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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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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 mousse 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 2136 μg 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 μg 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.

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, 1995, 1996a; MacKinnon et al., 2006; Roach et al., 2009), pinnatoxins (Chou et al., 1996a,b; Takada et al., 2001a; Uemura et al., 1995), pteriatoxins (Takada et al., 2001b), prorocentrolides (Hu, 1996; 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 (Fig. 1). Later on, two hydroxilated 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 LD_{50} for Gymnodimine-A in mice estimated from intraperitoneal (*i.p*) injection was firstly estimated about 450 (Seki et al., 1995) and 700 μg kg^{-1} 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^{-1} 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 and 15 min 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

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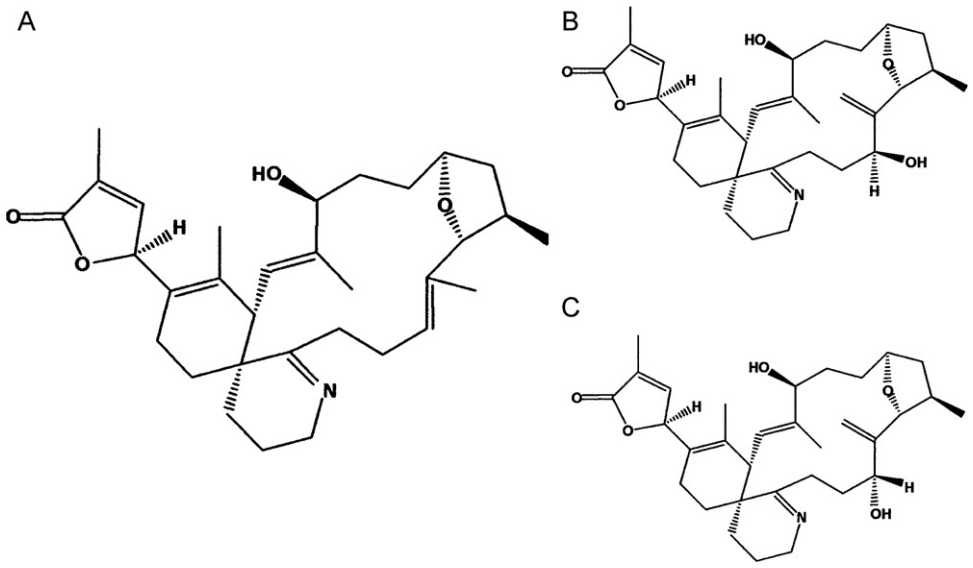

Fig. 1. Chemical structures of gymnodimines: (A) gymnodimine A; (B) gymnodimine B and (C) gymnodimine C.

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 (Fonfría et al., 2010; 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 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) has been proposed to prevent acute or chronic toxicity, respectively. A low risk might be presumed taking into account the low oral toxicity in mice (LD_{50} for GYM-A of 775 and ca. 7500 μg kg^{-1} 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. LD_{50} (*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 New 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 1300 km of coastline and considerable resources for shellfish production. The Gulf 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), shellfish 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 these 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 (Fig. 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,