

Original research paper

PSP toxins in Moroccan shellfish by mouse bioassay, high-performance liquid chromatography and Receptor Binding Assay (2004-2016)

ABSTRACT

Paralytic phycotoxins or PSP (Paralytic shellfish poisoning) are secondary metabolites of phytoplankton toxic species, the consumption of shellfish accumulating these toxins can cause neurological symptoms and even death. Within the framework of the program of surveillance of safety in littoral environment, the investigation of this toxin was conducted from 2004 to 2016 in south Moroccan's shellfish, mussels from south Agadir region and solen from Dakhla bay. The determination was carried out bi-monthly to weekly using the official method AOAC 959.08 by the mouse bioassay (MBA). In parallel, monitoring of toxic phytoplankton in water was conducted. In the aim to determine the shellfish toxin profile, high-performance liquid chromatography with fluorescence detection (HPLC-FD) was performed. The Receptor Binding Assay (RBA) also was used for determination of PSP toxins in Agadir's mussels.

In both regions, the analysis of sea water revealed the presence of the toxic algae *Alexandrium* spp during the toxics events. At Agadir coast, the PSP toxins were accompanied by the presence of *Alexandrium cf. minutum* in seawater, these toxic events were widely distributed in time and space and mainly detected during the summer and fall seasons. In some samples concentrations were exceeding the sanitary threshold (ST) 800 µg eq STX /kg. HPLC analysis revealed that the toxin profile was dominated by Saxitoxin and Gonyautoxins. The comparison between different methods showed a strong uphill (positive) linear relationship, a correlation coefficient $r=0.79$ for MBA and HPLC and $r = 0.809$ for MBA and RBA.

Keywords

Paralytic shellfish poisoning, *Alexandrium minutum*, Morocco, HPLC, RBA, MBA.

1. INTRODUCTION

PSP toxins form a family of more than 20 molecules, chemically close to each other, all derived from Saxitoxin by substitution of various radicals of this molecule [1], [2].

PSP toxins were detected repeatedly in the bivalves of Moroccan coasts and can represent a real danger for the consumers. The first case of intoxication by shellfish was recorded in 1961. 1971, 1975, 1981. The year 1994 was marked by many episodes of human intoxication [3], [4].

From 1998 to 2016, several episodes of shellfish toxicity were detected in Moroccan Mediterranean's bivalves [5], [6], [7] and in the Atlantic coasts bivalves [8]. Through a marine biotoxin monitoring system established by National Institute for Fisheries Research (INRH) no case of PSP human intoxication had been reported during this period.

The aim of this work is to present a spatio-temporal evolution of toxic phytoplankton in waters and PSP toxicity of south Moroccan shellfish, in mussels from the south of Agadir and in solen from Dakhla bay. Moreover, an investigation of PSP toxin profile was conducted by HPLC-FD. RBA method was also used for the detection of these toxins; the results of different methods were compared by presenting the advantages and disadvantages of each test. This the first report concerning used of a nuclear method (RBA) for detection of PSP toxins in Moroccan shellfish.

2. MATERIEL and METHODS

2.1. Sampling site

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Two shellfish species were taken from two sampling sites of south Atlantic Moroccan coasts, mussels from Agadir coast and Solen from Dakhla bay. At Agadir (Dar sfint site), Mussels were collected weekly from January 2010 to December 2016, more than 362 samples were studied. 286 samples of solen were harvested at Dakhla bay (Pk25 site) from 2004 to 2010, Three kg of shellfish were taken at low tide and kept in coolers during transport to the laboratory. In parallel, Water samples are collected weekly at each site. Sea water Samples were taken at high tide (± 2 hours of peak) with net (20 μ m) and with a bottle (1 L). The samples are kept in a cooler during the transport to the laboratory.

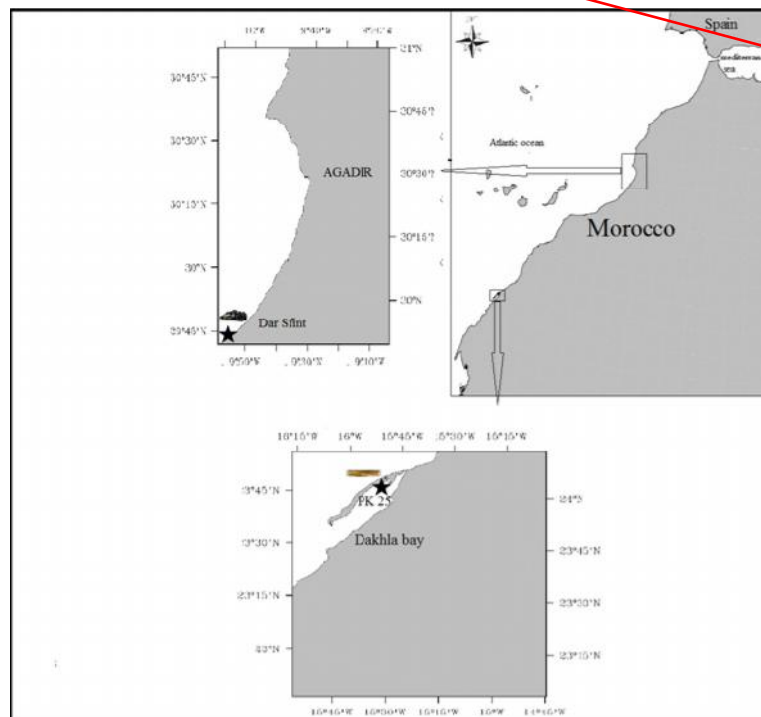


Fig1. Sampling sites

2.2. Toxin analysis

2.2.1 MBA

The MBA procedure was carried out following AOAC official method 959.08 [9] 100 $\pm$ Yg shellfish flesh were homogenate in 100 ml 0.1 M HCl. Three mice (20 $\pm$ 2 g) were injected with 1 ml of the shellfish extract. The method was standardised using a certified reference standard of STX obtained from, National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada). Toxicity was correlated with death time and converted to μ g STX_{eq} per kg of shellfish flesh using a conversion factor of laboratory 0.2. These analyzes were carried out at the marine biotoxins laboratory of the National Institute for marine Research (INRH), Agadir, Morocco.

2.2.2. HPLC-FD

Paralytic shellfish poisoning toxins profiles were screened by liquid chromatography (HPLC/FD) on contaminated mussel's tissues using post column oxidation with periodic acid as described by Thielert et al. [10] and modified by Hummert et al [11] and Yu et al [12]. Saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins (GTX1, 2, 3, 4) standards were purchased from the National Research Council Canada (NRC-PSP- 1B). The standards solutions were diluted with acetic acid 0.03 N as required. These analyzes were carried out at the food chemistry laboratory of Friedrich-Schiller University in Jena, Germany.

Please add information, how the samples for HPLC were performed (solvent used for extraction, speed of centrifugation etc. Add information about the condition of HPLC procedure; type of used column, temperature of separation, flow rate, concentration and composition of mobile phase

2.2.3. Receptor Binding Assay RBA

The extracts of Agadir's samples taken during toxics events from 2010 to 2016 were analyzed by the RBA method. Extracts obtained by the AOAC 959.08 method are filtered using a 0.45 filter paper and stored at -20 °C. These analyzes were conducted at the Environmental Laboratory of the International Atomic Energy Agency in Monaco under Technical cooperation program of IAEA RAF 7012 and 7014 Projects. The determination of PSP toxins by RBA was made according to the protocol of Van Dolah et al [13]. This method is based on the competition between a radioligand (STX-tritium) and the different dilutions of the samples on the same receptors. Thus, the amount of radioligand complexes decreases with increasing concentrations of the extract. The counting of the completed radioactivity is performed by liquid scintillation counter which gives a competition curve. This curve showing the percentage of the complex formed according to the toxins added, this allows the determination of total amount of the toxin present in the sample.

2.2.4. Identification and phytoplankton counting

Observations were carried out using an inverted microscope, in the sedimentation tank 10 or 25 ml, according [14]. The determination is made through appropriate systematic identification keys. The counting is performed with horizontal scanning. The results densities are expressed on number of cells per liter (cell.L^{-1}).

3. Results and discussion

3.1. MBA analysis and toxic species of phytoplankton investigation

In Agadir (Dar sfint area), five periods of PSP toxicity were recorded during the studies period from 2010 to 2016, toxins were found in 23 of 362 analyzed samples (6.62%) (Fig2). The year 2012 recorded the longest period of toxicity (7 weeks) against the shortest one was noted in 2013 (1 week). But in 2015, the highest concentration of toxins in mussels in this region was recorded (6 weeks of toxicity).

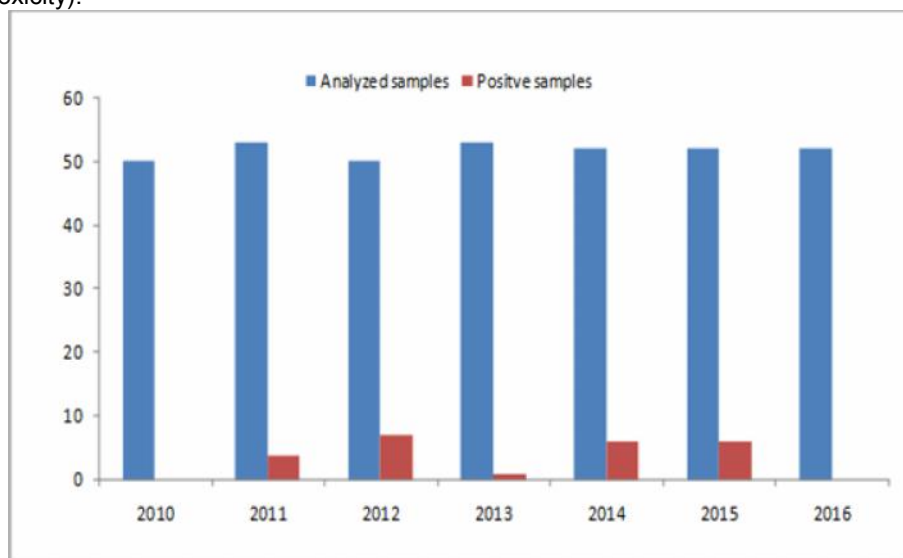


Figure 2: PSP positives samples in southern Agadir coasts 2010 to 2016.

The monitoring of toxic phytoplankton *Alexandrium* spp shows a significant spatial and temporal variability. Indeed, the species were present at low concentrations between winter 2010 and fall 2011 to increase in winter 2011. The maximum concentration reached $7.2 \cdot 10^3 \text{ Cell.L}^{-1}$ recorded in the fall. *Alexandrium* remain in waters, with low densities that reach high values in late spring-early summer 2012 ($2 \cdot 10^3 \text{ Cell.L}^{-1}$), then they proliferate in July with a maximum of $4 \cdot 10^3 \text{ Cell.L}^{-1}$ then, no detection of

this species until the end of spring 2014 with a maximum in June of $1.5 \cdot 10^3 \text{ Cell.L}^{-1}$; after this species remain present throughout the year in superficial waters, but at low concentrations ranging between 10^2 and $9 \cdot 10^2 \text{ Cell.L}^{-1}$. In the spring of 2015, *Alexandrium* reached concentrations around $8 \cdot 10^2 \text{ Cell.L}^{-1}$ to mark a major efflorescence in August with $25 \cdot 10^3 \text{ Cell.L}^{-1}$, bloom spread through the waters until the beginning of fall 2015. The concentrations of *Alexandrium* fell gradually to $7 \cdot 10^2$ and 40 Cell.L^{-1} , recorded respectively in spring and winter 2016.

The analysis of PSP toxins by MBA shows that, many samples contains high levels of toxins compared to the regulatory limit ($800 \mu\text{g STX eq.kg}^{-1}$), maximums noted were 845, 1145, 357, 2109, and $7068 \mu\text{g STX eq.kg}^{-1}$ respectively in fall 2011, in summer 2012, in fall 2013, in the end of spring 2014 and in summer 2015 (Fig. 3). This toxicity is explained by the high concentrations of *Alexandrium* recorded in seawater during these episodes.

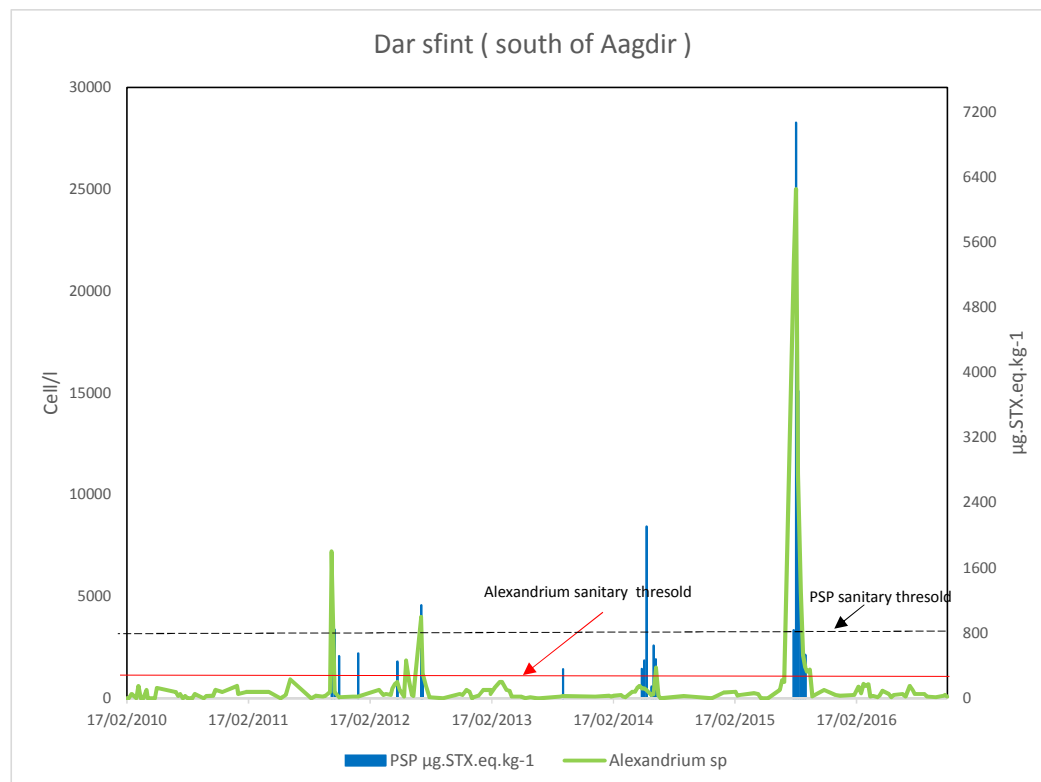


Figure 3: Evolution of *Alexandrium* spp in waters and PSP toxins in mussels from Dar sfint (2010- 2016)

At the Dakhla bay (Pk25 area), just one period of PSP toxicity was recorded from 2004 to 2010, toxins were found in 6 analyzed samples (2.09%). The year 2006 recorded 5 weeks of toxicity (Fig4). No case of PSP was detected before winter 2006; in December 2006, the toxicity was exceeding sanitary threshold with a maximum of $1215 \mu\text{g stx eq.kg}^{-1}$, in general concentrations were at STX levels near or below the regulatory between 395 and $600 \mu\text{g STX.eq.kg}^{-1}$ from October to November 2006. From January 2007 to December 2016, PSP toxins were not recorded in Solen. Toxins were detected in fall, during this season the temperature is high in this region between 18 and 20°C [15]. Toxic phytoplankton observation conducted from 2004 to 2006 shows that *Alexandrium* spp concentrations in seawater records a low spatial and temporal variability. Indeed, winter 2004 and spring 2006 showed an irregular presence of the species with low concentrations (ranging from 100 to 800 cell.l^{-1}), in May and June the concentrations exceeded the health threshold of *Alexandrium* (10^3 cell.l^{-1}) with $2.5 \cdot 10^3 \text{ cell.l}^{-1}$, after the concentrations increased sharply in fall-winter 2006. The maximum value reached $49.4 \cdot 10^3 \text{ cell.L}^{-1}$ registered in November 2006 (Fig. 4, 5). The detection of this toxic dinoflagellate coincided with the shellfish toxicity of this bay.

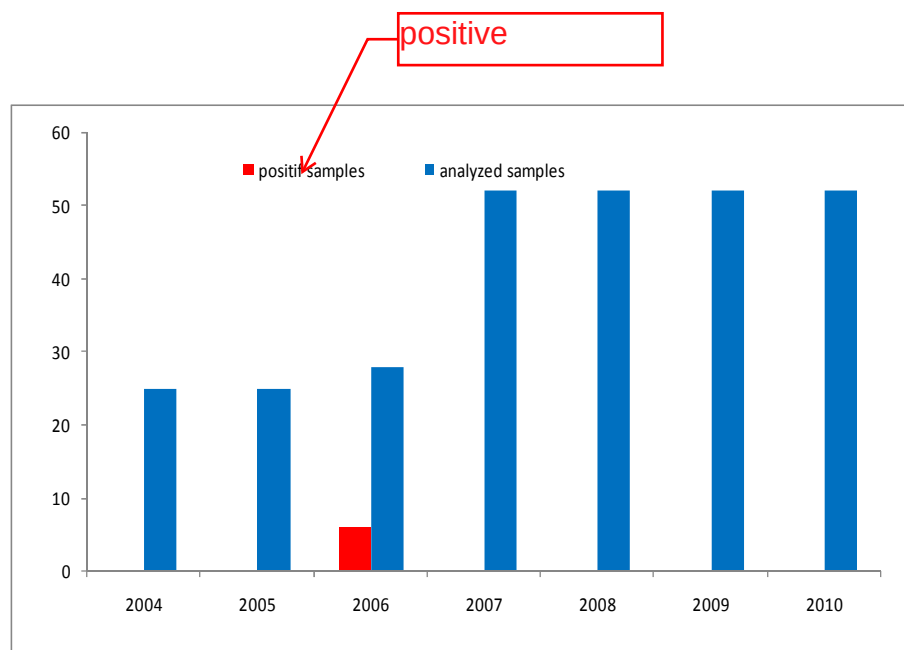


Figure 4: PSP positives samples in Dakhla bay coasts 2004 to 2006

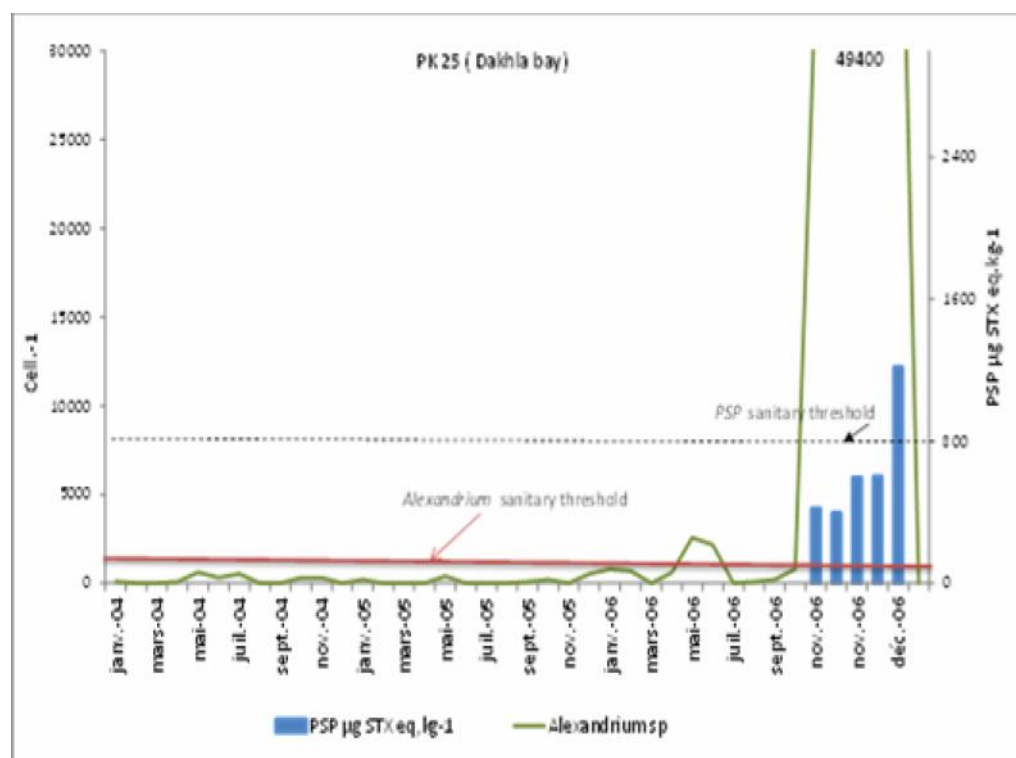


Figure 5: Evolution of Alexandrium spp in waters and PSP toxins in Solen from Dakhala Bay (2004-2006).

In the southern Moroccan Atlantic, PSP toxins were detected barely and with low concentrations, but in recent years these toxins became more frequent with very high concentrations. In general over the last decades the occurrence and intensity of HAB appears to be increasing on a global scale due to rising ocean temperatures and growing coastal eutrophication [16].

The dinoflagellates species from the genus *Alexandrium*, detected during the episodes of shellfish toxicity from 2004 to 2016, have been known as producers for the neurotoxins of PSP, which is one of the most dangerous marine toxins known [17].

This genus is most often associated with PSP toxins, as about 10 of the approximately 30 *Alexandrium* species are PSP producer. In south of Morocco, the shellfish toxicity records indicate that generally the typical "bloom season" for *Alexandrium* is between summer and full annually, but with high interannual variability. The same results was recorded in Salish sea [18], where *Alexandrium* blooms occurred from July to November. In Morocco, MBA is the official method for PSP monitoring of toxins; this method is used for many years to protect the public against this type of intoxication [19], [20].

HPLC-FD analysis

HPLC-FD analysis confirms the presence of PSP detected by MBA, the amounts detected didn't exceed $511 \mu\text{g STX.eq.Kg}^{-1}$. The PSP toxin profile of Dakhla's solen and Agadir's mussel samples showed the presence of Carbamate toxins (GTXs), and Saxitoxin (STX). The Main toxins that had been detected were GTX2, GTX3 and STX. (fig.6, 7).

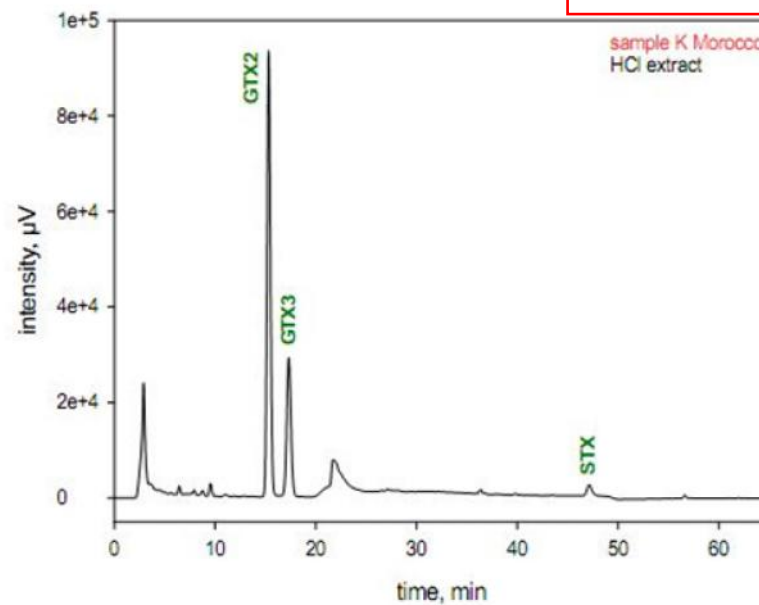
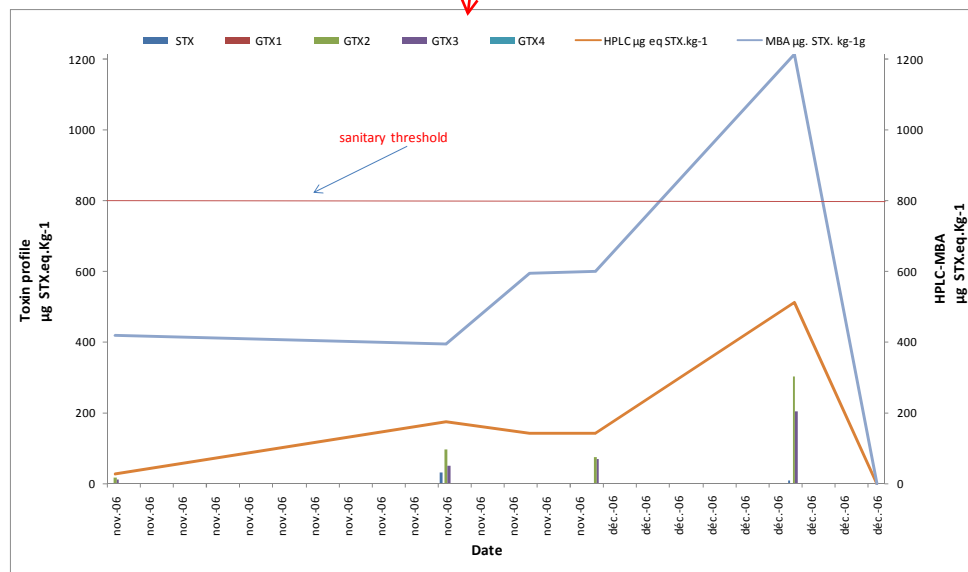


Figure.6 : PSP toxin profile of Agadir's mussels on june 2006

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Figure 7: PSP by MBA and HPLC with toxins profile of Dakhla's solen during toxic even of end 2006

Both in Agadir coast and Dakhla bay (North Africa), **Carbamats toxins** were the main toxins detected with majority of GTXs and minority of STX. Similar results have been reported [5] in wedge shells taken from the north of Agadir with a profile dominated by GTXs. This toxin profile is very close to that of *Alexandrium minutum*, according to [21] the **specie** collected from Cap Town (South Africa) **has a same profile with dominance of gonyautoxins** (GTX1-GTX4). Generally, the shellfish toxin profile is very close to that of ingested phytoplankton [22]. This dinoflagellate was detected in sea water during all periods of toxicity in Agadir coast and Dakhla bay from 2004 to 2016 [23], [24], what leads us to implicate *Alexandrium minutum* in PSP toxicities of shellfish of both regions, this species were reported as toxic dinoflagellate [25], [26], [27].

3.2. Comparison between the different methods used for PSP toxin analysis

Figs.8 and 9 illustrate comparison between toxicities of HPLC and MBA and that between RBA and MBA. The first information obtained there is a strong positive correlation the two different non-animal methods against the reference MBA, the comparative results are summarized in Table 1. Results yielded an r of 0.79, indicating that the MBA reports somewhat higher STX equivalents in shellfish, relative to the HPLC. Probably this can be explained by conversion of the n-sulfocarbamyl toxins (low relative toxicity) to the corresponding carbamate toxins (high relative toxicity) by hydrolysis of acid extraction used in MBA [28], [29]. The fact that the MBA reports somewhat higher toxicity than the HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. These conclusions are in agreement with that found in previous study [13]. When the data from MBA and RBA were compared, a good linear correlation was found $r = 0.809$, for high concentrations the RBA did not report any false negatives. For concentrations below or near to the regulatory limit, some differences between the two methods were found; this difference concerned 25% of analyses samples.

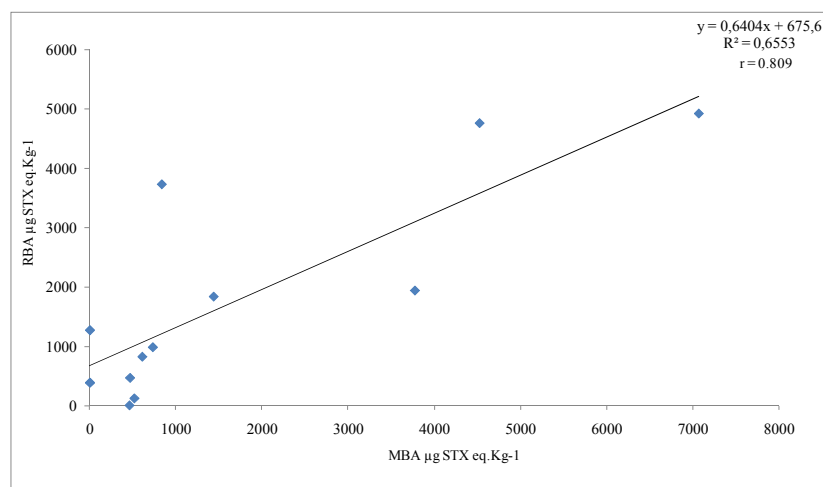


Figure Figure 8 : Correlation between MBA and HPLC-FD method for analysis of PSP in mussel ($n = 8$)

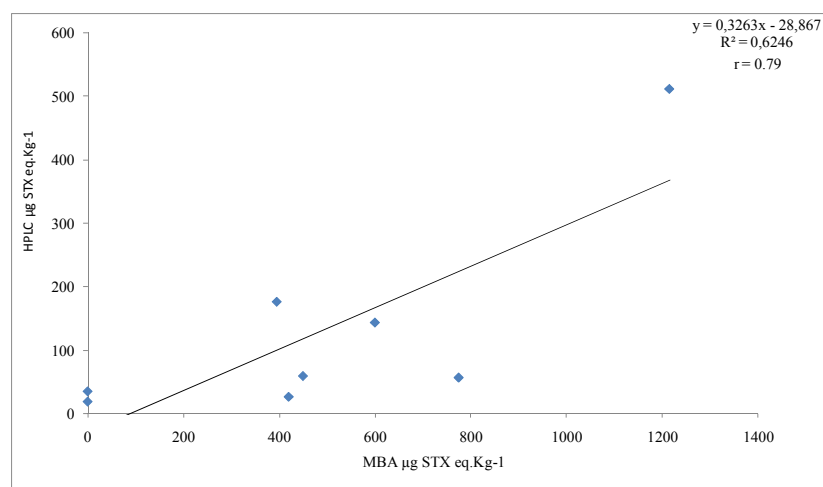


Figure.9: Correlation between MBA and RBA method for analysis of PSP in mussel ($n = 12$).

Table 1 : Summary of results in mussels and solen comparing HPLC-FD and RBA against the reference MBA

	RBA	HPLC-FD
Number of samples	12	8
Mean ratio to (RSD%)	61,41	104.6%
Pearson correlation coefficient (r)	0.809	0.79
Linear refression slope equation	$y=0.6404x+675.6$	$y=0.3263x-28.867$
Other method> ST ; MBA< ST	3 (25 %)	0
Other method< ST ; MBA> ST	0	1 (12.5%)
Other method and MBA both < or > ST	9 (75 %)	7 (87.5 %)

4. CONCLUSION

The comparison between three methods used for the analysis of PSP in shellfish from South Atlantic Moroccan coasts shows that HPLC was more sensitive and more specific than MBA and RBA. This chemical method allows detection of small amount and identification of the different compounds of PSP toxins present in samples of this region. The higher costs of HPLC testing (expensive equipment, purchase frequently several types of standard PSP) and longer testing time mean that many laboratories use the mouse bioassay for investigation of PSP toxins in shellfish. As with the MBA and RBA, total toxicity can then be communicated in micrograms of Stx equivalents per kilogram of shellfish tissue. But HPLC data can also be presented as a full toxin profile.

The RBA test has allowed detecting low concentrations, use of a minimal amount of the standard and analysis of several samples, but this method requires adequate facilities for the management of radioactive waste despite the low radioactive emission of tritium used in the marking of the STX.

For the MBA, among the known drawbacks for the use of laboratory animals and the high price of standard STX frequently used to standardize this biological test, it remains effective for monitoring PSP toxins. During this PSP investigation, using MBA, no human intoxications were reported.

Concerning investigation of toxic phytoplankton, *A. minutum* was identified and confirmed as dinoflagellate responsible for the production of PSP toxins. However, the relationship between shellfish toxicity levels distribution, occurrence and abundance of vegetative cells of *Alexandrium* in southern Moroccan coasts is complex.

In order to protect the consumer from PSP shellfish poisoning, an alert system (official monitoring program) is established for stopping the collection and commercialization of shellfish. These activities are permitted when the toxin concentrations are below the sanitary threshold value until the environment is purified.

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