

Weekly occurrence of *Gymnodinium catenatum* and Paralytic shellfish Poisoning in the Mediterranean shore of Morocco

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Abstract:

Weekly monitoring for the presence of *Gymnodinium catenatum* and PSP toxins in mussels (*Mytilus galloprovincialis*) 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 conducted using the light inverted microscope. Monitoring of PSP toxins levels in mussels was achieved using the mouse bioassay method and toxin's profile was performed by HPLC/FD in positive samples.

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 familie, 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

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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 (FAO, 2004).

Several species of dinoflagellates, such as *Alexandrium tamarense* (Lebour) Balech (Prakash, 1967), *A.catenella* (Whedon & Kofoid) Balech (Schantz et al., 1966; Proctor et al., 1975), *Pyrodinium bahamense* var.compressum (Maclean, 1977; Harada et al., 1982) and *Gymnodinium catenatum* H.W.Graham (Anderson et al., 1989; Oshima et al., 1993) 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 decarbamoyls toxins consisting of dcGTX1-dcGTX4, dcNEO and dcSTX.

(4) Deoxydecarbamoyls toxins composed of doSTX, doGTX2, do GTX3.

These groups possess different toxicities as determined by intraperitoneal 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 (Oshima 1995a).

Recently, three novel hydroxybenzoate saxitoxin analogues were isolated from Australian strains of *G. catenatum* and designated GC1 through GC3 (Negri, et al., 2003). 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 (Taleb et al., 2001).

In the Mediterranean, this contamination has been due to the presence of *G.catenatum* (Taleb et al., 2001), while in the Atlantic it has been due to the appearance of both *G.catenatum* and the genus *Alexandrium* (Taleb et al., 2003).

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 (Negri, et al., 2003).

2. Material 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 galoprovincialis*) 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.

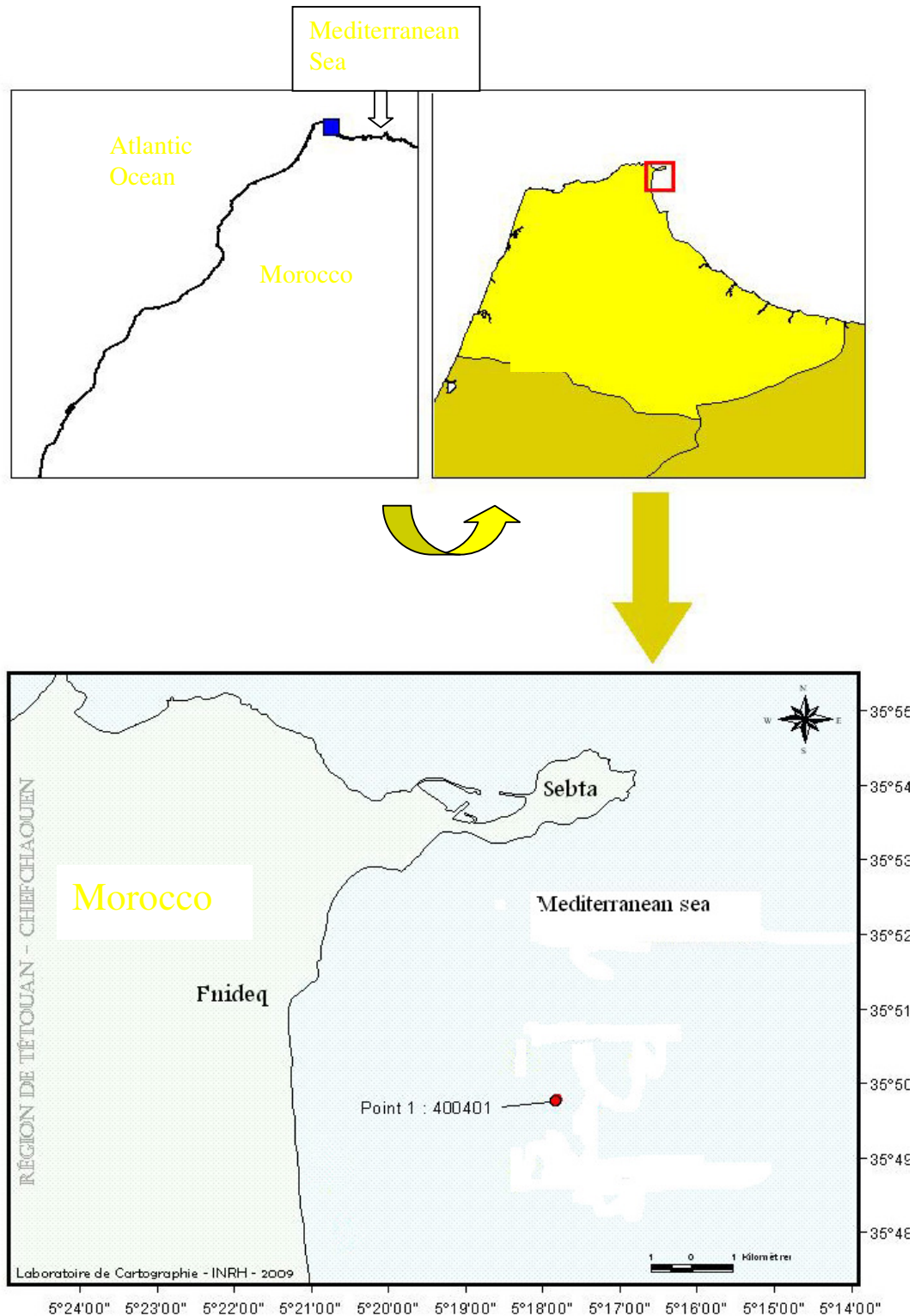


Fig. 1: Sampling site in Mediterranean coasts of Morocco.

2.2. Phytoplankton analysis

A potentially harmful species of dinoflagellates were analysed and identified by inverted microscopy according to the Utermöhl method (1958).

2.3. Mouse bioassay

Mussel samples were extracted according to the AOAC method (2000). Briefly, 100 g of homogenized tissues was mixed with 100 mL of hydrochloric acid 0.1M (pH adjusted to ca.3) and the extract boiled for 5 min; after cooling the pH was adjusted to 2-4 by HCl 5M or NaOH 0.1 M, then the mixture was transferred to a graduated cylinder, diluted to 200 mL by distilled water and centrifuged at 3000g for 5 min. One millilitre of the supernatant was injected intraperitoneally into three 20 g albinos mice. The values were expressed in µg STX equi./Kg of meat.

The sanitary threshold adopted by the National regulations is 800 µg STXeq./Kg of meat.

2.4. Liquid chromatography analysis

2.4.1. Reagents

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 Liquid chromatography separation followed by fluorescence detection.

The only mussel's sample analysed by HPLC was the sample of 16 September 2010.

2.4.3. Liquid chromatography with fluorescence detection (HPLC-FLD)

Liquid chromatography was performed on Hewlett-Packard (HP) equipment consisting of: Model 1050 quaternary pump and autosampler, Model 1046-A fluorescence detector and Model 1100 in-line degasser. 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 30 °C. 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, and from 2% to 8% eluent A from 5 to 10 min, followed by re-equilibration to the starting conditions for 5 min prior to the next oxidation cycle. The time for derivatizing plus the 5 min re-equilibration time added to a total

of 12 min for re-equilibration between consecutive injections. Flow rate was set at 0,5 mL/min. The GCs analogues were revealed with an increase in the acetonitrile gradient, as described in Vale (2008b).

The automated oxidising reactions with periodate were described in detail in (Vale, 2005), usually 5 µL of sample were injected. For better peak shape, manual peroxide oxidations were preferred, and 12.5 µL of the oxidised mixture reaction was injected on column.

Toxin identification for GC group, where no standards are available, was described in detail in Vale (2008b).

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 (fig2).

At the first period stretching between 04 January 2010 and 16 February 2010, there was a gap between the phase of increase in the concentration of *G.catenatum* and the level of PSP toxins in mussels (fig2 a). 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. 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 2 b). 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 (fig2 c). 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 concentrations of *G.catenatum*, we note that for figure 2 b, it took only 1010 cells L⁻¹ to reach a value of 15620 µg/Kg of meat, while for the figure 2 c, it took 10720 cells L⁻¹ to have a value close to 15620 (16370 µg/ Kg of meat).

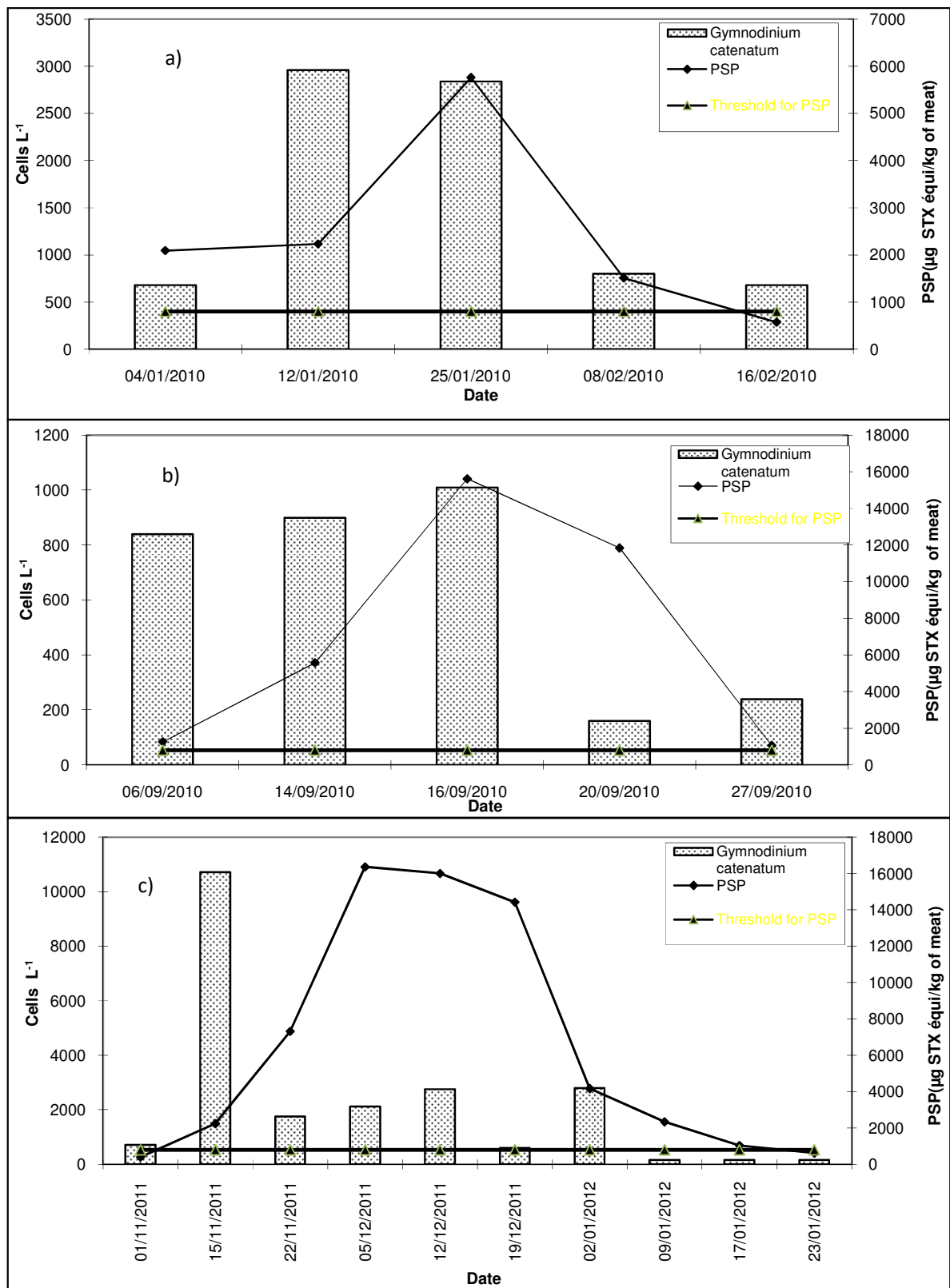


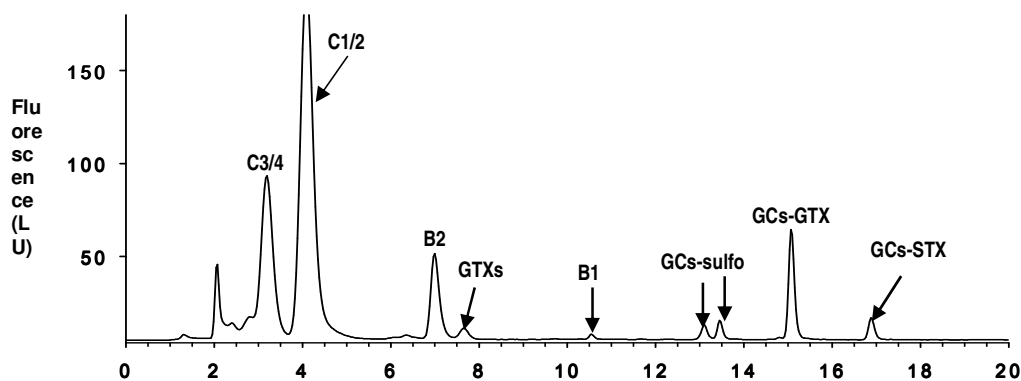
Fig 2: Evolution of bivalve toxicity in association with *G.catenatum* in:

a) first period b) second period c) third period

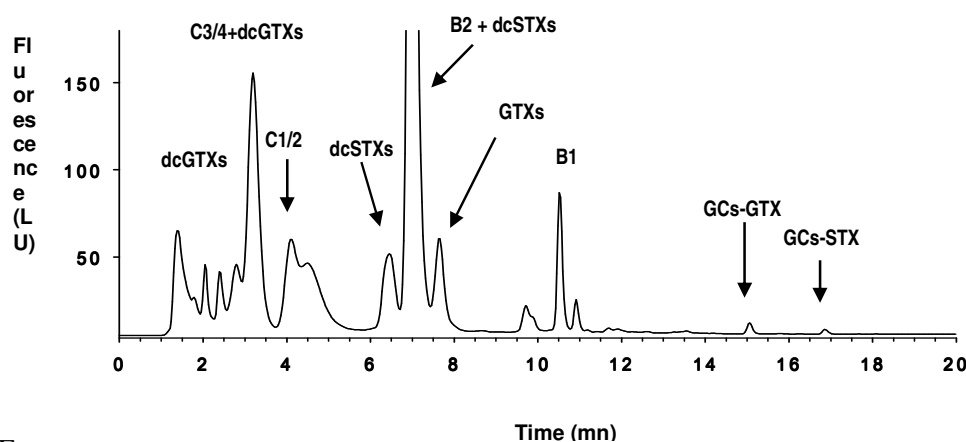
NB: The y-scales are not the same for the figures a, b and c.

3.2. Liquid Chromatography:

A)



B)



F

Fig.3: Chromatograms of PSP toxins in: A) a cultured *G. Catenatum* strain;
B) Moroccan Mussels.

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.3 B) 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 and the concentration of *G.catenatum* present in sea water.

Indeed, during the 3 years of follow-up we observed an increase in the rate of PSP 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*

G.catenatum is the main organism causing recurrent Paralytic Shellfish Poisoning (PSP) event in the western Mediterranean coast (Tahri-Joutei Leila 1998, Ribeiro *et al.* 2012, Maman *et al.* 2000, Gomez and Claustre 2001, Fernando Gomez 2003, Illoul *et al.* 2005, Frehi *et al.* 2007, Trainer *et al.* 2010, Taleb *et al.*, 2001 and 2003). 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.

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 which persist from November to March. However, seasonal reappearance in summer is very short and leads to bivalve molluscs contamination at relatively low levels usually below the accepted safety threshold. (Taleb *et al.* 1998, 2001, Tahri-Joutei Leila 1998, Ribeiro *et al.* 2012, Maman *et al.* 2000, Gomez and Claustre 2001, Fernando Gomez 2003, Illoul *et al.* 2005, Frehi *et al.* 2007, Trainer *et al.* 2010).

Maximum levels of PSP toxins depends not only on levels of toxic algae but also other parameters which play a key role such as cell toxicity and toxic algae on non-toxic ratio or distribution of toxic algae in the water which may be heterogeneous.

This study also has highlighted for the first time in Morocco, 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* (Negri *et al.* 2003), and later detected in strains from other regions (Negri *et al.* 2003, 2007; Vale, 2008; Bustillos-Guzman *et al.*, 2011; Gu *et al.*, 2013).

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 decarbamoyls analogues.

Similar conversion was first hypothesized in several species of Portuguese shellfish by Vale (2008a).

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.

References:

Anderson, D.M., Sullivan, J.J., Reguera, B., 1989. Paralytic shellfish poisoning in northwest Spain: the toxicity of the dinoflagellate *Gymnodinium catenatum*. *Toxicon* 27 (6), 665– 674

AOAC (Association of Official Analytical Chemists), 2000b. AOAC Official Method 959.08. Paralytic Shellfish Poison, Biological Method. Final Action. AOAC official methods of analysis, 17th ed., Gaithersburg, MD, USA, 59-61. AOAC 959.08 (*Source: AOAC, 2000, Chap. 49:59 - 61*)

Bustillos-Guzman, J., Vale, P., Band-Schmidt, C., 2011. Presence of benzoate-type toxins in *Gymnodinium catenatum* Graham isolated from the Mexican Pacific. *Toxicon*, 57 (6), 922-926.

FAO, 2004. Marine Biotoxins, FAO Food and Nutrition Paper, 80. Food and Agriculture Organization of the United Nations, Rome. 278 pp.

Frehi H., Couté A., Gérard Mascarell G., Catherine Perrette-Gallet C., Mebarek Ayada M., Kara M. H., 2007. Dinoflagellés toxiques et/ou responsables de blooms dans la baie d'Annaba (Algérie). *Comptes Rendus Biologies* 330, 615–628.

Gomez Fernando., 2003. The toxic dinoflagellate *Gymnodinium catenatum*: an invader in the Mediterranean Sea. *Acta Botanica Croatica*, 62: 65-72.

Gu, H., Liu, T., Vale, P., Luo, Z., 2013. Morphology, phylogeny and toxin profiles of *Gymnodinium inusitatum* sp. nov., *G. catenatum* and *G. microreticulatum* (Dinophyceae) from the Yellow Sea, China. *Harmful Algae*, 28, 97-107.

Harada, T., Oshima, Y., Kamiya, K., Yasumoto, T., 1982. Confirmation of paralytic shellfish toxins in the dinoflagellate *Pyrodinium bahamense* var. *compressa* and bivalves in Palau. *Bull. Jpn. Soc. Sci. Fish.* 48, 821–825.

Illoul, H., Maso, M., Figueroa, R.I., Van, Lenning K., 2005. Detection of toxic *G.catenatum*(Graham, 1943) in Algerian waters (SW Mediterranean Sea), *Harmful Algae* News 29.

- Lawrence, J.F., Niedzwiadek, B., Menard, C., 2005. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. *J. AOAC Internat.*, **88** (6), 1714-1732.
- Maclean, J.L., 1977. Observations on *Pyrodinium bahamense* Plate, a toxic dinoflagellate in Papua New Guinea. *Limnol. Oceanogr.* 22(2):234-254.
- Negri, A., Stirling, D., Quilliam, M., Blackburn, S., Bolch, C., Burton, I., Eaglesham, G., Thomas, K., Walter, J., Willis, R., 2003. Three novel hydroxybenzoate saxitoxin analogues Isolated from the dinoflagellate *Gymnodinium catenatum*. *Chem Res Toxicol* 16(8):1029–1033.
- Oshima, Y., Blackburn, S.I., Hallegraeff, G.M., 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *G. catenatum* from three different countries. *Mar Biol* 116:471–476.
- Oshima, Y., 1995a. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. *J AOAC Int* 78(2):528–532.
- Prakash A., 1967- Growth and toxicity of a marine dinoflagellate, *Gonyaulax tamarensis*. *J. Fish Res. Bd Canada* 24 (7) : 1589-1606.
- Proctor, N. H., Chan, S. L., and Trevor, A. J., 1975. Production of saxitoxin by cultures of *Gonyaulax catenella*. *Toxicon* 13: 1-9.
- Sagou, R., Amanhir, R., Taleb, H., Vale, P., Blaghen, M., Lotfi, M., 2005. Comparative study on differential accumulation of PSP toxin between cockle (*Acanthocardia tuberculatum*) and sweet clam (*Callista chione*). *Toxicon*, 46(6), 312-618.
- Schantz, E.J., Lynch, J.M., Vayada, G., Masumoto, K., Rapoport, H., 1966. The purification and characterization of the poison produced by *Gonyaulax catenella* in axenic culture. *Biochemistry* 5, 1191–1195.
- Tahri, L.J., 1998. *G. catenatum* Graham blooms on Moroccan waters. In: Reguera, B., Blanco, J., Ferná'ndez, M.L., Wyatt, T. (Eds.), *Harmful Algae*, Xunta de Galicia and IOC of UNESCO. Spain, pp. 66–67.
- Taleb, H., Idrissi, H., Blaghen, M., 1998. Seasonality of PSP toxicity in shellfish from the Atlantic and Mediterranean coasts of Morocco. In: Reguera, B., Blanco, J., Ferná'ndez, M.L., Wyatt, T. (Eds.), *Harmful Algae*. Xunta de Galicia and IOC of UNESCO, Spain, pp. 68–69.
- Taleb, H., Vale, P., Jaime, E., Blaghen, M., 2001. Study of paralytic shellfish poisoning along the Mediterranean shore of Morocco. *Toxicon*, 39, pp.1855-1861.
- Taleb, H., Vale, P., Blaghen, M., 2003. Spatial and temporal evolution of PSP toxins along the Atlantic shore of Morocco. *Toxicon*. 41, 199-205.

Trainer *et al.* 2010. In Gomez and Claustre 2001, Gómez Fernando and Claustre Hervé, 2001. Spreading of *G.catenatum* Graham in the western Mediterranean Sea. Harmful Algae News 22

Utermöhl, H., 1958. Zur Vervollkommung der quantitativen phytoplankton. Methodik. Mitt. Int. Verein. Theor. Angew. Limnol 9, 1–38.

Vale, P., Taleb, H., Food Addit. Contam. 22 (2005) 838.

Vale P. 2008a. Fate of benzoate paralytic shellfish poisoning toxins from *Gymnodinium catenatum* in shellfish and fish detected by pre-column oxidation and liquid chromatography with fluorescence detection. J Chromatogr A 1190(1–2): 191–197.

Vale, P., 2008b. Complex profile of hydrophobic paralytic shellfish poisoning compounds in *Gymnodinium catenatum* detected by liquid chromatography with fluorescence and mass spectrometry detection. *J. Chromatogr. A.*, **1195**, 85-93.

Vale, P., Rangel, I., Silva, B., Coelho, P., Vilar, A., 2009. Atypical profiles of paralytic shellfish poisoning toxins Ribeiro *et al.* 2012, shellfish from Luanda and Mussulo bays, Angola. Toxicon 53:176–183.