investigate PSP contamination in Green Mussel (*Perna viridis*) in Cambodia's marine water using the Mouse Bioassay Method (MBA). A monthly monitoring study would be conducted from June 2011 – May 2012. This study is part of the biotoxins watch programme in Cambodia's marine water, which contributes to the control of biotoxins contamination in Asian region.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

In this study, two sampling sites were identified and selected, Koh Preap and Tumnup Rolok, as shown in Figure C1. Koh Preap and Tumnup Rolok are bivalves-inhabiting locations and they are the favourite fishing grounds for local fishermen. These sampling sites are located in Preah Sihanouk province, one amongst the four provinces in coastal areas situated in the South-western part of the Kingdom of Cambodia.
This province is a productive biodiversity area in the Cambodia's Exclusive Economic Zone (EEZ) where it produces all types of seafood including bivalves and molluscs. Most of the fishery products in this area are captured from the wild, but some are from aquaculture farming. In contrast, this province is an industrial area that potentially post risks to the marine environment in this area. With reference to the Asia's mussel watch programme conducted during 1997 – 2001, implemented by the Centre for Marine Environmental Studies (CMES), Japan discovered some level of toxins contamination in mussels collected from this area, but PSP contamination in the species has not yet been investigated. Therefore, the selected sampling sites are suitable for this study to investigate for possible PSP contamination in Cambodian bivalves in this coastal province.

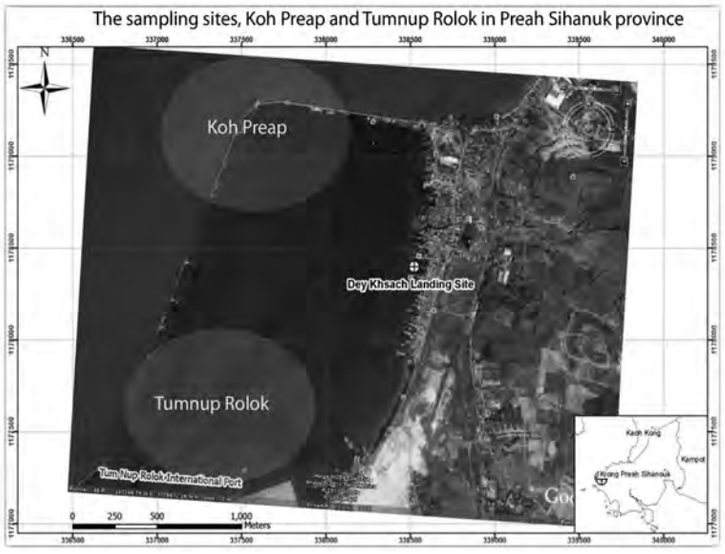

Figure C1. Map showing the sampling sites of the study

Green Mussels (*Perna viridis*) are widely distributed and more preferred by consumers amongst other types of bivalves. This type of bivalves is collected from the wild resource and widely marketed in Cambodia. In this study, Green Mussel (*Perna viridis*) is identified for investigation of PSP contamination.

Figure C2. Identification and measurements of the samples of Green Mussel (Perna viridis)

The samples were collected monthly, on the fourth week of each month starting from June 2011 - May 2012. Each time, 4 - 5 kg of Green Mussel (*Perna viridis*) samples were collected at the fishing grounds from each sampling site. Then, each piece of identified Green Mussel (*Perna viridis*) was recorded for its length and weight (Table C1). The samples were collected in live form and quickly kept in an icebox to transport to Phnom Penh city. At the laboratory in Phnom Penh, the samples were cleaned and washed several times with clean water before the shell was being cut opened. The whole tissues of weight between 350 - 500g were collected and kept in zip-locked plastic bag with proper labelling and stored in a freezer at -25°C until they are used for testing.

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				

Table C1. Average length and weight of collected samples, Green Mussel (Perna viridis)

b. Method of Analysis

350 - 500g of the whole tissue of Green Mussel (*Perna viridis*) was transferred into icebox for analysis at the laboratory of the Centre of Analytical Service and Experimentation (CASE), Department of Science and Technology of Ho Chi Minh City in Vietnam. The analytical method was conducted in accordance with the CASE laboratory standard protocol for PSP detection using a Mouse Bioassay Method (MBA).

In this study, MBA is used to test the PSP level in Green Mussel (*Perna viridis*). PSP is analyzed qualitatively for the amount of positive or negative toxins present. The internationally recognized method used for analysis of PSP toxins is the standard AOAC Mouse Bioassay method (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 40µg STXeq/100g wet weight of tissue. The procedures were:

1. Boil 100g of tissue with 100ml of 0.1 N HCl for 5 minutes.
2. Adjust the volume back to 200ml and pH to 3 (ideally).
3. Inject the intraperitoneal (i.p.) infection of the acidified extract 0.1N HCL solution into 3 white mice.
4. Observe white mice carefully for 1 hour.

Results are then 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 - 0.23µg STXeq). For standard MBA, 1ml of the solution was used and injected intraperitoneally into three male mice, where they were observed for symptoms and the number of times of death.

The median death time (i.e. period between injection and death) was used to calculate the number of MU. Toxicity level of the sample (MU g^{-1}) was determined from the dose–death time relationship (Japan Food Hygiene Association 2005), where 1MU is defined as the amount of toxin required to kill a 20g male mouse within 30 minutes of injection.

c. Limit of Detection & Limit of Quantification

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

d. National Regulatory Limits

No national regulatory limit has been set up yet in the Kingdom of Cambodia. Cambodia Fisheries Administration is mainly adopting limits based on the ASEAN and European Union (EU) Standards in complying with the international conformity for controlling safety of exported and imported fisheries products.

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

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b. Survey Results & Discussion

PSP in Cambodian Green Mussel (*Perna viridis*) was investigated throughout a period from June 2011 to May 2012. All samples were analysed for PSP contamination by using the Standard MBA. The results of this analysis have shown that PSP was Not Detected (negative) in all analysed samples of Green Mussels (*Perva viridis*) from the above-mentioned two sampling sites, Koh Preap and Tumnup Rolok, throughout the sampling period (Table C2).

Month and Year of Sampling	Method	No. of Replica Analysed	Average Sample Weight (g)	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)					

Table C2. Results of the detection of PSP contamination in Green Mussel (Perna viridis)

PSP in humans is caused by ingestion of shellfish containing PSP toxins. These PSP toxins are accumulated by shellfish grazing on algae and eventually producing these toxins. The PSP toxins are produced mainly by dinoflagellates belonging to the genus Alexandrium, which occur in marine environment, but are also produced in freshwater cyanobaceria and associations with calcareous red macroalgae have also 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 Food and Agricultural Organization of the United Nations (FAO)'s reports during the last 20 years, there seems to have been an increase in intoxications caused by PSP toxins. However, this is unclear yet as to whether the increase is real, or attributed to 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's 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's 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

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

also revealed that the environment where the Green Mussels are 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 present in this area. In contrast, the absence of PSP toxins is probably due to the limitations of the detection method used in this study that is not capable of quantifying low level of PSP toxins in this species because PSP toxins level in marine shellfish is probably below the limit detection of 40µg/100g, so it may still pose possible risks to human health in future.

Therefore, it is suggested that further study should be carried out to explore the use of quantitative methods to elucidate the PSP toxins level in the samples clearly. The results from our present study would provide insight for further researches about biotoxins contamination in fisheries products as well as in contributing to biotoxins monitoring programme in the ASEAN region.

c. Corrective Actions

PSP toxins contamination in the Cambodia's Green Mussel (*Perna viridis*) was Not Detected in all samples tested during the period from June 2011 - May 2012, so it is proven that it is safe for human consumption, and hence no corrective action needs to be taken.

V. Problems and Challenges Encountered

The problems and challenges encountered include:

- The samples were transported under long distances for analysis at the laboratory in Vietnam due to the lack of laboratory facilities in Cambodia, thus increasing the cost of analyses. Moreover, it is difficult to make clearance at check-point at times.
- There is limited financial support for the study.
- There is limited background information (references).
- There is limited human resource capacity in this field.

VI. Recommendations and Suggestions for Future Follow-Up Action

Cambodia's marine water may be potentially contaminated with hazardous chemical toxins due to increased 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, long term biotoxins monitoring programme should be set up and human resource capacity building is needed for Cambodia.

INDONESIA

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I. Introduction

Monitoring of shellfish and its environment (water quality) is aimed to protect the consumers from chemical and microbiological hazards that may arise from consuming shellfish products. Sufficient data on the safety status of shellfish is also aimed to increase the utilization of its resources for domestic and export markets.

The government of Indonesia has established several regulations, namely Regulation No. PER.01/MEN/2007 and Regulation No. KEP.01/MEN/2007 which lay down the requirements for Quality and Safety Assurance of Fishery products in general and DG Decree No. 010/DJ-P2HP/2007 with regards to the official control and monitoring of fishery product. These regulations could help to maintain and protect the area of shellfish growth from the domestic and industrial sewage which may contaminate shellfish. The regulation divides the shellfish harvesting area into 4 classes (A-D) based on the several parameters (Table 11). Some observations were done prior to the sampling time, to have an overview of shellfish' and the water quality status. The presence evidence of contamination should be followed by sampling of water and shellfish and laboratory analysis of the present contaminant. The area's status depends on the laboratory results. For example, samples that are taken from an approved area showed results that are not in accordance with the regulatory limits, a reevaluation action should be done and by that time they will be classified as prohibited area.

In Indonesia, the biotoxin monitoring activity which is also known as shellfish sanitation program has been conducted since 1997 as part of a general monitoring program designed to identify and evaluate biological toxins as well as chemical and microbiological contamination of shellfish and the water quality. This program has been scheduled as in Table 12, however the frequency of monitoring might be reduced or terminated when the results achieve satisfactory levels.

Class	Criteria
A (Approved)	• Not contaminated by domestic waste • PSP <80µg/100g • DSP negative • Most Probable Number (MPN) of faecal coliform <14/100 ml water sample and less than 10% of samples containing faecal coliform and not exceeding 43/100 ml water sample. • 5-tube MPN of E.coli < 230/100 g of meat, heavy metals not exceeding the requirements
B (Conditionally Approved)	• Not contaminated by domestic waste • PSP <80µg/100g • DSP negative • MPN of faecal coliform <14/100 ml water sample and less than 10% of samples containing faecal coliform not exceed 43/100 ml water sample. • 5-tube MPN of E.coli > 4,600/100 g of meat, heavy metals not exceeding the requirements
C (Restricted)	• Low content of domestic waste and pollutant • PSP <80µg/100g • DSP negative • MPN of faecal coliform 88/100 ml water sample air and less than 10% of samples containing faecal coliform not exceed 260/100 ml water sample. • 5-tube MPN of E.coli > 4,600/100 g of meat, heavy metals not exceeding the requirements
D (Prohibited)	• High content of domestic waste and pollutant • PSP ≥ 80µg/100g • ASP ≥ 2 mg/100g • heavy metals do not meet the requirements

Table II: Classification of shellfish harvesting area

No.	Parameter	Regulatory limit	Frequency
1	Biotoxin : a) PSP b) DSP c) ASP	 80µg/100g negative by Mouse Bioassay (MBA) 2 mg/100g	Once every 2 weeks during harvesting period, at sampling point
2	Heavy metals : a) Mercury (Hg) b) Lead (Pb) c) Cadmium (Cd)	 0.5 mg/kg 1.0 mg/kg 1.0 mg/kg	Once every 3 months during harvesting period, at sampling point

No.	Parameter	Regulatory limit	Frequency
3	Microbiology:		
	a) E. coli	< 3 MPN	Once every 2 weeks during harvesting period, at sampling point
	b) Salmonella	(-) /25g	

Table I2: Frequency and parameters of water for quality inspection

From the period of 1997-2012, there are several reported cases due to the consumption of contaminated shellfish. On June-September 2010, more than 30 people were hospitalized after consuming clams and fish from Teluk Lasongko and Bau-Bau. They showed similar symptoms, i.e. vomiting, diarrhea, tongue and lips paralysis. The presence of *Peridinium sp.* was confirmed from the fish caught in those area, however no reported data on the clinical sample of the patient. On 16 July 2010, another case has occurred in Lampung due to contaminated shellfish and fish consumption and cause nausea and vomiting. Phytoplankton of Pirodinium bahamense was identified as a cause of the shellfish poisoning.

Indonesia has two main PSP monitoring programme:

1. PSP Monitoring Conducted by National Center for Fish Quality Control (NCQC)

   PSP monitoring has been conducted by NCQC since 1997 and sampling is conducted in the shellfish production area. The samples were analyzed using validated Mouse Bioassay (MBA) method (Limit of Detection, LOD: 40µg STX/100g meat), however, due to limited budget, the sampling frequency could not match up with the procedures performed. In 2011, NCQC conducted PSP monitoring in Sidoarjo, East Java other than the PSP monitoring funded by Japanese Trust Fund II.

2. PSP Monitoring under Japanese Trust Fund II (JTF II) in Tanjung Balai, Asahan. The selection of this location as the project site because Tanjung Balai Asahan is the main shellfish production both for local consumption and export purpose.

II. Objectives and Goals

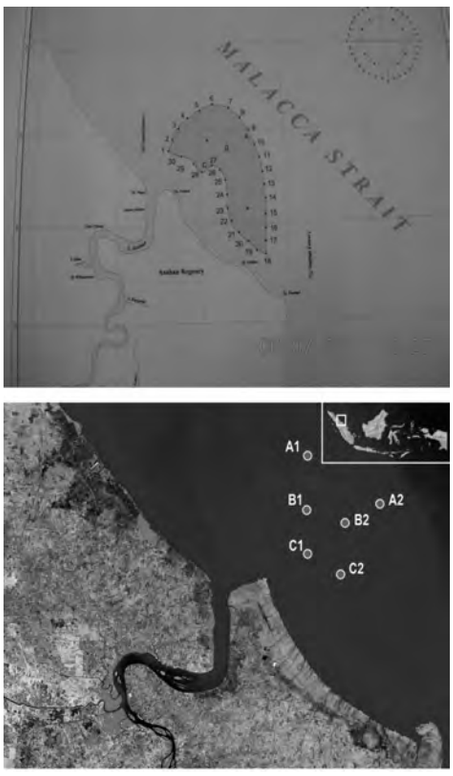
The objectives of shellfish sanitation program are to ensure the quality and safety of shellfish harvested in the production area, as well as to provide data and information required for setting policies or regulations in terms of implementation of quality assurance and safety of fishery products.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

Sampling for wild shellfish and water was conducted on sampling site of shellfish monitoring area of ±7500 ha, where the coordinates of the sampling sites are:

- A1 = 03°06’15” N/ 99°55’00” E
- A2 = 03°04’45” N/ 99°57’30” E
- B1 = 03°04’30” N/ 99°55’00” E
- B2 = 03°04’00” N/ 99°56’15” E
- C1 = 03°03’00” N/ 99°55’00” E
- C2 = 03°02’20” N/ 99°56’00” E



Description:

C = the nearest point to the coast,
B = mid-point of the area,
A = the farthest point

The distance from point C to point B = 3 km, the distance from point B to point A = 3 km

The depth of sea water from surface to bottom/sand depends on the condition of the tides. The depth of sea water for each coordinate of each point during sampling are as follows:

A1 : between 3 - 7 m
A2 : between 1.5 - 5 m
B1 : between 2 - 6 m
B2 : between 1.5 - 5 m
C1 : between 2 - 4 m
C2 : between 1 - 3 m

Figure II. Map of shellfish monitoring area and coordinates of sampling site

The targeted species for JTF II project is Baby Clam (*Meritrix meritrix*). However, as the size of Baby Clam does not meet the harvesting size for consumption during January and February, the shellfish cannot be harvested for analysis purpose. Therefore, Anadara inflata is used for samples during January and February considering that *Meritrix meritrix* grows in the same habitat as *Anadara inflate*, so the toxin content is assumed to be equal.

The samples were tested for the following:

- Shellfish sampling for PSP analysis is conducted every two weeks in 6 coordinates of sampling site with 6 samples per sampling time
- Shellfish is also analysed for heavy metals (Pb, Cd, Hg) every 2 months
- Sampling of water is conducted every 2 months for additional data such as field condition (weather, depth, temperature, pH, Salinity and DO), plankton identification and heavy metals (Pb, Cd and Hg)

b. Method of Analysis

For time of sampling, it was carried out every two weeks from January to December 2011, as follow:

Time of Sampling	Dominant Sample (s)	Remarks
January: • Week 2 (W2): 11 January 2011 • Week 4 (W4): 25 January 2011	kerang bulu (*Anadara inflata*).	-

Time of Sampling	Dominant Sample (s)	Remarks
February: • Week 2 (W2): 8 February 2011 • Week 4 (W4): 22 February 2011	kerang bulu (*Anadara inflata*).	In Week 4, water was carried out for fitoplankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 4 are also tested for heavy metals.
March: • Week 2 (W2): 9 March 2011 • Week 4 (W4): 23 March 2011	baby clam (*Meritrix meritrix*)	-
April: • Week 2 (W2): 11 April 2011 • Week 4 (W4): 29 April 2011	baby clam (*Meritrix meritrix*)	In Week 4, water was carried out for fitoplankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 4 are also tested for heavy metals.
May: • Week 2 (W2): 10 May 2011 • Week 4 (W4): 23 May 2011	baby clam (*Meritrix meritrix*)	-
June: • Week 2 (W2): 10 June 2011 • Week 4 (W4): 22 June 2011	baby clam (*Meritrix meritrix*)	In Week 4, water was carried out for fitoplankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 4 are also tested for heavy metals.
July: • Week 2 (W2): 12 July 2011 • Week 4 (W4): 25 July 2011	baby clam (*Meritrix meritrix*).	-
August: • Week 2 (W2): 9 August 2011 • Week 4 (W4): 22 August 2011	baby clam (*Meritrix meritrix*)	In Week 4, water was analyzed for field condition, plankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 4 are also tested for heavy metals.
September: • Week 2 (W2): 12 September 2011 • Week 4 (W4): 26 September 2011	baby clam (*Meritrix meritrix*).	-

Time of Sampling	Dominant Sample (s)	Remarks
October: • Week 2 (W2): 10 October 2011 • Week 4 (W4): 24 October 2011	baby clam (*Meritrix meritrix*)	In Week 4, water was analyzed for field condition, plankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 4 are also tested for heavy metals.
November: • Week 2 (W2): 8 November 2011 • Week 4 (W4): 21November 2011	baby clam (*Meritrix meritrix*).	-
December: • Week 1 (W1): 5 December 2011 • Week 3 (W3): 19 December 2011	baby clam (*Meritrix meritrix*)	In Week 3, water was analyzed for field condition, plankton and heavy metals (Pb, Cd and Hg) identification. In addition, shellfish in Week 3 are also tested for heavy metals.

Table I3: Time of sampling and dominant sample

The shellfish sampling was conducted through the following steps:

1. Sampling was carried out with the scallop shells dredge gear (scallop rakes) which consists of three types namely: *garuk, tojok* and *tanktailand*. In this monitoring, *garuk* is most often used.

2. After arriving at the area, garuk is lowered to scrape into the sand layer. The shaft is held firmly and then the boat turned around until the *garuk* contains shellfish.

3. *Garuk* is then removed from the water and the shellfish are washed to clean off sand and any other foreign matter by shaking the gear several times on the surface of sea water.

4. Shellfish are then shed on the deck of the boat waiting to be sorted. The sorted shellfish were washed again with clean sea water and put into a sack. Samples were collected at per sampling point and selected with individual weight of ± 50g/ shellfish (minimum weight accepted by company/exporter to get ± 1kg shellfish that yield > 100g of meat.

5. Samples were prepared according to AOAC, 2000. Shells were clean with fresh water and then opened. The inside was cleaned to remove foreign matter. Meat is separated from the shell and it was done carefully to prevent damage or truncation. Meat is then drained and frozen at -20°C until analysis.

Meat of shellfish for saxitoxin (STX) for Paralytic Shellfish Poisoning (PSP) analysis is extracted as follows:

1. 0.5g sample was weighed in centrifuge tube, 1ml of 0.1M HCl was added and vortex for 3 minutes at maximum speed

2. Heated in waterbath at 85°C for 5 minutes

3. Vortex for 1 minute, centrifuge for 10 minutes at 4000rpm

4. Transferred 0.5ml of clear supernatant to a new tube

5. Diluted 20 times with sample extraction buffer prior to testing

6. Analyzed using ELISA method (the results are obtained in units of ng/g and converted to µg/100 g of meat)

For water sampling:

- Sampling site for water is the same as the shellfish sampling site coordinates.
- Water sampling for phytoplankton identification uses plankton net.
- Water sampling for heavy metals done by using Niskin bottle.

c. Limit of Detection & Limit of Quantification

PSP testing uses validated ELISA method:

Limit of Detection (LoD):0.153µg/100g (1.53ng/g)

Limit of Quantification (LoQ):0.236µg/100g (2.36ng/g)

CCβ:< 40µg/100g (400ng/g)

Concentration (ng/g)	Repeatability (%RSD)	Recovery (%)
400	8	121
800	7	115
1200	3	98

Table I4: Repeatability and recovery(%) for various concentration

d. National Regulatory Limits

PSP: maximum 80µg STX/100g (800ng/g) of meat

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

In 2011, NCQC, in coordination with National Accreditation Body of Indonesia participated in PSP proficiency testing provided by Asia Pacific Laboratory Accreditation Cooperation (APLAC). To date, no result is available yet.

b. Survey Results & Discussion

PSP monitoring conducted by NCQC shows that high level of PSP is always detected in Lampung and Ambon and low level in Bangka. Other sites show no detected PSP. Data of PSP conducted by NCQC from 1997 – 2011 is provided in Table I5.

No	Location	Time	Testing method	Samples		(µg/100g of meat)		
				Number	Positive	Minimum	Maximum	Average
1	Ambon	May-1997	MBA	8	4	40	128	96.7
2	Banda Aceh	September-1997	MBA	8	-	ND	ND	-
3	Teluk Hanura & Gebang, Lampung	September-1997	MBA	8	3	47	76	61.3

No	Location	Time	Testing method	Samples		(µg/100g of meat)		
				Number	Positive	Minimum	Maximum	Average
4	Bali	October-1997	MBA	8	-	ND	ND	-
5	Jakarta	December-1997	MBA	8	-	ND	ND	-
6	Tj. Balai, North Sumatera	April-1999	MBA	8	-	ND	ND	-
7	Kep. Riau	March-1999	MBA	8	-	ND	ND	-
8	Kep. Babel	March-1999	MBA	8	-	ND	ND	-
9	Teluk Hanura & Gebang, Lampung	April-1999	MBA	8	2	52	60	56.0
10	Jakarta	March-1999	MBA	8	-	ND	ND	-
11	Cirebon	July-1999	MBA	8	-	ND	ND	-
12	Ambon	August-1999	MBA	8	2	130	135	132.5
13	Tj. Balai, North Sumatera	May-2000	MBA	8	-	ND	ND	-
14	Bandar Lampung	June-2000	MBA	8	2	49	61	55.0
15	Tj. Balai, North Sumatera	April-2001	MBA	12	-	ND	ND	-
16	Musi Banyu Asin, South Sumatera	March-2001	MBA	12	-	ND	ND	-
17	Teluk Hanura & Gebang, Lampung	May-2001	MBA	12	-	ND	ND	-
18	Perairan Blurukidul, East Java	September-2001	MBA	12	-	ND	ND	-
19	Pantai Makassar, South Sulawesi	September-2001	MBA	12	-	ND	ND	-
20	Tj. Balai & Belawan, North Sumatera	April-2002	MBA	12	-	ND	ND	-
21	Teluk Hanura & Pangkep, Lampung	May-2002	MBA	12	-	ND	ND	-
22	Kenjeran & Uj.Pangkah, East Java	July-2002	MBA	12	-	ND	ND	-
23	P.Satando & Batang, South Sulawesi	August-2002	MBA	12	-	ND	ND	-
24	Tj. Balai, North Sumatera	June-2003	MBA	12	-	ND	ND	-
25	Tj. Balai, North Sumatera	August-2003	MBA	12	-	ND	ND	-

No	Location	Time	Testing method	Samples		(µg/100g of meat)		
				Number	Positive	Minimum	Maximum	Average
26	Perairan Sidoarjo, West Java	July-2003	MBA	12	-	ND	ND	-
27	Perairan Sidoarjo, West Java	September-2003	MBA	12	-	ND	ND	-
28	Tj. Balai, North Sumatera	June-2004	MBA	6	-	ND	ND	-
29	Tj. Balai, North Sumatera	August-2004	MBA	6	-	ND	ND	-
30	Teluk Hanura, Lampung	April-2004	MBA	6	-	ND	ND	-
31	Teluk Hanura, Lampung	August-2004	MBA	6	-	ND	ND	-
32	Bangka Belitung	September-2004	MBA	6	-	ND	ND	-
33	Bangka Belitung	December-2004	MBA	6	-	ND	ND	-
34	Sedati & Uj.Pangkah, East Java	July-2004	MBA	6	-	ND	ND	-
35	Sedati & Uj.Pangkah, East Java	October-2004	MBA	6	-	ND	ND	-
36	Tj. Balai, North Sumatera	August-2005	MBA	6	-	ND	ND	-
37	Teluk Hanura & Tj. Putus, Lampung	May-2005	MBA	10	6	64.2	948.5	747.9
38	Perairan Bangka Belitung	July-2005	MBA	6	-	ND	ND	-
39	Sidoarjo & Uj.Pangkah, East Java	September-2005	MBA	12	-	ND	ND	-
40	Musi Banyu Asin, South Sumatera	October-2005	MBA	6	-	ND	ND	-
41	Per. Mangkang, Central Java	October-2005	MBA	6	-	ND	ND	-
42	Tj. Balai, North Sumatera	May-2006	MBA	9	-	ND	ND	-
43	Tj. Balai, North Sumatera	October-2006	MBA	9	-	ND	ND	-
44	Teluk Hanura, Lampung	September-2006	MBA	9	-	ND	ND	-
45	Teluk Hanura, Lampung	December-2006	MBA	9	-	ND	ND	-
46	Per. Sidoarjo & Pasuruan, East Java	September-2006	MBA	9	-	ND	ND	-

No	Location	Time	Testing method	Samples		(µg/100g of meat)		
				Number	Positive	Minimum	Maximum	Average
47	Per. Sidoarjo & Pasuruan, East Java	December-2006	MBA	9	-	ND	ND	-
48	Per. Baguala, Ambon	May-2006	MBA	9	9	674.9	864.5	778.8
49	Per. Baguala, Ambon	August-2006	MBA	9	9	49.9	76.5	61.1
50	Per. Berau, East Kalimantan	April-2006	MBA	9	-	ND	ND	-
51	Per. Berau, East Kalimantan	September-2006	MBA	9	-	ND	ND	-
52	Per. Kawal, Kep. Riau	May-2006	MBA	9	-	ND	ND	-
53	Per. Kawal, Kep. Riau	October-2006	MBA	9	-	ND	ND	-
54	Tj. Balai, North Sumatera	June-2007	MBA	9	-	ND	ND	-
55	Tj. Balai, North Sumatera	December-2007	MBA	9	-	ND	ND	-
56	Per. Blurukidul, Sidoarjo, East Java	April-2007	MBA	9	-	ND	ND	-
57	Per. Blurukidul, Sidoarjo, East Java	August-2007	MBA	9	-	ND	ND	-
58	Per. Baguala, Ambon	August-2007	MBA	9	7	55.84	70.52	60.8
59	Tj. Balai, North Sumatera	May-2008	MBA	9	-	ND	ND	-
60	Tj. Balai, North Sumatera	September-2008	MBA	9	-	ND	ND	-
61	Per. Desa Itam, Bangka Belitung	May-2008	MBA	9	1	46	-	46
62	Per. Desa Itam, Bangka Belitung	November-2008	MBA	9	-	ND	ND	-
63	Teluk Hanura, Lampung	March-2008	MBA	9	-	ND	ND	-
64	Teluk Hanura, Lampung	November-2008	MBA	9	-	ND	ND	-
65	P. Panggang, Kep. Seribu	March-2008	MBA	9	-	ND	ND	-
66	P. Panggang, Kep. Seribu	September-2008	MBA	9	-	ND	ND	-
67	Per. Blurukidul, Sidoarjo, East Java	April-2008	MBA	9	-	ND	ND	-

No	Location	Time	Testing method	Samples		(µg/100g of meat)		
				Number	Positive	Minimum	Maximum	Average
68	Per. Blurukidul, Sidoarjo, East Java	September-2008	MBA	9	-	ND	ND	-
69	Per. Baguala, Ambon	May-2008	MBA	9	-	ND	ND	-
70	Per. Baguala, Ambon	October-2008	MBA	9	-	ND	ND	-
71	Tj. Balai, North Sumatera	May-2009	MBA	9	-	ND	ND	-
72	Tj. Balai, North Sumatera	August-2009	MBA	9	-	ND	ND	-
73	Teluk Hanura, Lampung	April-2009	MBA	9	-	ND	ND	-
74	Teluk Hanura, Lampung	October-2009	MBA	9	-	ND	ND	-
75	P. Panggang, Kep. Seribu	April-2009	MBA	9	-	ND	ND	-
76	P. Panggang, Kep. Seribu	September-2009	MBA	9	-	ND	ND	-
77	Per. Blurukidul, Sidoarjo, East Java	March-2009	MBA	9	-	ND	ND	-
78	Per. Blurukidul, Sidoarjo, East Java	August-2009	MBA	9	-	ND	ND	-
79	Per. Gerupuk, West Nusa Tenggara	May-2009	MBA	9	-	ND	ND	-
80	Per. Gerupuk, West Nusa Tenggara	October-2009	MBA	9	-	ND	ND	-
81	Per. Baguala, Ambon	May-2009	MBA	9	-	ND	ND	-
82	Per. Baguala, Ambon	August-2009	MBA	9	-	ND	ND	-
83	Per. Blurukidul, Sidoarjo, East Java	March-2011	ELISA	9	-	ND	ND	-
84	Per. Blurukidul, Sidoarjo, East Java	June-2011	ELISA	9	1	0.247	ND	0.247
85	Per. Blurukidul, Sidoarjo, East Java	September-2011	ELISA	9	3	0.278	0.303	0.29

ND: Not Detected

Table I5. Data of PSP conducted by NCQC from 1997 – 2011

Meanwhile, for PSP Monitoring under JTF II, PSP was detected in samples of *Anadara inflata* with results between 0.567 - 1.770µg/100g of meat and between 0.635 - 1.334µg/100g of meat in January and February respectively. Only one out of whole samples of *Meritrix meritrix* gave detected results in March 2011 with 0.237µg/100g of meat. The detected results are above Limit of Quantitation (LOQ). Detailed data of PSP funded by JTF II from January – December 2011 is provided in Table I6.

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
1	ELISA	January	*Anadara inflata* Jan W2 – A1	1.770
			Anadara inflata Jan W2 – A2	1.318
			Anadara inflata Jan W2 – B1	1.136
			Anadara inflata Jan W2 – B2	1.140
			Anadara inflata Jan W2 – C1	1.087
			Anadara inflata Jan W2 – C2	1.152
			Anadara inflata Jan W4 – A1	1.239
			Anadara inflata Jan W4 – A2	1.208
			Anadara inflata Jan W4 – B1	0.977
			Anadara inflata Jan W4 – B2	0.885
			Anadara inflata Jan W4 – C1	0.700
			Anadara inflata Jan W4 – C2	0.567
2	ELISA	February	*Anadara inflata* Feb W2 – A1	1.314
			Anadara inflata Feb W2 – A2	1.256
			Anadara inflata Feb W2 – B1	0.804
			Anadara inflata Feb W2 – B2	1.165
			Anadara inflata Feb W2 – C1	0.635
			Anadara inflata Feb W2 – C2	0.897
			Anadara inflata Feb W4 – A1	1.247
			Anadara inflata Feb W4 – A2	1.334
			Anadara inflata Feb W4 – B1	1.109
			Anadara inflata Feb W4 – B2	1.030

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
			Anadara inflata Feb W4 – C1	0.987
			Anadara inflata Feb W4 – C2	0.968
3	ELISA	March	*Meritrix meritrix* Mar W2 – A1	ND
			Meritrix meritrix Mar W2 – A2	ND
			Meritrix meritrix Mar W2 – B1	ND
			Meritrix meritrix Mar W2 – B2	ND
			Meritrix meritrix Mar W2 - C1	ND
			Meritrix meritrix Mar W2 – C2	ND
			Meritrix meritrix Mar W4 – A1	ND
			Meritrix meritrix Mar W4 – A2	ND
			Meritrix meritrix Mar W4 – B1	0.237
			Meritrix meritrix Mar W4 – B2	ND
			Meritrix meritrix Mar W4 – C1	ND
			Meritrix meritrix Mar W4 – C2	ND
4	ELISA	April	*Meritrix meritrix* Apr W2 – A1	ND
			Meritrix meritrix Apr W2 – A2	ND
			Meritrix meritrix Apr W2 – B1	ND
			Meritrix meritrix Apr W2 – B2	ND
			Meritrix meritrix Apr W2 - C1	ND
			Meritrix meritrix Apr W2 – C2	ND
			Meritrix meritrix Apr W4 – A1	ND
			Meritrix meritrix Apr W4 – A2	ND
			Meritrix meritrix Apr W4 – B1	ND
			Meritrix meritrix Apr W4 – B2	ND
			Meritrix meritrix Apr W4 – C1	ND
			Meritrix meritrix Apr W4 – C2	ND

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
5	ELISA	May	*Meritrix meritrix* May W2 – A1	ND
			Meritrix meritrix May W2 – A2	ND
			Meritrix meritrix May W2 – B1	ND
			Meritrix meritrix May W2 – B2	ND
			Meritrix meritrix May W2 - C1	ND
			Meritrix meritrix May W2 – C2	ND
			Meritrix meritrix May W4 – A1	ND
			Meritrix meritrix May W4 – A2	ND
			Meritrix meritrix May W4 – B1	ND
			Meritrix meritrix May W4 – B2	ND
			Meritrix meritrix May W4 – C1	ND
			Meritrix meritrix May W4 – C2	ND
6	ELISA	June	*Meritrix meritrix* Jun W2 – A1	ND
			Meritrix meritrix Jun W2 – A2	ND
			Meritrix meritrix Jun W2 – B1	ND
			Meritrix meritrix Jun W2 – B2	ND
			Meritrix meritrix Jun W2 - C1	ND
			Meritrix meritrix Jun W2 – C2	ND
			Meritrix meritrix Jun W4 – A1	ND
			Meritrix meritrix Jun W4 – A2	ND
			Meritrix meritrix Jun W4 – B1	ND
			Meritrix meritrix Jun W4 – B2	ND
			Meritrix meritrix Jun W4 – C1	ND
7	ELISA	July	*Meritrix meritrix* Jul W2 – A1	ND
			Meritrix meritrix Jul W2 – A2	ND

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
			Meritrix meritrix Jul W2 – B1	ND
			Meritrix meritrix Jul W2 – B2	ND
			Meritrix meritrix Jul W2 - C1	ND
			Meritrix meritrix Jul W2 – C2	ND
			Meritrix meritrix Jul W4 – A1	ND
			Meritrix meritrix Jul W4 – A2	ND
			Meritrix meritrix Jul W4 – B1	ND
			Meritrix meritrix Jul W4 – B2	ND
			Meritrix meritrix Jul W4 – C1	ND
			Meritrix meritrix Jul W4 – C2	ND
8	ELISA	August	*Meritrix meritrix* Aug W2 – A1	ND
			Meritrix meritrix Aug W2 – A2	ND
			Meritrix meritrix Aug W2 – B1	ND
			Meritrix meritrix Aug W2 – B2	ND
			Meritrix meritrix Aug W2 - C1	ND
			Meritrix meritrix Aug W2 – C2	ND
			Meritrix meritrix Aug W4 – A1	ND
			Meritrix meritrix Aug W4 – A2	ND
			Meritrix meritrix Aug W4 – B1	ND
			Meritrix meritrix Aug W4 – B2	ND
			Meritrix meritrix Aug W4 – C1	ND
			Meritrix meritrix Aug W4 – C2	ND
9	ELISA	September	*Meritrix meritrix* Sep W2 – A1	ND
			Meritrix meritrix Sep W2 – A2	ND
			Meritrix meritrix Sep W2 – B1	ND
			Meritrix meritrix Sep W2 – B2	ND

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
			Meritrix meritrix Sep W2 - C1	ND
			Meritrix meritrix Sep W2 – C2	ND
			Meritrix meritrix Sep W4 – A1	ND
			Meritrix meritrix Sep W4 – A2	ND
			Meritrix meritrix Sep W4 – B1	ND
			Meritrix meritrix Sep W4 – B2	ND
			Meritrix meritrix Sep W4 – C1	ND
			Meritrix meritrix Sep W4 – C2	ND
10	ELISA	October	*Meritrix meritrix* Oct W2 – A1	ND
			Meritrix meritrix Oct W2 – A2	ND
			Meritrix meritrix Oct W2 – B1	ND
			Meritrix meritrix Oct W2 – B2	ND
			Meritrix meritrix Oct W2 - C1	ND
			Meritrix meritrix Oct W2 – C2	ND
			Meritrix meritrix Oct W4 – A1	ND
			Meritrix meritrix Oct W4 – A2	ND
			Meritrix meritrix Oct W4 – B1	ND
			Meritrix meritrix Oct W4 – B2	ND
			Meritrix meritrix Oct W4 – C1	ND
			Meritrix meritrix Oct W4 – C2	ND
11	ELISA	November	*Meritrix meritrix* Nov W2 – A1	ND
			Meritrix meritrix Nov W2 – A2	ND
			Meritrix meritrix Nov W2 – B1	ND
			Meritrix meritrix Nov W2 – B2	ND
			Meritrix meritrix Nov W2 - C1	ND
			Meritrix meritrix Nov W2 – C2	ND

No	Testing Mehod	Period	Sample ID. (species, month, week, coordinat)	Concentration (µg/100g of meat)
			Meritrix meritrix Nov W4 – A1	ND
			Meritrix meritrix Nov W4 – A2	ND
			Meritrix meritrix Nov W4 – B1	ND
			Meritrix meritrix Nov W4 – B2	ND
			Meritrix meritrix Nov W4 – C1	ND
			Meritrix meritrix Nov W4 – C2	ND
12	ELISA	December	*Meritrix meritrix* Dec W1 – A1	ND
			Meritrix meritrix Dec W1 – A2	ND
			Meritrix meritrix Dec W1 – B1	ND
			Meritrix meritrix Dec W1 – B2	ND
			Meritrix meritrix Dec W1 - C1	ND
			Meritrix meritrix Dec W1 – C2	ND
			Meritrix meritrix Dec W3 – A1	ND
			Meritrix meritrix Dec W3 – A2	ND
			Meritrix meritrix Dec W3 – B1	ND
			Meritrix meritrix Dec W3 – B2	ND
			Meritrix meritrix Dec W3 – C1	ND
			Meritrix meritrix Dec W3 – C2	ND

ND: not detected

Table I6. Data of PSP from Tanjung Balai, Medan funded by Japanese Trust Fund II from January – December 2011

PSP monitoring conducted by NCQC during 1997 – 2011 shows that the PSP content of shellfish obtained from Teluk Hanura and Gebang, Lampung and Baguala, Ambon exceeds the requirements several times. Teluk Baguala, Ambon has been closed at 1997, 1999 and 2005 and the shellfish harvesting area has been changed as prohibited. Any shellfish harvesting activity has been prohibited in the areas of Teluk Hanura and Gebang since 2005 and they have been labelled as restricted area in year 1997 and 1999. Both areas experience frequent red tide and the waters are closed to shellfish harvesting area. The government has also banned people for eating shellfish from waters of Teluk Hanura and Gebang, Lampung and Baguala, Ambon.

Sample for PSP analysis sponsored by the JTF II is *Anadara inflate* during January and February 2012 and *Meritrix meritrix* during March until December 2012.

No PSP toxin exceed the requirements found in the 144 shellfish samples obtained from Tanjungbalai Asahan waters in North Sumatra which serve as the sampling location in 2011 in biotoxin monitoring activities sponsored by the JTF II. Even if any PSP toxins were detected by ELISA, it was still far below the requirements in the range of 0.237 - 1.77µg/100g of meat. There were PSP toxin found in small concentrations in January and February on *Anadara inflata* and one sample of *Meritrix meritrix* in March. *Anadara inflata* has a larger size than *Meritrix meritrix*, so the presence of PSP toxin could be more accumulated in this species.

Sampling history in Tanjungbalai Asahan waters since 1998 showed that there were no samples that exceed the requirements.

c. Corrective Actions

Some samples give positive results using the ELISA method particularly samples of Anadara inflata. To illustrate, PSP results between 0.567 - 1.770µg/100g of meat in January and between 0.635 - 1.334µg/100g of meat in February. Only one out of whole sample of Meritrix meritrix give detected results in March 2012 with 0.237µg/100g of meat. The detected results are above LOQ, however these results are not confirmed as the results are far below national regulatory standards (maximuml 80µg STX/100g (800ng/g) of meat.

V. Problems and Challenges Encountered

No significant problems were encountered except for the sampling process. The long distance between the sampling location and laboratory testing requires the samples to be frozen and sent by air so the testing is conducted within the schedule.

The selection of species that serve as a sample is dependable on the availability in nature at the time of sampling. Shellfish are used as the sample as an individual with the whole weight of ±50g (acceptable for canning companies and exporters), so the smaller sizes should be returned back into the water.

VI. Recommendations and Suggestions for Future Follow-Up Action

This project should be continued periodically in the future to build a shared understanding of the potential of PSP toxins in regional waters. It is also important to establish shellfish reference laboratory in ASEAN region in order to strengthen laboratories that have recently developed monitoring shellfish. In addition it is also important to set up a community of Harmful Algae Blooms (HAB) at the regional level as a forum for sharing knowledge and exchanging information related to shellfish toxin and also to learn about other toxins that are present in the shellfish, including the methods of analysis.

LAO PDR

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Department of Livestock and Fisheries for Lao PDR

I. Introduction

The Department of Livestock and Fisheries (DLF) is responsible for fisheries and aquaculture throughout the country. Under DLF, there is one Fisheries Division (FD) and there is one center known as Namxouang Aquaculture Development Center (NADC) and the center is responsible for the development of aquaculture techniques to farmers and the newest addition to the centre is the Product Quality Control Laboratory, which is responsible for verifying the quantity and quality of fisheries products.

The Fisheries Law is approved by the National parliament in 2009. According to the Fisheries Law, fish processing development and other aquatic animal products, including the verification of the quality and quantity of fisheries products should be carried out before disseminating to the consumers to ensure the quality and safety of fish and fisheries products for food security within the country.

Lao PDR does not have adequate facilities and there are insufficient equipmentas well as limited human resources to carry out biotoxins analysis. However, under the collaboration of Japanese Trust Fund II (JTF II) with Southeast Asian Fisheries Development Center/ Marine Fisheries Research Department (SEAFDEC/ MFRD), Singapore, the Biotoxin Monitoring in ASEAN project was organized. The survey on biotoxins monitoring was conducted in Lao PDR, together with collaboration work done with the Department of Fisheries, the Fish Inspection and Quality Control Division.

II. Objectives and Goals

The objectives and goals of this project are to strengthen the ASEAN capacity in detecting the outbreak of biotoxins in ASEAN countries and to enhance the laboratory for the analysis of biotoxins in bivalve molluscs.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

With regards to the sampling collection in Lao PDR, most marine products are imported from neighbouring countries. Therefore, the collection of imported Green Mussels (*Perna viridis*) samples is of great importance for laboratory analysis, especially under the project Biotoxins Monitoring in ASEAN.

Green Mussel (*Perna viridis*) samples are being collected from the market area. The selected sampling site is the Thongkankham market located in Vientiane, Lao PDR. It is a big wholesale commercial market which sells mainly imported marine products. The target species are imported Green Mussels (*Perna viridis*). Other than the green mussels, blood cockle is also one of the species that is imported the neighbouring countries. 12 samples were

collected and the sampling size was 1kg per sample.

b. Method of Analysis

The outside of the shellfish sample (Green Mussel (*Perna viridis*)), which is collected from the market, is cleaned by rinsing with tap water. Thereafter, the shellfish is pried open and the inside is rinsed with tap water. The tissue is first removed from the shellfish with a knife, then placed in a sieve to drain out the excess water and transferred to a suitable container. The tissue is blended till homogeneous and then stored at temperature ≤-18°C.

The two methods of analysis used are Paralytic Shellfish Poisoning (PSP) by Mouse Bioassay (MBA) and PSP by High Performance Liquid Chromatography (HPLC). PSP by MBA is used for the screening of samples and the reference method used is AOAC 1995, Volume II.

For MBA method, quality control is ensured by first inoculating three mice with reagent blank, and then inoculates five mice with diluted Saxitoxin (STX) standard for the collection of conversion factor. The death time obtained for the mice should be between 5 - 7 minutes.

PSP by HPLC is then used as the confirmation method for the detection of biotoxins in samples. The reference method used is AOAC official method 2005.06. Quality control is ensured through reagent blank. Spiked sample is prepared and duplicate testing is conducted.

c. Limit of Detection & Limit of Quantification

Limit of Detection (LOD) = 0.006µg/g (STX)

Limit of Quantification (LOQ) = 0.06µg/g (STX)

d. National Regulatory Limits

PSP in shellfish = 0.80µg/g (STX)

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

Inter-Laboratory Proficiency Participation	% RSD_R
1. Inter-lab comparison test for PSP by MBA with National Oceanic and Atmospheric Administration (NOAA); United States of America (USA)	18.3
2. Inter-lab comparison test for PSP by MBA with Department of Fisheries Laboratory and Central Laboratory (Thailand) Co., Ltd	8.6

Table L1: Summary of Inter-Laboratory Proficiency Testing Participation

b. Survey Results & Discussion

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Thongkhank-hammarket, Vientiane Capital, Lao PDR.	September - December 2011	PSP (MBA)	5	Not Detected	< 35µg STX eq/ 100g	< 35µg STX eq/ 100g
		PSP (HPLC)	2	Not Detected	-	Not Detected

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Thongkhank-hammarket, Vientiane Capital, Lao PDR.	January -May 2012	PSP (MBA)	7	Not Detected	< 35µg STX eq/ 100g	< 35µg STX eq/ 100g
		PSP (HPLC)	3	Not Detected	-	Not Detected

Table L2: Survey Results

No biotoxins were detected in all 12 samples of Green Mussel (*Perna viridis*). All bivalve mollusc products are imported from neighbouring countries; hence there is a need to continue to conduct surveys on the biotoxin monitoring activity to ensure food safety, particularly on fisheries products and to ensure that they are liberated from biotoxins contamination.

c. Corrective Actions

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The suggestions include:

- To establish networks in the Asian region so as to develop the method of analysis and to share information among ASEAN member countries.
- To continue the training course on biotoxins activity to strengthen the capacity of the government authorities in ASEAN countries.

V. Problems and Challenges Encountered

There is a trend of lesser consumption of Green Mussels (*Perna Viridis*) in Lao PDR. As green mussel is not the most popular type of shellfish consumed in Lao PDR, and the imported quantity is based on the amount of customer's order, thus the collection of sample is restricted. During the period of conducting the survey, the maximum order is 10kg per week and the minimum order is 3kg per week. Due to time constraint, the frequency of the sample collection will be required once every month and the dates and times for sample collection could not be fixed.

VI. Recommendations and Suggestions for future Follow-up action

There should be establishment of network in the Asian region to develop methods of analysis and sharing information within ASEAN countries. Training course on Biotoxins activity should be conducted to strengthen the capacity of the central and local government officers particularly the laboratory staffs in ASEAN member countries. There should be a training courses on the analysis of Biotoxins and multiple shellfish species should be targeted for analysis.

MALAYSIA

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Department of Fisheries

I. Introduction

Since 1995, the Department of Fisheries, Malaysia has carried out an annual identification of planktons from seawaters in the representative sampling sites along the Peninsular's coastal areas through the Sanitary and Phytosanitary (SPS) Programme. However, Sabah (a coastal state in Borneo Island) already has a similar monitoring programme for shellfish and waters since the early 1970s. Occasional outbreaks of biotoxins, especially Paralytic Shellfish Poisoning (PSP) due to *Pyrodinium bahamense* (a dinoflagellate), have been reported in the west-coast of Sabah waters in the 1970s. This was due to the annual occurrence of algae blooms, but isolated reports were not well documented for various reasons.

In 1991, the first PSP outbreak case reported were caused by *Gymnodinium catenatum* which could be found in farmed mussels located in Sebatu, Melaka. In 2001, six school children were hospitalized after consuming benthic clams (lokan-*Polymesoda*) contaminated with algal toxins (*Alexandrium minutum*) in brackish water lagoons off Tumpat, Kelantan, which confirmed the contamination of Saxitoxins (STXs) toxins. In 2002, blooms of *Prorocentrum minimum*, was observed in South Johor waters at the Water Front City in the months of July, August and September.

Thus far, a comprehensive biotoxin monitoring system / procedures have yet to be established. Nevertheless, through the Japanese Trust Fund II (JTF II) Programme for Biotoxin Survey in 2011, six samples of Green Mussels (*Perna viridis*) from Pasir Gudang, Johor have been analyzed for the presence of PSP. This report elaborates on the work done under this programme.

II. Objectives and Goals

The objectives are to analyse the PSP levels in fish products, mainly from Green Mussels (*Perna viridis*), to identify the distributions and toxicity level of PSP in fish products and to ensure fish products are free from PSP contamination.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

Random sampling method is used for the survey. The sampling site is Pasir Gudang located in Johor and targeted species are Green Mussels (*Perna viridis*), with one sample done every month. The sampling size is 10 - 20 individuals per sample.

b. Method of Analysis

The sample preparation method used is Oshima method. The analytical method used is High

Performance Liquid Chromatography (HPLC) post column fluorescent detector method which is also known as the Oshima method. The quality control measure used is by conducting one quality check for every ten samples.

c. Limit of Detection & Limit of Quantification

	Limit of Detection (LOD) (pico mol/g)
GTX 4	19.7
GTX 1	12.08
GTX 5	10.83
GTX 3	2.71
GTX 2	3.57
Neo-STX	65.6
Dc STX	49.6
STX	52.0
C1	1.70
C2	1.80

Table MA1: Limit of Detection (LOD) for various biotoxins

d. National Regulatory Limits

There are currently no established national regulatory limits but international standards were used as regulatory purpose.

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

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b. Survey Results & Discussion

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Johor	26 April 2011	Green Mussel	1	0.40	-	-
Johor	2 June 2011	Green Mussel	1	48.20	-	-
Johor	26 July 2011	Green Mussel	1	11.40	-	-
Johor	25 August 2011	Green Mussel	1	3.48	-	-
Johor	30 October 2011	Green Mussel	1	2.20	-	-
Johor	30 November 2011	Green Mussel	1	4.00	-	-

Table MA2: Survey Results

All the results showed that the concentrations of PSP in Green Mussel (*Perna viridis*) samples did not exceed the 80µg eq. STX /100g limits. This implied that the Green Mussels are safe for consumption throughout the period of April - November 2011.

c. Corrective Actions

No corrective action is deemed necessary at this stage.

V. Problems and Challenges Encountered

The problems encountered during the survey are lack of manpower and budget constraints in executing monthly sampling. At times, sampling could not be carried out in designated sampling sites due to the contamination of water quality which led to little production of Green Mussels. During the analyses of PSP, there were difficulties in identifying the peak and retention time for the samples as compared to the standards, and also in getting the limit of detection for each PSP, including their calculations.

VI. Recommendations and Suggestions for Future Follow-Up Action

Shellfish Sanitation Monitoring Programme, which also includes the Biotoxins Survey, is a very important programme in ensuring the safety and quality of bivalve or shellfish for both local consumption and export purposes. In the long run, such programme would help to sustain the industry. As such the monitoring of PSP and other biotoxins in Green Mussel, Cockle and Oyster cultured areas needs to be carried out annually and regularly, at both national and ASEAN's coastal countries level.

It is recommended that the provisions for specific budget, staff capacity and staff capability to analyze samples can be reconsidered. Technical difficulties such as peak and retention time determination as well as calculation for LOD would require further elaborations and trainings so as to ascertain that accurate results (i.e. true value) are achieved consistently. This would eventually help in arriving at a better management decision.

MYANMAR

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Analytical Laboratory Section of FIQC
Department of Fisheries

I. Introduction

For the previous year, no biotoxins monitoring system and procedures were carried out in Myanmar. Most of the species of shellfish are distributed in the coastal region area and people who consume shellfish are usually located in these coastal regions. However, majority of the people in Myanmar do not prefer to eat shellfish. There is no history and evidence of any biotoxin incidents or outbreaks in Myanmar as there were no monitoring practice and surveillance programme for biotoxins. The country's Department of Fisheries (DoF) just started participating in the "Biotoxins Monitoring in ASEAN" programme in year 2009, under the Japanese Trust Fund II (JTF II) Project on Chemical and Drug Residues in Fish and Fish Products in Southeast Asia, led by the Marine Fisheries Research Department (MFRD), Southeast Asian Fisheries Development Center (SEAFDEC).

Under this progamme, a survey was conducted and the monitoring period was classified into two quarters, with the first quarter from January to June 2011 and the second quarter from July to December 2011. Toxins such as Saxitoxin for Paralytic Shellfish Poisoning (PSP), Okadaic Acid for Diarrhetic Shellfish Poisoning (DSP) and Domoic Acid for Amnesic Shellfish Poisoning (ASP) in mussels and hard clams are being monitored. The monitoring areas were Kawthaung District and Tanintharyi Region which were located in the Southern parts of Myanmar. Samples were collected at least one month before analysis by DoF officers in the Tanintharyi region.

II. Objectives and Goals

The objectives are to

- increase the knowledge about biotoxins
- raise awareness to the people in Myanmar
- protect the consumers in the event of any biotoxins outbreak
- practice and control biotoxin outbreaks by surveillance and monitoring programme
- train skilful personnel to assist laboratory staffs to carry out biotoxins analysis
- ensure uniformity and compliance with the standards of biotoxins monitoring in all member states.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

The sampling sites were Kawthaung District and Tanintharyi Region, which were located in the southern parts of Myanmar, along the coastal region areas. The targeted species for biotoxins monitoring are Green Mussels (*Perna viridis*) and Hard Clam (*Metretrix casta*). 10 samples were collected for duplicate determination.

Samples were collected directly from sampling

sites and put into insulated boxes. These samples were kept in chilled conditions, where temperatures were controlled between 0 to 4oC and then sent to the analytical laboratory in Yangon by air.

b. Method of Analysis

The testing procedures of ABRAXIS chemical test kit from Netherlands was used for sample preparation and the ELISA method was used for analysis. Quality control measures were achieved by ensuring that:

- every batch of analysed samples used the standard series for calibration curve,
- each sample went through duplicate analysis,
- spiked sample was used for percentage recovery,
- zero standard was used for OD,
- acceptance criteria for co-efficient, r, must be ≥ 0.98,
- duplicate analysis (with separate extraction) was done for every 10% sample and
- percentage of RPD must be ≤ 30.

c. Limit of Detection & Limit of Quantification

	1st Quarter	Limit of Detection (LOD)	Limit of Quantification (LOQ)
PSP	(G.M)	0.948683µg/100g	3.1622776µg/100 g
	(H.C)	1.4491376µg/100g	4.8304589µg/100g
DSP	(G.M)	0.9486832µg/100g	3.1622776µg/100g
	(H.C)	1.264911µg/100g	4.2163702µg/100g
ASP	(G.M)	5.0596442µg/100g	16.8654808µg/100g
	(H.C)	6.32455µg/100g	21.081851µg/100g

	2nd Quarter	Limit of Detection (LOD)	Limit of Quantification (LOQ)
PSP	(G.M)	1.2649110µg/100g	4.2163702µg/100g
	(H.C)	1.5811388µg/100g	5.2704628µg/100g
DSP	(G.M)	1.4491377µg/100g	4.8304590 µg/100g
	(H.C)	1.5491933µg/100g	5.1639778µg/100g
ASP	(G.M)	4.4271887µg/100g	14.7572958µg/100g
	(H.C)	6.9570109µg/100g	23.1900362µg/100g

d. National Regulatory Limits

There are no established national regulatory limits for PSP, DSP and ASP in Myanmar yet. Myanmar DoF complies with European Union (EU) Standard and other importing countries requirements.

The maximum tolerance levels established by the EU and their criteria are as follows:

- PSP: 40 - 80µg/100g edible
- DSP: 16µg/100g
- ASP: 2000µg/100g

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

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b. Survey Results & Discussion

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Kawthaung Tanintharyi Region	January – June 2011	PSP	GM 10	0	1	0.9
			HC 10	0	1	0.3
		DSP	GM 10	0	1	0.1
			HC 10	0	1	0.2
		ASP	GM 10	0	6.0	3.2
			HC 10	2	6.0	4.0
	July – December 2011	PSP	GM 10	0	1	0.8
			HC 10	0	1	0.5
		DSP	GM 10	0	1	0.3
			HC 10	0	1	0.4
		ASP	GM 10	2	6.0	4.2
				0	6.0	3.4

Table MY1: Survey results

All the test results including the 1st and 2nd half of Biotoxins Survey concluded that each average concentration of PSP, DSP and ASP was less than EU limits. Furthermore, all test results could be concluded as "Not Detected (ND)" because the average concentration for PSP, DSP and ASP are less than the LOD.

c. Corrective Actions

Corrective actions required were:

- verification of the analysis method
- participation in the Proficiency Testing programme
- verification of sampling method

V. Problems and Challenges Encountered

Problems and challenges encountered included:

- Insufficient project funding for overall survey charges. At present, only ELISA method was used for biotoxins survey in place of High Performance Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) as the latter method is more expensive than the ELISA method. Thus LC/MS/MS method is not used.
- Sampling problems.
- Transportation problems.

VI. Recommendations and Suggestions for Future Follow-Up Action

This project should be conducted annually and future trainings that were to be conducted could be structured to allow better understanding on biotoxins analysis as well as the principles and procedures of biotoxins monitoring system. There should be more manpower to carry out the future trainings to maximise the laboratory's capability in biotoxins testing. Further investigations to be carried out on aquatic fish species such as Grouper, Spanish mackerel and John's snapper to detect other potentially toxic substances such as Ciguatera Fish Poisoning (CFP). It is recommended to set up a new ASEAN reference laboratory which focuses on Biotoxin analysis in the ASEAN region. It is also recommended for all ASEAN member countries to be contacted with only one Biotoxins Proficiency testing service provider.

PHILIPPINES

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I. Introduction

The Bureau of Fisheries and Aquatic Resources (BFAR) implements a marine biotoxin monitoring programmes in support to the regulatory functions of the Bureau and other allied agencies including local government units. This monitoring programme aims to migitate the health issues (shellfish poisoning episodes) and economic losses through effective and efficient monitoring and management of marine biotoxins, provide quality control for trade of shellfish both in the domestic and international markets, and aid formation of appropriate policies. Proper regulatory actions can be done to address Harmful Algal Blooms (HABs) when information is acquired in a timely manner through the monitoring program. It also supports the information and education campaign against adverse effects of HABs. The BFAR central office laboratory serves as the reference laboratory to designated BFAR regional laboratories and some local government run units.

The core of the monitoring activities is the shellfish toxicity analysis. Collected shellfish samples are analysed for toxicity using mouse bioassay (MBA). Toxicity analysis results served as criteria in determining which areas are to be considered positive or free from toxin. Monitoring data from surveys are consolidated as basis for the regular issuance of shellfish bulletins and timely shellfish advisories. Bulletins are issued twice a month and are published in two newspapers of national circulation. On the other hand, advisories are issued when an area is positive for Harmful Algal Bloom (HAB), and when the HAB has dissipated to a toxicity level below the national regulatory standard.

II. Objectives and Goals

The monitoring programme aims to mitigate the health issues (shellfish poisoning episodes) and economic losses through effective and efficient monitoring and management of marine biotoxins. In addition, information on marine toxicity data obtained can be considered as basis for policy formulation.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

Five sampling stations were established within Sorsogon Bay, as shown in Figure P1 and Table P1. These sites were shellfish farms and Green Mussels (*Perna viridis*) samples were collected from the bamboo stakes within each station. Approximately 20 marketable sized Green Mussels (*Perna viridis*) were collected.

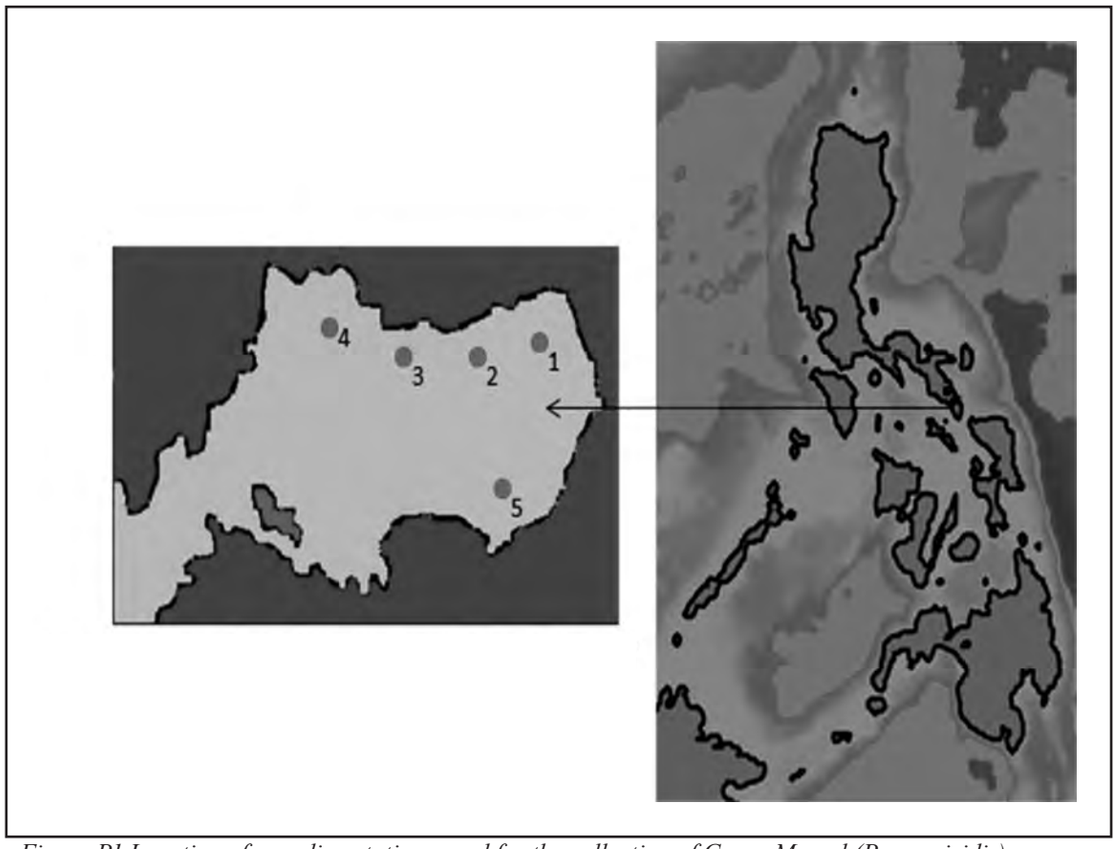

Figure P1 Location of sampling stations used for the collection of Green Mussel (Perna viridis)

Sampling Area	Latitude	Longitude
1. Pier site, Sorsogon City	12° 57' 23.3 N	124° 00' 17.3 E
2. Cambulaga	12° 57' 01.9 N	123° 59' 39.7 E
3. Waray dahon	12° 56' 51.9 N	123° 57' 57.6 E
4. Rizal	12° 57' 59.9 N	123° 54' 34.5 E
5. Casiguran	12° 53' 03.7 N	124° 00' 10.1 E

Table P1: Description of sampling area

Green Mussels (*Perna viridis*) were prepared according to AOAC Method No. 959.08 Section D. The Green Mussels (*Perna viridis*) were cleaned to remove all foreign matter, the 100 - 150g of shellfish meat was collected and the excess water was drained off. The shellfish meat was subsequently homogenized at high speed until no lumps were visible.

Toxins from the Green Mussels (*Perna viridis*) were extracted according to AOAC Method No. 959.08 Section E. 100g of homogenized meat was weighed and added with 100ml of 0.10N HCl. The final mixture was weighed. The pH of the mixture was also measured and adjusted to fall in the range of pH 3 - 4. The mixture was gently boiled for 5 minutes and then allowed to cool, with re-adjustments of pH made whenever necessary. Thereafter, distilled water was added to achieve the original measured weight. The mixture was then centrifuged at 4000rpm for 10 minutes to obtain the supernatant which would be used for MBA.

b. Method of Analysis

Method	Method's Information	
1. Mouse Bioassay (MBA)	Reference Method	AOAC Official Method No. 959.08 (Paralytic Shellfish Poison)
	Materials	• Saxitoxin dihydrochloride standard solution • International Cancer Research (ICR) strain mice calibrated against standard saxitoxin • Hydrochloric Acid (HCl) • Sodium Hydroxide (NaOH)
	Determination of Calibration Factor (CF)	1. Saxitoxin (STX) working standards ranging from 0.10 - 0.80µg/mL were prepared. 2. Each standard was injected into the mice and 10 trials were performed for various concentrations. 3. CF was computed for concentrations that yielded median death time between 5 - 7 minutes. 4. The average CF was determined and the computed average was used for routine analysis.
	Extraction Procedure	1. 100g of homogenized shellfish meat was mixed with 100mL of 0.10N HCl. 2. The mixture was boiled for 5 minutes and left to cool. 3. The pH was adjusted to be between 3 and 4. 4. Supernatant solution was collected and used for bioassay.
	Bioassay Method	1. 1ml of shellfish extract was injected intraperitoneally into the test mouse and observed for 1 hour for PSP symptoms and death of mouse. 2. The lethal time was recorded. 3. Three trials were conducted for each sample. 4. The mouse units were calculated for each of the lethal times by using Sommer's Table (dose: death time table). Individual mouse units were corrected for the weight of mice used and the median corrected mouse unit was used to compute for PSP toxin level. Formula: µgSTXeq/100g = CMU x CF x DF x 200 where: CMU = corrected mouse units (MU/mL) CF = Calibration Factor DF = Dilution Factor Limit of Quantification = 45µgSTXeq/100g Limit of Detection = 31µgSTXeq/100g
2. High Performance Liquid Chromatography (HPLC)	Reference Method	AOAC Official Method No. 2005.06
	Materials	• STX Standards • HPLC system with fluorescence detector • Solid Phase Extraction (SPE) C18 Column

Method	Method's Information	
	Extraction Procedure	1. 5g of homogenized meat was mixed with 3ml of 1% acetic acid in a 50ml Polypropylene (PP) tube and mixed in a vortex mixer. 2. The mixture was heated in boiling water bath for 5 minutes, mixed in vortex mixer and cooled. 3. The supernatant solution was obtained by centrifugation at 4500rpm for 5 minutes. 4. The supernatant solution was collected in a 15ml conical tube. 5. The extraction was performed twice. 6. The supernatant solution was pooled and volume adjusted to 10ml with water.
	SPE Clean-Up	1. A 3ml SPE C18 cartridge was conditioned with 6ml of methanol followed by 6ml of water. 2. 1ml of shellfish extract was added at a flow rate of 2 to 3ml/ minute and the effluent was collected. 3. The cartridge was washed with 2 ml water and the effluent was combined. 4. pH was adjusted to 6.5 and solution was adjusted to a final volume of 4.0ml.
	Peroxide Oxidation	1. In a 1.5ml microcentrifuge tube, add 250µl 1M NaOH, 25µl 10% Hydrogen Peroxide (H2O2), and 100µl standard/sample after SPE effluent was mixed and then allowed to react for 2 minutes. 2. 20µl of concentrated acetic acid was added.
	HPLC Analysis Condition	• Sample/Standard Volume Injected: 25µl • Column: C18 reversed phase o 150mm x 4.6mm internal diameter (i.d). x 5µl particle size • Detector: Fluorescence o Excitation: 340nm o Emission: 395nm • Column Oven Temperature: 30°C • Pump: binary gradient • Flow rate: 1ml/ min • Mobile Phase A: 0.1M ammonium formate • Mobile Phase B: 0.1M ammonium formate in 5% acetonitrile • Gradient: o 0 - 5 minutes : 0% – 5% B o 5 – 9 minutes : 5% - 70% B o 9 - 11 minutes : 0% B o 11– 14 minutes : 0% B

Table P2: Summary of Methods

c. Limit of Detection & Limit of Quantification

Limit of Detection (LOD) for MBA = 31µgSTXeq/100g

Limit of Detection (LOD) for HPLC = 2.25µg/100g

d. National Regulatory Limits

Philippine Regulatory limit is set at 60µg/100g

(Reference Source: Fisheries Administrative Order 235 s.2010)

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

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b. Survey Results & Discussion

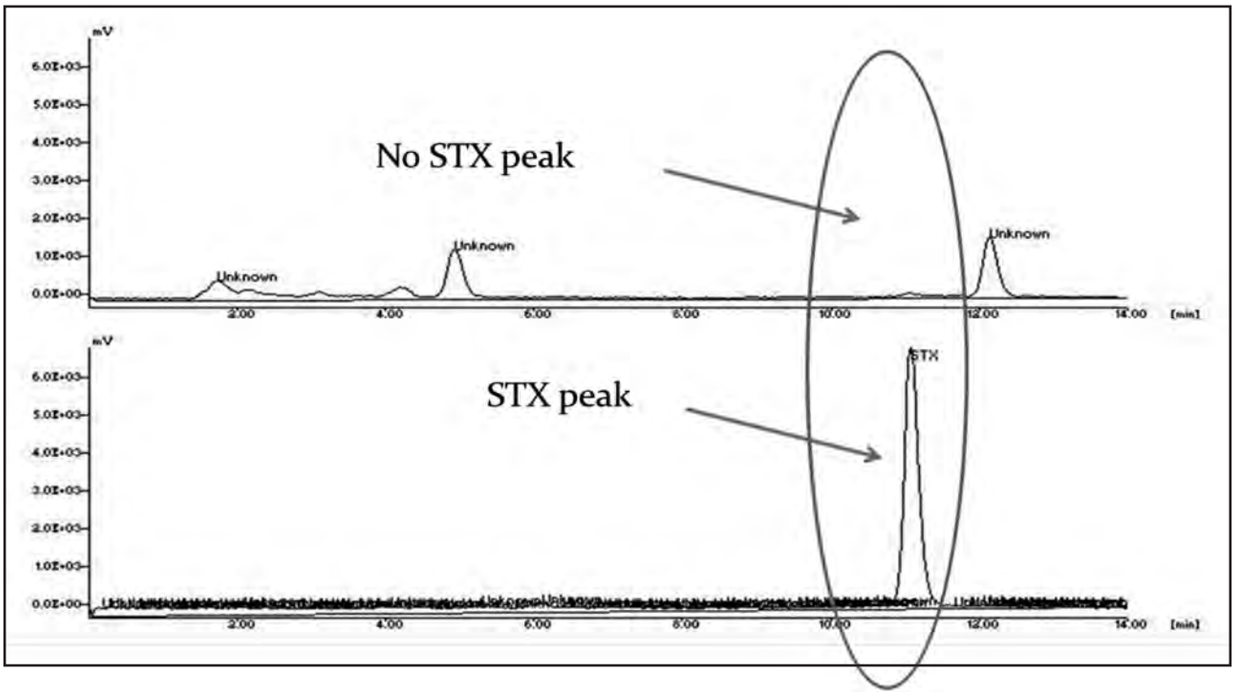
Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Sorsogon Bay	January – December 2011	Saxitoxin (STX)	60	MBA: < 40ug/100	MBA: 63ug/100	MBA: 56.8ug/100
				HPLC: < 2.25ug/100g	HPLC: 52.7ug/100g	

Table P3: Survey Results

Mouse bioassay procedures involved the extraction of whole shellfish meat using dilute hydrochloric acid followed by intraperitoneal injection in test mice. The test mice were then observed for symptoms associated with PSP namely thrashing, jumping, gasping for air and death by respiratory arrest. The lethal time was used as the basis of quantifying the toxin. Quantity of the toxin was determined using the Sommer's Table which correlates the PSP toxin dosage and mouse lethal time. Thereafter, toxicity was indicated in terms of mouse units (MU) and converted to µg STX equivalent/100g.

Results obtained from MBA are highly dependent on the strain of mice used. In this project, all analysis conducted made use of international cancer research (ICR) strain mice obtained from the Philippine's Food and Drug Authority (FDA) and private mouse farm (MT). Both mice sources were calibrated against standard STX. Standard toxin, saxitoxin dihydrochloride, was obtained from the National Institute of Standards and Technology (NIST). From the calibration procedures, a CF of 0.2859 MU/µg and 0.2895 MU/µg were calculated for FDA and MT ICR mice, respectively. These values were used for calculations.

All samples were analyzed for PSP using MBA and HPLC methods. Bioassay analysis results showed six positive samples for PSP. The presence of saxitoxin in these samples was further confirmed by HPLC. Likewise, saxitoxin was not detected through HPLC in the samples that were negative using MBA.



FigureP2. Sample of chromatogram of extract negative for PSP

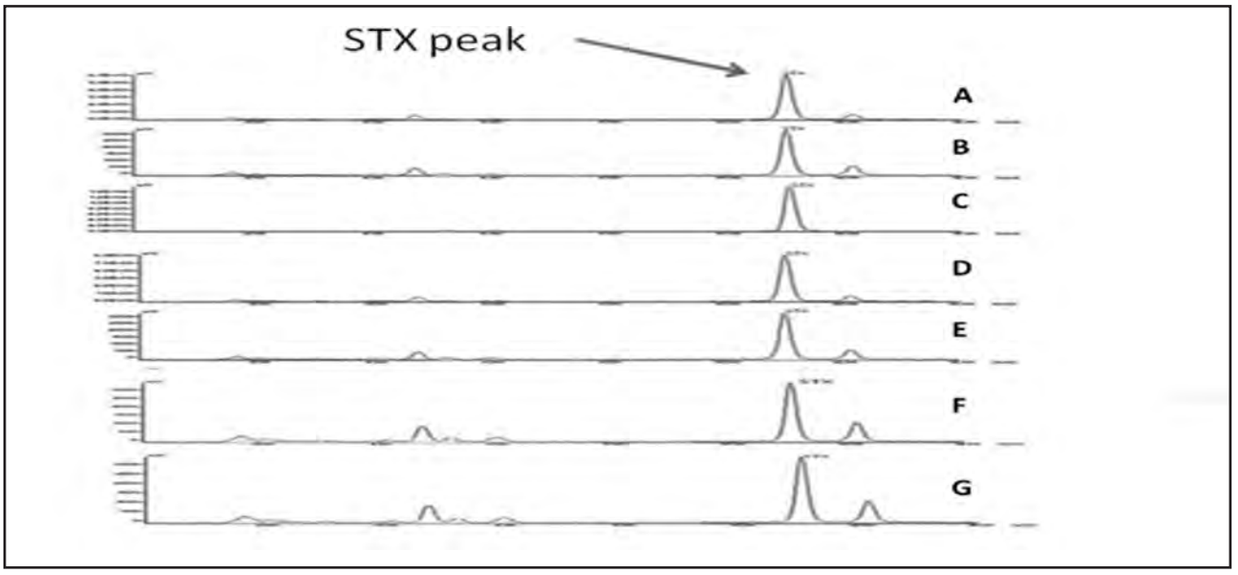


Figure 3. Chromatogram of extract that is positive for PSP

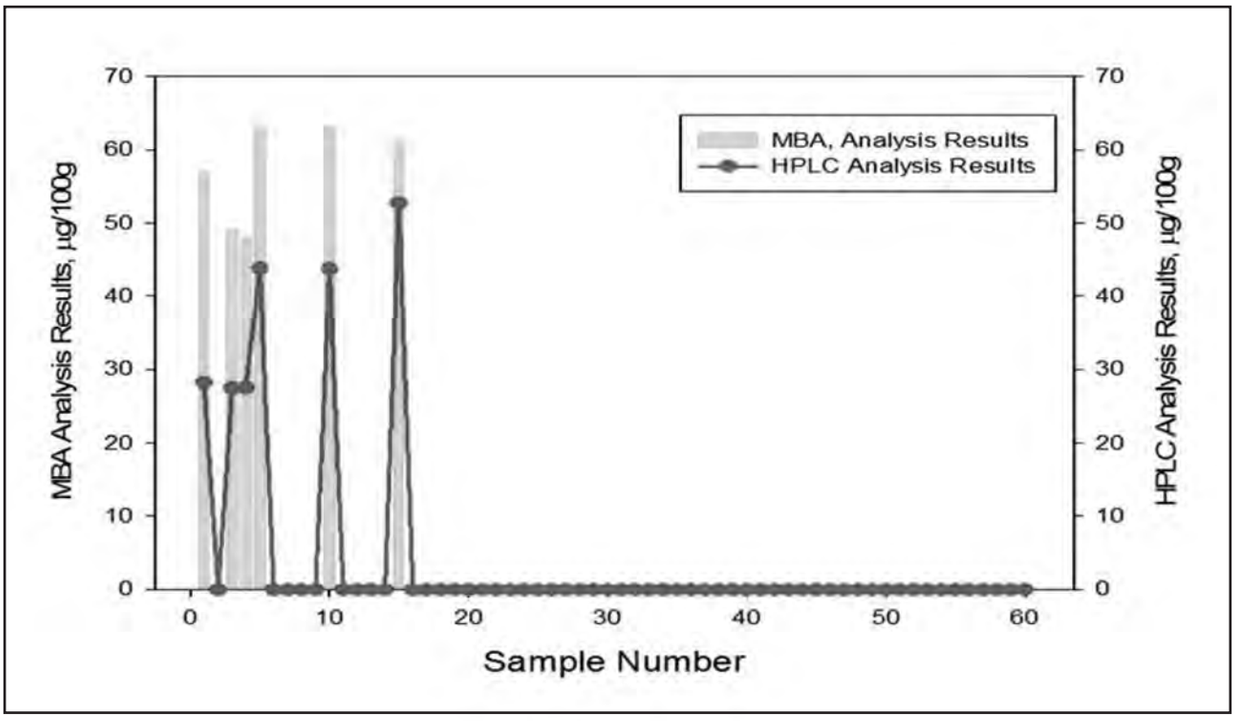


Figure P4. Confirmatory test for STX using HPLC

a. Corrective Actions

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I. Problems and Challenges Encountered

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II. Recommendations and Suggestions for future Follow Up Action

Based from the study, the HPLC would be the best method in determining toxicity in the shellfish since it has a lower limit of detection compared with that of the MBA. However, the method would entail a lot of laboratory time, personnel, and funds for equipment operation and maintenance. Considering time, financial and personnel constraints, BFAR would still have to use the MBA method. Nonetheless, monthly verification of MBA results with the results obtained from the HPLC method would be done to confirm the presence of STX and other PSP toxin analogues.

SINGAPORE

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Microbiology Division
Agri-Food and Veterinary Authority of Singapore (AVA)

I. Introduction

As Singapore is a small city state, much (about 90%) of its food is imported, including seafood / shellfish. There is a monitoring programme in place for the screening of imported shellfish for biotoxins.

On local production, Singapore has several coastal floating mussel farms. A biotoxin monitoring programme is also in place as an early alert strategy, to ensure that contaminated products are not harvested and sold for human consumption.

II. Objectives and Goals

This biotoxin monitoring programme is part of the AVA's shellfish sanitation monitoring programme. The objective of the programme is to monitor Singapore coastal mussels for biotoxins as an early alert strategy and to ensure that any contaminated mussels are not harvested and sold for human consumption.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

For sampling method, one sample was taken from each farm and three samples were taken every month. Samples were collected from the Singapore coastal farms. The target species used for this survey were Green Mussels (*Perna viridis*). Each time, the sampling size as per sample used was about 1.5 to 2kg of Green Mussels.

b. Method of Analysis

Mussel samples were taken from selected aquaculture farms off the coasts of Singapore. The samples were de-shelled and homogenized and the whole shellfish was analyzed. The extraction was carried out as in accordance to the laboratory's protocol for the various biotoxins – Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP).
Various techniques were used. For PSP, samples were screened using an ELISA test kit and suspect the samples confirmed by High Performance Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS). DSP test is carried out using the LC/MS/MS and ASP is by High Performance Liquid Chromatography (HPLC) method. As the use of animals for laboratory testing / experiments is controlled in Singapore, the MBA technique is not used as a routine testing method.

c. Limit of Detection & Limit of Quantification

Biotoxin	Limit of Detection (LOD)	Limit of Quantification (LOQ)
PSP	50ppb	-
ASP	5ppm	10ppm
DSP	5ppb	10ppb

Table S1: LOD and LOQ for various biotoxins

d. National Regulatory Limits

Guidelines applied:

PSP: 80µg/100g flesh

ASP: 20ppm

DSP: 0.2ppm

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

The laboratory took part in one round of inter-laboratory proficiency testing programme organized under Asia Pacific Laboratory Accreditation Cooperation (APLAC) on PSP using the MBA method. Report of results has not been received at the point of the submission of this technical compilation.

b. Survey Results & Discussion

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Local farms off the shores of Singapore	January – June 2011	PSP, DSP & ASP	24	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	July – December 2011	PSP, DSP & ASP	14	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	January – June 2012	PSP, DSP & ASP	18	Not Detected	Not Detected	Not Detected

Table S2: Survey results – mussel samples from coastal farms of Singapore

On average, two to three samples were tested for PSP, DSP and ASP each month. The samples were taken randomly from Green Mussel farms located off the shores of Singapore. No biotoxins (PSP, DSP and ASP) were detected in the 56 Green Mussel (*Perna viridis*) samples surveyed during the period January 2011 - June 2012.

c. Corrective Actions

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V. Problems and Challenges Encountered

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VI. Recommendations and Suggestions for Future Follow-Up Action

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THAILAND

Mrs Supanoi Subsinserm
Senior Food Technologist
Fish Inspection and Quality Control Division
Department of Fisheries

I. Introduction

The Department of Fisheries (DOF) holds the legal authority for classifying and approving fisheries' harvesting areas including bivalves. DOF has imposed the Notification on Classification of bivalve harvesting areas under the authority of Fisheries Act B. E. 2490 (1947). The major objective is to ensure that fishery products exported from Thailand have been harvested from approved areas and further processed in a safe, clean and wholesome manner by approved establishments.

DOF laboratories perform the analyses required in Council Directives 91/493/EEC, 91/492/EEC and 79/923/EEC. Samples are taken to the laboratory for the DOF control of sanitary quality of fishery products as well as for the monitoring of marine biotoxins and bacteriological contamination of bivalve molluscs.

Since 1997, DOF has been submitting reports on bivalve production and sanitation program to the European Union (EU). The report comprises of monitoring results of bivalve molluscs' flesh from the approved harvesting areas for biotoxin contents like Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP).

In 2002, the Commission Decision of European Union established detailed rules for the implementation of Council Directive 91/492/EEC specifying official control of microbiological classification of bivalve molluscs' production areas and maximum permitted levels which include methods of analysis for certain marine biotoxins in bivalve molluscs, echinoderms, tunicates and marine gastropods. In addition, the Commission Decision lays down limits used for the maximum levels of other toxins such as Yessotoxin (YTX), Pectenotoxin (PTX) and Azaspiracids (AZA).

Since 2003, DOF started to perform biotoxin analysis such as PSP, ASP, DSP, PTX, YTX and AZA and results thus far showed that no biotoxins were detected in all bivalve mollusc samples.

II. Objectives and Goals

The objectives are to strengthen the ASEAN capacity to detect and control the outbreak of biotoxins in ASEAN countries and also to strengthen the capacity of the laboratory for analysis of biotoxins in bivalve molluscs.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

The sampling site would be the Trad province, which is an approved harvesting area. The target species would be Green Mussels (*Perna*

viridis). At the approved harvesting areas, the shellfish samples are taken from the top, middle and bottom of the lines. The total weight of the flesh samples should not be less than 300g at each sampling point and the total amount of samples need to be at least 36 samples.

b. Method of Analysis

There are two methods of analysis- PSP by Mouse Bioassay (MBA) and PSP by High Performance Liquid Chromatography (HPLC). The PSP by MBA method, reference method AOAC 1995, Vol II, is used for the screening of samples. Quality control is ensured by first, inoculations of one mice with reagent blank, innoculation of three mice for each sample with diluted Saxitoxin (STX) standard for the collection of conversion factor. The death time of the mice should be obtained within 5 - 7 minutes. This was performed once every 3 months.

The second method of analysis, PSP by HPLC, is used for the confirmation of detection of biotoxins in samples. The reference method used is AOAC official method 2005.06. Quality control is ensured by using a reagent blank, spiked sample to determine the percentage of recovery and perform duplicate testing for the samples.

c. Limit of Detection & Limit of Quantification

Limit of Detection (LOD) = 0.006ug/g (STX)
Limit of Quantification (LOQ) = 0.06ug/g (STX)

d. National Regulatory Limits

PSP = 0.80ug/g (STX)

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

Inter-Laboratory Proficiency Testing Programme	Results (% RSD R)
Inter-lab comparison test for PSP by Mouse Bioassay with National Oceanic and Atmospheric Administration (NOAA); United States of America (USA)	18.3
Inter-lab comparison test for PSP by Mouse Bioassay with Department of Fisheries Laboratory and Central Laboratory (Thailand) Co.,Ltd	8.6

Table T1: Participation in Inter-Laboratory Proficiency Testing

b. Survey Results & Discussion

Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
At the approved harvesting area (Trad province)	April – June 2011	PSP (MBA)	12	Not Detected	< 35µg STXeq/ 100g	< 35µg STX eq/ 100g
	April – June 2011	PSP (MBA)	12	Not Detected	< 35µg STXeq/ 100g	< 35µg STX eq/ 100g
		PSP (HPLC)	3	Not Detected	-	Not Detected
	July – December 2011	PSP (MBA)	24	Not Detected	< 35µg STXeq/ 100g	< 35µg STX eq/ 100g
		PSP (HPLC)	6	Not Detected	-	Not Detected

Table T2: Survey Results

The findings of this survey indicated that no biotoxins were detected in the bivalve mollusc samples. However, there is still a need to continue the monitoring on the level of biotoxins to ensure that the bivalve mollusc products are free from biotoxins contamination.

c. Corrective Actions

V. Problems and Challenges Encountered

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VI. Recommendations and Suggestions for Future Follow-Up Action

The recommendations for future follow up action are for the project to focus on updating and training on new analytical methods, specifically on multi-residues analysis for all concerned analytical method, to improve the testing capabilities on fish quality and safety in Southeast Asia and for Southeast Asian Fisheries Development Center (SEAFDEC) to provide inter-laboratory comparison tests among ASEAN countries.

The suggestions for the project are the need to establish a networking system to develop the method of analysis for fish and fishery products among ASEAN countries and also training for laboratory staff should be carried out continuously to update their knowledge and skills on the new techniques and technologies.

VIETNAM

Mr Ngo Hong Phong
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National Agro-Forestry and Fisheries Quality Assurance Department (NAFIQAD)
Ministry of Agriculture and Rural Development (MARD)

I. Introduction

In the integration into the world economy, together with technology innovation for higher productivity and lowered price, Vietnam is also interested in improving product quality to protect consumers' interests and health as well as to meet the technical and food safety requirements of importing countries. With regards to food production in general, particularly in fishery production, technical barriers are mainly focused on environment and ecology system protection and food hygiene and safety requirements to protect consumers' health.

To protect local consumers' health as well as to overcome technical barriers given by big consumers of bivalve mollusc products such as the European Union (EU) and North America, the Sanitation Monitoring Programme for Bivalve Mollusc (BM) production areas in Vietnam has been set up and implemented as a pilot project with Baby Clams (*Meretrix lyrata*) in Tien Giang and Ben Tre since July 1997. So far, the programme has been expanded to include Ho Chi Minh city, Tra Vinh, Kien Giang, Binh Thuan, Thai Binh, Nam Dinh and Quang Ninh provinces, which comprises a total of 18 EU-approved production areas with different species such as Baby Clam (*Meretrix lyrata*), White Baby Clam (*Meretrix Meretrix*), Yellow Clam (*Paphia sp.*), Antique Ark (*Anadara antiquata*) and Blood Clam (*Tegillarca granosa*).

The implementation of the programme is in accordance with the regulation on sanitation monitoring in bivalve mollusc production areas, Manual of the Sanitation Monitoring Programme for BM production areas and amending document thereof of the National Agro-Forestry-Fisheries Quality Assurance Department (NAFIQAD) is in compliance with European Commission (EC) regulations.

II. Objectives and Goals

The objectives and goals look into:

- Protecting consumer's health
- Reducing damages due to harvest of BM from insanitary production areas
- Promoting export of BM
- Contributing to sustainable development of BM resources

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species, Number of Samples & Sampling Size

In 2011, the Monitoring Programme was applied continuously to 15 EU approved bivalve mollusc production areas located in the following eight provinces/cities in Vietnam:

- Thai Binh
- Nam Dinh
- Tien Giang
- Ben Tre

- Ho Chi Minh City
- Binh Thuan
- Tra Vinh
- Kien Giang

The sampling method and size was conducted in accordance to that stated on the Manual of the Sanitation Monitoring Programme for Bivalve mollusc production areas. Meanwhile, the species sampled includes Baby Clam (*Meretrix lyrata*), Yellow Clam (*Paphia sp.*), Blood Clam (*Tegillarca granosa*), Antique Ark (*Anadara subcrenata*), Antique Ark (*Anadara antiquata*) and Scallop (*Chlamys nobilis*). A total of 1,277 samples were analyzed in the Monitoring Programme.

b. Method of Analysis

No.	Criteria	Sampling frequency	Maximum Permitted Limits (MRLs)	Analysis method
1	Lipophilic toxins	Twice (*) or 4 times (**) per month	Negative or: • Total Okadaic acid + Dinophysis toxins + Pecteno toxins: 160 µg/kg • Yessotoxins: 1mg/kg • - Azaspiracids: 160 µg/kg	Mouse Bioassay (MBA) or High Performance Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS)
2	Paralytic Shellfish Poisoning (PSP)	Twice (*) or 4 times (**) per month	Negative or 800 µg /kg mollusc flesh and intra-valvular liquid	MBA or LC/MS/MS
3	Amnesic Shellfish Poisoning (ASP)	Twice (*) or 4 times (**) per month	20 mg domoic acid/kg mollusc flesh and intra-valvular liquid	High Performance Liquid Chromatography (HPLC) or Liquid Chromatography Mass Spectrometer (LC/MS)
(*): half-day tidal production areas *(**): production areas where harvesting is done for the whole month*				

Table V1: Analysis method and MRLs for toxins

c. Limit of Detection & Limit of Quantification

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d. National Regulatory Limits

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IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

In 2011, 3 labs had participated in the programme of QUASIMEME for ASP. All of the three lab results are optimal.

b. Survey Results & Discussion

Parameters	Analysis	Number of positive samples	Value	MRLs	Number of samples with detection level exceeding MRLs
PSP (Neg- Pos)	875	0	• 0	Neg	0
Lipophylic (Neg- Pos)	1236	185	Pos	Neg	185
ASP (µg/g)	899	5	3.7-5.5 (µg/g)	20	0

Table V2: Survey results

PSP had not been detected in all the samples collected from the 15 production areas. For lipophilic toxins, 185 analysis were positive with lipophilic toxins among 1236 analysis (including samples from intensified sampling) analyzed samples, making up 14.97%. For some areas, lipophilic was detected in whole BM (in both mollusc flesh and intra-valvular liquid). However, testing result for lipophilic was negative for adductor (of scallop) or flesh (of Antique Ark) sample. Meanwhile, five out of 899 samples were found to contain 3.7 – 5.5µg/g of ASP, but this is much lower than warning limit of 20µg/g.

c. Corrective Actions

The lipophilic content was found to exceed MRL. After the positive detection of this toxin by MBA in BM, NAFIQAD notified for a stop-harvesting from the above-mentioned areas and required relevant local CA to intensify the sampling to test for this toxin and take water samples to survey the variation of alga.

V. Problems and Challenges Encountered

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VI. Recommendations and Suggestions for Future Follow-Up Action

As the budget for biotoxins monitoring activity is limited, the collected data are insufficient to reflect the real impact of biotoxins in BM. It is recommended that the new project could focus on building up capacity in food safety risk analysis and traceability of fish and fishery products in South-East Asia countries.

Summary

A total of 9 member countries, namely Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam, participated in this JTF II project on biotoxins monitoring in ASEAN.

From the survey results of the Paralytic Shellfish Poisoning (PSP) in Green Mussels (Perna virdis) and Baby Clams (Meritrix meritrix) conducted by the above-mentioned member countries, it was noted that six samples have exceeded the permitted regulatory limits. Nonetheless, it could be concluded that the shellfish products from these member countries are generally free of PSP toxin.

From this survey, it was observed that four countries had participated regularly in recognised inter-laboratory proficiency testing programmes but not on a regular basis. Active participation in inter-laboratory proficiency testing is strongly encouraged as it ensures credibility of the test results produced by the laboratory beside enhancing the confidence level of the laboratory personnel.

Some of the major challenges faced by the participating member countries include inadequate sufficient funds for sampling and analysis, limited laboratory resources such as personnel with the scientific know-how and for some countries, poor logistic arrangement which resulted in undesirable preservation of samples collected from distant sampling area. To address these issues, the recommendations suggested include: (1) the extension of investigation scope to cover other potential toxin hazards in fish and fish products, (2) the establishment of a monitoring scheme under the comprehensive quality management system, (3) the upgrading of human resource and laboratory's capability in biotoxins testing and lastly, the introduction of advanced reliable rapid test method to shorten the test cycle time.

In conclusion, this project has assisted the participating countries in developing methodologies for biotoxins analyses and establishing a monitoring scheme for biotoxins detection. This is particularly beneficial to the participating countries which do not have such system in place previously. The information gathered from this project would be useful for individual countries in their future planning of the monitoring programmes. In addition, this project has provided a platform for knowledge-sharing and networking amongst the participating countries in the course of obtaining a better understanding on the frequency of biotoxins occurrences and incidences in shellfish within ASEAN.

Annexes Section

Annex I

Administrative Report for Regional Technical Consultation, 2009

Report of the REGIONAL TECHNICAL CONSULTATION ON JAPANESE TRUST FUND II CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA (BIOTOXINS MONITORING IN ASEAN)

26 – 28 AUGUST 2009
Singapore

I. Introduction

1. At the invitation of the SEAFDEC Marine Fisheries Research Department (MFRD), the Regional Technical Consultation on "Japanese Trust Fund II Chemical and Drug Residues in Fish and Fish Products in Southeast Asia (Biotoxins Monitoring in ASEAN)" was held from 26-28 August 2009 in Singapore.

2. The meeting was attended by representatives from SEAFDEC member countries, an expert from Japan, the Chief of MFRD as well as staff from MFRD. The list of participants appears in Annex 1.

II. Opening Of The Meeting By Chief, MFRD

3. The Chief of the Marine Fisheries Research Department (MFRD), Mrs Tan-Low Lai Kim, welcomed the participants and thanked the Government of Japan for the funding of the project and the Japanese expert, Dr Toshiyuki Suzuki, for participating in the meeting and sharing his experience and knowledge.

4. The meeting was conducted with the objective of identifying biotoxins that should be surveyed, the training needs of each member country and the training sites that had the facilities to conduct on-site training. As budget was limited, countries would have to prioritize the scope of monitoring and the need to increase capability for biotoxins monitoring in ASEAN. The Chief mentioned that if there was a need, member countries could request SEAFDEC for a continuation of the project beyond 2012.

5. The Deputy Head of the Fish Quality & Management Technology Branch, Miss Tan Lu Hsia, presented the Agenda of the meeting for discussion and the agenda was adopted. The agenda appears in Annex 2.

III. Presentation On The Project Overview By The Marine Fisheries Research Department

6. The meeting was informed that the project is a continued project with a new focus area beginning from 2009 until 2012 and would be implemented under Japanese Trust Fund II, on a cost-sharing basis, to develop methodologies in biotoxins

analyses. The project overview, which included project background and rationale, project objectives, project management and administration, expected project outputs, project schedule and project methodology was presented by MFRD for the benefit of the meeting participants.

7. The Chief informed the meeting that the first regional training course would be held at the Veterinary Public Health Laboratory (VPHL) of AVA in Singapore in 2010 with one sponsored participant from each member country. The Chief thanked AVA/VPHL for offering their laboratory as the venue for the regional training course in 2010. Depending on the space available, countries are welcomed to send more participants sponsored by their government. The three other venues for on-site training would be decided based on the availability of appropriate facilities available to conduct the training.

8. The meeting noted that as MFRD did not have the capability to conduct biotoxins analysis, experts from Japan or from other member countries would be identified to conduct the training.

IV. Country Paper Presentations On Status Of Biotoxins Monitoring System

9. Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam presented their country papers on the status of biotoxins monitoring system in the fisheries industry in their countries.

10. It was noted that the frequency of sampling for biotoxins monitoring varies among countries. For example, Indonesia monitors biotoxins twice a year to protect domestic consumers. Countries exporting to European Union (EU) like Vietnam monitors biotoxins once a week, according to European Commission (EC) requirements.

11. Cambodia are currently setting up their plan for biotoxins monitoring and in the case of Myanmar, they would embark on the proposed plan in their presentation once approval is given. Lao PDR informed that there is currently no biotoxins analysis facility in their country.

12. Singapore informed the Meeting that there is no proficiency testing scheme for biotoxins available commercially although request has been made to FAPAS before. The Chairman commented that as it was difficult for service providers to provide stable test materials, any information on proficiency tests providers could be shared by all participants in the meeting.

13. With regards to the use of ELISA method, Philippines raised that the ELISA method is yet to be accredited/approved by AOAC.

14. Philippines informed that currently biotoxins surveillance is conducted for both wild stocks and aquaculture farms. The provincial laboratories had developed capabilities in conducting plankton identification and two of the provincial laboratories had also developed capabilities in biotoxins analysis.

15. With regards to an enquiry raised by Vietnam, Singapore informed the meeting that Singapore's requirements for the import of fish and fishery products is available at the Agri-Food and Veterinary Authority (AVA) website (www.ava.gov.sg).

16. Vietnam informed the meeting that EU conducts on-site inspection for bivalve mollusks monitoring programme once every two years with the requirements for sampling frequency being once every week.

17. Philippines highlighted to the meeting

that certain bivalves were not good accumulators of biotoxins and this was a point to consider when selecting samples. It was also brought up by Thailand that from the results presented in their country paper, the occurrence of marine biotoxins was dependent on area and not on the species.

18. Philippines highlighted that Nha Trang University of Science in Vietnam had staff trained in plankton identification in Denmark. Vietnam (NAFIQAD) could contact the university for future collaboration.

19. Philippines informed the meeting that the Intergovernmental Oceanographic Commission (IOC) created a portal with information on the management of harmful algae bloom in SEA and the monitoring situation. The Chairman commented that the portal could be a good information source. For the benefit of participants, the portal website address is as follows: http://portal.unesco.org/habsea.

V. Biotoxins Monitoring System – Japanese Experience By Dr Toshiyuki Suzuki

20. Dr Toshiyuki Suzuki presented a paper on 'Biotoxin Monitoring System – A Japanese Experience.'

21. Dr Suzuki provided the meeting with findings from Japan's laboratories on developments in screening and instrumental methods. He mentioned that some of the methods developed were currently used in Japan only and were not commercially available. The rapid screening methods introduced for PSP and DSP toxins in shellfish are OA PP2A assay kit, YTX ELISA kit, PTX ELISA kit and PSP ELISA kit.

22. With respect to analytical methods used, Dr Suzuki said that HPLC methods were more accurate and reliable as compared to the Mouse Bioassay (MBA) method. However, MBA is useful for routine monitoring and the detection of unknown toxins.

23. Dr Suzuki also mentioned that there are currently no standards available for Ciguatera toxins; otherwise LC-MS-MS is useful for the quantitative determination of Ciguatera toxins.

VI. Deliberation On Scope And Implementation Of The Project: Implementation Mechanism And Identification Of Training Needs And Testing Facilities In Member Countries

24. Member countries were requested to list down and submit their current testing capabilities for biotoxins, available facilities in their laboratories as well as their training needs. The list submitted by member countries is attached in Annex 3.

25. With deliberation, member countries felt a need to learn more methods within a training course, rather than having on-site training courses. As such, the meeting agreed to organise one Regional Training Course of about 8 days duration at the Veterinary Public Health Laboratory (VPHL) in Singapore. The targeted period for conducting the course is in June/July 2010.

26. For the regional training course, only one participant would be sponsored for each country. Respective countries would have to bear the costs if they were interested to send additional participants. As the VPHL would be able to accommodate about 20 participants, additional participants would be accepted on a first-come-first serve basis.

27. For this regional training course, the tests covered would be multi-component DSP and lipophilic toxins analysis using LC-MS-MS and PP2A kit; PSP analysis using HPLC and a commercially available ELISA method; and TTX analysis using HPLC or LC-MS-MS. As per Cambodia's request, the Japanese expert, Dr Suzuki, would also present a lecture on the setting up of a monitoring system (with additional information on export requirements to the EU) and also come up with a sample plan based on Japan's experience.

28. As Dr Suzuki mentioned, it would be useful to learn the extraction method for both shellfish and seawater samples, it was decided that seawater analysis would be included and participants would be given the chance to perform the extraction of different samples as long as time is available during the course.

29. Dr Suzuki agreed to bring positive test samples (PSP, DSP, TTX) of the meat for the training. Singapore would help to facilitate customs clearance for bringing in the samples.

30. The DSP rapid method was requested, but due to the high cost of the test kit, it would be demonstrated to the participants during the training course if there is a budget for procurement of the test kit.

31. Training on analysis of Ciguatera toxins was also requested but Dr Suzuki commented that there would be difficulty in obtaining standards for the training and hence, it was agreed that it would not be possible to conduct the training.

32. Dr Suzuki recommended Dr Oshima to be present at the training course as he is a leading expert for PSP analysis. The Chairperson commented that this would be considered if the budget was sufficient.

33. In response to requests for training on phytoplankton identification and taxonomy to identify harmful algae, Dr Suzuki mentioned that he would be able to recommend experts in these areas if there is a need.

VII. Deliberation On Scope And Implementation Of The Project: Identification Of Scope Of Biotoxin Survey In Member Countries

34. The meeting agreed that the objectives of the project are as follows:
    (i) Establish protocols for harmonization
    (ii) Encourage member countries without monitoring system to establish their own system
    (iii) Establish a directory of reference of experts and responsible persons
    (iv) Enhance analysis capability to a acceptable confidence level with the 1-year survey

35. Dr Suzuki commented that for such a survey, it would be more useful to focus on only one type of species, but increase the frequency of sampling. Sampling once a week would be good but budget constraints would have to be taken into consideration.

36. The meeting agreed that PSP is most crucial to human health and hence, would be the target analyte for the survey. The method of analysis would be up to individual countries, although they would be encouraged to use the methods learnt during the regional training.

37. The meeting agreed that all countries would use green mussels (*Perna viridis*) as the samples, except for Vietnam and Indonesia, who would use baby clams (*Meritrix spp*).

38. The meeting agreed that the sampling site should be an area near harvesting sites or an area with history of shellfish

contamination. Information of sampling protocols will be shared at the regional training course.

39. It was agreed that the sampling frequency would be weekly. The sample size would comprise of at least 10 individual shellfish with not less than 100g of flesh per sample at each sampling point. As the toxins are stable, it is possible to freeze the sample and analyze the sample within the year.

40. The budget given to each country for the survey of US$2,000 would be for the purchase of samples and consumables, or for the sending of samples to other countries as in the case of Cambodia and Lao PDR. It was agreed that Cambodia would send their samples to Vietnam while Lao PDR would send their samples to Thailand for the survey.

41. Due to the tight budget, member countries would also need to use their national budget to ensure adequate data for a comprehensive survey. These results could be included in the final technical compilation at the end of the survey.

42. The meeting decided that the units for reporting would be µg/100g of meat. If Mouse Bioassay (MBA) is used, it would be possible to report in µg/100g if a standard was available, otherwise the results would be in mouse units.

43. Dr Suzuki suggested that grouping and analysing results from comparable methods would be more useful when conducting an analysis of the results at the end of the survey.

44. Participants were informed that the methods used for the survey should be validated before use as the limit of quantification (LOQ), limit of detection (LOD), survey methodology and national regulatory limits were also required to be reported.

VIII. Deliberation On Scope And Implementation Of The Project: Reporting Structure And Financial Matters

45. Member countries submitted the tentative names of Key Project Leaders (KPLs), and MFRD would officially write to Council Directors for nomination.

46. The meeting was informed that key project leaders are required to submit half yearly reports to MFRD on the progress of the survey as requested by the Trust Fund Manager. The first report will be due on 10 June 2011 for January to June, and the second report due on 9 December 2011 for July to December. The same deadlines apply for the statement of accounts to be submitted half-yearly with original receipts if possible.

47. The project proposal for the survey from individual countries is to be submitted to MFRD by 31 October 2009.

48. The KPLs would be required to detail the physical parameters of the samples in their reports as it was agreed that member countries do not need to submit the raw data sheet.

IX. Any Other Matters

49. The Chairperson informed the meeting that SEAFDEC would be organising an ASEAN-SEAFDEC conference in 2011. In preparation for the conference, MFRD would be organising a Regional Technical Consultation (RTC) meeting in 2010. The purpose of the RTC is to gather stakeholders' feedback on the needs and direction of the ASEAN fisheries industry with regards to food safety and post-harvest technology.

X. Talks by Suppliers

50. Talks by suppliers were arranged for participants to understand the latest developments in instruments available for biotoxin analysis.

51. Two representatives from Shimadzu Asia Pacific Pte Ltd, delivered presentations on 'Automated On-Line GPC-GCMS for Analysis of Multi-residual Pesticides/ Hazardous Substance' and 'A rapid MS-based method for microbial identification.'

52. A representative from Waters Asia Pte Ltd, delivered a presentation on 'Analysis of BFRs, PFCs and Other Emerging Contaminants- Analytical Approaches and New Developments.'

53. A representative from Agilent Technologies Singapore, delivered a presentation on 'The Analysis of Marine Biotoxins Using Liquid Chromatography Combined with Mass Spectrometry.'

XI. Closing of the Meeting

54. The Chairperson concluded the Meeting and thanked all participants for their contributions to the Meeting.

55. The participants from the SEAFDEC member countries and Japan expressed their heartfelt appreciation to MFRD for the warm hospitality accorded to them and the excellent arrangements made for the Meeting and to the Government of Japan for making this Meeting possible.

56. The Meeting was held in the traditional spirit of SEAFDEC co-operation and cordiality.

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AGENDA OF THE MEETING

Agenda 1: Opening of the Meeting

Agenda 2: Background and Introduction of Japanese Trust Fund II Project

Agenda 3: Country Paper Presentation of Status of Biotoxins Monitoring System in Fisheries Industry

Agenda 4: Biotoxins Monitoring System – Japanese Experience by Dr Toshiyuki Suzuki

Agenda 5: Deliberation on Scope and Implementation of the Project

Agenda 6: Talk by Suppliers

Agenda 7: Adoption of Administrative Report

Agenda 8: Closing of the Meeting

SCOPE AND IMPLEMENTATION OF PROJECT

A. Information of Biotoxins Monitoring System in Member Countries

COUNTRY	BIOTOXINS ANALYSED AND REGULATORY LIMITS	METHODS OF ANALYSIS	FREQUENCY OF SAMPLING AND SAMPLING METHODS	DEPARTMENT IN-CHARGE
CAMBODIA	NOT APPLICABLE	NOT APPLICABLE	NOT APPLICABLE	DIRECTORATE OF FISHERY ADMINISTRATION
INDONESIA	PSP, DSP, ASP	PSP – OIOASSAY (AOAC 1996) DSP – OIOASSAY (IOC 2003) ASP-HPLC (IOC2003)	BI-YEARLY FROM 2003 TO 2009. EXCEPT IN 2005, WHEN SAMPLING IS DONE ONCE.	DIRECTORATE GENERAL OF FISH PROCESSING AND MARKETING – NATIONAL CENTER FOR FISH QUALITY CONTROL (NCQC)
LAO P.D.R	N.A.	N.A.	N.A.	N.A.
MALAYSIA	TETRADOTOXIN (TTX), PSP	PSP – OIOASSAY (AOAC 1996) TTX-GCMS	700 SAMPLES TAKEN FROM 2004	FISHERIES BIOSECURITY DIVISION
MYANMAR	ASP, DSP, NSP, PSP	ELISA (AOAC 2006) HPLC (JSPP21) LCMSMS (JOURNAL OF ROMATOGRAPHY)	NIL (NO TRAINED PERSONNEL)	DEPARTMENT OF FISHERIES – FISH INSPECTION AND QUALITY CONTROL SECTION (KEY LAB)

COUNTRY	BIOTOXINS ANALYSED AND REGULATORY LIMITS	METHODS OF ANALYSIS	FREQUENCY OF SAMPLING AND SAMPLING METHODS	DEPARTMENT IN-CHARGE
PHILIPPINES	PSP (60UG/100G)	MOUSE BIOASSAY	2X A WEEK SPECIFICALLY FOR MANILA BAY FOR PROVINCIAL AREAS: ONCE A MONTH DURING NON-HAB OCCURRENCES AND WEEKLY DURING HAB OCCURRENCES	MARINE BIOTOXINS MONITORING SECTION FISHERIES RESOURCE MANAGEMENT DIVISION OF THE BUREAU OF FISHERIES AND AQUATIC RESOURCES (UNDER THE DEPARTMENT OF AGRICULTURE)
	ASP (20UG PER GRAM)	HPLC-UV		
	DSP (DEATH OF 2 MICE OF 3 W/IN 24HRS)	MOUSE BIOASSAY		
	CFP (POSITIVE W/ BLUE COLORATION ON TEST KIT)	IMMUNOASSAY (CIGUA-CHECK TEST KIT)		
	POLYCAVERNOSIDE (PRESENT WHEN DEATH OF 1 MOUSE IN 24 HRS THAT = 2MU; IF 2 MICE ARE ALIVE AFTER 24HRS = NEGATIVE)	MOUSE BIOASSAY		
	TTX (ABSENCE OF TOXIN IN FISH)	MOUSE BIOASSAY		
	UNKNOWN SEA URCHIN TOXIN (PSP & CFP)	MOUSE BIOASSAY & IMMUNOASSAY		
SINGAPORE	PSP (80UG / 100G)	PSP – ELISA & BIOASSAY	(a) IMPORT – VARIES (EVERY CONSIGNMENT OR 1 IN 10 OR 1 IN 20) (b) LOCAL FARMS – ONCE A MONTH OR MOVE IF ALERT ARISES	AGRI-FOOD & VETERINARY AUTHORITY – VETERINARY PUBLIC HEALTH CENTRE
	DSP (0.2 - 0.4PPM)	DSP – LCMSMS		
	ASP (20PPM)	ASP-HPLC		

COUNTRY	BIOTOXINS ANALYSED AND REGULATORY LIMITS	METHODS OF ANALYSIS	FREQUENCY OF SAMPLING AND SAMPLING METHODS	DEPARTMENT IN-CHARGE
THAILAND	PSP (80UG / 100G)	PSP – DOUSE BIOASSAY, HPLC-FL	MONTHLY (MEAT ~ 1KG PER STATION)	DEPARTMENT OF FISHERIES – FISH INSPECTION AND QUALITY CONTROL DIVISION (FIQD)
	DSP - LIMIT FOR MOUSE BIOASSAY - 2 OUT OF 3 MICE DIE & LIMIT FOR LCMSMS - 0.I6UG/G	DSP – GOUSE BIOASSAY, LCMSMS	MONTHLY	FIQD
	ASP (20PPM)	ASP – HPLC	MONTHLY	FIQD
	LIPOPHILIC TOXINS – PTX (0.16UG/G) YTX (1UG/G) AZA (0.16UG/G)	LIPOPHILIC TOXINS – BIOASSAY, LCMSMS	MONTHLY	FIQD
	TETRADOTOXINS	TTX - HPLC	MONTHLY (PUFFER FISH)	DEPARTMENT OF FISHERIES – FISHERIES TECHNOLOGICAL DEVELOPMENT DIVISION (FTDD)
VIETNAM	PSP (80UG/100G)	PSP – POUSE BIOASSAY AND HPLC	2 WEEKS	MINISTRY OF AGRICULTURE & RURAL DEVELOPMENT – NATIONAL AGRO-FORESTRY – FISHERIES QUALITY ASSURANCE DEPARTMENT (NAFIQAD)
	ASP (20MG/KG)	ASP – LCMSMS OR HPLC		
	DSP (160UG/KG)	DSP - ROUSE BIOASSAY AND LCMSMS OR HPLC		

*EXTRACTED FROM PRESENTATION MATERIALS

B. Request for Information of Testing Facilities Available in Member Countries

COUNTRY	INSTRUMENTS AVAILABLE	METHODS OF ANALYSIS	METHODS OF ANALYSIS THAT COUNTRY WISHES TO LEARN	LABORATORY IN-CHARGE / CONTACT PERSON	REMARKS
CAMBODIA	NOT AVAILABLE	NOT APPLICABLE	ELISA TEST METHOD PSP, ASP & DSP ANALYSIS BY HPLC, LCMSMS & LCMS		TRAINING, SURVEYING
INDONESIA	1. HPLC-UV 2. LCMSMS 3. LCMS	PSP & DSP – MOUSE BIOASSAY	PSP- HPLC, ELISA (FROM GERMANY)	NATIONAL CENTER FOR FISH QUALITY CONTROL (NCQC) MURTININGSIH (HEAD OF TESTING DIVISION) NINGFISH@ YAHOO.COM	WISH TO LEARN ON CTX; PSP AND DSP USING HPLC
		ASP:HPLC	DSP:HPLC		
LAO P.D.R	NOT AVAILABLE / NOT APPLICABLE	NOT APPLICABLE	ELISA TEST METHOD	NAHC. DLF	TRAINING, SURVEY TRAINING ON SIMPLE METHODS SUCH AS ELISA

COUNTRY	INSTRUMENTS AVAILABLE	METHODS OF ANALYSIS	METHODS OF ANALYSIS THAT COUNTRY WISHES TO LEARN	LABORATORY IN-CHARGE / CONTACT PERSON	REMARKS
MALAYSIA	-	HPLC	TTX-HPLC METHOD PSP-HPLC METHOD	AZLAN MD. NOR ROSLINA AHMAD NAWAWI	HPLC PROCUREMENT (REPLACE EXISTING MOUSE BIOASSAY WITH HPLC METHOD)
MYANMAR	ELISA, HPLC, LCMSMS (ALREADY AVAILABLE IN LAB, BUT CURRENTLY USED TO TEST FOR OTHER TOXINS)	NOT AVAILABLE	LEARN ON: ELISA – PSP LCMSMS – PSP HPLC-ASP	U THAN WINN	TRAINING NEEDED REQUEST FOR METHODS OF REFERENCE AND METHODS OF VALIDATION
PHILIPPINES	MICROSCOPES (COMPOUND LIGHT, INVERTED) HPLC FREEZER CULTURE CHAMBER PLANKTON NETS, VARIOUS LAB APPARATUS (SEDGWICK RAFTER COUNTING CHAMBER, GLASSWARES, GRADUATED CYLINDERS, PIPETTES, ETC.)	MOUSE BIOASSAY IMMUNOASSAY HPLC-UV	CIGUATOXIN ANALYSIS ASIDE FROM CIGUA-CHECK DSP SCREENING METHOD	MRS ARCAMO SANDRA VICTORIA ROSALES OR MR. JUAN R. RELOX, JR	REQUESTS OR COMMUNICATIONS SHOULD BE COURSED THROUGH BFAR DIRECTOR ATTENTION FRMD CHIEF

COUNTRY	INSTRUMENTS AVAILABLE	METHODS OF ANALYSIS	METHODS OF ANALYSIS THAT COUNTRY WISHES TO LEARN	LABORATORY IN-CHARGE / CONTACT PERSON	REMARKS
SINGAPORE	HPLC, LCMSMS & ELISA READER AND OTHER SUPPORT EQUIPMENT	HPLC, LCMSMS & ELISA	MULTI-COMPONENT OF DSP AND LIPOPHILIC TOXINS (INCLUDING YTX AND PTX USING LCMSMS) CTX TTX AZA	DR. PAUL CHIEW OR MISS HELEN PHANG	AGRI-FOOD & VETERINARY AUTHORITY (AVA) – VETERINARY PUBLIC HEALTH LABORATORY
THAILAND	HPLC, LCMSMS, MOUSE BIOASSAY	SAME AS SECTION A	LIPOPHILIC TOXINS (DSP, PTX, YTX, AZA) BY LCMSMS PSP BY HPLC	FIQD	
			IDENTIFICATION OF PHYTOPLANKTON	MARINE FISHERIES DIVISION	
			CTX TTX BY LCMSMS	FTDD	

COUNTRY	INSTRUMENTS AVAILABLE	METHODS OF ANALYSIS	METHODS OF ANALYSIS THAT COUNTRY WISHES TO LEARN	LABORATORY IN-CHARGE / CONTACT PERSON	REMARKS
VIETNAM	LCMSMS	LCMSMS	TTX BY HPLC	NGO HONG PONG NAFIQAD BRANCH 1, 4, 6	LCMSMS (DSP, AZA, PTX, DTX, YTX)
	HPLC - ANCH	HPLC - ANCH IOC (PSP)			
	MOUSE BIOASSAY	MOUSE BIOASSAY IOC, AOAC (PSP, LIPOPHILIC)			

C. Request for Information of Proposed Survey Scope by Member Countries (for project implementation from 2011 to 2012)

COUNTRY	PROPOSED BIOTOXINS FOR SURVEY	PROPOSED BIOTOXINS SPECIES FOR SURVEY	METHODS OF ANALYSIS FOR PROPOSED BIOTOXINS TO BE SURVEYED ON	REMARKS
CAMBODIA	NOT APPLICABLE	1. PUFFER FISH 2. SHELLFISH 3. (GREEN MUSSEL, COCKLES & BABY CLAM)	PSP USING ELISA & HPLC ASP & DSP USING LCMS	WISH TO LEARN ON HOW TO SET UP BIOTOXINS MONITORING SYSTEM AND HOW TO DO SAMPLING AND ANALYSIS

COUNTRY	PROPOSED BIOTOXINS FOR SURVEY	PROPOSED BIOTOXINS SPECIES FOR SURVEY	METHODS OF ANALYSIS FOR PROPOSED BIOTOXINS TO BE SURVEYED ON	REMARKS
INDONESIA	PSP, DSP & ASP	1. PERNA VIRIDIS 2. ANADARA SPP 3. MERITRIX SPP 4. PAPHIA UNDULATA	ELISA (PSP), HPLC (PSP, DSP & ASP)	
	CTX	CORAL FISH	HPLC	
LAO P.D.R	N.A.	N.A.	N.A.	INTERESTED IN COLLABORATING WITH THAILAND FOR SURVEY OF FRESHWATER PUFFER FISH AND/ OR SHELLFISH BEHIND AREA OF LAO PDR AND THAILAND. SURVEY COCKLES, BABY CLAM, GREEN MUSSELS (IMPORTED FROM DIFFERENT PARTS, ESPECIALLY NORTHERN THAILAND)
MALAYSIA	TTX	PUFFER FISH OR RELATED SPECIES	HPLC METHOD	TRAINING NEEDED FOR HPLC METHOD
MYANMAR	ASP, DSP, PSP	1. GREEN MUSSEL (*PERNA VIRIDIS*) 2. CLAM (*VENERID CLAMS*) 3. SPOTTED BABYLON (*BABYLONIA SPIRALA*) [ALONG THE MARINE COASTLINE OF MYANMAR]	PSP, ASP, DSP	

COUNTRY	PROPOSED BIOTOXINS FOR SURVEY	PROPOSED BIOTOXINS SPECIES FOR SURVEY	METHODS OF ANALYSIS FOR PROPOSED BIOTOXINS TO BE SURVEYED ON	REMARKS
PHILIPPINES	PRIORITY WOULD BE PSP SO ALL SEAFDEC MEMBER COUNTRIES WOULD BE ON THE SAME LEVEL OF CAPACITY CIGUATOXIN (SECONDARY PRIORITY)	BIVALVE SHELLFISH I.E. MUSSELS, OYSTERS, COCKLES, CERTAIN CLAMS ID (IDENTIFICATION) OF CIGUATERA CAUSATIVE ORGANISM/S (JAPAN RECOMMEND TO USE LCMS METHOD, BUT ITS NOT AOAC METHOD YET)	MOUSE BIOASSAY AND INTERNATIONALLY ACCEPTED SCREENING METHODS ANY OTHER METHOD ASIDE FROM CIGUA-CHECK	CAN THE PROJECT FACILITATE IF NOT PROCURE TOXIN STANDARDS LIKE CIGUATERA, ASP AND DSP TOXINS FOR BIOTOXIN MONITORING IN THE ASEAN? CAN SHARE WITH MEMBERS OF PHYTOPLANKTON ID USING MOUSE BIOASSAY
SINGAPORE	1. PSP, DSP, ASP 2. YTX, AZA	SHELLFISH (OYSTERS & MUSSELS)	EITHER HPLC OR LCMSMS OR ELISA	BOTH ON IMPORT AND LOCALLY PRODUCED SAMPLES
THAILAND	PSP	PSP (SHELLFISH, BABY CLAM, MUSSEL)	PSP (MOUSE BIOASSAY AND HPLC)	
	TTX	TTX (PUFFER FISH)	TTX (HPLC)	

COUNTRY	PROPOSED BIOTOXINS FOR SURVEY	PROPOSED BIOTOXINS SPECIES FOR SURVEY	METHODS OF ANALYSIS FOR PROPOSED BIOTOXINS TO BE SURVEYED ON	REMARKS
VIETNAM	PSP, LIPOPHILIC TOXINS, ASP, DSP	BABY CLAM, BLOOD CLAM, ANTIQUE ARK (USE THE SAME METHOD AS ABOVE)	ASP: HPLC PSP: MOUSE BIOASSAY	ALGAE (PROPOSED BIOTOXINS SPECIES FOR SURVEY) PROPOSE TAXONOMY TRAINING COURSE TO IDENTIFY HARMFUL ALGAE

CONSOLIDATED TABLE FOR PROJECT IMPLEMENTATION

D. Training Course

Tests Requested	Countries Requested for Tests	Current Capability of Member Countries for Regional / On-site
Multi-component of DSP and lipophilic toxins (YTX, PTX) using LCMSMS method & Japan rapid method (~US$800 for 30 samples in triplicates) (Optional) Proposed for Regional Training Course in Singapore – June/ July 2010	1. Indonesia (DSP using HPLC) 2. Myanmar (DSP using LCMSMS) 3. Philippines (DSP screening method) 4. Thailand (DSP, PTX, YTX, AZA by LCMSMS) 5. Singapore (using LCMSMS)	1. Indonesia (HPLC-UV, LCMSMS, LC-MS) 2. Myanmar (ELISA, HPLC and LCMSMS) 3. Philippines (Mouse bioassay, Immunoassay and HPLC-UV 4. Thailand (HPLC, LCMSMS, Mouse bioassay) 5. Singapore (HPLC, LCMSMS, ELISA)
PSP using HPLC and ELISA Proposed for Regional Training Course in Singapore – June/July 2010	1. Indonesia (HPLC and ELISA) 2. Lao P.D.R (ELISA) 3. Malaysia (HPLC) 4. Myanmar (ELISA) 5. Thailand (HPLC)	1. Indonesia (HPLC-UV, LCMSMS, LC-MS) 2. Lao P.D.R (ELISA) 3. Malaysia (pending HPLC procurement) 4. Myanmar (ELISA, HPLC and LCMSMS) 5. Thailand (HPLC, LCMSMS, Mouse bioassay)

Tests Requested	Countries Requested for Tests	Current Capability of Member Countries for Regional / On-site
CTX Limitations of standards	1. Indonesia (using HPLC) 2. Philippines (besides Ciguacheck) 3. Thailand 4. Singapore	1. Indonesia (HPLC-UV, LCMSMS, LC-MS) 2. Philippines (Mouse bioassay, Immunoassay and HPLC-UV) 3. Thailand (HPLC, LCMSMS, Mouse Bioassay) 4. Singapore (HPLC, LCMSMS, ELISA)
TTX Proposed for Regional Training Course in Singapore – June/ July 2010	1. Malaysia (using HPLC) 2. Thailand (LCMSMS) 3. Vietnam (HPLC) 4. Singapore	1. Malaysia (LCMSMS and pending HPLC procurement) 2. Thailand (HPLC, LCMSMS, Mouse bioassay) 3. Vietnam (LCMSMS, HPLC, Mouse Bioassay) 4. Singapore (HPLC, LCMSMS, ELISA)
AZA	1. Singapore	
ASP using HPLC	1. Myanmar	
Identification of phytoplankton	1. Thailand	
Taxonomy to identify harmful algae	1. Vietnam	

E. For Biotoxins Survey

Tests	Species	Countries	Remarks
PSP (HPLC)	*PERNA VIRIDIS* *ANADARA SPP* *MERITRIX SPP* *PAPHIA UNDULATA*	Indonesia	Cambodia wish to learn on how to set up biotoxins monitoring system and how to do sampling and analyzing (to be covered in Regional Training Course in Singapore) Both on import and locally produced samples.
	Oyster and mussel	Singapore	Lao PDR is interested in collaborating with Thailand for survey of shellfish behind area of Lao PDR and Thailand.
	Shellfish, baby clam and mussel	Thailand	Survey cockle, baby clam, green mussel (import from different parts, especially Northern Thailand) Cambodia wish to learn on how to set up biotoxins monitoring system and how to do sampling
PSP (Mouse Bioassay)	Mussel, oyster, cockle and certain clam	Philippines	
	Shellfish, baby clam and mussel	Thailand	Lao PDR is interested in collaborating with Thailand for survey of freshwater puffer fish and/or shellfish behind area of Lao PDR and Thailand. Survey cockle, baby clam, green mussel (import from different parts, especially Northern Thailand)
	Baby clam, blood clam and antique ark	Vietnam	Propose: • Algae (biotoxin species for survey)
PSP	Green mussel, clam, spotted Babylon	Myanmar	
DSP (HPLC)	*PERNA VIRIDIS* *ANADARA SPP* *MERITRIX SPP* *PAPHIA UNDULATA*	Indonesia	
	Shellfish (oyster and mussel)	Singapore	Both on import and locally produced samples.

Tests	Species	Countries	Remarks
DSP (LCMSMS)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples
DSP (ELISA)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples
DSP	GREEN MUSSEL, CLAM, SPOTTED BABYLON BABY CLAM, BLOOD CLAM AND ANTIQUE ARK	Myanmar Vietnam	 Propose: • Algae (biotoxin species for survey) • Taxonomy training course to identify harmful algae
ASP (HPLC)	*PERNA VIRIDIS* *ANADARA SPP* *MERITRIX SPP* *PAPHIA UNDULATA* SHELLFISH (OYSTER AND MUSSEL) BABY CLAM, BLOOD CLAM AND ANTIQUE ARK	Indonesia Singapore Vietnam	 Both on import and locally produced samples. Propose: • Algae (biotoxin species for survey) • Taxonomy training course to identify harmful algae
ASP (LCMSMS)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples.
ASP (ELISA)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples.
ASP	GREEN MUSSEL, CLAM, SPOTTED BABYLON	Myanmar	
CTX (HPLC)	CORAL FISH	Indonesia	

Tests	Species	Countries	Remarks
CTX (any other methods except ciguacheck)	CIGUATERA CAUSATIVE ORGANISMS	Philippines	Can the project facilitate, if not procure toxins standards like ciguatera, ASP and DSP toxins for biotoxins monitoring in the ASEAN? Philippines can share with members of phytoplankton ID using mouse bioassay Lao PDR is interested in collaborating with Thailand for survey of freshwater puffer fish
TTX (HPLC)	PUFFER FISH OR RELATED SPECIES PUFFER FISH	Malaysia Thailand	Training needed for HPLC method. Lao PDR is interested in collaborating with Thailand for survey of freshwater puffer fish and/or shellfish behind area of Lao PDR and Thailand. Survey cockle, baby clam, green mussel (import from different parts, especially Northern Thailand)
Lipophilic toxins	BABY CLAM, BLOOD CLAM, ANTIQUE ARK	Vietnam	Propose: • Algae (biotoxin species for survey)
YTX (HPLC, LCMSMS, ELISA)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples.
AZA (HPLC, LCMSMS, ELISA)	SHELLFISH (OYSTER AND MUSSEL)	Singapore	Both on import and locally produced samples.

Annex 2: Regional Training Course Participants' List, 2010

No.	Name	Designation	Country	Office	Department/ Division	Office Address	Telephone No.	Fax No.	Email Address
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Annex 3

Administrative Report of End-of-Project Seminar

Report of the END-OF-PROJECT (EOP) SEMINAR ON JAPANESE TRUST FUND II CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA (BIOTOXINS MONITORING IN ASEAN)

20 – 21 NOVEMBER 2012
Singapore

I. Introduction

1. At the invitation of the SEAFDEC Marine Fisheries Research Department (MFRD), the End-of-Project (EOP) Seminar on "Japanese Trust Fund II Chemical and Drug Residues in Fish and Fish Products in Southeast Asia (Biotoxins Monitoring in ASEAN)" was held from 20-21 November 2012 in Singapore.

2. The meeting was attended by representatives from SEAFDEC member countries, Technical Coordinator from SEAFDEC Secretariat, the Chief of MFRD Programmes as well as the Deputy Director for Post-Harvest Technology Division (PHTD) of the Agri-Food & Veterinary Authority of Singapore (AVA) and other staff from PHTD as the collaborating center for MFRD Programmes. The list of participants appears in Annex 1.

II. Opening Of The Meeting By Chief, MFRD Programmes

3. The Chief of the MFRD Programmes, Mr Yeap Soon Eong, welcomed the participants to the End-of Project seminar to share the outcome of the project and to discuss the new project on Biotoxins.

4. The Deputy Director for PHTD, Ms Khoo Gek Hoon, on behalf of Agri-food and Veterinary Authority of Singapore (AVA) delivered the opening remarks to the participants of the seminar.

5. The Chairperson and MFRD JTF II Project Leader, Ms Neo Shan Yu, presented the agenda of the seminar and it was adopted by the meeting. The agenda appears in Annex 2.

III. Presentation On The Introduction Of Japanese Trust Fund II Project By The Marine Fisheries Research Department

6. The MFRD JTF II Project Leader presented the project overview, which included project background and rationale, project objectives and progress of the project activities.

7. The Chief informed the meeting that Brunei was unable to attend the meeting due to unforeseen circumstances. He added that Brunei also did not participate

in the Biotoxins monitoring survey.

IV. Country Paper Presentations On Outcome Of Biotoxins Monitoring System

8. Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam presented their reports on the outcomes of biotoxins monitoring survey in their respective countries.

Cambodia

9. The Chief enquired if the analytical laboratory (Centre of Analytical Service and Experimentation (CASE) of Vietnam) that Cambodia sent samples for analysis is a government or private laboratory. Vietnam responded that CASE is an ISO-accredited laboratory operated by private company.

Indonesia

10. Cambodia enquired if the different sampling sites were used as replicates. Indonesia clarified that the sampling sites were not replicates and each site was situated about 3 km apart from one another.

11. The Chief enquired about the reason for Ambon to have high level of Paralytic Shellfish Poisoning (PSP) biotoxins. Indonesia responded that the area is a bay which is more enclosed, thus resulting in higher concentration of biotoxins.

12. The Chief requested the meeting to share with Indonesia the source of reference material for Amnesic Shellfish Poisoning (ASP). Singapore shared that the reference materials could be obtained from National Research Council of Canada (NRC) Halifax.

Lao PDR

13. Lao PDR shared that the green mussels samples used in the Biotoxins Survey were imported from Thailand and the samples were sent to a collaborating laboratory in Thailand for testing. Collaboration was done with the Fish Inspection and Quality Control Division of the Department of Fisheries in Thailand.

14. The Chief enquired on the popular types of shellfish in Lao PDR. Lao PDR highlighted that initially they were targeting the blood cockles as they are the most popular shellfish in Lao PDR. However, blood cockles do not fall within the target species for this project, therefore green mussels were analysed for this project. The Chief asked if there are any historical records of poisoning cases due to ingestion of blood cockles. Lao PDR replied that there were cases of poisoning but the figures were low.

Malaysia

15. Malaysia shared that the Oshima method used to test for PSP in their samples referred to the method which they had learnt from the Regional Training Course held in 2010 in Singapore.

16. The Chairperson enquired about the source of the regulatory limits of PSP that was used as a reference for the project. Malaysia responded that they used international regulatory limits for their purpose.

17. Malaysia shared with the meeting that the monitoring programme for PSP would be finalized by the end of this year and would hopefully be implemented in 2013. The Chief expressed his concern for the initiation of biotoxin monitoring programme for Malaysia because of the multiple challenges that were raised during the presentation. With regards to

the enquiry by Chief about the details of the monitoring programme, Malaysia responded that the plan has not been released by the headquarters.

Myanmar

18. Singapore enquired if Myanmar used the same ELISA test kits for all the three biotoxins testing (PSP, Diarrhetic Shellfish Poisoning (DSP) and Amnesic Shellfish Poisoning (ASP)). Myanmar clarified that three different test kits were used.

19. Philippines enquired if the ELISA test kit used by Myanmar is approved by AOAC as a reference method for PSP. Myanmar was uncertain if the testing procedure is of AOAC standard. Philippines shared that the ELISA ABRAXIS test kit is approved by AOAC as a screening method. Indonesia agreed with Philippines' comment and added that the data obtained from ELISA test kit is more qualitative than quantitative.

20. Indonesia enquired Myanmar about the data presentation as it was observed that the values were reflected even when it is below the Limit of Detection (LOD). Singapore clarified that if the results were below the Limit of Detection, it should be considered as "Not Detected".

21. Myanmar expressed interest in knowing the preferred methodology for PSP testing. Singapore informed that the use of the RidaScreen ELISA test kit, which was one of the methods taught during the Regional Training Course in 2010, could be adopted as a screening method. The HPLC method that was taught during the course could be used as a confirmatory test.

22. The Chairperson informed the meeting that the procedures for the laboratory methods taught in the Regional Training Course in 2010 could be found in the annexes of the Technical Compilation.

Philippines

23. Philippines shared with the meeting about a project on AOAC approved screening methods that was funded by the Japan Ministry of Agriculture Forestry and Fisheries, in collaboration with the North Pacific Marine Science Organisation (PICES) on some AOAC approved screening methods.

24. Indonesia enquired on the actions taken by the Philippines government when a sample was detected to exceed the safety limit. Philippines explained that a shellfish advisory would be issued when an area is found to be positive for Harmful Algae Bloom (HAB) and a the issuance of a localized ban on shellfish gathering, harvesting and trading would be coursed through the local government executive. In addition, the checks would be intensified on a weekly basis during the period of the ban. The ban on the harvesting of shellfish in the affected area would only be lifted when results are negative or below the Philippines Regulatory Standard for three consecutive weeks.

Singapore

25. Myanmar enquired if ELISA or Mouse Bioassay is a better method for screening. Singapore shared that the ELISA technique could be used as a screening test but positive sample should be confirmed by an acceptable reference method such as Mouse Bioassay, High Performance Liquid Chromatography (HPLC) or High Performance Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS).

26. The Chief enquired if European Union (EU) allows the use of Mouse Bioassay methods. Singapore responded that

the Mouse Bioassay is currently still recognized as an approved method by the EU.

Thailand

27. The Chief clarified on the recommendation provided by Thailand on MFRD as an independent agency to provide inter-laboratory and/or Proficiency Test (PT) for the ASEAN countries. Thailand added that currently they are aware that only QUASIMEME from Netherlands can provide the PT service for Biotoxins test.

28. In response to Thailand's recommendation on MFRD as an independent agency for inter-laboratory and/or PT for the ASEAN countries, the Chief informed the meeting that a similar suggestion had also been raised in the previous Japan Trust Fund projects on pesticides and heavy metals. However, MFRD does not have the expertise to produce reference materials for PT.. Singapore added that the Asia Pacific Laboratory Accreditation Cooperation (APLAC) had conducted a round of PT for Mouse Bioassay recently. In addition, Food Analysis Performance Assessment Scheme (FAPAS) which conducts PT for laboratories collects feedbacks on the type of analytes for PT. Thailand explained that they have been requesting for Biotoxins PT from FAPAS since 5 years ago but to no avail. Chief of MFRD sought for all member countries to input request for biotoxins PT to FAPAS.

Vietnam

29. In response to the budget enquiry, the Chief explained that JTF projects were carried out on a cost-sharing basis and were meant to help the country to initiate the programme. The Chief added that although the project timeline was short, he still hoped that the countries would continue the programme even after the project has ended.

30. With regards to the proposed recommendation by Vietnam on traceability, the Chief informed that a traceability workshop was held last year in Vietnam and another workshop would be held next year in Thailand. He welcomed all interested countries to attend the workshop. Vietnam clarified that they were aware of the project on "JTF V Traceability Systems for Aquaculture Products in the ASEAN Region" but would like to see more activities in this area.

31. The SEAFDEC technical coordinator, Mr Tadahiro Kawata, mentioned that the Japanese Government supports many other programmes besides the Japanese Trust Fund projects. He hopes that the member countries would continue to support and implement the projects even though the budget is limited.

V. Technical Compilation – Comments and Recommendations

32. The Chairperson informed the meeting that the revised Technical Compilation would be disseminated to all member countries after having incorporated all the comments and recommendations from the meeting.

33. The Chief proposed to include a new annex to list the national laboratories involved in biotoxins testing in member countries and requested for all countries to provide the name and the address of the laboratories to the MFRD JTF II Project Leader.

34. The SEAFDEC technical coordinator requested to include the administrative report for Regional Technical Consultation (RTC), the participants' list for the Regional Training Course, and

the administrative report for the End-of-Project (EOP) Seminar into the annexes of the Technical Compilation.

IV. Deliberation And Discussion On Scope Of The New Project Extension On Biotoxins

35. The MFRD JTF II Project Leader presented the new proposed project extension under JTF VI, "JTF II Chemical and Drug Residues in Fish and Fish Products in Southeast Asia – Biotoxins Monitoring in ASEAN (ASP, AZA and BTX). This included the project overview, which comprised of the project background and rationale, project objectives, project management and administration, expected project outputs, project schedule and proposed activities.

36. Singapore queried on how the three new biotoxins, Azaspiracids (AZA), Amnesic Shellfish Poisoning (ASP) toxin and Brevetoxins (BTX) were chosen. The Chief responded that these three biotoxins were previously shortlisted by the member countries at the last RTC of JTF II that was held in 2009.

37. Philippines enquired about the relevance of BTX as this biotoxin is not common in the ASEAN region and therefore may not be applicable. In view of this, Philippines proposed ciguatoxins as one of the targeted biotoxins for JTF VI project as there are currently no available rapid methods to detect ciguatoxins. Singapore also shared that the current difficulty in ciguatoxin analysis is the unavailability of commercial ciguatoxins standards.

38. Cambodia enquired if the new JTF VI project could also include drug residues as part the monitoring purpose. The Chief shared that antibiotics residues, pesticides residues and heavy metals were already covered in the previous JTF projects in 2004. Therefore, this coming project would only focus on biotoxins.

39. Malaysia enquired on the procurement of biotoxins reference materials. Singapore replied that these reference materials are commercially available.

40. Indonesia enquired about the methodologies and proposed equipments for the project and would like to request for any project information to be given early enough so that they could plan for inclusion into their agency budget and be in time for the upcoming project. The Chief explained that these details would be elaborated at the RTC, which is to be held in 2013.

41. Philippines noted that Mouse Bioassay is the most common method used among the countries other than HPLC and LC/MS/MS, and hence suggested to have a common methodology for the new JTF VI project across the ASEAN countries to facilitate information exchange. The Chief explained that the main rationale of the project's extension is on the inclusion of new biotoxins.

42. Singapore informed the meeting that in the Commission Regulation (EU) No 15/2011 of 10 Jan 2011, Official Journal of EU, L6, Vol 54, 11 Jan 2011, the Mouse Bioassay for DSP would be replaced by LC/MS/MS with effect from 2014. Thailand updated the meeting that since 2011, HPLC method has been the reference method for PSP in EU.

43. In response to Myanmar's query on the rationale of focusing on only shellfish for biotoxins monitoring, the Chief explained that with the exception of ciguatoxins, biotoxins occurs mostly in shellfish and less common in fishes. Indonesia also explained that shellfish, being filter feeders, has higher tendency of accumulating biotoxins as compared

to fishes. Singapore added that other than ciguatoxins, there are also puffer fish toxins.

44. Lao PDR proposed to target multiple species of shellfish in the upcoming JTF VI project so as to allow the inclusion of blood cockles. The Chief agreed on the inclusion of other popular species of shellfish for the next project.

45. Singapore proposed for the inclusion of algae identification in the new project and this was supported by Philippines and Indonesia. Indonesia commented that they have been doing simple monitoring of algae in the water using microscopy. Philippines agreed on the importance of algae monitoring and suggested experts to share the knowledge. The Chief requested for Philippines to provide the contacts of Japanese algae experts. Singapore added that New Zealand may also have experts in algae monitoring.

46. The Chief updated the meeting that the JTF VI project has been approved and hoped that the experience gained from the current JTF II project could help member countries to better implement the next JTF VI project.

XI. Closing Of The Seminar

47. The Chairperson concluded the Seminar and thanked all participants for their contributions to the Seminar.

48. The participants from the SEAFDEC member countries expressed their heartfelt appreciation to MFRD for the warm hospitality accorded to them and the excellent arrangements made for the Seminar and to the Government of Japan for making this Seminar possible.

49. The Seminar was held in the traditional spirit of SEAFDEC co-operation and cordiality.

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AGENDA OF THE MEETING

PROVISIONAL AGENDA & TIMETABLE

20 November 2012, Tuesday

0845 – 0900	Registration
0900 – 1000	**Agenda 1:** Opening of the Meeting
	Welcome Remarks by Chief, MFRD Programmes
	Opening Remarks by Deputy Director, PHTD
	Agenda 2: Introduction of Japanese Trust Fund II Project
1000 – 1030	Group Photography Session & Coffee Break
1030 – 1230	**Agenda 3:** Country Report Presentation
	1030 – 1100: Cambodia
	1100 – 1130: Indonesia
	1130 – 1200: Lao PDR
	1200 – 1230: Malaysia
1230 – 1400	Lunch Break
1400 – 1530	**Agenda 3:** Country Report Presentation (*cont.*)
	1400 – 1430: Myanmar
	1430 – 1500: Philippines
	1500 – 1530: Singapore
1530 – 1600	Coffee Break

Agenda 3: Country Report Presentation (*cont.*)

1600 – 1630: Thailand

1630 – 1700: Vietnam

1700 - 1730 Final Remarks on Country Reports and Survey Results

21 November 2012, Wednesday

0900 - 1000 **Agenda 4:** Technical Compilation – Comments and Recommendations

1000 – 1030 Coffee Break

1030 – 1200 **Agenda 5:** Presentation and Discussion on New Project on Biotoxins

1200 – 1330 Lunch Break

1330– 1430 **Agenda 6:** Summary Discussion

1430 – 1500 **Agenda 7:** Closing Remarks

~Coffee Break and End ~

Annex 4

Determination of Diarrhetic Shellfish Poisoning and Lipophilic Toxins Using Liquid Chromatography – Mass Spectrometry (LC-MS) Method

1. Extraction of Toxins

1.1 Shellfish samples

1.1.1 To 1 g of homogenate, add 9 mL of MeOH/distilled water (9:1 v/v).

1.1.2 Extract with a homogenizer for 3 minutes.

1.1.3 Centrifuge at 3000 rpm for 5 minutes.

1.1.4 Pass an aliquot of supernatant through a 0.5 μm filter.

1.1.5 Inject a 5 μL aliquot of the filtrate into the LC-MS.

1.2 Plankton net samples

1.2.1 Keep condensed plankton sample collected by plankton net in a –30°C freezer.

1.2.2 Ultra-sonicate for 30 seconds.

1.2.3 Filter with No. 5A paper filter

1.2.4 Add 5 mL of 200mM phosphate buffer (pH 5.8) to 50mL of filtrate.

1.2.5 Condition the C18 Plus Sep-pak (Equilibrate with 5mL of MeOH, followed by 5mL of 20 mM phosphate buffer at pH 5.8.)

1.2.6 To the sep-pak, add in the 50 mL of filtrate with added phosphate buffer (from 1.2.4), followed by 10 ml of distilled water and 5ml of MeOH.

1.2.7 Collect the 5 mL of MeOH eluate and evaporate the solvent.

1.2.8 Dissolve residues in 500 μL of MeOH.

1.2.9 Inject a 5 μL aliquot of the solution into the LC-MS.

2. Preparation of Reagents

2.1 200mM phosphate buffer (pH 5.8)

a) 15.6 g of NaH_2PO_4/ $2H_2O$ is dissolved in 500 mL distilled water.

b) 35.8 g of Na_2HPO_4/ $12H_2O$ is dissolved in 500 mL distilled water.

c) (b) is added to (a) to adjust the pH to 5.8.

2.2 Preparation of mobile phases

2.2.1 Stock solution (1M HCOOH, 40mM HCOONH4 stock solution)

a) Weigh out HCOOH (MW 46.03). The amount is 12.79g for 90% HCOOH, 11.99g for 96% HCOOH and 11.74g for 98% HCOOH.

b) Weigh out 0.6306g of HCOONH4 (MW 63.06).

Dissolve (a) and (b) in 250mL of distilled water, then adjust pH to 2.3 ± 0.05 with conc. NH_4OH solution.

2.2.2 LC-MS mobile phases

Mobile phase A: 50mM HCOOH, 2mM$HCOONH_4$ solution

Mix distilled water and stock solution in the ratio of 95:5 vol/vol (475mL:25mL)

Mobile phase B: 50mM HCOOH, 2mM$HCOONH_4$ in 95% MeCN solution

Mix Acetonitrile (MeCN) and stock solution in the ratio of 95:5 vol/vol (475mL:25mL)

3. LC-MS Conditions

Column: Hypersil BDS-C8 (2.1 id x 50 mm) (Keystone Scientific)

Column temperature: 20°C

Gradient: 20% B to 100% B for 10minutes

100% B for 15minutes

Flow rate: 0.2ml/min

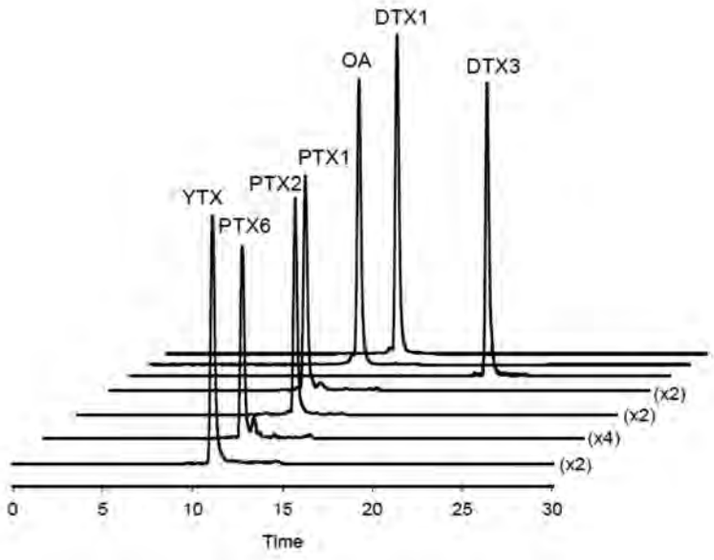


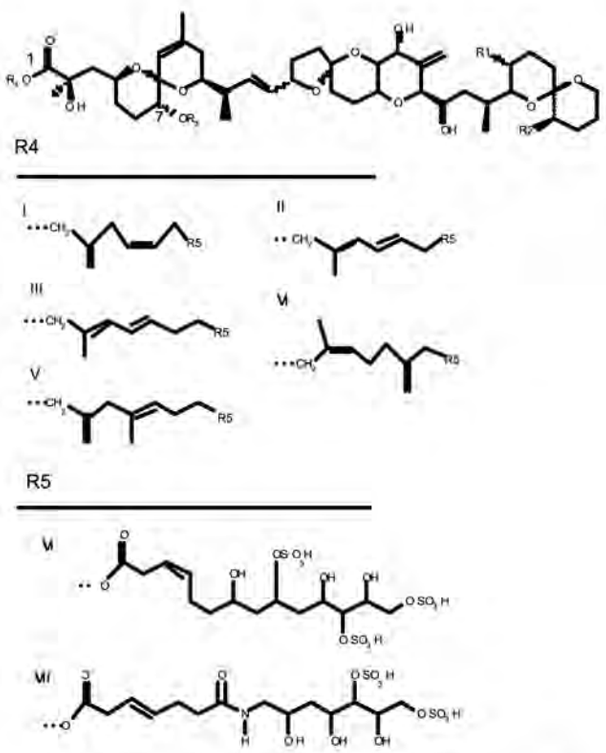
Fig. 1. LC-MS chromatogram

4. References

1) T. Suzuki, T. Jin, Y. Shirota, T. Mitsuya, Y. Okumura, and T. Kamiyama: Quantification of lipophilic toxins associated with diarrhetic shellfish poisoning (DSP) in Japanese bivalves by liquid chromatography-mass spectrometry (LC/MS) and comparison with mouse bioassay (MBA) as the official testing method in Japan. Fish. Sci., 71, 1370-1378 (2005).

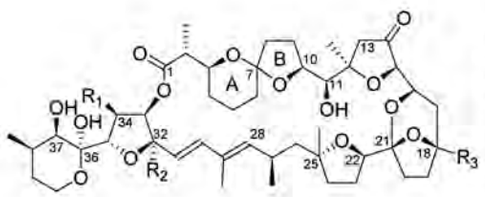
2) T. Suzuki, A. Miyazono, K. Baba, R. Sugawara, and T. Kamiyama: LC-MS/MS analysis of okadaic acid analogues and other lipophilic toxins in single-cell isolates of several Dinophysis species collected in Hokkaido, Japan. Harnful Algae, 8, 233-238 (2009).

5. Appendix

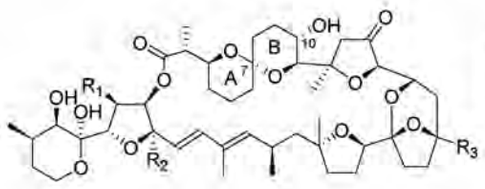


	R1	R2	R3	R4	R5	EMW
OA	CH_3	H	H	H	-	804.5
DTX1	CH_3	CH_3	H	H	-	818.5
DTX2	H	CH_3	H	H	-	804.5
DTX3		(H or CH_3)	Acyl	H	-	
OA-D7a	CH_3	H	H	I	OH	914.6
OA-D7b	CH_3	H	H	II	OH	914.6
OA-D8	CH_3	H	H	III	OH	928.6
OA-D9a	CH_3	H	H	IV	OH	942.6
OA-D9b	CH_3	H	H	V	OH	942.6
DTX4	CH_3	H	H	III	VI	1472.6
DTX5a	CH_3	H	H	II	VII	1392.6
DTX5b	CH_3	H	H	III	VIII	1406.6

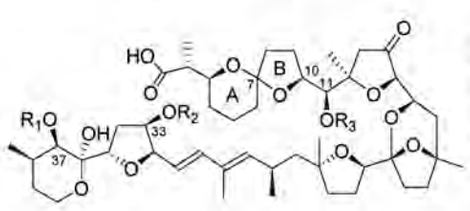
Fig. 1 Structure of OA and DTX1 analogues



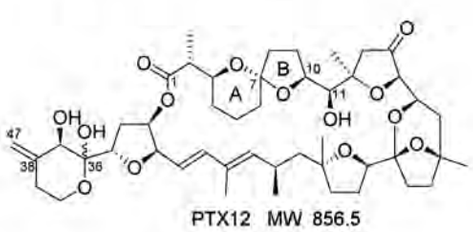
	C7	R1	R2	R3	MW
PTX2	*R*	H	H	CH_3	858.5
PTX2b	*S*	H	H	CH_3	858.5
PTX1	*R*	H	H	CH_2OH	874.5
PTX4	*S*	H	H	CH_2OH	874.5
PTX3	*R*	H	H	CHO	872.5
PTX6	*R*	H	H	COOH	888.5
PTX7	*S*	H	H	COOH	888.5
PTX11	*R*	OH	H	CH_3	874.5
PTX11b	*S*	OH	H	CH_3	874.5
PTX13	*R*	H	OH	CH_3	874.5



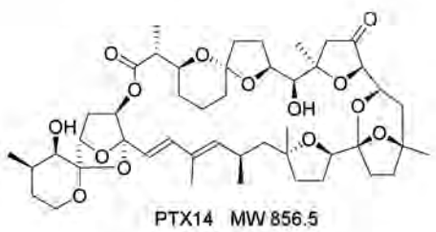
	C7	R1	R2	R3	MW
PTX2C	*S*	H	H	CH_3	858.5
PTX8	*S*	H	H	CH_2OH	874.5
PTX9	*S*	H	H	COOH	888.5
PTX11C	*S*	OH	H	CH_3	874.5



	C7	R1	R2	R3	MW
PTX2sa	*R*	H	H	H	876.5
7-*epi*-PTX2sa	*S*	H	H	H	876.5
37-*O*-acyl PTX2sa	*R*	acyl	H	H	
33-*O*-acyl PTX2sa	*R*	H	acyl	H	
11-*O*-acyl PTX2sa	*R*	H	H	acyl	

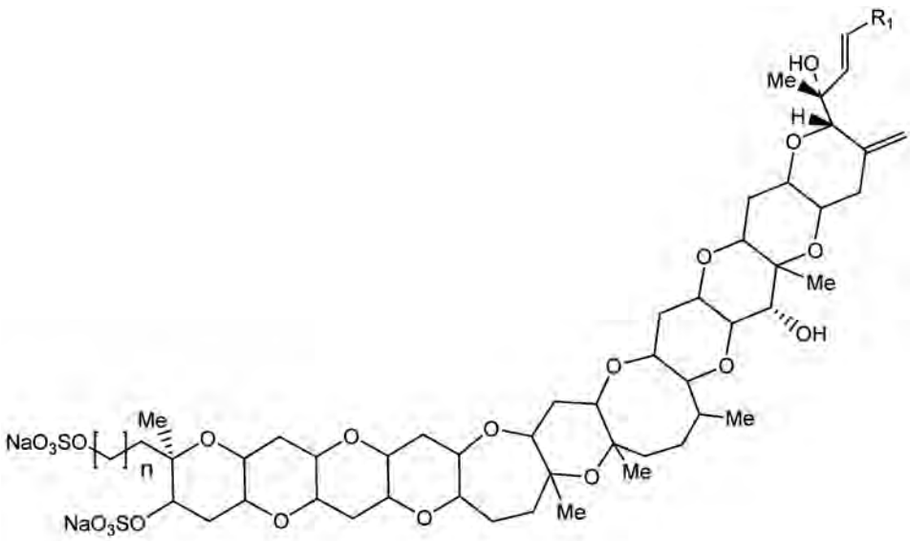


PTX12 MW 856.5



PTX14 MW 856.5

Fig. 2 Structure of PTX analogues



	n	MW	R_1
YTX	1	1186.5	
1-homoYTX	2	1200.5	
45-hydroxyYTX	1	1202.5	
45-hydroxy-1-homoYTX	2	1216.5	
45,46,47-trinorYTX	1	1146.5	
45,46,47-trinor-1-homoYTX	2	1160.5	

Fig. 3 Structure of YTX analogues

Table 1. DSP and other lipophilic toxins data

Toxins	Molecular formula	Molecular weight	Toxicity (µg/kg)	µg/MU
OA	$C_{44}H_{68}O_{13}$	804.5	200	4.0
DTX1	$C_{45}H_{70}O_{13}$	818.5	160	3.2
DTX2	$C_{44}H_{68}O_{13}$	804.5		
DTX3*1	$C_{61}H_{100}O_{14}$	1056.7	250	5.0
PTX1	$C_{47}H_{70}O_{15}$	874.5	250	5.0
PTX2	$C_{47}H_{68}O_{14}$	858.5	230	4.6
PTX6	$C_{47}H_{68}O_{16}$	888.5	500	10.0
PTX2SA	$C_{47}H_{72}O_{15}$	876.5		
YTX	$C_{55}H_{80}O_{21}S_2Na_2$	1186.4	100	2.0
45OHYT	$C_{55}H_{80}O_{22}S_2Na_2$	1202.4	500	10.0

*1 DTX3: 7-*O*-palmitoyl-DTX1

Data from Yasumoto et al., J AOAO Int 78:574-582 (1995)

Table 2. Ions detected in LC/MS for DSP and other lipophilic toxins

Toxins	$[M+H]^+$	$[M+H_4]^+$	$[M+Na]^+$	$[M+H]^-$	$[M+CH_3COOH-H]^-$	$[M+HCOOH-H]^-$
OA	805.5	822.5	827.5	803.5		
DTX1	819.5	836.5	841.5	817.5		
DTX2	805.5	822.5	827.5	803.5		
DTX3*1	1057.7	1074.7	1079.7	1055.7		
PTX1	875.5	892.5	897.5	873.5	933.5	919.5
PTX2	859.5	876.5	881.5	857.5	917.5	903.5
PTX6	889.5	906.5	911.5	887.5	947.5	933.5
PTX2SA	877.5	894.5	899.5	875.5	935.5	921.5
YTX				1141.5*2		
45OHYT				1157.5*2		

*1 DTX3: 7-*O*-palmitoyl-DTX1

*2 $[M-2Na+H]^-$

Annex 5

Diarrhetic Shellfish Poisoning Rapid Method

DSP Rapid Kit 96 Ver.2010.2.26

A colorimetric phosphatase inhibition assay

(DSP: Diarrhetic Shellfish Poisoning)

Introduction

Diarrhetic Shellfish Poisoning (DSP) is caused by the ingestion of shellfish contaminated by toxic dinoflagellates. DSP has been observed worldwide and caused problems to public health and the shellfish industry. To detect DSP toxins, a colorimetric phosphatase inhibition assay was developed using a highly purified recombinant human PP2A C-subunit. The assay is very sensitive, fast, easy, accurate and reproducible to detect DSP toxins (OA group) in the shellfish. It does not require a high level of skills. The lowest quantifiable limit of OA is 0.05 μg/g in shellfish whole meat.

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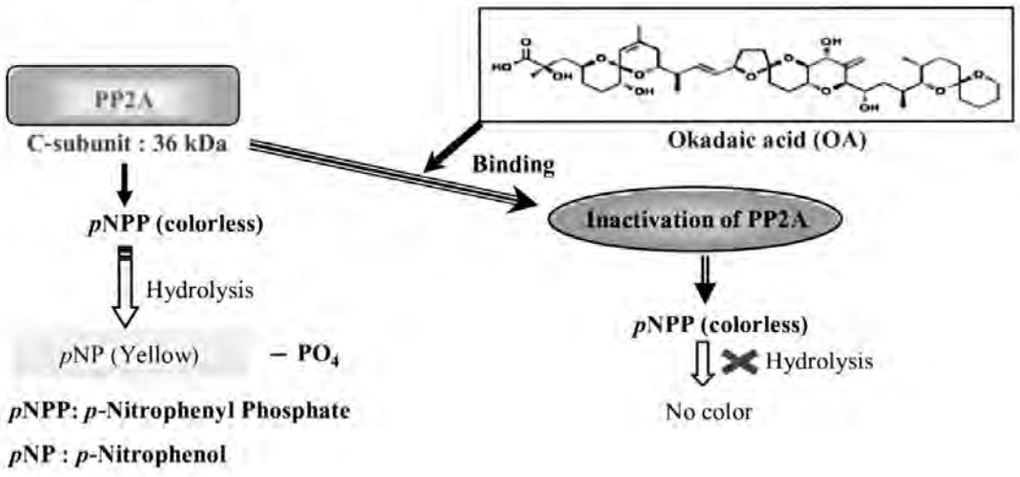
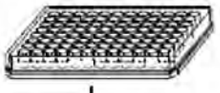
1

Assay Principle

The assay is based on the inhibition of the protein phosphatase 2A (PP2A) by DSP toxins (OA and DTXs). PP2A can hydrolyze a colorless artificial substrate, *p*-nitrophenyl phosphate (*p*-NPP), and produces the yellow color of *p*-nitrophenol (*p*-NP) in the alkaline solution. The intensity of the color is proportional to the enzyme activity and the absorbance is measured at 405 nm. The concentration of DSP toxins in the sample is calculated from the standard curve produced using known concentrations of OA.



Summary of Assay



96 Well plate

← Add 50 µl of **sample** and **OA Reference Solution**

← Add 100 µl of **PP2A Working Solution**

← Add 100 µl of **Substrate Working Solution**

Mix for 1 min by a microplate mixer

↓

Incubate for 30 min at 36℃

↓

Measure absorbance at 405 nm against 490 or 492nm as reference

2

Kit Components

	Item	Amount	Storage
1	Okadaic Acid (OA) Reference Solutions (OA R1~OA R8)	8 x 0.5 ml	-20°C
2	Sample Buffer	40 ml	-20°C
3	1.25 N NaOH Solution	1.5 ml	-20°C
4	1.25 N HCl Solution	1.5 ml	-20°C
5	PP2A Buffer A	11 ml	-20°C
6	PP2A Buffer B	1 ml	-20°C
7	PP2A Stock Solution (0.5 ml tube)	80 µl	-20°C
8	Substrate Buffer	12 ml	-20°C
9	Substrate Tablet (*p*-NPP)	20 mg tablet	-20°C
10	1 x 8 wells x 12strips	1	
11	Adhesive Film	1	
12	containing Excel spread sheet*		

***The Excel spreadsheet is on the website(http://www.ttc.co.jp/dsp/).**

Caution: This kit contains strong alkali and acid solutions, and toxin (Okadaic acid). Wear disposable gloves, eye protection and protective clothing when preparing and handling reagents and samples.

The PP2A Stock solution of this kit might contain a small amount of recombinant Baculovirus because of its manufacturing method.

Additional Materials Required

- Microplate reader capable of measuring absorbance at 405 nm against 490 or 492nm as reference
- Microplate mixer
- Incubator for use at 36°C
- Heating apparatus: water bath or heating block
- Vortex mixer
- Reagent reservoirs
- Micro pipettes (20-200 µl and 200-1000 µl) with disposable tips.
- Multichannel pipette
- 15 ml and 50 ml Polyethylene tubes
- 1.5 ml Microcentrifuge tubes
- 2 ml Screw capped tubes
- Deionized or distilled water

Sample Preparation

Sample for Free OAs (OA, DTX1, and DTX2)

Collect the whole meat of shellfish.

↓ Remove excess water by placing on filter papers.

Mince the whole meat and weigh 2 g into a 50 ml centrifuge tube.

↓ ← Add 18 ml of 90% methanol. (9 times of weighed minced sample)

Homogenize for 1 min with Polytron homogenizer or an equivalent (any other capable homogenizer) at room temperature.

↓ Centrifuge at 2,300 × g for 10min. at room temperature.

Transfer the middle part of supernatant to a suitable tube.
(the supernatant is designated **Extract Supernatant**)

↓ Keep the Extract Supernatant at room temperature.

Pipette 50 µl of Extract Supernatant into a 1.5 ml microcentrifuge tube and add 950 µl of the Sample Buffer **(Sample for Free OAs)**.

Note: Sample Buffer may contain precipitates that can be dissolved by shaking at room temperature.
Extract supernatant storage : store at -20 ℃
Extract supernatant return to the room temperature and dissolve precipitation before using. (Do NOT heat !)

Sample for Total OAs (OA, DTX1, DTX2 and DTX3)

Pipette 500 µl of the **Extract Supernatant** into a screw-capped 2 ml tube.

↓ Add 100 µl of 1.25 N NaOH solution, tighten the cap and mix.

Heat at 80℃ for 40 min.

↓

Cool the tube to room temperature and add 100 µl of 1.25 N HCl to neutralize the solution.

↓

Pipette 50 µl of the neutralized solution into a 1.5ml microcentrifuge tube and add 950 µl of the Sample Buffer **(Sample for Total OAs)**.

4

Preparation of Working Solution

Before preparing Substrate Working Solution and PP2A Working Solution, defrost OA Reference Solutions, PP2A Buffer A, PP2A Buffer B and Substrate Buffer (Kit Components #: 1, 5, 6 and 8).

Keep OA Reference Solutions (#1) at room temperature, and keep the other solutions (#5, 6, and 8) at 4℃ or on ice.

PP2A Stock Solution (#7) should be taken out from -20 ℃ right before the PP2A Working Solution preparation and kept at 4℃ or on ice.

1. Substrate Working Solution

Substrate buffer + Substrate Tablet (*p*-NPP)

Dissolve the Substrate tablet in Substrate Buffer. Wrap the tube with aluminium foil and keep at 4℃ or on ice.

2. PP2A Working Solution

PP2A Buffer A + PP2A Buffer B + PP2A stock solution

First, spin down PP2A Buffer B and PP2A Stock Solution (#6 and 7).

Add PP2A Buffer B (#6; 1ml) to PP2A Buffer A (#5; 11ml) and mix well (invert slowly!).

Then, add PP2A Stock Solution (#7; 70µl) to the solution (Buffer A and B mixed) and mix well (invert slowly!).

The PP2A Working Solution should be kept at 4℃ or on ice and should be used on that day.

5

Assay Procedure

An example for placing OA Reference Solutions and test samples in the 96-well (1 x 8 wells x 12strips) microplate is shown below.
TRIPLICATES of OA Reference Solutions and samples are recommended for the higher reliability of data.
We recommend not to use the most outer wells because of their inconsistency.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
C		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
D		OA R1	OA R2	OA R3	OA R4	OA R5	OA R6	OA R7	OA R8			
E		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
F		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
G		SF1	SF2	SF3	SF4	SF5	ST1	ST2	ST3	ST4	ST5	
H												

EXAMPLE OF PLATE ARRENGEMENT

SF: Sample for Free OAs **ST: Sample for Total OAs**

1. Add 50 µl of the OA Reference Solutions and Samples to each well.
2. Add 100 µl of PP2A Working Solution. A multichannel pipette is recommended to use .
3. Add 100 µl of Substrate Working Solution. A multichannel pipette is recommended to use.
4. Seal the plate with the Adhesive Film and mix for 1 minute with a microplate mixer.
5. **Incubate the plate for 30 minutes at 36°C** and get the microplate reader ready.
6. After incubation, carefully remove the Adhesive Film and measure absorbance with a microplate reader at 405 nm against 490 or 492 nm as reference.

6

Quality Assurance

Quality of the performed assay is assured by the following criteria.

1. The absorbance value for OA-R1 should reach 0.4 or over.
2. The absorbance value for OA-R8 should be smaller than 0.15.
3. The relative error** must be smaller than 10%, if a possible outlier is excluded from the three run data in the assay.
   **(data A – data B)/(data A + data B), where data A > data B.

Data Calculation

The data calculation is performed semi-automatically on a Microsoft Excel spreadsheet.* (* cf. page 3)

1. Input the net-absorbance values obtained for OA Reference Solutions into cells colored light blue in the Table placed at the top of the sheet.
2. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded.
3. The standard curves for the net-absorbance values and percent of residual enzyme activity are drawn at the middle left and right of the sheet, respectively.
4. Input the net-absorbance values obtained for the sample solutions to the corresponding cells which are tabulated at the bottom of the sheet and colored light green. The sample numbers with prime denote hydrolyzed samples.
5. Check the relative standard deviation values. If the value exceeds 10%, a possible outlier should be excluded by erasing the cell for the outlier.
6. The calculation results expressed in terms of OA-equivalents are shown in the right end column in μg/g. Values less than 0.1 μg (OA-equivalent)/g tissues are expressed as <0.1 μg/g. For calculation of the total OA-equivalent, a factor "1.4" is used to calibrate the value increase due to added alkali and acid solutions.

References

1. Ikehara T, Shinjo F, Ikehara S, Imamura S, Yasumoto T.: Baculovirus expression, purification, and characterization of human protein phosphatase 2A catalytic subunits alpha and beta. Protein Expr Purif. 45(1):150-6, 2006.
2. Tubaro A, Florio C, Luxich E, Sosa S, Della Loggia R, Yasumoto T.: A protein phosphatase 2A inhibition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. Toxicon 34(7):743-52, 1996.
3. Takai A, Mieskes G.: Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. Biochem J. 275:233-9, 1991

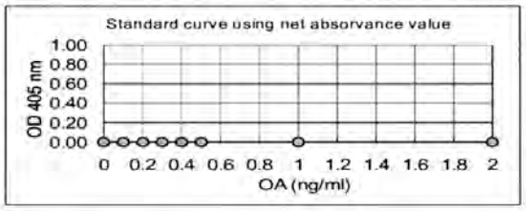
Example of Data Calculation Sheet

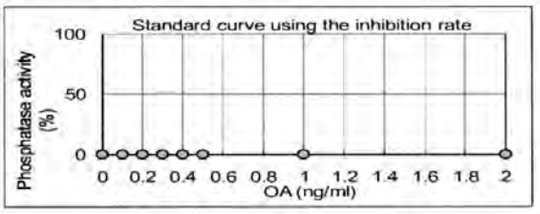
<Data calculation sheet>

DATE 2008/ / ( )
Laboratory:

Net absorbance values

OA reference solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (ng/ml)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1								
run 2								
run 3								
Average	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
relative standard deviation	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Phosphatase activity (%)	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (µg/g)*2
1					#DIV/0!	#DIV/0!	#DIV/0!
2					#DIV/0!	#DIV/0!	#DIV/0!
3					#DIV/0!	#DIV/0!	#DIV/0!
4					#DIV/0!	#DIV/0!	#DIV/0!
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.
*2 The result is expressed as the concentration in the sample in µg/g.

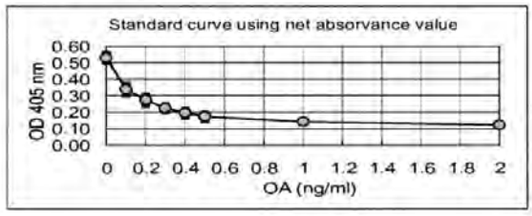
Calculation Example

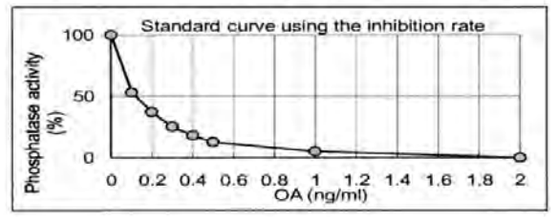
<Data calculation sheet>

DATE 2007/10/15 (Mon)
Laboratory: TTC

Net absorbance values

OA reference solutions	R1	R2	R3	R4	R5	R6	R7	R8
OA concentration (ng/ml)	0	0.1	0.2	0.3	0.4	0.5	1	2
run 1	0.549	0.351	0.279	0.232	0.203	0.180	0.147	0.124
run 2	0.522	0.327	0.265	0.221	0.194	0.172	0.143	0.124
run 3	0.520	0.339	0.281	0.225	0.194	0.173	0.143	0.123
Average	0.5303	0.3390	0.2750	0.2260	0.1970	0.1750	0.1443	0.1237
relative standard deviation	3.1%	3.5%	3.2%	2.5%	2.6%	2.5%	1.6%	0.5%
Phosphatase activity (%)	100.0	53.0	37.2	25.2	18.0	12.6	5.1	0.0





sample name	Hydrolysis*1	run 1	run 2	run 3	average	relative standard deviation(%)	result (µg/g)*2
1					#DIV/0!	#DIV/0!	#DIV/0!
2					#DIV/0!	#DIV/0!	#DIV/0!
3					#DIV/0!	#DIV/0!	#DIV/0!
4					#DIV/0!	#DIV/0!	#DIV/0!
5					#DIV/0!	#DIV/0!	#DIV/0!
6					#DIV/0!	#DIV/0!	#DIV/0!
1'	h				#DIV/0!	#DIV/0!	#DIV/0!
2'	h				#DIV/0!	#DIV/0!	#DIV/0!
3'	h				#DIV/0!	#DIV/0!	#DIV/0!
4'	h				#DIV/0!	#DIV/0!	#DIV/0!
5'	h				#DIV/0!	#DIV/0!	#DIV/0!
6'	h				#DIV/0!	#DIV/0!	#DIV/0!

*1 For hydrolysis sample input "h" in the column indicated "hydrolysis" to enable automatically calibrate the result.
*2 The result is expressed as the concentration in the sample in µg/g.

8

Annex 6

HPLC Analysis of PSP Toxins (Post-Column Derivatization Fluorescent HPLC)

Stock solutions for the mobile phase and oxidizing reagent

(1) 100 mM sodium 1-heptanesulfonate: Dissolve 2.02 g of 1-heptanesulfonate (C7H15SO3Na, for ion-pair chromatography, MW 202.3) in 100 ml of distilled water. Keep it in a refrigerator to prevent bacterial growth.

(2) 500 mM phosphoric acid solution: Dissolve 28.8 g of concentrated phosphoric acid (85% aqueous solution, analytical grade, MW 98.0) in distilled water and make up to 500 mL.

(3) 1 M ammonium hydroxide solution: Dilute ammonia water (25%, analytical grade) with 13 volumes of distilled water.

(4) Acetonitrile: CH_3CN in HPLC grade of equivalent.

(5) 1.0 M tetrabutylammonium dihydrogen-phosphate solution*: HPLC grade solution is available from companies (ie Aldrich).

(6) 50 mM acetic acid solution: Dissolve 1.5 g of acetic acid (glacial, analytical grade, MW 60.0) with distilled water and make up to 500 mL.

(7) 350 mM periodic acid solution: Dissolve 7.98 g of periodic acid dihydrate ($HIO_4.2H_2O$, analytical grade, MW 227.94) in 100 mL of distilled water.

(8) 250 mM dipotassium phosphate solution: Dissolve 21.77 g of K_2HPO_4 (analytical grade, MW 174.18) in distilled water and make up to 500 mL.

(9) 1 M potassium hydroxide solution; Dissolve 5.6 g of KOH (analytical grade, MW56.1) in 100 mL of distilled water.

Mobile phases (each 500 mL)

(a) For the analysis of **GTX group** (GTX1-GTX5, dcGTX2, dcGTX3):
(2 mM heptanesulfonate in 10 mM ammonium phosphate buffer containing 1 % acetonitrile)

Dissolve 10 mL of 100 mM sodium 1-heptanesulfonate **(1)** and 10 mL and of 500 mM phosphoric acid solution **(2)** in 450 mL of distilled water. Adjust (raise) pH to 7.0 by adding 1 M NH4OH **(3)** with stirring. Add 5 mL of acetonitrile **(4)** and make up to 500 mL with distilled water. Degas the solution by gentle aspiration in a sonication bath.

(b) For the analysis of **STX group** (STX, neoSTX, dcSTX):
(2 mM heptanesulfonate in 30 mM ammonium phosphate buffer containing 5 % acetonitrile)

Prepare as (a), but change the volume of **(2)** phosphoric acid to 30 mL, and **(4)** acetonitrile to 25 mL.

(c) For the analysis of **C-toxin group** (C1-C4):
(2 mM tetrabutylammonium in acetate buffer pH 6.)

Dissolve 1 mL of 1 M tetrabutyl ammonium solution **(5)** and 1 mL of 500 mM phosphoric acid in 450 mL of distilled water. Adjust pH to 6.0 by carefully adding 1 mM NH_4OH solution. **(3)**. Make up to 500 mL with distilled water.

Oxidizing reagent (500 mL):

(7 mM periodic acid in 10 mM sodium phosphate buffer pH 9.0)

Add 10 mL of 350 mM periodic acid solution **(7)** and 100 mL of 250 mM K_2HPO_4 **(8)** to 300 mL of distilled water. Adjust to pH 9.0 by adding 1.0 M KOH **(9)** and make up to 500 mL

Acidifier: 500 mM acetic acid (500 mL)

Dissolve 15 g of glacial acetic aid (analytical grade) in distilled water and make up to 500 mL.

Table 1. Preparation of reagents for PSP analyzer (each for 500 mL except for acidifier)

Mobile phase	**Stock sol**		**pH adjustment**
C1/C4	1.0 M tetrabutyl ammonium phosphate	1 mL	pH 6.0 with
	0.5 M H_3PO_4	1 mL	1 M NH4OH or
	Distilled water	450 mL	diluted NH_4OH
GTXs	0.1 M Na 1-heptanesulfate	10 mL	pH 7.0 with
	0.5 M H_3PO_4	10 mL	1 M NH_4OH
	Distilled water	450 mL	
	CH_3CN	5 mL	
STXs	0.1 M Na 1-heptanesulfate	10 mL	pH 7.0 with
	0.5 M H_3PO_4	30 mL	1 M NH_4OH
	Distilled water	400 mL	
	CH_3CN	25 mL	
Oxidizing reagent			
7 mM periodate	0.35 M periodic acid	10 mL	pH 9.0 with
	0.25 M K_2HPO_4	100 mL	1.0 M KOH
	Distilled water	350 mL	
Acidifier			
0.5 M acetic acid	CH_3COOH glacial	29 mL	
	Distilled water	971 mL	

Operation of HPLC

(a) Start-up procedure

1) Run 25% acetonitrile through column for 5 min at 0.8 mL/min and then the mobile phase for 15 min.

2) Prime post-column reaction pumps with the oxidizing reagent and acidifier and run at 0.4 mL/min.

3) Heat up water-bath to 65°C.

4) Operate fluorescent detector set excitation wavelength at 330 nm, emission wavelength at 390 nm, and wait for 15 min.

5) Inject 10 mL of the toxin standard mixture repeatedly until retention times and peak areas of all the toxin become constant.

(b) Sample analysis

1) Inject 10 mL of samples and record peak hights or peak areas.

2) Inject the standard solution after every 2-4 samples to ensure the system is working properly.

3) Use the nearest standard chromatogram for calculation of toxin concentration in the sample.

(c) Shut down procedure

1) Shut down fluorescent monitor and water bath. Take out reaction coil from the water bath, if possible.

2) Prime pump for oxidizing reagent with distilled water, flow water at least 10 min and stop the pump.

3) After washing the reaction system with water for 5 min, prime HPLC main pump with 25% acetonitrile, and run the pump at least for 15 min. Do not leave the mobile phase.in the column.

(d) Others

1) To analyze the other toxin group (eg from GTXs to STXs or STXs to GTXs), just change the mobile phase. However, for changing from C-toxin to other toxin group or from other toxin group to C-toxin group, flush the column with 25% acetonitrile at least 15 min between the two mobile phases.

2) Be careful not to run the column dry. If the column is not used for longer period, flush the column well with 50% acetonitrile and cap it tightly.

Preparation of test solution from shellfish extract

In analyses of actual shellfish samples, fluorescent compounds sometime interfere the analysis. In scallop extract, a huge peak of unknown compound appeared near GTX4. Thus, cleanup method for the extracts was explored using a reversed-phase cartridge column (Sep-Pak plus C18). Dry cartridge column did not retaine the interfering substance but the one regenerated with methanol and equilibrated with water could remove it from the extract. The first part of the eluate was diluted with water remaining in the column and the interfering substance started to appear after 2.0 ml. Thus 1.5 to 2.0 ml fraction was applied for the further purification by ultrafiltration. The dilution factor by glycerol used as lubricant of the ultrafiltration membrane was negligible when tested with 50 mL toxin solution. Treatment of the extract with Sep-Pak C18 cartridge column was also effective in removing peaks eluting after C1-C4 and STX, and analysis time could be shortened significantly. The cleanup procedure was also effective in elongating the life time of column.

1. Prepare the 0.1 N HCl extract of shellfish

according to the AOAC mouse bioassay method.

2. Centrifuge of filter the extract, if it contained too much particles.

3. Wash Sep-Pak plus C18 cartridge column with 10 mL of methanol using glass syringe.

4. Wash the column with 10 mL of distilled water using syringe. Plastic one can be used.

5. 5Purge the column by passsing air through it.

6. Take 3 mL of the shellfish extract in a dry syringe, plug it to SepPak and push gently.

7. Collect the first eluate of 1.5 mL and discard.

8. Take next 0.3-0.5 mL of the eluate into an ultrafiltration unit.

9. Centrifuge the unit at the maximum speed allowed for the kit. (usually 8,000 rpm)

10. Inject 10mL of the liquid passed through the membrane into HPLC.

11. SepPak cartridge can be reused after washed with 10 mL of distilled water.

Preparation of test solution from plankton sample

A. By centrifugation

1. Collect the sample by vertical tow of plankton net of 20 mm pore size. Take aside a part of the sample, and fix it for counting cell numbers later).

2. Take certain volume of suspension into pre-weighed centrifuge tube.

3. Centrifuge gently (i.e. 3,000 rpm, 5 min).

4. Remove supernatant as much as possible without disturbing sediment

5. Add 300 – 500 mL of 1 N acetic acid (not necessary to be accurate)

6. Take weight of the centrifuge tube.

7. Tightly seal the tube and keep frozen until use or for transportation.

8. Disrupt sediments in acetic acid by a rod type sonicator.

9. Centrifuge at the highest speed allowed.

10. Transfer the supernatant to the ultrafiltration unit.

11. Centrifuge the unit at the maximum speed allowed for the kit. (usually 8,000 rpm)

12. Inject 10mL of the liquid passed through the membrane into HPLC.

From the weight of sample, which indicates the total volume of extract assuming the desity is 1, and total number of cells applied for centrifugation, you can calculate equivalent cell number in the extract and also toxin content in one cell (femtomole/cell) if toxins are detected.

B. Concentration with charcoal column

1. Freeze and thaw a plankton ample collected by vertical taw of 20 mm plankton net from the bottom to surface (suspension around 100 mL suspension).

2. Remove particles by filtration after sonication or vigorous shaking.

3. Wash activated charcoal (for chromatography, Wako Pure chemicals, Tokyo) with distilled water several times, removing small particles by decantation.

4. Pack 5 mL of charcoal in a glass column of 7 mm id, by poring suruary.
5. Pass the filtered plankton net sample through the column.
6. Wash column with 10 mL distilled water.
7. Elute the column with 20 mL of 2% acetic acid in 50 % ethanol.
8. Evaporate the eluate to near dryness.
9. Dissolve the solid in 300 mL of distilled water.
10. Pass the solution through ultrafiltration unit by centrifugation.

Column used at Biotxoins Training Course

Mightysil RP-8-GP, 4.6 x 150 mm +	Inertsil AX, 4.6×33 mm
Cica reagent, Tokyo	GL-Science, Tokyo
(C8 reversed phase column)	(Anion exchange colum)

Annex 7

Determination of Paralytic Shellfish Poison and Related Algae Toxins in Mussels using RIDASCREEN FAST PSP SC

RIDASCREEN FAST PSP SC is a competitive enzyme immunoassay for the quantitative analysis of Paralytic Shellfish Poison (PSP) and related algae toxins in mussels.

All reagents required for the enzyme immunoassay – including standards – are contained in the test kit. The test kit is sufficient for 48 determinations (including standards).

1. Reagents (Provided in Test Kit)

1.1 Microtiter plate with 48 wells (6 strips with 8 removable wells each)
- Coated with capture antibodies against anti-PSP antibodies

1.2 PSP standard solution, 1.3 ml
- 0 ppb (zero standard), in aqueous solution, ready to use
- Only standard 1 (0 ppb) is included in the test kit. The standard curve (B/B0) is provided with the certificate of the test kit.

1.3 Conjugate, 0.3 ml (red cap)
- Peroxidase conjugated PSP, concentrate

1.4 Anti-PSP antibody, 0.3 ml (black cap)
- Concentrate

1.5 Red Chromogen, 10 ml (brown cap)
- (Substrate/chromogen solution), stained red
- Contains tetramethylbenzidine

1.6 Stop solution, 14 ml (yellow cap)
- Contains 1 N sulfuric acid

1.7 Buffer, 50 ml
- Sample, conjugate and antibody dilution factor

2. Sample Preparation

The mussel samples should be stored in a cool place, protected from the light.

2.1 Remove the mussel shell.

2.2 Wash the mussel meat with water and homogenize.

2.3 Mix 10 g of homogenized mussels with 10 ml of HCl (0.1 M) and boil for 5 minutes while stirring.

2.4 Centrifuge the mixture for 10 minutes at approximately 3500 g at 4°C.

Note: Possible clouding of the supernatant after centrifugation has no effect on the test result.

2.5 Adjust the pH of the supernatant with 5 N HCl to ≤ 4.0 after centrifuge.

2.6 Use 100 µl of the supernatant and fill up to 1 ml (1:10 dilution) with sample dilution buffer.

Note: For higher contaminated samples, which are not lying in the linear range of the standard curve, we recommend a

further dilution of 1:10 (1+9) with sample dilution buffer. The dilution factor is then 200 instead of 20 and has to be taken into account for the results.

2.7 Employ 50 µl per well in the assay.

3. Test Procedure

Carefully follow the recommended washing procedure. Do not allow micro-wells to dry between working steps.

3.1 Insert sufficient number of wells into the micro-well holder. Record the positions of the standard and sample.

3.2 Add 50 µl of standard or prepared sample into separate wells; use a new pipette tip for each standard or sample.

3.3 Add 50 µl of diluted enzyme conjugate (red cap) to each well.

3.4 Add 50 µl of diluted anti-PSP antibody solution (black cap) to each well. Mix gently by shaking the plate manually and incubate for 15 minutes at room temperature (20-25°C).

3.5 Dump the liquid out of the wells into a sink. Tap the micro-well holder upside down onto a clean filter towel (three times in a row) to remove all remaining liquid from the wells.

3.6 Using a wash bottle or multi-channel pipette, fill the wells (250 µl per well) with distilled or deionized water. Repeat the washing step two more times.

3.7 Add 100 µl of substrate / chromogen (brown cap) to each well. Mix gently by shaking the plate manually and incubate for 15 minute at room temperature (20-25°C) in the dark.

3.8 Add 100 µl of stop solution (yellow cap) to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm. Read within 10 minutes after addition of stop solution.

4. Results Calculation

For calculation of results, transfer the measured absorbance for standard 1 (0 ppb) and B/B_0 values for standards 2 – 6 (2.5 / 5 / 10 / 20 / 40 ppb) into the RIDA SOFT WIN. This software program would calculate the standard curve and the content of the PSP in the samples.

To obtain the saxitoxin concentration (in µg/kg) of a sample, multiply the concentration read from the calibration curve by the corresponding dilution factor.

Annex 8

Determination of Tetrodotoxins using Liquid Chromatography- Mass Spectrometry (LC-MS) Method (ed. by T. Suzuki and Y. Oshima)

1. Extraction of toxins

1.1 Shellfish samples

1.1.1 To 1 g of homogenate, add 4 mL of 0.1% acetic acid.

1.1.2 Heat at 100°C for 10 minutes

1.1.3 Centrifuge at 18,600g for 15 minutes at 4°C.

1.1.4 Condition the C18 Plus Sep-pak (Equilibrate with 5mL of MeOH, followed by 5mL of distilled water.).

1.1.5 To the sep-pak, add in the 2 mL. Discard the first 1.5 mL, Collect the following 0.5 mL.

1.1.6 Ultrafiltrate the solution with Ultrafree-MC, 10,000 NMWL at 5300g for 15 minutes at 4°C.

1.1.7 Inject a 5 µL aliquot of the eluate into the LC-MS.

2. Preparation of reagents

2.2 0.1% aceitic acid

a) 0.1 mL of acetic acid is made up to 100 mL by distilled water.

2.2 Preparation of mobile phases

2.2.1 Stock solution (160mM $HCOONH_4$ stock solution, pH 5.5)

a) 160mM HCOONH4 : Weigh out 2.5224g of HCOONH4 (MW 63.06) and dissolved in 250 mL distilled water.

b) 160mM HCOOH: Weigh out 1.8784g (1.539 ml) (98%)of HCOOH (MW 46.03) and dissolved in 250 mL distilled water.

b) is added to (a) to adjust pH to 5.5 ± 0.05.

2.2.2 LC-MS mobile phases

Mobile phase A: 16mMHCOONH4 solution

Mix distilled water and stock solution in the ratio of 90:10 vol/vol (450mL:50mL)

Mobile phase B: MeCN

3. LC-MS Conditions

Column: TSKgel Amide 80 (2.0 id x 150 mm) (Tosoh, Tokyo, Japan)

Column temperature: 25°C

Gradient: Isocratic A:B (3:7 v/v)

Flow rate: 0.2ml/min

MRM conditions for TTX analogues by positive LC-MS/MS

	Parent ion *m/z*	Product ion *m/z*	Retention time Min
TTX	320	162	17.04
4-*epi*TTX	320	162	15.15
4.9-*anhydro*TTX	302	162	11.75
5-*deoxy*TTX	304	162	10.82
11-*deoxy*TTX	304	162	11.50
6,11-*dideoxy*TTX	288	224	10.04
5,6,11-*trideoxy*TTX	272	162	6.24

4. Quarantine Level

MBA: 10 MU/g fish meat
LC/MS: 2 μg/g fish meat (Conversion factor 5 MU/ug TTX)
0.4 μg/mL solution

5. References

1) T. Nakagawa, J. Jang, M. Yotsu-Yamashita: Hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry of tetrdotoxin and its analogs. *Anal. Biochem*., **352**, 142-144 (2006).

2) J.H Jang, J.S.Lee, M. Yotsu-Yamashita: LC-MS analysis of tetrodotoxin and its deoxy analogus in the marine puffer fish Fugu niphobles from the Southern Coast of Korea, and in the Brackishwater Puffer fishes Tetradon nigroviridis and Tetradon biocellatus from Southeast Asia. *Mar. Drugs*, **8**, 1049-1058 (2010).

Annex 9

Information on Key Project Leader for JTF II Project

Country	Name of Key Project Leader (KPL)	Designation	Organization	Office's Address	Telephone Number	Fax Number	Email Address
Cambodia	Dr Chhoun Chamnan	Acting Director	Department of Fisheries Post-Harvest Technologies and Quality Control (DFPTQ), Fisheries Administration	186 Norodom Blvd., Phnom Penh 12301, Cambodia P.O. Box 582	+855-23-224 800	+855-23-224 800	chhounchamnan@gmail.com/ chhounchamnan@fia.gov.kh
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Lao PDR	Ms Sisamouth Phengsakoun	Technical Staff	Fish Culture Standard Control and Development Centre, Department of Livestock and Fisheries, Ministry of Agriculture and Forestry	Phontongchoumany Village, Phontongchoumany Road, Chanthabouly District, Vientiane Capital, Lao PDR	+856-2121 5242	+856-2121 5141	sphengsakoun@yahoo.com
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Country	Name of Key Project Leader (KPL)	Designation	Organization	Office's Address	Telephone Number	Fax Number	Email Address
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Annex 10

Information on National Reference Laboratory for Biotoxins Monitoring

No	Country	Name of Laboratory	Address	Phone No.	Fax No.	Email	Contact Person
1	Indonesia	National Centre for Fish Quality Control	Jalan Raya Setu No 70, Setu – Cipayung, Jakarta Timur, 13880, Indonesia	6221 84997969 / 6221 84998429	6221 99360	-	Mr Harinto Wibowo / Ms Prihastini Ngudi Lestari
2	Malaysia	Fisheries Research Institute	Jalan Batu Maung, 11960 Batu Maung, Penang, Malaysia	04-6263925 / 04-6263981	04-6262210	-	Mr Ismail Bin Ishak
3	Philippines	Marine Biotoxins Laboratory, Fisheries Resource Management Division, Bureau of Fisheries and Aquatic Resources	860 Arcadia Building, Quezone Avenue 1100 Manila, Philippines	(632) 3320210	-	bfar_hab@ yahoo.com	-
4	Singapore	Veterinary Public Health Centre, Toxins Section	Agri-Food & Veterinary Authority 10Perahu Road Singapore 718837	6795 2822 / 6795 2829	6861 9491	-	Ms Helen Phang

No	Country	Name of Laboratory	Address	Phone No.	Fax No.	Email	Contact Person
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		Fish Inspection and Quality Center, Fish Inspection and Quality Control Division, Department of Fisheries	Suratthani Fish Inspection and Research Center, Department of Fisheries 20/62 moo 7, Takham, Phunphin, Suratthani, Thailand 84130	+66-77-274230-2 (Mobile: +66-81-3262869)	+66-77-274231	pratumwan.c@dof.mail.go.th	Mrs Pratumwan Charernporn
6	Vietnam	National Agro-Forestry-Fisheries Quality Assurance Department (NAFIQAD) Branch 1	51 Le Lai street, Ngo Quyen District, Hai Phong city	(+84) 31.3.837508 - 3.837124	(+84) 31.3.837507	branch1.nafi@mard.gov.vn	Tran The Phong (Director) (+84) 913392591
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