TECHNICAL COMPILATION OF BIOTOXINS MONITORING IN THE ASEAN REGION

Japanese Trust Fund VI: Chemical and Drug Residues in Fish and Fish Products in Southeast Asia 2015 - 2017

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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 VI 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 Viet Nam. SEAFDEC has five departments, namely, the Marine Fisheries Research Department (MFRD) in Singapore, the Training Department (TD) in Thailand, the Aquaculture Department (AQD) in Philippines, the Marine Fishery Resources Development and Management Development (MFRDMD) in Malaysia, and the Inland Fishery Resources Development and Management Department (IFRDMD) in Indonesia.

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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 increase in number of cases of human poisoning 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 the risk of marine biotoxins.

For this reason, the SEAFDEC/MFRD programmes through the Post-Harvest Technology Centre of Agri-Food and Veterinary Authority of Singapore, implemented the project "Chemical and Drug Residues in Fish and Fish Products in Southeast Asia – Biotoxins Monitoring and Harmful Algae Blooms in the ASEAN region" from 2009 to 2019 to expand and improve initiatives in monitoring, detecting and sharing of information on marine biotoxins in order to reduce the public health risks associated with the consumption of contaminated shellfish and fish.

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 safe and quality seafood for the Southeast Asian region.

Ms. Khoo Gek Hoon
Chief, MFRD Programmes

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Introduction

Background

Marine biotoxins represent a significant and expanding threat to human health in many parts of the world. The impact is visible in terms of human poisoning or even death following the consumption of contaminated shellfish or fish, as well as mass killings of fish and shellfish, and the death of marine animals and birds.

The Codex Alimentarius Code of Practice for Fish and Fishery Products (CAC/RCP 52-2003) defined biotoxins as poisonous substances naturally present in fish and fishery products or accumulated by the animals feeding on toxin producing algae, or in waters containing toxins produced by such organisms.

Monitoring seafood for toxicity is essential to manage possible risks. However, there are several limitations in monitoring for toxicity such as varying toxin content between individual shellfish, different detection and extraction methods for the various toxins, deciding on which toxins to test for and frequency of sampling to ensure that toxicity does not rise to dangerous levels in temporal or spatial gaps between sampling times or locations. Furthermore, the growing harvest of non-traditional shellfish (such as moon snails, whelks, barnacles, etc.) may increase human health problems and management responsibilities.

MFRD Biotoxins Monitoring Programme

MFRD programmes conducted a project on biotoxins monitoring in ASEAN from 2009 to 2019 to increase awareness and focus on expanding and improving initiatives to monitor, detect and share information on marine biotoxins to reduce public health risks associated with the consumption of contaminated shellfish and fish. The first phase of the project, JTFII (2009 to 2012), covered training in analytical methods for Diarrhetic Shellfish Poisoning (DSP) toxins, lipophilic toxins, Paralytic Shellfish Poisoning (PSP) toxins and Tetradotoxin (TTX), as well as a monitoring survey on PSP toxin in ASEAN-SEAFDEC Member Countries. The second phase of the project, JTFVI (2013 to 2017), addressed the needs of Member Countries and continued with capability building in biotoxins and monitoring, focusing on other biotoxins like the Amnesic Shellfish Poisoning (ASP) toxin (Domoic Acid) and Azaspiracid (AZA) toxin. Brevetoxins (BTX) which causes Neurotoxic Shellfish Poisoning (NSP) was also recommended to be included in the new project phase as ASP, AZA and BTX, along with DSP and PSP, are regulated according to CODEX for shellfish.

From 2015, MFRD programmes also incorporated new activities under this project to enhance regional capabilities for the identification of toxic HAB species, strengthening the Member Countries' capability for biotoxins monitoring. The project was extended for two more years (2018-2019) upon request from the Member Countries during the Regional Training Course on Identification of HAB Species in the ASEAN Region in 2016. For MFRD to organize additional training courses to enhance the region's capabilities in managing toxic HAB incidences and to give participating countries more time to complete the biotoxins monitoring surveys.

The key stakeholders/beneficiaries of this project are the relevant agencies in the fisheries department of the ASEAN-SEAFDEC Member Countries which are responsible for ensuring the safety of fish and shellfish for consumption and for the monitoring and testing of fish and shellfish; aquaculture farmers and harvesters of the fish and shellfish; as well as both international and domestic consumers/buyers.

Resolution and Plan of Action

This project is in line with the following Resolution and Plan of Action as endorsed at the ASEAN-SEAFDEC Conference of 2011:

Resolution 21: Improve technologies and facilities to ensure fish quality assurance and safety management systems, taking into account the importance of traditional fishery products and food security requirements, and promote the development of fishery products as an alternative supplementary livelihood for fisheries communities.

Plan of Action D61: Strengthen fish quality and safety management systems that support the competitive position of ASEAN fish products on world markets, including moving towards ISO/IEC 17025 accreditation of national fish inspection laboratories, strengthening capacity and acknowledging the recognized national laboratories, risk analysis and equivalence agreement such as the Mutual Recognition Agreement (MRA) and promote the implementation of the quality and safety management systems among small and medium enterprises in the ASEAN region.

Plan of Action D63: Promote and conduct training programs and develop training materials to upgrade the technical skills and competencies of personnel in the public and private sectors on fisheries post-harvest technology and food safety management system.

Programme Activity Line-up

This project is also in line with the SEAFDEC Program Thrust II on Enhancing Capacity and Competitiveness to Facilitate International and Intra-Regional Trade.

The project comprised of 5 activities as follows:

Activity 1: Regional Technical Consultation on Biotoxins (ASP, AZA and BTX) Monitoring in ASEAN Region (Year 2013)

A Regional Technical Consultation (RTC) Meeting was held in Singapore from 24-25 July 2013 to initiate the project and plan for all project activities. A total of 19 participants from ASEAN-SEAFDEC member countries attended the meeting. The meeting came to a consensus on the national project leader (key project leader, KPL) for each country as well as the methods to be taught for the training course.

Activity 2: Regional Training Course on Biotoxins Analyses (Year 2014)

The 5-days Regional Training Course was successfully organised from 2-6 June 2014 in Singapore. This training course was jointly conducted by MFRD, SEAFDEC and the Veterinary Public Health Laboratory, Agri-Food & Veterinary Authority of Singapore, together with two biotoxins expert, Dr Toshiyuki Suzuki from the National Research Institute of Fisheries Science, Fisheries Research Agency, Japan and Dr Dao Viet Ha from the Institute of Oceanography, Viet Nam.

It was attended by 21 participants from all ASEAN member countries and covered a series of lectures including the general introduction of marine toxins, analytical tools for detection, characterisation and quantification of AZA, BTX and ASP via instrumental analysis, as well as practical sessions on sample preparation and the use of High Performance Liquid Chromatography Tandem Mass Spectrometer (LC/MS/MS) method and High Performance Liquid Chromatography method for the detection of AZA, BTX and ASP. A laboratory tour to various laboratories within the Veterinary Public Health Centre was also conducted for the participants.

Activity 3: Biotoxins Monitoring Survey (Year 2015 - 2017)

The survey involved monitoring ASP, AZA and BTX biotoxins occurrences and incidences in fish and fisheries products in the ASEAN region. Biotoxins that were already covered in the training course in 2010 (for example Diarrhetic Shellfish Poisoning, DSP and lipophilic toxins, TTX) could also be included in the survey if Member Countries were interested.

Initially, the Biotoxins Monitoring Survey was to be conducted over a period of one and a half years, during the 3rd and 4th year of project (2015 and 2016). However, due to manpower and technical constraints faced by some of the Member Countries, the survey period was officially extended till the end of 2017. Meanwhile, Cambodia also formally requested to withdraw from the survey. The other 9 participating countries submitted their progress reports on a quarterly basis during this two-year period.

Activity 4: Regional Technical Consultation on Toxic HAB species Identification (Year 2015)

The Regional Technical Consultation (RTC) on Harmful Algal Blooms (HABs) in the ASEAN region was successfully organized and conducted in Singapore from 5-6 August 2015. Each ASEAN-SEAFDEC Member Countries sent two representatives to attend the RTC and presented country reports on toxic HAB occurrences and incidences, as well as the management of toxic HABs in their waters. The meeting agreed that Dr Yasuwo Fukuyo will be the principal expert trainer for the Regional Training Course which was conducted in Singapore the following year. The meeting also discussed the individual training needs of each Member Country which would be taken into consideration in the planning of the Regional Training Course. Finally, the meeting identified the Key Project Leaders for each country and initiated the process of establishing a network or directory of responsible national authorities and HAB experts in the region.

Activity 5: Regional Training Course on Identification of HAB Species in the ASEAN Region (Year 2016)

The Regional Training Course was successfully organized in Singapore on 18-22 July 2016 in collaboration with IOC-WESTPAC and included 3 Japanese and 2 regional experts who conducted the training. There were a total of 22 participants from the 10 ASEAN Member Countries. The course programme included lectures and practical sessions as well as a field trip/sampling session. Positive feedbacks were received from the participants, indicating that the programme was well conducted and beneficial to their work. As an additional output of the training course, the participants agreed to form a team headed by Philippines and consists of a representative from each ASEAN Member Country to prepare 2 posters on red tide causing species and HAB species. Member Countries would provide photos of the species for the posters which would be distributed to the Member Countries for knowledge sharing.

Activity 6: Regional Training Course on Specimen Preservation and its Application in HAB Monitoring and Studies (Year 2017)

The Regional Training Course was successfully conducted in collaboration with the Institute of Ocean & Earth Science (IOES), University of Malaya (UM) at its Bachok Marine Research Station (BMRS) in Kelantan from 10 - 13 July 2017. The training course included both lectures and practicals on specimen preservation methods and techniques, use of fluorescence and electron microscopy and flowcytometry. A total of 22 participants from the 9 ASEAN-SEAFDEC Member Countries (except Myanmar) attended the training. The participants provided positive feedback that the course was well conducted and beneficial to their work.

Activity 7: Regional Training Course on Culturing for HAB Species Identification and Toxin Characterization (Year 2018)

The 7-days Regional Training Course was successfully conducted in collaboration with the Institute of Ocean & Earth Science (IOES), University of Malaya (UM) at its Bachok Marine Research Station (BMRS) in Kelantan from 8 - 14 July 2018. This training course is last of the series of three regional training courses which were conducted on HAB since 2016. It comprised of both lectures and hands-on practical sessions, designed to expose participants to several aspects of harmful algae bloom monitoring and studies and also included fundamental knowledge of microalgal culturing and maintenance, species identification and detection using fluorescence and electron microscopy and flow cytometry. A total of 20 participants from 10 ASEAN Member Countries attended the training. The participants were satisfied with the course outline in general and they commented that the trainers were knowledgeable and willing to share information. They also felt that the materials and information provided during the course was beneficial to their work.

Activity 8: Technical Compilation (Year 2019)

The results from the three-years Biotoxins Monitoring Survey were compiled for finalization at the End-of-Project (EOP) meeting in 2019.

Activity 9: End-of-Project (EOP) Meeting (Year 2019)

An End-of-Project (EOP) Meeting was conducted in the 3rd quarter of 2019, to present and discuss the reports and/or results of the biotoxins monitoring surveys carried out by the respective Member Countries. They were encouraged to share the challenges faced during the project implementation and discuss the plans for future projects or activities nationally and regionally. Lastly, the Member Countries would finalize the Technical Compilation for publication.

Survey of

Biotoxins in ASEAN Region

1) Indonesia
2) Malaysia
3) Myanmar
4) Philippines
5) Singapore
6) Thailand
7) Viet Nam

INDONESIA

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I. Introduction

In Indonesia, since 1997-2016, there were several reported food poisoning cases due to the consumption of contaminated shellfish. Between June – September 2010, more than 30 people were hospitalized after consuming clams and fish from Teluk Lasongko and Bau-Bau in Southeast Sulawesi. 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 areas, there was a lack of clinical data from patients. On 16 July 2010, another case of food poisoning in Lampung due to consumption of contaminated shellfish and fish' resulted in nausea and vomiting. Phytoplankton *Pirodinium bahamense* was identified as the species causing the shellfish poisoning.

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

The government of Indonesia has established several regulations, namely, The Government Regulation No. 57/2015 and Decree No. 52A/KEPMEN-KP/2013 which lay down the requirements for Quality and Safety Assurance of Fishery Product. These regulations could help to maintain and protect the culturing area of shellfish from the domestic and industrial sewage which may contaminate shellfish.

In Indonesia, the biotoxin monitoring activity which is also known as shellfish sanitation program has been conducted since 1997 as part of general monitoring program designed to identify and evaluate biological toxins as well as chemical and microbiological contamination of shellfish and the water quality.

National Regulatory Limits for Analytes tested:

No	Parameter	Regulatory Limit	Frequency
1.	ASP *(Domoic Acid)*	2 mg/100 g	Once every 2 months, at sampling point
2.	AZP *(Azaspiracid)*	1,6 µg/100 g	
3.	NSP *(Brevetoxin)*	0,8 mg/100 g	

Indonesia has a monitoring programme for ASP, AZP and NSP under the Japanese Trust Fund VI (JTF VI) in Tanjung Balai Asahan (North Sumatra) and Teluk Lampung (Lampung). The project is located in Tanjung Balai Asahan because this is largest production area in Indonesia while according to the previous monitoring result, there has been PSP occurrence around Lampung area.

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 (include scientific name), Number of Samples and Sampling Size

Sampling for shellfish was conducted on sampling site of shellfish monitoring area of ± 7500 ha in Tanjung Balai Asahan where the coordinates of the sampling sites are chosen on largest production area:

- 1 = 3°01'59.6"N 99°51'48.9"E
- 2 = 3°02'45.4"N 99°52'23.1"E
- 3 = 3°03'14.2"N 99°52'59.2"E
- 4 = 3°03'08.8"N 99°53'35.5"E
- 5 = 3°02'48.2"N 99°53'52.1"E
- 6 = 3°02'11.8"N 99°54'15.2"E

In Teluk Lampung, sampling for shellfish was conducted on sampling site of shellfish monitoring area where the coordinates of the sampling sites are chosen according to the previous monitoring result i.e. positive result for PSP:

- 1 = -5.4793737, 105.2537337
- 2 = -5.4805782, 105.2608285
- 3 = -5.4665515, 105.2608285
- 4 = -5.4651093, 105.2615561

The targeted species for JTF VI project is Baby Clam (*Meritrix meritrix)* in Tanjung Balai Asahan and Green Mussel (*Perna viridis*) in Teluk Lampung. The samples were tested for the following toxins ASP, AZP, and NSP.

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

The shellfish sampling for Baby Clam (*Meritrix meritrix*) in Tanjung Balai Asahan was conducted through the following steps:

1. Sampling was carried out with scallop shells dredge gear (scallops rakes) which consists of two types namely : garuk or tojok[1]. In this monitoring, garuk is most often used.

2. After arriving at the sampling area, garuk was lowered to scrape into the sand layer. The shaft was held firmly and the boat was turned until the garuk contained the shellfish.

3. Garuk was then removed from the water and the shellfish were washed to clean off sand and any other foreign material by shaking the gear several times on the surface of sea water.

4. Shellfish were then placed on the deck of the boat waiting to be sorted. The sorted shellfish were washed again with clean sea water and packed into a sack. Samples were collected at per sampling point and selected with individual weight of ± 50 g/

[1] Traditional sampling equipment

shellfish (minimum weight accepted by company/exporter to get ± 1kg shellfish that yield > 100 g of meat.

5. Samples were prepared according to AOAC (2000). Shells were cleaned and opened. The inner part was also cleaned to remove any foreign material. Meat was handled carefully to prevent damage or truncation. Meat was then drained and frozen at -20° C until analysis.

The shellfish sampling for Green Mussel (*Perna viridis*) in Teluk Lampung was conducted through the following steps:

1. Sampling was carried out from the ropes in floating net on area of marine culture.
2. Shellfish were then placed on the deck of the boat waiting to be sorted. The sorted shellfish were washed again with clean sea water and packed into a sack. Samples were collected at per sampling point and selected with individual weight of ± 50 g/ shellfish (minimum weight accepted by company/exporter to get ± 1kg shellfish that yield > 100 g of meat.
3. Samples were prepared according to AOAC (2000). Shells were cleaned and opened. The inner part was also cleaned to remove any foreign material. Meat was handled carefully to prevent damage or truncation. Meat was then drained and frozen at -20° C until analysis.

Meat of shellfish for Azapiracids and Domoic Acid analysis were extracted as follows:

1. 9 ml of MeOH/distilled water was added to 1 g of shellfish meat (9:1 v/v)
2. Extract was homogenized for 3 minutes.
3. Homogenized extract was then centrifuged at 3000 rpm for 5 minutes.
4. The aliquot of supernatant was passed through a 0.5 µm filter.
5. 5µL aliquot of the filtrate was injected into the LC-MSMS.

Meat of shellfish for Brevetoxins analysis were extracted as follows:

1. Meat were removed from their shells, washed with deionized water, thoroughly dried and homogenized (Waring blender, Polytron or equivalent).

2. 1.0 gm of homogenized mussels was then placed in a 40 mL glass vial.
3. 9.0 mL of a methanol/deionized water solution was added (9:1 v/v).
4. Vial was capped and hand shaken vigorously for 2 minutes.
5. Mixture was then centrifuged for 10 minutes at 3000 rpm. Supernatant was collected.
6. 20 uL of collected extract was diluted to 1.0 mL with Sample Diluent (equals a 1:50 dilution).
7. Diluted extracts were analyzed as samples.
8. ELISA method was used for analysis (the result were obtained in units of ng/g and converted to µg/100 g of meat.

c. Limit of Detection and Limit of Quantification

No	Parameter	Analytical Method	LOD	LOQ	Recovery
1	ASP *(Domoic Acid)*	LC-MSMS	0,1 µg/g	0,2 µg/g	94,0%
2	AZP *(Azaspiracid)*	LC-MSMS	0,01 µg/g	0,02 µg/g	91,9 %
3	NSP *(Brevetoxin)*	ELISA	22,5 ng/g	45 ng/g	90,0%

d. National Regulatory Limits

No	Parameter	Regulatory Limits
1	ASP *(Domoic Acid)*	2 mg/100g of meat
2	AZP *(Azaspiracid)*	1.6 µg/100 g of meat

3	NSP *(Brevetoxin)*	0.8 mg/100 g of meat

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

Did not participate in inter-laboratory proficiency testing.

b. Survey Results and Discussion

Table for Survey Results

Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
May 2015	TL1	28	Not Detected	Not Detected
	TL2	29	Not Detected	Not Detected
	TL3	27	Not Detected	Not Detected
	TL4	35	Not Detected	Not Detected
	TL5	16	Not Detected	Not Detected
	TL6	27	Not Detected	Not Detected
	TL7	21	Not Detected	Not Detected
	TL8	26	Not Detected	Not Detected
	TL9	29	Not Detected	Not Detected
	TJB1	30	Not Detected	Not Detected
	TJB2	11	Not Detected	Not Detected
	TJB3	10	Not Detected	Not Detected
	TJB4	10	Not Detected	Not Detected
	TJB5	18	Not Detected	Not Detected
	TJB6	19	Not Detected	Not Detected
	TJB7	18	Not Detected	Not Detected
	TJB8	26	Not Detected	Not Detected
	TJB9	20	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
TJB = Code of sample from Tanjung Balai Asahan

Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
July 2015	TL10	13	Not Detected	Not Detected
	TL11	12	Not Detected	Not Detected
	TL12	12	Not Detected	Not Detected
	TL13	13	Not Detected	Not Detected
	TL14	11	Not Detected	Not Detected
	TL15	9	Not Detected	Not Detected
	TL16	13	Not Detected	Not Detected
	TL17	16	Not Detected	Not Detected
	TL18	17	Not Detected	Not Detected
	TJB10	Not Detected	Not Detected	Not Detected
	TJB11	Not Detected	Not Detected	Not Detected
	TJB12	141	Not Detected	Not Detected
	TJB13	37	Not Detected	Not Detected
	TJB14	130	Not Detected	Not Detected
	TJB15	74	Not Detected	Not Detected
	TJB16	86	Not Detected	Not Detected
	TJB17	104	Not Detected	Not Detected
	TJB18	23	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
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Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
October 2015	TL19	34	Not Detected	Not Detected
	TL20	37	Not Detected	Not Detected
	TL21	33	Not Detected	Not Detected
	TL22	48	Not Detected	Not Detected
	TL23	27	Not Detected	Not Detected
	TL24	19	Not Detected	Not Detected
	TL25	34	Not Detected	Not Detected
	TL26	15	Not Detected	Not Detected
	TL27	19	Not Detected	Not Detected
	TJB19	Not Detected	Not Detected	Not Detected
	TJB20	Not Detected	Not Detected	Not Detected
	TJB21	Not Detected	Not Detected	Not Detected
	TJB22	Not Detected	Not Detected	Not Detected
	TJB23	Not Detected	Not Detected	Not Detected
	TJB24	Not Detected	Not Detected	Not Detected
	TJB25	Not Detected	Not Detected	Not Detected
	TJB26	Not Detected	Not Detected	Not Detected
	TJB27	Not Detected	Not Detected	Not Detected

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Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
December 2015	TL28	12	Not Detected	Not Detected
	TL29	11	Not Detected	Not Detected
	TL30	12	Not Detected	Not Detected
	TL31	9	Not Detected	Not Detected
	TL32	10	Not Detected	Not Detected
	TL33	13	Not Detected	Not Detected
	TL34	12	Not Detected	Not Detected
	TL35	12	Not Detected	Not Detected
	TL36	11	Not Detected	Not Detected
	TJB28	11	Not Detected	Not Detected
	TJB29	12	Not Detected	Not Detected
	TJB30	12	Not Detected	Not Detected
	TJB31	20	Not Detected	Not Detected
	TJB32	18	Not Detected	Not Detected
	TJB33	17	Not Detected	Not Detected
	TJB34	26	Not Detected	Not Detected
	TJB35	18	Not Detected	Not Detected
	TJB36	20	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
TJB = Code of sample from Tanjung Balai Asahan

Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
February 2016	TL37	24	Not Detected	Not Detected
	TL38	26	Not Detected	Not Detected
	TL39	18	Not Detected	Not Detected
	TL40	29	Not Detected	Not Detected
	TL41	31	Not Detected	Not Detected
	TL42	31	Not Detected	Not Detected
	TL43	18	Not Detected	Not Detected
	TL44	31	Not Detected	Not Detected
	TL45	27	Not Detected	Not Detected
	TJB37	22	Not Detected	Not Detected
	TJB38	15	Not Detected	Not Detected
	TJB39	13	Not Detected	Not Detected
	TJB40	23	Not Detected	Not Detected
	TJB41	28	Not Detected	Not Detected
	TJB42	10	Not Detected	Not Detected
	TJB43	26	Not Detected	Not Detected
	TJB44	38	Not Detected	Not Detected
	TJB45	24	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
TJB = Code of sample from Tanjung Balai Asahan

Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
April 2016	TL46	35	Not Detected	Not Detected
	TL47	31	Not Detected	Not Detected
	TL48	27	Not Detected	Not Detected
	TL49	36	Not Detected	Not Detected
	TL50	26	Not Detected	Not Detected
	TL51	17	Not Detected	Not Detected
	TL52	31	Not Detected	Not Detected
	TL53	14	Not Detected	Not Detected
	TL54	19	Not Detected	Not Detected
	TJB46	51	Not Detected	Not Detected
	TJB47	20	Not Detected	Not Detected
	TJB48	19	Not Detected	Not Detected
	TJB49	13	Not Detected	Not Detected
	TJB50	20	Not Detected	Not Detected
	TJB51	29	Not Detected	Not Detected
	TJB52	56	Not Detected	Not Detected
	TJB53	30	Not Detected	Not Detected
	TJB54	55	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
TJB = Code of sample from Tanjung Balai Asahan

Time of Sampling	Code of Sample	Concentration (µg/100g of meat)		
		ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
June 2016	TL55	35	Not Detected	Not Detected
	TL56	31	Not Detected	Not Detected
	TL57	27	Not Detected	Not Detected
	TL58	36	Not Detected	Not Detected
	TL59	26	Not Detected	Not Detected
	TL60	17	Not Detected	Not Detected
	TL61	31	Not Detected	Not Detected
	TL62	14	Not Detected	Not Detected
	TL63	19	Not Detected	Not Detected
	TJB55	51	Not Detected	Not Detected
	TJB56	20	Not Detected	Not Detected
	TJB57	19	Not Detected	Not Detected
	TJB58	13	Not Detected	Not Detected
	TJB59	20	Not Detected	Not Detected
	TJB60	29	Not Detected	Not Detected
	TJB61	56	Not Detected	Not Detected
	TJB62	30	Not Detected	Not Detected
	TJB63	55	Not Detected	Not Detected

Note : TL = Code of sample from Teluk Lampung
TJB = Code of sample from Tanjung Balai Asahan

Time of Sampling	Sampling Location	Code of Sample	Concentration (µg/100g of meat)		
			ASP (Domoic Acid)	AZP (Azaspiracid)	NSP (Brevetoxin)
April 2017	Teluk Lampung	TL1	Not Detected	Not Detected	Not Detected
		TL2	Not Detected	Not Detected	Not Detected
		TL3	Not Detected	Not Detected	Not Detected
		TL4	0,017	Not Detected	Not Detected
		TL5	Not Detected	Not Detected	Not Detected
		TL6	0,011	Not Detected	Not Detected
May 2017	Tanjung Balai Asahan	TJ1	0,667	Not Detected	Not Detected
		TJ2	0,169	Not Detected	Not Detected
		TJ3	0,606	Not Detected	Not Detected
		TJ4	0,705	Not Detected	Not Detected

Note: TL = Code of sample from Teluk Lampung
TJB = Code of sampling from Tanjung Balai Ashan

Discussion Results

Samples of Green Mussel (*Perna viridis*) and Baby Clam (*Meritrix meritrix*) were analyzed for Domoic Acid, Azaspiracid, Paralitic and Brevetoxin from April to May 2017. The results showed that **Brevetoxins and Azaspiracid were not detected** in the shellfish samples from Tanjung Balai Asahan and Teluk Lampung which serve as the sampling site. Domoic Acid was detected with LCMSMS in six locations, but it is still below the regulatory limits in the 2 mg/100g of meat.

c. Corrective Actions

In the future, shellfish sanitation program need to be done more frequently.

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 to ensure that the testing is conducted within the schedule. The selection of species that serve as samples depended on its availability in nature at the time of sampling. Shellfish samples with individual whole weight of ± 50g were collected for the analysis, the smaller size shellfish were returned back into the water.

VI. Recommendations and Suggestions for Future Follow- Up Action(s)

This project should be continued periodically in the future to build a shared understanding of the occurence of ASP, AZP and NSP in regional waters. It is also important to establish shellfish reference laboratory in ASEAN region in order to strengthen laboratories that have recently developed the shellfish monitoring programme.

MALAYSIA

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I. Introduction

Paralytic Shellfish Poisoning (PSP) is among the main intoxication cases reported due to marine biotoxin besides puffer fish poisoning in Malaysia (Table 1 and 2). Therefore, the capability and capacity for marine biotoxin analysis in Malaysia centred on PSP toxin or Saxitoxin (STX) and Tetrodotoxin (TTX) (Table 3 and 4). So far, there are no confirmed cases of poisoning due to Amnesic Shellfish Poisoning (ASP), Diarrhetic Shellfish Poisoning (DSP) and Neurotoxic Shellfish Poisoning (NSP) caused by Domoic acid, Azaspiracid acid (AZA) and Brevitoxin (BTX) respectively.

In Malaysia, HAB monitoring (harmful algae identification and quantification in sea water samples from major wild and cultured shellfish areas) was carried out by the Department of Fisheries (DOF) Malaysia since the year 2000 under the Sanitary and Phyto Sanitary (SPS) Program (2000-2012). Biotoxin analysis particularly STX was performed on an ad hoc basis or upon demand using mouse bioassay, ELISA (Takata Kit) and later HPLC. In 2013, HAB monitoring was placed under the National Shellfish Sanitation Program (NSSP) until present. Under the NSSP, biotoxin (particularly PSP toxin) analysis will be carried out if the cell counts are high in water samples. Domoic acid, Azaspiracid acid (AZA) and Brevitoxin (BTX) are not prioritised under the NSSP because to date there are no reported cases of ASP, DSP or NSP in Malaysia. However, studies pertaining to the presence of the responsible groups or toxin producers of ASP, DSP and NSP, especially *Pseudo-nitzschia* sp. have been carried out (Table 5).

Table 1: Reported cases of PSP in Malaysia

	Year	Location	Toxic alga/species	Notes	Reference
1.	1976	West Coast of Sabah	*Pyrodinium bahamense* var *compressum*	7 deaths	Roy (1977)
2.	1976-1988	Sabah	*P. bahamense* var *compressum*	31 deaths	Ting and Wong (1989)
3.	1991	Sebatu, Melaka	*Alexandrium tamiyavanichii Gymnodinium catenatum*	3 person hospitalized	Anton et aI. (2000) Usup et aI. (2002)
4.	2001	Tumpat, Kelantan	*Alexandrium minutum*	1 death, 6 hospitalized;	Lim et aI. (2004)
5.	2009	Kota Kinabalu, Sabah	*P. bahamense*	No data	DOF, Sabah (2009)
6.	2013	Sepangar Bay or Kuala Penyu, Sabah	*P. bahamense* var *compressum*	4 deaths	Suleiman et al. (2017)
7.	2013 2014	Kuantan, Pahang	*A. tamiyavanichii*	10 hospitalized	Normawati et al. (2017)

Table 2: Reported cases of pufferfish poisoning in Malaysia

	Year	Location (cases)	Reference
1.	1985	Sabah (4)	Lyn (1985)
2.	1987	Sabah (9)	Kan et al. (1987)

3.	1997	Terengganu (1)	Loke & Tan (1997)
4.	2008	Johor (34), Sabah (1), Sarawak (1)	Chua and Chew (2009)
5.	2009	Sabah 6, Terengganu (5), Sarawak (3)	Murali (2009), Razak et al. (2009)
6.	2013	Sarawak	Pers. Comm.

Table 3: Capabilities and facilities for biotoxin analysis in Malaysia

	Agency	**Toxin**	**Technique**
1.	Likas Fisheries Research Centre, DOF Sabah	STX	Mouse Bioassay
2.	Fisheries Research Institute (FRI), Batu Maung, Penang (DOF)	STX, TTX	HPLC, LC-MS/MS
3.	Fisheries Biosecurity Laboratory, Kuantan, Pahang (DOF)	STX, ASP, AZA	HPLC, LC-MS/MS
4.	Fisheries Research Institute (FRI), Bintawa, Sarawak	BTX	ELISA Kit
5.	Public Health Laboratory, Johor (Ministry of Health)	TTX	LCMS
6.	National Poison Centre, Universiti Sains Malaysia (USM),	TTX	GCMS
7.	Dept. of Aquatic Science, UNIMAS, Sarawak	TTX, STX	HPLC

Table 4: Capabilities and facilities for HAB work

	Agency	**Activities**
1.	FRI, Batu Maung, Penang	Monitoring, R&D
2.	Fisheries Biosecurity Laboratory, DOF - Kuantan, Pahang - Kuala Lumpur - Bintawa, Sarawak	Monitoring
3.	Likas Fisheries Research Centre, DoF Sabah	Monitoring
4.	Public Universities (UM, UNIMAS, UKM, IIUM)	R&D

Table 5: Reported ASP, AZA and BTX producer algae (toxic species) in Malaysia

	Species	**Locations**	**Reference**	**Note**
Domoic acid producer				
1.	*Pseudo-nitzschia batesiana*	Miri, Telok Batik	Lim et al. 2013, Teng et al. 2016	Newly described *Pseudo-nitzschia*
2.	*P. abrensis*	Miri	Teng et al. 2016	
3.	*P. kodamae*	Telok Batik, Port Dickson, Johor strait, Bintulu, Miri, Pulau Banggi, Semporna	Teng et al. 2013, 2014, 2016	Newly described *Pseudo-nitzschia*
4.	*P. lundhomiae*	Telok Batik, Miri	Lim et al. 2013, Teng et al. 2016	Newly described

				Pseudo-nitzschia
5.	*P. subfraudulenta*	Telok Batik, Port Dickson, Grigat, Miri, Pulau Banggi	Teng et al. 2013, 2016	
Neurotoxic Shellfish Poisoning/ BTX producer				
1.	*Karenia brevis*	Johor strait	Leong et al. 2015, Tan et al. 2016,	First report along the Johor strait

II. Objectives and Goals

The objectives of this project are to develop the capability to analyze ASP, AZA and BTX in shellfish tissue and to determine the level of AZA, ASP and BTX in shellfish samples from Malaysia.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species *(include scientific name)*, Number of Samples and Sampling Size

Random sampling method was adopted by the State Fisheries Assistant under the NSSP program. Locations of the sampling included major natural and cultured shellfish areas. Shellfish samples collected for PSP testing under the NSSP Program were used in this project. Figure 1 illustrates the sampling locations determined under this program. Samples examined for ASP, AZA and BTX determination in this project consist of clams (*Polymesoda expansa*), cockles (*Anadara granosa*), oysters (*Crossostrea sp.*) and green mussels (*Perna viridis*). About 1-2 kg of commercial size shellfish was collected from each sampling location and were transported to the laboratory in ice cooled insulated box immediately.

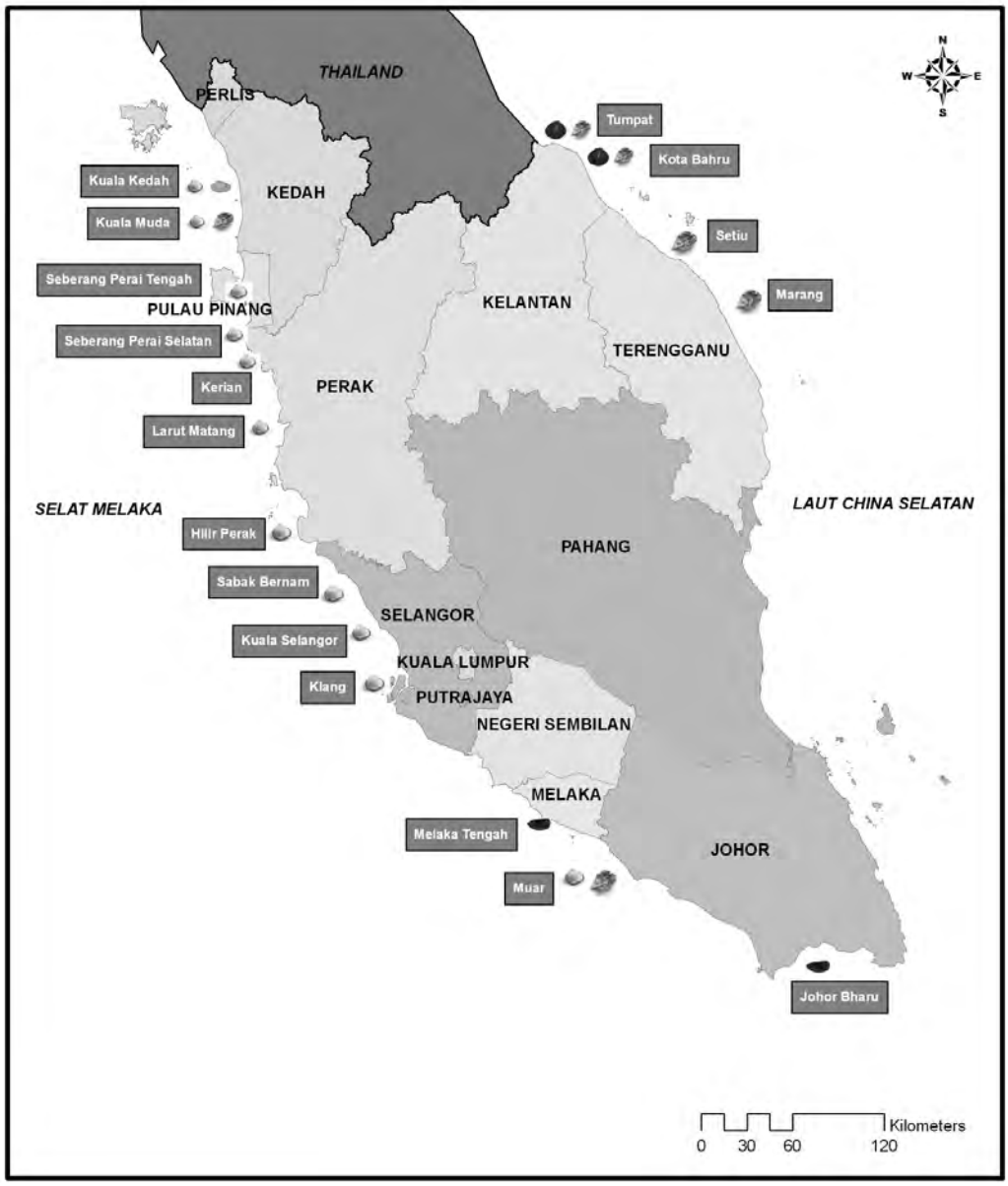

Figure 1: Sampling locations under the NSSP

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

AZA and *ASP*

Sample preparation was carried out according to Suzuki et al. 2005. Shellfish tissue was minced and a small portion (1 g) was extracted with 9 ml of methanol/distilled water (9:1 v/v). The samples were homogenized for 3 min followed by centrifuging at 3,000 rpm for 5 min (Eppendorf 5430, Hamburg, Germany). The supernatant obtained was filtered through a

0.45 µm PTFE membrane filter before being analysed using LC-MS/MS. Mass spectrometry was performed using a TSQ Quantum Discovery MAX model from Thermo Electron, USA consisting of a MS Surveyor pump with auto sampler coupled to a Mass Spectrometer equipped with an electrospray ionisation interface (ESI). Toxin was separated on a Hypersil BDS C8 column (5 µm, 150 mm x 2.1 mm inner diameter) at a flow rate of 200 µl/min. The injection volume was 5 µl. LC was performed with mobile phase A consisting of 2mM ammonium formate and 50 mM formic acid in water. Mobile phase B contained 2mM ammonium formate and 50 mM formic acid in acetonitrile/water (95/5, v/v). The gradient elution started with 20% B and increased to 100% B within 0.1 min, held over 10 min, then stayed stable for 15 min. Certified Reference Materials (ASP-Mus-e, AZA 1, AZA 2, AZA 3) were obtained from National Research Council Canada.

Brevetoxins

BTX analysis was carried out in accordance to the instruction from the manufacturer of the ELISA kit, PN 520026, Microtiter Plate (96T), Abraxis, Warminster, USA. Firstly, shellfish tissues were removed from the shells and washed with deionized water. Samples were homogenized using a blender (Waring) and 1.0g portion of the homogenized tissue was placed in a glass vial and added with 9.0ml of methanol/deionized water solution (9:1 v/v). The vial was vigorously shaken for 2 min. The mixture was centrifuged for 10min at 3000g. The supernatant was collected and 20ul of the collected extract was diluted with 1ml diluent. Diluted extracts were assayed according to the manufacturer's instructions.

c. Limit of Detection and Limit of Quantification

Since the data obtained specifically for this analysis is still limited, LOD and LOQ of this method could not be determined.

d. National Regulatory Limits

There are currently no established national regulatory limits in Malaysia. International standards were used as references which are 20 mg/kg for ASP (European Committees (EC) Regulation 853/2004), 160 µg/kg for AZA (EC Regulation 853/2004) and 0.8 mg/kg for BTX (FDA, 2000).

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

Did not participate in inter-laboratory proficiency testing.

b. Survey Results and Discussion

Table 6 presents the level of ASP in the shellfish samples analysed. In general, the levels of ASP in clams, oysters and cockle samples are way below the permissible level of 20 mg/kg. Shellfish samples from the east coast of Peninsular (Johor, Kelantan and Terengganu) showed low levels of ASP. The highest amount of ASP (min 4.80 mg/kg) was detected in clam's samples from Johor. Most of the shellfish samples from Kelantan also harboured detectable levels of ASP.

Table 6: ASP (Domoic acid) levels in shellfish samples

Location	**Date of sampling**	**Samples**		**Level (mg/kg of meat)**
		Type	**Number**	**Average**
Johor	1/9/2015	Clams	2	4.80
Kelantan	10/9/2015	Clams	1	1.05
	21/9/2015	Clams	3	0.60
	26/10/2015	Clams	1	0.82
	28/10/2015	Clams	1	0.79
	3/11/2015	Clams	1	0.34
	7/11/2015	Clams	3	0.41
	3/11/2015	Oysters	1	0.52
	18/1/2016	Clams	3	0.24
	22/2/2016	Clams	3	0.90
	29/3/2015	Clams	3	0.14

	25/4/2016	Clams	3	0.95
	30/5/2016	Clams	3	Not detected
Kedah	26/4/2016	Cockles	2	Not detected
Terengganu	3/11/2015	Oysters	1	0.35
Perak	25/5/2016	Cockles	2	Not detected
	26/5/2016	Cockles	2	Not detected

Table 7 indicates the level of AZA in the shellfish samples analysed. Similarly, like ASP, the level detected for AZA is way below the permissible level of 160 µg/kg. Less than 5% of the samples showed level of AZA. The highest amount of AZA detected is only 0.003 mg/kg in oysters from Kelantan. The source of AZA was not determined in this study.

Table 7: Azaspiracids acid (AZA) levels in shellfish samples

No	**Location**	**Date of sampling**	**Samples**		**Level (mg/kg of shellfish meat/tissue)**
			Type	Number	
1.	Terengganu	6/4/2015	Oysters	1	Not detected
2.	Johor	23/6/2015	Mussels	1	Not detected
		30/6/2015	Mussels	2	Not detected
		27/7/2015	Cockles	1	Not detected
		1/9/2015	Clams	3	Not detected
		9/9/2015	Mussels	2	Not detected
		9/9/2015	Oysters	1	Not detected
		9/9/2015	Cockles	3	Not detected
		28/10/2015	Clams	1	Not detected

3.	Pahang	20/3/2015	Oysters	1	0.001 (AZA 1,3)
		22/4/2015	Oysters	1	Not detected
		28/4/2015	Mussels	1	Not detected
		29/5/2015	Oysters	1	0.001 (AZA 1,3)
		26/10/2015	Clams	1	Not detected
4.	Kelantan	24/3/2015	Oysters	1	0.003 (AZA 1,3)
		12/5/2015	Clams	1	Not detected
		10/9/2015	Clams	1	Not detected
		21/9/2015	Clams	3	Not detected
		3/11/2015	Clams	1	Not detected
		3/11/2015	Oysters	2	Not detected
		17/11/2015	Clams	3	Not detected
		18/1/2016	Clams	3	0.002 (AZA 1,3)
		22/2/2016	Clams	3	Not detected
		29/3/2016	Clams	3	Not detected
		25/4/2016	Clams	3	Not detected
		30/5/2016	Clams	3	Not detected
5.	Kedah	25/4/2016	Cockles	5	Not detected
		26/4/2016	Cockles	5	Not detected
6.	Perak	25/5/2016	Cockles	5	Not detected
		26/5/2016	Cockles	5	Not detected

The levels of BTX in shellfish samples collected from Perak, Penang, Kedah, Sabah and Sarawak are presented in Table 8. In general, the levels of BTX in clams, oysters and cockle samples are way below the permissible level of 0.8 mg/kg.

Table 8: BTX levels in shellfish samples

No	Location	Date of sampling	Samples		Level (ppb)
			Type	Number	
1.	Perak	5/5/2016 3/8/2016	Cockles Cockles	9 2	2.00 <1.00
2.	Bako Sarawak	15/7/2016	Cockles	1	0.121
	Sematan, Sarawak	16/5/2016	Cockles	1	2.00
	Sematan, Sarawak	16/5/2016	Clam	1	2.00
	Samarahan, Sarawak	3/5/2016	Cockles	1	2.00
	Bintulu, Sarawak	15/5/2016	Clam	1	2.00
	Semera, Sarawak	10/8/2016	Cockles	2	<1.00
3.	Penang	3/7/2016	Cockles	5	<1.00
4.	Kedah	24/7/2016	Cockles	10	<1.00
5.	Kota Kinabalu, Sabah	10/8/2016	Cockles	2	<1.00

c. Corrective Actions

No corrective action is deemed necessary at this stage.

V. Problems and Challenges Encountered

The main problem encountered in this project was the insufficient funding. The reference standards for ASP and AZA as well as the maintenance of LC-MS/MS are expensive. The ASP, AZA and BTX analysis are also new to the lab and need to be improved continuously. Technical difficulties such as peak and retention time determination as well as calculation for

LOD and LOQ would require further elaborations and trainings so as to ascertain that accurate results (i.e. true value) are achieved consistently.

VI. Recommendations and Suggestions for Future Follow- Up Action(s)

a. To suggest that JTFP organize a split sample testing program/inter-laboratory testing for ASP, AZA and BTX.
b. To try alternative method such as rapid kits for the monitoring program since it is not as expensive as the LC-MS/MS method.
c. To carry out method validation for the current LC-MS/MS method if there is enough funding and to establish the LOQ and LOD of the methods.

MYANMAR

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I. Introduction

Biotoxin Monitoring Survey and procedure has been carried out in Myanmar since 2009 by Department of Fisheries (DoF) through SEAFDEC, MFRD under the Japanese Trust Fund II (JTF II) Program. Myanmar participated in the 1st project (2009 – 2012) as well as the 2nd project under the Japanese Trust Fund VI (JTF VI) Program (2013 – 2017).

Most of the shellfish species are located and distributed along the coastal regions of Myanmar. Although most people in Myanmar do not prefer to eat shellfish, many visitors from overseas eat them at the coastal beaches. There is also a small amount of shellfish being exported, but there has been no outbreak of poisoning concerning Biotoxins in Myanmar.

In the 2nd Biotoxin monitoring project (JTF VI), Myanmar divided the monitoring period into three quarters and used Green Mussel and Oyster to test for Amnesic Shellfish Poisoning (ASP) Domoic Acid.

Monitoring period:

- 2015 June to August (1st Quarter)
- 2015 October to December (2nd Quarter)
- 2016 April to June (3rd Quarter)
- Domoic Acid for Amnesic Shellfish Poisoning(ASP) in Green Mussel and Oyster are monitored.

Monitoring Area - Kyune Su Township, Tanintharyi Region (Southern parts of Myanmar)

Sample Collection - Wild catch of Green Mussel and Oyster from the market area of Kyune Su Township were collected to test ASP.

II. Objectives and Goals

Our Objectives and Goals are as follows;

- To analyse the ASP (Domoic acid) levels in shellfish especially Green Mussel and Oyster
- To practice and train skillful laboratory staffs for the analysis of Biotoxin in bivalves molluscs
- To protect and raise awareness of consumer's health in the event of any Biotoxins outbreak.
- To ensure harmonization and compliance in accordance to the standard of Biotoxins in ASEAN countries

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species (include scientific name), Number of Samples and Sampling Size

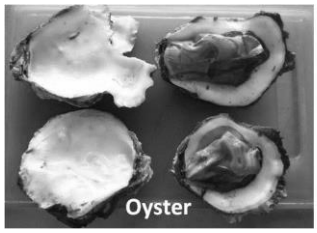
Sampling Method – Sample collection, collect the sample directly at sampling site and place into insulated box, control the temperature about 0˚C to +4˚C (chilled) and send to Analytical Laboratory Unit (Yangon Region) by air.

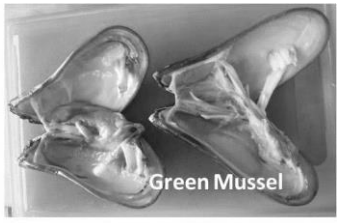
Sampling Site – Kyune Su Township, Tanintharyi Region (Southern Parts of Myanmar) Coastal Regions Area (wild caught).

Target Species – Common Name (Scientific Name)

Green Mussel (Perna viridis)

Oyster (Crassostrea beicheri)





Number of samples and size – 10 samples per testing and marketable size.

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

Sample Preparation Method:

According to the testing procedure of Bioo Scientific Chemical Test Kit, U.S.A for sample preparation of Mussels:

1. Remove the mussel tissue samples from shells, wash with deionized water.
2. Drain the excess liquid and homogenize the sample to a soupy texture.
3. Weigh out 0.5 g of homogenized tissue, add 2 mL of 50% methanol (in water) and vortex for 5 minutes.
4. Centrifuge tube for 10 minutes at 4,000 rpm.
5. Transfer 0.5 mL of supernatant to a new tube, heat the sample 75°C for 5 minutes.
6. Centrifuge 10 minutes at 4000 rpm.
7. Transfer 50 µL of the clear supernatant to a new tube, add 950 µL of 1X Sample Extraction Buffer/Methanol (90/10, V/V), mix well. The sample is ready for the assay.

Analytical Method Used:

Bio Scientific Test Kit by ELISA Method

ELISA Testing Protocol

1. Add 50 µL each Domoic Acid Standard into different wells.
2. Add 50 µL of each sample in duplicate into different sample wells.
3. Add 50 µL of Domoic Acid-HRP Conjugate to each well.
4. Add 50 µL of Anti-Domoic Acid Antibody to each well **IMMEDIATELY FOLLOWING MIXING IN THE WELL BY PIPPETING UP AND DOWN ONCE**. After add the Antibody to all wells, mix wells by gently rocking the plate manually for 1 minute.
5. Incubate the plate for 30 minutes at room temperature (20 - 25°C / 68 - 77°F) in the dark.
6. Wash the plate 3 times with 250 µL of 1X Wash Solution. After the last wash, invert the plate and gently tap the plate dry on paper towels.
7. Add 100 µL of TMB substrate. Time the reaction immediately after adding the substrate. Mix the solution by gently rocking the plate manually for 1 minute while

incubating. Incubate for 15 minutes at room temperature (20 - 25°C / 68 - 77°F) in the dark.

8. After incubation, add 100 µL of Stop Buffer to stop the enzyme reaction.
9. Read the plate as soon as possible following the addition of Stop Buffer on a plate reader with 450 nm wavelength.

Quality Control Measures:

1. Every batch used standard series for calibration curve
2. Duplicate analysis for each sample (with separate extraction)
3. Zero (0) standard used for OD (standard blank)
4. Sensitivity (LOD = Detection Limit) – ng/g (or) ppb
    - Mussels – 30 (according to Bio Scientific Chemical Test Kit)
5. The European Commission Directive 2002/226/EC implemented a domoic acid (ASP), the Maximum Permitted Level (MPL) of 20µg/g (ppm) in shellfish intended for human consumption.

c. Limit of Detection and Limit of Quantification

1st quarter		LOD	LOQ
ASP	(G.M)	1.34015 µg/100g	4.46716 µg/100 g
	(O)	4.59141 µg/100g	15.30468 µg/100g

2nd quarter			
ASP	(G.M)	0.09487 µg/100g	0.31623 µg/100g
	(O)	0.50596 µg/100g	1.68655 µg/100g

3rd quarter			
ASP	(G.M)	1.16790 µg/100g	3.89301 µg/100g
	(O)	3.63112 µg/100g	12.10372 µg/100g

d. National Regulatory Limits

There are currently no national regulatory limits for ASP in Myanmar. Department of Fisheries (DoF) is mainly adopting and complying the limits based on ASEAN and EU Standards, following the international conformity for food safety of imported and exported fishery products. The maximum tolerance levels established by the EU criteria is as follows;
ASP is 20 µg/g (ppm) = 2000 µg/100g

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

Did not participate in inter – laboratory proficiency test.

b. Survey Results and Discussion

Table for Survey Results

Sampling Location	Month & Year of Sampling (MM/YYYY)	Analyte Tested	No. of Samples Analysed	Min Concentration (ug/100g of meat)	Max Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Kyune Su Township, Tanintharyi Region, Southern Parts of Myanmar, Coastal Region Area (wild)	June – August, 2015	ASP	GM 10	Not Detected	1.4	0.18
			O 10	Not Detected	4.6	0.67
	October– December, 2015	ASP	GM 10	Not Detected	0.1	0.01
			O 10	Not Detected	0.4	0.08
	April- June, 2016	ASP	GM 10	Not Detected	0.9	0.24
			O 10	Not Detected	2.7	0.75

Discussion Results

The average concentration of Domoic acid (ASP) for all test results including 1st, 2nd and 3rd quarters of Biotoxins survey, are less than the maximum concentration stipulated by EU. In addition, all the test results can be considered as Not Detected (ND) because each average concentration of Domoic acid (ASP) is less than LOD. The findings of this survey suggested

that ASP toxins were not detected for the bivalve mollusc samples in Kyune Su Township, Tanintharyi Region of Myanmar. In the last survey project (JTF II, 2009-2012) at Kawthaung Township, Tanintharyi Region, ASP toxins were shown to be higher than current results (JTF VI, 2013-2017) at Kyune Su Township, Tanintharyi Region. However, all results are Not Detected (ND) since they are under the EU limit. According to the survey results, the coastal area is considered non-hazardous and safe for human consumption.

c. Corrective Actions

Since ASP toxins were not detected in all green mussel and oyster samples tested for, no corrective actions were required.

Problems and Challenges Encountered

At present, we could only use ELISA method of biotoxins analysis for these survey as LC-MSMS method is more expensive than ELISA method.

Project funding is very small and insufficient to cover the whole monitoring survey, therefore Myanmar decided to test only ASP (Domoic acid) but not AZA and BTX.

V. Recommendations and Suggestions for Future Follow- Up Action(s)

We would like to suggest to continue this project with more Biotoxins Training. Through the training offered in this project, it has equipped the laboratory staff with testing experiences and helped developed capability for the laboratory. We suggest the training to be provided on an annual basis for biotoxin analysis and principle and procedure of biotoxin monitoring system.

We have participated in two Biotoxins monitoring surveys, (2009- 2013) and (2013 – 2017). We would like to continue the research as there are still other species of shellfish/ bivalves not covered. We hope to conduct Biotoxins testing for these seafood and that MFRD conduct more Proficiency Tests (PT). We need to conduct research study in other coastal regions such as Rakhine, where most tourists concentrate. This is to ensure that all shellfish/ bivalves are safe for human consumption. HABs trainings conducted were also very useful to identify types of algae and red tide and others. We would like to do research with other ASEAN countries.

PHILIPPINES

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I. Introduction

The Philippines has a long history of blooms of toxic algae particularly those caused by *Pyrodinium bahamense* variety *compressum*. A number of Philippine bays and coastal waters continue to declare positive results for paralytic shellfish toxins (PSTs). A monitoring system for bivalves contaminated with saxitoxins is in place.

Monitoring for PSTs was part of the Biotoxin Monitoring in the ASEAN Region under the Japanese Trust Fund II (2009 - 2012). Equally important in the monitoring are algal toxins such as domoic acid and brevetoxins. These toxins are part of the BFAR algal toxin monitoring though not as extensive as PSTs.

Manila Bay was chosen as the study site as it has an extensive shellfish farming, particularly for green mussels. High annual production (Figure 1) was reported based on data published by the Philippine Statistics Authority. For this reason, Manila Bay was chosen as sampling area for the implementation of the Biotoxins Monitoring in ASEAN Region: ASP, AZA and BTX. Green mussel (*Perna viridis*) growing areas in Metro Manila, Cavite and Bataan were selected and sampling stations were established for routine sample collection.

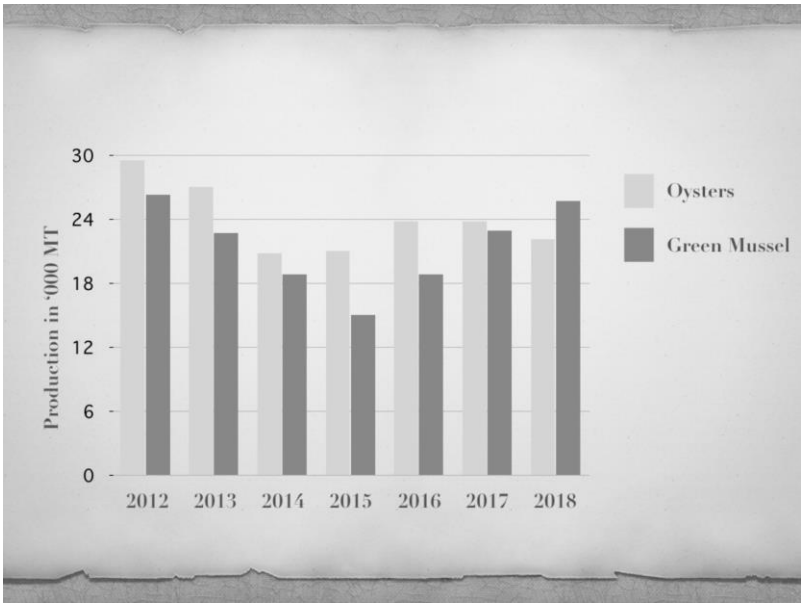

Figure 1: Shellfish production in the Philippines

II. Objective and Goals

Pseudonitzschia spp. and *Karenia brevis* are the identified phytoplankton that produce domoic acid (DA) and brevetoxin (PbTX), respectively. Blooms of these phytoplankton with corresponding toxicity cases have been reported. Pseudonitzia spp. has been reported in Southeast Asian waters particularly in Philippines, Viet Nam and Malaysia and contamination of tropical bivalves with domoic acid has been reported. In contrast, information on brevetoxin in tropical bivalves is rather limited. The primary objective of the monitoring activities was to establish a baseline data for the possible occurrence of domoic acid (DA) and brevetoxin (PbTX) in bivalves from tropical waters.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species (include scientific name), Number of Samples and Sampling Size

Sample Collection

One of the strategies for designing a monitoring strategy is through the use of a suitable bioindicator species for the detection of low level toxins. In the case of Manila Bay, the likely choice of species is the green mussel (*Perna viridis*). In previous studies involving saxitoxins, green mussel was successfully used as indicator species. This bivalve mollusk is of an epifaunal habitat, therefore there is a greater chance of exposure to possible toxic

phytoplankton in the water column. Sample collection was done on a monthly basis at the pre-determined sampling sites. The sampling stations and marketable sized green mussels (approximately 20 pcs) were collected at random within the vicinity of the sampling stations. Figures 2 and 3 shows the sampling collection sites and green mussel samples, respectively.

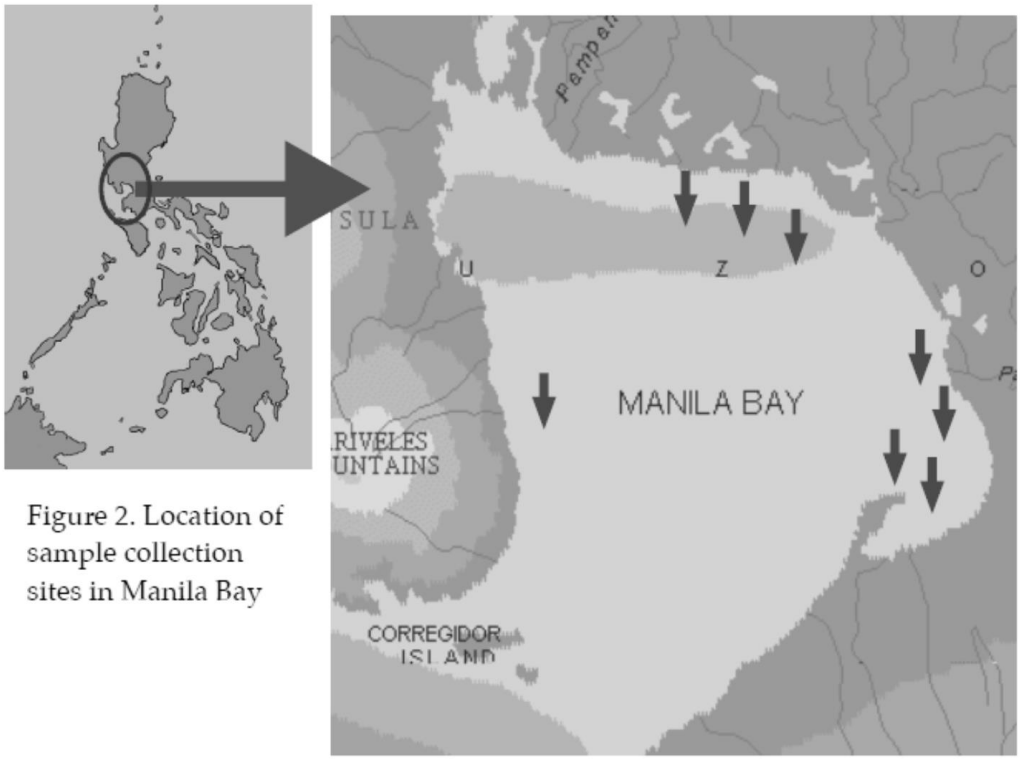

Figure 2. Location of sample collection sites in Manila Bay

Figure 3. Green mussel sample collected from Manila Bay

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

Sample Preparation

Domic Acid

Green mussels were prepared according to AOAC Method No. 959.06 section D. Shells were cleaned to remove foreign materials. Approximately 100g-150g of shellfish meat was homogenized at high speed until no lumps are visible. Domoic acid from green mussels was extracted according to AOAC Method No. 959.08 Section E [11]. 100g of homogenized shellfish meat was measured and 100 mL of 0.10N HCl was added. The combined weight was measured. The pH of the mixture was measured and adjusted to pH 3 - 4 when necessary. The mixture was gently boiled for five (5) minutes and allowed to cool to room temperature. Re-adjustment was done when measured pH was out of the prescribed range. Distilled water was used to adjust the weight of the solution to the original. The mixture was centrifuged at 4000 rpm for 10 minutes. The resulting supernatant solution was used for analysis.

Brevetoxin

Brevetoxin was extracted according to the method described in the Manual of Harmful Marine Microalgae IOC Manuals and Guides No. 33 UNESCO 1995. Brevetoxin was extracted through a series of partitioning and cleaned up using organic solvents. Green mussel extracts were first extracted with acetone and the mixture was concentrated using a rotary evaporator. Aqueous methanol and n-hexane were added. The methanol layer was collected and concentrated. Diethyl ether was added to the concentrated methanol fraction. The ether layer was collected and evaporated to dryness. The final residue was dissolved in tween solution in preparation for the assay.

Analytical Methods Used

Domoic Acid

A commercially available screening method employing a lateral flow immunochromatographic was used for screening purposes. This is a “dip-stick” antibody based method. Toxicity analysis results are interpreted based on color development in the control and test lines. Instrumental method of analysis using high performance liquid

chromatography (HPLC) following AOAC Method No. 991.26 was used to quantify the domoic acid in green mussel extract. Balances and pH meters used for analysis were calibrated before the start of each analysis. Certified reference materials purchased from National Research Council of Canada were used for calibration standards.

Brevetoxin

Mouse bioassay method was used to determine the presence of brevetoxin in shellfish. ICR strain mice from the Food and Drug Authority of the Philippines were used. Green mussel extracts were injected intraperitoneally to mice. The test mice were observed for toxicity symptoms associated with brevetoxins. Death of two out of three mice is considered positive for brevetoxins. Calculations were also based on dose-response table published in Manual of Harmful Marine Microalgae.

c. Limit of Detection and Limit of Quantification

Limit of detection (LOD) was determined for each of the analysis conducted. LOD was calculated based on the lowest concentration of domoic acid used which is 1.0 ug/mL. The LOD was calculated as 3 times the standard deviation. Based on experiments conducted, LOD is as low as 1.08 ug/g (Figure 5).

d. National Regulatory Limits

The Philippine regulatory limit for domoic acid as listed in the Philippine National Standard (PNS/BAFPS 89:201, ICS 67.120.30 is ⩽20 milligrams domoic acid per kilogram shellfish meat.

For brevetoxin, national standard has not been set yet. But for this case, national standards from Codex Alimentarius is applied. For brevetoxin, it is ⩽ 200 mouse units per kilogram or (0.8 milligrams BTX2 equivalent).

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

Did not participate in inter – laboratory proficiency test.

b. Survey Results and Discussion

Domoic acid

Analysis of domoic acid is part of the harmful algal bloom monitoring of the Bureau of Fisheries and Aquatic Resources. HPLC was used for analysis but with the increasing demand for domoic acid analysis in response to food safety issues and as a requirement for commercial transport, a different approach was implemented. In the new system, shellfish are first screened for domoic acid. Confirmatory test using HPLC is used in the case of green mussels that tested positive. In 2015 and 2016 the method used was HPLC and in 2017, screening-confirmatory test was applied.

Toxin analysis using HPLC is based on retention time of domoic acid standard and calculation are based on interpolation from a standard curve. Figure 4 shows a sample calibration curve used in the analysis. Graph shows a linear response between calibration solutions and peak area. However, for economic reasons, single point calibration was used for routine analysis.

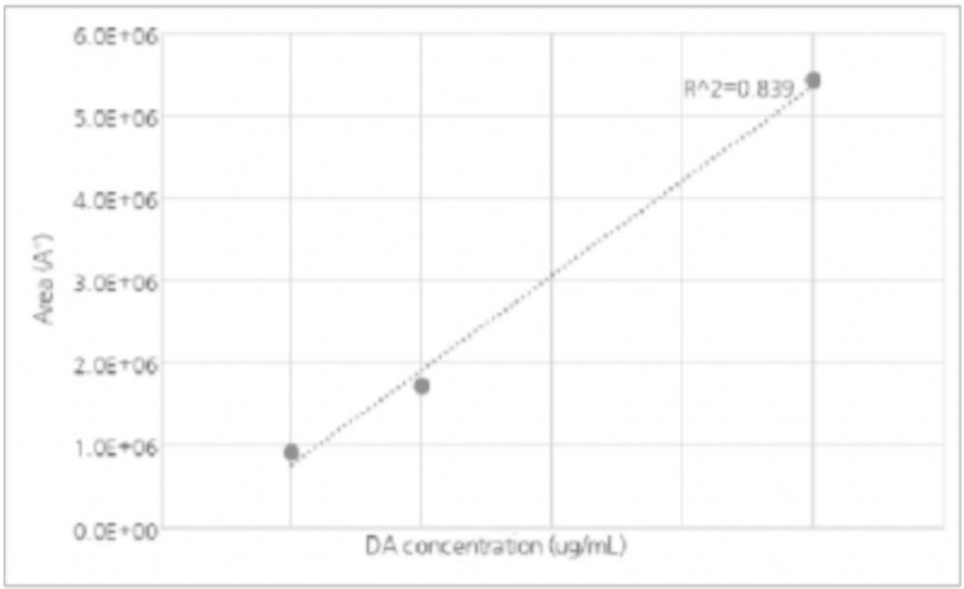

Figure 4. Linear response between domoic acid concentration and peak area

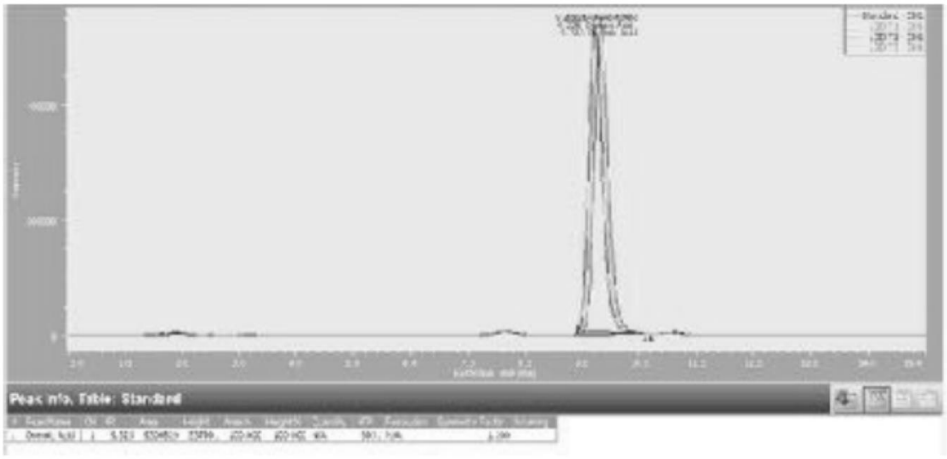

Figure 5. Sample chromatograms of standards used in the analyses

Detection of domoic acid is based on retention time. Typically, domoic acid has a retention time between 9 - 10 minutes. Domoic acid peak from standard chromatogram with identical retention to peaks at the sample chromatogram suggests the presence of toxin. Figure 6 shows a sample chromatogram that is negative for domoic acid. The absence of peak at around 9.2 minutes (see Figure 6) is indicative of the absence of domoic acid.

Reveal2 for ASP was the commercially available antibody based screening method used for the 2017 monitoring. The method was evaluated using a certified reference material (muscle tissue with known amount of domoic acid) following the manufacturer's instructions. Several dilutions were prepared with values above and below the expected limit of detection. Test strips showed positive results for at least 18 ug/g domoic acid. This is considered as false positive but is not an issue for these can be confirmed using HPLC. Results are summarized in Table 1. Based on the monitoring conducted, domoic acid were below detection limits of the method. Results are summarized in Table 2. Sample test strips are shown in Figure 7.

Figure 6. Sample chromatogram of a sample negative for domoic acid

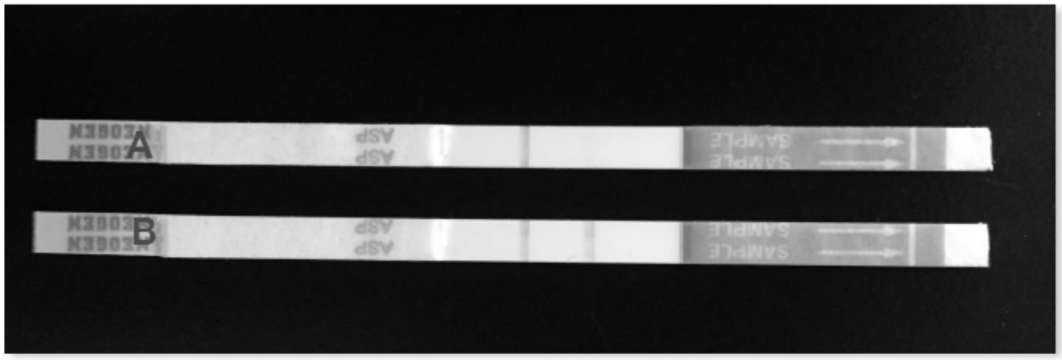

Figure 7. Sample test strips used in screening for domoic acids showing positive (A) and Negative (B) results

Table 1. Evaluation of Reveal2 for ASP using certified reference material	
Concentration (ug/g)	Results
24.05	Positive
23.05	Positive
21.20	Positive
20.10	Positive
18.10	Positive
10.50	Negative

Table 2. Summary of domoic acid monitoring results				
Year	Number of samples analyzed	Results	Method	Limit of Detection , LOD (ug/g)
2015	96	< LOD	HPLC	1.08
2016	96	< LOD	HPLC	1.08
2017	96	< LOD	Reveal2 for ASP	20

Brevetoxin

In the analysis of brevetoxin using mouse bioassay, test mice were observed for 24hours for telltale symptoms of brevetoxin. Extended observation for four (4) days were done to check for toxin below the detection level. Weight loss for four consecutive days suggests the presence of toxins in low quantities. During the conduct of monitoring, death in tests mice were not observed. Characteristic symptoms associated with brevetoxin were also not observed during the standard and extended observation period for all samples. Thus samples collected for this period is negative for brevetoxin. Results are summarized in Table 3.

Table 3. Summary of brevetoxin monitoring results				
Year	Number of samples analyzed	Results	Method	Limit of Detection , LOD (ug/g)
2015 Jan - June	48	< LOD	MBA	10MU/100g
2015 Jul - Dec	48	< LOD	MBA	10MU/100g
2016 Jan - June	48	< LOD	MBA	10MU/100g

c. Corrective Actions

Methods such as the mammalian assay or mouse bioassay, particularly for brevetoxin, is not ideal for several reasons. Firstly, there is limited sensitivity for the method which is only quantitative. Secondly, the influence of individual variation in terms of reaction to toxins makes the results difficult to interpret. Thirdly, there are ethical issues inherent in tests using live animals.

Receptor binding assay (RBA) was one of the test methods that can be used in lieu of mouse bioassay. The method is quantitative and the detection limit is less than that of mouse assay. BFAR partnered with the Philippine's Department of Science and Technology's Philippine Nuclear Research Institute to develop the capability of using RBA for toxins detection.

The monitoring of brevetoxin was stopped at the first stage due to limited resources available and the prioritization of the use of test mice for paralytic shellfish toxins. The plan was to shift to a method that is quantitative. The method of choice is RBA. The method of choice is still in an experimental stage but in terms of investment, RBA is the choice since the machine needed can be used for paralytic shellfish toxins, domoic acid, ciguatoxins and brevetoxins.

RBA can be used for ciguatoxin and brevetoxin analysis. This means that both biotoxin analyses methods share the standards, membranes and tracers. The RBA method is operational as of June 2018. However, the demand for analysis for ciguatoxin is greater than that of brevetoxin. The analytical demand comes from partner offices within BFAR who are in charge of the implementation of food safety regulations. For this reason and logistical constraints in the procurement of RBA test kits, test for ciguatoxins were prioritized.

V. Problems and Challenges Encountered

Participation in proficiency testing is one way of measuring the performance of a laboratory in terms of quantitative detection of toxins. In the case of this study, particularly that of domoic acid and brevetoxin, difficulties were encountered when finding for a suitable proficiency. An alternative would be an inter-laboratory testing but at the time of this project until the present, only BFAR Central Office has the capability to analyze domoic acid and brevetoxin. BFAR regional laboratories were more focused on the development of their capability for the detection of paralytic shellfish toxins (PSTs). This is because algal blooms that are associated with PSTs is a persistent and recurring event in Philippine bays and coastal waters.

Another challenge encountered is the procurement of marine toxins standards that are required for analysis. Standards used in analysis are not available in the Philippines. Domoic acid calibration standards and certified reference materials can be procured from National Research Council of Canada while that of brevetoxin of (RBA analysis) is from American Radiolabeled Chemicals. There was difficulty finding a Philippines supplier capable of handling the procurement of the required standards since Philippines laws are rather stringent for direct purchases from foreign distributor/ manufacturers.

Resources available for testing was another challenge encountered, particularly for brevetoxin analysis. The method is operational as of June 2018. However, the RBA method for brevetoxin detection in shellfish is the same as that for ciguatoxin. This means that both methods share standards, membranes and tracers. However, the demand for ciguatoxin analysis is greater than that of brevetoxin. The analytical demand comes from partner offices within BFAR who are in charge of the implementation of food safety regulations.

VI. Recommendations

Diatoms that are potential producers of domoic acid have been reported in the Philippines. *Pseudonitzschia* species has been reported in Manila and San Pedro Bays, in the Philippines [13,14]. *Nitzschia navis-varingica* which is a rather newly discovered domoic acid producing diatom was also reported in several bodies of water in the Philippines. Among the *Pseudonitzschia* species, P. *pungens* was reported as the most dominant. Previous study also showed that P. *pungens* do not produce domoic acid. However, studies also show that exposure of *Psuedonitzschia* spp to varying salinity conditions can induce the production of domoic acid.

In addition, domoic acid in tropical bivalves has been reported in the Philippines. *Karenia* spp. on the other hand, has yet to be reported in the Philippines.

Therefore, it is recommended that the monitoring of domoic acid and brevetoxins to be continued. In the interest of food safety and public health, expanding coverage in terms of bays and coastal waters monitored is also suggested. The data obtained in this study can serve as a point of reference should there be occurrences of algal blooms involving *Pseudonitzschia* spp and *Karenia* spp. In the case of domoic acid, monitoring several monitoring approaches geared towards predictive approach for the early warnings of domoic acid events. Monitoring data gathered will be of vital use. Azaspiracids were not included in the monitoring and the development of the capability to analyse these toxin is also recommended.

Karenia spp and *Azadinium* spp., are toxic algal species yet to be reported in Philippines bays and coastal waters. A risk based approach is recommended for the monitoring strategies for the toxins produced. The role of top management of agencies tasked with monitoring and management is emphasized. Top management support is necessary for the implementation of monitoring strategies and in sustaining the needs in terms of required equipment and supplies needed for testing.

SINGAPORE

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Toxins Section, Laboratory Group
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Agri-Food and Veterinary Authority of Singapore

I. Introduction

The Agri-Food and Veterinary Authority (AVA) of Singapore is the agency responsible to ensure a resilient supply of safe food, safeguard the health of animals and plants; and facilitate agri-trade for Singapore. As Singapore is a small city state, much (about 90%) of its food is imported, including seafood / shellfish. A stringent monitoring programme is already in place for the screening of imported seafood/shellfish for biotoxins to ensure that they are safe for consumption.

On local production of food fish, Singapore has several coastal floating fish farms that supplies about 8 % of our food fish consumption. In recent years, these food fish farms have been affected by HAB occurrence, resulting in massive destruction of the food fish stock and large monetary losses to the livelihood of the local fish farmers.

The biotoxin monitoring programme that AVA implemented for the local fish farms is to provide early warnings to the local fish farmers and so that they can be prepared and implement measures to mitigate the effects of a HAB occurrence to a minimum. The AVA biotoxin monitoring programme has the following objectives:

a) to monitor the imported seafood / shellfish for biotoxins,
b) to monitor local farms and to ensure that contaminated products are not harvested and sold for human consumption.

All the biotoxins testing are carried out at the AVA's official food safety testing laboratory, the Veterinary Public Health Laboratory (VPHL).

II. Objectives and Goals

This ASEAN biotoxin monitoring project is to monitor products for the biotoxins Azaspiracids (AZA) 1,2 and 3, Amnesiac Shellfish Poisons (ASP) and Brevetoxins (PbTx) 2,3 and 6 in Green Mussels (*Perna viridis*). The results will provide useful database on the levels of these biotoxins in the seafood and for assessment if there is any food safety concern.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species (include scientific name), Number of Samples and Sampling Size

For sampling method, mussel samples were taken from various aquaculture farms near and around the coastal region of the Singapore waters. At least three samples were taken monthly under normal non-HAB alert situations. However, during any alert of HAB occurrence near the costal food fish farms due to an elevated algae count, the sampling process will be increased. 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.

On average, 4 to 10 samples were tested for ASP, Brevetoxins and AZA each month according to AVA's surveillance program. The samples were taken randomly from local fish farms located off the shores of Singapore. No biotoxins (ASP, Brevetoxins and AZA) were detected in the Green Mussel (*Perna viridis*) samples surveyed during the period Jan 2015 – Dec 2017.

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

On arrival at the laboaratory, the samples were de-shelled and the whole shellfish was homogenized for use in analysis. The extraction was carried out as in accordance to the laboratory's protocol for the various biotoxins – Azaspiracids (AZA) 1,2 and 3, Amnesiac Shellfish Poisons (ASP) and Brevetoxins (PbTx) 2,3 and 6 method. High Performance Liquid Chromatography coupled to a Triple Quadrupole Mass Spectrometer (LC-MS/MS). technique was used for the testing of the 3 groups of biotoxins. The methods used are adopted from the EC reference methods. All the methods are fully validated and accredited to ISO / IEC 17025.

c. Limit of Detection and Limit of Quantification

Biotoxin	Limit of Detection (LOD)	Limit of Quantification (LOQ)
AZA1 , 2 and 3	2ppb	4ppb
ASP	200ppb	400ppb
Brevetoxins 2	160ppb	200ppb
Brevetoxins 3	80ppb	200ppb
Brevetoxins 6	160ppb	200ppb

d. National Regulatory Limits

The following limits were used:

Marine Biotoxins	Guidelines applied
AZA1 , 2 and 3	160µg/kg flesh
ASP	20 mg/kg flesh
Brevetoxins 2,3 and 6	80µg/100g flesh

IV. Results and Discussion

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

The laboratory participated in 2 rounds of inter-laboratory proficiency testing (PT) programme organised by WEPAL, QUASIMEME for **ASP toxins** in May 2016 and Oct 2017 respectively. Reports of proficiency test results that were released on July 2016 and Feb 2017, showed that our testing results were within the z-score of 2 for the 2 PT rounds ie results were satisfactory

The laboratory also participated in 2 rounds of inter-laboratory proficiency testing programme for AZA1,2 and 3 toxins. organised by the same organiser in October 2016 and

Oct 2017 respectively . For the Oct 2016 round, results of 9 out of 10 analytes screened were within the z-score of 2. For the Oct 2017 round, results of 9 out of 11 analytes were within z-score of 2 i.e. satisfactory performance.

There are no PTs rounds currently for Brevetoxins 2,3,6.

Summary of Proficiency Testing (PT) for ASP and AZA

PT Provider	**Round**	ASP	AZA1,2 and 3 toxins
WEPAL, QUASIMEME	May 2016 Oct 2017	3/3 satisfactory 3/3 satisfactory	- -
WEPAL, QUASIMEME	Oct 2016 Oct 2017		9/10 satisfactory 9/11 satisfactory

b. Survey Results and Discussion

Table for Survey Results

Sampling Location	Month & Year of Sampling (MM/YYYY)	Analyte Tested	No. of Samples Analysed	Min Concentration (ug/100g of meat)	Max Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Local farms off the shores of Singapore	Jan-Mar/2015	ASP Brevetoxins 2,3,6	9 9	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Apr-Jun/2015	ASP Brevetoxins 2,3,6 AZA 1,2,3	22 22 22	Not Detected	Not Detected	Not Detected

Local farms off the shores of Singapore	Jul-Sep/2015	ASP Brevetoxins 2,3,6 AZA 1,2,3	9 9 9	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Oct-Dec/2015	ASP Brevetoxins 2,3,6 AZA 1,2,3	13 13 13	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Jan-Mar/2016	ASP Brevetoxins 2,3,6 AZA 1,2,3	16 16 16	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Apr-Jun/2016	ASP Brevetoxins 2,3,6 AZA 1,2,3	12 8 12	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Jan-Mar/2017	ASP Brevetoxins 2,3,6 AZA 1,2,3	4 6 4	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Apr-Jun/2017	ASP Brevetoxins 2,3,6 AZA 1,2,3	33 30 33	Not Detected	Not Detected	Not Detected

Local farms off the shores of Singapore	Jul-Sep/2017	ASP Brevetoxins 2,3,6 AZA 1,2,3	38 38 38	Not Detected	Not Detected	Not Detected
Local farms off the shores of Singapore	Oct-Dec/2017	ASP Brevetoxins 2,3,6 AZA 1,2,3	18 18 18	Not Detected	Not Detected	Not Detected
TOTAL		ASP Brevetoxins 2,3,6 AZA 1,2,3	174 169 165	Not Detected	Not Detected	Not Detected

Discussion Results

A total of 174 shellfish samples were tested for ASP, 169 shellfish samples for Brevetoxins 2,3,6 and 165 shellfish samples for AZA1,2,3. ASP, Brevetoxins and AZA were not detected in all the samples tested.

d. Corrective Actions

Results of proficiency testing rounds have been good overall, with only 3 out of the 27 analytes screened outside of the satisfactory z-score of +/2. Corrective action have been taken and results of re-testing with newly purchased PT samples from the previous 2 rounds were within the acceptable z-score ie satisfactory performance.

V. Problems and Challenges Encountered

There is no PT provider providing PT rounds for Brevetoxins 2,3,6.

VI. Recommendations and Suggestions for Future Follow- Up Action(s)

1. To source for PT providers for Brevetoxins
2. To initiate a monitoring programme for Ciguatoxins in reef fish in order to assess if there is any food safety concern

THAILAND

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Food Technologist
Fish Inspection and Quality Control Division
Department of Fisheries

I. Introduction

The Department of Fisheries (DOF) has legal authority for classifying approved and harvesting areas of fishery include bivalves. DOF has imposed the Notification on Classification of bivalve harvesting areas under the authority of Fisheries Act.1947. The main 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 and for the monitoring of marine biotoxins, trace elements and bacteriological contamination of bivalve mollusks. Since 1997, DOF has been submitting reports on bivalve production and sanitation program to the EU which covers the monitoring results of bivalve mollusks flesh from the approved harvesting areas. The reports on biotoxin contents including PSP (Paralytic Shellfish Poisoning), DSP (Diarrhetic Shellfish Poisoning), ASP (Amnesic Shellfish Poisoning) and Lipophilic toxins such as Yessotoxin (YTX), Pectenotoxin (PTX) and Azaspiracids (AZA).

These monitoring programs are for routine surveillance testing of bivalve mollusks, improving our knowledge and understanding on the levels of biotoxins occurrences in the ASEAN region, and enhancing our capabilities to prevent of marine biotoxins occurrence to ensure that fish & fishery products are safe for human consumption.

II. Objectives and Goals

1) To strengthen the laboratory capacity to detect and control outbreak of Biotoxins in Thailand and ASEAN region.
2) To strengthen the laboratory capacity for analysis of Marine Biotoxins in Bivalve mollusks.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species *(include scientific name)*, Number of Samples and Sampling Size

The samples are taken from the approved harvesting area in Chonburi province. Green mussel samples are taken from the top, middle and bottom regions. The total weight of flesh samples should be not less than 300 g at each sampling point.

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

Sample Preparation

Sample was collected from the approved harvesting area in Chonburi province. Clean the outside of the shellfish with fresh, running water, if necessary. Open by cutting the adductor muscles. Rinse inside, only if necessary (e.g. the excessive presence of sand), with fresh, running water. Remove the tissue from the shell with a suitable knife. Collect required weight of tissue (approx. 150g) and place on a sieve to drain. Transfer to suitable container for weighing (beaker). Transfer to blender and blend the tissue until homogeneous**.**

Determination method for ASP by HPLC

Reference method: In house method based on M.A. Quilliam. 2003. Chemical methods for domoic acid, the amnesic shellfish poisoning (ASP) toxic. In: G.M. Hallegraeff, D.M. Anderson & A.D. Cembella (Eds), Manual on Harmful Marine Microalgae, Monographs on Oceanographic Methodology, Vol. II. Chapter 9. Intergovernmental Oceanographic Commission (UNESCO), Paris, 247 – 266.

Sample extraction

Homogenized samples (approximately 4.0 g) were extracted with MeOH/H2O (1:1, 16 mL) at 10,000 rpm for 5 min. After 40 min of centrifugation at 4,000 rpm, supernatant (5 mL) was filtered through a 0.45 μm membrane filter. Then sample solutions were analyzed immediately by HPLC with PDA at WL 242 nm.

HPLC Condition:

- Column: Symmetry C18, 3.9x150 um
- Mobile phase: 10% Acetonitrile: 0.1% TFA
- Flow rate: 10 ml/min
- Column Temperature: 40 degrees Celsius
- 5 Injection volume: 20 µl

Quality Control:

1) Reagent Blank
2) Spiked sample
3) Duplicate sample

Determination method for AZA by LC/MS/MS

Reference method: D. Faulkner, S.A. Hewitt and J. Cooper. 2012. The determination of Lipophilic (DSP) shellfish toxins in shellfish species by LC-MS-MS. Veterinary Sciences Division. Agri-Food and Biosciences Institute (AFBI). Standard Operating Procedure CSD 379 Version 4.

Sample extraction:

Weigh individual aliquots (2.00 ± 0.05 g) of each sample into 50 ml screw capped plastic centrifuge tubes. Add 9 ml of methanol to each tube and vortex mix for 3 minutes**.** Centrifuge at 2800 rpm for 10 minutes and transfer the supernatants to individually labelled 20 ml volumetric flasks. Add a further 9 ml of methanol to the sample tube and homogenise for 1min**.** Combine with the previous supernatants. Make up to the 20 ml graduation with methanol. Filter and transfer to vials and now ready for LCMSMS analysis as un-hydrolysed.

For hydrolysed, pipette 1.0 ml of each extract to individually labelled vials. Then pipette 125µl of 2.5M NaOH into each vial. Vortex mix. Heat the mixture at 76°C ±3°C for 40 minutes in a water bath. Pipette 125µl of 2.5M HCl into each vial, re-cap and vortex mix for 10 seconds. Centrifuge the vials for 5 minutes at 5000 rpm. The hydrolysed extract is now ready for LC-MS-MS analysis.

HPLC Condition:

- Column: Waters X-Bridge C18 (3.5µm), 100 x 2.1mm
- Mobile phase: 10% Acetonitrile: 0.1% TFA
- Flow rate: 0.3 ml/min
- Column Temperature: 20 degree Celcius
- Injection volume: 10 µl

Quality Control:

1) Reagent Blank
2) Spiked sample
3) Duplicate sample

c. Limit of Detection and Limit of Quantification

ASP: LOD = 0.03 ug/g and LOQ = 0.25 ug/g.

AZA: LOD = 0.003 ug/g and LOQ = 0.01 ug/g.

d. National Regulatory Limits

ASP = 20 ug/g

AZA = 0.16 ug/g

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results *(if any)*

1. Proficiency test for ASP by HPLC with QUASEMEME; The Netherland
   Results: Z score = - 0.40 to 0.60

2. Proficiency test for AZA by LC/MS/MS with QUASEMEME; The Netherland
   Results: Z score = 1.05 to 2.24

b. Survey Results and Discussion

Table for Survey Results

Sampling Location	Month & Year of Sampling (MM/YYYY)	Analyte Tested	No. of Samples Analysed	Min Concentration (ug/100g of meat)	Max Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
At Chonburi province	October 2014 – December 2017	ASP (HPLC)	28 samples	0.00 ug/100g (Not detected)	0.00 ug/100g (Not detected)	0.00 ug/100g (Not detected)
		AZA (LC/MS/MS)	28 samples	0.00 ug/100g (Not detected)	0.00 ug/100g (Not detected)	0.00 ug/100g (Not detected)

Discussion Results

The results of this survey during October 2014 – December 2017 indicated that ASP and AZA are not detected in all bivalve mollusk samples. However, the region still needs to continue monitoring the level of Biotoxins to ensure that the bivalve mollusk products are free from contamination of Biotoxins.

c. Corrective Actions

No corrective action is deemed necessary for this survey.

V. Problems and Challenges Encountered

No problems occurred during survey.

VI. Recommendations and Suggestions for Future Follow-Up Action(s)

Shellfish Monitoring Programme which also includes the Biotoxins Survey, is a very important programme for ensuring the safety and quality of bivalve or shellfish for both local consumption and export purposes. The future follow up action for this project should be focused on updating and training on new analytical methods, especially quantitative method for all biotoxins, in order to improve the testing capabilities on quality and safety of fishery products in Southeast Asia.

In addition, it is important to set up a networking system of Harmful Algae Blooms (HAB) at this regional level to support the sharing of knowledge and exchanging information related to shellfish toxins.

VIET NAM

Mr. Nguyen Thanh Binh
Deputy Director
Department of Conservation and Aquatic Resources Development
Directorate of Fisheries
Ministry of Agriculture and Rural Development

I. Introduction

Bivalve molluscs are important aquatic products in Viet Nam. There are 4 species (baby clam, *Metrix lyrata*; Undulated surf clam/ yellow clam, *Paphia undulata*; Antique ark, *Anadara antiquata*; and scallop, *Chlamys nobilis*) that are mainly harvested for domestic consumption and export. Among these species, undulated surf clam, *Paphia undulata*, has been monitored since 2000 for biotoxin contents in clam meat. The samples were collected from wild undulated surf clam in southern province of Kien Giang according to sampling scheme of the Sanitation Monitoring Program for Bivalve Molluscs Production areas in Viet Nam. Ba Lua harvesting area has been observed to have higher probability of ASP and marine lipophilic toxins detected in bivalve molluscs.

II. Objectives and Goals

The objective of this project is to monitor marine biotoxins including ASP, AZA in bivalve molluscs in harvesting areas in 8 provinces/cities in Viet Nam.

III. Survey Methodologies

a. Sampling Method, Sampling Site, Target Species (include scientific name), Number of Samples and Sampling Size

1. Setup of sampling plan
    - Define sampling date upon tidal zone
    - For production areas on tidal flat, sample shall be collected on day of largest tidal range (highest and lowest tide on a day)
    - For offshore production areas, sampling date may be any weekday.
    - Samples were not collected during the period from June to November due to closed season for resource protection purpose.

- Define sample collecting time

- For inshore production area:
    - Water sample collecting time: at highest tide of a day (±1 hour);
    - Bivalve mollusc sample collecting time: at lowest tide of a day.
- For offshore production area: sample (water and bivalve molluscs) collecting time: at any time.

- Identify bivalve mollusc species and size of sampling:

Bivalve mollusc samples shall be at commercial size, as follows:

TT	Species	Minimum size (mm –in length)
1.	Baby clam (*Meretrix lyrata*)	30
2.	Blood clam (*Tegillarca granosa / Arca granosa*)	30
3.	Antique ark (*Anadara antiquata*)	55
4.	Scallop *(Chlamys nobilis)*	60

- Sampling points

- Sampling point for biotoxin: at representative sampling point (defined on map and at field).

- Sample volume and quantity:

- Bivalve mollusc volume need to meet testing requirements but must be sufficent for extracting at least 50g of flesh and include at least 10 LBM pieces.
- Bivalve mollusc sampling for analysis of biotoxins: 01 sample per representative sampling point.

2. Bivalve mollusc sample collecting:

- For inshore production area: sample shall be collected by manual methods (by hand or wooden rake).
- For offshore production area: sample shall be collected by hand (diving to pick), trawl or other appropriate means.
- Undulated surf clam samples were collected for testing ASP and marine lipophilic toxins including AZA and transferred to the National Agro-Forestry-Fisheries Quality Assurance Department (NAFIQAD) Branch 6 in Can Tho city for analysis.

- Sample collection was carried out in compliance with the Guideline for implementation of the Sanitation Monitoring Program for Bivalve Molluscs Production areas adopted by NAFIQAD as above mentioned procedures. Total number of collected samples is 71 (2016: 24 samples, 2017: 22 samples, 2018: 25 samples).
- Sample preparation was carried out by the method developed and validated by NAFIQAD.

b. Method of Analysis *(e.g. sample preparation method, analytical method used, quality control measures)*

- ASP was analysed by the HPLC method (European Union Reference Laboratory for Marine Biotoxins, "EU-harmonised Standard Operating Procudure for determination of Domoic acid in shellfish and finfish by RP-HPLC using UV detection", version 1, June 2008).
- Lipophilic toxins were analysed by the LCMS/MS method. (European Union Reference Laboratory for Marine Biotoxins, "EU-harmonised Standard Operating Procudure for determination of Lipophilic marine biotoxins in molluscs by LC-MS/MS", version 5, January 2015).
- PSP was analysed by Mousebioassay method (AOAC 959.08).

c. Limit of Detection and Limit of Quantification

- Limit of detection for ASP by HPLC is 0.2 mg/kg. Limit of quantification is 0.6 mg/kg.
- Limit of detection for lipophilic toxins by LCMS/MS.

Biotoxin	**LOD (µg/kg)**
45 OH Yessotoxin (45 OH YTX)	10 µg/kg
45OH homo Yessotoxin (45 OH homoYTX)	10 µg/kg
Azaspiracid 1 (AZA1)	1 µg/kg
Azaspiracid 2 (AZA2)	1 µg/kg
Azaspiracid 3 (AZA3)	1 µg/kg
Dinophysistoxin 1 (DTX1)	20 µg/kg

Dinophysistoxin 2 (DTX2)	20 µg/kg
Homo Yessotoxin (homoYTX)	10 µg/kg
Okadaic acid (OA)	20 µg/kg
Pectenotoxin 1 (PTX1)	5 µg/kg
Pectenotoxin 2 (PTX2)	5 µg/kg
Total OA and ester/DTXs	20 µg/kg
Yessotoxin (YTX)	10 µg/kg

- Limit of detection for PSP by LCMS/MS is 400µg/kg.

d. National Regulatory Limits

Sampling frequency, testing criteria and maximum permitted levels for biotoxins thereof in the Sanitation Monitoring Program are showed in the following table:

No.	Criteria	Sampling frequency	Maximum Permitted Limits (MPLs)	Reference analysis method
1	Diarrhea-causing toxins (Lipophilic toxins)	Twice a month (1) Once a week (2)	Negative, or - Total Okadaic acid + Dinophysis toxins + Pecteno toxins: 160 µg/kg - Yessotoxins: 3,75 mg/kg - Azaspiracids: 160 µg/kg	LC-MS/MS
2	Muscle-paralyzing toxins (Paralytic Shellfish Poison - PSP)	Twice a month (1) Once a week (2)	Negative, or 800 µg/kg	Mousebioassay or LC-MS-MS
3	Dementia-causing toxins (Amnesic Shellfish Poisoning - ASP)	Twice a month (1) Once a week (2)	20 mg domoic acid/kg	HPLC

(1): Production areas affected by tides (with tidal flat)

(2): Production areas not affected by tides (without tidal flat)

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing and Results (if any)

Did not participate in inter-laboratory proficiency testing.

b. Survey Results and Discussion

Table for Survey Results (samples detected with marine biotoxin only)

Sampling Location	Month & Year of Sampling (MM/YYYY)	Analyte Tested	No. of Samples Detected	Average Concentration (ug/100g of meat)
Kien Giang	Jan 2016	PTX2	01	13.05 µg/kg
Ben Tre	Mar 2016	ASP	01	970 µg/kg (9.7mg/kg)
Kien Giang	May 2016	PTX2	01	149.63 µg/kg

Discussion Results

Of the 71 samples collected for analysis of ASP and marine lipophilic toxins (including AZA), only one (01) sample (ID: S17) was detected to contain ASP (970 µg /100g of meat) in 2016. However, the detected value was under the regulatory limit of 20 mg/kg. Among all samples (298 in 2016, 289 in 2017, 295 in 2018) collected in the other bivalve molluscs harvesting areas, there is no sample detected with ASP even though there were several times toxin-producing planktons, *Pseudonitzschia spp.* and *Dinophisis caudata*, was detected to exceed warning limits of 100,000 cell/l and 500cell/l respectively, in water samples in 2017 and 2018.

It was also noticed that AZA was not detected in undulated surf clam sample collected in Ba Lua harvesting area of Kien Giang province even though 2 samples were detected to contain pectenotoxins (PTX2) at lower levels (13.05µg/kg in January 2016 and 149.63 µg/kg in May 2016) than regulatory limit (160 µg/kg).

c. Corrective Actions

When the concentration of marine biotoxins exceed the maximum regulatory limits, NAFIQAD, as an inspection body, shall:

- Deliver warning to concerned production area, in which requires the following handling measures:

- Bivalve molluscs not meeting biotoxin criteria are not allowed to harvest and placed on the market.
- Local Competent Authorities in charge of fishery quality assurance (monitoring body): conduct intensive sampling for toxin-producing plankton and biotoxins with the sampling frequency of every 2-3 days at sampling points of bivalve molluscs detected with unsatisfactory biotoxin level.
- Handling/processing estabishments: consignments processed from bivalve molluscs harvested before sampling occasion are placed on the market or exported only when the biotoxin testing results are satisfactory.

- Update status of concerned production area on the its website.
- Bivalve molluscs in alert production areas may be harvested (accompanied with Certificate of Origin) for futher process if analysis results of toxin-producing plankton and biotoxins are satisfactory in 02 consecutive supplementary sampling occasions.
- Update harvesting regime and post-harvesting handling on its website.

V. Problems and Challenges Encountered

The monitoring program of marine biotoxins in bivalve molluscs and harmful algae are comprehensive and consecutive, and require resources to implement.

VI. Recommendations and Suggestions for Future Follow-Up Action(s)

Capacity building is necessary for people, particularly fishermen, to provide understandings of HABs and measures taken for the occurrence of HABs.

Advanced methods and equipment are recommended for quantitative analysis of harmful algae.

Member States should formulate a monitoring program for marine biotoxin in bivalve molluscs in order to ensure food safety of bivalve mollusc products for domestic consumption and export.

Summary

A total of 7 Member Countries, namely Indonesia, Malaysia, Myanmar, Philippines, Singapore, Thailand and Viet Nam participated in the JTFVI Biotoxins Monitoring in the ASEAN region. However, it was noted that only Indonesia, Malaysia and Singapore managed to test all 3 biotoxins (ASP, AZA and BTX).

From the survey results, it was observed that the level of biotoxins were under the limit of detection and hence, no corrective actions were deemed necessary. Nonetheless, member countries are still encouraged to continue the biotoxins monitoring programmes, including the increase of monitoring locations as well as the establishment of national biotoxin monitoring system.

Through this survey, participating countries have highlighted that one of the main challenges faced is the limited funding for sampling and analysis and insufficient laboratory resources.

Member countries too agreed upon the following recommendations:

1) To develop nation-wide biotoxin monitoring plans as a step-up of existing localized biotoxin monitoring plans for both domestic and exported seafood products within the SEAFDEC member countries
2) To explore the production of biotoxin as standard reference materials for biotoxin analysis that are not readily available
3) To continue strengthening network of experts and responsible personnel involved in biotoxins and HABs monitoring and management through information sharing, conducting proficiency tests and HAB species identification:
    i. Contact information of National Reference Laboratory and responsible center/ personnel (expert) to be updated
    ii. Proficiency Testing for Member Countries to be conducted by ASEAN Food Reference Laboratory for Marine Biotoxins and Scombrotoxins
    iii. Contact of Experts from International Organisation (eg. IOC-Westpac, EU)

In conclusion, this project has successfully enhanced regional capabilities for the testing of ASP, AZA and BTX biotoxins as well as the identification of toxic HAB species. Member countries now have a greater understanding and knowledge on the occurrences and incidences of biotoxins (i.e. fish and shellfish) and HAB species in the ASEAN region.

Annex Section

(1)	**Administrative Report - Regional Technical Consultation on Biotoxins (ASP, AZA and BTX), 2013**
(2)	**Participants' List - Regional Training Course in Biotoxins (ASP, AZA and BTX) Analyses, 2014**
(3)	**Administrative Report - Regional Technical Consultation on Toxic HAB species Identification, 2015**
(4)	**Administrative Report - Regional Training Course on Identification of HAB Species in the ASEAN Region, 2016**
(5)	**Administrative Report - Regional Training Course on Specimen Preservation and its Application in HAB Monitoring and Studies, 2017**
(6)	**Administrative Report - Regional Training Course on Culturing for HAB Species Identification and Toxin Characterization**
(7)	**Administrative Report - End-of-Project Meeting, 2019**
(8)	**Determination of domoic acid using High Performance Liquid Chromatography with Ultraviolet Detector (UV-HPLC) Method**
(9)	**Preparation of Domoic Acid Standard Solution and Homogenate fortified bivalve extract with Domoic Acid**
(10)	**Determination of Azaspiracids and Brevetoxins Using Liquid Chromatography – Mass Spectrometry (LC-MS) Method**
(11)	**Preparation of Azaspiracids Standard Solution and Homogenate fortified with Azaspiracids**
(12)	**Information on Key Project Leader for Biotoxins Monitoring**
(13)	**Information on National Reference Laboratory for Biotoxins Monitoring**
(14)	**Information on Key Project Leader for HABs Monitoring**
(15)	**Information on National Reference Laboratory for HABs Monitoring**

ANNEX 1

Administrative Report for Regional Technical Consultation, 2013

REGIONAL TECHNICAL CONSULTATION ON JAPANESE TRUST FUND VI CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA – BIOTOXINS MONITORING IN ASEAN REGION: ASP, AZA AND BTX

24 - 25 JULY 2013
SINGAPORE

I. INTRODUCTION

1. At the invitation of the SEAFDEC Marine Fisheries Research Department (MFRD) programme, the Regional Technical Consultation on "Japanese Trust Fund VI Chemical and Drug Residues in Fish and Fish Products in Southeast Asia - Biotoxins Monitoring in ASEAN: ASP, AZA and BTX" was held from 24-25 July 2013 in Singapore.

2. The meeting was attended by representatives from 10 SEAFDEC member countries, Senior Expert and Assistant Japanese Trust Fund Manager from SEAFDEC Secretariat, Japanese Expert, the Chief of MFRD Programmes as well as the Director for Post-Harvest Technology Department (PHTD) of the Agri-Food & Veterinary Authority of Singapore (AVA) and staff from PHTD which is the Collaborating Center of SEAFDEC 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 Regional Technical Consultation (RTC) which was held to discuss and plan for the new project that aims to address the needs of member countries in capability building in biotoxins' analyses and monitoring, with the focus on Amnesic Shellfish Poisoning (ASP) toxin, Azaspiracids (AZA) and Brevetoxin (BTX).

4. The 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 VI Project Leader, Ms Neo Shan Yu (Scientist of PHTD), presented the agenda of the RTC and it was adopted by the meeting. The agenda appears in Annex 2.

III. PRESENTATION ON THE INTRODUCTION OF JAPANESE TRUST FUND VI PROJECT BY THE SEAFDEC SECRETARIAT AND MARINE FISHERIES RESEARCH DEPARTMENT

6. The Senior Expert and Assistant Japanese Trust Fund Manager from SEAFDEC Secretariat, Mr Hidenao Watanabe, presented the overview of the Japanese Trust Fund programme.

7. The chairperson, Ms Neo Shan Yu, presented on the background and workplan/ activities of the project on biotoxins monitoring.

8. MFRD would be responsible for the coordination of the project activities which would be implemented by the member countries.

9. The Chief informed the meeting that the duration of the project activities on regional training course would be five days and the monitoring survey would be 1.5 years.

10. Myanmar enquired about training on monitoring of harmful algae bloom (HAB). The Chief replied that a new project on this has been proposed and pending approval by SEAFDEC.

IV. COUNTRY PRESENTATIONS AND DISCUSSION ON STATUS AND/OR PLANS OF BIOTOXINS MONITORING FOR ASP, AZA AND BTX

11. Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam presented the status on biotoxins monitoring for ASP, AZA and BTX in the fisheries industries in their respective countries.

Brunei Darussalam

12. Brunei queried if she could use fish samples or imported shellfish instead of local shellfish for testing as Brunei do not have a shellfish industry. The Chief replied that the imported shellfish could also be used.

13. Malaysia queried on the nature of the fermented product which was tested. Brunei responded that it was fermented shrimp paste, known as *Belachan*, locally.

14. Cambodia and Vietnam asked about the species of algae which were considered for the red tide management. Brunei responded that *Pyrodinium* was monitored for the presence of saxitoxin.

15. Dr Toshiyuki Suzuki mentioned that Paralytic Shellfish Poison (PSP) detection in fish was quite rare and enquired if the toxin was detected in the fish's meat or organs. Brunei responded that tests were done on fish's gills and gut.

16. Indonesia queried on the use of the phrase "Not Detected" in the result reporting. Indonesia commented that this might be misleading as it does not indicate if the value complied with the safety limits. The meeting came to the consensus that the phrase "Not Detected" referred to the results below the detection limit of the method and not to any safety limits.

Cambodia

17. The Chief asked if a biotoxin monitoring plan was present. Cambodia responded that adhoc testing was done only when a biotoxin-related issue was detected. Cambodia added that the Fisheries Administration, is setting up a laboratory in their Department of Fisheries, Post-Harvest and Quality Control to improve safety for fish and fishery product and trade development. In line with this, biotoxin monitoring would be included in the future plan.

Indonesia

18. The Chairperson enquired about the gap in ciguatera toxin monitoring and if it's under a monitoring programme or ad-hoc surveillance. Indonesia mentioned that the ciguatoxin monitoring was absent due to the lack of funding.

19. Malaysia enquired about the safety limits for PSP in shellfish and if the PSP standard of 800 micrograms per kilogram is correct. This figure was confirmed by Dr Toshiyuki Suzuki to be correct.

20. The Philippines asked if Indonesia had been approved by the European Union (EU) for shellfish export to EU. Indonesia replied that she has not been approved by EU.

Lao PDR

21. The Chief enquired if there were any continuation of biotoxin monitoring after the end of the previous JTF II project in 2012. Lao PDR replied that she had taken the biotoxin monitoring programme into consideration, under the Namxouang Aquaculture Development Center (NADC), Department of Livestock and Fisheries, as she imports shellfish from neighbouring countries.

22. The Philippines asked if Lao PDR only tested on imported shellfish. Lao PDR responded that as Lao PDR is a landlocked country with no access to the sea, all their shellfish are imported.

Malaysia

23. Dr Toshiyuki Suzuki enquired about the cost of the PSP ELISA kit. Malaysia confirmed that each box costs about USD 800. Dr Suzuki added that the cost of the ELISA kit was slightly more expensive than in Japan.

24. The Philippines enquired about how the monitoring areas were classified into risk categories. Malaysia responded that the information was obtained from the local officers from Sabah and determined based on cell count, species of plankton and toxins detected.

Myanmar

25. The Philippines enquired on the number of species (76) identified for being responsible for the discoloration of seawater and why the people in Myanmar were not accustomed to shellfish consumption. Myanmar responded that few people lived near the coastal region and were not used to eating shellfish, and that the discoloured seawaters were not safe for aquaculture.

26. The Chief enquired about the identity of the natural toxins in shellfish. Myanmar responded that she did not know the exact identity.

Philippines

27. No questions were raised.

Singapore

28. Malaysia enquired if the length of the turnaround time referred to the length of the analysis or the length of time until the release of the test report. Singapore responded that the duration indicated for the turnaround time was the duration until the time the result is released.

29. The Chairperson indicated that the meeting has taken into consideration that specific types of BTX and AZA toxin had to be identified for monitoring and this will be discussed under Agenda 5. The outcomes of the discussion under Agenda 5 are as attached in Annex 3(D).

Thailand

30. The Philippines enquired why dioxin and Poly-aromatic hydrocarbon (PAH) were monitored. Thailand responded that it's in accordance to EU's regulation for shellfish.

31. Cambodia enquired why monitoring was only done in the four approved areas. Thailand responded that monitoring was done for the areas where harvests were designated for export: baby clams in Trat province and Suratthani province, green mussel in Chumporn province, and last year green mussels were extended to a new harvesting area in the eastern and central part of Thailand.

32. Indonesia enquired if there was a monitoring system for shellfish from other provinces that did not export to EU. Thailand responded that only products from approved harvesting areas were monitored and allowed for export. Indonesia noted that the monitoring frequency is still on a weekly basis even though the programme had been on-going for 12 years. Thailand responded that biotoxins were not detected in previous test results but Food and Veterinary Officer (FVO) recommended weekly sampling to be done to gather analytical data for risk assessment.

33. Myanmar enquired whether tests were done for cooked products. Thailand responded that EU set the criteria for *E. coli* and that they conducted the tests based on EU guidelines. Thailand added that she has to fulfill the EU requirements on cooked products for exports to EU; while for the monitoring system, only live samples need to be tested.

34. Malaysia enquired on the species of shellfish that were exported to EU. Thailand responded that the main products were green mussels and baby clams. Blood cockles and oysters were still in the progress of being monitored.

35. Indonesia enquired about the procedures to determine the biotoxin situation of the products from the non-approved harvesting areas. Thailand responded that no monitoring was done on those products as they were not exported. Indonesia enquired whether the shellfish from the approved harvesting areas were wild caught or from aquaculture. Thailand responded they were obtained from both methods.

36. Malaysia enquired on the location of the approved harvesting areas. Thailand responded that all the areas were located in the Gulf of Thailand.

37. Singapore enquired on the need to register the shellfish farms with EU for export. Thailand responded that registration of the shellfish farms is only a domestic requirement and only the processing plants had to be registered with EU. Malaysia confirmed that there is no need to register the shellfish farms with EU and that EU relies on the Competent Authority of the country with regards to shellfish farms registration.

Vietnam

38. Cambodia enquired about the number of zones under monitoring. Vietnam responded that there were 20 zones monitored in 12 provinces.

39. Indonesia enquired about the singular sampling point, the number of samples and the frequency of sampling, whether 23 samplings were conducted for a whole year or a month; and whether the samples were only for shellfish or both water and shellfish. Vietnam responded that samples were taken for both shellfish and water and that the indicated frequency was for the whole year. Vietnam also added that more testing could be done if required.

40. Indonesia enquired on the action taken by government on products that contain biotoxins above the legal limit. Vietnam responded that the products would typically be detained and not released for human consumption and used for other non-human consumption purposes, such as fertilizer under government supervision. Vietnam had not detected any biotoxin exceeding the regulatory limits in the products in processing plant.

41. The Philippines enquired on the term 'Satisfactory' for the results. Vietnam responded that this would mean that the result is negative or lower than the detection limit. The Philippines further enquired on the double sampling points and when the intensified monitoring would cease. Vietnam responded that intensified sampling would stop when there were 2 consecutive satisfactory results.

42. Malaysia enquired about the classification of the production areas. Vietnam responded that the categories were A, B and C and were based on the *E. coli* and *Salmonella* counts in bivalve shellfish.

43. Cambodia enquired about the testing method for ASP. Vietnam responded that the current method was based on the LCMS method and they were moving on to develop the LCMS/MS method.

44. The Chief enquired about the difficulty in obtaining standard solutions. Vietnam responded that it referred to the difficulty in obtaining standards for some of the lipophillic biotoxins. Singapore added that there are different types of biotoxin under each biotoxin family that may have different availability as well as the high costs of the standards.

45. Indonesia suggested that the setting up of protocols to prevent contaminated products from being released to the market, for instance, it may be possible for the respective governments to offer compensation or to buy back the products.

V. PRESENTATION BY RESOURCE SPEAKER, DR TOSHIYUKI SUZUKI

46. Dr Toshiyuki Suzuki presented on the situation of shellfish contamination with ASP, AZA and BTX toxins in Japan; and the respective biotoxin's detection and analytical methods.

47. Singapore enquired about the recovery of the toxin and when the spiking of the sample was done. Dr Toshiyuki Suzuki responded that the spiking was done in the methanol extract.

48. Myanmar enquired about the differences between the spiking methods before and after extraction. Dr Toshiyuki Suzuki responded that the current spiking method could be employed if the extraction efficiency is high. However, if the sample is contaminated, a few extractions had to be carried out before analyzing the content.

49. Singapore enquired about the amount of shellfish sample necessary for the analysis. Dr Toshiyuki Suzuki responded that 1 gram would be sufficient. Singapore enquired about the conversion of mouse unit (MU) to a metric mass for BTX. Dr Toshiyuki Suzuki responded that such data is available but he would have to check.

50. Cambodia enquired on the way to differentiate between similar retention time compounds, in the event that one of them is a compound of interest. Dr Toshiyuki Suzuki responded that the two compounds could be differentiated based on their mass spectrum with the LCMS/MS method. However, if HPLC method was employed, optimization of method would be necessary to attain sufficient separation.

VI. DELIBERATION ON SCOPE AND IMPLEMENTATION OF THE PROJECT

51. Member countries were requested to provide the names of their respective Key Project Leader (KPL). Philippines would inform MFRD on the KPL after she has consulted her Council Director. The nominated KPLs for the other countries are as attached in Annex 3(A). The Chief requested that the member countries send their official nomination letter for the KPL to MFRD.

52. Member countries were requested to update their current testing capabilities for biotoxins, available facilities in their laboratories as well as their training needs. The updated list is attached in Annex 3(C).

53. Malaysia enquired if blood cockle would be a suitable shellfish for biotoxins monitoring as their green mussels production is declining. Dr Toshiyuki Suzuki explained that the accumulation of biotoxin in blood cockle is usually very low, hence it might not be a good substitute for biotoxin monitoring. Dr Suzuki enquired if Malaysia has blue mussels.

Malaysia responded that they do not have blue mussels, hence monitoring would continue with green mussels. However, she might face the problem of unavailability of samples during the scheduled monitoring period. Dr Toshiyuki Suzuki further enquired if Malaysia has any data of the blood cockle. Malaysia responded that the on-going project with Japan International Research Center for Agricultural Sciences (JIRCAS) has so far detected very low level of biotoxins in cockles.

54. The Chief clarified that the monitoring should be carried out on species which best represents the country's production and this decision can be made at a later stage during the monitoring plan phase. Malaysia suggested that the monitoring should also relate to the plankton population.

55. The Philippines enquired about the availability of standards for ASP, AZA, and DSP. Dr Toshiyuki Suzuki responded that his institute had standards for DSP and PSP which could be provided to member countries at no cost. The Chief clarified that the request should be done on a government-to-government basis. The Chief would disseminate the contact in the Ministry of Foreign Affairs, Japan, once Dr Suzuki has provided it to him. Member countries can write to this contact to request for the biotoxin standards from Dr Suzuki's institute.

56. Thailand and Vietnam suggested inter-laboratory comparison test/proficiency testing for member countries. Dr Toshiyuki Suzuki commented that it might be difficult to conduct such inter-laboratory comparison test/proficiency testing due to the lack of reference materials.

57. Singapore informed the meeting that with effect from 1st January 2015, the European Council (EC) would be replacing the mouse bioassay method with the LCMS/MS method as the reference method for lipophilic biotoxin testing.

58. The Chief announced that participants for the training course might need to share hotel rooms, due to budget constraints; and hence reminded member countries to take this into consideration for their nomination process.

59. The Philippines enquired if the training course could allow more than two participants per country. The Chairperson replied that member countries could send more than two participants but the additional participants would have to be at the countries' own cost.

60. The Chairperson informed member countries that the biotoxins monitoring survey would be for 1.5 years and a budget of USD 2,000 would be allocated for each country. She added that that the expenditure of the project would be on a reimbursement basis and countries were allowed to claim up to a maximum of USD 2,000 within the monitoring period.

61. Brunei enquired about the basis of reimbursement. The Chairperson replied that requesting country had to attach valid receipts/invoices in order to receive the reimbursement and the amount would usually be paid through Telegraphic Transfer (TT).

62. Brunei raised a query on the lack of an official bank account for reimbursement purpose. The Chief informed that if there were no official bank account, member country had to submit an official letter from the Council Director to inform MFRD that the survey expenditure is to be credited to the officer's bank account.

63. Vietnam queried on the duration taken from the submission of the receipts to the receiving of reimbursements. The Chairperson replied that it would normally take about a month.

64. The Philippines suggested harmonizing the results reporting format. Singapore suggested the inclusion of reporting of limit of detection. The Chairperson informed the meeting that the Limit of Quantitation (LOQ) and Limit of Detection (LOD) would have be included in the survey report, as what was done for the previous technical publication of the JTFII Biotoxins Monitoring in ASEAN project.

65. Malaysia suggested a Standard Operating Protocol (SOP) for test method and the handling of samples etc. The Chief explained that different test methods would have different modes of sample handling. Dr Toshiyuki Suzuki explained that SOP could only be developed for LCMS/MS. Mouse Bioassay and other ELISA test kits have different manufacturers and users should follow the manufacturers' instructions on sample treatment, etc. Singapore supported Dr Toshiyuki Suzuki comments and suggested doing recovery test to determine the method's performance.

VII. ANY OTHER MATTERS

66. Presentations by suppliers were arranged for participants to understand the latest developments in instruments available for biotoxin analyses.

67. Mr Venkatesha from Agilent Technologies delivered a presentation on “A Modern, Sensitive and Reliable Approach for the Screening and Identification of Marine Biotoxins”.

68. Dr Goh Lin-Tang from Alpha Analytical (S) Pte Ltd delivered a presentation on “Contaminants & Marine Toxins Analysis by Orbitrap-based High Resolution MS”.

VIII. CLOSING OF THE SEMINAR

69. The Chairperson concluded the meeting and thanked all participants for their contributions to the meeting.

70. The participants from the SEAFDEC member countries expressed their heartfelt appreciation to PHTD of AVA and 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.

71. The meeting was held in the traditional spirit of SEAFDEC co-operation and cordiality.

Participants of the Meeting

No	Name	Designation	Country	Organisation	Office Address	Telephone No.	Fax No.	Email Address
1	Hajah Laila Haji Hamid (Ms)	Head of Agrifood Safety Division	Brunei Darussalam	Ministry of Industry and Primary Resources	Agrifood Safety Division, Fish Landing Complex, Muara	+673-2770234		fiqc17025@gmail.com; laila.hamid@hotmail.com
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Agenda of Meeting

24 July 2013, Wednesday

0830-0900	Registration
0900- 0910	**Agenda 1**: Opening of the Meeting Welcome Remarks by Chief, MFRD Programmes Opening Remarks by Director, Post-Harvest Technology Department Adoption of Meeting Agenda
0910- 0940	**Agenda 2**: Overview of Japanese Trust Fund VI Programme Background and Introduction of Project on Biotoxins Monitoring
0940-1000	Group Photography Session and Coffee Break
1000-1200	**Agenda 3:** Country Presentation and Discussion on Status and/or Plans of Biotoxins Monitoring for ASP, AZA and BTX 1000 - 1010: Brunei Darussalam 1010 - 1020: Cambodia 1020 - 1030: Indonesia 1030 - 1040: Lao P.D.R 1040 - 1050: Malaysia 1050 - 1100: Myanmar 1100 - 1110: Philippines 1110 - 1120: Singapore 1120 - 1130: Thailand 1130 - 1140: Vietnam 1140 - 1200: Discussion
1200-1330	Lunch

24 July 2013, Wednesday (Continue)

1330-1500	**Agenda 4**: Presentation by Resource Speaker, Dr Toshiyuki Suzuki 1) ASP, AZA and BTX Toxins and Situation of Bivalve Contamination with these Toxins in Japan 2) Detection and Analytical Methods for ASP, AZA and BTX Toxins
1500-1520	Coffee Break
1520-1630	**Agenda 5**: Deliberation on Scope and Implementation of the Project
1630-1700	Any Other Matters

25 July 2013, Thursday

0900-1020	**Agenda 5**: Deliberation on Scope and Implementation of the Project *(Continue)*
1020-1040	Coffee Break
1040-1200	**Agenda 5**: Deliberation on Scope and Implementation of the Project *(Continue)*
1200-1300	Lunch Break
1300-1430	**Agenda 6**: Presentations by Laboratory Equipment Suppliers 1300 - 1345: Agilent Technologies 1345 - 1430: Alpha Analytical (S) Pte Ltd
1430-1450	Coffee Break
1450-1650	**Agenda 7**: Adoption of Administrative Report
1650-1700	**Agenda 8**: Closing of the Meeting

Information of Biotoxins Monitoring System in Member Countries

Abbreviations used:

ASP: **A**mnesic **S**hellfish **P**oisoning

DSP: **D**iarrhetic **S**hellfish **P**oisoning

PSP: **P**aralytic **S**hellfish **P**oisoning

CFP: **C**iguatera **F**ish **P**oisoning

TTX: **T**etrodo**tox**in

Country	Biotoxins Analysed and Regulatory Limits	Methods of Analysis	Frequency of Sampling	Department in-Charge
Brunei Darussalam	ASP DSP PSP	ELISA, LCMS/MS, HPLC	Monthly	Agrifood Safety Division
Cambodia	Not Applicable	Not Applicable	Not Applicable	Fishery Administration
Indonesia	PSP DSP ASP	PSP – Bioassay and ELISA (AOAC 1996) DSP – Bioassay and ELISA (IOC 2003) ASP – ELISA (IOC 2003)	Bi-yearly	Fish Quarantine and Inspection Agency/ Directorate General of Aquaculture
Lao P.D.R	Not Applicable	Not Applicable	Not Applicable	Namxouang Aquaculture Development Center / Department of Livestock and Fisheries
Malaysia	PSP	PSP – Mouse Bioassay (AOAC 1996)	Monthly	Fisheries Biosecurity

		HPLC post column ELISA		Division, Department of Fisheries
Myanmar	ASP DSP PSP	ELISA (AOAC 2006)	Biyearly	Department of Fisheries -Fish Inspection and Quality Control Division (Analytical Lab Section)
Philippines	PSP (60ug/100g)	Mouse Bioassay, HPLC post column, Jellet Rapid Test Strip	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, Bureau of Fisheries and Aquatic Resources, Fisheries Resource Management Division
	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 , LCMSMS	(a) Import: varies (every	Agri-Food & Veterinary

	DSP (0.2 - 0.4ppm)	DSP – LCMSMS	consignment or 1 in 10 or 1 in 20) (b) Local Farms – once a month or move if alert arises	Authority / Veterinary Public Health Centre
	ASP (20ppm)	ASP – HPLC		
Thailand	PSP (80ug / 100g)	PSP – Mouse Bioassay, HPLC-FL	Weekly (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 LCMS/MS - 0.16ug/g	DSP – Mouse Bioassay, LCMSMS	Weekly	FIQD
	ASP (20ppm)	ASP – HPLC	Weekly	FIQD
	Lipophilic toxins – PTX (0.16ug/g) YTX (1ug/g) AZA (0.16ug/g)	Lipophilic Toxins – Bioassay, LCMS/MS	Weekly	FIQD
Vietnam	PSP (80ug/100g)	PSP – Mouse Bioassay and LCMSMS	2 to 4 times per month	Ministry of Agriculture & Rural Development – National Agro-Forestry – Fisheries Quality National Agro-Forestry – Fisheries Quality /Assurance Department
	ASP (20mg/kg)	ASP – LCMS and HPLC		
	Lipophillic Biotoxins (160ug/kg)	DSP – Mouse bioassay, LCMSMS		

Request for Information of Testing Facilities Available in Member Countries

Abbreviations used:

ASP: **A**mnesic **S**hellfish **P**oisoning

AZA: **Aza**spiracid

DSP: **D**iarrhetic **S**hellfish **P**oisoning

PSP: **P**aralytic **S**hellfish **P**oisoning

CFP: **C**iguatera **F**ish **P**oisoning

TTX: **T**etrodo**tox**in

Country	Instruments Available	Methods of Analysis	Methods of Analysis that Country Wishes to Learn	Laboratory in-charge / Contact Person	Remarks
Brunei	1. HPLC 2. ELISA	Not Applicable	1. HPLC 2. ELISA 3. LCMS/MS	Seafood Analytical Lab/ Zuriana Kepli	Do not have a biotoxins laboratory yet but would participate in the monitoring survey
Cambodia	Not Available	Not Applicable	1. ELISA 2. PSP: HPLC, LCMSMS 3. ASP: HPLC, LCMSMS 4. DSP: HPLC, LCMSMS	Not Applicable	Do not have a biotoxins laboratory yet but would participate in the monitoring survey
Indonesia	1. HPLC-UV 2. LCMS/MS, ELISA	PSP, DSP: Mouse Bioassay and ELISA ASP: HPLC, ELISA	1. PSP: HPLC, LCMS/MS 2. ASP: LCMS/MS, HPLC	Fish Quarantine and Inspection Standard Examination Lab/	Would participate in the monitoring survey

			3. BTX: LCMS/MS, HPLC 4. AZA: LCMS/MS, HPLC 5. CTX: LCMS/MS, HPLC 5. DSP: LCMS/MS, HPLC	Asep Dadang Koswara Main Center Mariculture Development Laboratory/ Kurniastuti	
Lao P.D.R	Not available / Not applicable	Not applicable	1. ELISA Test method 2. ASP: HPLC, LCMS/MS 3. PSP: HPLC, LCMS/MS 4. DSP: HPLC, LCMS/MS	Namxouang Aquaculture Development Center / Department of Livestock and Fisheries Fisheries Division/ Department of Livestock and Fisheries / Sisamouth Phengsakoun	Would participate in the monitoring survey
Malaysia	1. HPLC 2. LCMS/MS 3. ELISA	PSP: Mouse Bioassay, HPLC post column, ELISA ASP, AZA, BTX: LCMS/MS	1. TTX: HPLC 2. DSP: HPLC 3. AZA: LCMS/MS 4. ASP: LCMS/MS 5. BTX: LCMS/MS	Fisheries Biosecurity Center, Kuantan/ Azlan Md. Nor Fisheries Research Center, Likas Sabah /Ainah Puyong Fisheries Biosecurity Center, Kuala Lumpur/ Hamdan Jaafar	Would participate in the monitoring survey Proficiency testing for PSP
Myanmar	1. ELISA	ELISA HPLC	All biotoxins analysis based on	Department of Fisheries/ Fish Inspection and	Would participate in the

	2. HPLC - fluorescence detector) 3. LCMS/MS		ELISA, LCMS/MS, HPLC	Quality Control Division/Analytical Lab Section/ Thet Naing	monitoring survey
Philippines	1. HPLC-UV/ fluorescence post column 2. ELISA	Mouse bioassay ELISA HPLC-UV/fluorescence post column	1. DSP and ASP screening method 2. AZA by LCMS 3. BTX: Mouse Bioassay or any analytical method 4. Identification of causative organism for ASP, AZA and BTX	Bureau of Fisheries and Aquatic Resources/ Marine Biotoxin Monitoring Section / Juan R. Rolox, Jr. The National Fisheries Research Institute /Dr Ulysses Montojo	Would participate in the monitoring survey Training on analytical methods for BTX, ASP and AZA Request for standards for ASP, AZA, DSP
Singapore	1. HPLC 2. LCMSMS 3. ELISA reader and other support equipment	1. HPLC 2. LCMS/MS 3. ELISA	1. AZA * 2. BTX* 3. ASP* 4. CTX 5. Algae identification	Agri-Food & Veterinary Authority (AVA) /Veterinary Public Health Laboratory/ Dr. Paul Chiew or Ms Helen Phang	Would participate in the monitoring survey * Interest in multi component analysis AZA, BTX and ASP
Thailand	1. HPLC 2. LCMSMS 3. ELISA	Same as Section A	1. DSP: LCMS/MS 2. PTX: LCMSMS 3. YTX: LCMSMS	Fish Inspection and Quality Control Division / Supanoi Subsinserm	Would participate in the monitoring survey Request for interlab

			4. AZA: LCMSMS 5. BTX: HPLC or LCMS/MS 6. CTX 7. TTX by LCMS/MS	Fish Inspection and Quality Control Division /Bordin Iddhibongsa	comparison / proficiency testing for biotoxin
			8. Identification of phytoplankton	Marine Fisheries Division/ Ms Renu	
Vietnam	1. LCMS/MS 2. HPLC-UV 3. LCMS	1. LCMS/MS (IOC) 2. HPLC - AOAC, 3. IOC) (ASP) 4. Mouse bioassay IOC, AOAC 5. (PSP, Lipophilic)	1. TTX: HPLC, LCMS/MS 2. Lipophillic toxins: LCMS/MS 3. PSP: LCMS/MS	Would participate in the monitoring survey Request for interlab comparison / proficiency testing for biotoxin	Would participate in the monitoring survey Request for interlab comparison / proficiency testing for biotoxin

Request for Information of Proposed Survey Scope by Member Countries

Country	Proposed Biotoxins For Survey	Proposed Biotoxins Species For Survey	Methods of Analysis for Proposed Biotoxins To Be Surveyed On	Remarks
Brunei	ASP BTX AZA	Clams Cockles Scallops Green mussels Oyster	LCMS/MS HPLC ELISA (ASP)	Shellfish samples will be imported products.
Cambodia	ASP AZA BTX	Hard clams Green mussels Oysters Cockles	Mouse bioassay HPLC ELISA LCMS/MS	
Indonesia	ASP AZA BTX	Green mussels Cockles Baby clams	ELISA LCMS/MS* HPLC*	*If CRM is available
Lao P.D.R	ASP	Cockles Green mussels	HPLC ELISA (ASP)	In collaboration with Fish Inspection and Quarantine Control Division/ Department of Fisheries, Thailand Shellfish will be imported samples.
Malaysia	ASP AZA BTX	Green mussels/cockles	ELISA LCMS/MS Mouse bioassay*	Dependent on availability of green mussels

				*Oysters, if FRC is participating
Myanmar	ASP AZA	Green mussels Oysters	ELISA HPLC	Locally produced samples. If CRM is available for method validation/verification.
Philippines	ASP AZA BTX	Green mussels Oysters Baby clams Thorny oysters	HPLC-UV ELISA (ASP) LCMS/MS Mouse bioassay (BTX)	LCMS/MS in collaboration with Food Development Center
Singapore	ASP AZA BTX	Green mussels	LCMS/MS HPLC	EC will be replacing the mouse bioassay (reference) method with the LCMS/MS method from 1st January 2015.
Thailand	ASP AZA	Green mussels Baby clams	HPLC (ASP) Mouse bioassay (AZA) LCMS/MS (AZA)	
Vietnam	ASP AZA	Baby clams Scallops Antique ark	LCMS/MS HPLC	

Proposed Scope for Regional Training Course

Date	Mid-June 2014 (5 days)
Venue	Veterinary Public Health Laboratory Biotoxins Laboratory 10 Perahu Road Singapore
Experts/Trainers	Dr Toshiyuki Suzuki Dr Ryuchi Watanabe (To be confirmed) Trainer for ASP ELISA test kit (To be confirmed)
Methods of Interest for each Biotoxins	LCMS/MS method for simultaneous detection of ASP, AZA, BTX HPLC-UV/DAD method for ASP (AOAC method) ELISA test kit method for ASP (Optional)

ANNEX 2

Regional Training Course in Biotoxins (ASP, AZA and BTX) Analyses Trainers & Participants' List, 2014

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ANNEX 3

Administrative Report for Regional Technical Consultation, 2015

REGIONAL TECHNICAL CONSULTATION ON JAPANESE TRUST FUND VI CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA – BIOTOXINS (ASP, AZA and BTX) AND HARMFUL ALGAL BLOOMS (HABS) IN THE ASEAN REGION

5 - 6 AUGUST 2015

SINGAPORE

I. INTRODUCTION

1. The Regional Technical Consultation (RTC) on HABs in the ASEAN Region was held from 5-6 August 2015 in Singapore.

2. The meeting was opened by the Group Director for Technology and Industry Development (TIDG) of the Agri-Food and Veterinary Authority (AVA) Mr Foo Siang Ming, and attended by representatives from SEAFDEC member countries, Technical Coordinator from the SEAFDEC Secretariat Mr Tsuyoshi Iwata, Japanese experts Dr Yasuwo Fukuyo and Dr Hiroshi Oikawa, Chief of MFRD Programmes and Deputy Director (Regional Programmes) for the Planning and Management Department (PMD) of the Agri-Food and Veterinary Authority of Singapore (AVA) Mr Yeap Soon Eong, and Director for the Post-Harvest Technology Department (PHTD) of AVA Ms Khoo Gek Hoon. The meeting was also attended by other staff from PHTD of AVA. The list of participants appears at **Annex 1**.

II. AGENDA 1 AND 2 - OPENING OF THE MEETING AND OVERVIEW OF PROJECT COMPONENT ON HABs

3. Chief of MFRD Programmes Mr Yeap Soon Eong welcomed the participants to the RTC, which was held so that member countries could update each other on the status and information of HAB occurrences and incidences in the region, and to identify the

training needs for identification of toxic HAB species as well as finalizing the details for a regional training course in 2016. The meeting would also appoint Key Project Leaders for each member country and initiate a network or directory of HAB experts in the region.

4. Group Director of TIDG Mr Foo Siang Ming delivered the opening remarks to the participants of the meeting, on behalf of AVA.

5. MFRD JTF VI Project Manager Mr Liu Yankai, as Chairman of the RTC meeting, presented the agenda of the RTC for discussion by the meeting. The adopted agenda appears in **Annex 2**.

6. Mr Liu Yankai further presented an overview of the project component on biotoxin-producing HAB species.

7. Malaysia enquired about its involvement in project activities on implementation of biotoxins survey. Chief, MFRD Programmes responded that Malaysia was already involved in the biotoxins monitoring component of the project.

8. Dr Fukuyo enquired whether non-toxic HAB species would be covered under the scope of the project. The Chairperson responded that the enquiry would be deliberated in Agenda 5.

9. A round of self-introduction was conducted prior to taking a group photograph.

III. AGENDA 3 - COUNTRY PRESENTATIONS

10. Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam presented their respective country reports.

Brunei Darussalam

11. Dr Fukuyo noted that there were two common types of HABs (*Pyrodinium bahamense* and *Cochlodinium polykrikoides*) in Brunei and he commented that the management approach for each type of HAB needed to be different. Brunei responded that a similar approach for both types of HABs was used as the public was unaware of the difference in the species of HABs.

12. Dr Fukuyo highlighted that Paralytic Shellfish Poisoning (PSP) toxins can also be acquired through sources other than shellfish, such as through bioaccumulation by

herbivorous fish species. Dr Fukuyo suggested the inclusion of fish species in the biotoxin monitoring. Brunei responded that they have been monitoring biotoxins in fish species as well and the results were negative.

13. Malaysia informed the meeting that red tides in Brunei could have originated from Sabah. Malaysia also enquired on the impact of red tide in Brunei to the offshore region. Brunei said the red tides occurring in inland waters were mainly caused by *Cochlodinium polykrikoides*. Brunei did not rule out the possibility of other species of HABs as the cause, as Brunei did not have the capabilities to detect other species.

Cambodia

14. Dr Fukuyo enquired about the type of toxins that Cambodia wanted to detect in order to satisfy EU regulations. Cambodia responded that it would take into consideration Dr Fukuyo's comments before deciding on the type of toxins to detect.

Indonesia

15. Vietnam updated Indonesia on EU's maximum allowable limits for marine biotoxins in shellfish products.

16. Malaysia asked for more information on *Pyrodinium* causing death in Lampung. Dr Fukuyo clarified that the image shown in Indonesia's presentation might have been mislabeled and it should have been *Cochlodinium* instead.

17. In view of the amount of effort required for biotoxins monitoring, Dr Fukuyo suggested the importance for evaluating the priority of monitoring activity of the toxins, as not all species produced toxins.

18. Ms Khoo suggested that the HABs monitoring data should include the environmental conditions and water quality. Indonesia responded that conditions such as water temperature and fecal coliform in water have been measured.

19. Ms Khoo further enquired on the handling procedures of massive fish kills caused by HABs. Indonesia responded that the dead fish were either burnt or buried. Chief, MFRD Programmes invited other countries to share their government policy on the handling procedures for massive fish kills. Dr Fukuyo responded that Japan buried the dead fish and warned the public about the danger of the consumption of affected fish, even fish

that were near death. Vietnam said that its government would issue an order to bury the affected fish and warn the public.

Japan

20. Dr Hiroshi Oikawa gave a special presentation on how Japan handled its HAB occurrences and incidences.

21. Malaysia enquired if *Heterocapsa circularisquama* could kill oysters as Malaysia recently encountered a massive shellfish kills in the Semenanjung region. Dr Oikawa responded that *Heterocapsa circularisquama* was harmless to humans but could kill oysters. Malaysia further enquired if *Heterocapsa circularisquama* could be found in tropical waters. Dr Fukuyo responded that there was a high possibility of its tropical origin based on its high tolerance and growth under higher temperatures. He further explained how he had found a blooming of the species in Hong Kong prior to the occurrence in Japan.

22. Philippines and Thailand enquired about the reopening of closed shellfish aquaculture areas. Dr Oikawa responded that Japan monitored toxin levels in the areas by collecting weekly data for three weeks. If the toxins level did not exceed the allowable limits, the fishing area would be reopened. Dr Fukuyo added that there were exceptions made for some regions to allow earlier reopening of some areas based on scientific research data accumulated for a long time (more than 10 years). Malaysia shared its policy on the reopening of fishing areas affected by HABs in Sabah.

23. Vietnam asked why the monitoring period of toxic plankton blooms was between February and May. Dr Oikawa responded that the blooms occurred during that period.

24. Vietnam further enquired about the water sampling methodology and the sampling depth of toxic plankton. Dr Oikawa responded that the depth of sampling was between 5 and 10 m. Dr Fukuyo explained that there were two schools of thoughts for water sampling. The first involved sampling from all depths at 2 meter intervals, while the other involved sampling at depths near where shellfish grew. He added that the depth of sampling was dependent on the species of HABs as some would move towards sunlight and some changed their depth at different times of the day. Thailand shared that they learnt from the EU that water samples should be collected from different depths.

25. Dr Fukuyo enquired about the difference between caution and warning issued by the government and opined that government warnings should not be based on plankton concentration but should be based on toxin levels. Dr Oikawa explained that a caution was issued when a low level of plankton is detected. However, no further action would be taken. A warning would be issued when a higher level of plankton was detected. At the same time, the government would educate the public on the potential toxin. Dr Oikawa added that this form of management for the caution and warning levels applied only to the Osaka region.

Lao PDR

26. Dr Fukuyo commented that he intended to include freshwater HABs for the training course in order to take into account the fact that Lao PDR did not have coastal waters. Dr Fukuyo enquired if Lao PDR faced mortality caused by HABs in inland ponds. Lao PDR responded that mortality data was not available as Lao PDR did not have any authorities in charge of HAB.

27. Dr Fukuyo requested Lao PDR to check the incidences of livestock and/or people falling sick due to drinking freshwater affected by HABs. He highlighted that toxins in drinking water were gaining worldwide concern as there was increasing use of desalinated water which may be processed from HAB-contaminated seawater.

Malaysia

28. Dr Fukuyo enquired if Malaysia observed any harmful effects of *Notiluca scintillans*. Malaysia responded that there were no fish kills caused by *Notiluca scintillans*, only discolouration. Dr Fukuyo added that there was a possibility for *Notiluca scintillans* to cause mortality through oxygen depletion and increased ammonia concentration in the water. The ammonia is produced by *Noctiluca* for increasing buoyancy.

29. Dr Fukuyo enquired about the effectiveness of easy-to-understand leaflets on guidelines and precautions in public education. Malaysia responded that the leaflets might be helpful in raising awareness among the literate, but may not be effective for young children or the illiterate. Dr Fukuyo shared with the meeting a case study on how Japan conducted outreach to children on HABs.

30. Brunei asked if there had been 2 cases of HABs causing PSP toxins in Sabah in April 2015. Malaysia said it would check and respond to Brunei subsequently.

31. To Vietnam's enquiry about the detection of other species of HAB, Malaysia responded that as resources were limited, the main focus was on PSP. Dr Fukuyo added that it was impossible to test for all toxins caused by all HAB species and countries should focus on the highest priority species of HABs.

32. Thailand enquired about the time required to disseminate public warnings. Malaysia shared that with the availability of mobile phones, information dissemination was very fast because of instant messaging apps like WhatsApp.

Myanmar

33. Dr Fukuyo enquired if there had been any harm caused from a HAB event. Myanmar responded that due to a lack of monitoring, harmful HABs could not be determined. Myanmar was looking into the monitoring the effects of HABs.

34. Vietnam asked if samples were kept alive or frozen, saying that there were concerns from the EU that freezing might reduce the level of toxins in the sample. Myanmar said that as the sampling location was far from the testing laboratories, samples were frozen. Dr Fukuyo explained that the claim that freezing would somehow reduce the level of toxins in a sample was unverified, although he conceded that there could be further contamination of the sample by dripping from toxic to non-toxic parts during thawing. Vietnam added that the EU required live samples to be analysed within 24 hours. Myanmar suggested that Vietnam could refer to EU Regulation Number 1331.

Philippines

35. Dr Fukuyo asked whether there was a relationship between blooms of *Pyrodinium bahamanse* and shellfish transplantation in coastal waters. Philippines responded that the cause for country-wide expansion of toxic plankton was unknown. Philippines attempted to investigate this relationship by transplanting the infected shellfish seeds to a non-infected area, but HABs did not occur as a result.

36. Dr Fukuyo felt that the international standard of 80µg/100g for PSP toxicity levels was already stringent, and asked Philippines for the basis of setting an even more stringent standard of 60µg/100g. Philippines explained that PSP toxins had been controlled with a standard of 40µg/100g, which was subsequently raised to 60µg/100g.

Singapore

37. Mr Iwata enquired about the type of shellfish used in Singapore for the monitoring of biotoxins. Singapore responded that green mussels were used for biotoxins monitoring as these were farmed in Singapore.

38. Malaysia enquired if Singapore conducted monitoring of *Karlodinium veneficum* since February 2015. Singapore responded that the monitoring was on-going.

Thailand

39. To the queries from Vietnam, Philippines and Japan, Thailand responded that sampling was conducted every day once phytoplankton levels exceeded the maximum limit. Sampling frequency would be reduced once a noticeable downward trend was observed.

40. Dr Fukuyo said that the Department of Marine and Coastal Resources (DMCR) of Thailand was interested in conducting training courses on HABs. He suggested that the Department of Fisheries could work with DMCR on the training.

41. Malaysia suggested to Thailand to consider cooperation amongst research institutes and government agencies to deal with HAB issues.

Vietnam

42. Dr Fukuyo informed Vietnam that advance methodology was available for on-site real-time monitoring. However, monitoring was species-specific and dependent on the DNA molecular probes that were available, which made the cost of this method very high. He recommended focusing on the detection of toxins, rather than specific HAB species.

43. In response to Malaysia's query on the reason for the adoption of EU guidelines for HAB, Vietnam said it was for the export to EU. Dr Fukuyo suggested that Vietnam could monitor HAB conditions in its waters and set up its own standards instead of adopting the EU standard.

IV. AGENDA 4 - PRESENTATIONS BY MR TSUYOSHI IWATA, TECHNICAL COORIDNATOR, SEAFDEC SECRETARIAT, AND DR YASUWO FUKUYO, EMERITUS PROFESSOR, THE UNIVERSITY OF TOKYO

44. Mr Iwata gave a presentation on the administrative system for monitoring and regulating toxins for bivalves in Japan.

45. Dr Fukuyo asked about financial assistance provided by the Japanese government to fishermen when fishing grounds were closed due to red tides. Mr Iwata responded that he was not aware of any assistance currently provided by the Japanese government, but he could check on this query.

46. Dr Fukuyo commented that the Japanese information dissemination system was effective as all fishermen worked under the cooperatives. The fish from the affected fishing area could easily be prevented from reaching the market by the control of fisheries cooperatives. He told the meeting that this system was quite unique to Japan as it was established based on traditional fishing communities which could not be found in other member countries.

47. Vietnam asked Mr Iwata about guidelines for water sampling. Mr Iwata explained that the sampling methodology was covered in the guidelines and the safety limits for the toxic levels were defined by the responsible organization.

48. Singapore enquired about the actions taken if there was a massive fish kill without the detection of toxins. Dr Oikawa responded that the local prefecture would issue cautions or warnings based on cell density. Dr Fukuyo added that in most cases of massive fish kills, toxins could not be detected from the water. Malaysia concurred with Dr Fukuyo's comment on using cell density. However, Malaysia added that the limit was dependent on species of HABs. Singapore commented that there was no correlation between cell density and toxin levels.

49. Philippines enquired about the management of human health and economic impact in Japan. Mr Iwata explained that the regulation existed to protect human health as it was of upmost importance to the Japanese government. On the other hand, the Japanese Government would also attempt to minimize the economic impact by closely monitoring the situation to quickly allow the lifting of the ban on affected fishing areas.

50. To Vietnam's request for the English version of the guidelines to be disseminated to the SEAFDEC member countries, Mr Iwata informed the meeting that the English version of the guidelines was not available at present.

51. Dr Fukuyo gave two presentations – one on the overview of HAB occurrences and situation in Southeast Asia and one on the techniques for identification of HAB species.

52. Vietnam enquired about the addition of preservatives to water samples. Dr Fukuyo explained that the addition of preservatives was not necessary in all cases as it might affect the physical structure of the algae. He advised to observe the effect of preservative on a small amount of the sample before applying to the entire sample.

53. Myanmar invited Dr Fukuyo to Myanmar to share his experience on HAB. Lao PDR also sought for training in the area of HABs due to lack of expertise in their country.

V. AGENDA 5 - DELIBERATION ON SCOPE AND IMPLEMENTATION OF THE PROJECT

54. Member countries were requested to provide the names of their respective Key Project Leader (KPL), briefly describe their capabilities to identify HABs and test for their toxins, and to highlight their training needs. The responses are consolidated in **Annex 3**.

55. The meeting endorsed the following details pertaining to the training course:

    I. Venue: AVA Veterinary Public Health Centre, 10 Perahu Road, Singapore 718837 (tbc)

    II. Date: Q2 2016 (tbc)

    III. Trainer: Dr Yasuwo Fukuyo

    IV. Duration: 5 days

56. In addition, the meeting also noted that MFRD Programmes was considering exploring the possibility of working with the Tropical Marine Science Institute of the National University of Singapore to jointly conduct this training course.

57. Chief, MFRD Programmes reiterated that the RTC aimed to identify the training needs of the region with regard to the identification of toxic HABs. Additional details pertaining to the training course would be worked out at a later stage.

58. Chief, MFRD Programmes highlighted that the training course would take into account the differing levels of knowledge on HABs among all the ASEAN-SEAFDEC member countries. He added that MFRD Programmes would continue to follow-up with Dr Fukuyo to design the training course in a way that would incorporate the needs of all member countries. Dr Fukuyo commented that he expected the participants of the training course to be familiar with this field, so that he need not go over the basics.

59. Lao PDR requested for an official letter requesting for the nomination of a Key Project Leader. Chief, MFRD Programmes said that MFRD Programmes could send such a letter.

60. Cambodia said that should SOPs on the detection of HABs be developed, draft SOPs should be circulated to member countries for comments. Chief, MFRD Programmes clarified that the development of SOPs was not within the scope of this project component, although some guidelines for the sampling and identification of HABs would be discussed.

VI. AGENDA 6 - FINAL REMARKS AND CONCLUSION OF THE MEETING

61. The Chairperson concluded the meeting and thanked all participants for their contributions.

62. 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 meeting and to the Government of Japan for making the meeting possible.

63. The meeting was held in the traditional spirit of SEAFDEC co-operation and cordiality.

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Agenda of Meeting

5 August 2015, Wednesday

0830 Registration

0900 Agenda 1: Opening of the Meeting

- Welcome remarks by Chief, MFRD Programmes
- Opening remarks by Group Director, Technology and Industry Development Group, AVA
- Adoption of meeting agenda

0915 Agenda 2: Overview of project component on identification of biotoxin-producing HAB species

0945 Group photography and coffee break

1015 Agenda 3: Country Reports

- Brunei Darussalam
- Cambodia
- Indonesia
- Japan – Presentation by Dr Hiroshi Oikawa, Senior Researcher, Harmful Algal Blooms Group, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency
    - Current situation of HAB occurrences and monitoring programmes in Japan

1215 Lunch

1330 Agenda 3 (continued): Country Reports

- Lao PDR
- Malaysia
- Myanmar
- Philippines

1530 Coffee break

1600 Agenda 3 (continued): Country Reports

- Singapore
- Thailand
- Vietnam

1730 End of Day 1

6 August 2015, Thursday

0900 Agenda 4: Presentation by Mr Tsuyoshi Iwata, Technical Coordinator, SEAFDEC Secretariat

- Administrative System for Monitoring & Regulation of Toxin in Bivalves (case in Japan)

0945 Agenda 4 (continued): Presentation by Dr Yasuwo Fukuyo, Project Professor, Graduate School of Agriculture Life Sciences, The University of Tokyo

- Overview of HAB occurrences and situation in Southeast Asia

1030 Coffee break

1100 Agenda 4 (continued): Presentation by Dr Yasuwo Fukuyo, Project Professor, Graduate School of Agriculture Life Sciences, The University of Tokyo

- Methodologies and techniques for identification of HAB species

1200 Lunch

1330 Agenda 5: Deliberation on scope and implementation of the project

1500 Coffee break

1530 Agenda 6: Final remarks and Conclusion of the Meeting

1630 End of Day 2

Scope and Implementation of Project

Summaries of discussion on the scope and implementation of project

Abbreviations used:

ASP: **A**mnesic **S**hellfish **P**oisoning
AZP – **A**zaspiracid **S**hellfish **P**oisoning
BTX- Brevetoxin
DSP: **D**iarrhetic **S**hellfish **P**oisoning
PSP: **P**aralytic **S**hellfish **P**oisoning
TBC- **T**o **B**e **C**onfirmed

Member Countries	Training Needs		KPL	Responsible Organisation	Other Remarks
	HAB species	Lab Facilities			
Brunei Darrusalam	Common toxic HAB	Light microscope only	TBC	Captured Fisheries Development Division, Department of Fisheries	• No expertise in the field of HAB. Hence, it was difficult to narrow down specific species for the training needs. • Interested to learn about the common toxic HAB in ASEAN that affect

					public health and socio-economy
Cambodia	Not identified.	Microscope for microalgae observation	Dr. Chea Tharith	Marine Fisheries Research and Development Institute, Fisheries Administration	• Interested in SOP for species identification
Indonesia	Toxic *Cochlodinium* spp, *Pyrodonium* spp	• Light microscope • ELISA • HPLC • LC-MS (lacks reference for toxin standards)	TBC	1. Director General of Aquaculture, Fish Quarantine and Inspection Agency	Required reference materials for HAB detection
Lao PDR	Common harmful HAB	Light microscope	Ms. Sisamouth Phengsakoun (TBC)	Namsouang Aquaculture Development Centre, Ministry of Agriculture and Forestry	• No basic knowledge on HAB. Requested for basic information for inland HAB during the training.
Malaysia	Species causing PSP, DSP, ASP, Ciguatera, fish death	• Mouse bioassay, HPLC, ELISA for PSP • 1 LCMS-MS for TDX analysis • Receptor binding assay (RBA) in Universiti Kebangsaan Malaysia	Ms. Yong Ai Hua (TBC)	Department of Fisheries Malaysia, Fisheries Biosecurity Division	• Some personnel have no basic knowledge on HAB. • Some laboratories have facilities to handle HAB • Lacked personnel to identify HAB species

		• Inverted microscope for plankton analysis (Fluorescence and Differential interference contrast) • Electron microscope from universities • UniMAS has flowcam to analyse shape of plankton			
Myanmar	*Alexandrium* spp., *Prorocentrum* spp.	• Microscope • ELISA kit for ASP, DSP, PSP • LCMS-MS	Dr. Su Myo Thwe	Department of Fisheries, Research and Development Division, Analytical Control Laboratories	
Philippines	Species causing ASP, DSP, BTX, AZP, fish death	• Fluorescence microscope • Light microscope	TBC	Marine Biotoxin Section, BFAR; Resource and Ecological Assessment Division, NFRDI	
Singapore	Species causing fish death	Ample facilities for identification of HAB species	TBC	Agri-Food and veterinary Authority of Singapore (AVA)	

Thailand	• Toxic phytoplankton identification • Risk assessment and management of HAB • Conditions encouraging phytoplankton to produce toxin	• Light microscope • Inverted microscope • Fluorescence microscope • Electron microscope from universities • HPLC • LCMS-MS	Ms Noppawan Muanmee	Marine Fisheries Research and Development Division, Department of Fisheries	
Vietnam	*Dinophysis* spp., *Prorocentrum* spp., *Alexandrium* spp., *Gymnodium* spp., *Presudo-nitzchia* spp., *Protoceratium* spp	• Microscopes • HPLC • LCMS-MS	Mr Nguyen Thanh Binh (TBC)	National Agro-Forestry-Fishery Quality Assurance Department Directorate of Fisheries	Additional training needs: - Water sampling methods for quantitative and qualitative analysis with different samplers. - Proficiency test programmes

ANNEX 4

Administrative Report for Regional Training Course on Identification of HAB species, 2016

JAPANESE TRUST FUND VI: CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA – BIOTOXINS (ASP, AZA and BTX) AND HARMFUL ALGAL BLOOMS (HABS) IN THE ASEAN REGION
REGIONAL TRAINING COURSE ON IDENTIFICATION OF HAB SPECIES IN ASEAN REGION
18 – 22 JULY 2016
SINGAPORE

I. INTRODUCTION AND OPENING CEREMONY

1. The Regional Training Course on Identification of HAB species in ASEAN Region was held from 18-22 July 2016 on St John's Island, Singapore.

2. The Training Course was conducted in collaboration with the IOC-WESTPAC (Tropical Marine Science Institute (TMSI)

3. At the Opening Ceremony of the Training Course on 18 July 2016, Prof Wong Sek Man, Director of TMSI and Mr Yeap Soon Eong, Chief of MFRD Programmes gave the Opening and Welcome Remarks respectively. In attendance were the course participants from ASEAN-SEAFDEC Member Countries, Japanese experts Dr Yasuwo Fukuyo, Dr Kazumi Wakita, Dr Mitsunori Iwataki and regional experts, Dr Lim Po Teen and Dr Lim Hong Chang from Malaysia, Dr Serena Teo Lay Ming, Deputy Director of TMSI, and Ms Alice Tan, Deputy Director, Product Innovation and Quality Section, Post-harvest Technology Department of Agri-Food and Veterinary Authority of Singapore (AVA). The list of participants, trainers and AVA staff appears as Annex 1, Annex 2 and Annex 3 respectively.

4. The MFRD JTF VI Project Manager, Mr Calvin Lee presented an overview of the project component on identification of HAB species, the project activities and schedule.

5. Ms Lim Lay Peng Research Assistant of TMSI presented the Training Program for the next 5 days followed by a safety briefing and tour of the TMSI facilities. The Training Program appears as Annex 4.

II. DAY 1: LECTURES AND COUNTRY PRESENTATIONS

6. The first lecture was given by Dr Fukuyo and it covered 2 types of HAB (red tide and toxic algae blooms) within the South East Asia region as well as Japan, the variety of toxic algae and their ecology, red tide blooming mechanism and its relation to environmental parameters, mass mortality mechanism, algal distribution expansion mechanism, Countermeasures and Management of HABs.

7. Following that, the Training Course then proceeded with the country presentations, namely Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam.

III. DAY 2: LECTURES AND LAB WORK

8. Day 2 started off with a lecture from Dr Lim Hong Chang, Senior Lecturer of Tunku Abdul Rahman University College. The topic covered was Taxonomy of Diatoms and consisted of basic morphologies and characteristics of the different species. The lecture then proceeded to highlight the mechanism of fish kill which was caused by some Diatoms, as well as to emphasize on the toxin producing species.

9. Following the lecture on Diatoms, 3 lab sessions were carried out. These lab sessions aimed to better equip the participants and introduce them to the field equipment and sampling techniques which would be used during the sampling trip the next day. In addition, the participants were briefed on proper use of a light microscope. The use of observation equipment, such as field microscopes, and proper sample preparation techniques were taught during the lab sessions. Prepared specimens of different HAB species were provided for the lab sessions.

10. A lecture on Taxonomy of Dinoflagellates was presented by Dr Lim Po Teen of Institute of Ocean and Earth Sciences, University of Malaya. The lecture covered the basic morphology of the Dinoflagellates as well as characteristics of the different species.

11. The last activity for the day was another lab session which was to further allow the participants to practise the techniques that they had learnt earlier on in the day as well as to identify some of the species from samples that they were given. In addition, the participants were briefed on the planned schedule for tomorrow as it involved the splitting of the participants into 3 different groups for the field trip.

IV. DAY 3: FIELD TRIP AND LAB WORK

12. The training program for Day 3 had to be delayed and changes made due to bad weather. As the field trip was scheduled to be conducted along the Straits of Johor the bad weather posed hazardous conditions.

13. While waiting for the weather to improve, the participants were briefed on and shown some more equipment that could be used for sampling as well as different equipment that could be used for the identification of the different HAB species.

14. Once the weather had cleared up, the participants were taken to the Republic of Singapore Yacht Club in 3 pre-determined groups for the field trip.

15. During the field trip, participants were first shown the proper techniques of sampling using plankton nets. 2 different sized nets where shown and benefits of either were explained. The participants were then tasked with collecting their own samples using the techniques taught under supervision of the TMSI staff. Other monitoring equipment was also shown to the participants and its uses explained to them.

16. Once done with sampling, the participants were brought back to TMSI and were shown the prepared samples which the TMSI staff had gathered during the field trip.

17. The final activity of the day was a lecture given by Dr Sandric Leong Chee Yew, Senior Research Fellow of TMSI. The topic was on Freshwater Harmful Algal Blooms which are of importance to member countries that have large bodies of freshwater such as Cambodia, Lao PDR and Thailand.

V. DAY 4: LECTURES AND LAB WORK

18. Day 4 started off with a lecture from Associate Professor of Asian Natural Environmental Science Center (ANESC), the University of Tokyo, Dr Mitsunori Iwataki. The topic covered was on the Taxonomy of Unarmoured Dinoflagellates and

Raphidophytes. Similar to the earlier taxonomy lectures, the lecture consisted of the basic morphology, as well as characteristics of them and the mechanism of fish kill.

19. Following the lecture, the participants had a lab session which covered the analysis and observation of the samples that they had gathered during the field trip the day before. During the lab session, the participants were required to prepare the samples in accordance to what was taught to them before. They were also required to enumerate the number of cells gathered through methods taught to them.

20. An additional presentation by Mr Valeriano Meneses Borja, Aquaculturist II of Resource and Ecological Assessment Division, National Fisheries Research and Development Institute, Bureau of Fisheries and Aquatic Resources in the Philippines, was given on the some of his findings during the course of his work. In the presentation he also mentioned the different techniques that are being used in the Philippines for the sampling and analysis of the HAB species and made mention of a "Practical Guide on Paralytic Shellfish Poisoning Monitoring in the Philippines". The Guide consists of information on the different types of microorganisms that cause paralytic shellfish poisoning as well as methods for collections and analysis of the samples.

21. Two more lab session followed in which the participants were required to identify any HAB species that they might have gathered during the sampling and to observe their movements.

22. Following the lab sessions, a discussion led by Dr Fukuyo was held to wrap up what had been learnt so far as well as to address any questions or comments that the participants had. Mr Saadon bin Kasmon of Malaysia mentioned that he would like to have more training courses if possible on areas such as the use of a fluorescent microscope.

23. The final activity of the day was a discussion session among the participants who were divided into 3 groups on issues that their countries were facing.

24. A short presentation was given by a representative of the groups and it consisted of examples of issues that the countries were facing, as well as needs or other measures to be taken.

25. Some of the issues highlighted were how to ensure food safety of seafood in general, how to ensure monitoring and identification skill of technical staff/government officials, need for standard operating procedure(SOP) for identifying HAB species and a poster of common HAB species in Southeast Asia.

VI. DAY 5: CLOSING PRESENTATION AND FINAL DISCUSSION

26. Dr Kazumi Wakita, Associate Professor of School of Marine Science and Technology, Tokai University, gave a presentation on "HAB issues, concerns and management: towards future steps." The presentation consisted of a summary of what was learnt over the past 4 days as well as issues that some of the countries highlighted during the discussion.

27. The presentation also covered suggestions for future steps for HAB management such as ideas on how to mitigate the impact of red tide occurrence. It also covered key conditions in determining success of policy implementation as well as posed a question to the participants on to think of what are they major plankton species that cause shellfish poisoning in their respective countries.

28. This was followed by a discussion on the HAB management as well as suggestions from the participants. Dr Iwataki mentioned that previously, many international workshops had been conducted by Dr Fukuyo and himself. But due to budget constraints, they are unable to hold one now. He also added that member countries who are planning to conduct training courses, can contact Dr Iwataki and Dr Fukuyo and they will discuss on how best to help the country.

29. Dr Fukuyo mentioned that an international conference by WESTPAC will be held in April 2017 where there will be a section on HAB. He also said that training courses should have a specific goal as the duration to cover everything on HAB would be too long and even a week might not be enough. Dr Fukuyo also offered to bring preserved specimens for training courses if needed. He also mentioned that member countries can go through SEAFDEC, WESTPAC or even directly contacting him for more info on HAB when an emergency occurs.

30. Dr Fukuyo also mentioned that he had requested WESTPAC to set up a website of photos of HAB species.

31. A question was raised on the best method to preserve a specimen and Dr Lim Hong Chang shared about a method to preserve diatom and dinoflagellates using Lugol's solution.

32. Dr Lim Hong Chang went on to suggest that a team consisting of 1 representative from each member country be formed to create the poster that was suggested earlier. He proposed that Mr Valeriano Meneses Borja of the Philippines take the lead as he has the most experience among all the participants.

33. Mr Borja agreed to form a poster development team to develop two posters, one on photosynthetic dinoflagellates and another on red tide. The team members agreed to try to send to him photos of dinoflagellates and red tide causing species. Mr Borja mentioned that the posters will be sent to experts for comments. Mr Borja also mentioned that he would be asking other experts/researchers to contribute photos to be featured in the posters. The titles of the posters will be decided later. The list of participants in the poster development team is in Annex 5.

34. There being no ther matters to discuss the Regional Training Course on Identification of HAB species in ASEAN Region was concluded with closing remarks by Mr Yeap Soon Eong. This was followed by a certificate presentation ceremony for the participants and tokens of appreciation for the trainers. A group photo was taken thereafter. A farewell dinner was also hosted by MFRD in the evening.

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Training Program

Day 1: 18 July 2016 (Mon)

10:00 – 10:30	Depart Marina South Pier (MSP) to St. John's Island (SJI)
10:30 – 11:15	Check-in to room / free time
11:15 – 12:15	Official welcome and opening address (Prof. Wong SM)

- Introduction to SJI Marine Laboratory (Dr Teo SLM)
- Welcome address from AVA (Mr Yeap SE)
- Introduction to project background (Mr Lee CW)
- Introduction to trainers and committee members (Ms Lim LP)
- Safety briefing + tour of the facilities (Ms Lim LP, Mr Lai QW)

12:15 – 13:00	Lunch
13:00 – 14:30	Lecture 1: Introduction to HABs Part 1 (Prof. Fukuyo Y)
14:30 – 15:00	Tea break
15:00 – 16:00	Lecture 2: Introduction to HABs Part 2 (Dr Wakita K)
16:00 – 17:45	Seminar: Presentation by Trainees (10 min per country)
18:00 – 18:30	Depart SJI (trainers)
18:00 – 20:00	Dinner
20:00	End of program for Day 1

Day 2: 19 July 2016 (Tue)

08:00 – 08:30	Depart MSP (trainers)
08:00 – 09:00	Breakfast
09:00 – 10:00	Lecture 3: Taxonomy 1-Diatoms (Dr Lim HC)
10:00 – 10:30	Lab 1: Introduction to sampling & field equipment (Mr Kok JWK, Ms Lim LP, Ms Lim VJW, Dr Leong SCY)

10:30 – 11:00	Tea break
11:00 – 12:00	Lab 2: Introduction to visualisation methods (Ms Lim LP, Dr Lim HC)
12:00 – 13:00	Lunch
13:00 – 15:00	Lab 3: Cell observation (Ms Lim LP, Prof. Lim PT)
15:00 – 15:30	Tea break
15:30 – 16:30	Lecture 4: Taxonomy 2-Dinoflagellates (Prof. Lim PT)
16:30 – 17:15	Lab 4: Sample observations / preparation and briefing for field trip
	(Mr Kok JWK, Prof. Iwataki M)
17:30 – 18:00	Depart SJI (trainers)
18:30 – 20:00	Dinner
20:00	Lab-time (optional)

Day 3: 20 July 2016 (Wed)

07:00 – 08:00	Breakfast
08:00 – 13:00	Sampling at RSYC (Gp 1)
	Depart SJI for MSP at 8:00
	Sampling 10:00-11:30
	Informal lunch + field microscope observation with trainers
	Depart MSP for SJI at 15:30
11:00	Arrive at RSYC (trainers)
	(Prof. Iwataki M, Prof. Lim PT, Dr Lim HC, Dr Wakita K, Dr Leong SCY)
11:00 – 18:00	Sampling at RSYC (Gp 2)
	Depart SJI for MSP at 11:00
	Sampling 12:30-14:00
	Informal lunch + field microscope observation with trainers
	Depart MSP for SJI at 17:30

11:00 – 18:00	Sampling at RSYC (Gp 3)
	Depart SJI for MSP at 11:00
	Informal discussion and lunch with trainers
	Sampling 15:00-16:30
	Depart MSP for SJI at 17:30
18:30 – 20:00	Dinner
20:00	End of program for Day 3

Day 4: 21 July 2016 (Thur)

08:00 – 08:30	Depart MSP (trainers)
08:00 – 09:00	Breakfast
09:00 – 10:00	Lecture 5: Taxonomy 3-Dinoflagellates + raphidophytes (Prof. Iwataki M)
10:00 – 11:00	Lab 5: Preparation, quantification & examination of field samples (Ms Wong LY, Prof. Iwataki M)
11:00 – 12:00	Discussion 1: HAB taxonomy (Prof. Iwataki M, Dr Lim HC)
12:00 – 13:00	Lunch
13:00 – 14:00	Lab 6: Examination of field specimens (Ms Lim LP, Prof. Lim PT)
14:00 – 15:00	Lab 7: Examination of specimens (Ms Lim VJW, Prof. Fukuyo)
15:00 – 15:30	Tea break
15:30 – 16:30	Discussion 2: HAB identification and sampling (Prof. Lim PT, Dr Leong SCY)
16:30 – 18:30	Lab 8: Examination of specimens (Mr Kok JWK, Dr Leong SCY)
17:30 – 18:00	Depart SJI (trainers)
18:30 – 20:00	Dinner
20:00	Phytoplankton videos (optional)

Day 5: 22 July 2016 (Fri)

08:00 – 08:30	Depart MSP (trainers)
08:00 – 08:45	Breakfast
08:45 – 10:00	Discussion 3a: HAB issues and concerns (Prof. Fukuyo Y and Dr Wakita K)
10:00 – 10:30	Tea break
10:30 – 12:30	Discussion 3b: HAB management (Prof. Fukuyo Y and Dr Wakita K)
12:30 – 14:00	Lunch and informal discussion
14:00 – 15:30	General Q&A with trainers (all trainers)
15:30 – 16:00	Wrap-up / photo session (Ms Wong LY)
16:00 – 16:45	**Tea break**
17:00 – 17:30	**Depart SJI / end**

Poster Development Group

No	Name	Country
1	Ms Hajah Zuliza Binti Haji Jolkifli	Brunei Darussalam
2	Mr Tit Phea Rak	Cambodia
3	Mr Aris Sasono	Indonesia
4	Mr Saddon Bin Kasmon	Malaysia
5	Ms Ae Myo Latt	Myanmar
6	Mr Valeriano Menese Borja	Philippines
7	Mr Mohammad Sairi Bin Amir	Singapore
8	Ms Noppawan Muanmee	Thailand
9	Ms Nguyen Vu Mai Anh	Vietnam

ANNEX 5

Administrative Report for Regional Training Course on Specimen Preservation and its Application in HAB Monitoring and Studies, 2017

JAPANESE TRUST FUND VI: CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA – BIOTOXINS (ASP, AZA and BTX) AND HARMFUL ALGAL BLOOMS (HABS) IN THE ASEAN REGION
REGIONAL TRAINING COURSE ON SPECIMEN PRESERVATION AND ITS APPLICATION IN HAB MONITORING AND STUDIES
10 – 13 JULY 2017
KELANTAN, MALAYSIA

I. INTRODUCTION AND OPENING CEREMONY

1. The Regional Training Course on Specimen Preservation and its Application in HAB Monitoring and Studies was held from 10-13 July 2017 in collaboration with the the Institute of Ocean & Earth Science (IOES), University of Malaya (UM) at its Bachok Marine Research Station (BMRS) in Kelantan, Malaysia.

2. At the Opening Ceremony of the Training Course on 10 July 2016, Assoc. Prof. Lim Po Teen, Head of BMRS, on behalf of Director of IOES, and Mr Ong Yihang, Senior Scientist and MFRD JTF VI Project Manager, on behalf of Mr Yeap Soon Eong, Chief of MFRD Programmes gave the Opening and Welcome Remarks respectively. In attendance were the 20 course participants from nine ASEAN Member Countries except Myanmar, Dr Leaw Chui Pin, Dr Hii Kieng Soon, 2 MFRD staff and the Japanese expert, Dr Mitsunori Iwataki. The list of participants, expert trainers and MFRD/PHTD staff appears as **Annex 1**, **Annex 2** and **Annex 3** respectively.

3. Assoc. Prof. Lim Po Teen presented the Training Program for the next 4 days followed by group photography. The Training Program appears as **Annex 4.**

II. DAY 1: LECTURES AND DEMONSTRATIONS

4. The first two lectures were delivered by Assoc. Prof. Lim Po Teen covering the introduction to HAB and the challenges in HABs monitoring. Following that, there was a demonstration of different tools and equipment used in collecting samples by Dr Leaw Chui Pin, Senior Research Fellow. The demonstration aimed to introduce them to the field equipment and sampling techniques. A lecture on methods in qualitative and quantitative phytoplankton analyses was done by Assoc. Prof. Lim Po Teen before the day end.

Welcome Dinner

5. A welcome dinner was hosted by Mr Ong Yihang, on behalf of Chief MFRD Programmes, at Restoran Sri ChengMai Sdn Bhd. All the participants and trainers for the training course were present at the welcome dinner.

III. DAY 2: LECTURES AND LAB WORK

6. Day 2 started off with a lecture from Dr Mitsunori Iwataki on Microscopic Observation of Harmful Algae covering the 4 different types of microscopy and the characteristic observations that can be made under the different microscopy.

7. Following the lecture, the participants were briefed on proper use of a light microscope. The use of observation equipment, such as field microscopes, and proper sample preparation techniques were taught during the lab sessions. Prepared specimens of different HAB species were provided for the lab sessions. They were allowed to do hands-on the light microscope to observe the phytoplankton. Participants were also asked to draw their observation on a worksheet. The laboratory session aimed to better equip the participants with microscopy observation techniques which would be used for species identification.

8. A lecture on Counting Phytoplankton Cells was presented by Dr Leaw Chui Pin. The lecture covered the different techniques available for counting the phytoplankton cells. This was followed by a practical session on microscopy based cells counting where all the participants were given the chance to practise the techniques that they had learnt in the lecture.

9. The participants were also given a tour of the facilities during the break time.

IV. DAY 3: LECTURES AND DEMONSTRATIONS

10. The day began with a lecture on species identification by epi-fluorescene microscopy by Dr Hii Kieng Soon. This lecture provided greater depth of the principle of epi-fluorescene microscopy, components of an epi-fluorescene microscope, observation and sample preparation required. Demonstrations on epi-microscopy was conducted after the lecture to reinforce their learning through actual samples.

11. The focus on the second half of the day was on one of the molecular detection of HAB species - Flow Cytometry by Dr Leaw Chui Pin. The lecture described how a flow cytometry works and how to detect HAB species using flow cytometry. The aim of the lecture was to give participants insights on available technology to detect HAB species. Demonstrations on the use of the flow cytometry were conducted by the demonstrators.

VII. DAY 4: LECTURE, LAB WORK AND DISCUSSION

12. Dr Leaw Chui Pin delivered the last lecture on "Molecular Characterisation of HABs Species" The presentation covered on molecular diagnostic in HAB species recognition, molecular detection tools in HAB research, and genomic perspective and implications in HAB research. Following the lectures, the participants had a chance to practise hands-on doing single cell Polymerase Chain Reaction (PCR), Quantitative Polymerase Chain Reaction (qPCR) and gel electrophoresis.

13. There was a slight rearrangement in the programme due to the waiting time for the PCR to process; the discussion session was conducted during the PCR process to minimise the waiting time.

14. The discussion session kicked off with Dr Iwataki sharing a brief overview of the programme by WESTPAC for HAB. Participants were then encouraged to provide feedback on the training and propose the focus for the training next year. All participants agreed that the training was well organised and the trainers were very knowledgeable and helpful. The trainers recommended for member countries to send participants with relevant background in HAB as the training as it is an advanced training course.

15. The tentative period for the training course in 2018 would be in July. The original proposed topic was on HAB species culturing for species identification and toxin characterisation. Some participants suggested to conduct more in depth training on molecular detection techniques while others found the HAB species culturing for species identification and toxin characterization are important. In order to fulfil both request, it was further suggested to conduct the training in two separate parallel tracks whereby each member countries would have 1 participant joining each track. Mr Ong Yihang and Assoc. Prof. Lim Po Teen would further discuss the feasibility of conducting the course in this manner.

16. There being no other matters to discuss the Regional Training Course on Specimen Preservation and its Application in HAB monitoring and Studies was concluded with closing remarks by Dr Lim Po Teen. This was followed by a certificate presentation ceremony for the participants. A group photo was taken thereafter.

17. The group went back into the laboratory to make the observations of the results from the gel electrophoresis before departing from the training venue.

Trainers and Participants of the Training Course

No	Name	Designation	Country	Organisation	Office Address	Telephone No.	Fax No.	Email Address
1	Ms Hajah Zuliza Haji Jolkifli	Fisheries Officer / Head of Fisheries Ecology Section	Brunei Darussalam	Department of Fisheries	Department of Fisheries Muara Fisheries Complex, SPG 287-53 Jalan Peranginan Pantai Serasa Muara, BT1728 Brunei Darussalam	+ 673 2770066; +673 2770067	+673 2770065	zuliza.jolkifli@fisheries.gov.bn
2	Ms Nurul Zuraiedah HJ Ibrahim	Laboratory Assistant	Brunei Darussalam	Department of Fisheries	Department of Fisheries Muara Fisheries Complex, SPG 287-53 Jalan Peranginan Pantai Serasa Muara, BT1728 Brunei Darussalam	+ 673 2770066; +673 2770067	+673 2770065	zuraiedah.ibrahim@fisheries.gov.bn

3	Mr Ny Pisith	Officer of Department of Fisheries Post-Harvest Technologies and Quality Control	Cambodia	Post-Harvest Technologies and Quality Control	186 Preach Norodom Blvd, Sangkat Tonle Bassac, Khan Chamcar Mon, Phnom Penh, Cambodia	+855 968 55 55 21		pisethny88@yahoo.com
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5	Mr Ngurah Nyoman Wiadnyana	Senior Researcher	Indonesia	Research Center for Fisheries	Gedung Balitbang KP 2 Lantai 2, Jl. Pasir Putih II, Ancol Timur Jakarta	+62 21 64700928; +62 81 121106119		ngurahwiadnyana14@gmail.com
6	Mr Didik Wahju Hendro Tjahjo	Senior Scientist	Indonesia	Research Institute for Fisheries Enhancement	Jl. Cilalawi no. 1, Purwakarta, West Java	+62 8128403545		didikwht@yahoo.com
7	Ms Manichit Lathichak	Unit Head of Aquaculture Development Centre, DLF	Lao PDR	Department of Livestock and Fisheries (DLF)	Khounta Village Sikhodtabong district, Vientiane, Lao PDR District	+856 21 215243	+856 21 215141	manichteep@gmail.com dalasean@hotmail.com

8	Ms Monenaly Keokhamdy	Deputy Chief of Fisheries Inspector Section, Division of Fisheries, DLF	Lao PDR	Department of Livestock and Fisheries (DLF)	Khounta Village Sikhodtabong district, Vientiane, Lao PDR	+856 21 217869	+856 21 217869	monenaly@outlook.com
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10	Mr Saadon bin Kasmon	Assistant Research Officer	Malaysia	Kuala Lumpur Fisheries Biosecurity Centre	Kuala Lumpur Fisheries Biosecurity Centre Lot 82, Jalan Caruthers, off Jalan Sultan Salahuddin 50480 Kuala Lumpur, Malaysia	+60 13 337 5321	+60 3 2202 8856	saadon@dof.gov.my donikan1963@yahoo.com.my
11	Mr Azlan bin Salleh	Assistant Science Officer	Malaysia	Kuantan Fisheries Biosecurity Centre	Lot 20755 Jalan Tanah Putih 25150 Kuantan, Pahang	+60 9 516 4460	+60 9 516 4452	alan_art1286@yahoo.com
12	Ms Elsa Fugen Furio	Supervising Aquaculturist	Philippines	National Fisheries Research and Development Institute	National Fisheries Research and Development Institute 101 Corporate	+63 2 376 1178 +63 926 027 2862	+63 918 416 4215	efurio2010@yahoo.com; efurio2010@gmail.com

					Bldg., Mother Ignacia Avenue Barangay South Triangle, Quezon City 1103, Philippines			
13	Ms Norvida Cruz Gatdula	Aquaculturist I	Philippines	National Fisheries Research and Development Institute	National Fisheries Research and Development Institute, 101 Corporate Bldg., Mo. Ignacia Ave., Brgy. So. Triangle, Quezon City, Philippines	+63 918 322 4119	+63 915 482 2420	norviecruzgatdula@gmail.com
14	Ms Leyau Yu Lee	Senior Scientist	Singapore	Agri-Food & Veterinary Authority of Singapore	Veterinary Public Health Centre, 10 Perahu Road, Singapore 718837	+65 6795 2816	+65 6861 9491	LEYAU_Yu_Lee@ava.gov.sg
15	Mr Mohammad Sairi Bin Amir	Scientist	Singapore	Agri-Food & Veterinary Authority of Singapore	Sembawang Research Station, Lorong Chencharu, Singapore 769194	+65 6751 9838	+65 6752 3242	mohammad_sairi_amir@ava.gov.sg
16	Seow Hui Ching	Scientist	Singapore	Agri-Food & Veterinary Authority of Singapore	Sembawang Research Station, Lorong Chencharu, Singapore 769194	+65 6751 9850	+65 6752 3242	seow_hui_ching@ava.gov.sg

17	Ms Noppawan Muanmee	Fishery Biologist, Practitioner Level	Thailand	Fishing Ground Inspection and Certification Group	Fishing Ground Inspection and Certification Group, Upper Gulf Fisheries Research and Development Center (Samut Prakan), 49, Pharatchawiriyaphorn 16 Alley, Bang Phueng Sub-district, Phra Pradaeng District, Samut Prakan Province 10130, Thailand	+66 2 462 6988	+66 2 462 6988	m.noppawan55@gmail.com
18	Mr Pongsatorn Arkarakittikul	Fishery Biologist, Practitioner Level	Thailand	Eastern Gulf Fisheries Research and Development Center (Rayong)	Eastern Gulf Fisheries Research and Development Center (Rayong) 2, Village no.2, Phe Sub-district, Muang District, Rayong Province 21160, Thailand	+66 3 865 1764	+66 3 865 1763	knot_arkara@yahoo.com
19	Thai Thi Kim Thanh	Researcher	Vietnam	Research Institute for Marine Fisheries	2B/174 Phu Thuong Doan street,	+84 0934292762	+84 22531 3836812	thaithanhrimf@gmail.com

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21	Dr Lim Po Teen	Associate Professor/ Head of Station	Malaysia	Institute of Ocean and Earth Sciences	Institute of Ocean and Earth Sciences, Deputy Vice Chancellor(Research & Innovation), University of Malaya, 50603 Kuala Lumpur, MALAYSIA	+609 7785003 +6013 8181820	+609778 5006	poteenlim@gmail.com , ptlim@um.edu.my
22	Dr Chui Pin Leaw	Senior Research Fellow	Malaysia	Bachok Marine Research Station	Institute of Ocean and Earth Sciences University of Malaya 16310 Bachok, Kelantan, Malaysia	+609 778 5001 +6013 8181802	+609778 5006	cpleaw@um.edu.my chuipinleaw@gmail.com
23	Prof. Mitsunori Iwataki	Associate Professor	Japan	Asian Natural Environmental Science Center	1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan	+81-3-5841-8798		iwataki@anesc.u-tokyo.ac.jp

				(ANESC), the University of Tokyo				
24	Dr Hii Kieng Soon	Research Fellow	Malaysia	Bachok Marine Research Station	Institute of Ocean and Earth Sciences University of Malaya 16310 Bachok, Kelantan, Malaysia	+6016 8126110	+609778 5006	
25	Ong Yihang	Senior Scientist	Singapore	Agri-Food & Veterinary Authority of Singapore	Post-Harvest Technology Department, 2 Perahu Road, Singapore 718915	+65 6790 7973	+65 6861 3196	ong_yihang@ava.gov.sg
26	Chan HuiLi	Senior Technologist	Singapore	Agri-Food & Veterinary Authority of Singapore	Post-Harvest Technology Department, 2 Perahu Road, Singapore 718915	+65 6790 7973	+65 6861 3196	chan_huili@ava.gov.sg

Training Program

Day 1: 10 July 2017 (Mon)

07:00 – 8:00	Breakfast at hotel
08:00 – 9:00	Depart from hotel to BMRS
09:00 – 9:15	Participant Registration
09:15 – 9:30	Welcome Remarks • Welcome address by Chief, MFRD Programmes (Mr Ong YH) • Opening Remarks by Director, IOES, UM (Dr Lim PT)
09:30 – 10:30	Lecture 1: Introduction to Harmful Algal Bloom (HAB) (Dr Lim PT)
10:30 – 11:00	Tea break
11:00 – 12:00	Lecture 2: Monitoring program in HAB (Dr Lim PT)
12:00 – 13:00	Lunch
13:00 – 14:00	Practical: Exposure to systematic sampling techniques in HAB monitoring • Handling sampling devices (Dr Lim PT, Dr Leaw CP, Dr Hii KS)
14:00 – 15:00	Short Lecture 1: Methods in quantitative and qualitative phytoplankton analysis (Dr Leaw CP) Practical: Sample handling and preparation • Type of preservatives, • Preparation of preservatives, • Sample preservation
15:00 – 15:30	Tea break
15:30 – 17:30	Practical: Sample handling and preparation (continued) (Dr Leaw CP) • Sample storage • Sample concentration
17:30 – 18:30	Depart from BMRS to Hotel
18:30 – 21:30	Welcome Dinner

Day 2: 11 July 2017 (Tue)

07:00 – 08:00	Breakfast at hotel
08:00 – 09:00	Depart from hotel to BMRS
09:00 – 09:30	Short Lecture 2: Microscopy-based techniques in HAB monitoring (Dr Leaw CP)
09:30 – 10:30	Practical: Identification of HAB species by LM (Dr Leaw CP, Dr Lim PT) • Observe and identify thecated HAB species: *Alexandrium Gonyoulax, Dinophysis* etc.
10:30 – 11:00	Tea break
11:00 – 12:00	Practical: Identification of HAB species by LM (continued) (Dr Leaw CP, Dr Lim PT) • Techniques in observing athcated HAB species • Observe and identify thecated HAB species: *Karlodinium, Karenia, Chattonella* etc.
12:00 – 13:00	Lunch
13:00 – 14:00	Short Lecture 3: The know-how in phytoplankton cell enumeration (Dr Lim PT)
14:00 – 15:00	Practical: Cell enumeration by counting chamber methods (Dr Leaw CP, Dr Lim PT) • Preparation of Sedgewick Rafter counting slides • Enumerating cells from field samples
15:00 – 1530	Tea break
15:30 – 17:30	Practical: Cell enumeration by counting chamber methods (continued) (Dr Leaw CP, Dr Lim PT) • Calculating results and data analysis • Method validation
17:30 – 18:30	Depart from BMRS to Hotel
18:30	End of Day 2

Day 3: 12 July 2017 (Wed)

07:00 – 08:00	Breakfast at hotel
08:00 – 09:00	Depart from hotel to BMRS
09:00 – 10:30	Practical: Species identification by epi-fluorescence microscopy (Dr Leaw CP, Dr Lim PT) • Sample preparation for fluorescent staining Short Lecture 4: How epi-fluorescence microscope works?
10:30 – 11:00	Tea break
11:00 – 12:00	Practical: Species identification by epi-fluorescence (continued) (Dr Leaw CP, Dr Lim PT) • Sample observation by fluorescence microscope
12:00 – 13:00	Lunch
13:00 – 14:00	Lecture 3: Introduction to molecular-based techniques in HAB monitoring (Dr Leaw CP)
14:00 – 15:00	Practical: Flow cytometry (Dr Leaw CP, Dr Lim PT) • How it works? Principle and practical considerations • Demonstration on flow cytometry application
15:00 – 15:30	Tea break
15:30 – 17:30	Practical: Flow cytometry (continued)
17:30 – 18:30	Depart from BMRS to Hotel
18:30	End of Day 3

Day 4: 13 July 2017 (Thur)

07:00 – 08:00	Breakfast at hotel
08:00 – 09:00	Depart from hotel to BMRS
	Short Lecture 5: Single-cell PCR – how it works? (Dr Leaw CP)
09:00 – 10:30	Practical: Single-cell PCR (Dr Leaw CP)
	• Sample handling and preparation for molecular work • Technique in single-cell isolation – micropipetting technique
10:30 – 11:00	Tea break
11:00 – 12:00	Practical: Single-cell PCR (continued) (Dr Leaw CP)
	• Running PCR • Amplification conditions, primer choices
12:00 – 13:00	Lunch
13:00 – 14:00	Practical: Setting up qPCR assay (Dr Leaw CP)
	Short lecture 6: Quantitative real-time PCR detection of HAB species
	• qPCR chemistries • Optimization strategy and practical considerations
14:00 – 15:00	Practical: Running qPCR assay (Dr Leaw CP)
15:00 – 15:30	Tea break
15:30 – 17:00	Round up and discussion
17:00 – 17:30	Closing ceremony
	Presentation of certificates
17:30 – 18:30	Depart from BMRS to Hotel
18:30	End of Day 4

ANNEX 6

Administrative Report for Culturing for HAB Species Identification and Toxin Characterization, 2018

JAPANESE TRUST FUND VI: CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA – BIOTOXINS (ASP, AZA and BTX) AND HARMFUL ALGAL BLOOMS (HABS) IN THE ASEAN REGION
REGIONAL TRAINING COURSE ON SPECIMEN PRESERVATION AND ITS APPLICATION IN HAB MONITORING AND STUDIES
8 – 15 JULY 2018
KELANTAN, MALAYSIA

I. INTRODUCTION AND OPENING CEREMONY

1. The Regional Training Course on Culturing for HAB Species Identification and Toxin Characterization was held from 8-15 July 2018 in collaboration with the Institute of Ocean & Earth Science (IOES), University of Malaya (UM) at its Bachok Marine Research Station (BMRS) in Kelantan, Malaysia.

2. At the Opening Ceremony of the Training Course on 8 July 2018, Assoc. Prof. Lim Po Teen, Head of BMRS, on behalf of Director of IOES, and Mr Tay Siang Hong, Scientist and MFRD JTF VI Project Manager, on behalf of Mr Yeap Soon Eong, Chief of MFRD Programmes gave the Opening and Welcome Remarks respectively. In attendance were 20 course participants from 10 ASEAN Member Countries, Dr Leaw Chui Pin, Dr Hii Kieng Soon, Dr Lim Hong Chang, Prof Gu Haifeng, Dr Mitsunori Iwataki, 1 MFRD staff and postgraduate students from University of Malaya (Names of the participants, expert trainers and MFRD/PHTD staff are listed in **Annex 1, 2 & 3**, respectively).

3. A/Prof. Lim Po Teen presented the Training Program **(Annex 4)** for the next 7 days followed by a group photograph.

4. At the start of the course, each participant was given some training material and a sampling tool kit. They were also presented with a book titled “Marine Phytoplankton of the Western Pacific” the book was published by Westpac – HAB.

5. The first lecture was delivered by Associate Professor Lim Po Teen. The title of the lecture was regarding microalgae research specifically on Harmful Algal Bloom (HAB). After lunch, Dr Leaw Chui Pin, Senior Research Fellow introduced various principles on culturing of microalgae before the participants were grouped and taken for practical classes on,
   a) Preparation for culturing
   b) Cleaning glassware to culture requirement
   c) Sterilization Techniques

6. After tea break, the participants continued their practical classes on topics regarding preparation of culture media and were taught the types of media, nutrient stock preparation and sterilization and storage of the media.

7. By then, it was end of the day and the participants were transported back to the hotel for their welcome dinner which was hosted by Mr Tay Siang Hong, on behalf of Chief MFRD Programmes at Chao Yuan Restaurant Sdn Bhd.

II. DAY 2: LECTURES AND LAB WORK

8. Day 2 started off with a lecture from Prof. Gu Haifeng on identifying harmful species of microalgae. Following the lecture, the participants were briefed on proper techniques in culture establishment. The participants isolated cells from already prepared water samples.

9. After tea break, the participants practiced their cell isolating techniques and was further briefed on some techniques and procedures to culture microalgae.

10. After lunch, more lecture topics on culturing techniques were taught and the participants were taught to scale up and maintain their culture in optimum conditions to carry out their research. Activities for the second half of the day focused mainly on building participants' competency in media preparation and culture maintenance.

11. Before the day ended, Dr Teng, introduced to the participants some identification technique using a software called Image J. The software allowed the participants to measure the length of the microalgae and comparing the data with a database to quickly identify the unknown species. The method was quick and effective in identifying the specie of microalgae.

III. DAY 3: LECTURES AND LAB WORK

12. The day began with a lecture on species identification of harmful species of microalgae by Dr Iwataki. The specie of focus was dinoflagellates. Participants were also taught staining techniques to view the thecal plates of the cell to identify dinoflagellates.

13. Lab practical on measuring culture density followed and the participants learnt to prepare the samples for enumeration under the light microscope. The process was very tedious.

14. After lunch, Dr Leaw Chui Pin introduced some culture study tools such as flow cytometry. The focus on the second half of the day was on one of the molecular detection of HAB species - Flow Cytometry by Dr Leaw Chui Pin. The lecture described how a flow cytometry works and how to detect HAB species using flow cytometry. The aim of the lecture was to give participants insights on available technology to detect HAB species. Demonstrations on the use of the flow cytometry were conducted by the Dr Leaw.

IV. DAY 4: LECTURE AND LAB WORK

15. The day began with a lecture on molecular rapid detection of HAB species and toxins and its application. During this lecture, techniques on DNA isolation were introduced to the participants.

16. After which, the participants proceeded to the laboratories to practice isolating DNA from pre-prepared samples. This step was a prelude to gene amplification using a technique known as polymerase chain reaction (PCR). As the process of very tedious, participants spend the whole day performing the tasks.

V. DAY 5: LECTURE AND LAB WORK

17. Dr Teng started the day with a lecture on principles of Polymerase Chain Reaction (PCR). He explained the practical applications of PCR and various considerations to make when selecting primers for targeted DNA amplification.

18. After which the participants went to retrieve their amplified DNA samples and also learnt to prepare the agarose gel for gel electrophoresis. Once the gel had been prepared, the participants injected the gels with their sample and allow the DNA to be separated. Gel electrophoresis is a technique to determine the quality of the amplified DNA.

Separation of the prepared DNA into various bands indicated that the DNA has been successfully spliced.

19. After lunch, the participants transferred their samples and prepared them for gene amplification by PCR. At the end of the day, the samples were placed in the PCR machine and the DNA was allowed to replicate for a night before analysis the next day.

VI. DAY 6: LECTURES AND LAB WORK

20. Dr Hii started the morning by giving a lecture on Quantitative PCR (qPCR). Quantitative PCR also known as Real Time PCR, monitors the targeted DNA amplification as it is happening unlike conventional PCR which monitors it at the end. The participants also learnt how to construct a calibration curve and to determine the relative amount of A-T and C-G base pairs using a melting curve analysis.

21. After lunch, Assoc Prof. Lim Po Teen gave a lecture on Paralytic Shellfish Toxins (PSTs) and how to detect them.

22. During the practical, the participants were introduced to various techniques to extract PSTs for analysis using an Enzyme Linked Immunosorbent Assay (ELISA) kit for toxin detection.

VII. DAY 7: LECTURES AND FINAL REMARKS

23. The final day of the course started with a lecture by Dr Lim and Dr Teng. They shared with the participants on DNA barcoding of HAB species and basic phylogenetic analysis.

24. To perform this analysis, DNA sequencing had to be performed first, after which the sequence can be searched on a database using Basic Local Alignment Search Tool (BLAST). Finally, a phylogenetic tree was created to show the similarities of the gene sequence with various species of microalgae. The closest match would be the identity of the unknown sequence.

25. Due to cancellation of the morning flight out of Kota Bahru on 15 July 2018 by Malaysian Airlines, the flight of 2 Vietnamese participants & 2 Laotian participants were brought forward to the evening of Day 7 (14 July 2018) at 1800 hrs

26. Dr Lim Po Teen and Mr Ong Yihang gave the closing remarks to mark the end of the training course. And the participants and trainers were all given a certificate of participation, respectively.

27. During his closing remarks, Mr Ong Yihang mentioned for the participants to remind their colleagues back in their home country to send their bio-toxin monitoring report to MFRD so MFRD can compile and publish a technical report. He also informed them that there would be an End of Project meeting next year.

28. After lunch, the trainers organized for a mini excursion to one of the beaches of Kota Bahru for the participants to take some photographs and enjoy their final day of the course.

Follow-up

	Follow-up	Timeline	Action By	Assessment by Group Director
a	To explore how molecular methods like qPCR can be incorporated and used during field sampling in order to reduce time required for cell identification and enumeration. This can be tried out during actual monitoring works and high risk period.	2018 -2020	Md Sairi bin Amir	
b	To familiarise with cell preservation and how to culture the toxic species in order to obtain its toxins for detection. Hence to procure the necessary chemicals and apparatus for the cell preservation and culture work.	2019 – 2020	Ms Leyau Yu Lee	
c	To test out the new PSP kit, a lateral flow type that is currently in development stage when is ready to be available commercially.	TBC	Ms Leyau Yu Lee	
d	To develop training materials e.g. brochures on Harmful Algae commonly found in the ASEAN region.	2018 - 2019	MFRD	
e	To collate bio toxin monitoring reports from various ASEAN countries and produce a Technical Publication	2018 - 2019	MFRD	

Trainers and Participants of the Training Course

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Training Program

Day 1: 8 Jul 2018 (Sun)

Workshop session 1: Techniques in microalgae culturing and maintenance

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 0930	Participant Registration
0930 - 1000	***Welcome Remarks:*** • Opening Remark by the Chief, MFRD Programmes o Delivered by Tay Siang Hong on behalf of Chief, MFRD Programmes • Opening Remark by the Director, IOES, UM • Briefing of project by Tay Siang Hong, Project Leader
1030 – 1100	***Tea break + photo session***
1100 – 1200	***Specific Lecture 1:*** Overview of microalgae research with emphasis on Harmful Algal Bloom (HAB) – Dr. Po Teen Lim
1200 - 1300	***Lunch break***
1300 - 1400	***Lecture 1:*** ***Introduction to culturing of microalgae: principles and fundamentals***
1400 - 1600	***Lab Practical:*** *Exposure to basic techniques in microalgae culturing* • Preparation for culturing • Cleaning glassware to culture requirement • Sterilization techniques
1600 – 1630	***Tea break***

1630 - 1730 ***Lab Practical:***
Preparation of culture media
- types of media
- nutrient stock solution preparation
- sterilization and storage

Troubleshooting session:
Questions of microalgae culturing

1730 - 1830 Depart from BMRS to dinner venue
1830 - 2130 ***Welcoming Dinner***

End of Day 1

Day 2: 9 Jul 2018 (Mon)

0700 - 0800 Breakfast at hotel

0800 - 0900 Depart from hotel to BMRS

0900 - 1000 ***Specific Lecture 2:***
Identifying harmful species of microalgae - Dr. Haifeng Gu

1000 - 1045 ***Lab Practical:***
Techniques in culture establishment
- Obtaining cells from field samples
- Cell isolation method
- Sterile technique in working with lamina flow hood

1045 - 1100 ***Tea break***
1100 - 1130 ***Lecture 2:***
Techniques and procedures in culturing microalgae
1130 - 1200 ***Lab Practical:***
Techniques in culture establishment (continued)
- Cell isolation method

1200 - 1300 ***Lunch break***
1300 - 1600 ***Lecture 3:***
Culture maintenance and scaling up

Lab Practical:
Techniques in maintaining clonal cultures
- Sub-culturing – transfer technique, interval
- Handling and maintaining cultures – media choice, light, temperature

- Assessing culture condition
- Managing culture stocks

1600 – 1630	***Tea break***
1630 - 1800	***Troubleshooting session:*** • media preparation • algae culturing • algae maintenance Dis***cussion session***: Sharing of various culturing techniques by researchers
1800	Depart from BMRS to Hotel

End of Day 2

Day 3: 10 Jul 2018 (Tue)

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 1000	***Specific Lecture 3:*** Identifying harmful species of microalgae - Dr. Mitsunori Iwataki
1000 - 1100	***Lab Practical:*** *Measuring culture density and growth rate by microscopic techniques* • Cell enumeration • Data analysis
1100 - 1115	***Tea break***
1115 - 1200	***Lecture 4:*** ***Tools in culture studies: microscopy and flow cytometry***
1200 - 1300	***Lunch break***
1300 - 1600	***Lab Practical:*** *Measuring culture density and growth rate by microscopic techniques* • Flow cytometry demonstration
1600 – 1630	***Tea break***

1630 - 1800	***Lab Practical:*** *Measuring culture density and growth rate by microscopic techniques (continued)* • data analysis on cell density and growth rate estimation ***Troubleshooting session:*** Data analysis on cell density and growth rate estimation
1800	Depart from BMRS to Hotel

End of Day 3

Day 4: 11 Jul 2018 (Wed)

Workshop session 2: Techniques in molecular characterization and detection of HAB species and toxins

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 1000	***Lecture 1:*** ***Overview of molecular rapid detection of HAB species and toxins and its application***
1000 - 1030	***Tea break***
1030 - 1200	***Lab Practical:*** *Exposure to basic molecular techniques and DNA isolation* • sample preparation • conventional DNA isolation
1200 - 1300	***Lunch break***
1300 - 1600	***Lab Practical:*** *Exposure to basic molecular techniques and DNA isolation (Continued)* • Commercial DNA isolation kit
1600 – 1630	***Tea break***
1630 - 1800	***Lab Practical:*** *Exposure to basic molecular techniques and DNA isolation (continued)* • other DNA isolation techniques
1800	Depart from BMRS to Hotel

End of Day 4

Day 5: 12 Jul 2018 (Wed)

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 0930	***Lecture 2:*** ***Principles of conventional polymerase chain reaction (PCR)***
0930 - 1030	***Lab Practical:*** *Performing polymerase chain reaction (PCR)* • practical considerations • setting up for PCR • amplification conditions, primer choices
1030 - 1100	***Tea break***
1100 - 1200	***Lab Practical:*** *Performing polymerase chain reaction (PCR) (continued)* • Running PCR
1200 - 1300	***Lunch break***
1300 - 1600	***Lecture 3:*** ***High specificity and sensitivity detection by qPCR*** ***Lab Practical:*** *Performing polymerase chain reaction (PCR) (continued)* • Running gel electrophoresis
1600 – 1630	***Tea break***
1630 - 1800	***Lab Practical:*** *Performing polymerase chain reaction (PCR) (continued)* • Result interpretation ***Troubleshooting session:*** • DNA isolation and PCR
1800	Depart from BMRS to Hotel

End of Day 5

Day 6: 13 Jul 2018 (Fri)

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 0930	***Lecture 4:*** ***DNA barcoding of HAB species***
0930 – 1000	***Lab Practical:*** *Running qPCR* • The instrument platform • Setting up of qPCR • qPCR chemistries
1000 - 1030	***Tea break***
1030 - 1200	***Lab Practical:*** *Running qPCR (continued)* • Optimization strategy and practical considerations
1200 - 1300	***Lunch break***
1300 - 1600	***Lab Practical:*** *qPCR data analysis* • Calibration curve construction • Melt curve analysis
1600 – 1630	***Tea break***
1630 - 1800	***Lab Practical:*** *qPCR data analysis* • Principle in qPCR quantification (MIQE Guideline) ***Troubleshooting session:*** • qPCR assay and the analysis
1800	Depart from BMRS to Hotel

End of Day 6

Day 7: 14 Jul 2018 (Sat)

0700 - 0800	Breakfast at hotel
0800 - 0900	Depart from hotel to BMRS
0900 - 1000	***Short lecture:***
	Interpretation of nucleotide sequences
	Lab Practical:
	Sequence data interpretation
	• perform sequence assembly
1000 – 1015	***Tea break***
1015 - 1200	***Lab Practical:***
	Sequence data interpretation (Continued)
	• perform sequence assembly • proofreading
1200 - 1300	***Lunch break***
1300 - 1600	***Lab Practical:***
	Sequence data interpretation (Continued)
	• BLAST search • Basic phylogenetic analysis
1600 – 1630	***Tea break***
1630 – 1800	***Round up and discussion***
	Closing ceremony
	Presentation of certificates
	Photo session
	Closing remark
1800	Depart from BMRS to Hotel

End of Day 7

Day 8: 15 Jul 2018 (Sun)

0645 - 1700	Departure of participants and airport transfer

End of Day 8

ANNEX 7

Administrative Report for End-of-Project Meeting, 2019

END-OF-PROJECT MEETING ON JAPANESE TRUST FUND VI CHEMICAL AND DRUG RESIDUES IN FISH AND FISH PRODUCTS IN SOUTHEAST ASIA –BIOTOXINS (ASP, AZA and BTX) AND HARMFUL ALGAL BLOOMS (HABS) IN THE ASEAN REGION
14 – 15 AUGUST 2019
SINGAPORE

I. INTRODUCTION

1. At the invitation of the SEAFDEC Marine Fisheries Research and Development (MFRD) programme, the End of Project (EOP) meeting for JTFVI: Chemical and Drug Residues in Fish and Fish Products in Southeast Asia – Biotoxins (ASP, AZA and BTX) and Harmful Algal Blooms (HABs) in the ASEAN Region was held from 14-15 August 2019 in Singapore.

2. The meeting was attended by representatives from SEAFDEC member countries, experts from Japan, Dr Yasuwo Fukuyo and Dr Mitsunori Iwataki; and from Singapore, Senior Research Fellow of the National University of Singapore (NUS) Tropical Marine Science Institute (TMSI) Dr Sandric Leong; Chief of MFRD Programmes and Director for the Standards Development and Promotion Department (SDPD) of Singapore Food Agency (SFA) Ms Khoo Gek Hoon; the SEAFDEC Alternate Council Director of Singapore and Director for Aquaculture Department, Urban Food Solutions Group (UFS) of SFA Mr Lim Huan Sein; Project Manager and Assistant Director for the Agri-Food & Innovation Department (AFID) of UFS Mr Ong Yihang; and other staff from SFA. The list of participants appears at **Annex 1**.

II. AGENDA 1 AND 2 - OPENING OF THE MEETING AND BACKGROUND AND INTRODUCTION OF JAPANESE TRUST FUND VI PROJECT

3. The Chief of MFRD Programmes, Ms Khoo Gek Hoon, welcomed the participants to the EOP meeting of the Japan Trust Fund (JTF) VI project, which is the extended phase of the JTF II project since 2009 that aims to develop the diagnostic and monitoring capabilities for major marine biotoxins and Harmful Algal Bloom in the Southeast Asia region. The EOP meeting would compile useful information derived from the project, in particular the results of the biotoxin survey conducted by SEAFDEC member countries into a technical publication for dissemination to benefit the policy makers, regulators, scientist and researchers in the fishery and aquaculture sectors.

4. Mr Lim Huan Sein, the Director for Aquaculture Department which is the collaboration center for the MFRD programmes with effect from 1 April 2019 following the formation of SFA, delivered the opening remarks to the participants of the meeting. He reiterated SFA's continual commitment of the collaborating center for MFRD programme to address the emerging challenges concerning seafood safety and quality in the regional fisheries and aquaculture sectors.

5. Mr Ong Yihang, Project Manager and Chairperson of the EOP meeting presented the agenda of the EOP **(Annex 2)** for discussion and the agenda was adopted.

6. The meeting was informed of the project overview, which included the project background, rationale, objectives, completed activities and outputs of the JTFII and JTFVI Projects and the remaining expected output for JTFVI project, by Mr Ong for the benefit of the participants of EOP meeting.

III. AGENDA 3 - COUNTRY REPORT PRESENTATIONS

7. Indonesia, Malaysia, Myanmar, Philippines, Singapore, Thailand and Viet Nam who participated in the biotoxin monitoring survey presented their country reports on biotoxin monitoring results, HAB occurrence and identification efforts and recommendations. Chief of MFRD Programmes encouraged the rest of the countries who opted out of the survey to provide inputs for the compilation of technical report.

Indonesia

8. Dr Fukuyo enquired on the plan for a single country-wide marine toxins monitoring system since Indonesia is utilizing their coastal areas not only for captured fishery but also shellfish production. Indonesia informed that it is currently monitoring 2 locations and would be adding 2 more next year onwards. Indonesia would consult their government regarding funding for setting up of additional locations for the marine biotoxin monitoring.

9. To Dr Iwataki's query on the HAB species identified in Tanjung Balai Asahan, Indonesia would respond following the clarification from its research centre in Tanjung Balai Asahan.

Malaysia

10. Viet Nam asked if Malaysia used the same method for the analysis (e.g. TTX) throughout all the sampling points. Malaysia replied that the analysis methods used are not harmonised as the samplings are for R&D and not for monitoring purpose. Malaysia would harmonise the methods for analysis if there are multiple labs conducting monitoring activities.

11. To Malaysia's survey report on AZA detection, Dr Iwataki commented that the HAB species that causes AZA is limited in Southeast Asia. It would be good for Malaysia to identify the suspected HAB species that produced AZA as this information would help other countries in their biotoxin monitoring programme.

12. In addition, Dr Fukuyo shared with the meeting that the expert who is able to identify the HAB species is very limited in this region. Currently, a few experts like Dr Iwataki, Dr Lim Po Teen (Malaysia) and Dr Sandric should be consulted to further identify the HAB to species level and if needed.

13. Dr Iwataki enquired on the type of AZA that Malaysia detected in shellfish from Kuantan. Malaysia responded that the AZA-1 and AZA-3 were detected.

14. Chief of MFRD Programmes sought clarification on the "international standards for the biotoxins" specified in the survey report, and the season which the samples were collected for the biotoxin analysis. She requested SEAFDEC Member Countries to

reflect this information clearly in the survey reports e.g. the CODEX or EU standards for biotoxin.

Myanmar

15. Dr Fukuyo advised Myanmar to further identify and determine the quantitative level and effects of different HAB species before concluding the association with the biotoxin detected. He cited that not all red tides were caused by dinoflagellates, and the importance to validate the identified HAB species with experts.

Philippines

16. To Dr Fukuyo's queries, Philippines explained the challenge to conduct country-wide biotoxin monitoring programme due to many aquaculture establishments across their archipelago country. In the past, there was only one central laboratory in Manila for biotoxin testing and it was too slow in responding in time of HAB outbreak. At present, the regional laboratories have established the biotoxin testing capability to test samples at the regional level.

17. Philippines shared its difficulty in accreditation of biotoxin testing as well as certifying trained personnel for HAB identification. Dr Fukuyo commented that Japan had faced the same issues.

18. The meeting recognized that ISO 17025 is applicable to biotoxin analysis for accreditation of laboratory to reflect the competence of testing methodology and personnel but is not applicable to laboratory with HAB identification competency. Dr Fukuyo further added that each SEAFDEC member country needs to have more technician with such HAB identification skill in order to strengthen the HABs experts network in this region.

19. Malaysia queried if it is acceptable to conduct mouse bioassay as one of the biotoxin monitoring method. Dr Fukuyo shared that Japan has been using mouse bioassay technique for biotoxin monitoring purposes in the past due to the lack of alternative methods. Thus, it depends on the availability of alternative methods and situation in each country.

Singapore

20. To Singapore's recommendation to prepare toxin standard, Dr Fukuyo remarked that having standard toxins for biotoxin monitoring would be beneficial for the region. He recommended to prepare the toxin standard from contaminated shellfish. Singapore expressed interest on the collaboration in these areas as well as working towards obtaining such standards. Malaysia shared that it has started to culture *Alexandrium minutum* for PSP toxin. The mass culture and purification method are still being tested. Country members could communicate with Dr Azman from Malaysia if more information or collaboration on this biotoxin production is of interest.

Thailand

21. Malaysia queried on the best way to conduct sampling test for shellfish monitoring. Thailand shared that for their green mussel, they follow their sampling guideline to collect samples from top, middle and bottom of the culture line. Dr Fukuyo shared that the time of the day affects the concentration and distribution of phytoplankton which could also differ inside a fish farm, nearby to it or far away. The key step would be to determine what area is to be taken as a representative. There are also different methods to conduct analysis on the collected samples. in Japan the water taken from different depth were mixed up and analyzed. However, it depends on each countries' capabilities and resources to determine the sampling procedures.

22. To Thailand's recommendation to enhance its capability in biotoxin analysis and participate in biotoxin proficiency testing, Singapore expressed interest to offer such service as an ASEAN Food Reference Laboratory for Marine biotoxin and scombrotoxins. Singapore shared its intention to conduct an ASEAN-wide survey to gather more information from SEAFDEC member countries regarding type of biotoxins proficiency testing required.

Viet Nam

23. Dr Iwataki requested Viet Nam to share the specific information on correlated HAB species with the Lipophilic toxin and AZA-2 detection. To which, Viet Nam would analyze the raw data and reply to Dr Iwataki.

24. The three countries which did not participate in the biotoxin monitoring survey updated the meeting on their biotoxin monitoring programme/ plan.

Brunei Darussalam

25. Brunei Darussalam gave an introduction on their "National Red Tide Level Plan" and shared some of the challenges for this programme including commitment across different agencies, lack of training for officials and lack of predictive modelling programme.

26. In addition, Brunei Darussalam shared its future plans to include the biotoxin monitoring plan as part of their National Red Tide Level Plan.

Cambodia

27. Cambodia shared that while biotoxins occurrence in their country is not a major problem, they are looking to further develop and refine their fish quality control system to meet EU requirements. It would initiate a risk assessment and develop sampling plan for biotoxin in Oct 2019.

Lao PDR

28. Lao PDR made comments that they lack capability for HAB monitoring and biotoxin testing due to insufficient manpower resources and that they are a land-locked country. Lao PDR shared that the acquired knowledge from JTF II and VI on biotoxin and HAB are useful for them to put in a HAB monitoring plan to support the Chinese investment for freshwater aquaculture in their country.

IV. AGENDA 4 – SHARING BY EXPERTS

"HAB and Fish Kills" by Dr Sandric Leong, Senior Research Fellow of the National University of Singapore (NUS) Tropical Marine Science Institute (TMSI)

29. Dr Leong gave a presentation on the types of HAB species and related impact, and the HAB incidents in Singapore. He highlighted that increasing impacts of climate change and accompanying marine heatwave might result in more HAB incidents in the future.

30. To enhance biotoxin monitoring method, Dr Leong shared that TMSI is collaborating with the Pharmacy Department of NUS in developing and refining the molecular based technique of Karlotoxin detection.

31. Mr Sairi of SFA queried if there were any faster methods to process high-resolution data as significant time is required for processing which may not be practical for operational use. Dr Leong responded that the high-resolution detection was more for prediction of HAB events instead of operational use as it was more important to have a predictive modelling data.

"Identification of small harmful dinoflagellates which were recently detected from Southeast Asia and East Asia" by Dr Mitsunori Iwataki, Associate Professor of the Asian Natural Environmental Science Centre

32. Dr Iwataki gave an introduction on the common HAB species found in Asia and their unique morphological features which could aid in their identification. He highlighted that many of these HAB species are very small (<20micron). He pointed out that the detected species are much smaller than the previously identified harmful species and may not be identifiable under light microscopy.

33. Dr Fukuyo enquired on the technique to capture the cell morphology under microscope for such small species. Dr Iwataki shared that the photos were taken using a 100x lens with oil. In addition, he would offer his expertise to member countries who require his assistance in this aspect.

34. Dr Fukuyo questioned if there are any suggestions for a practical monitoring method if there are 2-3 species together in the sample. Dr Iwataki said that Fluorescent In-situ Hybridization (FISH) is a good method for species-specific identification.

"Case study on action after HAB species detection in Japan" by Dr Mitsunori Iwataki.

35. Dr Iwataki provided another presentation on the actions taken by Japan when faced with HAB incidents and its HAB monitoring programme.

36. Mr Sairi queried how Japan's monitoring for critical ("beware") levels of HAB could occur once a week upon detection as the bloom could occur within 1-2 days. Dr Iwataki explained that frequency of HAB monitoring depends on certain factors unique to the location.

37. Malaysia requested if Japan would be able to share their existing Standard Operation Procedure for HAB monitoring as they would like to make reference to. Dr Iwataki elaborated that the HAB numbers were decided based on experience as the actions varied differently for different prefectures. Brunei Darussalam shared their experience in handling red tide occurrence.

"Suggestion for precautionary monitoring system development against poisoning and fish mortality" by Dr Yasuwo Fukuyo, Guest Professor (Tokai Univ.) Emeritus Professor (Univ. of Tokyo)

38. Dr Fukuyo shared the challenges to consider in monitoring toxic plankton bloom versus red tide bloom and provided suggestions on how to monitor each type of bloom. He also highlighted that the condition for toxic plankton bloom differs greatly from red tide plankton bloom and hence monitoring conditions have to be accommodated accordingly.

39. Cambodia queried if they could develop models for prediction in the future for toxin monitoring from their sampling data. Dr Fukuyo remarked that Japan had attempted in doing a predictive model. He added that the acquisition of parameters is very costly and time consuming. Furthermore, the accuracy of the model is not very high, which is not worth the cost.

40. Philippines proposed to raise awareness of the public and engage fishermen to strengthen the biotoxin monitoring effort in this region.

V. AGENDA 5 – TECHNICAL COMPILATION – COMMENTS AND RECOMMENDATIONS

41. The meeting discussed and agreed on the proposed contents, summary and recommendations for the technical compilation.

VI. AGENDA 6 – ADOPTION OF ADMINSTRATIVE REPORT

42. The administrative report was presented for discussion and final amendments. The finalized administrative report would be adopted and incorporated into the Technical Compilation Report.

VII. AGENDA 7 - FINAL REMARKS AND CONCLUSION OF THE MEETING

43. Chief of MFRD Programmes delivered the closing remarks in which she thanked SEAFDEC member countries for their participation in this EOM and commitment in the JTFVI project. She remarked that it was heartening to see countries progressing in their development of HAB and biotoxin monitoring guidelines and expressed hopes that SEAFDEC Member Countries could continue to work together to build capacity and knowledge and strengthen the network in this area.

44. 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 meeting and to the Government of Japan for making the meeting possible.

45. The meeting was held in the traditional spirit of SEAFDEC co-operation and cordiality.

Participants of Meeting

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Agenda of Meeting

5 August 2015, Wednesday

0830 Registration

0900 Agenda 1: Opening of the Meeting

- Welcome remarks by Chief, MFRD Programmes
- Opening remarks by Group Director, Technology and Industry Development Group, AVA
- Adoption of meeting agenda

0915 Agenda 2: Overview of project component on identification of biotoxin-producing HAB species

0945 Group photography and coffee break

1015 Agenda 3: Country Reports

- Brunei Darussalam
- Cambodia
- Indonesia
- Japan – Presentation by Dr Hiroshi Oikawa, Senior Researcher, Harmful Algal Blooms Group, National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency
    - Current situation of HAB occurrences and monitoring programmes in Japan

1215 Lunch

1330 Agenda 3 (continued): Country Reports

- Lao PDR
- Malaysia
- Myanmar
- Philippines

1530 Coffee break

1600 Agenda 3 (continued): Country Reports

- Singapore
- Thailand
- Vietnam

1730 End of Day 1

6 August 2015, Thursday

0900 Agenda 4: Presentation by Mr Tsuyoshi Iwata, Technical Coordinator, SEAFDEC Secretariat

- Administrative System for Monitoring & Regulation of Toxin in Bivalves (case in Japan)

0945 Agenda 4 (continued): Presentation by Dr Yasuwo Fukuyo, Project Professor, Graduate School of Agriculture Life Sciences, The University of Tokyo

- Overview of HAB occurrences and situation in Southeast Asia

1030 Coffee break

1100 Agenda 4 (continued): Presentation by Dr Yasuwo Fukuyo, Project Professor, Graduate School of Agriculture Life Sciences, The University of Tokyo

- Methodologies and techniques for identification of HAB species

1200 Lunch

1330 Agenda 5: Deliberation on scope and implementation of the project

1500 Coffee break

1530 Agenda 6: Final remarks and Conclusion of the Meeting

1630 End of Day 2

ANNEX 8

Determination of domoic acid using High Performance Liquid Chromatography with Ultraviolet Detector (UV-HPLC) Method

1. Chemicals and lab wares:

   - Toxin standard: Domoic acid standard:
     - ✓ CRM-DA-f (code 200712050425): 0.5 mL of solution with 327 µM DA in acetonitrile/water (1:19, v/v) (a certified calibration solution for domoic acid).
     - ✓ 1 mg of freeze dried domoic acid/ampule: TOCRIS Bioscience, UK, code: 0269, batch No.: 25A/96987, Cat. No. 14277-97-5
     - ✓ 1 mg of freeze dried domoic acid/ampule: WAKO, CAS [14277-97-5], Cat. No. 536-51281, page 599 Wako Pure Chenical Industries, Ltd., 2012
   - Acetonitrile (HPLC grade)
   - Distilled water
   - Formic acid (99%)
   - HPLC column: Wakosil C18-II 2.0x150mm 3µm (WAKO, Japan): WAKO Cat. no. 239-01211 (page 1440 of WAKO 2012)
   - Blender
   - Pipette Pasteur
   - Millipore: Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane
   - Centrifuged tubes (15 mL)
   - Cylinder 100 mL, 1000 mL
   - Glass beaker 50 mL, 100 mL, 1000 mL
   - Micro-pipette 20 µL, 100 µL, 200 µL and 1000 µL and suitable tips
   - Injected serine for HPLC (max. 20 µL)

2. Preparation of solutions:

   - 50% MeOH: MeOH/distilled water (D.W.): 1/1 v:v: for 100 mL of MeOH: 50 mL MeOH + 50 mL of D.W., mixed well.

- Mobile Phase: Acetonitrile/ D.W./Formic acid (9:90.8:0.2): For 1000 mL:

  Acetonitrile (HPLC grade): 90 mL

  Distilled water: 908 mL

  Formic acid (99%): 2 mL

  Mix well, degas before using to HPLC (long last about 2-3 days).

- Toxin standard working solution:

  Dissolve 1 mg of DA.STD in 1 mL of D.W.: 1000 µg/mL (Sol. A)

  10 µL of Sol. A + 90 µL of D.W.: 100 µg/mL (Sol. B)

  10 µL of Sol. B + 90 µL of D.W.: 10 µg/mL (Sol. C)

  10 µL of Sol. C + 90 µL of D.W.: 1 µg/mL (Sol. D): Working solution of DA.STD.

  Note:

  - ✓ Keep all series solutions in -20 degree and dark condition (can be used for several months).
  - ✓ Amount of sol. D should be just enough for use in 1 week, then make the new one.

3. Extraction of Toxins

3.1 Shellfish samples

1.1.1 5 g of shellfish homogenate add 20 mL of 50% MeOH

1.1.2 Homogenize for 1 min

1.1.3 Centrifuge at 3,000 rpm for 5 min

1.1.4 Transfer to 500 µL of Supernatant for Ultrafree-MC cartridge (NMWL 10,000)

1.1.5 Centrifuge at 10,000 rpm for 15 min

1.1.6 Collect supernatant

1.1.7 Inject 5 µL of supernatant into UV-HPLC system.

3.2 Plankton samples

3.2.1 Planktons sample collected by plankton net.

3.2.2 Plankton cell harvest by centrifuge at 2,000 rpm for 15 min

3.2.3 Remove a supernatant carefully by pipette Pasteur

3.2.4 Weight cell pellets by electric balance, add equal volume of distilled water

3.2.5 Boil at 5 min and cool down until room temp.

3.2.6 Centrifuge at 10,000 rpm for 5 min

3.2.7 Transfer 500 µL Supernatant to Ultrafree-MC cartridge (NMWL 10,000)

3.2.8 Centrifuge at 10,000 rpm for 15 min

3.2.9 Collect supernatant

3.2.10 Inject 5 µL of supernatant into UV-HPLC system.

4. Chromatographic conditions

- ✓ Column: Wakosil C18-II 2.0x150mm 3µm (WAKO, Japan)
- ✓ Mobile phase: Acetonitrile/ D.W./Formic acid (9:90.8:0.2)
- ✓ Column temp: 30 ^{0}C
- ✓ Elution: Isocratic
- ✓ End time: 20 min
- ✓ Injection volume: 5 µL
- ✓ Flow: 0.2 mL/min
- ✓ Detection: UV detector (242nm)

5. Reference

Takata, Y., Sato, S., Dao, V.H., Montojo, U.M., Lirdwiayaprasit, T., Kamolsiripichaipon, S., Kotaki, Y., Fukuyo, Y., Kodama, M. 2009 Occurrence of domoic acid in tropical bivalves. Fisherires Science 75 473-480.

DACS-1D. 2012. NRC Certified Reference Materials Program. 1-8. NRC-CNRC Institute for Marine Biosciences.

ANNEX 9

Preparation of Domoic Acid Standard Solution and Homogenate fortified bivalve extract with Domoic Acid

1. Preparation of standard solution:

   Dilute stock solution (1 mg DA/ mL or 1000 µg/mL of D.W.) to following calibration solutions (dilute with MeOH):

   - ✓ 10 µg/mL: 100 times (1)
   - ✓ 20 µg/mL: 50 times (2)
   - ✓ 50 µg/mL: 25 times (3)
   - ✓ 100 µg/mL: 10 times (4)

2. Preparation of bivalve extracts fortified with DA:

2.1.1 To 1 g of homogenate, add 100 µL standard solution (1-4) and kept for 5 -10 min at room temperature. The contents of DA in the homogenate are 1.0, 2.0, 5.0, and 10 µg/g, respectively.

2.1.2 Add 4 ml of 50% MeOH.

2.1.2 Extract with a homogenizer for 3 minutes.

2.1.3 Centrifuge at 10,000 rpm for 5 minutes.

2.1.4 Pass an aliquot of supernatant through a Millipore.

2.1.5 Inject a 5 µL aliquot of the filtrate into the HPLC.

Note: The best way of calibration:

- ✓ Domoic acid calibration solutions: DACS-1C (certified 100 µg/mL), and accurate dilutions of DACS-1C in injection diluent to give 1.0, 2.5, 10.0, 25.0 µg/mL.
- ✓ Mussel tissue reference material: MUS-1B (36 µg/g).

ANNEX 10

Determination of Azaspiracids and Brevetoxins
Using Liquid Chromatography – Mass Spectrometry (LC-MS) Method

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 Condition the C18 Plus Sep-pak (Equilibrate with 5mL of MeOH, followed by 5mL of distilled water, 5mL 50mM HCOOH, 2mM$HCOONH_4$ solution (Mobile phase A))

1.2.5 To the sep-pak, add in the 50 mL of filtrate with added 2.5 mL 1M HCOOH, 40mM $HCOONH_4$ solution (Stock solution), followed by 10 ml of 50mM HCOOH, 2mM$HCOONH_4$ solution (Mobile phase A) and 5ml of MeOH.

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

1.2.7 Dissolve residues in 500 μL of MeOH.

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

2. Preparation of reagents

2.1 Preparation of mobile phases

2.1.1 Stock solution (1M HCOOH, 40mM $HCOONH_4$ 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 $HCOONH_4$ (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.

2.1.2 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 conditions

Column: Hypersil BDS-C8 (2.1 id x 50 mm) (Keystone Scientific)
Column temperature: 20°C
Gradient: 5% B to 100% B for 10 minutes
100% B for 5 minutes
Flow rate: 0.2ml/min

4. MS conditions (Sciex QTrap™ 3200 MS/MS system)

Ionization; ESI positive
Ionization Voltage;
Azaspiracids 5.5 kV
Brevetoxins 5.5 kV
Domoic acid 4.5 kV

Ionization Temperature;

Azaspiracids 600 °C

Brevetoxins 400 °C

Domoic acid 600 °C

Table 1. MS parameters

Toxins	Quantification (Q1/Q3)	Confirmation (Q1/Q3)	DP Voltage (V)	Quantification CE(V)	Confirmation CE(V)
Domoic acid	312/266	312/91	56	21	75
Brevetoxin-1	867/221	-	60	30	-
Brevetoxin-2	895/301	895/319	50	40	40
Brevetoxin-B2	1034/88	-	136	95	-
DeoxyBrevetoxin-B2	1018/186	1018/204	150	80	70
Brevetoxin-3	897/85	897/725	71	85	45
Brevetoxin-B5	911/299	911/317	50	50	50
Azaspiracid-1	842/362	-	81	73	-
Azaspiracid-2	856/362	-	81	73	-
Azaspiracid-3	828/362	-	81	73	-

DP; Declusterizaiton Energy

CE; Collision Energy

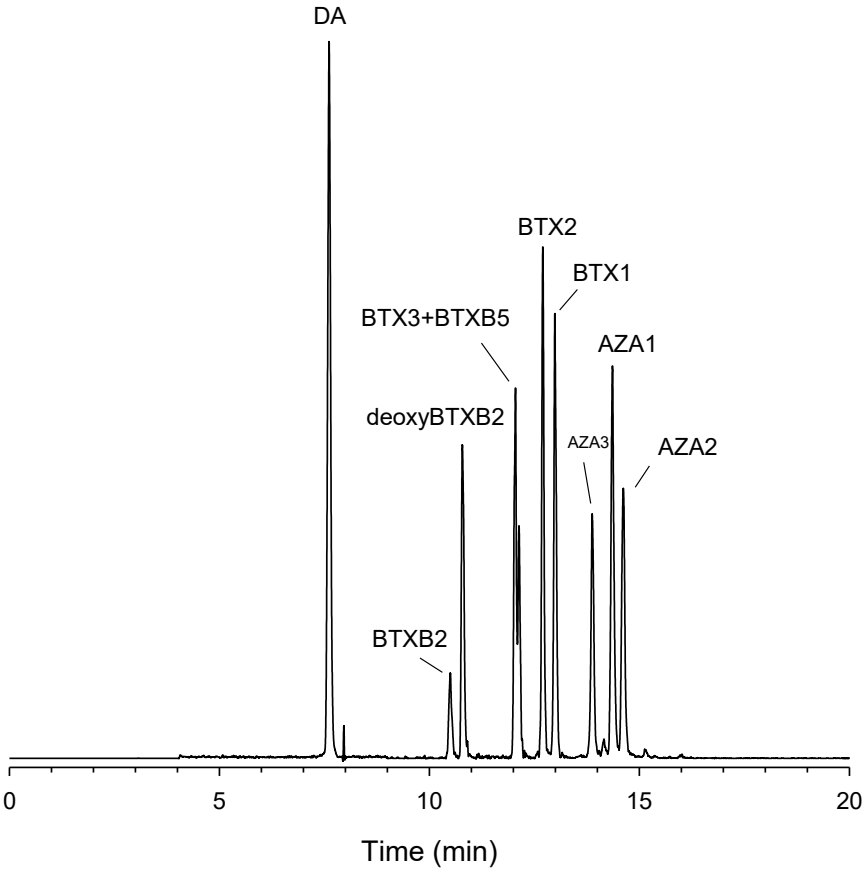
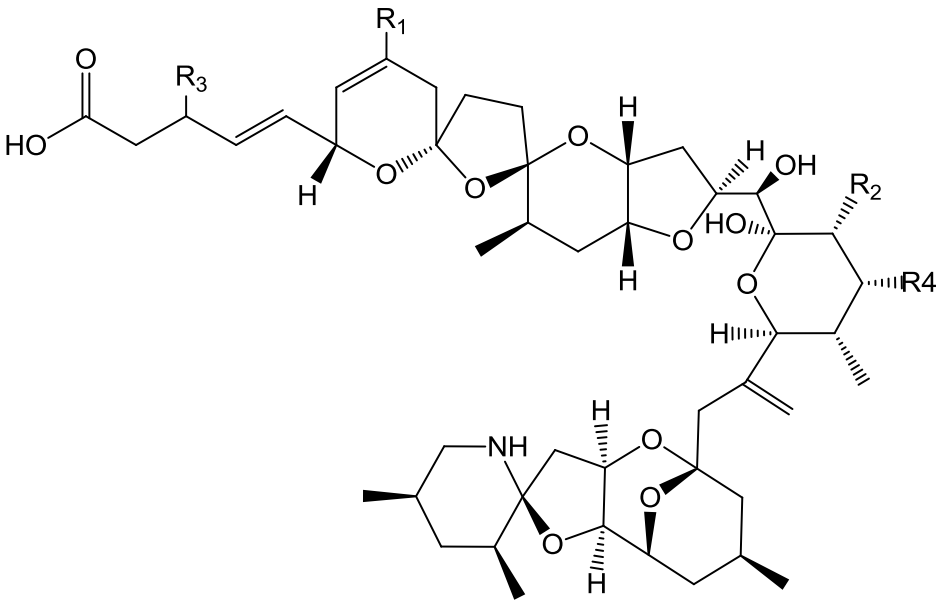


Fig. 1. LC-MS chromatogram

3. 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).



	R_1	R_2	R_3	R_4
AZA1	H	CH_3	H	H
AZA2	CH_3	CH_3	H	H
AZA3	H	H	H	H

Fig. 1 Structure of azaspiracid analogues

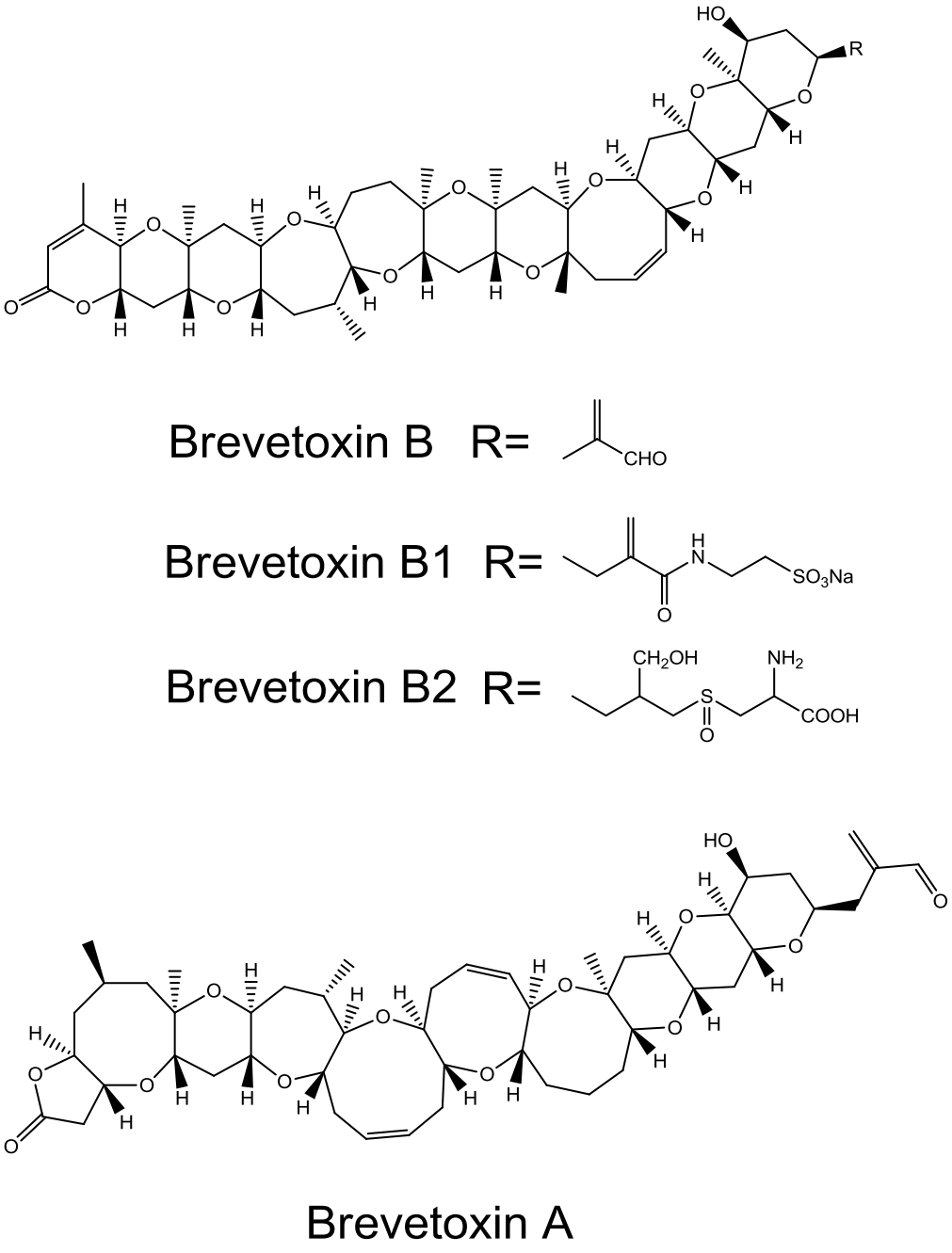
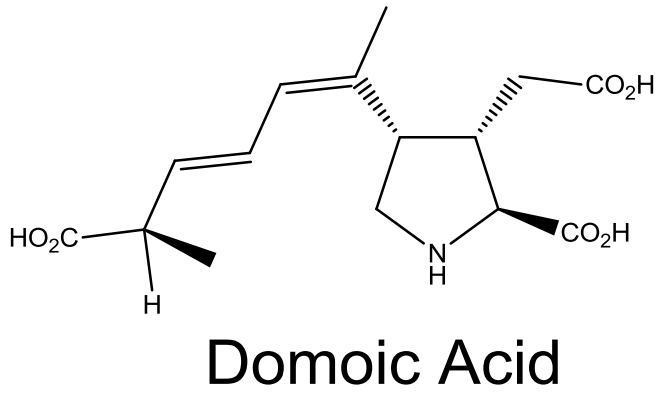


Fig. 2 Structure of brevetoxin analogues



Domoic Acid

Fig. 3 Structure of domoic acid

ANNEX 11

Preparation of Azaspiracids Standard Solution and Homogenate fortified with Azaspiracids

Preparation of standard solution

NRC CRM AZA1 (1240 ng/ml) (1)
NRC CRM AZA2 (1280 ng/ml) (2)
NRC CRM AZA3 (1040 ng/ml) (3)

50 uL of each (1) (2) and (3) are dissolved in 850 uL MeOH (4).
The concentration of AZA1,2,3 in the standard solution (4) are 62, 64 and 52 ng/mL, respectively.

The standard solution (4) are diluted to 1/50, 1/100, 1/200 and 1/400 with MeOH for calibration solution. The concentration (ng/ml) of each AZA are as follows:

	1/50	1/100	1/200	1/400
AZA1	1.24	0.62	0.31	0.155
AZA2	1.28	0.64	0.32	0.16
AZA3	1.04	0.52	0.26	0.13

2. Preparation of bivalve extracts fortified with AZA1,2,3

2.1.1 To 1 g of homogenate, add 50 µl standard solution (4) and kept for 1 minute at room temperature. The contents of AZA1,2,3 in the homogenate is 3.1, 3.2, and 2.6 ng/g, respectively.

2.1.2 Add 9 ml of MeOH/distilled water (9:1 v/v).

2.1.2 Extract with a homogenizer for 3 minutes.

2.1.3 Centrifuge at 3000 rpm for 5 minutes.

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

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

ANNEX 12

Information on Key Project Leader for Biotoxins Monitoring

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ANNEX 13

Information on National Reference Laboratory for Biotoxins Monitoring

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	Aquatic Toxicology Laboratory Section				marclawrence503@gmail.com	Mr. Marc Lawrence J. Romero
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ANNEX 14

Information on Key Project Leader for HABs Monitoring

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ANNEX 15

Information on National Reference Laboratory for HABs Monitoring

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	Aquatic Toxicology Laboratory Section				marclawrence503@gmail.com	Mr. Marc Lawrence J. Romero
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