Prevalence and persistence of gymnodimines in clams from the Gulf of Gabes (Tunisia) studied by mouse bioassay and LC-MS/MS.

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Field Code Changed

Abstract

In this work we studied the toxicity in clams from the Gulf of Gabes, Tunisia (Southern Mediterranean). Samples from two stations (M2 and S6) were collected monthly from January 2009 to September 2010, and analyzed by the official control method of mouse bioassay (MBA) for lipophilic toxins. All samples were also analyzed with the LC-MS/MS method for the determination of lipophilic toxins, namely: okadaic acid group, pectenotoxins, yessotoxins and azaspiracids, spirolides and gymnodimines (GYMs). The results showed prevalence of GYMs since it was the only toxin group identified in these samples with a maximum of 2,136 $\mu\text{g GYM-A kg}^{-1}$ (February 2009 at M2). Furthermore, GYMs showed persistence in the area, with only one blank sample below the limit of detection. Interestingly, this blank sample was found in June 2009 after an important toxic episode which supports the recent findings regarding the high detoxification capability of clams, much faster than that reported for oysters. In comparison, good agreement was found among MBA, the LD_{50} value of 80-100 $\mu\text{g kg}^{-1}$ reported for GYM-A, and quantitative results provided by LC-MS/MS. On the contrary to that previously reported for Tunisian clams, we unambiguously identified and quantified by LC-MS/MS the isomers GYM-B/C in most samples. Phytoplankton identification and enumeration of *Karenia selliformis* usually showed higher densities at site M2 than S6 as expected bearing in mind toxin results, although additional results would be required to improve the correlation between *K. selliformis* densities and quantitative results of toxins. The prevalence and persistence of GYMs in this area at high levels strongly encourages the evaluation of the chronic toxic effects of GYMs. This is especially important taking into account that relatively large quantities of GYMs can be released into the market due to the replacement of the official control method from mouse bioassay to the LC-MS/MS for lipophilic toxins (Regulation (EU) No 15/2011), and the lack of Regulation for this group of toxins.

Keywords: marine toxins; gymnodimine; mouse bioassay; LC-MS/MS; shellfish; Tunisia.

1. Introduction

Gymnodimines (GYMs) are marine toxins that belong the spirocyclic imine ring group linked ether moieties of toxins together with spirolides (Ciminiello et al., 2007; Hu et al., 2001; Hu et al., 1995; Hu et al., 1996a; MacKinnon et al., 2006; Roach et al., 2009), pinnatoxins (Chou et al., 1996a; Chou et al., 1996b; Takada et al., 2001a; Uemura et al., 1995), pteriatoxins (Takada et al., 2001b), prorocentrolides (Hu et al., 1996b; Torigoe et al., 1988) and spiro-prorocentrimine (Lu et al., 2001). Gymnodimine (GYM-A) was isolated from oysters (Seki et al., 1995; Stewart et al., 1997) showing a unique structure with butenolide, spiro and cyclic-imine moieties (Figure 1). Later on, two hydroxylated analogs, gymnodimine-B (GYM-B) and gymnodimine-C (GYM-C) were also elucidated (Miles et al., 2000, 2003). The dinoflagellate *Karenia selliformis* (formerly *Gymnodinium selliforme*) was identified as the biogenic origin of gymnodimines (Haywood et al., 2004). However, a new derivative 12-methylgymnodimine has been recently identified in the distantly related dinoflagellate *Alexandrium peruvianum* (Van Wagoner et al., 2011). The LD₅₀ for Gymnodimine-A in mice estimated from intraperitoneal (*i.p.*) injection was firstly estimated about 450 (Seki et al., 1995) and 700 µg/kg b.w. (Stewart et al., 1997), although more accurate estimations obtained from pure standards ranged between 80 (Kharrat et al., 2008) and 96 µg/kg b.w. (Munday et al., 2004). Symptoms in mice after *i.p.* injection of GYM-A include hyperactivity, jumping, paralysis of hind legs, and severe dyspnea. The death, related with a neurological impairment, occurs rapidly between 6-15 minutes after injection being the reason why they have been called fast-acting toxins. The spirocyclic imine ring has been proposed as responsible of the pharmacological activity (Kharrat et al., 2008; Stewart et al., 1997) because the similarity in toxicity with other cyclic imines such as spirolides (Munday et al., 2004). Gymnodimines target muscular and neuronal nicotinic acetylcholine receptors for which they show strong affinity (Kharrat et al., 2008). However, complete recovery occurs in mice that do not die without post-exposure symptoms. This suggests the reversible blockage of nicotinic acetylcholine receptors by GYMs (Fonfria et al.; Kharrat et al., 2008; Munday et al., 2004). Apparently, GYM-B is 10-fold less toxic than GYM-A (Kharrat et al., 2008), and a potential synergistic effect with other toxins has been proposed since GYMs were able to sensitize Neuro2a cells to other marine toxins such as okadaic acid (Dragunow et al., 2005). Nowadays, GYM-A has shown a worldwide distribution including New Zealand coastlines (Stirling, 2001b), Tunisia (Biré et al., 2002; Marrouchi et al., 2010), Australia (Takahashi et al., 2007), Europe & North America coast (Kharrat et al., 2008), and more recently South Africa (Krock et al., 2009) and China (Liu et al., 2011). They may persist in oysters for years (MacKenzie et al., 2002), although an enhanced detoxification capability has been recently demonstrated in clams (Medhioub et al., 2010). Gymnodimines have also been observed in many other species of contaminated shellfish, including greenshell mussel, blue mussel, scallop, cockle, surfclam, oyster and abalone (MacKenzie et al., 2002; Stirling, 2001a). The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain has assessed last year the risk of cyclic imines (EFSA, 2010) for humans due to shellfish consumption. Unfortunately, this expert panel concluded that both toxicological and exposure information is still scarce, and therefore the risk could not be assessed because the large uncertainty of its estimation. As a result, neither an acute reference dose (ARfD) nor a tolerable daily intake (TDI) have been proposed to prevent acute or chronic toxic, respectively. A low risk might be presumed taking into account the low oral toxicity in mice (LD₅₀ for GYM-A of 775 and ca. 7,500 µg/kg b.w. for gavage and voluntary consumption (Munday et al., 2004), and the similar

pharmacological action than that observed for other cyclic imines toxins such as spirolides. However, concentrations of GYMs may reach levels much higher in shellfish than those reported for spirolides (Stirling, 2001a). The European Union has established regulation for 13 marine lipophilic (EU, 2004), while cyclic imines are not yet legislated. Intoxications in humans caused by GYM and SPXs have not been reported, however. In recent years much effort has been dedicated to the development and validation of the LC-MS/MS method for multi-toxin analysis of lipophilic toxins, being the reference method in the EU since July 1st, 2011 (EU, 2011). Until this date, the mousse bioassay (MBA) allowed identification of GYMs in shellfish, then avoiding commercialization of shellfish containing GYMs above the ca. LD₅₀ (*i.p.*) in mice. As there is no a Maximum Permitted Level (MPL) for GYMs established (EU, 2004), it is expected that the replacement of the MBA by the LC-MS/MS method will lead to release in the market several hundreds of Ton/year of shellfish containing GYMs only coming from Tunisia. As the information about the persistence of GYMs is still limited apart from oysters in [NZNew Zealand](#), the magnitude of the potential arrival of GYMs in the market is difficult to assess. In this work, we studied the causative origin of toxicity in clams from the Gulf of Gabes, Tunisia, where the presence of GYMs has been previously reported (Biré et al., 2002; Marrouchi et al., 2010). Tunisia has 1,300 km of coastline and considerable resources for shellfish production. The [gGulf of Gabes](#) is an important area for natural stocks of the grooved carpet shell (*Ruditapes decussatus*, Linnaeus, 1758) with 79% of the total of the Tunisian production. [According to the Directorate General of Veterinary Services of Tunisia \(DGSV\)](#), [sShellfish](#) are exported mainly to France, Italy, Spain and Portugal with over 650 Ton/year and a domestic consumption over 500 Ton/year. The aim of this work is to study the prevalence of GYMs compared to other groups of lipophilic toxins in the Gulf of Gabes, and to provide a survey on the persistence of GYMs in this area, increasing the database on levels of this toxins in shellfish and consequently, improving the assessment of exposure in humans. To achieve this goal, clams were firstly analyzed with the MBA and with the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method, both for lipophilic toxins. Additionally, identification and enumeration of phytoplankton species present in water samples was conducted.

2. Materials and Methods

2.1. Samples

Clam samples of grooved carpet shell species (*Ruditapes decussatus*) were collected monthly from January 2009 to September 2010 at sampling stations S6 and M2 located at the south of Sfax and Boughrara lagoon, respectively (Figure 2). Sampling was carried out manually by randomly picking up clams off the coast. The sampling process was supervised by the *Commissariat Régional du Développement Agricole de Médnine et de Sfax (CRDA)*, Southern Tunisia. At least 4 kg of clams were collected per sample, and routed through the network of monitoring marine toxins to the officially approved laboratory analysis CRRV Sfax, responsible for the monitoring of shellfish in the Gulf of Gabes. A minimum of 100 g fresh flesh was taken of each sample and stored at -20°C. Phytoplankton samples were taken weekly using a 1-L Van Dorn bottle at 1m depth in sampling sites from S1 to S6, G1 to G3 and M1 to M5 shellfish area production (Figure 2), and fixed with lugol's solution.

2.2. Certificate Reference Materials

For LC-MS/MS analysis, Certificate Reference Materials (CRMs) of lipophilic toxins were purchased from the Institute for Marine Biosciences, National Research Council (Halifax, Canada). Namely: gymnodimine A (CRM-GYM) $5.0 \pm 0.2 \mu\text{g}\cdot\text{mL}^{-1}$; 13-desmethyl spirolide C (CRM-SPX-1) $7.0 \pm 0.4 \mu\text{g}\cdot\text{mL}^{-1}$; okadaic acid (CRM-OA) $24.1 \pm 0.8 \mu\text{g}\cdot\text{mL}^{-1}$; yessotoxin (CRM-YTX) $5.5 \pm 0.3 \mu\text{g}\cdot\text{mL}^{-1}$; pectenotoxin-2 (CRM-PTX-2) $8.6 \pm 0.3 \mu\text{g}\cdot\text{mL}^{-1}$; azaspiracid-1 (CRM-AZA-1) $1.24 \pm 0.07 \mu\text{g}\cdot\text{mL}^{-1}$.

2.3. Reagents

For the mouse bioassay, analytic quality acetone (RPH, Carlo Erba) and Tween 60 1% (Sigma-Aldrich, St. Louis, MO, USA) were used. For LC-MS/MS analysis, gradient-grade methanol, gradient grade formic acid, and hypergrade acetonitrile were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA). Ammonium formate ($\geq 99.995\%$ metal basis), sodium hydroxide pellets ($\geq 99\%$) and hydrochloric acid 37% for analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA), Riedel-de Haën (Seelze, Germany) and Panreac (Barcelona, Spain), respectively.

2.4. Identification and enumeration of phytoplankton in water samples

Phytoplankton identification and enumeration on water samples was performed with an IMT2 inverted Olympus microscope, after fixation with Lugol solution and the sedimentation of subsamples in chambers following Utermöhl's method (Utermöhl, 1958). Identification of toxic thecate species was conducted in an Olympus epifluorescence microscope.

2.5. Official control method for lipophilic marine toxins: mouse bioassay (MBA).

The mouse bioassay (MBA) for lipophilic toxins was performed according to the method reported by Yasumoto et al. (Yasumoto et al., 1978). Twenty grams of digestive glands from each clam sample were extracted with 50 mL acetone, homogenized with Ultraturrax (IKA, Staufen, Germany) for 10 min, and filtered through a 113 Whatman filter paper ($>30\mu\text{m}$ cut-off). Extraction was repeated twice for each sample, and the resulting extracts were combined and placed in a rotary evaporator (Büchi Rotavapor R-124, Switzerland). After complete evaporation, the dry residue was suspended in 4 mL Tween 60 1% by stirring with glass beads, being a 1 mL-aliquot administered to each mouse. Injection was performed *i.p.* to three [Swiss male mice](#) (~~Swiss males~~) of 19-21g weight. [The source of mice was the Centre Regional de Recherche Vétérinaire \(CRRV\) of Sfax \(Tunisia\), from a broodstock imported in 2005 supplied by Charles River laboratory in France.](#) Three control mice were also *i.p.* injected with 1 mL 1% Tween 60 solution. As soon as inoculation had been made, mice were carefully observed paying special attention to the symptoms occurring within the first 15 min, and the time of death was recorded. The MBA was considered positive if at least two out of three mice died within 24 h.

2.6. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for lipophilic toxins.

1 All the instrumental analyses were performed at IRTA's laboratory. Sample preparation
 2 for LC-MS/MS analysis involved double methanolic extraction. A 2-g portion of clam
 3 homogenate from at least a 100-g pool was extracted with 9 mL MeOH by using an
 4 Ultraturrax (IKA, Staufen, Germany). The supernatant was centrifuged and the
 5 extraction was repeated with 5 mL MeOH. The resulting supernatants were combined
 6 and made up to a final volume of 15 mL in a volumetric flask. Additionally, alkaline
 7 hydrolysis of samples was also performed following standardized procedures
 8 (Mountfort et al., 2001; Villar-González et al., 2008) in order to evaluate the presence of
 9 OA-ester derivatives. Briefly, an aliquot of 1.25 mL of crude extract was vortex-mixed
 10 with 125 μ L of 2.5 M NaOH and heated at 76 °C for 40 min in a Multi-Blok® heater
 11 (Lab-Line Instruments, Inc., Melrose Park, IL). After cooling to room temperature,
 12 reaction was stopped by addition of 125 μ L of 2.5 M HCl. Liquid chromatography-
 13 tandem mass spectrometry method was applied under acidic chromatographic
 14 conditions. An Agilent 1200 LC (Agilent Technologies, Santa Clara, USA) was coupled
 15 with a 3200 QTRAP mass spectrometer *via* an atmosphere pressure ionization
 16 electrospray ion source (TurboV®, Applied Biosystems, Foster City, CA, USA).
 17 Chromatographic separations were performed on a Luna C8(2) column (50 $\times$ 1 mm, 3
 18 μ m), equipped with a SupelcoGuard C8(2) cartridge (4 $\times$ 2 mm, 3 μ m), both from
 19 Phenomenex (Torrance, CA, USA). Separations were carried out at 30 °C and 200 μ L
 20 min⁻¹ flow rate using a binary gradient elution. Mobile phases consisted of 100% water
 21 (A) and 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM
 22 formic acid. The chromatographic gradient started at 90% A increasing up to 80% B
 23 over 6 min. Then, it increased to 90% B for 6 min and afterwards up to 100% B for 2
 24 min, holding it for additional 2 min. Finally, the gradient came back to the initial
 25 conditions in 0.5 min and equilibrated for 8.5 min before the next run. Injection volume
 26 was 5 μ L, and the syringe was washed for 1 second with 100% methanol at the flush
 27 port to avoid carry-over. The auto sampler was set at 4 °C. The flow was diverted to
 28 waste by a 10-port Valco valve for the first 1.5 min of each run to keep the ion source
 29 clean. Mass spectrometry detection was carried out both in positive and negative mode,
 30 by independent chromatographic analysis, with a triple quadrupole analyzer in Multiple
 31 Reaction Monitoring (MRM) mode. Two MRM transitions (precursor > product ion)
 32 were selected for each toxin except for ~~gymnodimines~~ GYMs for which 3 MRM
 33 transitions were selected per compound. The conditions for MS/MS detection and the
 34 limits of detection and quantification obtained for reference materials are summarized in
 35 Table 1. Source/gas parameters were optimized for lipophilic toxin determination:
 36 curtain gas: 20 psi; ion spray voltage: 5500 V (positive) or -4500 V (negative);
 37 temperature: 500 (positive) or 400 °C (negative); nebulizer gas: 50 psi; heater gas: 50
 38 psi; collision gas: 4 (positive) or medium (negative), interface heater: on. A nitrogen
 39 generator NM20Z (Peak Scientific, Renfrewshire, Scotland) supplied all operation
 40 gases. Resolution of both Q1 and Q3 quadrupoles were set at unit and the detector
 41 channel electron multiplier (CEM) worked at 2400 V. Analyst software v1.4.2 was used
 42 for the entire MS tune, instrument control, data acquisition and data analysis. For data
 43 processing, default Gaussian smooth was applied with default values of 100% filter
 44 width (% of minimal distance between acquired points) and 10 as a limit filter (number
 45 of minimal distance between points). Calibration was performed from 5 to 60 ng mL⁻¹
 46 (corresponding to 37.5 to 450 μ g kg⁻¹) for OA, PTX-2, AZA-1, GYM-A, SPX-1, and
 47 from 12.5 to 150 ng mL⁻¹ (corresponding to 94 to 1125 μ g kg⁻¹) for YTX. For
 48 quantification of ~~gymnodimines~~GYMs, a linearity equation was obtained for GYM-A, y
 49 = 1528x + 208 (R² = 0.9961), assuming an equimolar response by -MS/MS detection for
 50 GYM-B and/or GYM-C. For the latter, the 3 MRM transitions of GYM-A were

substituted with specific transitions for GYM-B/-C, set after the corresponding product ion experiments, and then re-analyzed by LC-MS/MS. Product ion experiments involved the study of fragmentation pattern for both precursor ions (*i.e.* 508.3 *m/z* for GYM-A and 524.3 *m/z* for GYM-B/-C). Product ion spectra were acquired between 75-525 *m/z*. This mode was only used for qualitative analysis. The total amount of GYMs equivalents was calculated with a Toxicity Equivalent Factor (TEF) of 0.1 for GYM-B/C (Kharrat et al., 2008).

3. Results and Discussion

In the analysis of shellfish samples with the MBA mice showed similar symptoms to those previously described for ~~gymnodimines-GYMs~~ poisoning in many samples: hyperactivity, jumping, paralysis of hind legs and severe dyspnea. When occurred, death was recorded rapidly between 6 and 15 minutes after *i.p.* injection, suggesting a severe neurological impairment related with the action of fast acting toxins (FAT). Nevertheless, some samples were not lethal even though they caused these characteristic symptoms over the first 10 minutes. In those cases the symptoms gradually disappeared and the mice recovered a normal state usually after ca. 30 min. Similar observations have been described by Seki *et al.* (Seki et al., 1995) , and Munday *et al.* who referred to these symptoms as sub-lethal (Munday et al., 2004). In our study most samples from the M2 site were positive (76%) with a rapid time of death ranging between 3 and 8 min, except for two samples with a time of death of 60 min. In site S6 less positive samples were obtained in relation to the M2 site (25%) with a time of death between 6 and 480 min.

The results obtained with LC-MS/MS method showed that from the total of 17 lipophilic toxins analyzed belonging to 6 different subclasses (OA-group, AZAs, PTXs, YTXs, SPXs and GYMs), only GYMs were present in samples for the total period studied, without traces of other toxins above the limits of detection. These results confirmed the previous reports of presence of ~~gymnodimines-GYMs~~ in the Gulf of Gabes (Biré et al., 2002; Marrouchi et al., 2010), but also showed the great prevalence of this toxin group in the area. According to our results, synergistic effects with other lipophilic toxins proposed by Dragunow and co-workers (Dragunow et al., 2005) are unlikely. Quantitatively, the Boughrara lagoon (M2) was identified as a *hot-spot* of ~~gymnodimines-GYMs~~ with values as high as 2,136 $\mu\text{g GYM-A kg}^{-1}$ (sample of February 2009 at M2) and with 13 out of 21 samples with GYMs levels above the LD₅₀ value of 80 $\mu\text{g kg}^{-1}$ (62% of the total). In this area, GYMs should be considered as persistent contaminants and therefore a continuous source of shellfish containing GYMs. As a consequence, we may expect that molluscs containing GYMs will be released to the market once the MBA is replaced. Consequently, studies on chronic toxicity should be conducted in order to carry out a proper risk assessment for GYMs. Regarding the identification of gymnodimine-B and or -C (GYM-B/-C), additional experiments were conducted to obtain the enhance product ion (EPI) spectrum for GYM-B/-C. Figure 3 shows the EPI spectra for both the CRM-GYM-A (Figure 3a) from the precursor ion $[\text{M}+\text{H}]^+$ at 508.3 *m/z* and for the precursor ion of GYM-B/-C (Figure 3b) at $[\text{M}+\text{H}]^+$ at 524.3 *m/z* acquired from the sample of February 2009 at M2. It is clearly shown both $[\text{M}+\text{H}]^+$ ions with 16 amu higher for GYM-B/-C due to the presence of an additional exocyclic methylene at C-17 and an allylic hydroxyl group at C-18 (Miles et al., 2000, 2003). The fragmentation gave the loss of water as a primary fragmentation product, but also a series of common ions with parent GYM-A that can be used for confirmation purposes: 462.3; 304.3; 216.2; 202.2; 174.2 and 136.1 *m/z*.

1 Thus, we confirmed the presence of GYM-B/-C in high contaminated samples by direct
 2 comparison of the EPI spectrum with the reference CRM-GYM-A. Since there was a
 3 lack of literature with a description of specific triple quadrupole analysis (*i.e.* MRM) for
 4 GYM-B/-C, three specific MRM transitions (524.3 > 506.4/ 488.4/ 202.4) were built for
 5 identification of GYM-B/-C in low contaminated samples and for quantification. The
 6 loss of water as a primary product ion for both GYM-A and GYM-B/-C made suitable
 7 to take this transition for quantification assuming an equimolar response (Figure 3). All
 8 samples were re-analyzed and then the concentration of the derivatives GYM-B/-C
 9 successfully obtained. Unfortunately, the analogs GYM-B and -C, if both were present,
 10 could not be distinguished by MS/MS detection nor resolved chromatographically with
 11 the multi-toxin gradient. The results showed that GYM-B/-C were present in all samples
 12 except samples taken on June 2009 at M2 and January 2010 at S6. This also
 13 demonstrates the persistence of these analogs in the area contrarily to what was
 14 previously reported by Biré et al. (Biré et al., 2002), but in agreement with Marrouchi
 15 et al. (Marrouchi et al., 2010). Besides, a constant ratio between the concentration of
 16 GYM-A and GYM-B/-C could not be established. The relative abundance between
 17 GYM-A and GYM-B/-C ranged from 5% in February 2009 at M2 to 54% in September
 18 2009 at S6. This variability makes necessary to determine also the hydroxylated analogs
 19 for an accurate determination of the total equivalents of [gymnodiminesGYMs](#), because
 20 their contribution may be not negligible even though a 10-fold lower toxicity in GYM-
 21 B/-C has been reported in relation to GYM-A (Kharrat et al., 2008). Figure 4 shows
 22 examples of LC-MS/MS chromatograms for the standard of GYM-A (Figure 4a), for
 23 GYM-A in sample from February 2009 at M2 (Figure 4b) and GYM-B/-C from the
 24 same sample (Figure 4c). Interestingly, other peaks appeared in the chromatogram with
 25 the same transitions than GYM-A, though at different retention time having a more
 26 lipophilic nature. From those signals, a few peaks also showed the same relative
 27 abundance among product ions meaning that these could be compounds related with
 28 parent GYM-A. The acquisition of the EPI spectrum from the precursor 508.3 *m/z*
 29 showed the same spectrum at 11.5 min than that acquired for GYM-A (6.61 min), which
 30 demonstrated that this compound is structurally-related to the parent GYM-A (data not
 31 shown). One hypothesis is that it could be a new isomer of GYM-A, however, it seems
 32 unlikely according to the difference of polarity. Other possibility is that it could be a
 33 derivative or aggregate weakly bonded that releases free GYM-A in the ion source.
 34 Further experiments need to be conducted to confirm or reject this hypothesis.
 35 Metabolization of lipophilic toxins in shellfish has been extensively reported for other
 36 subclasses of toxins. However, it has not been described yet for [gymnodiminesGYMs](#).
 37 If confirmed, derivatives might be of importance to evaluate the total amount of
 38 [gymnodiminesGYMs](#) in shellfish samples, and eventual bio-transformations should be
 39 taken into account. The quantitative results obtained by LC-MS/MS analysis were
 40 compared with the results obtained by the official MBA and with the reported LD₅₀ of
 41 80 µg kg⁻¹ as a threshold level. A good agreement was found between LC-MS/MS and
 42 MBA (Figure 5), showing much higher concentrations in sampling site M2 than in S6.
 43 Only 4 samples from a total of 21 positive results by the MBA were below 80 µg kg⁻¹
 44 (September 2009 at S6 and January 2009, April 2009 and September 2010 at M2),
 45 while all negative samples (n=20) according to the MBA were always below 80 µg kg⁻¹.
 46 However, bad correlation was obtained between the concentration of total equivalents of
 47 GYMs and the time of death ($y = -1.1384x + 505.3$; $R^2 = 0.0613$) for positive samples,
 48 especially when the concentration of GYMs were below 250 µg GYM-A equivalent kg⁻¹.
 49 That means that the 'YES/NO' response provided by MBA is not a good tool to
 50 obtain reliable measurements of GYMs close to the threshold level. Therefore,

quantitative results cannot be inferred from the time of death in a similar way that it is carried out for other toxins such as PSP toxins according to the Sommer's Table (AOAC, 1984).

Regarding to the harmful algae blooms, they are recurrent and widespread in the Gulf of Gabes, though only 10 species (*Alexandrium minutum*, *Karenia selliformis*, *Coolia monotis*, *Protoceratium reticulatum*, *Prorocentrum lima*, *Gymnodinium catenatum*, *Karlodinium veneficum*, *Osteopsis siamensis*, *Dinophysis sp.* and *Prorocentrum minimum*) and the cyst of *K. selliformis* were confirmed toxic after bioassay tests. Time series of phytoplankton monitoring (1995-2010) reveal that blooms of *Karenia selliformis* in the Gulf of Gabes are often (Feki et al., 2008; Hamza et al., 2010). Figure 6 shows the spatio-temporal distribution of *K. selliformis* in the sampling sites of the Gulf of Gabes. This species is frequently found in all coastal waters of the area with especially remarkable blooms in the south along the year. Site M2 located at the inner part of the Boughrara lagoon showed high abundance of *K. selliformis* with a permanent bloom lasting almost all period studied and where cell densities can reach over 4×10^6 cells L⁻¹. This species can also form blooms outside the Lagoon, especially from G1 to G3. On the contrary, more diversity compared to other toxic species is found in S6 outside of the Lagoon (Table 2), though in this site *K. selliformis* is also able to bloom at high densities (e.g. September 2009). Regarding the toxin content in shellfish provided by both MBA and LC-MS/MS, poor agreement was found with cell densities of *K. selliformis*. It might be related with an active population dynamics for this species involving rapid rising and falls of blooms in few days. Other possibilities are that parameters such as different toxicity of strains responsible for the blooms or environmental factors eventually determine the amount of toxin produced. Furthermore, the enhanced capabilities for detoxification recently reported in clams (Medhioub et al., 2010) encourages increasing the sampling rate of phytoplankton and shellfish in order to get a better correlation between both parameters. To clarify these hypotheses, toxin analysis in *K. selliformis*, in shellfish, as well as *K. selliformis* cell abundance should be monitored over smaller timeframe.

From these results, it has been demonstrated that the presence of cyclic imines in shellfish from specific areas may not be a punctual fact, but a source of persistent contamination. As GYMs are not presently regulated by the EU or by international standards such as the CODEX alimentarius (CODEX, 2008), consumers may therefore be exposed to GYMs in areas such as the ones studied. It is encouraged to define a preventive MPL for GYMs, otherwise the change of official control method from MBA to LC-MS/MS (EU, 2011) will not prevent the exposure to GYMs by consumers at levels as high as in the part per million range. Despite the fact the EFSA has assessed the risk for other cyclic imines like spirolides as low, the assessment took into account the the 95th percentile of the concentration of SPXs of 9, 15 and 7 µg SPXs/kg shellfish meat for mussels, oysters and clams, respectively, which is much lower than the concentrations found in this study for the Mediterranean Sea and those reported in literature ranging between 14.8 to 23,400 µg GYM/kg in New Zealand (Stirling, 2001a). Additionally, this study has contributed to the characterization of the distribution of GYMs in the Gulf of Gabes, which could be relevant as a permanent source of raw material containing GYMs useful to obtain reference materials or contaminated material needed for chronic toxicity studies.

4. Conclusions

1
2 We have confirmed the prevalence of GYMs in the Gulf of Gabes (Tunisia) since they
3 were the only toxins present in samples. Therefore, synergistic of GYMs with other
4 marine toxins are unlikely expected in this area. The inner side of the Boughrara lagoon
5 has been identified as a *hot-spot* for GYMs where the concentrations can reach levels
6 above 2 mg GYMs/kg in clams. Moreover, GYMs may be considered at this site a
7 persistent contaminant and consequently chronic studies are still needed to perform a
8 correct risk assessment. We unambiguously confirmed GYM-B/-C in most samples
9 meaning these derivatives have to be included in studies involving GYMs in order to
10 get reliable estimation of the total GYMs equivalents. Despite the lack of regulation, it
11 is encouraged that GYMs were included in all monitoring programs. It would allow
12 increasing the database of GYMs presence in shellfish and consequently being able to
13 assess the risk provided the correct exposure of consumers to these toxins. Moreover, it
14 would improve the agreement between MBA and LC-MS/MS during the transition
15 period towards the official new reference method (till 31st Dec, 2014) favoring the
16 international trade. Once the risk was properly assessed, a preventive maximum
17 permitted level should be fixed by food safety authorities to prevent consumers from
18 being exposed to large amounts of GYMs

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26

27 **Conflict of interest**

28 The authors declare that there are no conflicts of interest.
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Table 1. Summary of the toxin ions analyzed by LC-MS/MS.

Toxin	ESI polarity	Precursor ion	Q1 (m/z)	Q3 ^a (m/z)	LOD (µg kg ⁻¹)	LOQ (µg kg ⁻¹)
Desmethyl spirolide C (SPX-1)	+	[M+H] ⁺	692.5	444.2 426.3	<u>3</u>	<u>10</u>
Gymnodimine A (GYM-A)	+	[M+H] ⁺	508.3	490.3 392.3 202.3	<u>5</u>	<u>15</u>
Gymnodimine B and C (GYM-B/-C)	+	[M+H] ⁺	524.3	506.3 488.3 202.3	=	=
Pectenotoxin-2 (PTX-2) and 7- <i>epi</i> -pectenotoxin-2	+	[M+NH ₄] ⁺	876.5	823.5 213.3	<u>11</u>	<u>38</u>
Pectenotoxin-1 (PTX-1)	+	[M+NH ₄] ⁺	892.5	821.5 213.3	=	=
Pectenotoxin-2 seco acid (PTX-2sa)	+	[M+NH ₄] ⁺	894.5	823.5 213.3	=	=
Azaspiracid 1 (AZA-1)	+	[M+H] ⁺	842.5	362.3 462.5	<u>3</u>	<u>9</u>
Azaspiracid 2 (AZA-2)	+	[M+H] ⁺	856.5	362.3 462.5	=	=
Azaspiracid 3 (AZA-3)	+	[M+H] ⁺	828.5	362.3 448.5	=	=
Okadaic acid (OA) and dinophysistoxin-2 (DTX-2)	-	[M-H] ⁻	803.5	255.2 113.1	<u>5</u>	<u>16</u>
Dinophysistoxin-1 (DTX-1)	-	[M-H] ⁻	817.5	255.2 113.1	=	=
Yessotoxin (YTX)	-	[M-2Na+H] ⁻	1141.5	855.2 713.2	<u>26</u>	<u>88</u>
45-hydroxyessotoxin (45-OHYTX)	-	[M-2Na+H] ⁻	1157.5	855.2 713.2	=	=
Homoyessotoxin (homoYTX)	-	[M-2Na+H] ⁻	1155.5	869.2 727.2	=	=
45-hydrosyhomoyessotoxin (45-OHhomoYTX)	-	[M-2Na+H] ⁻	1171.5	869.2 727.2	=	=

^aHighest abundance ions are presented in bold letters.

Table 2. Summary of the results on microalgae identification and counts, mouse bioassay, and LC-MS/MS analysis.

Sampling date (month-year)	Dominant microalgae species in seawater ^a	Cell densities (cells L ⁻¹)	MBA ^b (died/total)	Time of death ^c (min)	GYMs (µg kg ⁻¹) ^d	
					GYM-A	GYM-B/-C
Sampling Site M2						
Jan-09	<i>Karenia selliformis</i>	421,600	3/3	4	N.Q.	N.Q.
Feb-09	<i>Karenia selliformis</i>	24,800	3/3	3	2,136	113
Mar-09	<i>Karenia selliformis</i>	4,500	3/3	4	194	215
Apr-09	<i>Karenia selliformis</i>	120,000	3/3	4	N.Q.	N.Q.
May-09	<i>Karenia selliformis</i>	1,600	3/3	5	157	146
Jun-09	<i>Karenia selliformis</i>	352,000	0/3	-	N.D.	N.D.
	<i>Coolia monotis</i>	300				
Jul-09	N.R.	-	0/3	-	N.Q.	N.Q.
Aug-09	<i>Karenia selliformis</i>	214,900	0/3	-	N.Q.	N.Q.
Sep-09	<i>Karenia selliformis</i>	200,900	3/3	4	210	217
Oct-09	<i>Karenia selliformis</i>	450,000	3/3	5	1,044	182
Nov-09	<i>Karenia selliformis</i>	615,000	3/3	60	530	55
Dec-09	<i>Karenia selliformis</i>	46,000	3/3	4	608	135
Jan-10	<i>Karenia selliformis</i>	500,000	3/3	3	479	38
Feb-10	<i>Karenia selliformis</i>	3929,600	3/3	4	265	180
Mar-10	<i>Karenia selliformis</i>	600	3/3	6	253	196
Apr-10	<i>Karenia selliformis</i>	88,000	3/3	8	1,129	186
May-10	N. S.	-	0/3	-	N.Q.	N.Q.
Jun-10	<i>Karenia selliformis</i>	17,400	2/3	60	208	178
Jul-10	<i>Karenia selliformis</i>	2,000	1/3	-	N.Q.	N.Q.
Aug-10	<i>Karenia selliformis</i>	225,600	3/3	6	126	101
Sep-10	<i>Karenia selliformis</i>	10,800	3/3	5	N.Q.	N.Q.
Sampling Site S6						
Jan-09	N.R.	-	0/3	-	N.Q.	N.Q.
Feb-09	N.R.	-	0/3	-	N.Q.	N.Q.
Mar-09	N.R.	-	0/3	-	N.Q.	N.Q.
Apr-09	<i>Coolia monotis</i>	300	0/3	-	N.Q.	N.Q.
May-09	<i>Karenia selliformis</i> (cysts)	400	0/3	-	N.Q.	N.Q.
Jun-09	<i>Karenia selliformis</i>	400	0/3	-	N.Q.	N.Q.
Jul-09	N.R.	-	0/3	-	N.Q.	N.Q.
Aug-09	<i>Karlodinium verificum</i>	200	0/3	-	N.Q.	N.Q.
Sep-09	<i>Karenia selliformis</i>	123,000	3/3	6	47	55
Oct-09	<i>Alexandrium minutum</i>	500	0/3	-	N.Q.	N.Q.
Nov-09	<i>Karenia selliformis</i> (cysts)	100	3/3	180	91	78
Dec-09	<i>Karenia selliformis</i>	100	3/3	40	127	129
Jan-10	<i>Alexandrium spp.</i>	100	0/3	-	N.Q.	N.D.
Feb-10	<i>Coolia monotis</i>	300	0/3	-	N.Q.	N.Q.
Mar-10	<i>Karlodinium verificum</i>	100	0/3	-	N.Q.	N.Q.
Apr-10	<i>Alexandrium minutum</i>	600	0/3	-	N.Q.	N.Q.
May-10	<i>Alexandrium spp.</i>	200	0/3	-	N.Q.	N.Q.
Jun-10	<i>Alexandrium minutum</i>	200	0/3	-	N.Q.	N.Q.
Jul-10	<i>Prorocentrum lima</i>	100	3/3	60	81	82
Aug-10	<i>Karenia selliformis</i>	200	3/3	480	103	105

a: N.R.: Nothing remarkable; N.S.: Not sampled.

b: A positive result is considered when the mice ratio died/total is $\geq 2/3$

c: Longest time of in the MBA.

d: Quantitative results by LC-MS/MS. N.D.: not detected ($<5\mu\text{g kg}^{-1}$); N.Q.: not quantified ($5\leq x\leq 37\mu\text{g kg}^{-1}$).

Figure captions:

Figure 1: Chemical structures of gymnodimines: **aA)** Gymnodimine A; **Bb)** Gymnodimine B and **Ce)** Gymnodimine C.

Figure 2: Sampling sites at the shellfish production areas in the Gulf of Gabes, Tunisia

Figure 3: Product ion spectra for: **aA)** Certificate reference material of gymnodimine A (GYM-A) from the molecular ion $[M+H]^+ = 508$ m/z selected as precursor ion. Mass spectrum extracted from chromatographic peak (6.6 to 7.0 min), and **Bb)** Sample M2/02/09 from the precursor ion $m/z = 524$, extracted from the chromatographic peak from 6.4 to 6.6 min. Collision energy was 55 V.

Figure 4: LC-MS/MS analysis of: **Aa)** gymnodimine-A standard (CRM-GYM) of 60 ng mL^{-1} . MRM transitions were as follows (precursor ion > product ion 1/ 2/ 3): 508 > 490 (blue) / 392 (red) / 202 (green); **bB)** sample M2/02/09, diluted (1:5) in MeOH, quantified as 2,136 μg GYM-A kg^{-1} . MRM transitions as in a); and **cC)** sample M2/02/09, with no dilution, quantified as 113 μg GYM B/C kg^{-1} . MRM transitions were in this case 524 > 506 (blue) / 488 (red) / 202 (green).

Figure 5: Evolution in time of the gymnodimines content and toxicity in mice for the two sampling sites, M2 (Boughrara) and S6 (Bousaid). Legend should be read as follows: **■**: **■**: Concentration of gymnodimine-A; **■**: **■**: Concentration of gymnodimine-B and/or gymnodimine-C; **-----**: **---**: LD_{50} reported by Kharrat et al. (Kharrat et al., 2008); **x** : * : MBA (Number of mice dying from a total of three).

Figure 6: Spatio-temporal distribution (average since 1995 to September 2010) of *Karenia selliformis* ($\log x+1$) found in the monitored sites from the Gulf of Gabes. x = number of cells L^{-1} .

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