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Detoxification enhancement in the gymnodimine-contaminated grooved carpet shell, *Ruditapes decussatus* (Linné)

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Abstract:

In the Gulf of Gabès (Tunisia, Eastern Mediterranean sea), the grooved carpet shell *Ruditapes decussatus* has been seen to contain persistent levels of gymnodimine (GYM) for several years. The present experimental work represents the first attempt to assess detoxification kinetics of fast-acting toxins (FAT) in marine molluscs fed specific diets of non-toxic algae (*Isochrysis galbana*).

To find an optimal detoxification method, two experiments were performed in which clams were first fed the toxic dinoflagellate *Karenia selliformis* to artificially contaminate them with GYM, thus simulating the effect of natural toxic episodes. As a second step, the same clams were fed a non-toxic algae, *I. galbana*, to speed up the detoxification process.

Changes in toxin content over the whole experiment were assessed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis.

The first results revealed (i) faster detoxification rates in digestive gland (DG) when clams were fed on *I. galbana* compared with a starved control (no food) and (ii) a typical detoxification pattern, i.e. a rapid drop in toxin content within the first days followed by a secondary slower decrease. GYM levels could be reduced approximately to less than 5% within 7–8 days in clams fed *I. galbana*, according to the initial toxin levels of 1400 and 9400 µg GYM/kg of DG, respectively. At the end of the second experiment, DSP mouse bioassay was negative when GYM was less than 100 µg/kg DG.

Keywords: Detoxification; Toxicity; Gymnodimine; *Karenia selliformis*; Grooved carpet shell; *Ruditapes decussates*

1. Introduction

At least five human syndromes are recognized to be induced by the consumption of phycotoxin-contaminated seafood worldwide. These fall into several groups: paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP) and ciguatera fish poisoning (CFP). There is, therefore, a growing consensus among scientists that the presence of toxic phytoplankton in coastal waters is of great significance for human health. The problem also causes serious economic losses due to periodic closure of commercial shellfisheries.

Gymnodimine (GYM) was isolated as a toxic substance from oysters and is unique in containing butenolide, a 16-membered carbocycle and cyclic imine moieties. GYM was chemically characterized by different investigators (Seki et al., 1995; Miles et al., 2000; Miles et al., 2003) (Fig. 1). The biogenetic origin of gymnodimine was identified as the dinoflagellate *Karenia selliformis*. Two analogs, gymnodimine-B and gymnodimine-C were also isolated from this dinoflagellate (Miles et al., 2000; Miles et al., 2003).

The presence of a spirocyclic imine indicated that this marine toxin belongs to the cyclic imines family, which already includes pinnatoxins (Uemura et al., 1995), pteriatoxins (Takada et al., 2001), prorocentrolides (Chou et al., 1996) and spirolides (Hu et al., 1995).

GYM was later on shown to be widely distributed along New Zealand coastlines, but generally at a low concentration (Stirling, 2001), and was recently identified in digestive-gland tissues of clams *Ruditapes decussatus* from Tunisia (Biré et al., 2002). GYM has also been observed in many other species of contaminated shellfish, including greenshell mussel, blue mussel, scallop, cockle, surfclam, oyster and abalone (Mackenzie et al., 1996; Stirling, 2001; Mackenzie et al., 2002). Furthermore, the toxin is not readily depurated from shellfish, and may persist for years, for example in oysters (Mackenzie et al., 2002).

More recently, GYM-A has been unequivocally detected in shellfish from European and North American coasts (Kharrat et al., 2008), and is considered as an emergent 'fast-acting' phycotoxin (FAT). Due to the rapid onset of neurological symptoms in mice and rapid death following intra-peritoneal (*i.p.*) injection (Mackenzie et al., 1996; Munday et al., 2004). GYM-A has also proved to be toxic to fish (Seki et al., 1995). However, when GYM administered to mice by oral route, toxicity appears to be quite low (Munday et al., 2004). As a result GYM is not considered as a hazard for humans in most concerned countries.

Recent studies tend to demonstrate that GYM-A targets the muscle nicotinic acetylcholine receptor, which could explain its neurotoxicity (Kharrat et al, 2008). When shellfish extracts containing GYM systematically give positive mouse bioassay results for lipophilic compounds, these shellfish cannot be safely consumed. Management of the carpet shell clam fishery requires drastic improvements, particularly the development of routine specific assays to monitor GYM and congeners (McNabb et al., 2005).

In the meantime, the development of detoxification processes could represent a useful option for reducing the final GYM concentration in shellfish meat to a level low enough to produce negative mouse assay results.

The purpose of this study was to determine the impact of non-toxic algal food on detoxification rates, testing the hypothesis that clams fed *I. galbana* detoxified faster than unfed (control) clams. Thus, the detoxification kinetics of *Ruditapes decussatus* experimentally contaminated with *K. selliformis* was studied in raceway-based recirculating or flow-through systems.

2. Materials and methods

2.1. Experimental contamination of clams fed on *Karenia selliformis* followed by detoxification with and without nontoxic food.

Experiment 1

Strain GM95GAB of *K. selliformis,* formerly referred to as *Gymnodinium maguelonnense* or *Karenia sp*. (Guillou et al., 2002, Shao et al., 2004), was isolated in 1995 (Arzul et al., 1995) from the Gulf of Gabès (North of Sfax) after an episode of mass fish mortality (Hamza and El-Abed, 1994, Hansen et al., 2004). Unialgal isolates are stored and batch cultured (250 mL) in f/2-medium (Guillard and Ryther 1962, Guillard, 1975) under alternation of light and shade 12 h / 12 h at 52 ± 4 µmole photons/m^2/s and 16 ± 0.5 °C. *Isochrysis galbana* "Tahiti strain" cultures, used as non-toxic live feed for control purposes, were also maintained under the same conditions. For the experiments, *K. selliformis* and *I. galbana* were grown in larger flasks, i.e. 4 L and 10 L flat-bottomed vessels, respectively, with (*I. galbana*) or without (*K. selliformis*) air supply. Toxic and non-toxic algal cells were harvested for feeding experiments at the end of the exponential growth phase (12 to 14 days after inoculation, respectively).

Algal-cell concentrations were quantified using a Nageotte hemocytometer.

In March 2007, *Ruditapes decussatus* clams with no history of phycotoxin contamination were collected from Noirmoutier island in France. Harvested clams were of commercial size, i.e. 37 ± 2 mm mean shell length (n = 10) and 0.44 ± 0.03 g mean tissue dry weight (n = 10). Samples (8 kg: 252 clams) were immediately transported to the IFREMER laboratory (Nantes, France), and acclimatized for 5 to 6 days in a raceway filled with 150 L seawater, fed on *Isochrysis galbana*, and maintained at 16 ± 0.5 °C (Table 2).

Sea water at a salinity of 35 psu was pumped through the raceways at a flow rate of 800 L/h and circulated in a closed system maintained at 16 ± 0.5°C. The experimental setting was similar to that previously described in Lassus et al. (1999). Seawater was totally renewed every two days to prevent an increase in ammonia concentration (dissolved ammonia levels were measured every two days using the method of Koroleff (1969).

During the contamination period, clams were continuously fed on toxic microalgae *K. selliformis* at a concentration of 200 cells/ml for six days; in such a way that available phytoplankton was automatically kept at a steady concentration (autoregulation through Labview ® software / datalogger / fluorescence detection). Clams were sampled for chemical analysis at day 0 and day 6 of contamination. The entire edible tissues and digestive gland of 10 clams were pooled, and their toxicity analysed according to the method described in 2.2.

During a 7-day detoxification, three experimental groups were prepared: in the first, 70 clams were fed *I. galbana* at 12 000 cells/ml with the same autoregulation protocol as that described for *K. selliformis*; in the second group, 70 clams were successively supplied with sea water alone for the three first days of detoxification and then fed *I. galbana* at the same concentration as group 1; and, in the last group, 70 clams were starved (sea water only) and thus served as a control. During detoxification, clams (n = 10) were sampled daily for GYM analysis, i.e. on days 0, 1, 2, 3, 4, 6 and 7. For each daily sample, tests were conducted on pooled digestive gland (n = 10) and on pooled remaining flesh fractions (n=10). Chemical analysis were performed three times for each sample (n=3).

No mouse bioassays were performed in this experiment.

Experiment 2

The same strain of *K. selliformis* was cultured in the INSTM hatchery of Monastir (Tunisia) using 40 L culture tanks of L1 medium (Guillard and Hargraves, 1993) and a 12 h / 12 h light-dark cycle with a light intensity of 40 µmole photons/m^2/s and temperature regulated at 18 ± 0.5°C. *Isochrysis galbana* "Tahiti strain" cultures were grown in 100 L polyethylene sheathes in f/2 medium (Guillard and Ryther 1962, Guillard 1975) and maintained under the same conditions.

Both toxic and non-toxic algae were harvested in late-exponential or early-stationary phase after 14-16 days (*K. selliformis*) and 6-8 days (*I. galbana*) growth.

In April 2008, *R. decussatus* clams with no history of phycotoxin contamination were collected from the Gulf of Gabès in Tunisia. These clams were of commercial size, i.e. 34 ± 2.3 mm mean shell length and 0.29 ± 0.07 g mean tissue dry weight (n=10). The clams (20 kg) were transported under dry and cold conditions to the laboratory where they were acclimatized for 5 to 6 days in a raceway filled with 150 L seawater at a temperature of 18 ± 0.1°C and salinity of 38 psu (Table 2).

During the contamination period, clams were fed the toxic microalgae *K. selliformis* (harvested in decline-phase after 30 days, 20 000 cell/ml) for seven days and sampled at days 0 and 7 of contamination for chemical analysis (n=10) and mouse bioassay (2 kg) (Table 2).

During the detoxification period, two experimental groups were prepared in an open-seawater system: three 150 L raceways, each containing 5 kg of clams with a temperature of 17 ± 0.3 °C, salinity of 37 psu and constant seawater flow-rate with total renewal of circulated seawater three times a day.

In the first two raceways, clams were fed on *I. galbana.* The daily algal food ration was 2 % DW algae / DW clam meat; clams in the last raceway were starved (sea water only) and thus served as controls.

During the detoxification period, clams were sampled, on days 0, 1, 2, 3, 4, 5, 7, 8 and 10 for GYM quantitative analysis and on days 7, 8 and 10 for mouse bioassay (2 kg).

For each daily sample, chemical analysis were conducted on pooled digestive gland (n=10). Chemical analysis were performed three times for each sample (n=3).

2.2 Extraction and LC-MS/MS analysis of gymnodimine

2.2.1 Extraction procedure

Clam toxin contents were monitored during the detoxification period of both experiments. Clam soft parts were dissected and then divided into two fractions: digestive gland and remaining tissues (including siphon, foot, gill, adductor muscle and mantle). These two fractions were drained for 2 h on a Büchner funnel, weighed, and then frozen at – 80°C.

Lipophilic toxins were extracted from 2 g of homogenized digestive gland or remaining tissue with 15 ml (3×5 ml) of a methanol/water (90/10) solution. After centrifugation (3000 g, 15 minutes, 4 °C) supernatants were combined and homogenized. A 2 ml sample was ultra-filtered by centrifugation through a 0.2 µm membrane (Whatman allipore filter) at 6000 rpm for 5 minutes. Five µl of the filtrate were injected into the LC-MS/MS system

2.2.2 LC-MS/MS analysis

The LC-MS/MS analysis were performed according to Amzil et al, (2007) using an Agilent 1100 LC model coupled to a triple-quadripole mass spectrometer (API 2000). Toxins were eluted in a 3-µm hyperclone MOS C8 column (50*2.0mm, Phenomenex) at 20 °C with a linear gradient set at 0.2 ml/min.

Analyses were carried out in multiple reaction monitoring (MRM) positive ion mode and the two most intense product ions per compound were selected. The transition conditions chosen for gymnodimine toxins are indicated in table 2.

2.3 Mouse bioassay

2.3.1 Toxin extraction

The mouse bioassay for the DSP toxins was performed according to the method of Yasumoto et al. (1978). 20 g digestive glands (DG) from each clam sample were extracted with 50 ml acetone, homogenized with ultra Turax, filtered and then placed in a rotary evaporator. This last step was repeated twice. Finally, after acetone / water evaporation, the dry residue was collected and stirred with glass beads and 4 ml 1 % Tween 60 before being stored at –80 °C until use.

2.3.2 Mouse inoculation

The residue was suspended in 1 ml 1 % Tween 60 solution and injected intra-peritoneally (i.p.) into three mice. Toxicity was determined by time until mouse death following the inoculation with clam extracts. Three control mice were also i.p. injected with 1 ml Tween standard solution. As soon as inoculation had been made, mice had to be carefully observed, paying special attention to the symptoms occurring within the first 15 min.

The bioassay was considered positive if at least two out of three mice died within 24 h.

2.4 Filtration rates

As soon as clam faeces (but not pseudo-faeces) were produced during the feeding period of experiment 1 they were detected and immediately removed with Pasteur pipettes twice a day. The amount of total particulate matter (TPM) and particulate inorganic matter (PIM) in seston (detritic and living particles) are expressed per unit of sampled sea water as:

$$TPM_{seston}(mg.l^{-1}) = PIM_{seston}+POM_{seston}$$

Biodeposits of the detoxification period (faeces and pseudo-faeces) amounts were determined by successively heating Whatman filters at 60 °C for 24 h and 450 °C for 1 h. The total particulate matter (TPM) of biodeposits was calculated using the following relationship:

$$TPM_{biodeposits}(mg.h^{-1}.ind^{-1}) = [(POM_{biodeposits}\ (mg) + PIM_{biodeposits}\ (mg)/\text{production time (h))/number of clams}]$$

Filtration rate (FR) was calculated using the following relationship (Hawkins et al., 1996; Urratia et al., 1996):

$$FR\ (mg.h^{-1}.g\ dmw^{-1}) = [(\text{mg inorganic matter issued from faeces and pseudofaeces } h^{-1}) \times [(\text{mg total particulate matter } l^{-1} \text{ seawater}) / (\text{mg inorganic matter } l^{-1} \text{ seawater})]/ \text{ g of dry meat weight (DMW) of clam.}$$

2.5 Condition index

Condition index (CI) was calculated using the relationship of dry meat weight (DMW) to dry shell weight (DSW) according to the following equation:

$$CI= DMW\times100/DSW$$

2.6 Siphon activity

The opening of siphon (*R. decussatus*) was monitored every half hour during contamination (clams exposed to *K. selliformis*) and detoxification periods (clams exposed to *Isochrysis galbana* or sea water) in experiment 1.The siphon activity was expressed as the ratio of the number of clams that opened their siphons to the number of clams used in the experiment.

2.7 Statistical analysis

Experimental data were analysed using *Statgraphics Centurion* software. During the three experiments, the impact of food in a raceway was assessed using multifactorial ANOVAs.

Data obtained from chemical analysis (toxicity level on the last days of detoxification compared with the safety threshold) were tested statistically using a T test.

For P-values less than 0.05, differences between toxin contents were considered statistically significant at a 95 % confidence level.

3. Results

3.1 Filtration rates during contamination and detoxification periods

Filtration rates (mg.h^{-1} g dmw^{-1}) for each treatment during experiment 1 are shown in Fig. 2. The filtration rate (FR) differed significantly during contamination and detoxification periods and for each diet tested during the experiment. The FR of clams fed *I. galbana* (groups 1 and 2) was higher during detoxification than during contamination. This difference can be correlated with the percentage clearance activity expressed as the percentage of clams with siphons open every half hour (Fig. 3). Diets containing the toxic dinoflagellate *Karenia selliformis* led to apparent reduced clearance activity (20 to 60 % of clams were active), whereas when non-toxic diets were used, an increase was seen in the number of actively filtering clams (60 to 80 % were active with *I. Galbana*). The clams that were fed (group 1 and 2) showed a higher FR compared with the starved clams. Overall, the differences in FR during the detoxification period indirectly confirmed that clams were feeding and ingesting food according to expectations of the experimental design.

Biodeposits (total particulate matter : $mg.h^{-1.}ind^{-1}$) for each group of clams during the detoxification period in experiment 1 is shown in Fig. 4. After 7 days detoxification, the amount of biodeposits (TPM) produced differed among treatments. During the first 3 days of detoxification, group 1, which was fed *I. galbana*, had a higher production than groups 2 and 3. On day 3, the group of clams that had received food showed an increase in TPM. In the treatment without food, only a minor increase in total fecal production was observed during the whole experiment, indicating that no further ingestion occurred (Fig. 4).

3.2 Distribution of GYM-A in carpet shell tissues during detoxification

In experiment 1, on the first day of detoxification, DG contained most of the GYM-A (97 %), whereas other tissues contained only 3 %. During the 7 days of detoxification, toxic content in DG decreased rapidly (Fig. 5) to weak level (1.93 %). Conversely, the toxin content of other tissues only decreased slightly during the detoxification period (1.24 %).

3.3 Detoxification kinetics in clams fed *K. selliformis* and *I. galbana* successively

Experiment 1

Detoxifications kinetics were determined from chemical assessment (LC-MS/MS) of toxin contents in the digestive gland (DG; Fig. 6). Maximal gymnodimine (GYM) levels were obtained at the end of the contamination period: 9491 ± 2110 µg eq GYM/kg DG. This concentration was reduced at day 7 detoxification to 153 ± 8, 320 ± 22 and 511 ± 18 µg eq

GYM/kg DG, respectively, for rations 1 (fed), 2 (unfed during the three first days) and 3 (starved control). At this time, the lowest concentration of GYM was observed in clams receiving food.

On day 1, the toxin concentration in clams receiving no food (group 2 and 3) was significantly (P=0.042) higher compared with clams feeding on *I. galbana* (group 1). On day 3, clams receiving food showed a significant decrease in toxicity (1476 ± 33 to 550 ± 28 µg eq GYM/kg DG at day 4).

After 7 days detoxification, DG of clams that had been fed on nontoxic algae since the first day of detoxification had lost 98 % of their toxin content, whereas clams fed on nontoxic algae since the third day of detoxification had lost 97 % and clams receiving no food (Table 2) only 95 %. At the end of detoxification, no differences in clam condition index were observed between the different feeding conditions, except some slightly lower values for unfed clams (day 7: IC=5.1±0.13).

In table 2, detoxification rates of *Ruditapes decussatus* are described by the general negative exponential equation $y_1 = y_0\, e^{-kt}$, which corresponds to a one compartment model (for the group fed on nontoxic algae and for the starved group) where t = detoxification period (day); y_0 = initial toxin level (µg eq Gym kg^{-1} of DG) and k represents the detoxification coefficient (day^{-1}). In this experiment, the ratio between the two detoxification coefficients was 1.37, with a coefficient of 0.37 d^{-1} using seawater and 0.51 d^{-1} with the *I. galbana* diet (Table 2).

Experiment 2

In experiment 2, the maximum GYM level in clam DG (1238 ± 159 µg eq GYM/kg DG) was observed after 7 days exposure to *K. selliformis* (Fig. 7). At the end of the detoxification period (10 days) toxin concentrations in starved clams (375 ± 26 µg eq

GYM/kg DG) was significantly higher than in clams fed *I. galbana* (54 ± 2.1 µg eq GYM/kg DG) representing 70 % and 96 % drops in toxin content, respectively (Table 2).

The ratio between the two detoxification coefficients in this experiment was 3.04, with a coefficient of 0.092 d^{-1} in seawater and 0.28 d^{-1} with the *I. galbana* diet (Table 2).

It was only on day 8 and 10 that detoxified clams gave a negative result with mouse bioassay (no mouse death). At the end of the detoxification period, differences in condition index between each treatments were observed; values were lower for unfed clams (day 10: IC = 7.5 ± 1.2 n = 10), in comparison to clams fed on *I. galbana* (day 10: IC = 9.2 ± 1.35 n = 10).

4. Discussion

Different feeding processes have been tested to see how they could speed up the elimination of paralytic and diarrheic toxins from bivalve molluscs. Croci et al. (1994) showed that treatment with ozone had no significant effect on diarrheic toxin detoxification. Temperature and salinity seem to have no effect (Blanco et al., 1999), and several authors reported that food supply has little positive effect either (Sampayo et al., 1990; Blanco et al., 1999), while starvation was found to increase the apparent detoxification rate (Svensson, 2003).

Field detoxification by moving mussels from toxic to nontoxic environments (relaying) was performed by Haamer et al. (1990), Marcaillou-Le Baut et al. (1993), Poletti et al. (1996) and Blanco et al. (1999). Moreover the availability of nontoxic food has been proposed by several authors to be the main factor affecting diarrheic detoxification in mussels (Haamer et al., 1990; Sampayo et al., 1990; Poletti et al., 1996; Blanco et al., 1999).

Studies on the effects of food on detoxification of contaminants in mussels have been investigated for other types of toxins. Novaczek et al., (1992) and (Wohlgeschaffen et al., (1992) found no difference in detoxification rate of the hydrophilic neurotoxin domoic acid among fed or starved mussels, *M. edulis*. Regarding the effect of environmental factors on paralytic shellfish toxin (PST) detoxification rates in *Mytilus galloprovincialis*, Blanco et al., (1997) found that phytoplankton concentration seemed to have no particular effects. Chen and Chou, (2001) observed that detoxification efficiency of (PST) in the purple clam, *Hiatula rostrata* was similar for clams fed nontoxic algae or starved.

No attempt had been made to detoxify GYM contaminated shellfish before the present study. In this work, our method consisted of transferring clams to waters free of toxic organisms, with environmental conditions that could promote accelerated detoxification.

The experiments aimed to test the effects of food on detoxification under controlled conditions. It was found that clams fed non toxic algae had the highest amount of fecal production compared with starved animals. This confirmed that ingestion rates differed among treatments according to predictions, and in this way the experiment was successful.

Moreover, during the contamination period the clams showed a significant reduction in filtration rate when fed *K. selliformis* at a concentration of 200 cells ml^{-1}. The FR increase when exposed to *I. galbana* at a concentration of 12000 cell ml^{-1} at the beginning of detoxification period. This suggest that *K. selliformis* diet is inappropriate and probably harmful for grooved carpet.

GYM detoxification kinetics were faster in clams fed on *I. galbana* (experiments 1 and 2) than in starved clams. These results are in agreement with other studies such as detoxification experiments with blue mussels, which showed that feeding mussels accelerated the detoxification process. Moreover, observations made by Sampayo et al. (1990) during several DSP episodes on the Portuguese coast, suggested that the detoxification rate increases with phytoplankton concentration, i.e with the main food for bivalve molluscs. These findings contrast with the experiments carried out by Svensson, (2003), who showed that detoxification of lipophilic phycotoxin (okadaic acid) in mussels was unaffected by food availability.

During the two experiments, detoxification rates were rapid (7 to 8 days) whatever non-toxic algal diets were used. Thus, in order to set up a process that will optimize detoxification, the presence of *I. galbana* is a very significant element to help reach the estimated safety threshold. These observations seem consistent with Bricelj and Shumway's (1998) classification of *R. decussatus* as a "fast detoxifier" with in the same time frame as *Crassostrea gigas* for paralytic toxins

In this study, the initial toxicity was much higher in experiment 1 (9491 ± 2110 µg eq GYM/kg DG) than in experiment 2 (1238 ± 159 µg eq GYM/kg DG). This difference can be attributed to the high number of clams and the low toxicity of *Karenia selliformis* used in experiment 2 (2.6 pg GYM/cell compared with 10.7 pg GYM/cell in experiment 1). Clam digestive gland accumulated most of the total GYM (97 %), and the remaining toxins were distributed in the other tissues. This disproportionate accumulation agrees with results obtained for other bivalve molluscs, like scallops (Cembella et al., 1993; Choi et al., 2003), mussels (Bricelj et al., 1990) and *Hiatula rostrata* (Chen and Chou, 2001). Most studies have concluded that bivalve viscera and DG accumulated most of the total toxin burden, despite the limited contribution of these organs to the total body burden (Bricelj et al., 1990; Cembella et al., 1993).

The different detoxification rates for each kind of tissue may cause an increase in the percentage contribution of the digestive gland to the whole shellfish toxin burden as detoxification progresses, e.g. in the case of *Argopecten irradians* the percentage increased to 95 % after two days of detoxification. In our study, it was observed that DG detoxification rate in clams (fed and unfed) was faster during the first two days (more than 84 % loss) but then subsequently slowed down. This suggests that toxins are distributed between these two compartments. Similar patterns were also observed for blue mussels (Marcaillou-Le Baut et al., 1993; Fernandez et al., 1998; Blanco et al., 1999) and also for scallops (Bauder et al., 1996). This last author found that the rapid loss of toxins during the first 3 days of detoxification coincided with the evacuation of toxin-producing algae from the viscera. Biphasic (fast and slow) detoxification kinetics were also found for other types of algal toxins in different shellfish species, like PSP in king scallop *Pecten maximus* (Lassus et al., 1989), or DSP in blue mussels (Marcaillou-Lebaut et al., 1993).

The slow detoxification rates shown in other tissues (including gill, mantle, siphon and foot) were in agreement with previous studies such those as in surfclams, *S. solidissima*. According to Bricelj and Cembella, (1995) the rank order of various tissue pools in terms of their detoxification rates is as follows: viscera > gill > mantle > siphon > foot > adductor muscle. The two experiments we carried out reveal that the detoxification of clams fed with *I. galbana* could be achieved in 7 or 8 days (experiments 1 and 2, respectively). As described in the “material and methods” section, mouse bioassay was done only in experiment 2. This experiment revealed that toxicities in the fed clams were 134 ± 11.34, 55 ± 5.6 and 54 ± 2.1 µg GYM/kg DG after 7, 8 and 10 days of detoxification, respectively, and negative mouse test results were only found after 8 and 10 days. In contrast, clams that were not fed during the detoxification period, which had final toxicities of 366 ± 66.46, 363 ± 20.43 and 375 ± 25.89 µg of GYM/kg DG after 7, 8 and 10 days of detoxification, respectively, showed a positive result in the mouse test.

The correspondence of these chemical analyses with the results of the mouse biotest showed that toxicity over 55 µg GYM/kg DG was responsible for the death of all three mice. Indeed, the presence of GYMs in mice appears through body stretching, hyperactivity, stiff tail, slowing of movements and paralysis of the rear limbs followed by rapid mortality. These symptoms are all neurological (Hu et al, 1996; Cembella et al, 2000; Takada et al., 2001). Moreover, GYM is listed among toxins with a fast action FAT (fast-acting toxins) since it results in the death of mice within a span of a few minutes (Rein and Borrone, 1999). Another significant toxicological characteristic of fast acting toxins lies in the differential expression of toxicity according to the toxin levels present in the extracts of tested mice. Indeed, these toxins present an “all or nothing” effect, (Hu and al., 1996; MacKenzie et al., 1996 ; Hu et al., 2001), initially observed by Tindall et al., (1984). This effect is characterized by a sharp disappearance of the mouse lethality once the injected extracts are diluted beyond a certain threshold (MacKenzie et al., 1996).

5. Conclusions

Controlled detoxification applied to GYM-contaminated shellfish could be a practical approach for the management of clam fisheries, particularly to ensure a continuous supply of safe clams for the market. The results obtained in this study have shown that detoxification occurs when a non-toxic alga is present in the detoxification system and that GYM levels can be reduced to approximately 5 % of initial toxin content within 7 to 8 days for laboratory-contaminated clams i.e. it is possible to obtain toxin levels below the safety threshold of 100 µg GYM/kg DG. To optimise the conditions for GYM detoxification in clams, feeding with nontoxic algae can be started some days after the beginning of the detoxification process (after 3 days of starvation, for instance). However, more information is needed about seasonal variability and the effect of salinity, specific toxicity of algal strains, bloom duration and cell concentration on the detoxification rates. Further experiments are also needed on naturally contaminated clams. Taking into consideration these different aspects should considerably reduce concerns about health risks related to the consumption of gymnodimine-contaminated *R. decussatus*.

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Table 1. m/z transition conditions for GYM-A and GYM-B. m/z (mass to charge ratio).

Toxins	**Transitions m/z**
GYM A	508.4>490.2/392.3
GYM B	524.4>506.4

Table 2. Summary of experimental conditions, exponential equation, comparison of bioassays and chemical analysis after detoxification period.

Clam group	Origin/Date/shell length	Acclimation period (days)	Contamination period (days)	Detoxification period (days) / Temperature		Initial toxin content/ Final toxin content (µg Gym/kg GD)		DSP Mouse bioassay	Exponential equation
Experiment 1									
Control	Island Noirmoutier (France) March 2007 37 ± 2 mm	5-6	6	7	16±0.5°C	9491	511	Not done	y = 4751 exp (-0.3722 t) R^2= 0.8581
With algal food*							320		y = 6412.8 exp (0.5113 t) R^2= 0.9083
With algal food							153		y = 2970 exp (-0.5044 t) R^2= 0.791
Experiment 2									
Control	Golf of Gabès (Tunisia) April 2008 34 ± 2.3 mm	5-6	7	8	17±0.3°C	1238	363	+	y = 733.25 exp (-0.0922 t) R^2= 0.5963
With algal food							55	-	y = 716.57 exp (-0.2803 t) R^2= 0.912

Control : Without feeding ;With algal food: *Isochrysis galbana* ;* *Isochrysis galbana* on the third day of detoxification; Mouse test = + : all mice died; - no death observed

Figures

Fig.1. Gymnodimine structure.

Fig.2. Filtration rates (mg/h^{-1} g dmw^{-1}) of clams exposed to (A) *Karenia selliformis* (contamination period) and (B) *Isochrysis galbana* or sea water during experiment 1 (detoxification period). Triangles (∆): starved clams, squares (■) : clams fed from the third day of detoxification, and circles (●) : clams fed throughout detoxification.

Fig.3. Percentage of clams with opened siphon (siphon activity) exposed to (A) *Karenia selliformis* (contamination period) and (B) *Isochrysis galbana* or sea water during experiment 1 (detoxification period). Triangles (∆) : starved clams, squares (■) : clams fed from the third day of detoxification, and circles (●) : clams fed throughout detoxification.

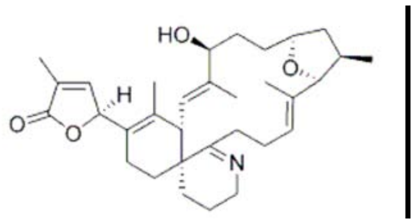
Fig.4. Biodeposits (total particulate matter production : mg.h^{-1}·ind^{-1}) of clams exposed to *Isochrysis galbana* or sea water during detoxification period of experiment 1. Triangles (∆) : starved clams, squares (■):clams fed from the third day of detoxification, and circles (●) : clams fed throughout detoxification.

Fig.5. Relative toxicity (%) between DG and other tissues during detoxification period according to clams receiving food during the experiment 1.

Fig.6. Clam detoxification kinetics according to the different detoxification diets used in experiment 1. Triangles (∆) : starved clams, squares (■) : clams fed from the third day of detoxification, and circles (●): clams fed throughout detoxification. Mean values ± S.E. (3 chemical analysis for any point and diet used).

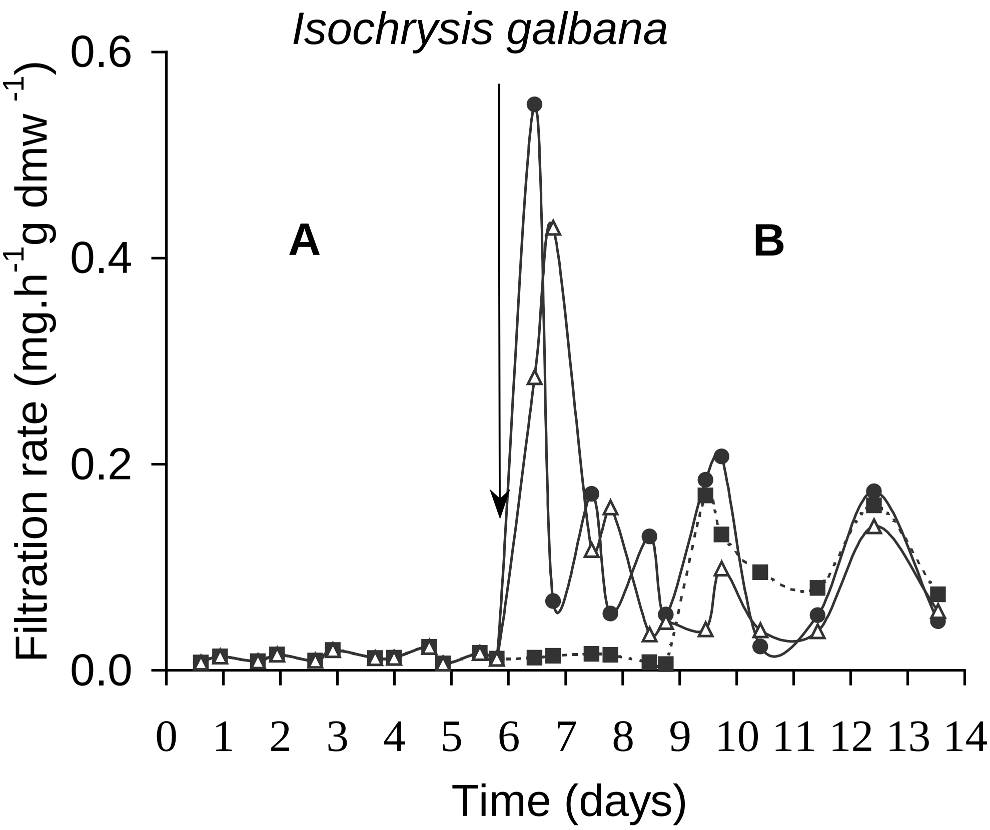
Fig. 7. Clam detoxification kinetics according to the different diets used in experiment 2. Triangles (∆) : starved clams, squares (■) : fed clams. Mean values ± S.E. (3 chemical analysis for any point and diet used).

Fig. 1



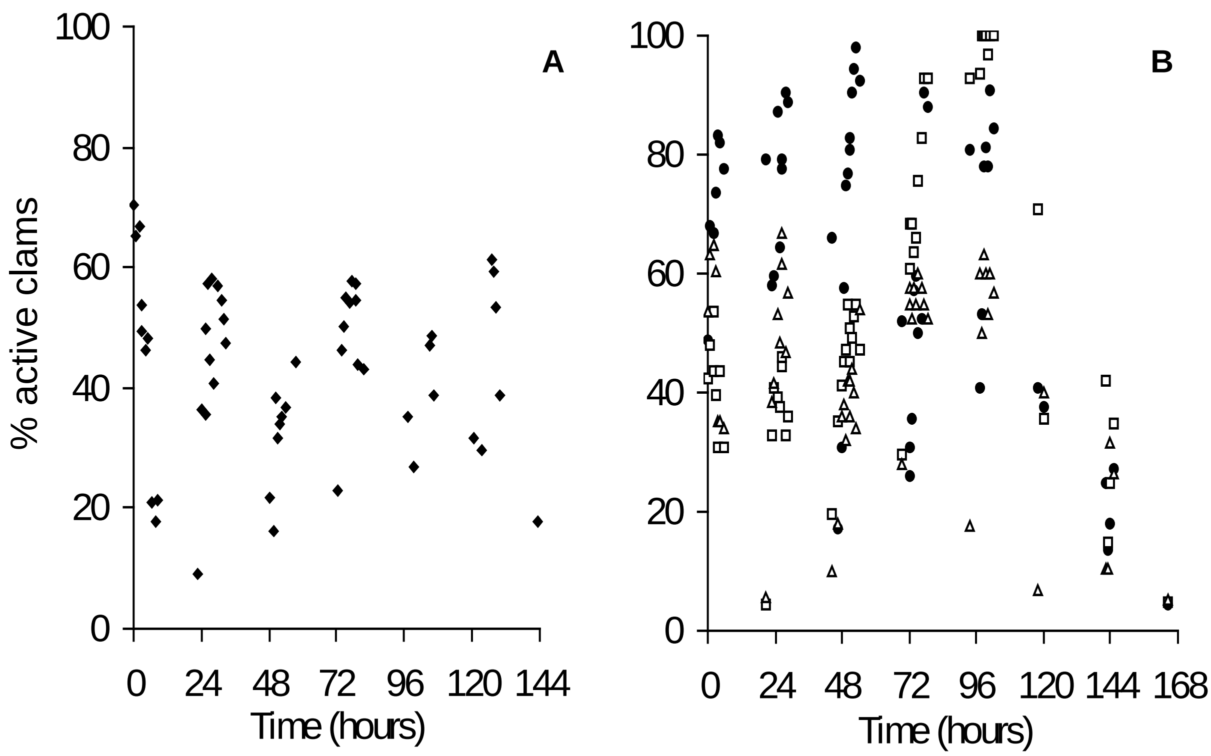
HO
O
H
O
O
N

Fig.2



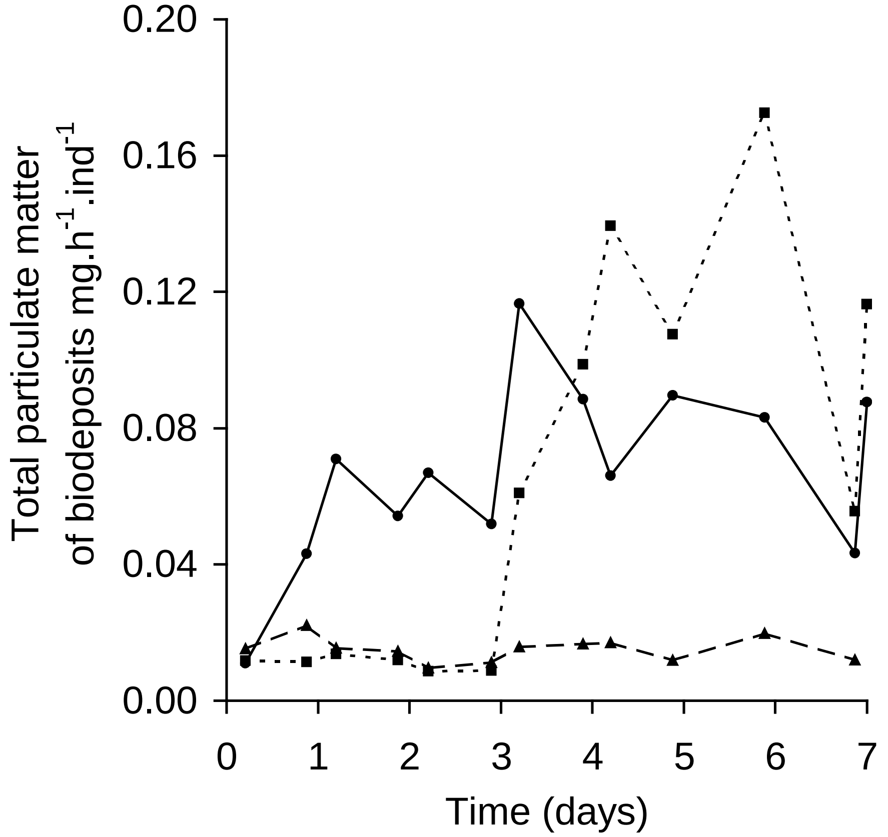
Isochrysis galbana
Filtration rate (mg.h-1g dmw -1)
0.0
0.2
0.4
0.6
A
B
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time (days)

Fig. 3



A
% active clams
100
80
60
40
20
0
0 24 48 72 96 120 144
Time (hours)
B
100
80
60
40
20
0
0 24 48 72 96 120 144 168
Time (hours)

Fig. 4



0.20
0.16
0.12
0.08
0.04
0.00
Total particulate matter
of biodeposits mg.h^{-1}.ind^{-1}
0
1
2
3
4
5
6
7
Time (days)

Fig.5

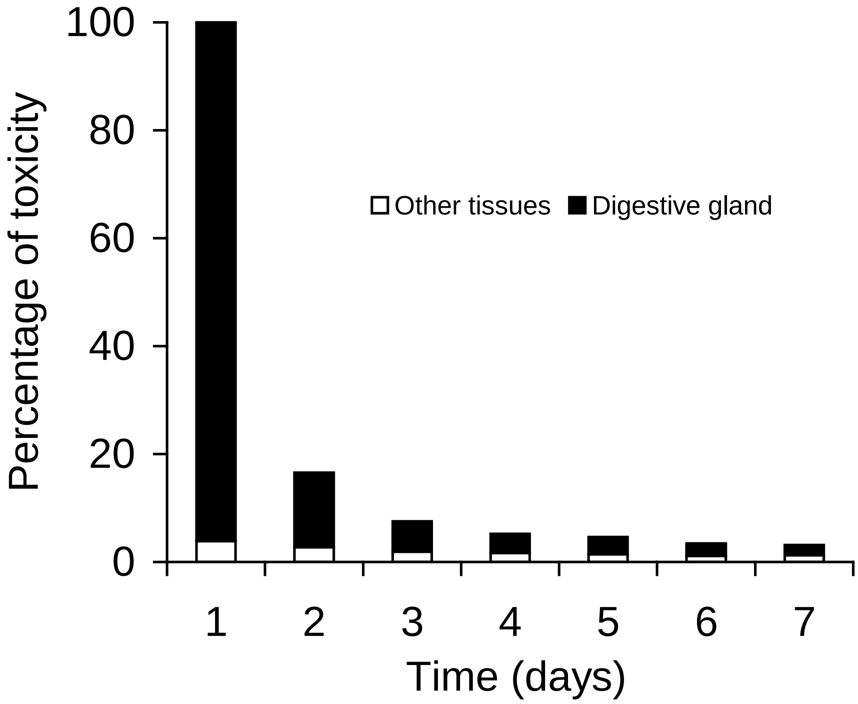
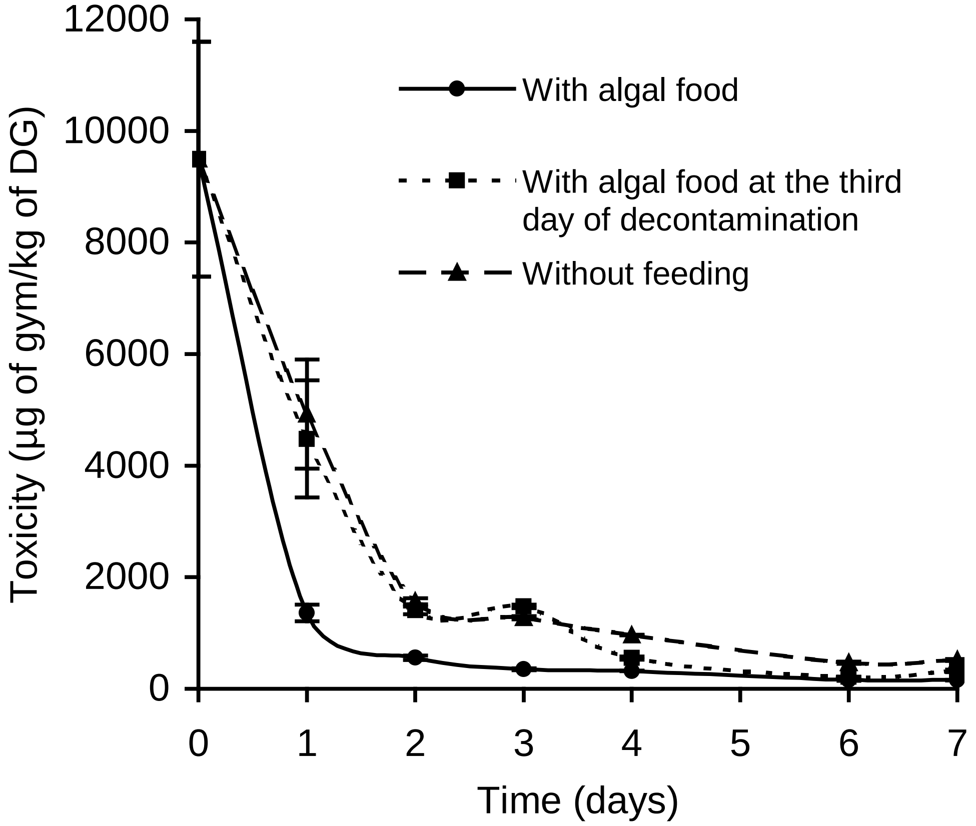
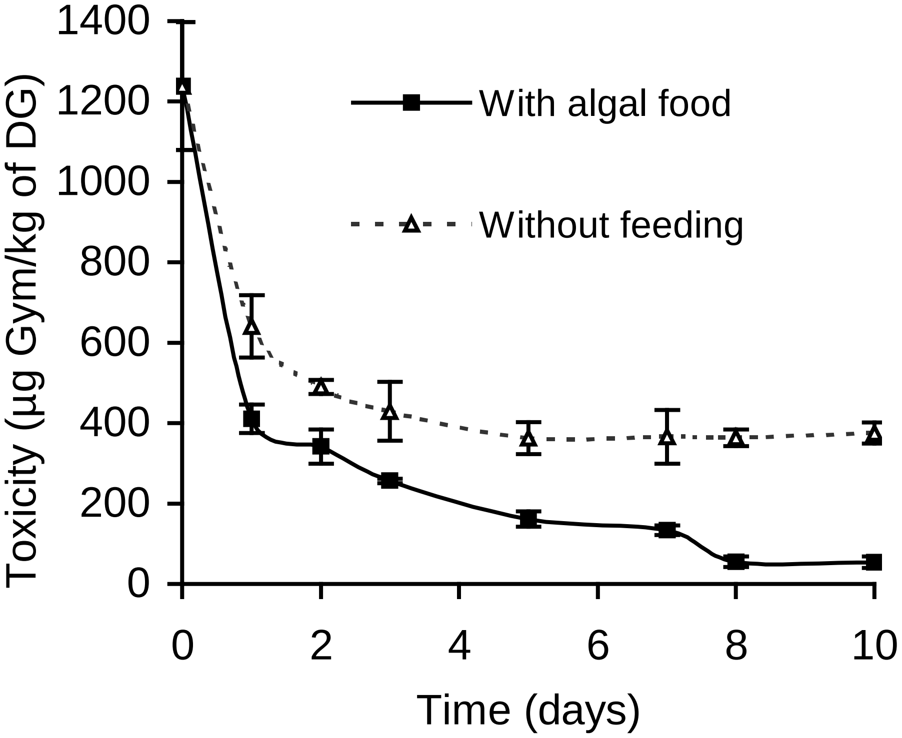

100
80
60
40
20
0
Percentage of toxicity
Other tissues
Digestive gland
1
2
3
4
5
6
7
Time (days)

Fig. 6



12000
10000
8000
6000
4000
2000
0
Toxicity (µg of gym/kg of DG)
0
1
2
3
4
5
6
7
Time (days)
With algal food
With algal food at the third day of decontamination
Without feeding

Fig. 7


1400
1200
1000
800
600
400
200
0
Toxicity (µg Gym/kg of DG)
With algal food
Without feeding
0
2
4
6
8
10
Time (days)