PHILIPPINES

Ms Sandra Victoria R. Arcamo
Chief
Fisheries Resource Management Division
Bureau of Fisheries and Aquatic Resources

I. Introduction

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

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

II. Objectives and Goals

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

III. Survey Methodologies

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

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

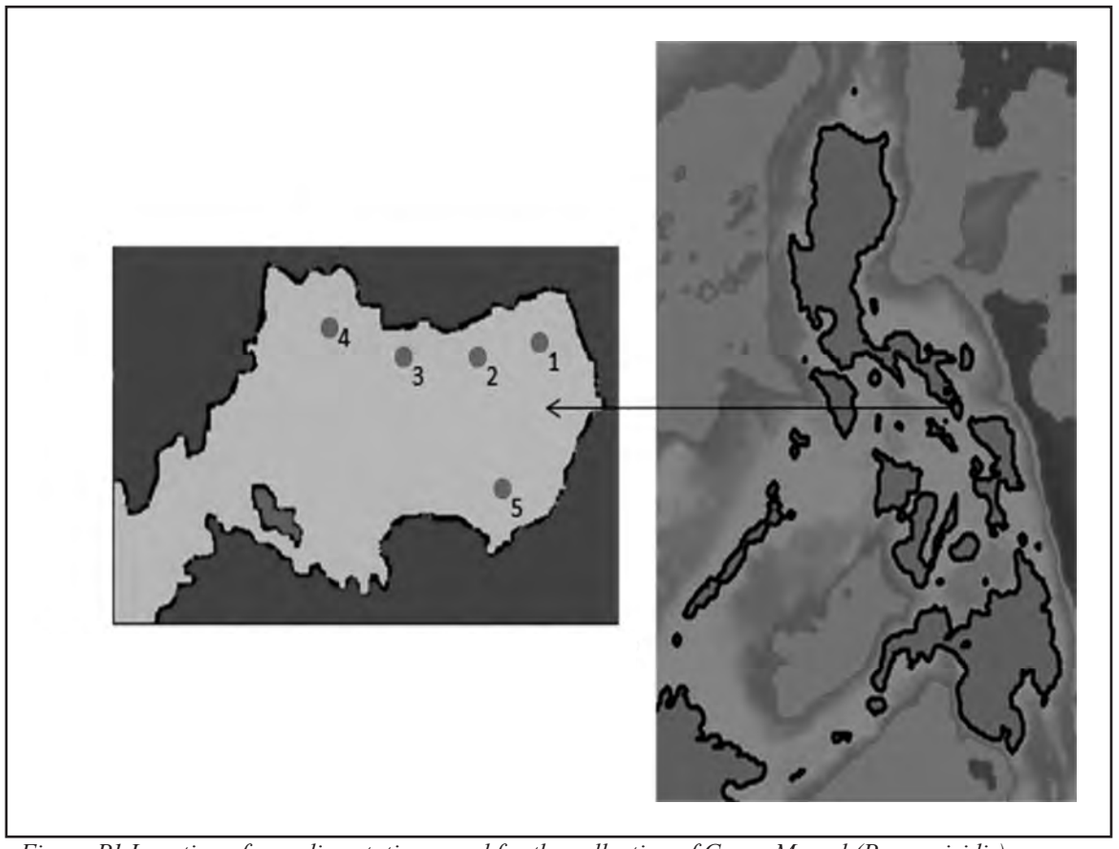


Figure P1 Location of sampling stations used for the collection of Green Mussel (Perna viridis)

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

Table P1: Description of sampling area

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

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

b. Method of Analysis

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

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

Table P2: Summary of Methods

c. Limit of Detection & Limit of Quantification

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

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

d. National Regulatory Limits

Philippine Regulatory limit is set at 60µg/100g

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

IV. Results and Discussions

a. Participation in Inter-Laboratory Proficiency Testing & Results

-

b. Survey Results & Discussion

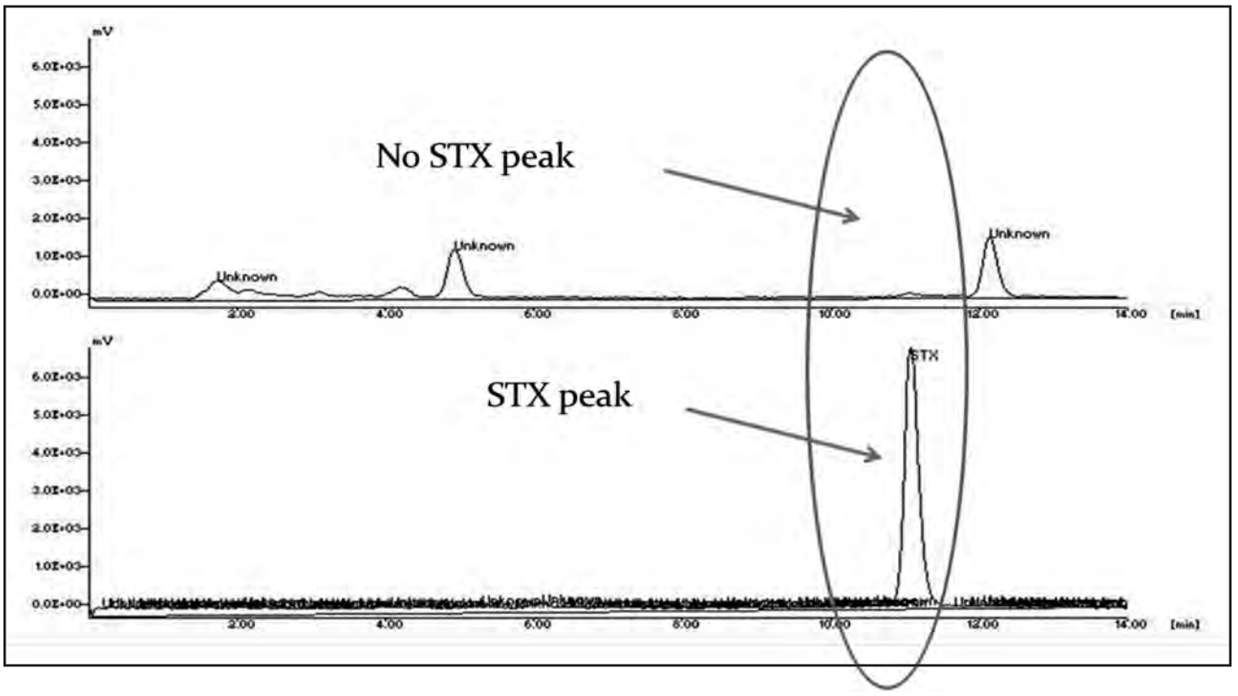
Sampling Location	Month & Year of Sampling (MM/YYY)	Analyte Tested	No. of Samples Analysed	Minimum Concentration (ug/100g of meat)	Maximum Concentration (ug/100g of meat)	Average Concentration (ug/100g of meat)
Sorsogon Bay	January – December 2011	Saxitoxin (STX)	60	MBA: < 40ug/100	MBA: 63ug/100	MBA: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

Table P3: Survey Results

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

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

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



FigureP2. Sample of chromatogram of extract negative for PSP

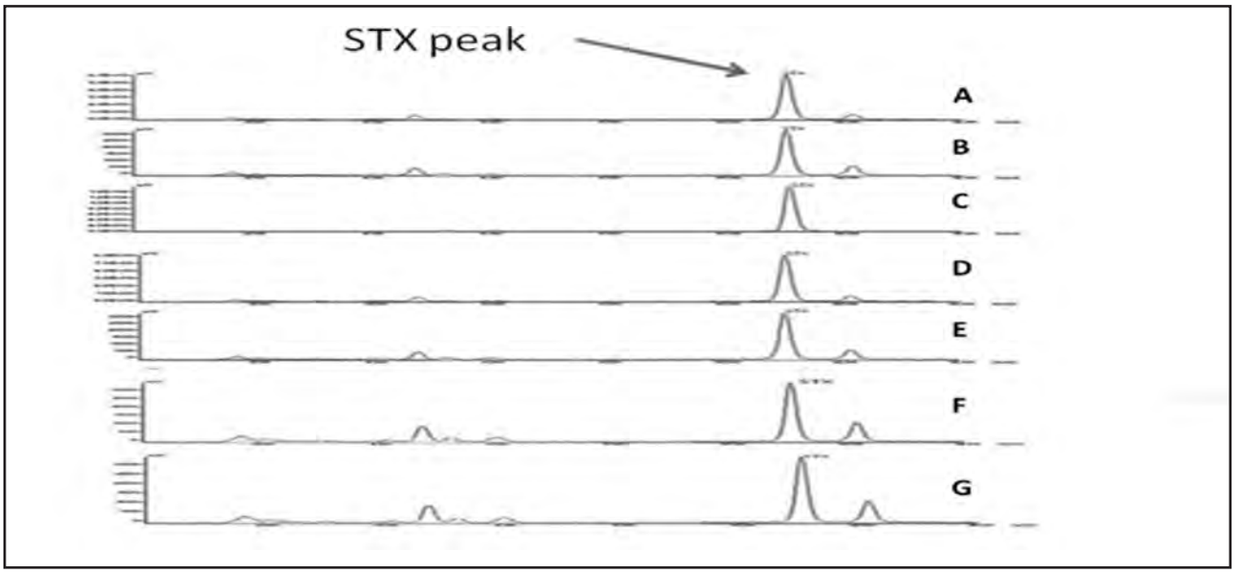


Figure 3. Chromatogram of extract that is positive for PSP

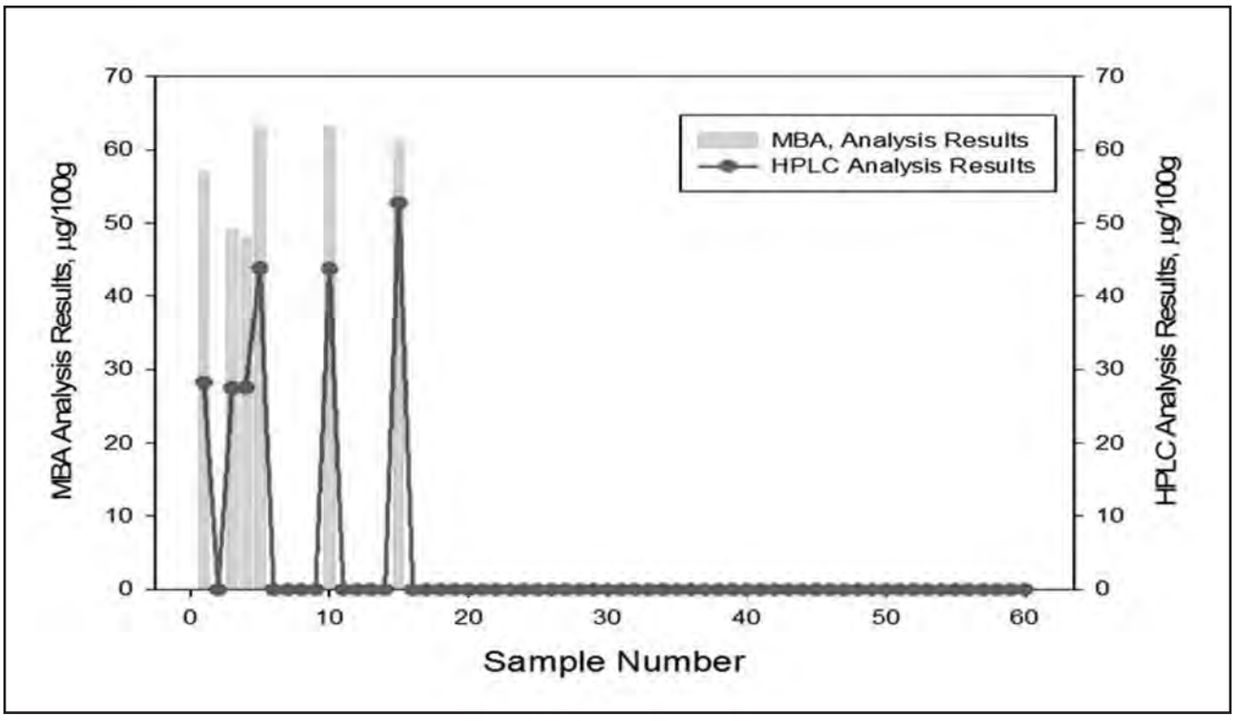


Figure P4. Confirmatory test for STX using HPLC

a. Corrective Actions

-

I. Problems and Challenges Encountered

-

II. Recommendations and Suggestions for future Follow Up Action

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