

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

Weekly monitoring of ????? :[1M]Comment

Abstract:

Weekly simultaneous monitoring of the presence of *Gymnodinium catenatum* and paralytic shellfish poisoning (PSP) toxins in mussels of *Mytilus galloprovincialis* harvested on the growing site in Fnideq on the Mediterranean Moroccan coast was carried out from 2010 until 2012 year. The enumeration of *Gymnodinium catenatum* cells in water was conducted using the inverted microscope. Monitoring of PSP toxins levels in mussels has been achieved using the mouse bioassay method and the PSP toxins profile was performed by HPLC/FD in positive samples.

The results showed a correlation between the evolution of *Gymnodinium catenatum* cells concentration and the PSP toxin level accumulated in mussels. The appearances of *Gymnodinium 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 *Gymnodinium catenatum* profile composed mainly of the N-sulfocarbamoyls toxins 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.

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 known as paralytic shellfish poisoning (PSP) and posed a serious health hazard in many areas of the world.

Several species of dinoflagellate, such as *Alexandrium tamarense* (Prakash, 1967), *A.catenella* (Schantz et al., 1966; Proctor et al., 1975), *Pyrodinium bahamense* var.compressum (Macleane, 1977; Harada et al., 1982) and *Gymnodinium catenatum* (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 gonyautoxins (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

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49 N-sulfocarbamoyl analogues the least toxic, while for the deoxydecarbamoyl analogues there
50 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 *Gymnodinium catenatum* (Taleb et al., 2001), while in the Atlantic it has been due to the appearance of *Gymnodinium catenatum* and the genus of *Alexandrium* sp. (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 *Gymnodinium catenatum* and PSP toxins accumulation rates in bivalve mollusc was carried out *in situ*. This study allowed also to characterize in Moroccan water the presence of hydroxybenzoates (GC toxins), a new compound belonging to the PSP toxins group that was 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 2010 and 2012 in the growing mussels area of Fnideq located on the Mediterranean coast (fig.1). Sea water sampling is done at high tide using the bottle at depths 0.5 m while mussels' samples were collected at low tide.

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Gymnodinium catenatum :[4M]Comment

?????the method used :[5M]Comment

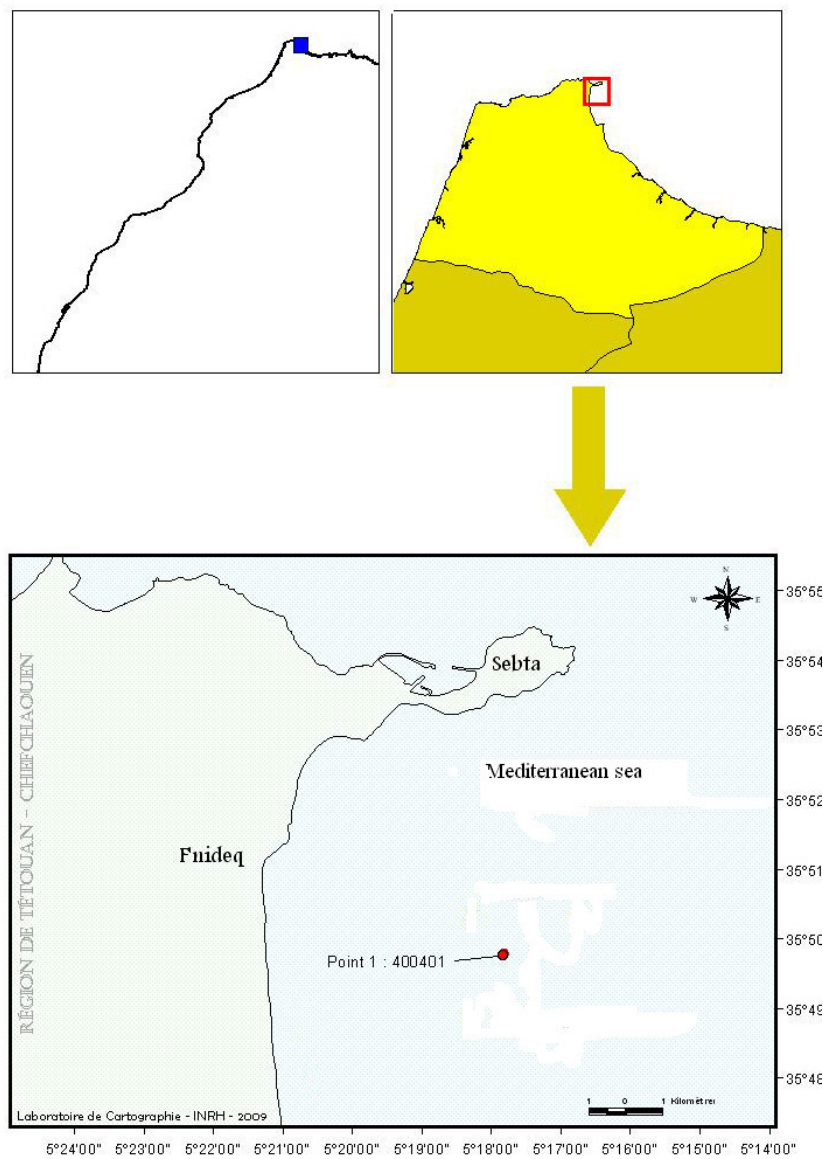


Fig. 1: Sampling site in Mediterranean coasts of Morocco.

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2.2. Phytoplankton analysis

Potentially harmful species of dinoflagellate *Gymnodinium catenatum* were analysed and identified by inverted microscopy according to the Utermöhl method (1958).

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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.

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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 quantitation.

2.4.2 Toxin extraction

Supernatant of shellfish samples obtained by mouse bioassay according to AOAC (2000) were used for Liquid chromatography separation followed by fluorescence detection.

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: 20mM ammonium formate with 1mM 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 10min, followed by re-equilibration to the starting conditions for 5 min prior to the next oxidation cycle. The time for derivatizing plus the 5min 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.

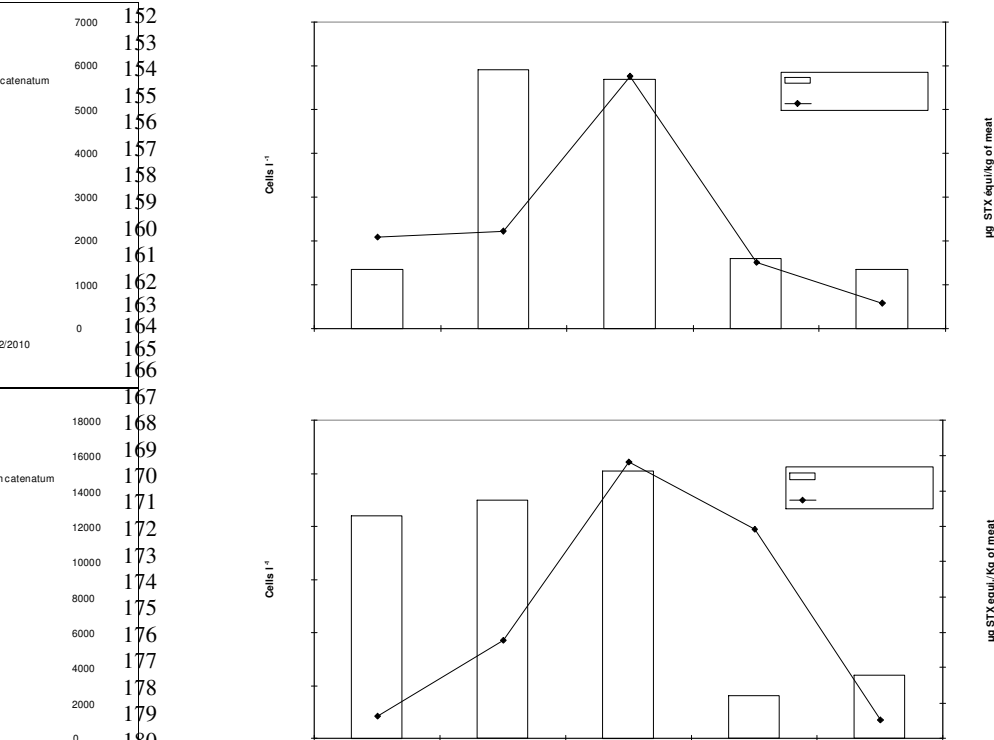
The automated oxidising reactions with periodate were described in detail in (Vale, P., 2005), usually 5_L of sample were injected. For better peak shape, manual peroxide oxidations were preferred, and 12.5_L of the oxidised mixture reaction was injected on column.

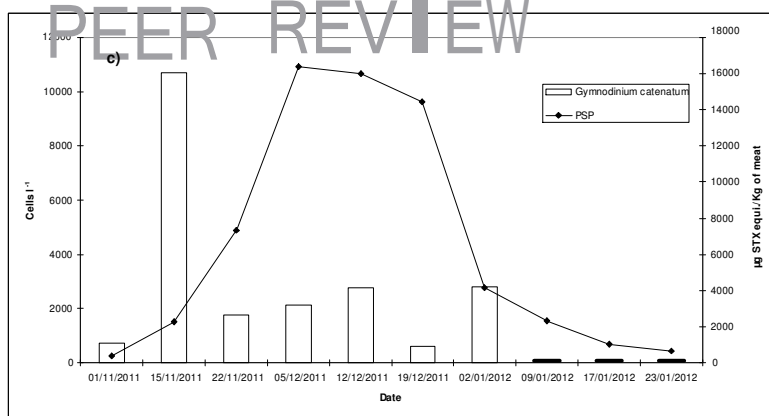
3. Results

3.1. Monitoring data:

During weekly monitoring of phytoplankton and the PSP toxins between January 2010 and January 2012 the concentration of *Gymnodinium catenatum* and the PSP toxins level were observed to increase three times.

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 *Gymnodinium catenatum* and the level of PSP toxins in mussels (fig 2 a). The maximum concentration of *Gymnodinium 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 with a lag of almost two weeks between both peaks. The stages of decay of the two parameters are progressive and are nearly identical.





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

However, the second period extending between 06 September 2010 and 27 September 2010 was marked by a proportionate increase in the concentration of *Gymnodinium catenatum* and the level of PSP toxins in mussels (fig 2 b). The maximum cells *Gymnodinium catenatum* concentration was 1010 cells L-1 where the PSP toxins values reached was 15620 µg STXequi./kg of meat. But there was a shift of one week during the decline phase of the two parameters.

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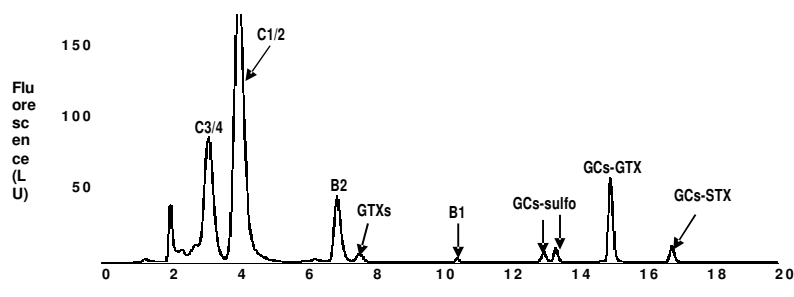
During the third period, between 01-11-2011 and 23-01-2012, evolution was quite different. The concentration of *Gymnodinium catenatum* was different from the rate of PSP in mussels (fig 2 c). The two phases of increase and decrease were quick for the concentration of *Gymnodinium catenatum* and progressive for level of PSP in mussels. In fact, the maximum value of the concentration of *Gymnodinium catenatum* (10720 cells L-1) was achieved in 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.

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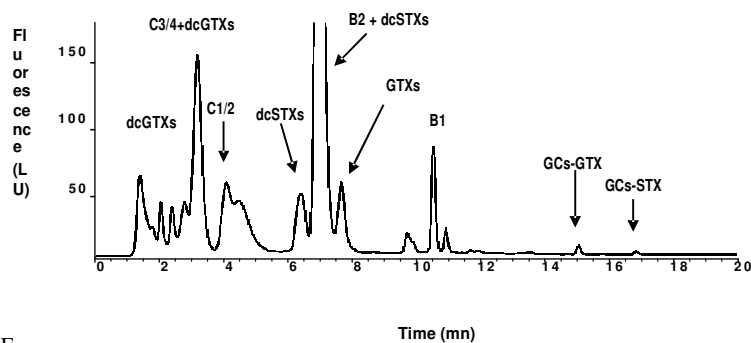
In addition, the maximum cell numbers of *Gymnodinium catenatum* will plummet in a week to reach a value of 1760 cells L-1 where the PSP toxins value in mussels decrease gradually over two months to reach a level of 620 µg STXequi./Kg of meat.

3.2. Liquid Chromatography:

A)



B)



F

Fig.3: Chromatograms of PSP toxins in: A) *Gymnodinium catenatum*; B) Mussels

Owing to the lack of *Gymnodinium catenatum* toxin profile from Moroccan coast, the comparison was carried out with *Gymnodinium catenatum* from the coast of Portugal (fig.3A).

In *Gymnodinium 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 mussels chromatogram (fig.3 B) showed a profile reflecting that of *Gymnodinium catenatum* (the presence of a sulfocarbamoyls toxins) but with the appearance of the decarbamoyl gonyautoxins (dcGTXs) and decarbamoyl saxitoxins (dcSTXs). After 11 min of the chromatography the GC-sulfo toxins disappeared and GCs- GTX and GCs-STX analogues constituted a minor component toxin content.

4. Discussion:

The present study allowed showing a relationship between the accumulation of PSP toxins in mussels and the concentration of *Gymnodinium 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 *Gymnodinium catenatum* cells. Maximum PSP levels in mussels were reached at least after one week of *Gymnodinium catenatum* cell maxima. Thus, the mussel's contamination was associated with the occurrence in sea water of *Gymnodinium catenatum*

Gymnodinium 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 *Gymnodinium 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, 200, 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 other algal species abundance or distribution of toxic algae in the water which may be heterogeneous.

This same study has highlighted also 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 *Gymnodinium catenatum* (Negri *et al.* 2003).

The comparison between the toxin profiles of *Gymnodinium catenatum* and mussels showed that the GCs-GTX and GCs-STX toxins that were majority after toxins C1/2 and C3/4 in *Gymnodinium catenatum* became minority in mussels. The appearance of the dcGTXs and dcSTXs and the disappearance of the GC-sulfo toxins in the mussels led to the suspicion of the transformation of these toxins in their similar decarbamoyls GC.

A conversion of GC toxins into decarbamoyl analogues was confirmed by *in vitro* incubations of bivalve's digestive glands with semi-purified GC toxins (Vale, P., 2008). Vale reported a widespread carbamoylase activity in shellfish, exclusively targeted at benzoate PSP analogues and that is heat-inactivated.

5. Conclusion:

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

The highest abundance of *Gymnodinium catenatum* and the maximum levels of PSP toxins were recorded in winter and summer.

This study is in fact the first attempt at an assessment of *Gymnodinium 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 *Gymnodinium catenatum* and minority in mussels. They are also appearance of the decarbamoyls toxins in mussels. Therefore further work needs undertaking for understand the mechanisms of the conversion of GC toxins into decarbamoyl analogues.

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