Bioluminescence of *Vibrio fischeri*: A novel application for PSP quantification

Mohamed Blaghen\(^1\,^2\), Abdel-hamid Abdallah Abakar\(^1\)

\(^1\)Laboratory of Microbiology, Pharmacology, Biotechnology, and Environment, Faculty of Sciences, Ain Chock, Hassan II University, Casablanca, Morocco.

\(^2\)Laboratory of Plant Biotechnology, Ecology and Ecosystem Valorization, Faculty of Sciences El Jadida, Chouaïb Doukkali University, El Jadida, Morocco.

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**ABSTRACT**

Paralytic Shellfish Poison (PSP) are the most harmful neurotoxins create a serious public health problem. It is important to assess PSP in Shellfish destined for human consumption. However, recommended methods have some limitations for example in the case of Mouse Bioassay (MBA) showed a low sensitivity and reproducibility, and undesirability for ethical reasons; while physicochemical techniques rest expensive and time-consuming. The main objective of this study, after discovering that PSP inhibited the luminescence of *Vibrio fischeri*, was the quantification of PSP by using Bioluminescence Inhibition Assay (BIA), and comparing the results obtained with those determined by MBA and LC-MS. Bivalve used were collected from Corniche Martil, Kabila, and Oued Laou, along with the Mediterranean coast of Morocco in Mars-2015. Results showed a weak correlation between LC-MS and MBA with \( r = 0.11 \), while, the correlation between LC-MS and BIA was very strong with \( r = 0.97 \), which suggests that, BIA could offer an interesting additional assessment of PSP risk. In addition, after seen its rapidity, ease, reliability, sensitivity, reproducibility, and cost-effectiveness, it would be eligible to use for monitoring in surveillance programs.

**1. INTRODUCTION**

Some species of dinoflagellates such as *Alexandrium*, *Gymnodinium*, and *Pyrodinium* produce Paralytic Shellfish Poisoning (PSP) [1,2]. PSP are ubiquitous along coasts, which pass by marine food chain through vector organisms accumulate them by feeding on dinoflagellates [3]. Among PSP, the Saxitoxin (STX) is discovered in 1975 [1] and is the most potent aquatic bioxotins, which caused a huge number of poisoning case in humanity due to respiratory failure [4-5]. Up to know, 57 analogs of STX are known, all exhibiting different toxicities [1]. Consequently, many countries have decided to ensure bivalve destined for consumption must not exceed the permitted threshold of PSP in shellfish, is 800 mg STXs equivalents (STX eq) per kg of shellfish tissue [5]. However, among the official methods used to monitor PSP were principally the Mouse Bioassay (MBA) and physicochemical techniques. MBA was discovered in the 1930s, and used by the Association of Official Analytical Chemists (AOAC) international, European Union (EU) legislation and the United States Food and Drug Administration as an official method for PSP in shellfish monitoring [6-8]. MBA method is easy to operate and rapid to assess a total toxicity [9-11]. However, this method has some limitations, including low sensitivity and reproducibility [12] and also its undesirability for ethical reasons [14], was conducted the search for alternative physicochemical techniques for PSP monitoring. However, these physicochemical techniques seem to be very expensive, useful mostly for identified PSP in samples because their operations depend on the sample toxicity, require well-trained persons for their operations, and consume more time particularly when analyzing a huge amount of samples [3,30-33]. Among these techniques, we have thin layer chromatography [13], capillary electrophoresis [15], immunological based method [16-18], High Performance Liquid Chromatography with either pre- or post-column derivatization with Fluorescence Detection (HPLC-FLD) [5,19,20], Liquid Chromatography coupled to Mass Spectrometry (LC-MS), Hydrophilic Interaction Liquid Chromatography coupled to tandem Mass Spectrometry (HILIC) LC-MS/MS [21-26]. HPLC-FLD called Lawrence method which the detection was based to the conversion of PSP into fluorescent derivatives [27], has been validated and adopted by AOAC as an official technique, This automated method was permitted sensitive and selective toxin determination [28]; LC-MS that has been adopted by EU as the standard method in PSP assessment, has attracted more attention for its high performance of qualitative, quantitative and selective analysis [29] to monitor PSP.

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in shellfish. This paper presents, after discovered that PSP inhibited the luminescence of *Vibrio fischeri* (*V. fischeri*), the utilization of Bioluminescence Inhibition Assay (BIA) for PSP quantification. This method was therefore based on the inhibition of the luminescence of *V. fischeri* by PSP since the rate of inhibition of bioluminescence depended on PSP concentration. According to the scientific literature, BIA has never been used to quantify PSP and, therefore we have used the study of the inhibition of bioluminescence by PSP for implementing a new technique for PSP quantification.

2. MATERIALS AND METHODS

2.1. Biological materials

Bacteria: *Vibrio fischeri* strain NRRL-B-11177.

Cockle: *Acanthocardia tuberculatum*.

2.2. Bacterial growth media

*V. fischeri* was grown in a modified Luria-Bertani Salt (LBS) medium as described in 2017 by A. A. Abdel-hamid *et al.* (10 g nutrient broth, 5 g yeast extract, 20 g NaCl, 50 ml 1 M Tris buffer, pH 7.5 ± 0.02, in one liter of distilled water at 25°C) [38]. All products were purchased from Biokar Diagnostics.

2.3. Bioassays and Statistical analysis

PSP assessment was carried out by MBA according to AOAC, LC-MS, and BIA.

BIA performed in 96-well microplate format using Luminoskan Ascent Luminometer (LAL). Bacteria growth was determined using spectrophotometer (UV-Visible) at the optical density of 600 nm (OD_{600}). All data were analyzed using Graph-PadTM Prism. Values are means ± standard errors. All experiments were done in triplicate. Bioluminescence inhibition was determined according to the equation below as described by A. A. Abdel-hamid *et al.* [38].

\[
\% \text{ light inhibition} = 100 - 100 \times \left( \frac{IT_{15}}{KF \times IT_0} \right)
\]

\[KF = \frac{IC_{15}}{IC_0}[38]\]

2.4. Sample

Cockles and other bivalve were collected from Kabila, Corniche Martil, and Oued Laou, along with the Mediterranean coast of Morocco in Mars 2015 (Fig. 1). Samples collected were kept at -20°C prior to PSP extraction and analysis. All samples were thawed at 4°C prior to PSP extraction.

An STX standard (66.3 μM) was obtained from Laboratory of the Marine Biotoxins, National Institute of Halieutic Research, Casablanca, Morocco.

2.5. Extraction of PSP

Extraction of PSP form cockles was done in accordance with the previously published protocol in 2010 by CK Wong *et al.* [3].

2.6. MBA

Albinos mice (20 ± 2 g) were obtained from Laboratory of the Marine Biotoxins, National Institute of Halieutic Research, Casablanca, Morocco. They were acclimated for 24 h and housed in a controlled environment at 24 ± 2°C, 54–56% humidity. PSP assessment by MBA was performed in accordance with the official AOAC procedure of PSP assessment [39].

2.7. LC-MS

PSP in cockles were quantified by LC-MS in accordance with the previously published protocol in 2005 by Staack *et al.* [40].

2.8. BIA

Dilutions (1:1.25, 1:1.5, 1:1.75, 1:2, 1:2.5, 1:3, 1:3.5, 1:4) of PSP of all samples were prepared in 2% NaCl at pH 3.5. 0.1 ml of each PSP dilution was added into different wells in duplicate, supplemented with 0.1 ml of bacterial suspension by automatic dispensing within LAL testing chamber. The bioluminescence was recorded after the microplate was automatically shacked. The signal of the light was measured in the first contact at 25°C in duplicate.

The quantification of PSP in cockles was done according to following procedure:

- Serial dilutions (1:1.25, 1:1.5, 1:1.75, 1:2, 1:2.5, 1:3, 1:3.5, 1:4) of PSP were prepared in 2% NaCl (pH 3.5).
- 2% NaCl (pH 3.5) served as a control and solution of dilution.
- Extract and diluted PSP were incubated at 25°C for 30 min.
- Luminescent bacteria *V. fischeri* were reconstituted and then incubated at 25°C, by shaking (250 rpm) and aerating (600 ml/min) for 22 h [38].
- 0.1 ml of each diluted PSP solution was added into different wells in duplicate, supplemented with the same volume of bacterial suspension by an automatic dispensing and shacked within LAL testing chamber.
- The signal of the light was directly and automatically measured.

The Standard of STX with different concentrations (0.16, 0.33, 0.99, 1.65 μM) was analyzed using the same procedure.

2.9. pH solutions

pH 1-14 solutions were prepared in 2% NaCl by using HCl 5N, NaOH 0.1N, HCl 0.1N and NaOH 5N.

3. RESULTS AND DISCUSSION

Nowadays the detection of toxins in bivalve is becoming more necessary and frequent. Therefore, it is very important to use a method that is easy, rapid, sensitive and cost-effective to evaluate PSP in bivalve. BIA is based on *V. fischeri* a marine gram-negative bacterium,
widely used to evaluate toxicity. The best conditions for intensity and stability of luminescence emission and growth of the bacteria were determined in accordance with the previously published work in 2017 by A. A. Abdel-hamid et al. [38]. These best conditions had let us to evaluate the toxicity of PSP in cockles.

The goal of this work was to investigate the applicability of the inhibition of *V. fischeri* by PSP for the PSP quantification. The Cockles were collected from Kabila, Corniche Martil, and Oued Laou, along with the Mediterranean coast of Morocco. 100 g of shellfish tissues of each sample was crushed then homogenized and boiled gently with stirring for 5 min and then brought to 200 ml with distilled water. Each boiled homogenate was centrifuged at 3000 x g for 10 min. supernatants were adjusted to pH 3.5 and kept at 4°C prior to testing.

Firstly, all samples were tested by MBA in accordance with the official AOAC procedures for PSP [39], before analyzing by LC-MS and BIA.

For MBA, three mice were weighed and injected intraperitoneally with 1 ml of PSP extract (the same extract used for LC-MS analysis and BIA). The treated mice were then closely observed. The median survival time was adopted for toxicity (MU) calculation. The values of toxicity were expressed in terms of μg STX eq/100 g tissues, based on AOAC method, thereafter converted to μM. All the three samples showed toxicity to mice. The most sample toxic to mice was that’s from Oued Laou, its calculated toxicity value was 12.80 μM, the less toxic extract was that from Kabila with a toxicity value of 6.30 μM, while Corniche Martil extract showed a toxicity (11.20 μM) near to the toxicity of Oued Laou (Table 1).

Secondly, all samples were analyzed by LC-MS. and STX at concentrations of 0.16, 0.33, 0.99, 1.65 μM was used to obtain the standard curve.

According to the results obtained using LC-MS, a sample from Corniche Martil showed the highest concentration (2.77 μM) of STX compared to those from Kabila and Oued Laou whose concentrations were 1.31 and 0.98 μM respectively (Table 1).

Before analyzing samples, all the samples were adjusted to pH 3.5 based on AOAC method [39]. It is important to see the effects of pH on the bacteria bioluminescence. Indeed pH between 1 and 14 was tested. Results showed that the pH 1, pH 13 and pH 14 inhibited totally the luminescence from the first contact, whereas pH 2 has begun to inhibit the light after 15 min of incubation, meanwhile from pH 3 to pH 12 did not inhibit the bioluminescence (Fig. 2).

Thirdly, all samples were analyzed using BIA. The STX standard was used to obtain the standard curve, Analyzed concentrations of the saxitoxin were 0.16, 0.33, 0.99, 1.65 μM (Fig. 3).

All PSP extracts from different sites showed bioluminescence inhibition. This inhibition decreased with dilution factor (Fig. 4).

The concentration of PSP of each sample was calculated according to the standard curve of STX. The result showed that the sample from Corniche Martil which its PSP concentration value was 2.26 μM of STX eq inhibited most the light of the bacteria. Samples from Kabila and Oued Laou showed the PSP concentration values of 1.56 and 1.1 μM of STX eq respectively, nearly the same inhibition of the bioluminescence while there was a significant difference between results obtained using LC-MS and MBA (Fig. 5) (Table 1).

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**Table 1**: STX equivalent values obtained using MBA, LC-MS and BIA. Values expressed in μM.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>MBA*</th>
<th>LC-MS</th>
<th>BIA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corniche Martil</td>
<td>11.20 ± 1.20</td>
<td>2.77</td>
<td>2.26 ± 0.01</td>
</tr>
<tr>
<td>Kabila</td>
<td>06.30 ± 0.70</td>
<td>1.31</td>
<td>1.56 ± 0.02</td>
</tr>
<tr>
<td>Oued Laou</td>
<td>12.80 ± 1.30</td>
<td>0.98</td>
<td>1.1 ± 0.01</td>
</tr>
</tbody>
</table>

*MBA*: Mouse Bioessay. **BIA**: Bioluminescence Inhibition Assay.

Figure 2: Measurements of luminescence of *V. fischeri* at different pHs. Measured directly after first contact, 5 min, 10 min, 15 min and 30 min.

Figure 3: Standard curve of STX obtained by BIA.

Figure 4: Measurements of luminescence of *V. fischeri* exposed to dilutions of PSP from Oued Laou, Kabila, and Corniche Martil. Measured directly after the first contact. 2% NaCl served as a control and diluent.

According to LC-MS, the results were 2.77, 1.31 and 0.98 μM STX for Corniche Martil, Kabila and Oued Laou respectively. Meanwhile, MBA results were 11.2, 6.3 and 128 μM STX equivalent for Corniche Martil, Kabila, and Oued Laou respectively (Fig. 5) (Table 1). Thus,
the correlation between LC-MS and MBA was very weak with $r = 0.11$ (Fig. 6).

![Figure 5](image)

**Figure 5**: Comparison of the three methods (BIA, LC-MS, and MBA) used for the assessment of PSP from Corniche Martil, Oued Laou, and Kabila. Values were expressed as μM of STX eq.

![Figure 6](image)

**Figure 6**: Correlation between LC-MS and MBA for analysis of PSP in cockles expressed in STX equivalent (Number of compared samples = 3).

![Figure 7](image)

**Figure 7**: Correlation between LC-MS and BIA for analysis of PSP in cockles expressed in STX eq (Number of compared samples = 3).

However, PSP concentrations obtained by BIA were 2.26, 1.56 and 1.1 μM STX equivalent for Corniche Martil, Kabila and Oued Laou respectively. Thus, these results were significantly comparable with those obtained using LC-MS with a strong correlation with $r = 0.97$. Based on these results, BIA additionally may be used for the assessment of PSP risk. Thus, its rapidity, ease, reliability, sensitivity, reproducibility, and cost-effectiveness would eligible its use for PSP monitoring in surveillance programs.

4. CONCLUSION

According to the results obtained during this study, all samples presented toxicity to mice, inhibited the light of *V. fischeri*. There was a significant difference between results obtained using LC-MS and MBA, the correlation was very weak with $r = 0.11$. However, the expressed PSP concentrations obtained by BIA were 2.26, 1.56 and 1.1 μM STX eq for Corniche Martil, Kabila and Oued Laou respectively with a very strong correlation with $r = 0.97$. Thus, it could offer an additional quantification of PSP in bivalves.

5. ACKNOWLEDGMENTS

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6. REFERENCES

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