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Lipophilic Toxin Profile in *Mytilus galloprovincialis* from the North Atlantic Coast of Morocco: LC-MS/MS and Mouse Bioassay Analyses

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Abstract: ***For the Moroccan Phycotoxins Monitoring that is part of the Safety of the Coastal Monitoring Network (RSSL), shellfish samples were harvested from different locations at North Atlantic of Morocco where harmful algae blooms were known to have occurred. For all shellfish samples found positive by the mouse bioassay for diarrheic shellfish poisoning (DSP) toxins, liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) in order to search the following lipophilic toxins: okadaic acid (OA), dinophysistoxins (DTXs), pectenotoxins (PTXs), azaspiracids (AZAs), yessotoxins (YTXs), spirolides (SPXs) and gymnodimines (GYMs). The results revealed different lipophilic toxin profiles as a function of the shellfish sampling location. It has been noticed that all the samples contained OA and its derivatives named dinophysistoxins (DTXs). In addition, other lipophilic toxins were found in shellfish samples: (YTXs), and pectenotoxins (PTX-2, PTX-2-seco-acid and 7- epi-PTX-2-seco-acid) on the North Atlantic coast .This paper reports on the first detection of YTXs , PTXs GYMs and their derivatives in Moroccan shellfish.***

Keywords: diarrheic toxins, lipophilic toxins, LC-MS/MS, mouse test, shellfish

1. Introduction

Lipophilic toxins is a severe gastro intestinal illness caused by consumption of seafood contaminated with toxigenic dinoflagellates [1] such as certain species of the genus Dinophysis, Prorocentrum, Lingulodinium and Azadinium algae. According to the European Commission, the lipophilic toxins are subdivided into four distinct families: acidic toxins, including okadaic acid (OA) and its derivatives named dinophysistoxins (DTXs) which were identified in toxic shellfish[1,2,3], neutral toxins consisting of polyether-lactones of the pectenotoxin group (PTXs) [4], sulphated compounds called yessotoxin and its derivatives (YTXs) [5,6]. YTXs have now been categorized separately because they do not induce diarrhea [7], and azaspiracids (AZAs) [8,9].

Other marine toxins were also identified – both spirolides (SPXs) and gymnodimines (GYMs) in lipophilic extracts of shellfish used for diarrheic toxin detection by mouse bioassay according to the European Union Reference Laboratory for Marine Biotoxins (EU-LR-MB) method [10]. These toxins have caused the rapid death for mice after intra peritoneal injected by lipophilic extract with neurotoxic symptoms (fast acting toxins, FAT). GYMs have been detected in shellfish in association with Karenea selliformis firstly in New Zealand [11,12] and subsequently in Tunisia [13]. In Morocco, among the toxic microalgae found along Moroccan coasts, four genera: Dinophysis, Gymnodinum, Alexandrium and Pseudo-nitzschia, have been specifically monitored [14, 15]. Phytoplankton and Phycotoxins Monitoring in charge of the Moroccan shellfish monitoring program, performs toxicity analysis on bivalves. Lipophilic toxins (LSP), are monitored weekly by Marine biotoxins laboratory using the mouse bioassays.

In 1998, the mouse bioassays test revealed the presence of diarrheal toxins (DSP) in Moroccan bivalves. The analysis of sea water of these regions showed the efflorescence of the species Lingulodinium polyedrum in the region of El Jadida causing a red color brick seawater (red tide). This species has been accompanied with Dinophysis acuta and Dinophysis accuminata [16]. Since 2003, DSP contamination was very important, and diarrheic toxins were detected in mussels, clams and oysters samples along the Moroccan Atlantic Littoral from El Jadida to Dakhla [17].

In Morocco, the latest works have shown the detection of these toxins in the North and South coast [18,19,20], the main identified toxins were the DTX -2, AO , DTX -1 as well as AZPs. The control of lipophilic toxins in shellfish samples harvested at monitoring areas of the North Atlantic of Morocco, showed the high concentrations of okadaic acid and its esters, with presence for the first time the new lipophilic toxins. Currently, a mouse bioassay is used in the Moroccan Monitoring Program.

In this study, we report the detection of lipophilic toxins in

mussels from the North Atlantic coast of Morocco during the period between 2014 and 2015 by using two methods: mouse bioassay and liquid chromatography-Tandem mass spectrometry (LC-MS/MS) for confirmation in the cases of positive results via the mouse bioassay. Currently, a mouse bioassay is used in the Moroccan Monitoring Program. The LC-MS/MS technique was performed at Ifremer Phycotoxins laboratory in Nantes. The results showed the high concentration of okadaic acid and its esters, with presence for the first time the new lipophilic toxins.

The main objective of this work was to evaluate the presence of lipophilic marine toxins in shellfish harvested from North Atlantic and to characterize the lipophilic toxin profile in positive shellfish samples by mouse bioassay.

2. Materials and Methods

2.1. Collection and preparation of raw bivalve molluscs samples

During 2014-2015, several shellfish samples were collected weekly at different production areas along the North Atlantic Coast of Morocco between El Jadida and Essaouira.(Fig.1) The shellfish samples were Mytilus edulis galloprovincialis. A minimum of 30 g of hepatopancreas (HP) of each samples was homogenized in a blender. 20 g were used for the DSP mouse bioassay according to the modified method of EU-LR-MB [10]. The remaining 10 g of HP homogenate were later used for LC-MS/MS analyses for mousse bioassay (MBA) positive samples.

2.2. Biological analysis for lipophilic toxins

2.2.1. Extraction procedure

Extractions were carried out according to the method of European Union Reference Laboratory for Marine Biotoxins (EU-LR-MB) [10]. The extraction from 20 g ± 0.2 g of HP was performed with 100 ml of acetone (99% or greater purity) at 3360×g with a homogenizer "Ultra-Turax" for 2 min, the mixture is filtered through paper for 10 minutes. Acetone is evaporated (temperature of the water bath must be maintained at 42°C ± 2°C). A volume of 10 ml of distilled water is added in the evaporation flask, followed by extraction the toxins with 3 times 50 ml of dichloromethane (DCM). The three organic extracts collected after separating via reparatory funnel are washed 2 times with 20 ml of distilled water. DCM is evaporated and the resultant residue in evaporation flask is suspended in a 4 ml aqueous solution of 1 % Tween.

2.2.2. Mouse bioassay

A volume of 1ml of the final extract (toxins + Tween 1%) is inoculated to each of three mice weighing 19g- 21g and observed for 24 hour. The bioassay is regarded as positive if at least two out of three mice die.

2.3. LC-MS-MS analysis of lipophilic toxins

2.3.1. Extraction procedure

In accordance with the European method validated internally Ifremer [21], lipophilic toxins were extracted from 2.00 g±0.05g of hepatopancreas homogenate of shellfish. Then the homogenate was extracted in duplicate with 9 mL methanol.

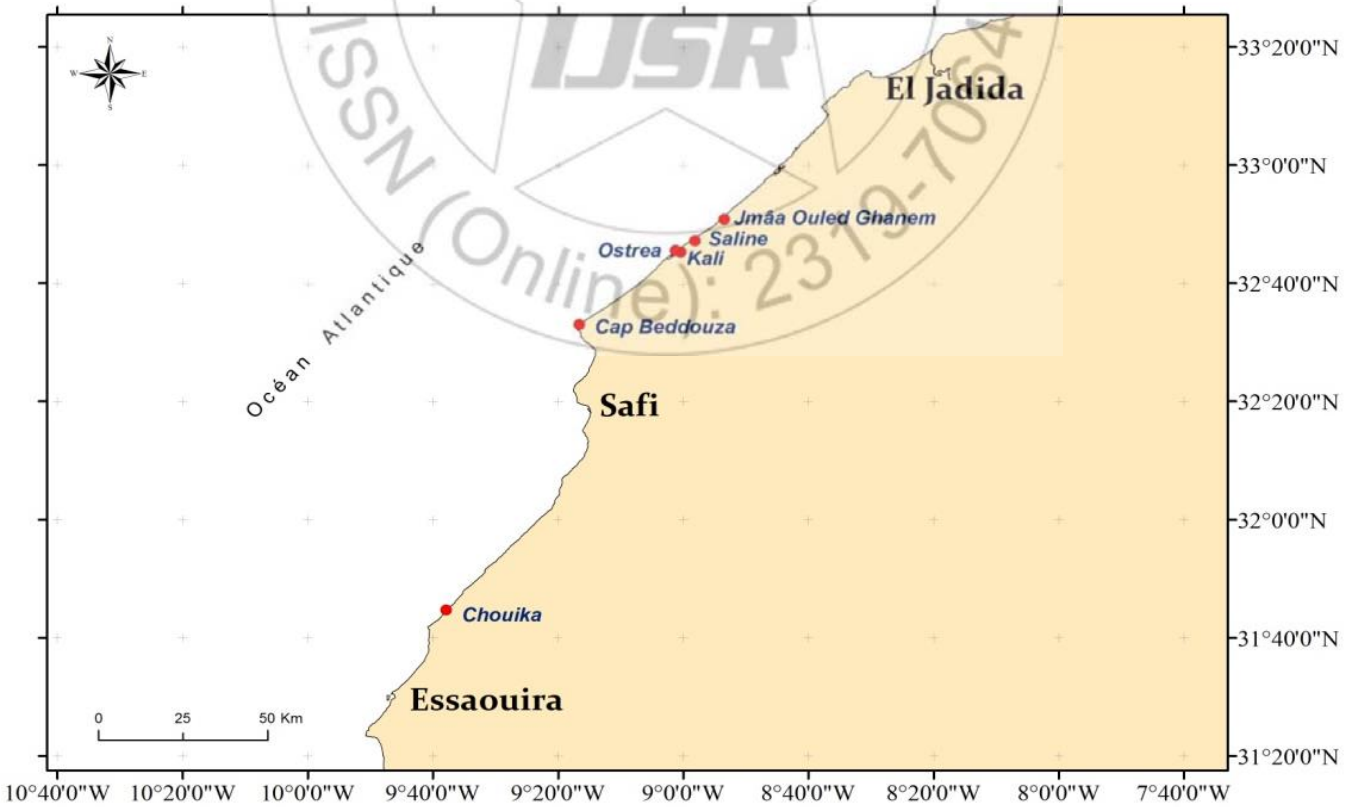

Figure 1: Location of sampling sites

After the first addition of methanol, the mixture was homogenised with an UltraTurax mixer (IKA Works, Wilmington, NC, USA) for 3 min at 10,000 rpm and the extract was centrifuged for 8 min at 8000 g, after the supernatant was transferred to a 20 mL volumetric flask. The second addition of methanol, the mixture was homogenized with an UltraTurax, and centrifuged for 8 min at 8000 g, after which the supernatant was combined with the first extract and methanol was added to reach a final volume of 20 mL.

One aliquot was used for lipophilic toxin analysis (with the exception of "DTX-3" acyl-esters), and a second aliquot for DTX-3 alkaline hydrolysis to release OA and/or DTX-1 and/or DTX-2, and subsequent analysis of DTX3.

2.3.2. LC/MS-MS analyses of lipophilic toxins

For each shellfish sample, LC-MS/MS analyses were performed on two aliquots (before and after hydrolysis). 5 µl of each aliquot was injected into the LC-MS/MS system using an LC system (UFLC XR, Shimadzu) coupled to a hybrid triple quadrupole/linear ion-trap mass spectrometer (API 4000 Qtrap, AB SCIEX) equipped with a Turbo V ion spray® source. Toxins were separated in a 3 µm MOS-Hyperclone C8 column (50 mm x 2.0 mm id, Phenomenex) at 25°C.

The electrospray ionization interface (ESI) was operated in both positive and negative modes using the following parameters: curtain gas: 20 psi (PM) and 30 psi (NM) ; temperature: 50 °C (PM) and 550°C (NM) ; gas 1: 50 psi (PM) and 40 psi (NM); gas 2: 50 (PM) and 50 psi (NM) (PM); CAD gas: medium; Ion spray voltage: 500V (PM) and -4500 V (NM); EP: 10 V (PM) and -13 V (NM). These parameters had been previously optimized using toxin standards. The mass spectrometer was operated in multiple reaction-monitoring (MRM), analyzing the two or three most intense product ions per compound. For ESI positive, the transitions selected were: [M+NH4] ions: PTX-2, m/z 876.5>823.5/841.5/805.5; PTX-2-sa and 7-epi-PTX-2-sa, m/z 894.5>823.5/805.5; [M+H] ions: SPX-13-desMe-C (SPX-1), m/z 692.5>444/164; SPX-A: 692.7>444/150; GYM-A, m/z 508.5>490.5/392.5, AZA-1, m/z 842.5>824.5/806.5/672.5; AZA-2, m/z 856.5>838.5/820.5; GYM-B: m/z 524.4>524.4/506.4; AZA-1, m/z 842.5>824.5/672.5/654.4; AZA-2, m/z 856.5>838.6/672.4/654.4; AZA-3,m/z 828.5>8792.5/658.4/640. For ESI negative mode, the transition selected was: [M–H] ions: OA and DTX-2, m/z 803.4>255.1/113.1; DTX-1, m/z 817.5>254.9/112.9; YTX, m/z 1141.5 >1061.5/855.5; Homo YTX, m/z 1155.5>1075.5/869.4; 45-OH-YTX, m/z 1157.5>1077/855.5; 45-OH-HomoYTX, m/z 1171.5> 1091.5/869.4.

The mobile phase was 100% water containing 2 mM ammonium formate, and 50 mM formic acid (channel A) and acetonitrile/water (95/5) containing 2 mM ammonium formate and 50 mM formic acid (channel B).

Certified reference materials provided by IMB-Halifax, NS, Canada: standard solutions of OA, DTX-2, DTX-1, AZA-1, AZA-2, AZA-3, YTX, PTX-2, SPX-13-desMeC and GYM-A. In addition, a blank and positive quality control (QC) prepared during extraction of the samples set.

3. Results and discussion

The mussel samples harvested during 2014 and 2015 at some monitoring areas from the North Atlantic of Morocco (Jamaâ Ouled Ganem, Ostréa, Cab Beddouza and Chouika) (Fig.1) were analyzed for the search of lipophilic toxins using the mouse bioassay (MBA). The positive mussel samples were analyzed by the chemical method (LC-MS-MS).

3.1 Mouse bioassay results

During the outbreak DSP, shellfish samples tested positive by mouse bioassay concerned especially mussels Mytilus galloprovincialis.

In 2014, Ostrea (OST) and Jamâa Ouled Ghanem (JOG) mussels were affected by diarrheic toxins (DSP) during autumn with the percentages of contaminated samples received monthly are respectively 25% and 75% (Fig.2).

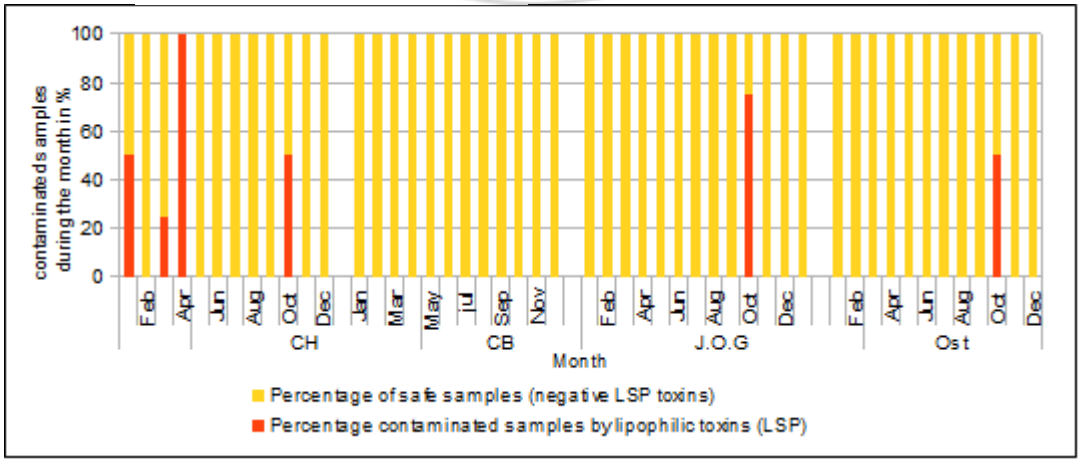

Figure 2: Seasonal evolution of percentage of contaminated samples collected in different areas from the North Atlantic Coast during 2014.

DSP outbreak in Chouîka (CH) was recorded in winter, spring and autumn. A maximum of the 100% of samples harvested at Chouika were affected in spring (April) (figure2).

In 2015, mussel production areas (CB, JOG, OST and CH) were affected by short episodes toxic LSP, and for each site, contaminated shellfish samples was not exceed 25 % of the total analyzed during the month. These episodes were occurred during summer and autumn in Cap Beddouza and in Ostrea, during summer in Jamâa Ouled ghanem (JOG), and spring, autumn in Chouîka (CH) (Fig.3). Ostrea (OST), Cap beddouza (CB), Jamaâ Ouled Ghanem (JOG) and Chouika (CH). LC/MS/MS results showed the presence of different lipophilic toxins (LSP) in mussel samples tested positive to the mouse bioassay. These toxins are especially OA and/or DTX-2, AZAs, PTXs YTXs, and GYMs (low quantities). The European Union (EU) has set a regulatory limit of 160 µg/kg for the sum of OA, DTXs, and pectenotoxins; 160 µg/kg, for AZAs [22] and 3.75 mg/kg for YTX [23].

Table.1 shows the lipophilic toxin profiles and maximum

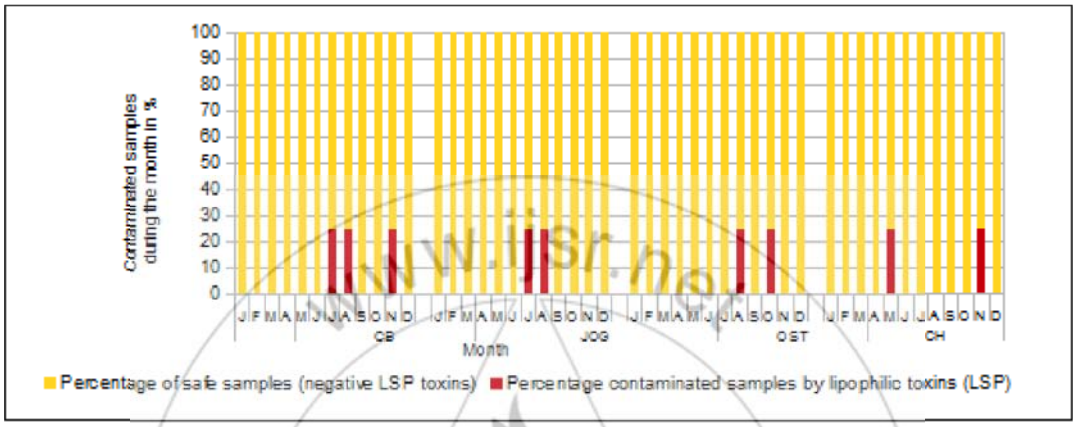

Figure 3: Seasonal evolution of percentage of contaminated samples collected in different areas from the North Atlantic Coast during 2015

3.2. LC-2.2 LCMS/MS results

Mussel samples testing positive via MBA were analyzed by LC-MS-MS-based approach which enabled us to investigate the presence of OA together with a wide range of lipophilic toxins distributed in the coastal region between El Jadida and Essaouira from Morocco with different location:

Table 1: Maximum amount of toxins in µg/kg of whole tissue found during 2014 and 2015 Monitoring program

North Atlantic coast				
Regions	El Jadida		Safi	Essaouira
Sites	OST	JOG	CB	CH
Species	Mussels (*M. galloprovincialis*)			
Lipophilic toxins				
AO free	1047	1801	1047	598
AO total (free and related forms)	1580	4114	215	1770
DTX2 free	<LQ	<LQ	<LQ	61
DTX2 total	<LQ	36	<LQ	70
AZA1	<LQ	<LQ	<LQ	<LQ
AZA2	223	399	210	17,9
YTX	53	38	38	52
Homo-YTX	117	82	101	527
45-OH YTX	37	<LQ	<LQ	48
45-OH Homo YTX	93	82	61	442
GYMA	<LQ	<LQ	<LQ	5,6
SPX desMe C	<LQ	<LQ	<LQ	<LD
PTX2sa	<LQ	8	<LQ	10,5
PTX2sa épi	<LQ	8	<LQ	6,8
COOH YTX	162	85	73	173
COOH HOMO-YTX	310	239	100	878

amounts obtained from mussels analyzed in three regions during the 2014-2015 monitoring program at North Atlantic coast. The toxicity profile varies as a function of the region: El Jadida (JOG and OST), Safi (CB) and Essaouira (CH). The limits of quantification (LOQ) and limits of detection (LOD) of some lipophilic limit of toxins are showed in table 2. During 2014 and 2015, OA (free or esterified) were found in mussels in four sites (OST, CB, JOG, CH) with the high concentrations, 215 to 4114 µg/Kg during autumn, exceeding the public health safety threshold of 160 µg/ Kg of edible tissues, which was associated with mouse bioassay positive results (Fig.2, Fig.3, Fig.5). The contamination depended on the period of collection and the highest level of DSP was registered in October, while the lowest level was found in July. During the toxic season, the percentage of DTX2 was low of total LSP toxins (Figure 5). OA, either free or esterified was the main toxin contaminating northern Atlantic molluscs in accordance with previous reports [16, 24, 25].

Table 2: Quantification limits (LOQ) and detection limits (LOD) for some lipophilic toxins

µg/Kg of edible tissues	LOD	LOD
OA+DTXs	10	30
PTXs	2	6
AZAs	2.5	7.5
YTXs	10	30
SPXs	2	4
GYMs	2	4

Figure 4 shows an example of LC-MS/MS analysis of mixture of lipophilic toxins multi-standards in negative mode.

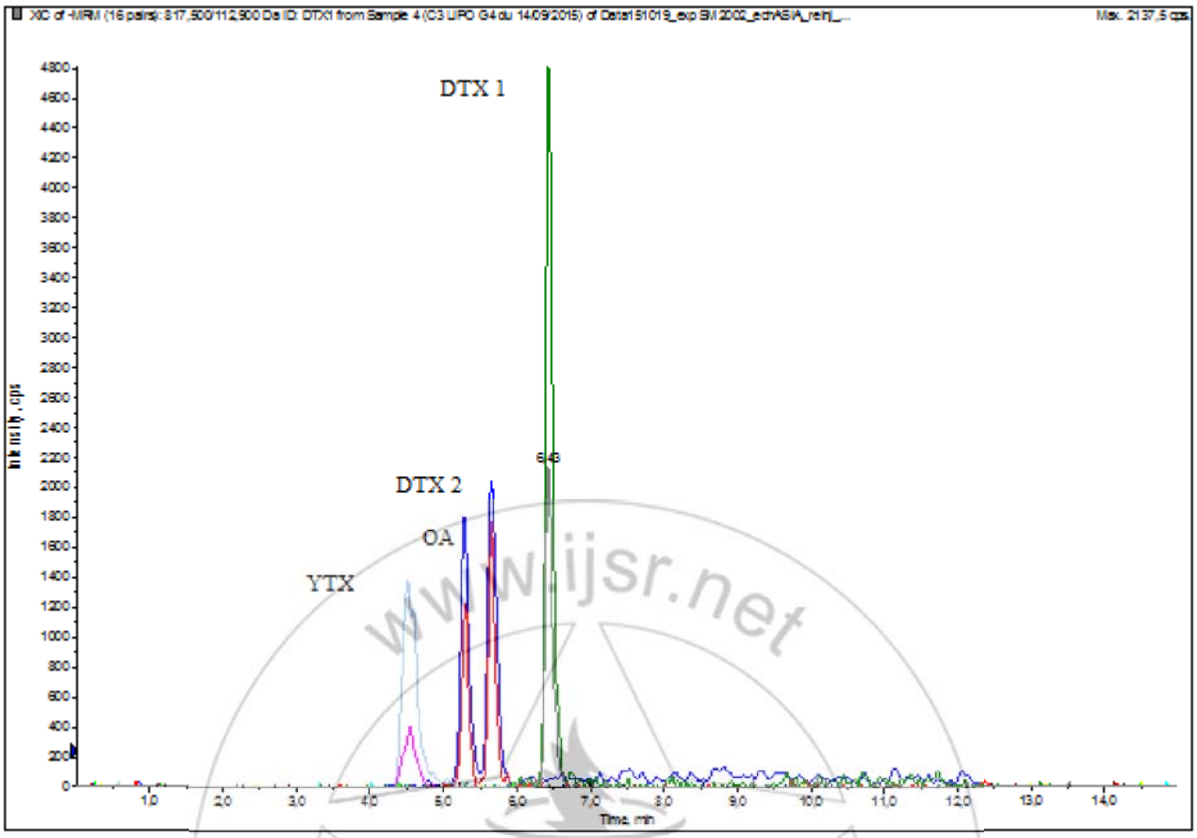

Figure 4: Example of analytical results in LC-MS / MS of a mixture of lipophilic toxins multi- standards : negative mode

DTX2 was also found in a low percentage of samples and the level registered did not exceed the safety threshold, very below to those of OA, according to authors who had detected the amounts of DTX2 in Morocco [19], in Galicia and Portugal [24,26,27]. Generally DTX2 is produced by D. acuta, and D. acumunata which typically appears in autumn on the Moroccan coast [20,16] and on the Galician coasts [28]. DTX-2 was present in both forms (free or esterified) in mussels harvested in Chouika and except in form esterified in mussels from Jamâa Ouled Ganem.

The presence of OA and DTX-2(free or esterified) could be related to the presence of D.acuta, and D. acumunata which was present in this episode but at low concentration not exceeded the statutory threshold [29].

LC-MS/MS technique identified the presence of PTXs (PTX2sa and epi-PTX-2-sa) for the first time in Morocco in mussels from Chouika and Jamaâ Ouled Ghanem during autumn (October). The concentrations of PTXs were very widely above the statutory thresholds (160 µg eq OA/Kg of shellfish tissue) (Fig.6).

PTX-2-sa and 7-epi-PTX-2-sa are the transformation products of PTX-2 via enzymatic hydrolysis [30]. The absence of PTX-2 could be explained by its complete conversion into PTX-2-sa and 7-epi-PTX-2-sa.

This contamination of shellfish by PTX-2 derivatives could be linked to the presence of D.acuta and D. acuminata, which is known to produce PTX-2 [31, 32].

Additionally, other regulated lipophilic toxins which can give positive DSP Mouse Bioassay like AZAs (AZA 2), were detected in mussels from all sampling sites at levels more significant 17.9-399 µg/kg. The maximum values were recorded during the summer (fig.6).

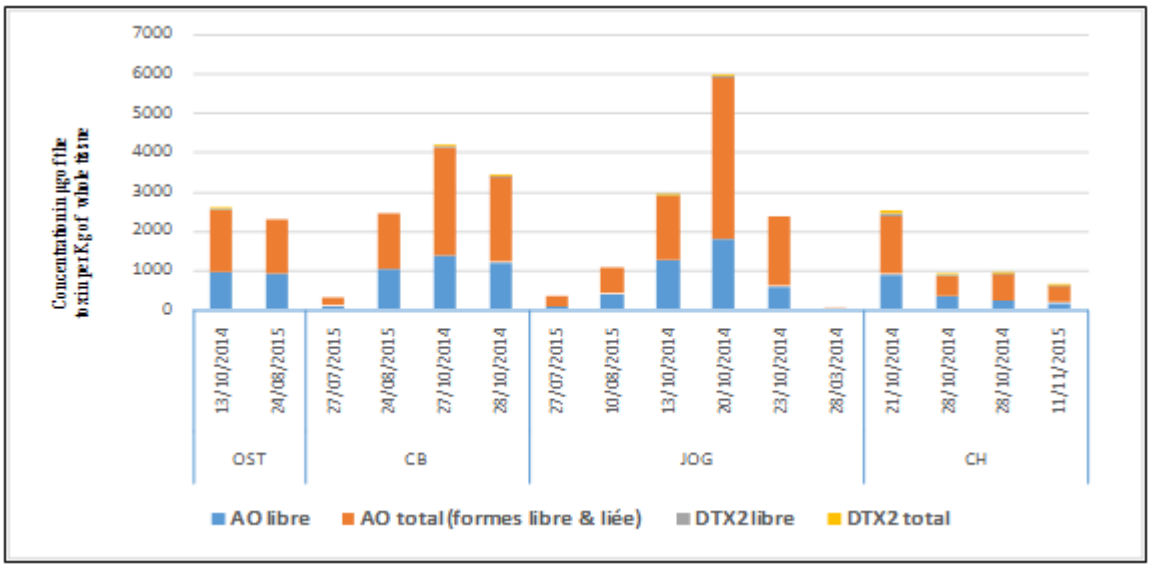

Figure 5: The spatiotemporal evolution of OA free and DTXs toxins in mussels during 2014-2015 monitoring programme

The presence of AZA2 as the dominant form of azaspiracid family in mussels collected in the Atlantic Coast of Morocco was reported for the first time during the summer of 2004 and 2005[18]. So far, AZP has been found only in northern

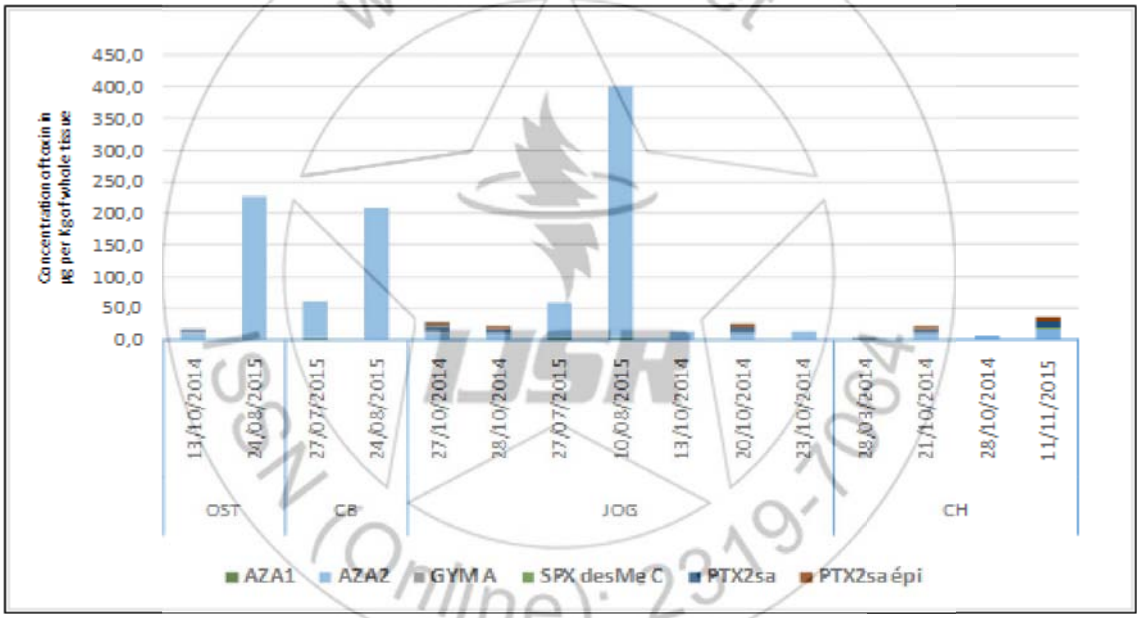

Figure 6: The spatiotemporal evolution of AZAs, GYMs and PTXs toxins in mussels during 2014-2015 monitoring program

European costs such as Ireland, England, Norway, and France, as well as in Galicia [33,34,35] and in Northwest coast of Portugal [27]. Since, dinoflagellate Azadinium *spinosum gen* and sp *nov. (Dinophyceae)* [36] which are identified as AZP source, have not been observed at abnormal concentrations in water column at this period.

Other toxins like Gymnodimine (GYM A), which are known as "fast-acting toxins", were recorded in Morocco mussels for the first time, especially at Jamâa Ouled Ganem with a low concentration of 5.6 µg/kg that not far from the limit of quantification (Table.1).

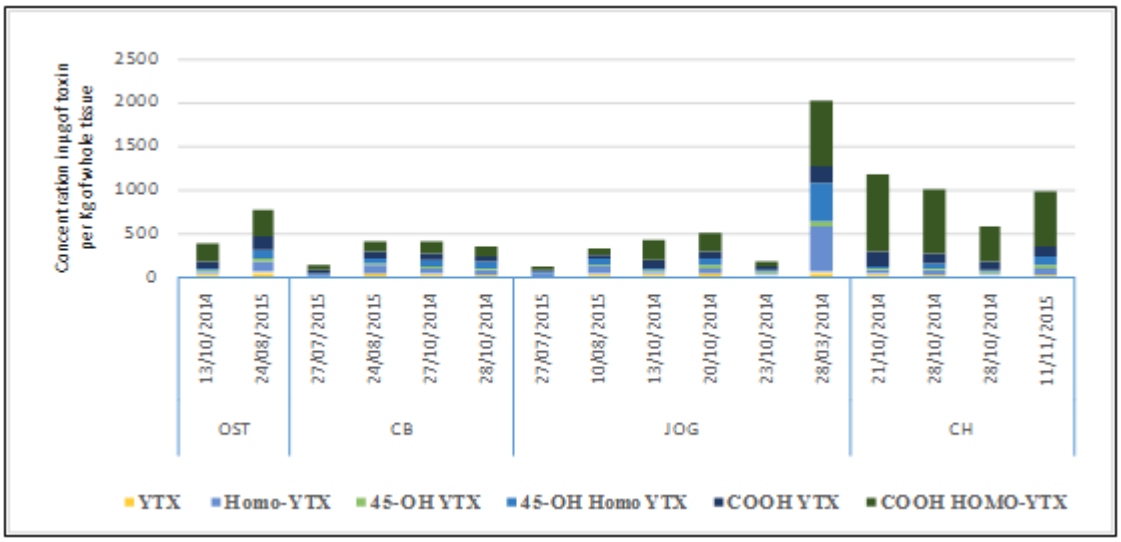

Figure 7: The spatiotemporal evolution of YTX toxins and its derivatives in mussels during 2014-2015 monitoring programme

The contamination of mussels by Gymnodimine could be related to the presence of Karenia selliformis. A wide distribution of Gymnodimine has been reported around the New Zealand coastline [37] and has recently been identified in shellfish from Tunisia [38, 39].For the first time in

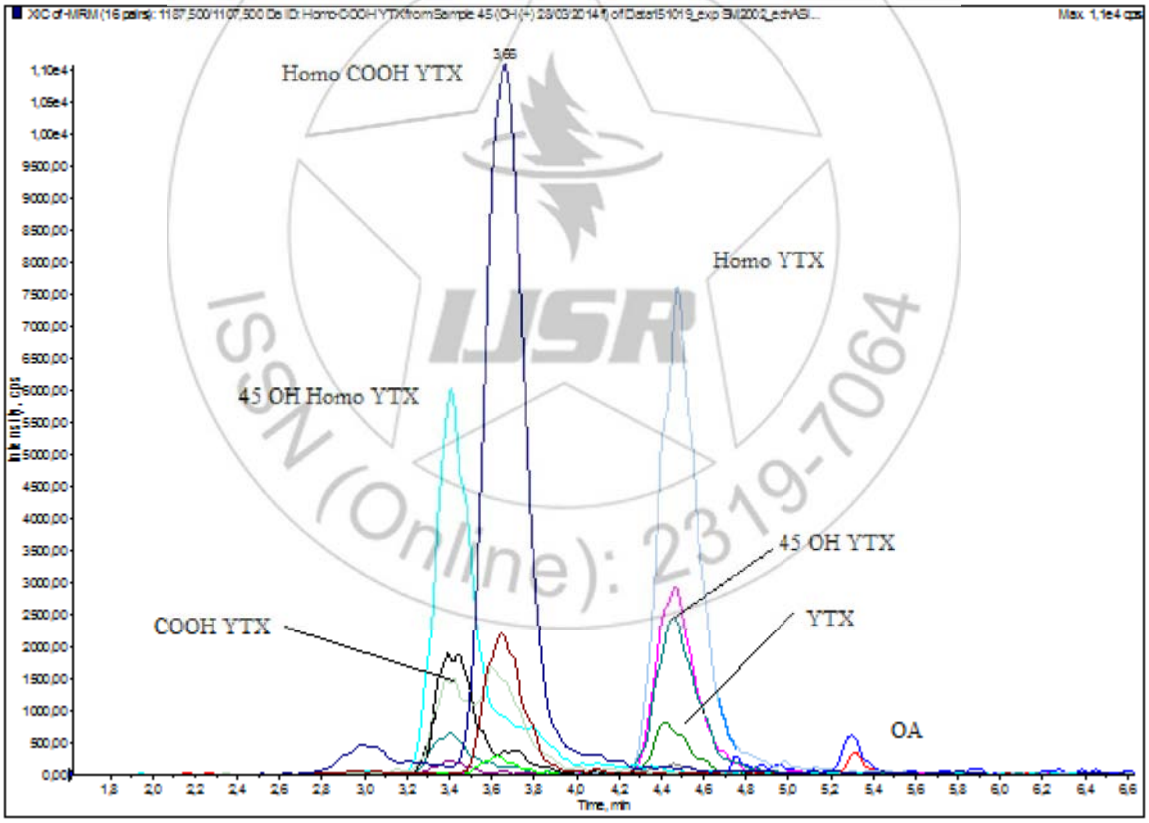

Figure 8: Example of LC-MS-MS analysis of YTXs in mussels from the Chouika

Morocco, YTXs have been detected at low concentrations in all mussel samples (Jamâa Ouled Ghanem, Cap Beddouza, Ostrea and Chouika) testing positive in the DSP bioassay (Fig.7). Recorded levels were not exceeding 878µg/kg very below the statutory thresholds (1mg YTXeq/kg).

The proportions of YTX and its analogues (Homo-YTX, 45-OHYTX, 45-OH Homo YTX, COOH YTX and COOH Homo-YTX) found, varied greatly between samples (Fig.7). COOH Homo-YTX had most abundant in this group followed by Homo-YTX and 45-OH Homo YTX, these results correlate with previous work regarding predominance of COOH Homo-YTX [40]. The maximum concentrations were recorded at Chouika (fig.7). Figure 8 shows an example of LC-SM/SM analysis of YTX and its analogues in M. galloprovincialis from North Atlantic Coast.

Yessotoxins (YTXs) are a group of structurally related polyether toxins produced by the dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum* and *Gonyaulax spinifera*. YTXs accumulate in shellfish and are toxic to mice by intraperitoneal injection [41, 42]. The setting evidence of YTXs in the Moroccan *M. galloprovincialis* could be associated with the presence of *Lingulodinium polyedrum* cells in sea water. Yessotoxin (YTX) was first isolated in 1986 in Mutsu Bay, Japan [43] from the digestive gland of *Patinopecten yessoensis*, a scallop that gave its name to the toxin. Since then, it has also been found in the mussel Mytilus edulis (blue mussel) in Norway [44], Mytilus galloprovincialis from the Adriatic Sea in Italy [45], and from Galicia (Spain) [46]. YTXs were first included within the DSP group due to their occurrence with DTX1 and DTX3 [47], but nowadays YTXs are independently regulated in the EU legislation [22,23] since it was demonstrated that these compounds display different mechanisms of action [48].

4. Conclusion

During the monitoring organic contaminants, particularly marine biotoxins, on the Moroccan coast by the monitoring network (RSSL) of INRH, analysis of diarrheal toxins (lipophilic toxins) by the mouse test on bivalve molluscs (mytillus galloprovincialis) collected in shellfish farming areas of the North Atlantic coast, have shown positive results. Since 1999. The DSP toxic episodes had occurred episodically during 1999-2012, for the long periods could last 2 to 3 months, contrary to what has been happened over the years between 2012 and 2015 while the toxic events by lipophilic toxins was short durations. Via the analysis by LC-MS or LC-MS / MS on the presumptive positive samples, the toxin profile of diarrheal toxin was especially marked by the presence of AD, DTXs (DTX1 and DTX2) and the AZPs since1999 - 2009. In recent years, from 2010-2015, DSP toxin profile proved very varied with the presence for the first time some toxins such as YTXs, GTXs and PTXs. The correlation between the accumulation of lipophilic toxins and concentrations of phytoplankton responsible for the production of these toxins was not always confirmed, this could be due to the method of water sampling, the phase difference between water sampling and analysis toxins on the molds, or the effect of enzymatic bioconversion by bivalve molluscs of certain toxins to more toxic forms.

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