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Contamination and depuration of Paralytic Shellfish Poisoning by *Acanthocardia tuberculata* cockles and *Callista chione* clams in Moroccan waters

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Abstract

This study of *Gymnodinium catenatum* was conducted across two sampling stations; M'diq bay and Oued Laou estuary during the period from July 2007 to May 2009. *Gymnodinium catenatum* blooms occurred after a rainfall event in autumn and early winter. Statically analyses showed a positive correlation with rainfall. During January 2008, the *G. catenatum* bloom resulted in contamination of tuberculate cockles and sweet clam by Paralytic Shellfish Poisoning (PSP) toxins. In the Oued Laou estuary, the levels of these toxins in shellfish went beyond the normative threshold for consumption of shellfish, (80 µg SXTeq. /100g of meat) and reached (710 ± 82.07) and (198 ± 6.56) µg SXTeq. /100g of meat in cockles and sweet clam respectively. In M'diq bay, concentrations of PSP toxins in the meat of these two shellfish were lower (256.57 ± 12.22 µg SXTeq. /100g and 80.66 ± 8.14 µg SXTeq. /100g of meat in tuberculate cockles and sweet clam respectively. An experimental test of depuration of tuberculate cockles and sweet clam contaminated by the PSP toxins was conducted in laboratory conditions in the first week of January 2008. The results showed partial and progressive elimination of PSP toxins in two shellfish species over time. In the tuberculate cockle, the elimination of PSP appears to be slower compared with the sweet clam; it took 120 days to reach levels of 80 µg SXTeq. /100g of meat, on the other hand only 3 days were needed to reach this safe concentration in the sweet clam.

1. Introduction

Gymnodinium catenatum appearances generate PSP toxins and human shellfish poisonings. Theses toxins form a group of 21 of chemically similar molecules, with the base toxin Saxitoxin (SXT) [1-23-24-25-32]. This toxin affects the nervous system by the inhibition of depolarization of the potential membrane and propagation of the potential action by blocking the Na^+ channel [2] which causes paralysis. Mainly the microalgae from the dinoflagellates produce PSP toxins: *Gymnodinium catenatum, Pyrodinium bahamense* and many species of the genus *Alexandrium* [3], the recent studies have shown the production of PSP toxin by cyanobacteria [26-33]. *G. catenatum* is the most common species related to PSP toxins in the Western Mediterranean coast of Morocco (unpublished). This species is widely distributed in the world; it is a cosmopolitan warm-water species [4-22].
The first report, referring to intoxicated persons, after ingestion of PSP contaminated shellfish was reported in 1793; this intoxication affected the crew of the Captain Vancouver on the West coast of Canada [5]. In Morocco, the first report of poisoning by seafood is dated November 1971 and October 1975 [6]. In October 1982, numerous intoxications and two deaths were reported [7]; the patients had generalized and localized allergic reactions, constriction of the throat with choking sensation of thirst, sweat, abdominal pain, paralysis at the level of the face or limbs, vomiting, tingling, headaches and anesthetic sensitivity type disorders [7].
The aim of this work is to study the spatio-temporal evolution of *G. catenatum* abundances and shellfish species contamination by Paralytic Shellfish Poisoning (PSP) toxins.

2. Material and Methods

2.1. Gymnodinium catenatum abundance

G. catenatum abundance was monitored at two stations located in the Moroccan Western Mediterranean coasts (Fig. 1). Station 1 (S1) (35° 27', 310N - 05° 05'06W) is located near the Oued Laou estuary. This estuary receives permanent inputs from Oued Laou streams. Station 2 (S2) (35° 41,646 N - 05° 19 075' W) is located in

M'diq bay. This Bay receives three rivers after torrential rains. The M'diq bay and Oued Laou estuary are important fishing areas for shellfish (*Acanthocardia tuberculata* and *Callista chione*). Furthermore, the M'diq bay is under the influence of anthropogenic activity. However, the estuary Oued Laou is less urbanized.

At stations S1 and S2, the *G. catenatum* abundance was monitored, in weekly seawater samples during the period from July 2007 to May 2009. The salinity, temperature and the pH of the water were measured respectively using Multipara-meter instrument temperature / salinity / conductivity WTW LF, 597, probe and pH meter field WTW pH, 197.

The species composition of phytoplankton (genus and species) was studied from samples collected during the third week of January 2008 in two sampling stations S1 and S2. The samples taken by Nansen bottles at a depth of 0.5 m, was fixed by acetic Lugol (2–3 ml per liter of seawater). Phytoplankton species were identified by following the Utermöhl method [8] and observed at a linear 100x, 200X, 400X magnification, by using an inverted microscope Leica DMLII. The count was made by using a settlement bottle of 25 mL capacity, following the Hasle method described in Sournia [9]. Phytoplankton identification was made according to the systemic groups using classical references (books and illustrations): [5-10-11]. During *G. catenatum* bloom, the relative dominance (Dr) of each species is the ratio between cell abundance (Aa) and the total phytoplankton abundance of the sample (At) expressed by: Dr (%) = (Aa / At) x 100.

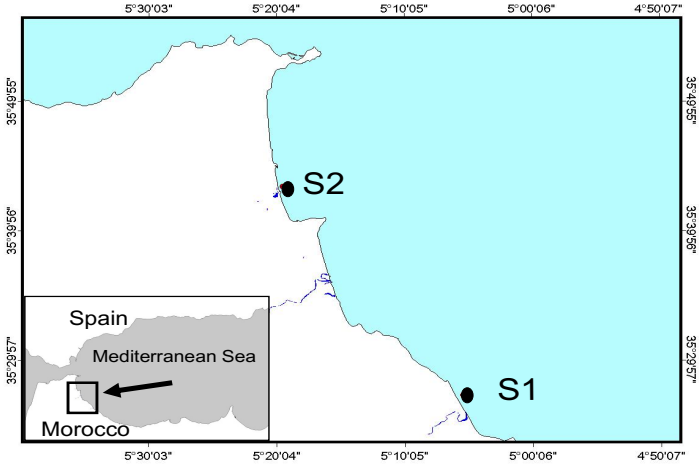

Figure 1: Geographical location of sampling in Oued laou estuary (S1) and M'diq Bay (S2), Morocco.

2.2. Toxins analyses

During January 2008, shellfish species (cockles and sweet clam) were sampled by dredging at S1 and S2 to measure the content of the PSP toxins in the flesh of the tuberculate cockle and sweet clam. The PSP toxins analyses were performed by the Biological Test mouse standardized method [12]. Meat of 15 individuals of shellfish were dissected and homogenized, 100g of meat (ground and homogenized) were added to 100 mL HCl (0.1 N), the sample was homogenized by Ultra-turrax, and then pH adjusted to 3. This sample was heated to boiling on a hot plate for 5 min. Distilled water was used to fill to 200 ml. After centrifugation at 3000 rpm for 5 min., 1 mL of the supernatant was injected into mice albino type. Three mice were injected per sample. The survival time of these mice was identified to evaluate the degree of toxicity.

2.3. Depuration experience

Tuberculate cockles and sweet clam PSP toxins contaminated naturally were studied in laboratory conditions. These samples were collected by dredging shellfish at S1 in the first week of January 2008. The collected individuals were kept in bins of 5 m^3 containing sand under the following conditions: i) permanent input of Sea water at 17°C and 36.5 psu of temperature and salinity respectively, ii) Oxygenation of water by dissemination of ambient pressure, iii) a daily administration (ad libidum) of food composed by non-toxic microalgae of a monospecific cultures of *Skeletonema*, *Thalassiosira* and *Nanochloropsis*. An analysis of PSP toxins was conducted monthly among tuberculate cockle. Concerning the sweet clam, the three first analyses were performed at an interval of three days and the three following analyses according to a weekly frequency.

2.4. Statistics

The Spearman's rank correlation test was performed for two stations, to evaluate the relationships between the *G. catenatum* abundances and various observed environmental parameters (rainfall accumulated over a 3-week period, temperature, salinity and pH). This test was performed using the software XLSTAT 2011, with the level of significance set at α equal to 0.05.

3. Results and discussion

At S1, during the period from July 2007 to May 2009 we registered proliferation of *G. catenatum* (Fig. 2). The first bloom was during: i) the third week of September 2007 until the first week of February 2008 (fall 2007 - early winter 2008), ii) the first week of October 2008 until the second week of February 2009 (fall 2008 - early winter 2009).

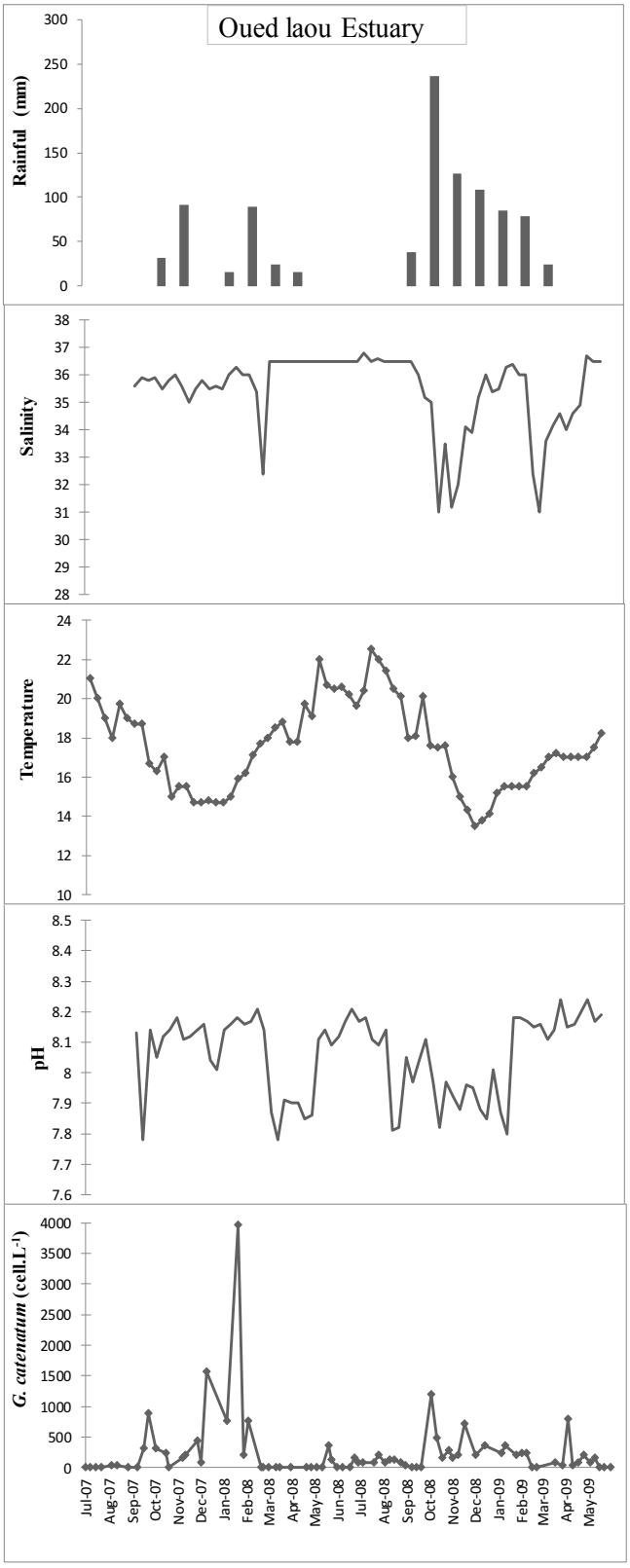

Figure 2: *Gymnodinium catenatum* abundance (cells.L^{-1}), Temperature, pH, Salinity and Rainfall, from September 2007 to May 2009 in Oued Laou Estuary (S1).

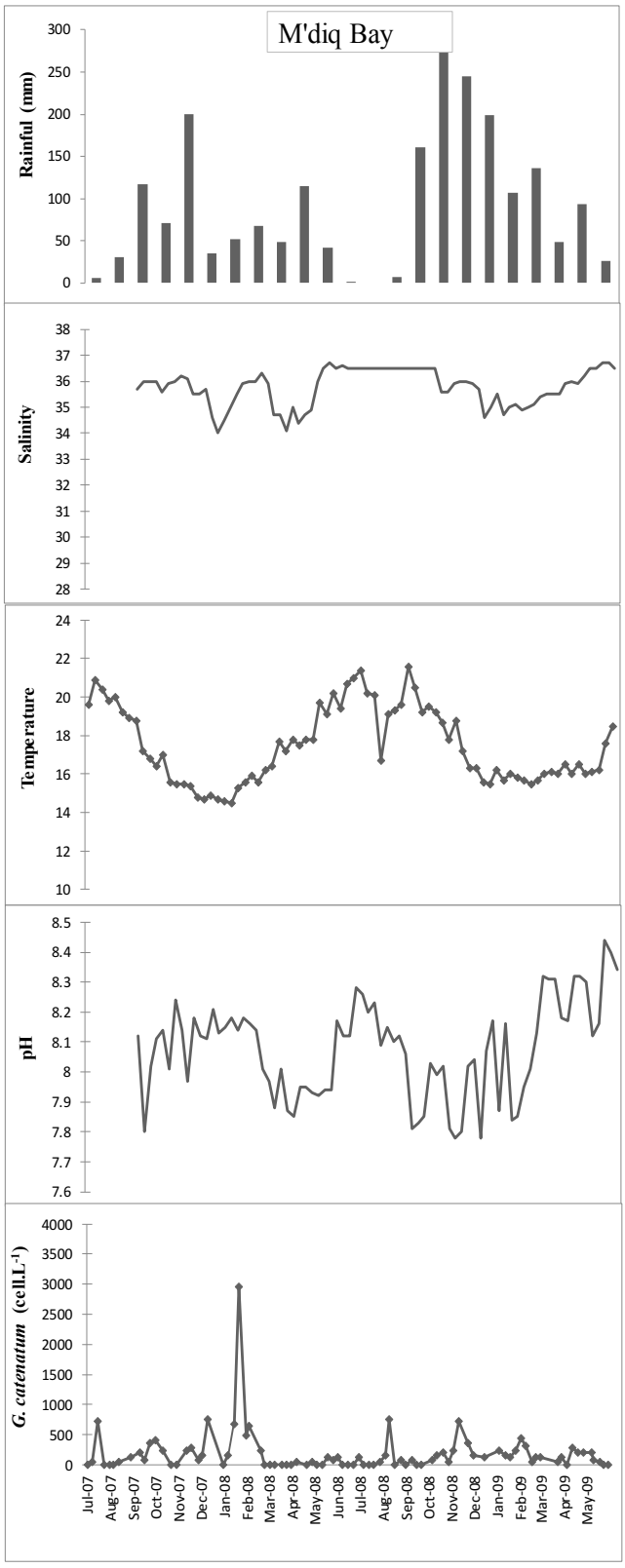

Figure 3: *Gymnodinium catenatum* abundance (cells.L^{-1}), Temperature, pH, Salinity and Rainfall, from September 2007 to May 2009 in M'diq Bay (S2).

The largest *G. catenatum* abundances were noted during the first bloom with a maximum of 3960 cells.L^{-1} recorded in samples of the third week of January 2008. However, abundances during the fall 2008 - early winter 2009 did not exceed 10^3 cells.L^{-1} with the exception of 1200 cells.L^{-1} recorded in the samples of the first week of October 2008.

At S2, the *G. catenatum* proliferation followed the same pattern as recorded in S1, with two bloom periods: fall 2007 - early winter 2008 and fall 2008 - early winter 2009 (Fig. 3). This species was registered at smaller abundances than S1 with a maximum of 2960 cells.L^{-1} in the samples of the third week of January 2008.

At S1 and S2 sites, during July-August 2007, mid-February - mid-September 2008 and mid-February - end of May 2009, the presence of *G. catenatum* in our samples remained very sporadic except in some samples. In S1, in the third week of May 2008 (360 cells.L^{-1}) and the first week of April 2009 (800 cells.L^{-1}). In S2, in the third week of July 2007 (720 cells.L^{-1}), the second week of August 2008 (760 cells.L^{-1}) and the second week of April 2009 (280 cells.L^{-1}).

Statistical correlations (Spearman ranks) between the appearance of *G. catenatum* and environmental parameters (rainfall accumulated over a 3-week period, temperature, salinity and pH) showed a positive correlation with rainfall in M'diq bay (0.306) and Oued Laou estuary (0.239), negative correlation with salinity in Oued Laou estuary (-0.412), and no correlation with temperature and pH in the two areas (Table 1 and 2). *G. catenatum* was registered at temperatures from 14.7 °C to 19°C and salinities from 34 to 36 psu. The bloom of this species was noted at temperature of about 15.5 °C and salinity of 35.6 psu.

Table 1: Spearman rank correlation coefficients between the variables studied in Oued Laou estuary (S1)

	G. catenatum	**Temperature**	**Salinity**	**pH**	**Rainfall**
G. catenatum	1.000				
Temperature	-0.139	1.000			
Salinity	**-0.412**	**0.421**	1.000		
pH	-0.232	-0.080	0.019	1.000	
Rainfall	**0.239**	**-0.490**	**-0.401**	**-0.289**	1.000

Bold, significant values (non-diagonal) in the alpha threshold = 0.050 (bilateral test)

Table 2: Spearman rank correlation coefficients between the variables studied in M'diq bay (S2)

	G. catenatum	**Temperature**	**Salinity**	**pH**	**Rainfall**
G. catenatum	1.000				
Temperature	-0.116	1.000			
Salinity	-0.222	**0.573**	1.000		
pH	0.016	-0.136	0.151	1.000	
Rainfall	**0.306**	-0.220	**-0.471**	-0.074	1.000

Bold, significant values (non-diagonal) in the alpha threshold = 0.050 (bilateral test)

In the third week of January 2008, we observed a *G. catenatum* bloom at stations S1 and S2. Phytoplankton belonged to four classes: Diatomophyceae (19 species), Dinophyceae (16 species), Dictyochophyceae (1 species) and Raphidophyceae (1 species) (Table 3).

At S1, we identified 27 taxa (genus and species) some of them are renowned to be producers of toxins, namely: *Gymnodinium catenatum, Alexandrium minutum, Chattonella sp*. and *Pseudo-nitzschia* spp.. The class of Diatomophyceae dominated the phytoplankton community (67% of the total species), followed by Dinophyceae (31%). The total abundance of phytoplankton community was 1.8 x 10^4 cells L^{-1}. Four species dominated this community: *Leptocylindrus* spp. (30%), *G. catenatum* (21%), *Eucampia zodiacus* (10%) and *Pseudo-nitzschia* spp. (9%).

At S2, we identified 30 taxa (genus and species); some of them able to produce toxins, namely: *Gymnodinium catenatum, Alexandrium minutum, Dinophysis acuminata, Gonyaulax spinifera* and *Pseudo-nitzschia* spp.. The total abundance of phytoplankton community was 3.4 x 10^4 cells.L^{-1}, dominated by Diatomophyceae (86%) and followed by Dinophyceae (12%). The dominants species was *Leptocylindrus* spp. (28%), *Thalassiosira* spp. (24 %), *Pseudo-nitzschia* spp. (15%), *Gymnodinium catenatum* (8%), *Chaetoceros* spp. (7%) and *Rhizosolenia stolterfortii* (6%).

Table 3: Phytoplankton Composition and population structure in *Gymnodinium catenatum* bloom (January 2008) in M'diq Bay and Oued laou estuary. S1-Oued Laou estuary, S2- M'diq Bay.

Species	Species (cells.L^{-1})		Relative dominance	
	S1	S2	S1	S2
Dinophyceae	5800	4440	31.25 %	12.85 %
Gymnodinium catenatum	3960	2960	21.34 %	8.56 %
Alexandrium minutum	80	120	0.43 %	0.35 %
Scrippsiella sp	1000	560	5.39 %	1.62 %
Heterocapsa sp	80	-	0.43 %	-
Amphidinium sp	-	120	-	0.35 %
Gyrodinium sp	-	160	-	0.46 %
Protoperidinium sp	80	80	0.43 %	0.23 %
Protoperidinium steinii	-	80	-	0.23 %
Dinophysis acuminata	-	40	-	0.12 %
Gonyaulax spinifera	-	40	-	0.12 %
Prorocentrum sp	320	120	1.72 %	0.35 %
Prorocentrum micans	40	-	0.22 %	-
Noctiluca scintillans	120	-	0.65 %	-
Ceratium fusus	40	80	0.22 %	0.23 %
Ceratium furca	80	40	0.43 %	0.12 %
Ceratium pentagonum	-	40	-	0.12 %
Diatomophyceae	12480	30040	67.24 %	86.92 %
Leptocylindrus spp	5640	9840	30.39 %	28.47 %
Rhizosolenia pungens	40	40	0.22 %	0.12 %
Rhizosolenia alata	120	40	0.65 %	0.12 %
Rhizosolenia styliformis	40	-	0.22 %	-
Rhizosolenia stolterfortii	920	2160	4.96 %	6.25 %
Pseudo-nitzschia spp	1720	5400	9.27 %	15.63 %
Coscinodiscus sp	40	80	0.22 %	0.23 %
Guinardia sp	-	640	-	1.85 %
Pleurosigma sp	80	80	0.43 %	0.23 %
Nitzschia spp	880	480	4.74 %	1.39 %
Navicula sp	40	120	0.22 %	0.35 %
Thalassiosira spp	560	8320	3.02 %	24.07 %
Thalassionema	-	80	-	0.23 %
Odentella sp	120	80	0.65 %	0.23 %
Chaetoceros spp	360	2600	1.94 %	7.52 %
Humiaulus sp	-	80	-	0.23 %
Eucampia zodiacus	1880	-	10.13 %	-
Rhabdonema sp	40	-	0.22 %	-
Dictyochophyceae	80	80	0.43 %	0.23 %
Dictyocha sp	80	80	0.43 %	0.23 %
Raphidophyceae	200	0	1.08 %	0.00 %
Chattonella sp	200	-	1.08 %	-
Total	18560	34560	100.00 %	100.00 %

At S1 and S2 during January 2008, analyses showed the presence of PSP toxins in the shellfish meat (Figure 4). At S1, during the first week of January, the PSP toxins exceeded the normative threshold (80 µg SXTeq. /100g of meat); they were (370 ± 26.47) µg SXTeq. /100g of meat in tuberculate cockle and (110 ± 7) µg SXTeq. /100g of meat in sweet clam. These concentrations were higher during the third week of January; they were (710

± 82.07) µg SXTeq. /100g of meat in tuberculate cockle and (198 ± 6.56) µg SXTeq. /100g of meat in the sweet clam. At S2, at the beginning of January 2008 levels of PSP toxins in the meat of these shellfish were less important than those recorded for S1. They were about (123.67 ± 6.03) µg SXTeq. /100g of meat in tuberculate cockle and (40 ± 4.36) µg SXTeq. /100g of meat in sweet clam. Levels of these toxins in shellfish increased due to the maximum spreading of *G. catenatum* during the third week of January 2008; they were about (256.57 ± 12.22) µg SXTeq. /100g of meat in tuberculate cockle and (80.66 ± 8.14) µg SXTeq. /100g of meat in sweet clam.

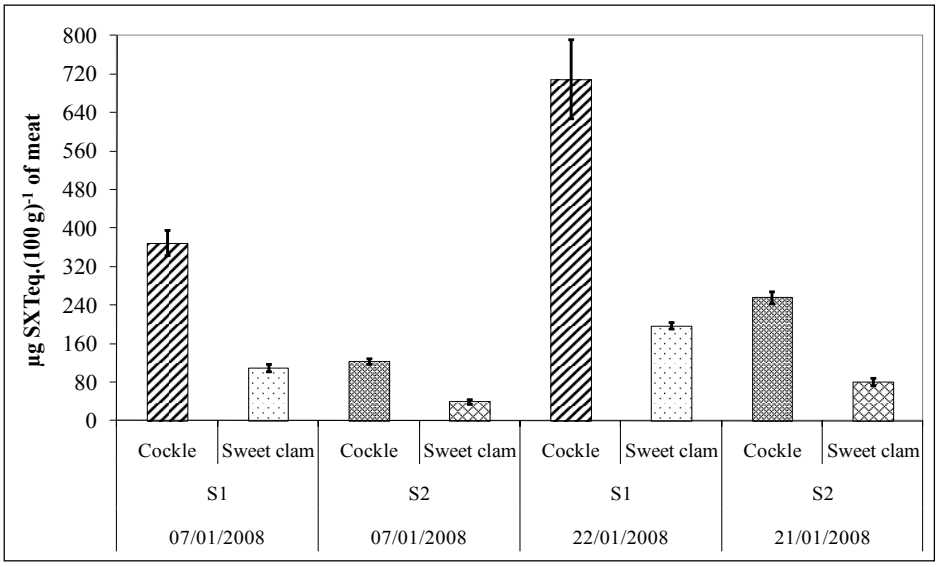

Figure 4: Results of analysis of PSP (Paralytic Shellfish Poisoning) toxins (µg SXTeq. /100g of meat) in shellfish *Acanthocardia tuburculata* (cockle) and *Callista chione* (sweet clam) in January 2008

Depuration experiments with tuberculate cockle and sweet clam from S1 showed the possibility of removal of PSP toxins in these two species. However, their elimination kinetics were variable; tuberculate cockle (Figure 5-A) took four months in the laboratory conditions reaching values below 80 µg SXTeq. /100g and 6 months to reach the minimum value of (41.33 ± 5.51) µg SXTeq. /100g. During the first two months, a fraction of PSP toxins was eliminated in a timely manner (regression equation: A = 433.37 – 65.5 T). This PSP toxins elimination rate decreased to experience progression to become very weak in its last stages (regression equation: A = 93.47 - 17. 97 T) (5th and 6th months).

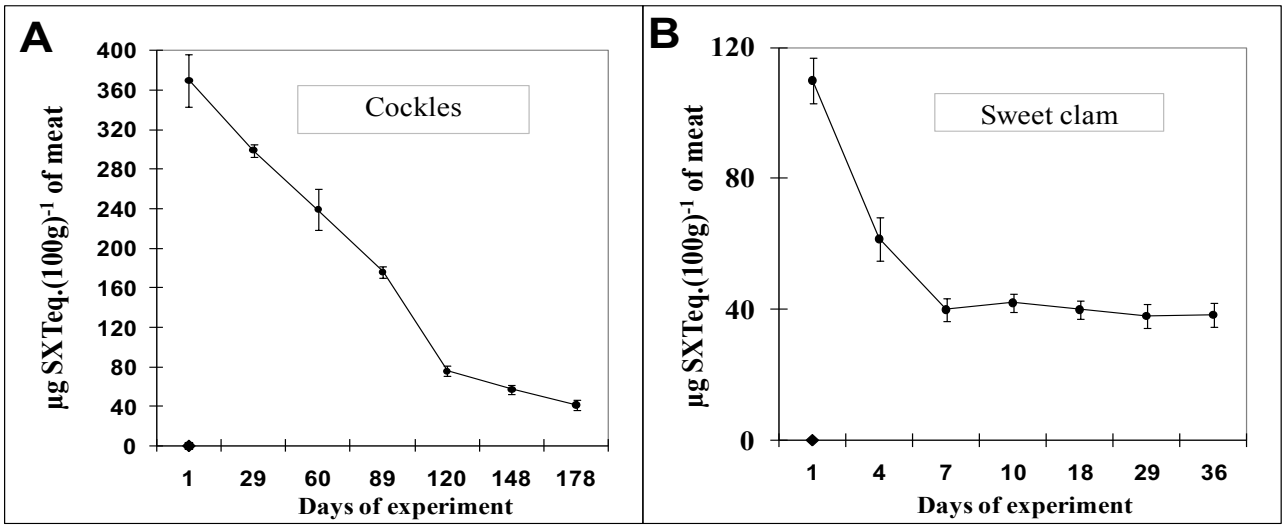

Figure 5: Concentration of PSP (Paralytic Shellfish Poisoning) toxins (µg SXTeq. /100g of meat). A- Experiment depuration of PSP toxins in cockle. B- Experiment depuration of PSP toxins in sweet clam.

Depuration of sweet clam (Figure 5-B) is much faster than tuberculate cockle. Indeed, during the first 6 days, the PSP toxins at sweet clam declined by 63.64%; decontamination is very fast (regression equation: A = 140.53 - 35 T). During the rest of the experience, sweet clam decontamination rate became very low (regression equation: A = 42.55 - 1.08 T). After a month, the PSP toxins in sweet clam became undetectable.

In the present study, *G. catenatum* blooms occurred during autumn and early winter (running period) similar to on the Spanish and Italian Mediterranean coasts. In the Spanish Mediterranean coast, the peak abundances of this species appear during the period February / March [5]. In Italy (Tyrrhenian coastal lagoon), the peak abundances of this species were observed from late spring until early autumn [14].
In our study, we found that the proliferation of *G. catenatum* is related to rainfall. *G. catenatum* bloom occurs in Moroccan Western Mediterranean coasts, after the period of high precipitation (and November - early January). Indeed, [27] shows an increase in bacterial activity during the wet season (autumn and winter) in Moroccan Western Mediterranean coasts. Have recently been demonstrated the influence of the accompanying bacterial community on the physiology and growth dynamic of the dinoflahgellate *G. catenatum* [28-29]. The growth and toxicity of *G. cetanatum* may be influenced by allopathic effect of other phytoplankton species. [30] demonstrates the allopathic effect of the raphidophyte *Chattonella marina var. marina* on the dinoflagellate *G. catenatum*. According [15], the seasonal variations in *G. catenatum* abundance are primarily controlled by water temperature, while interannual variations are thought to be related to differences in water temperature, rainfall patterns and wind stress [16]. In this study, *G. catenatum* was registered at temperatures ranging from 14.7 °C to 19°C and salinity from 34 to 36 psu. These results concord with those by [4] and [17], during *G. catenatum* blooms in Gulf of California and in Tasmanian waters respectively. On the other hand, [3-22-38-39] have shown the presence of a relationship between the blooms of *G. catenatum* and flows of fresh water in Tasmanian waters. The same was observed at the two sites in our study, with a correlation between the *G. catenatum* bloom and salinity decrease in estuary Oued Laou and its absence in Bay M'diq bay. In the Moroccan Western Mediterranean coasts, *G. catenatum* blooms may be related to humus-laden waters, since it appears after rainfall event. According to [17] the humic fractions do not markedly affect the growth rate but prolong the stationary phase allowing the species to achieve higher biomass levels. In an unpublished study, we have noted a sharp increase in the macronutrient concentrations (nitrogen and phosphorus) in the water of M'diq bay and Oued Laou estuary following rainfall events. Freshwater inputs will contribute nutrients from coastal waters and cause a freshening of sea water; it is these two environmental factors together with others (hydrology, climate, biological and chemical) that generate the *G. catenatum* bloom.
The shellfish from the study area showed that the rate of contamination was greater in tuberculate cockles than in sweets clam; it was higher in the Oued Laou estuary than M'diq bay. In contrast, amnesic shellfish contamination in sweet clam is greater than that detected in tuberculate cockles [18]. This could be related to several factors such as: i) the amount of seawater filtered by each shellfish species ii) the phytoplankton biomass and iii) the physiology of the shellfish species.
According to our previous studies, tuberculate cockle filter generally more seawater than sweet clam and are thus more likely to be contaminated. However [19] reported that shellfish filtration rate is related to food concentration; at high food concentrations, a decrease occurs in clearance rate and assimilation efficiency. This is frequently observed in shellfish as a response to satiation of the filtering and digestive system [20]. A decrease in clearance rate is often accomplished by reducing the shell opening [21]. In our study, phytoplankton biomass during the *G. catenatum* blooms in Oued Laou Estuary was lower than that recorded at M'diq bay. The relative dominance of *G. catenatum* in the phytoplankton population was larger in Oued Laou estuary than in M'diq Bay. This situation lead to a higher contamination of shellfish in Oued Laou estuary than in M'diq Bay.
PSP toxins elimination kinetics have shown that tuberculate cockle required 120 days depuration to reach a concentration of less than 80 µg SXTeq. /100g, and decontamination in the sweet clam needed only 3 days to achieve concentrations lower than 80 µg SXTeq. /100g. In the early stages of detoxification, PSP toxins decreased in a remarkably fast manner in the two shellfish species (cockle and sweet clam); this removal may be primarily associated with the elimination of accumulated toxins from the hepatopancreas. This variation in contamination/decontamination between species may be related to the transformation of toxins in tuberculate cockle. In *G. catenatum*, the toxin content is dominated by Sulfocarbamoyl toxins, but toxins from the carbamate groupe can also be found in trace levels [23-31-32]. In a study [35-34] it was demonstrated that the carbamoyl analogues being the most toxic, decarbamoyl analogues intermediate and the N-sulfocarbomyl analogues are the least toxic. Several studies have recently been published showing that the biotransformation to decarbamoyl toxins analogues would occur via the loss of the benzoyl analogues side chain by carbamoylase enzyme activity in shellfish [35-37]. The height contamination in tuberculate cockle, may be exploded by carbamoylase enzyme activity in this species. On the other hand, acid hydrolysis may induce conversion of N-sulfocarbamoyl toxins to the carbamoyl toxins[34-36].

Conclusion

Gymnodinium catenatum blooms in Moroccan Western Mediterranean coasts are observed during the fall and early winter. This species is responsible for recurring contamination of shellfish by the PSP toxins. Contamination of shellfish depends on algal biomass. Sites with low algal biomass are likely to generate

significant higher contamination of shellfish than sites with higher algal biomass. The high toxicity observed in tuberculate cockles is related to its process of contamination – decontamination; they filter higher sea water quantities than sweet clam which resulted in high contamination and its release of PSP toxins is very slow.

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