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Weekly Occurrence of *Gymnodinium catenatum* and Paralytic Shellfish Poisoning in the Mediterranean Shore of Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. Author RA designed the study, carried out some analyses of PSP by mouse bioassay and wrote the first draft of the manuscript. Author AB completed the remainder of PSP analyses by mouse bioassay. Author BRL analysed the phytoplankton. Author PV carried out the analysis of PSP by HPLC and corrected the manuscript. Author HT managed the literature searches and corrected the manuscript. Author MB supervised the work. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Weekly monitoring for the presence of *Gymnodinium catenatum* and PSP toxins in mussels (*Mytilus galloprovinciallis*) harvested on the growing site in Fnideq on the Mediterranean Moroccan coast was carried out from 2010 until 2012. The enumeration of *G. catenatum* cells in water was

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conducted using the light inverted microscope. Monitoring of PSP toxins levels in mussels was achieved using the mouse bioassay (MBA) method and toxin's profile was performed by HPLC/FD in a selected sample.
The results showed a correlation between the evolution of *G. catenatum* cells concentration and the PSP toxin level accumulated in mussels. The appearances of *G. catenatum* and PSP toxin concentration increases were observed at different periods, between January and February 2010, in September 2010 and between November 2011 and January 2012.
Liquid chromatographic analysis revealed a composition reflecting *G. catenatum* profile composed mainly of toxins from the N-sulfocarbamoyls family, as well as the presence for the first time in Morocco of the hydroxybenzoates analogues of saxitoxin, so called GC-toxins, which have been recently detected in Australia strains of *G. catenatum*.

Keywords: Morocco; M'diq; shellfish; Gymnodinium catenatum; hydroxybenzoates toxins.

1. INTRODUCTION

Seafoods, especially shellfishes contaminated by saxitoxin and its analogues (STXs) have caused the human poisoning syndrome known as paralytic shellfish poisoning (PSP) and posed a serious health hazard in many areas of the world [1].

Several species of dinoflagellates, such as *Alexandrium tamarense* (Lebour) Balech [2], *A. catenella* (Whedon & Kofoid) Balech [3,4], *Pyrodinium bahamense* var.compressum [5,6] and *Gymnodinium catenatum* H.W.Graham [7,8] are known to transmit their toxins to shellfish. These toxins are usually grouped into 4 groups:

(1) Carbamate toxins composed of gonyautoxines (GTX1-GTX4), neosaxitoxin (NEO) and saxitoxin (STX).
(2) N-sulfocarbamoyl toxins including C1 - C4, B1 and B2.
(3) The decarbamoyl toxins consisting of dcGTX1-dcGTX4, dcNEO and dcSTX.
(4) Deoxydecarbamoyl toxins composed of doSTX, doGTX2, do GTX3.

These groups possess different toxicities as determined by intraperitonial injection in mice, the carbamoyl analogues being the most toxic, decarbamoyl analogues intermediate and the N-sulfocarbamoyl analogues the least toxic, while for the deoxydecarbamoyl analogues there is no information [9].

Recently, three novel hydroxybenzoate saxitoxin analogues were isolated from Australian strains of *G. catenatum* and designated GC1 through GC3 [10]. The GC3 was found to be the 4-hydroxybenzoate ester derivative of decarbamoylsaxitoxin, while GC1 and GC2 were the epimeric 11-hydroxysulfate derivatives of GC3.

The Moroccan coast is not spared from this problem of PSP toxins. Indeed, several episodes of shellfish contamination by PSP toxins have been identified since 1969 in both Mediterranean and Atlantic coast [11].

In the Mediterranean, this contamination has been due to the presence of *G. catenatum* [11], while in the Atlantic it has been due to the appearance of both *G. catenatum* and the genus *Alexandrium* [12,13].

The novelty of the present study lies in the fact that this is the first time that a simultaneous surveillance of the presence of *G. catenatum* and PSP toxins accumulation rates in bivalve mollusc was carried out *in situ*. This study allowed also to screen in Moroccan shellfish the presence of hydroxybenzoates (GC toxins), new compounds belonging to the PSP toxins group that were first reported from Australia [10].

2. MATERIALS AND METHODS

2.1 Sample Collection

A weekly monitoring of phytoplankton and biotoxin was carried out between January 2010 and January 2012 in the mussel aquaculture place, in open waters of Fnideq (City located on the Mediterranean coast) (Fig. 1). Sea water sampling was done at high tide using the bottle at depths of 0.5 m while mussel's samples (*Mytilus galoprovinçialis***)** were collected at low tide in the same day.

The lack of samples in some days and some weeks was due to the problem of weather.

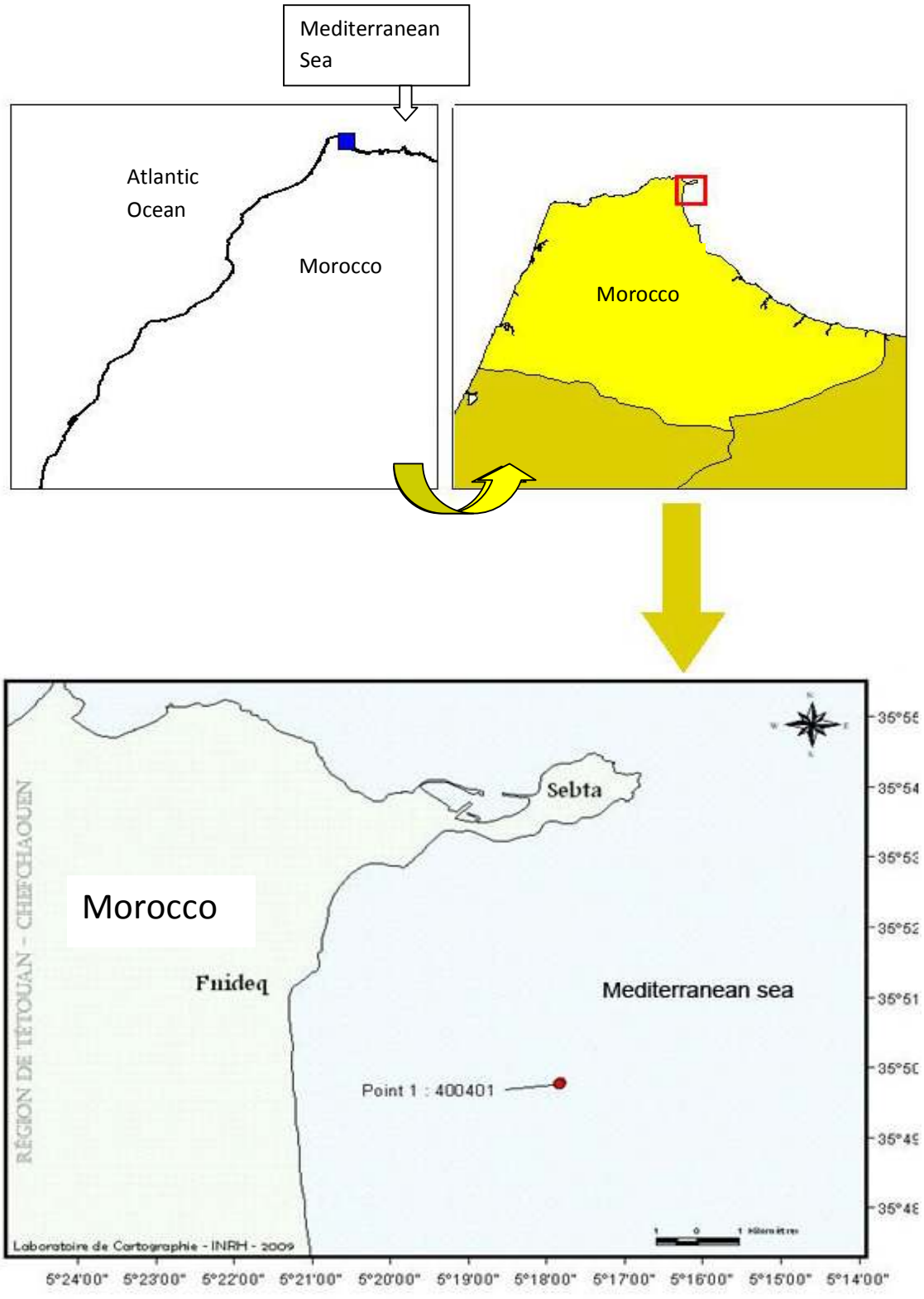


Fig. 1. Sampling site in mediterranean coast of Morocco

2.2 Phytoplankton Analysis

The seawater samples were fixed with Lugol's iodine solution. A sub-volume (2–5 mL) of fixed seawater samples was settled in sedimentation chambers for 24 h.

A potentially harmful species of dinoflgellates were identified by inverted microscopy according to the Utermöhl method [14].

2.3 Mouse Bioassay

Mussel analysed with mouse test (MBA) according to the AOAC method [15]. Briefly, 100 g of homogenized mussels tissues was mixed with 100 mL of hydrochloridric acid 0.1M,. The obtained mixture is adjusted to pH to ca.3 and boiled gently for 5 min. After cooling at the room temperature the pH was adjusted another time to value ranging between 2 and 4 by HCl 5M or NaOH 0.1 M solutions. The final homogenate was diluted to 200 mL by distilled water in the graduated cylinder. After a centrifugation at 3000 g for 5 min, one millilitre of the supernatant was injected intraperitoneally into three mice (Swiss albinos strain CD1) having a weight between 18 and 20 g. The time taken for the mice to die (death time) is taking into account for determination of sample toxicity expressed in mouse unit (MU) by Sommer Table [15].

A conversion factor (CF) is obtained by the calibration test using the saxitoxin standard (STX). The CF allows to convert the results from MU to µg STXeq. / Kg of meat. The calculation of this factor is detailed in AOAC, 2000. In our case the CF found is 0.2, determined with ideal concentrations of 0.322, 0.33 and 0.34 µg/ml of saxitoxin.

Standard solutions from STX were purchased from the Certified Reference Materials Program (CRMP) of the Institute for Marine Biosciences, National Research Council (Halifax, Canada).

The sanitary threshold adopted by the National regulations is 800 µg STXeq./Kg of meat. If toxin levels exceed the regulatory limit, the aquaculture sites and harvesting areas are closed until shellfish depuration.

2.4 Liquid Chromatography Analysis

2.4.1 Reagents and standards

Reagents were of analytical grade, except for acetonitrile that was LC grade. Water was purified using a Milli-Q 185 Plus system (Millipore, Bedford, MA, USA). Standard solutions from STX, GTX2+3, dcSTX, dcGTX2+3, B1 and C1+2 were purchased from the Certified Reference Materials Program (CRMP) of the Institute for Marine Biosciences, National Research Council (Halifax, Canada), and used for identification and quantification.

2.4.2 Sample preparation

Supernatant of shellfish samples obtained by mouse bioassay according to AOAC (2000) were used for analysis by pre-column oxidation followed by liquid chromatography separation and fluorescence detection, according to a methodology modified from Lawrence et al. [16].

The only mussel sample analyzed by HPLC was the sample from 16 September 2010.

2.4.3 Liquid chromatography with fluore-scence detection (HPLC-FLD)

Liquid chromatography was performed on an Agilent equipment consisting of Model 1050 quaternary pump, Model 1100 autosampler, and in-line degasser, Model 1200 fluorescence detector. The HP Chemstation software performed data acquisition and peak integration. Toxins were separated on a 125 mm x 3 mm id column packed with 5 µm Nucleosil 100-5 C18 (Macherey-Nagel, Duren, Germany), and equipped with a 4 x 4 mm id guard column packed with 5 µm Lichrospher 100 RP-18 (Merck, Darmstadt, Germany). Column temperature was kept at 30ºC. Detection wavelengths were set at 330 nm for excitation and 390 nm for emission, and gain set at 214.

The mobile phases were as follows: A: acetonitrile; B: 20 mM ammonium formate with 1 mM acetic acid. The column was equilibrated with 100% eluent B. During each analysis a gradient from 0 to 2% eluent A was run from 0 to 5 min, from 2% to 8% eluent A from 5 to 10 min and from 8% to 40% eluent A from 10 to 20 min, followed by re-equilibration to the starting conditions for 5 min prior to the next oxidation cycle. Flow rate was set at 0,5 mL/min.

In order to obtain fluorescent derivatives from PSP toxins, standards and extracts were oxidized in vials at room temperature with periodic acid.

The periodate mixture and reaction times were described in detail in Lawrence et al. [16].

Toxin identification for GC group, where no standards are available, was made by comparison with the same cultured *G. catenatum* strain described in detail by Vale [17]. Strain nº IO.13.04, from the algal library of the Instituto de Oceanografia (Lisbon University), was grown in-house with f/2 media without silica. Briefly, the GCs analogues eluted with an increase in the acetonitrile gradient. Confirmation was done by match of retention time, a confirmatory emission wavelength at 430 nm and fluorescence spectra whenever possible.

3. RESULTS

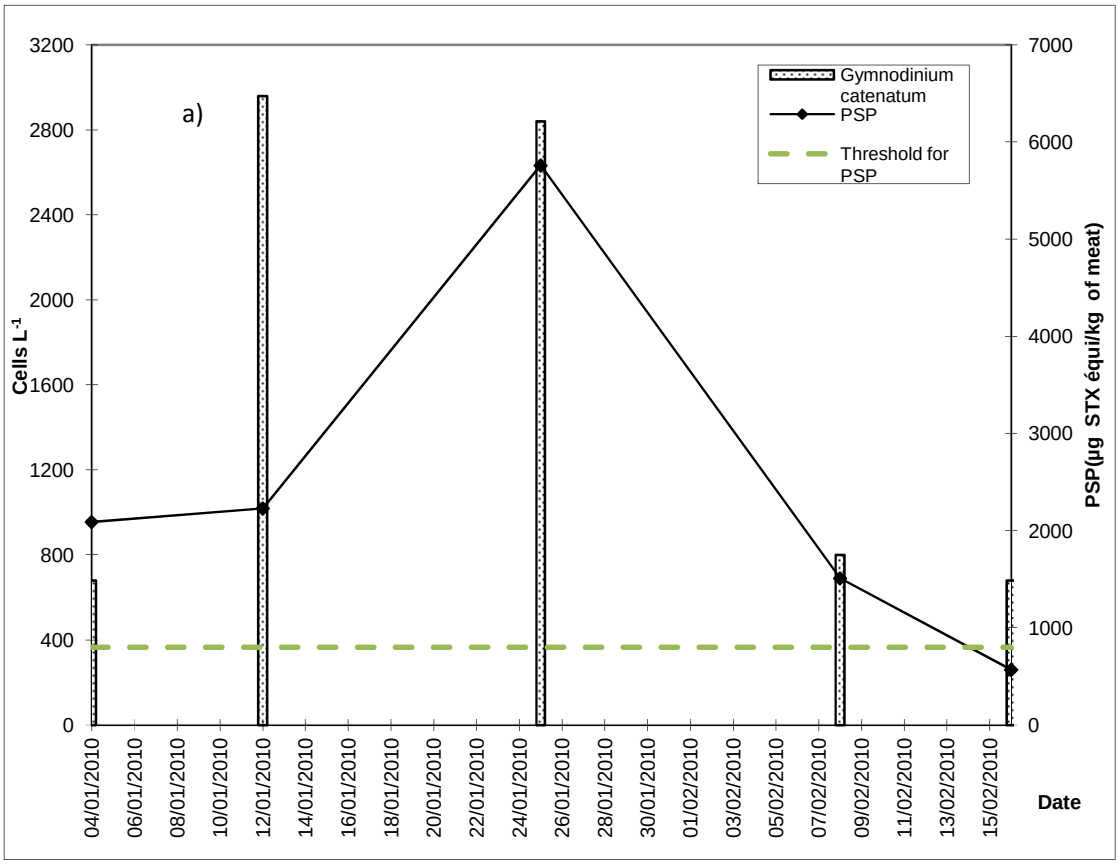
3.1 Monitoring Data

During weekly monitoring of phytoplankton and PSP toxins the concentration of *G. catenatum* and the PSP toxins level were observed to increase three times (Fig. 2).

The irregularity of sampling frequency is due to the non availability of samples in some days due to bad weather and accessibility at the side during the tide.

At the first period stretching between 04 January 2010 and 16 February 2010 (Fig. 2a). The maximum concentration of *G. catenatum* (3000 cells L^{-1}) was reached on 12 January 2010, while the maximum value of the amount of PSP toxins (5760 µg STX equi./Kg of meat) was reached on 25 January 2010. There is a lack of sampling between the days 12 and 25 January 2010. The stages of decay of the two parameters were progressive and were nearly identical.

However, the second period extending between 06 September 2010 and 27 September 2010 was marked by a proportionate increase in the concentration of *G. catenatum* and the level of PSP toxins in mussels (Fig. 2b). There was a lack of sampling between the weeks of 06 and 14 Septembre and sampling of 16 September was added to show the evolution of *G. catenatum* concentration and PSP toxins level during the same week.



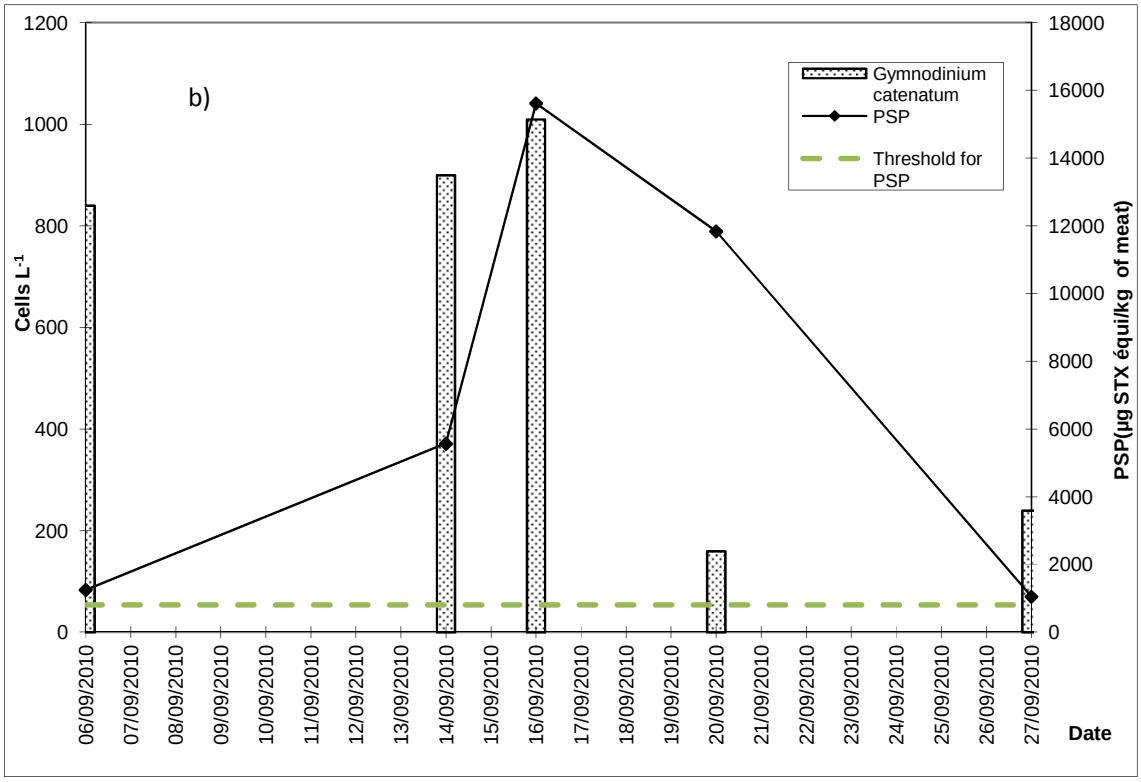


Fig. 2. Evolution of bivalve toxicity in association with *G. catenatum* in: a) first period b) second period c) third period

NB: The y-scales are not the same for the Figs. a, b and c.

The maximum *G. catenatum* concentration was 1010 cells L^{-1} where the PSP toxins values reached was 15620 µg STXequi./kg of meat.

But the *G. catenatum* concentration dropped in 4 days while the decrease in the rate of PSP was progressive and lasted 11 days.

During the third period, between 01-11-2011 and 23-01-2012, evolution was quite different. The concentration of *G. catenatum* was different from the rate of PSP in mussels (Fig. 2c). The two phases of increase and decrease were quick for the concentration of *G. catenatum* and progressive for the level of PSP in mussels. In fact, the maximum value of the concentration of *G. catenatum* (10720 cells L^{-1}) was achieved within two weeks, while the maximal toxin level of PSP (16370 µg STXequi./Kg of meat) was attained over month, with a phase shift of three weeks.

In addition, the maximum cell numbers of *G. catenatum* did plummet in a week to reach a value of 1760 cells L^{-1} where the PSP toxins value in mussels decreased gradually over two months to reach a level of 620 µg STXequi./Kg of meat.

By comparison of the maximum values of PSP level depending on the concentrations of *G. catenatum*, we note that for figure (Fig. 2c) it took about 10720 cells L^{-1} to reach PSP toxins value of 16370 µg / Kg of meat while for figure (Fig. 2b) only 1010 cells L^{-1} were necessary to reach the nearest PSP toxins value which is 15620 µg/Kg of meat.

3.2 Liquid Chromatography

Owing to the lack of *G. catenatum* toxin profile from Moroccan coast, the comparison was carried out with a cultured *G. catenatum* strain isolated from the coast of Portugal (Fig. 3A).

In the *G. catenatum* chromatogram (Fig. 3A), during the first 11 min of the chromatography eluted the hydrophilic STX analogues: C3/4, C1/2, B2, GTXs and B1.

After 11 min several fluorescent compounds were observed. The first couple of peaks were postulated to contain a sulphate group in the benzoate moiety instead of a hydroxyl group like GC1/2 or GC3. The next two most abundant peaks were produced by hydroxy-benzoate analogues, with a sulphate group at the eleventh position of the STX core present or absent (GCs-GTX and GCs-STX analogues, respectively).

The mussel's chromatogram (Fig. 3B) showed a profile partially reflecting that of *G. catenatum* (with the presence of N-sulfocarbamoyls toxins) but with the additional abundant presence of decarbamoyl gonyautoxins (dcGTXs) and decarbamoyl saxitoxins (dcSTXs). The decarbamoyl toxins produce two peaks, and the second one overlap with C3/4 (the decarbamoyl-gonyotoxins) or B2 (the decarbamoyl saxitoxins), respectively.

In the second part of the chromatogram, the GC-sulfo toxins were absent and the GCs- GTX and GCs-STX analogues constituted only a minor component of the fluorescent profile corresponding to paralytic shellfish poisoning analogues.

4. DISCUSSION

The present study showed a relationship between the accumulation of PSP toxins in mussels determined by MBA analysis and the concentration of *G. catenatum* identified by inversed microscope in sea water.

Indeed, during the 3 years of follow-up we observed an increase in the rate of PSP toxins in mussels always follows the increase in the number of *G. catenatum* cells. Maximum PSP levels in mussels were reached at least after one week of *G. catenatum* cell maxima. Thus, the mussel's PSP contamination was associated with the occurrence in sea water of *G. catenatum.*

In our case *G. catenatum* blooms as well as the mussels toxicity were recorded also in late fall, in winter and in summer.

Most authors who have worked on the region report that *G. catenatum* is the main organism causing recurrent Paralytic Shellfish Poisoning (PSP) outbreak in the western Mediterranean coast [18,19,20,21,22,23,24,25,11,12]. It is considered as an invasive species in the Mediterranean and its presence is due to geographic expansion of the Atlantic through the Strait of Gibraltar into the Alboran Sea and spread along the southern basin of the Mediterranean, Morocco, Algeria and Tunisia.

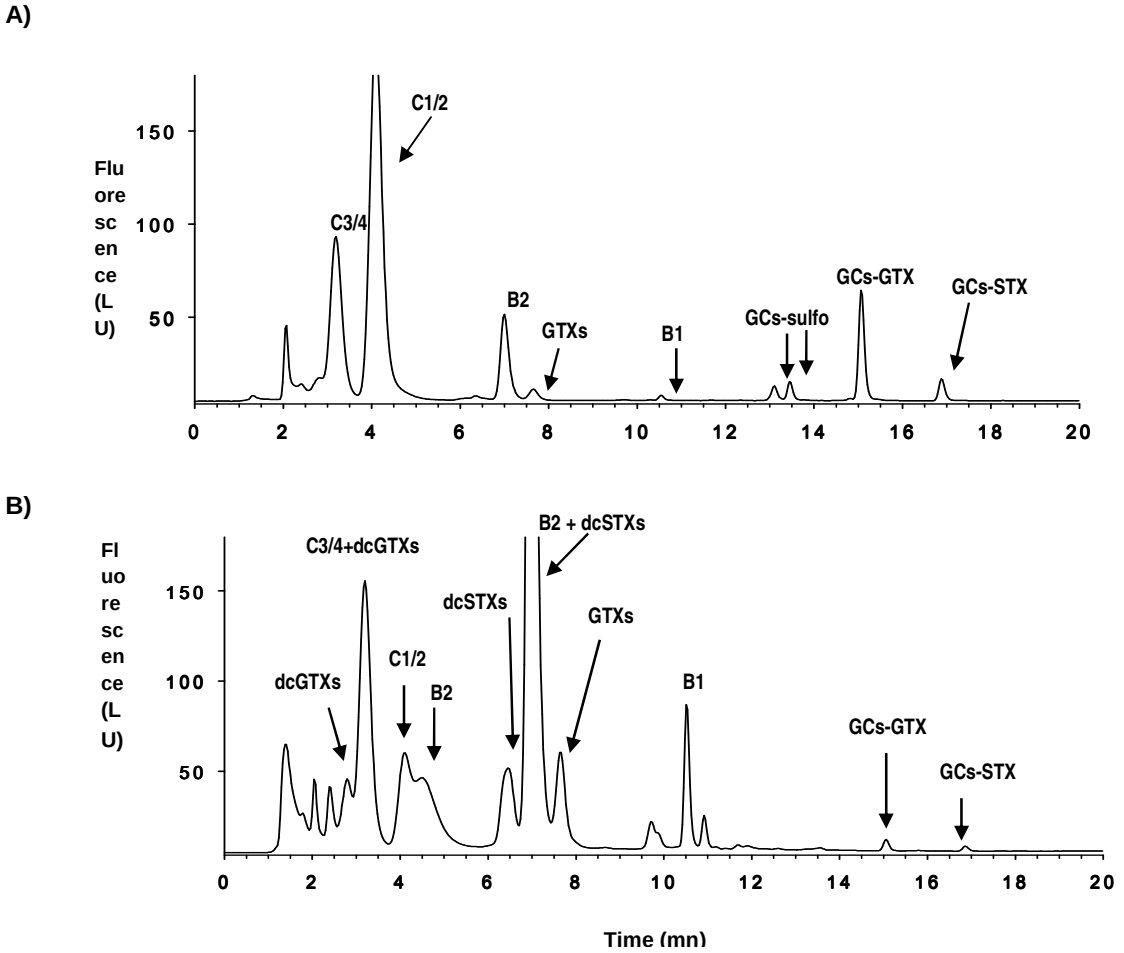


Fig. 3. Chromatograms of PSP toxins after periodate oxidation in: A) a cultured *G. catenatum* strain; B) Moroccan mussels

It is also reported that the frequency of *G. catenatum* blooms in this region of Morocco is seasonal and appear with annual trends, usually in late fall and early winter accompanied with high level PSP toxicity of bivalve molluscs [26,11,18], that confirm the autcomes of the present stady concerning the seasonnality outbreak occurrence of *G. catenatum* and the high toxicity of mussels.

The shellfish's toxicity depends not only on the concentration of the toxic algae but as well as of other factors, namely:

(i) The characteristics of the phytoplankton bloom (cell toxicity and toxic algae on non toxic ratio); (ii) individual variations within the same species and interspecies variation; (iii) the dynamics of food intake [31].

It is probably the reason that in our case, we get the same levels of PSP toxins with different concentrations of *G. catenatum*. Sometimes with 10 times concentration of *G. catenatum* we obtain almost the same level of toxicity.

This study has also highlighted for the first time in Morocco, using liquid chromatographic analysis, a new group of PSP toxins, the GC toxins which are the hydroxybenzoates analogues of saxitoxin. This group of PSP toxins was discovered for the first time in the world in an Australian strain of *G. catenatum* [10], and later detected in strains from other regions [10,27,28,29,30].

The comparison between the toxin profiles of *G. catenatum* and mussels showed the GCs-GTX and GCs-STX toxins, that were majority after

C1/2 and C3/4 in *G. catenatum* strains, became minor contaminants in mussels. The abundance of the dcGTXs and dcSTXs in the mussels led to the suspicion of these resulting from biotransformation of hydroxybenzoate analogues into their similar decarbamoyl analogues.

Similar conversion was first hypothesized in several species of Portuguese shellfish by Vale (2008a). Additionally, the proportion of B1 and B2 also increased by contrast to the microalgae. These changes result from loss of the sulfate group at C11, typically observed during shellfish detoxification [31,32].

Although little is known about their toxicity, these toxins do not seem to constitute a major portion of PST analogues in mussels. This knowledge is important for future replacement of biological testing methods by chemical methods. Further confirmation is needed resorting to liquid chromatography coupled to mass spectrometry detection. Standards for these toxins are also important, not only to calibrate chemical analysis, but also to accurately determine the toxicity of wide diversity of GC analogues produced by G. *catenatum*.

5. CONCLUSION

The present study showed that there is a correlation between the *G. catenatum* cells concentration and the PSP toxin level accumulated in mussels on the Moroccan Mediterranean Coast.

The highest abundance of *G. catenatum* and the maximum levels of PSP toxins were recorded in late fall and early winter.

This study is in fact the first attempt at an assessment of *G. catenatum* and PSP production in mussels on the Moroccan Mediterranean Coast.

Liquid chromatographic analysis revealed the presence for the first time in Morocco of the GC-toxins, hydroxybenzoates analogues of saxitoxin.

The toxin profiles showed that the GC toxins are abundant in *G. catenatum* and minority in mussels. They are also appearance of the decarbamoyl toxins in mussels. Therefore further work needs undertaking for understand the mechanisms of the conversion of GC toxins into decarbamoyl analogues.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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