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.

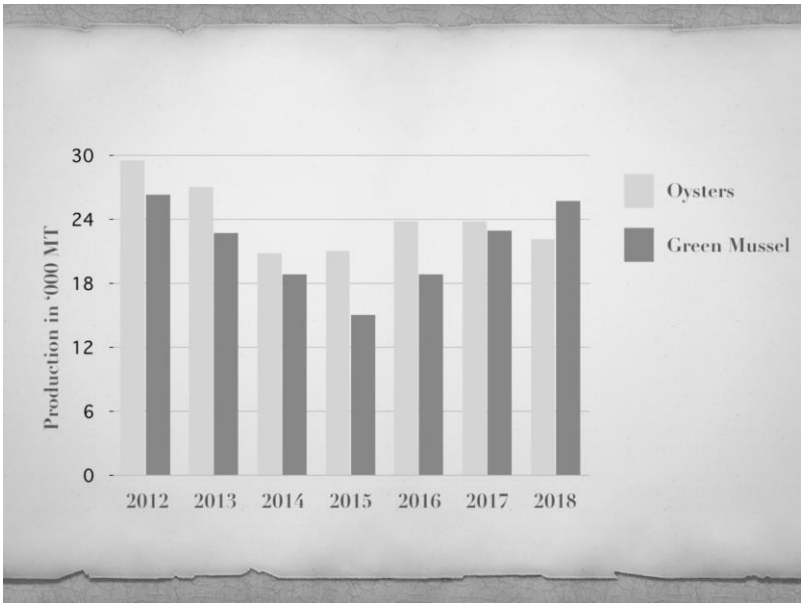

Figure 1: Shellfish production in the Philippines

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.

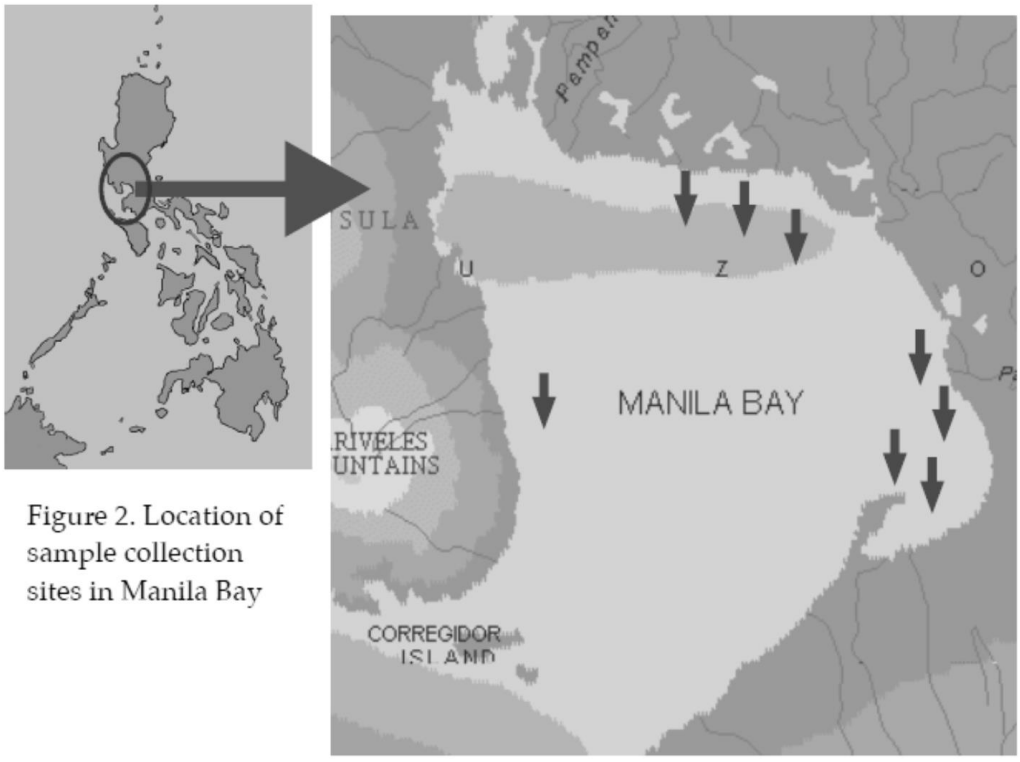

Figure 2. Location of sample collection sites in Manila Bay

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.

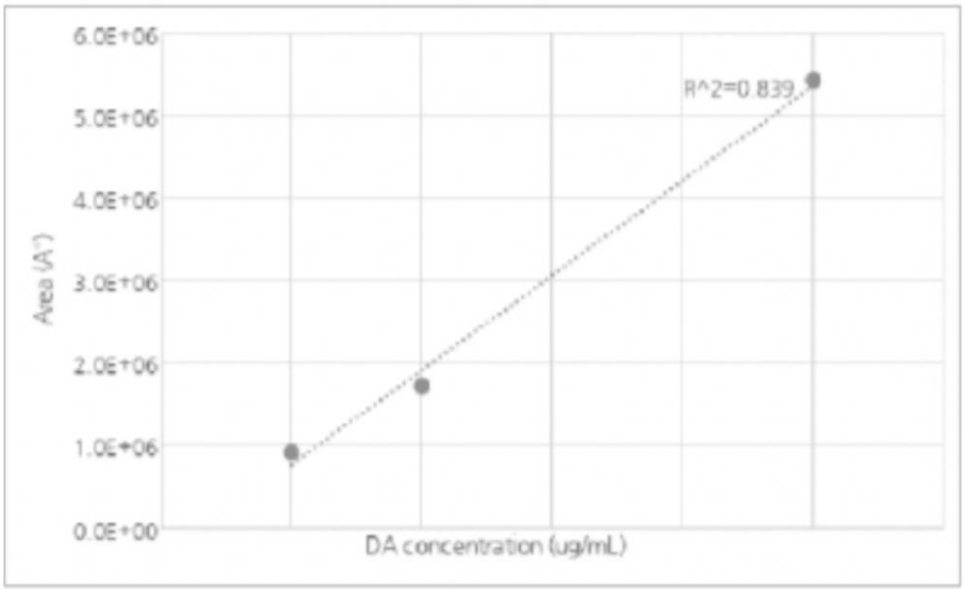

Figure 4. Linear response between domoic acid concentration and peak area

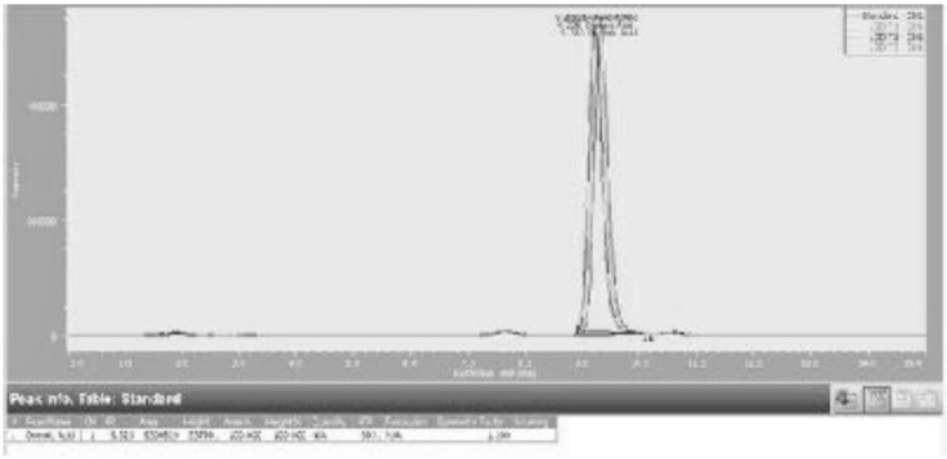

Figure 5. Sample chromatograms of standards used in the analyses

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

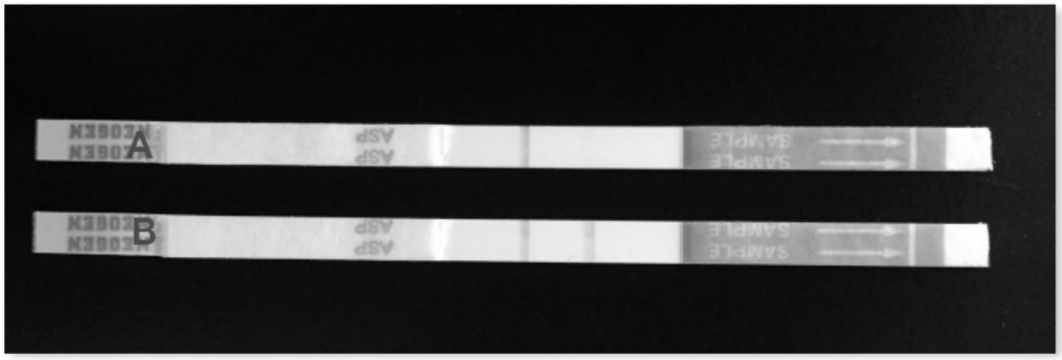

Figure 7. Sample test strips used in screening for domoic acids showing positive (A) and Negative (B) results

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.