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.

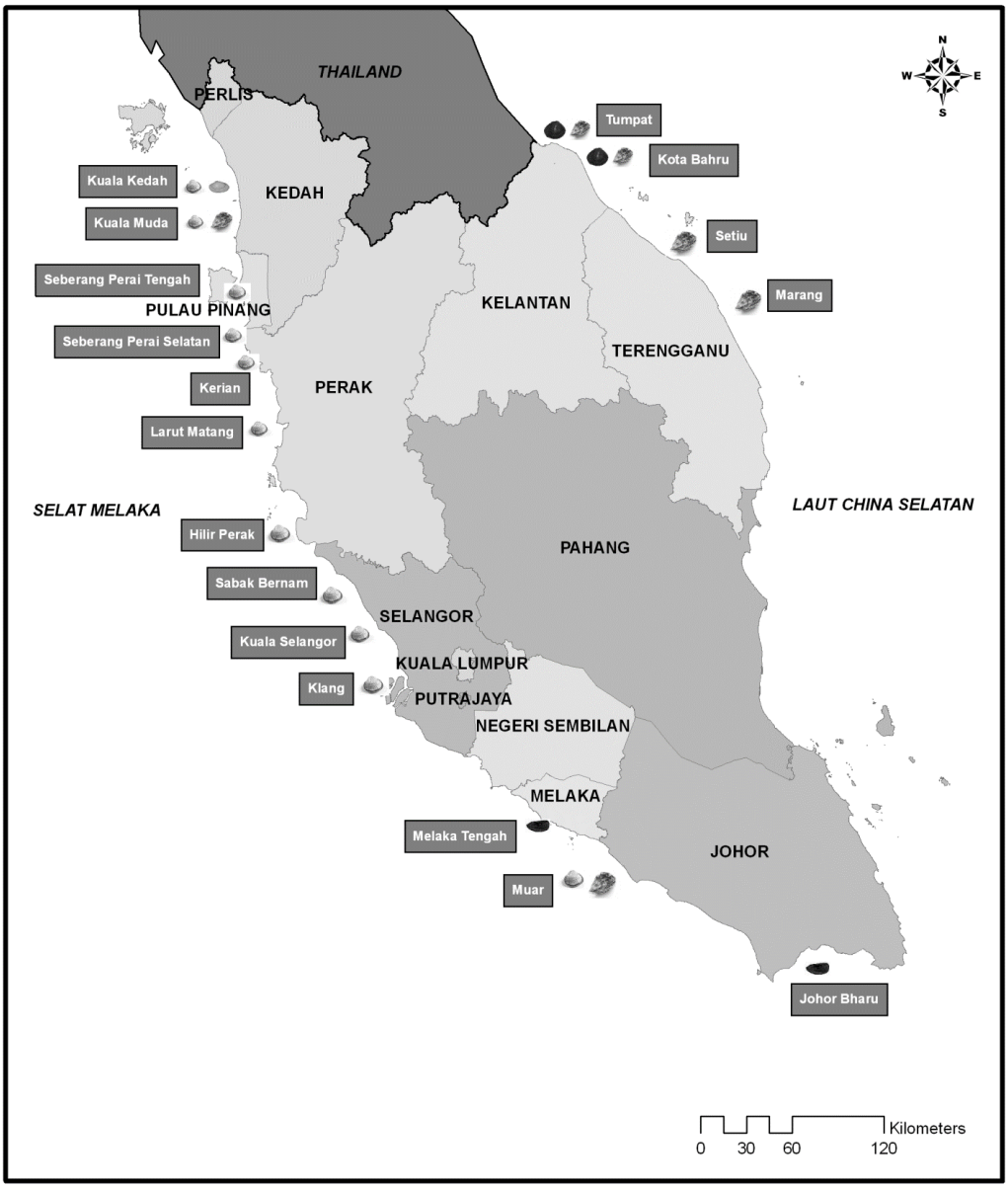

Figure 1: Sampling locations under the NSSP

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.