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Determination of PSP Toxins in Moroccan Shellfish by MBA, HPLC and RBA Methods: Links to Causative Phytoplankton *Alexandrium minutum*

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Authors' contributions

This work was carried out in collaboration between all authors. Author RA designed the study, performed the HPLC, MBA and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author AB wrote phytoplankton section and interpreted the results obtained from phytoplankton analysis. Author MYDB carried out the analysis of PSP toxins by RBA and corrected the manuscript. Author JEA managed the literature searches and data entry. Author MD analysed the phytoplankton from Dakhla bay. Authors AM, NI and MA performed the PSP toxins analyzes by MBA. Author SB supervised the work. All authors read and approved the final manuscript.

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ABSTRACT

Paralytic shellfish poisoning (PSP) toxins are secondary metabolites of the toxic species of phytoplankton. The consumption of shellfish accumulating these toxins can cause neurological symptoms and even death. Within the framework of the surveillance program of seafood safety

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along the Moroccan littoral environment established by National Institute of Fisheries Research (INRH), a study of PST was conducted from 2004 to 2016 in south Moroccan's shellfish, mussels from south Agadir region and Razor Shell from Dakhla bay. The surveillance was carried out bi-monthly or weekly using the AOAC official method of analysis (AOAC 959.08) mouse bioassay (MBA). In parallel, monitoring of toxic phytoplankton in water was conducted. With the aim to determine the shellfish toxin profile, ion-pair high-performance liquid chromatography with post-column derivatisation and fluorescence detection (HPLC-FD) was performed. The Receptor Binding Assay (RBA) also was used for determination of total toxicity of PSP toxins in Agadir's mussels.

In both regions, the analysis of seawater revealed the presence of the toxic algae *Alexandrium* spp during toxics events. Along the coast of Agadir, PSP toxins in shellfish were associated with 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 exceeded the sanitary threshold (ST) of 800 µg eq STX /kg. HPLC analysis revealed that Saxitoxin and Gonyautoxins dominated the toxin profile. The comparison between different methods showed a strong uphill (positive) linear relationship, with a coefficient correlation of r=0.79 between MBA and HPLC and r = 0.809 between MBA and RBA.

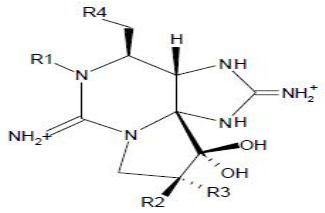
Keywords: Paralytic shellfish poisoning; Alexandrium minutum; Morocco; HPLC; RBA; MBA.

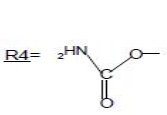
1. INTRODUCTION

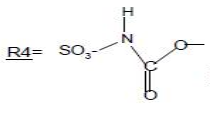
PSP toxins form a family of more than 20 molecules, chemically close to each other, all derived from Saxitoxin by substitution of various radicals of this molecule, the chemical structures are illustrated in Fig. 1. Shellfish filtering accumulates PSP toxins on algae producing toxins, mainly by dinoflagellates belonging to the genus Alexandrium, which may occur in the temperate and tropical climate regions [1,2].

PSP toxins were repeatedly detected in the bivalves of Moroccan coasts and can represent a real danger for the consumers of shellfish especially of mussels collected from natural beds along atlantic and Mediterranean coasts. The first case of intoxication by shellfish was recorded in 1961. The year 1994 was marked with many episodes of human intoxication [3,4].

From 1998 to 2016, several episodes of shellfish toxicity were reported, involving the Moroccan Mediterranean coasts [5,6,7] and in bivalves of the Moroccan Atlantic coasts [8] but no case of PSP human intoxication were reported during this period. In the aim to protect the consumer of this seafood, a phycotoxin and toxic phytoplankton monitoring system was established in the early nineties by the National Institute for Fisheries Research (INRH) along the Moroccan coasts.







R4= OH

R4=H

R_1	R_2	R_3	carbamate toxins	N-sulfo-carbamoyl toxins	decarbamoyl toxins	deoxy-decarbamoyl toxins
H	H	H	STX	GNTX5(B1)	dcSTX	doSTX
H	H	OSO_3^-	GNTX2	C1	dcGNTX2	
H	OSO_3^-	H	GNTX3	C2	dcGNTX3	
OH	H	H	neoSTX	GNTX6(B2)	dcneoSTX	doneoSTX
OH	H	OSO_3^-	GNTX1	C3	dcGNTX1	doGNTX1
OH	OSO_3^-	H	GNTX4	C4	dcGNTX4	

Fig. 1. Chemical structures of PSP toxins

The aim of this work is to present a spatio-temporal variation of toxic phytoplankton in waters and PSP toxin analysis in southern Moroccan shellfish (in mussels from the south of Agadir and in Razor Shell from Dakhla bay), the shellfish of these areas are collected for consumption by local populations. Moreover, an investigation of PSP toxin profile is presented and three detection methods were compared, the AOAC official method of analysis by mouse bioassay (MBA), HPLC-FD and the AOAC official method by receptor binding assay (RBA). The results of different methods were compared by presenting the advantages and disadvantages of each test. This is the first report concerning the use of the nuclear method (RBA) for detection of PSP toxins in Moroccan shellfish.

2. MATERIALS AND METHODS

2.1 Sampling Site

Two shellfish species were taken from two natural beds in the southern Atlantic Moroccan coasts, *mussel Perna perna* specie from Agadir coast (Dar sfint site : 29°40',404 N - 09°59',115 W) and razor shell solen marginatus specie form Dakhla bay (Pk25 site 23°54',746N - 15°46', 060W). . Mussels were collected weekly from January 2010 to December 2016, more than 362 samples were studied. 286 samples of Razor Shell were harvested from 2004 to 2010. Three kg of shellfish were taken at low tide,in parallel, 362 sea water samples were collected weekly, from the same sites, at high tide (± 2 hours of peak) with net (20 µm) and with a bottle (1 L). All samples were kept in a cooler box during the transport to the laboratory.

2.2 Toxin Analysis

2.2.1 MBA

The MBA used for analyses of PSP toxins was conducted under national monotoring. During this study, analysis were performed from 2004 to 2016 in shellfish of south of Morocco according to the AOAC method for paralytic shellfish poisons 959.08 [9] using white male mice weighing between 18 and 22 g. The mice were injected with 1 ml of extract obtained from fresh shellfish meat (100 g ± 1) extracted with 0.1 M HCl (100 ml ± 1), pH adjusted to 3 ± 0.1. The mice (3 mice per extract) were observed for 1 h, the mouse weight and mean survival time were used to calculate the toxicities expressed in µgSTX equi/kg by using a conversion factor (0.2). This factor was calculated by standardization of this method using a certified reference standard of Saxitoxin (STX) purchased from National Research Council Canada (IMB, NRCC, Halifax, Nova Scotia, Canada). 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

PSP Toxin profiles were performed by ion pair high-performance liquid chromatography with post column derivatization and fluorescence detection (HPLC/FD) in samples of Dakhla and Agadir collected during detection of PSP toxins in 2006. Analysis were based on the method of Thielert et al. [10] with modification discrebed by Yu et al. [11]. All solvents used were analytical grade, 4 ml acetic acid 0.03 M was used for 2 g of sample.

- Extraction of phycotoxins: the sample and the solvent were mixed in Vortex for 1 min and then homogenized during 10 min in an ultrasonic bath and finaly samples were centrifuged for 5 min at 140000 rpm. The supernatant was filtered by a 0.45 µm diameter filter syringe to obtain the filtrate ready for analysis by HPLC.
- Hydrolysis: For the determination of C-toxins (N-sulfocarbamates) a hydrolysis of the filtrate was performed. To 150 µl of the filtrate, 37 µl of 1 M HCl were added and the mixture was mixed in a vortex for 30 seconds and heated for 15 minutes at 90°C. The mixture was vortexed a second time for 30 seconds. Finaly the extract was neutralized with 75 µl of sodium acetate 1 N and mixed for 30 seconds to obtain the sample ready for HPLC analysis.
- Chromatography was conducted acording to conditions destripted by Diener [12]. The injection volume was 10 µl, column used was a Phenomenex type, Luna 5 µm RP-C18, 250 mm x 4.6 mm. The mobile phases were as follows: eluent A: 6 mM octanesulfonic acid, 6 mM heptanesulfonic acid, 40 mM ammonium phosphate, thepH is adjusted to 7 with H3PO4 and 0.75% tetrahydrofuran and eluent B: 7 mM octane sulfonic acid, 7 mM heptanesulfonic acid, 48 mM ammonium phosphate, the pH adjusted to 7 with H3PO4 and with 1% tetrahydrofuran and 10% acetonitrile. The flow rate was 1 ml/min.

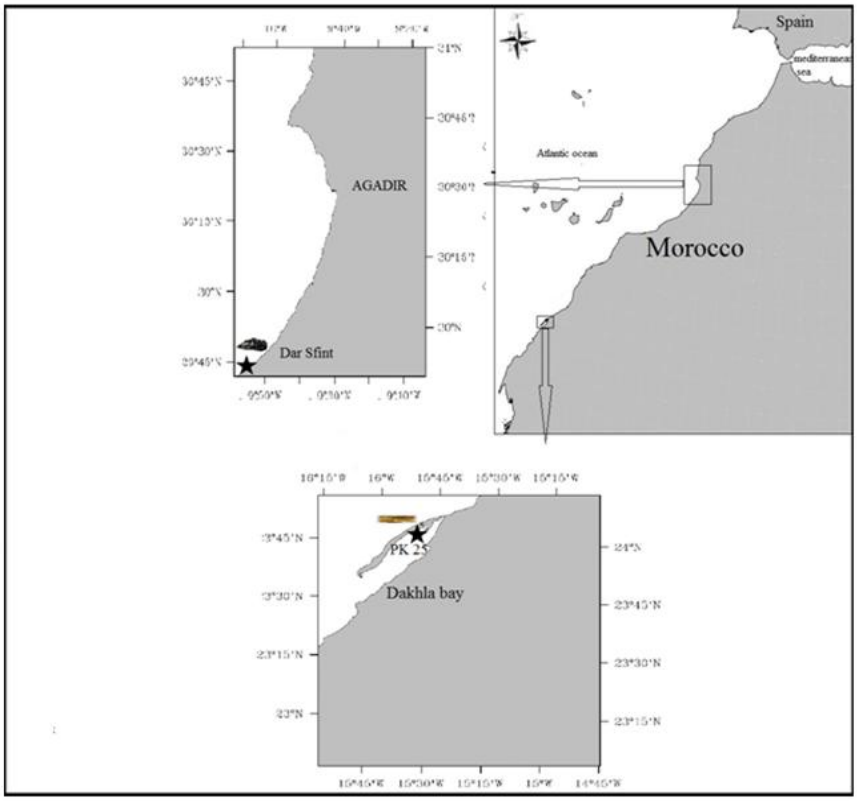


Fig. 2. Sampling sites

- The temperature set for the post-column derivatization was 50°C, a solution containing 10 mM periodic acid and 550 mM NH3 solution with a flow rate of 0.3 ml / min was used, pH was reduced to 2-3 by 0.75 M nitric acid with a flow rate of 0.4 ml/min. Detection wavelengths were set at 330 nm for excitation and 390 nm for emission. STX, neosaxitoxin (NEO), and 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.

2.2.3 Receptor binding assay RBA

The extracts of Agadir's samples taken during toxics events from 2010 to 2016 were analyzed following the procedure of the AOAC OMA 2011.27. Extracts obtained following the AOAC 959.08 method were filtered using a 0.45 filter paper and stored at -20°C. These analyzes were conducted at the Environment Laboratories of the International Atomic Energy Agency in Monaco. 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 toxin in 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, 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 to Uthermol method [14]. The determination was made through appropriate systematic identification keys. The counting was performed with horizontal scanning. The results densities were expressed as number of cells per liter (cells*L^{-1}).

2.2.5 Comparaison between methods

For the comparison between the RBA and MBA methods, the Agadir sample extracts taken during toxic events from 2010 to 2016 (number of samples n = 8) were analyzed by MBA and the same samples were filtered and analyzed by RBA. and for the comparison between HPLC-FD and MBA, Dakhla shell razor samples collected during the 2006 toxicity period (number of samples n=12) were analyzed by both the bilological and chemical methods. Results of two methods (RBA - MBA or HPLC - MBA) were compared by statistical analysis (Mean ratio RSD %, Linear regression slope equation, Pearson correlation coefficient).

3. RESULTS AND DISCUSSION

3.1 MBA Analysis and Toxic Species of Phytoplankton Investigation

The results of MBA analysis in Agadir mussels showed that five episodes of toxicity were recorded during this study from 2010 to 2016. Toxins were detected in 6.62% of the samples (23 MBA positive on a total of 362 samples) (Fig 3). The longest period of mussels toxicity was recorded by this biological method in 2012 with 7 weeks against the shortest one was noted in 2013 (1 week). But, the highest concentration of this toxin was recorded in 2015 with 7068 µg eq STX/kg.

The monitoring of toxic phytoplankton *Alexandrium* sp showed a significant spatial and temporal variability. Indeed, the species were present at low concentrations between February 2010 and September 2011 to increase in winter 2011. The maximum concentration reached $7.2*10^3$ cells*L^{-1} was recorded in the fall. *Alexandrium* sp remain in waters with low densities but in the late spring and early summer of 2012 high concentrations were recorded with values reaching $2*10^3$ cells*L^{-1}, then they proliferate with a maximum of $4*10^3$ cells*L^{-1} in July 2012, after this period no detection of this species until the spring 2014 with a maximum in June of $1.5*10^3$ cells*L^{-1}; after this species remain present throughout the year in superficial waters, but at low concentrations ranging between 10^2 and $9*10^2$ cells*L^{-1}. In the spring of 2015, *Alexandrium sp* reached concentrations around $8*10^2$ cells*L^{-1}at July to mark a major efflorescence in August with $25*10^3$ cells*L^{-1}, bloom spread through the waters until the beginning of fall 2015 (since October). The concentrations of *Alexandrium sp* fell gradually to $7*10^2$ and 40 cells*L^{-1}, recorded respectively in spring (March) and summer (July) 2016.

The analysis of PSP toxins by MBA shows that many samples contain high levels of toxins compared to the regulatory limit (800 µg STX equi/kg), maximums noted were 845, 1145, 357, 2109, and 7068 µg STX equi./kg respectively in

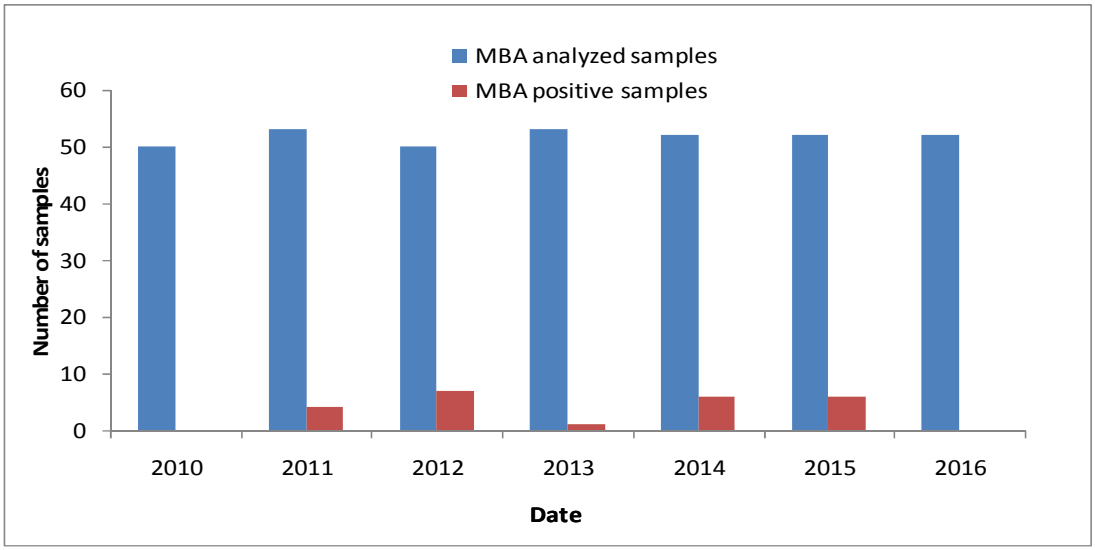


Fig. 3. MBA positives samples in southern Agadir coasts 2010 to 2016

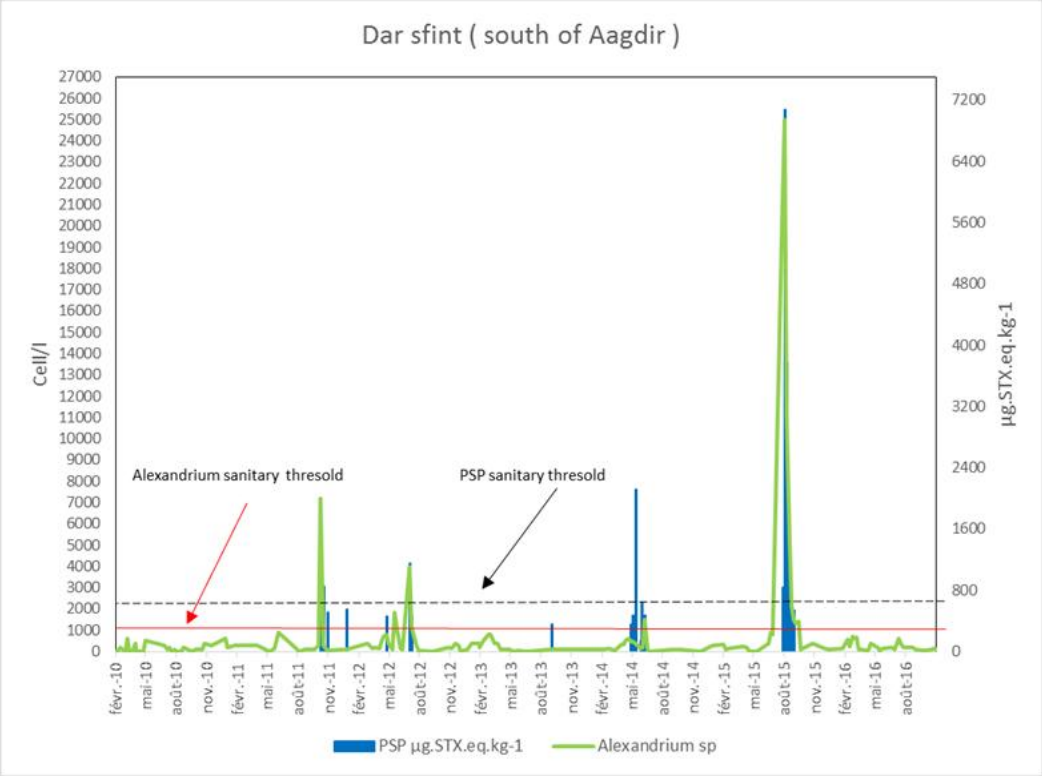


Fig. 4. Variation of *Alexandrium* spp concentrations in sea waters and PSP toxins by MBA in Agadir's mussels (Dar sfint area) (2010- 2016)

fall 2011, in summer 2012, in fall 2013, in the end of spring 2014 and in summer 2015 (Fig. 4). This toxicity is explained by the high concentrations of *Alexandrium* sp recorded in seawater during these episodes.

At the Dakhla bay (Pk25 area), the MBA analysis of PSP toxins in shellfish showed that one period was recorded from 2004 to 2010, where Razor Shell was toxic for 5 weeks, toxins were found in 2.09 % of total analyzed samples (Fig. 5). No case of PSP toxins was detected before winter 2006; in December 2006, the toxicity was exceeded sanitary threshold with a maximum of 1215 µg STX equi/kg, in general, from October to November 2006 the concentrations of PSP toxins were near or below the sanitary threshold between 395 and 600 µg STX.equi/kg. From January 2007 to December 2016, PSP toxins were not recorded in Razor Shell. Toxins were detected in fall, during this season the temperature was high in this region between 18 and 20°C [15]. Toxic phytoplankton observation conducted from 2004 to 2006 showed that *Alexandrium* sp concentrations in seawater recorded 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 cells*L^{-1}) in May and June the concentrations exceeded the health threshold of *Alxendrium* sp (10^3 cells*L^{-1}) with $2.5*10^3$ cells*L^{-1}, after the concentrations increased sharply in fall-winter 2006. The maximum value reached $49.4*10^3$ cells*L^{-1} registered in November 2006 (Figs. 5, 6). The detection of this toxic dinoflagellate coincided with the shellfish toxicity of this bay.

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 (harmful algal blooms) appears to be increasing on a global scale due to rising ocean temperatures and growing coastal eutrophication [16]. The species from the genus Alexandrium, detected during the episodes of shellfish toxicity from 2004 to 2016, have been known as producers for the neurotoxins PSP, which is one of the most dangerous marine toxins known [17]. This genus

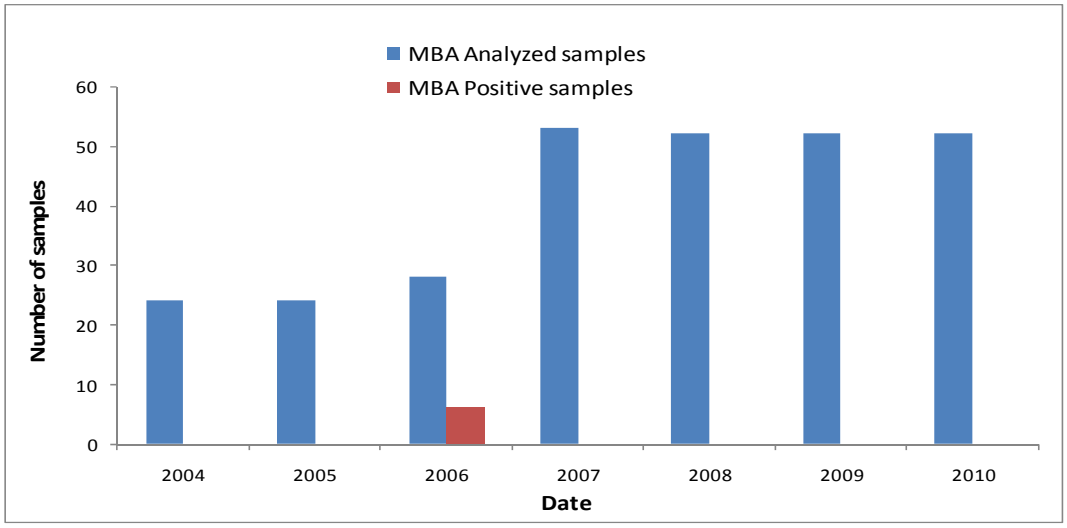


Fig. 5. MBA positives samples in Dakhla bay coasts 2004 to 2006

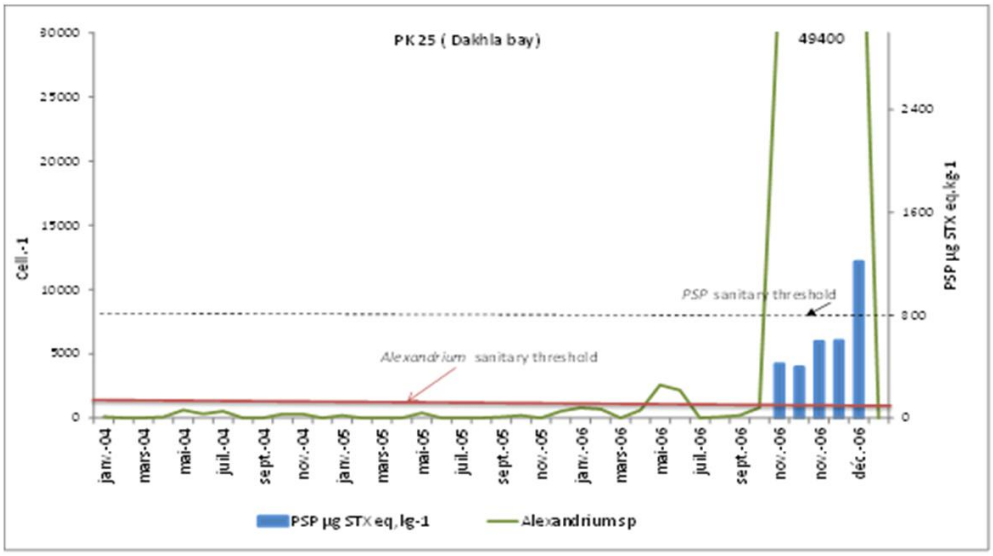


Fig. 6. Variation of *Alexandrium spp* concentrations in sea waters and PSP toxins in Razor Shell by MBA Dakhla's Razor Shell (Pk 25 area) (2004- 2006)

is most often associated with this type of toxins, as about 10 of the approximately 30 Alexandrium species are PSP toxins producer [18]. The Alexandrium species was identified as *Alexandrium minutum* and confirmed in the seawater of the southern region of Agadir (Dar sfint) and pk25 (Dakhla bay) by scanning microscopy and epifluorescence microscopy [19]. This species is primarily an inhabitant of environments with a high terrestrial influence, such as lagoons, bays and estuaries where nutrient levels are high, the water column is stratified, and disturbance low. In spite of this, *Alexandrium minutum* is able to grow in coastal areas where land-derived influences are low. An important factor in Alexandrium's presence in coastal areas is the requirement for a shallow water depth, as cysts which provide the seed population needed to initiate a bloom must be exposed to light for germination. In this study, these conditions were more widely present in Dakhla bay than in Dar sfint area which could explain why *Alexandrium* sp never dominated algal community and were presented the very low density and increase only in the bloom period. Also, At Dar sfint waters, the net sample

presented some temporary cysts of *Alexandrium* sp suspended in the water column. The annual and inter annual variation of *Alexandrium* sp bloom occurrence may be due to variations in the hydro-climatic conditions of coastal water in this region, where the water is warmer (17 to 22°C). The warmer period in the south of Atlantic Moroccan coasts starts generally from June to September [20]. *Alexandrium minutum* have preferential for more temperate waters. Statistical studies on all bloom of *Alexandrium minutum* highlight the evidence that temperature is a major factor controlling this efflorescence.

In many countries MBA is used for many years as an official method for PSP toxins monitoring for protection of the public health against this type of intoxication [21,22]. The results obtained from both the *Alexandrium* sp cell counts and the MBA analysis suggest a clear relationship between the high cell counts recorded and high concentrations of PSP toxins in shellfish. The shellfish toxicity events coincide with the typical "bloom season" for *Alexandrium* sp which generally occurred in summer and fall, but with high inter-annual variability. The same results were recorded by Guallar-Morillo [23] who reported that the period of appearance of *Alexandrium* sp was often between the end of spring and early summer, it's the time niche and risk period of PSP toxins contamination.

3.2 HPLC-FD Analysis

HPLC-FD analysis confirms the presence of PSP detected by MBA, the amounts detected did not exceed 511 µg STX.equi./kg. The PSP toxin profile of Dakhla's Razor Shell 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 (Figs. 7, 8).

In Both Agadir coast and Dakhla bay, carbamate 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* collected from Cap Town in South Africa, this species had a toxin profile dominated by gonyautoxins [24]. Generally, the shellfish toxin profile is very close to that of ingested phytoplankton [25]. This dinoflagellate was detected in sea water during all toxic event in Agadir coast and Dakhla bay from 2004 to 2016 [19,26], what leads us to implicate *Alexandrium minutum* in PSP toxicities of shellfish of both regions However several studies reported that this toxic specie of dinoflagellate was implicated in PSP toxin events [27,28,29].

3.3 Comparison between the Different Methods Used for PSP Toxin Analysis

Figs. 9 and 10 illustrate respectively the correlation between toxicities of HPLC and MBA and that between RBA and MBA. The first information obtained there is a strong positive correlation between the two different non-animal methods against the reference MBA; the

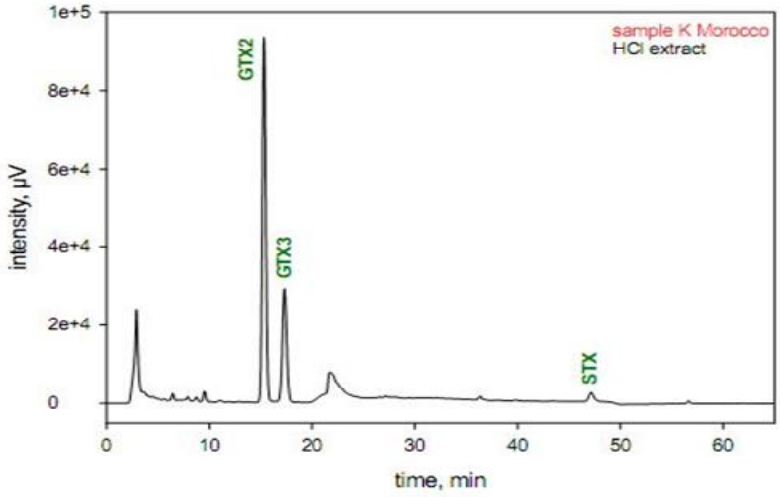


Fig. 7. PSP toxin profile of Agadir's mussels on June 2006

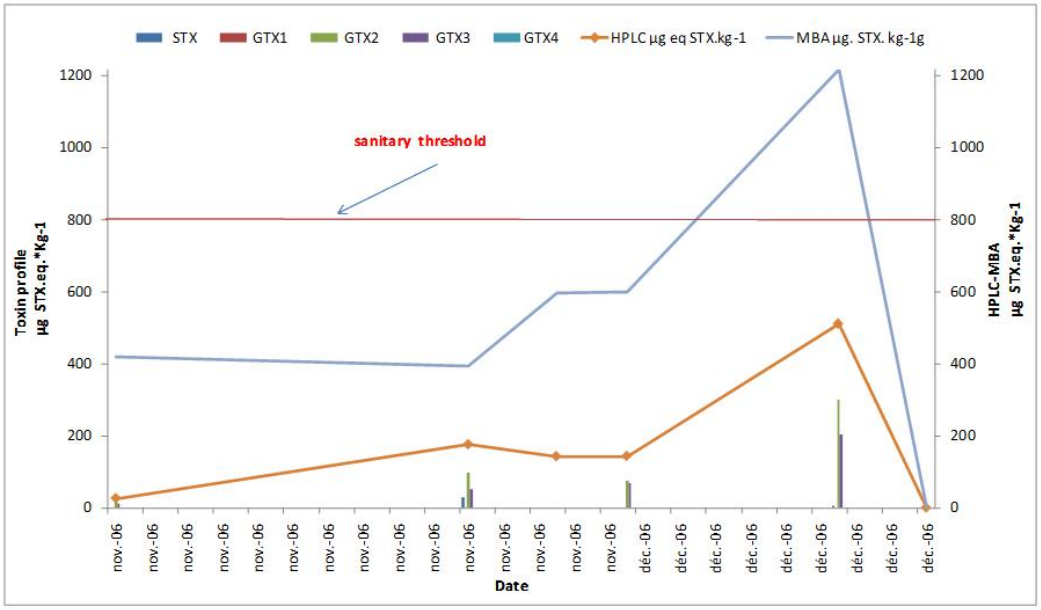


Fig. 8. PSP by MBA and HPLC with toxins profile of Dakhla's Razor Shell during toxic event of end 2006

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 [30]. 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 high linear correlation was found r = 0.809, this positive correlation means that the concentrations detected by the two methods vary in the same way, which means that there is a great resemblance in the results of those two methods. For high concentrations (above the sanitary threshold), the RBA reported no false results, with regard to the low concentrations, some small differences between the results of the two methods were found; this is probably due to the very high sensitivity of the radioactive assay to detect very low concentrations below the limit of detection of the bioassay.

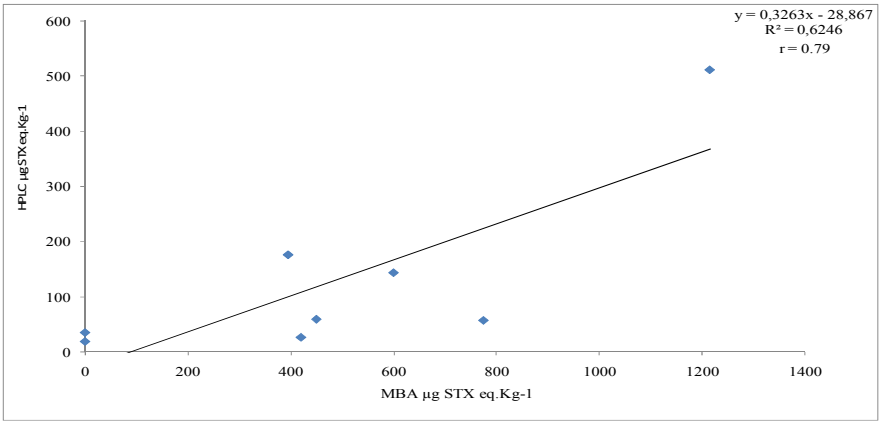


Fig. 9. Correlation between MBA and HPLC-FD method for analysis of PSP in mussel (Number of *compared samples* = 8)

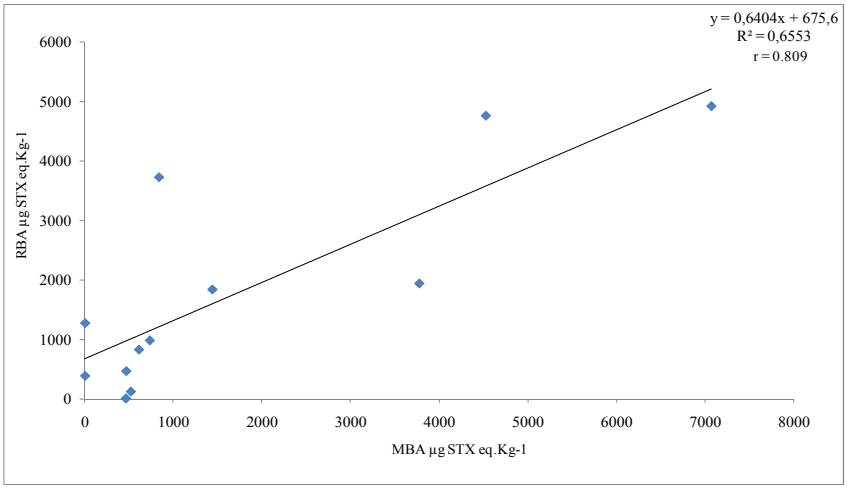


Fig. 10. Correlation between MBA and RBA method for analysis of PSP in mussel (Number of *compared samples* = 12)

Table 1. Summary of results in mussels and Razor Shell comparing HPLC-FD and RBA against the reference MBA (ST: Sanitary threshold, RSD: Relative standard deviation)

	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 toxins in shellfish from the outhern Atlantic Moroccan coasts shows that HPLC was more sensitive and more specific than MBA and RBA. This chemical method allows detection of a 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. The three methods MBA, HPLC and RBA report the total toxicity in µg STX equi/kg, but the advent of the chemical method, a complete toxin profile is obtained as well as the concentration of each component present in sample. The RBA test has allowed detecting low concentrations, with the 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 by using bioassay, no human intoxications were reported.

Concerning investigation of toxic phytoplankton, *Alexandrium 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 sp* in the southern Moroccan coasts is complex.

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

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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