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MARINE BIOTOXIN PROFILE AND *Karenia selliformis* AND *Alexandrium minitum* OCCURRENCE IN BOUGHRARA LAGOON DURING THE LAST DECADE

Abstract: As part of the routine control of the Tunisian network for the monitoring of the most problematic microalgae species *Karenia selliformis* and *Alexandrium minutum* and for the study of hydrophilic and lipophilic marine biotoxin profiles, clams and seawater samples from Boughrara lagoon were analysed during the past decade. The results showed that the abundance of *Karenia selliformis* and *Alexandrium minutum* in the Boughrara lagoon exhibited significant variability at annual scales. *K. selliformis* was recorded in all sampling years with interannual variability. Among the nine sampled years, 2010, 2012, 2016 and 2017 were, respectively, the most significant in terms of mean abundance $(2.85, 2.42, 1.38, 1.69) \cdot 10^5$ cells L^{-1}. *A. minutum* species were observed in the water column during all investigated years except in 2013, 2016 and 2017, but in low concentrations compared to *K. selliformis* ($\leq 7.4 \cdot 10^2$ cells L^{-1}). Using LC-FLD, traces of paralytic toxins (C1 and GTX-2) were detected in some clam samples. Their concentrations were largely below the regulatory limit. For the first time, N-sulfocarbamoyl gonyautoxin-2 (C-1) and gonyautoxin 2 (GTX-2) were detected in some clam samples from this region. LC-MS/MS analysis confirmed the presence of gymnodimines in some samples. The main peak corresponds to the GYM-A with high concentrations generally above 1 mg kg^{-1} of clams meat, which confirms the persistence of this neurotoxin in Boughrara lagoon.

Keywords: clams, toxicity, PSTs, GYM-A, *Karenia selliformis*, *Alexandrium minutum*

Introduction

In recent decades, a problem relating to phytoplankton in general and dinoflagellates in particular, has emerged in various coastal areas of the world following the consumption of contaminated marine products, in particular bivalve molluscs [1-5]. Many studies proposed to use bivalve molluscs as sentinel organisms for monitoring levels of environmental contaminants in water [6].

In fact, intoxications are due to these secondary metabolites produced by certain toxic species of phytoplankton during algal bloom called marine biotoxins [7, 8]. They are highly toxic, of low molecular mass and of non-protein nature.

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Marine biotoxins can be classified into eight groups including saxitoxins, okadaic acid and dinophysistoxins, domoïc acids, pectenotoxins, yessotoxins, brevetoxins, azaspiracids, and cyclic imines. These toxic secondary metabolites are produced by species of the *Alexandrium*, *Dinophysis*, *Gymnodinium* and *Pseudonitzschia* genus [9].

In order to protect the consumer health while preserving a considerable economic activity, several methods of detection and monitoring of these products have been developed [10, 11]. In fact, the mouse bioassay has been routinely applied to detect marine biotoxins in shellfish. For ethical and scientific reasons, analytical techniques for chemical analysis like liquid chromatography (LC) coupled with mass spectrometry (LC-MS) have been invented in order to provide qualitative and quantitative answers on marine toxins profile in shellfish [12, 13].

At the national level, massive mortalities of eels, cuttlefish and mullet were recorded in Boughrara lagoon (Gulf of Gabes, Southern Tunisia) early 90s [14]. Since then, same events have become more and more frequent and generalised at several sites of the Gulf of Gabes, which alone contributes 98 % of clam's national production. Clam samples, harvested from these sites, were toxic to mice when injected by intraperitoneal route. Investigations have shown that the acute neurotoxicity observed in mice was due to the presence of GYM-A [15]. Later, analyses carried out on seawater samples have shown the involvement of *Karenia selliformis* in these episodes of toxicity [16].

In this article, we present the results of 10 years of routine monitoring of Boughrara lagoon for the occurrence of *K. selliformis* and *A. minitum*, the most problematic species in the Gulf of Gabes, according to studies carried out by Abdennadher et al. [17].

As no studies have been conducted to investigate the toxin profiles in Boughrara lagoon over the past decade, we present in this work the results of a screening for hydrophilic and lipophilic marine biotoxins that contaminate clams harvested from this important area of wild clam's production.

Material and methods

Chemicals

LC/MS grade acetonitrile and water from Fisher Scientific (Illkirch, France). Milli-Q water was produced in-house to 18 MΩ/cm quality, using a Milli-Q integral 3 system (Millipore). HPLC grade methanol (MeOH) and formic acid (98%) from Sigma-Aldrich (Steinheim, Germany). Ammonium formate was from Fluka (St. Louis, MI, USA).

Certified reference toxins were obtained from the National Research Council (Halifax, NS, Canada) (saxitoxins (STXs), tetrodotoxin (TTX), C-toxins, gonyautoxins (GTXs), okadaic acid (OA), dinophysistoxins 1 and 2 (DTX1 and 2), 13-desmethyl spirolide C (13-desMe-C), pinnatoxin G (PnTX-G), pectenotoxin 2 (PTX2), gymnodimines (GYMs)).

Clam sampling

Sampling stations were selected through the Tunisian national monitoring network of phytoplankton and phycotoxins (REPHY), with consideration given to already-existing mollusc collection sites and followed up by field reconnaissance.

Samples of clams (*Ruditapes decussatus*) were collected from Boughrara lagoon between January 2010 and December 2019. Sampling was carried out manually by randomly picking up clams off the coast and controlled by the 'Commissariat Regional

au Developpement Agricole de Mednine' (CRDA, Southern Tunisia). Samples were kept at +4 °C until analysed in the Laboratory of Food Toxins at Pasteur Institute of Tunis.

Chemical analysis of hydrophilic toxins by liquid chromatography/fluorescence detection (LC/FLD)

Toxin contents were analysed out according to the method of Van De Riet et al. [18]. Briefly, 5 mL of 0.1 N hydrochloric acid were added, and the samples were mixed with a high-speed homogeniser (15,000 rpm) for 2 min. The pH was adjusted between 2.0 and 4.0, and then the samples were centrifuged at 4200 g for 10 min at +4 °C. The supernatants were filtered on 10-kDa polyethersulfone (PES) filters, and the using the liquid chromatography with fluorescence detection (LC/FD) PSP toxin analyses method of Van De Riet et al. [18]. The toxins GTXs, dc-GTXs, dc-STXs and STXs were separated using a reverse chromatography column (Zorbax Bonus RP, 3.5 µM, 4.6 · 150 mm, Agilent Technologies, Massy, France) with a flow rate of 0.8 mL · min^{-1}. The eluent pH and/or column temperature were optimised to separate dc-GTX3/GTX5/dc-GTX-2 and C1/C2. The toxin concentrations were determined using certified standards provided by CNRC (Halifax, NS, Canada). The identification of each toxin relied on extraction of their specific retention time, as obtained with injection of standard solutions. Toxin concentrations were calculated based on peak area.

Liquid chromatography mass spectrometry analyses of lipophilic toxins

Extraction procedure

In accordance with the European method validated internally Ifremer [19], lipophilic toxins were extracted from (2.00 ±0.05) g of hepatopancreas homogenate of clams. Then the homogenate was extracted in duplicate with 9 mL methanol. 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 homogenised 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.

LC/MS-MS analyses of lipophilic toxins

For each sample prepared according to the method detailed above, 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 · 2.0 mm id, Phenomenex) at 25 °C.

The electrospray ionisation interface (ESI) was operated in both positive and negative modes using the following parameters: curtain gas: 20 psi (1 psi = 6 894.76 Pa) (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: 500 V

(PM) and –4500 V (NM); EP: 10 V (PM) and –13 V (NM). These parameters had been previously optimised using toxin standards. The mass spectrometer was operated in multiple reaction-monitoring (MRM), analysing 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 > 792.5/658.4/640. 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; HomoYTX, *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.

Sampling and microalgae enumeration

Phytoplankton samples were collected from 2010 to Jun 2018 at Boughrara lagoon along the Southern Mednine coastline (Gulf of Gabes). This zone was selected from the Tunisian National Monitoring Network of Phytoplankton and Phycotoxins. Sampling was conducted weekly from October to May during the clam's harvest season and every two weeks during the rest of the year (June, July, August and September), but in the case of a bloom situation, sampling will be conducted weekly Water samples were collected with a Van Dorn bottle at 1 m depth. Phytoplankton cells were preserved with Lugol's iodine solution (4 % final concentration) and stored in the dark. Counting was conducted according to the Utermohl method [20] using 10 mL sedimentation chamber under an inverted microscope (Olympus CK40).

Results and discussion

Temporal variability of *K. selliformis* and *A. minutum* abundance in Boughrara lagoon

The box plots displayed in Figure 1 (boxplot) revealed that the abundance of *K. selliformis* and *A. minutum* in the Boughrara lagoon exhibited significant variability at annual scales. *K. selliformis* was recorded in all sampling years with interannual variability (Fig. 1a). Among the nine sampled years, 2010, 2012, 2016 and 2017 were the most significant in terms of mean abundance $2.85 \cdot 10^5$ cells L^{-1}, $2.42 \cdot 10^5$ cells L^{-1}, $1.38 \cdot 10^5$ cells L^{-1} and $1.69 \cdot 10^5$ cells L^{-1}, respectively. However, *A. minutum* species presented a different trend (Fig. 1). It was observed in the water column during all investigated years except in 2013, 2016 and 2017, but in low concentrations compared to *K. selliformis* ($\leq 7.4 \cdot 10^2$ cells L^{-1}).

From the temporal distribution of these two toxic species in the Boughrara lagoon (Fig. 1), it was apparent that *K. sellifromis* was more frequent and abundant than *A. minutum*.

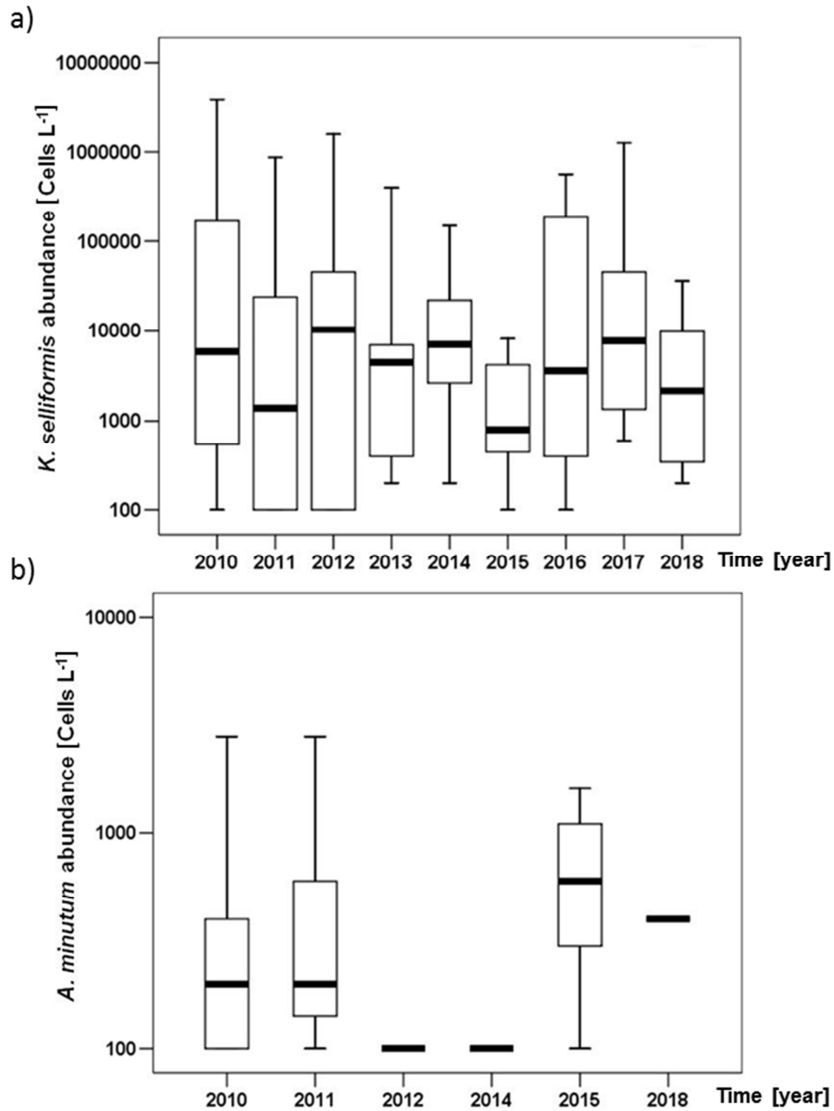


Fig. 1. Box plots reporting: a) *K. selliformis* and b) *A. minutum* distribution according to the years. Note the log-scale on the *y*-axis

The occurrence of *K. selliformis*, which represents 37 % of the toxic dinoflagellate population in the Gulf of Gabes [21] and *A. minutum* over nine years of observation suggests that these species were common in the Boughrara lagoon. This indicates that this species has been well adapted to survival in this area.

In fact, *K. selliformis* and *A. minutum* are well established in coastal waters of the Gulf of Gabes [21]. Feki et al. [22] showed a north-south gradient of *K. selliformis* occurrence

along the Gulf of Gabes coastline. Indeed, the Boughrara lagoon achieved the highest abundance [21]. *A. minutum* outbreaks occurred also in this confined lagoon. Nevertheless, *A. minutum* proliferation in this lagoon was less than that recorded along the north coast of the Gulf [17]. Furthermore, the Boughrara lagoon may be ideal not only for growth of vegetative cell [17, 22], but also as seeding bed for resting cysts which retain the capacity to germinate for several years [23, 24]. In fact, the initiation of future blooms and outbreaks of toxic dinoflagellates has often been shown to be linked with the existence of favorable harmful resting cysts seedbed [24-26].

Since 1994, *K. selliformis* is present throughout the year and it often blooming in all seasons and the blooms can last more than two months [27]. Bloom initiation occurs when density reaches 10^6 cells L^{-1} [27]. In fact, in this site, the maximum abundance of this endemic specie was due to several factors such as high salinity (which reach 52 on August), relatively low-temperatures (ranged between 13 °C and 16 °C) and high concentrations of phosphate [27].

In terms of abundance, *A. minutum* remains a much less problematic species compared to *K. selliformis*. Indeed, a study focused on the monitoring of this species in 20 sites in the Gulf of Gabes, including Boughrara lagoon during 1997-2006, showed that *A. minitum* abondunce, in this lagoon, was much lower than that recorded for *K. selliformis* [17], which is in accordance with our result.

Hydrophilic and lipophilic biotoxins detected in clam samples

Hydrophilic toxins N-sulfocarbamoyl gonyautoxin 3 (C-2), N-sulfocarbamoyl gonyautoxin 4 (C-3), N-sulfocarbamoyl gonyautoxin 5 (C-4), gonyautoxin 1 (GTX-1), gonyautoxin 3 (GTX-3), gonyautoxin 4 (GTX-4), gonyautoxin 5 (GTX-5), gonyautoxin 6 (GTX-6), decarbamoyl gonyautoxin 1 (dc-GTX-1), decarbamoyl gonyautoxin 2 (dc-GTX-2), decarbamoyl gonyautoxin 3 (dc-GTX-3), decarbamoyl gonyautoxin 4 (dc-GTX-4), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dc-NEO), saxitoxin (STX), decarbamoyl saxitoxin (dc-STX), decarbamoyloxy saxitoxin (do-STX), tetrodotoxin (TTX) were undetectable (below detection limits (LODs)).

N-sulfocarbamoyl gonyautoxin-2 (C-1) and gonyautoxin 2 (GTX-2) were detected in some clam samples (Fig. 2). It is interesting to note that C-1, by metabolic conversion via desulfonation and epimerisation in clams, can be converted to GTX-2 [28], thereby GTX-2 may be the product of *Alexandrium minutum* cells as it may be a metabolic derivative.

Concentrations of GTX-2 and C-1 were, respectively, less than 70 µg/kg and 40 µg/kg of clam meat. These levels were largely below the regulatory limit for paralytic shellfish toxins, which is 800 µg STX-eq/kg of tissue. The detection, at trace level, of these toxins is consistent with the low abundance of the causative organism.

To our knowledge, this is the first report of occurrence of GTX-2 and C1 toxin in Boughrara lagoon. These findings serve to draw attention to this potential threat and to provide the information necessary to ensure the biotoxin-monitoring program continue in order to produce a complete risk assessment especially if it is known that, recently, several severe cases of human intoxication have been recorded in Morocco following consumption of shellfish contaminated by paralytic shellfish toxins [29].

Lipophilic toxins; okadaic acid (OA), dinophysistoxin-1 (DTX-1), dinophysistoxin-2 (DTX-2), pectenotoxin-1 (PTX-1), pectenotoxin-2 (PTX-2), azaspiracide-1 (AZA-1), azaspiracide-2 (AZA-2), azaspiracide-3 (AZA-3), gymnodimine-B (GYM-B), desmethyl spirolide-C (desMe SPX-C), spirolide-A (SPX-A), spirolide-B (SPX-B), spirolide-C

(SPX-C), spirolide-D (SPX-D), desmethyl spirolide-D (desMe SPX-D), pinnatoxin-A (PnTX-A), pinnatoxin-G (PnTX-G), pectenotoxin-2 seco acid (PTX-2sa), 7-epi pectenotoxin-2 seco acid (PTX-2sa epi), pectenotoxin-6 (PTX-6) were undetectable (below detection limits (LODs)).

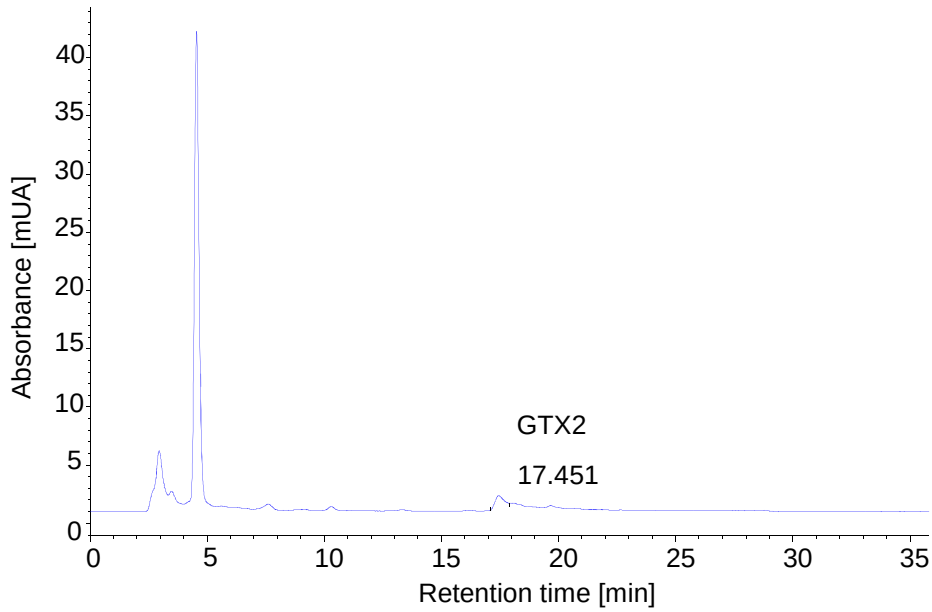


Fig. 2. LC/FD chromatogram of GTX-2 in clam meat (*Ruditapes decussates*)

LC-MS/MS analysis confirm the presence of gymnodimine-A (GYM-A) (Fig. 3) in samples with a molecular weight of 508 *m/z*. The different samples analysed showed high concentrations of GYM-A ranging between 906 µg/kg and 1331 µg/kg. These findings confirm the persistence of this fast-acting neurotoxin in Boughrara lagoon. These concentrations were almost the same recorded since the discovery of this toxin in the region [30], proving the stabilisation of the contamination level, explaining the high abundance of *Karenia selliformis* in this area and reflecting that this specie adapts well with the biotic and abiotic conditions parameters of the Boughrara lagoon [31, 32]. The closure of several sites has a negative socio-economic impact on the clam's production sector since the most of the local population depends on this activity, in particular 6000 'foot fishermen', the majority of whom are women [33].

In order to find practical solutions for this problem, works carried out on the depuration of contaminated clams in controlled medium have concluded that this process takes time, costly and generates other problems such as the reduction of animal weight and the accumulation of wastes [30]. Additional studies are needed to assess the risks of this neurotoxin in order to set an acceptable dose with no effects on humans. It should be noted that the GYM-A is still unregulated [34], therefore its regulation is a national priority to solve the clam contamination problem in the Boughrara lagoon.

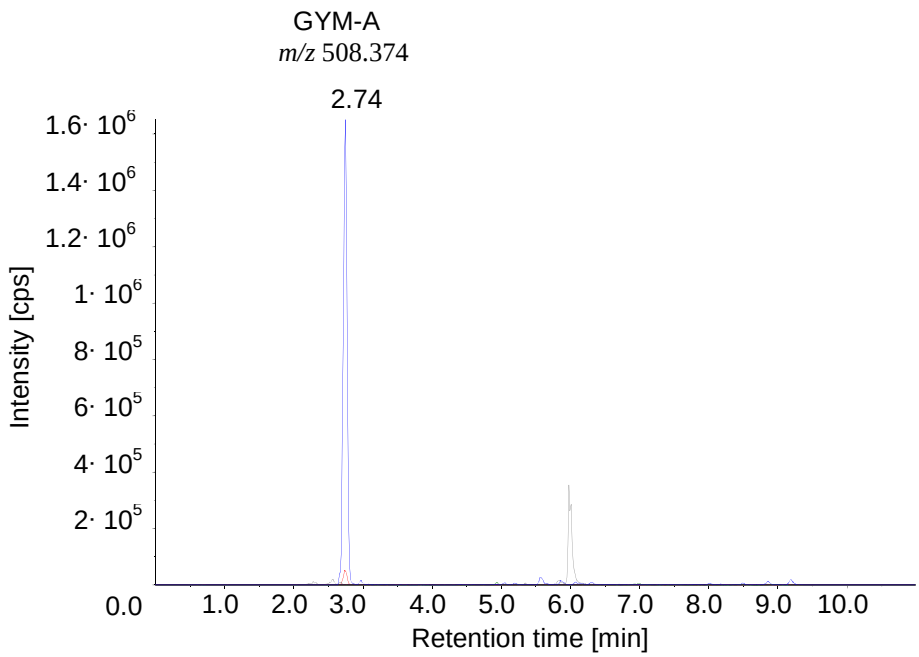


Fig. 3. LC-MS/MS chromatogram of GYM-A in digestive gland tissue of clams (*Ruditapes decussates*)

Conclusion

Karenia selliformis and *Alexandrium minutum* are the most problematic species in Boughrara lagoon. Their abundance exhibited significant variability at annual scales. *K. selliformis* was recorded in all sampling years with interannual variability. Its concentration in water can reach high values. *A. minutum* species were observed in the water column but in low concentrations.

For the first time, N-sulfocarbamoyl gonyautoxin-2 (C-1) and gonyautoxin 2 (GTX-2) were detected in some clam samples. Their levels were largely below the regulatory limit for paralytic shellfish toxins but vigilance must be maintained in order to limit human poisoning by this family of toxins. The study of lipophilic toxin profiles confirms the persistence of gymnodimine-A in clams from Boughrara lagoon. High concentrations were recorded, proving the stabilisation of the contamination level since the same values have been recorded previously. In conclusion, if paralytic shellfish toxins remain a potential danger in Boughrara lagoon, gymnodimine-A is a real threat. That is why its regulation remains a national priority to save the clam shellfishing sector.

Acknowledgments

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