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Fatty acid ester metabolites of gymnodimine in shellfish collected from China and in mussels (*Mytilus galloprovincialis*) exposed to *Karenia selliformis*

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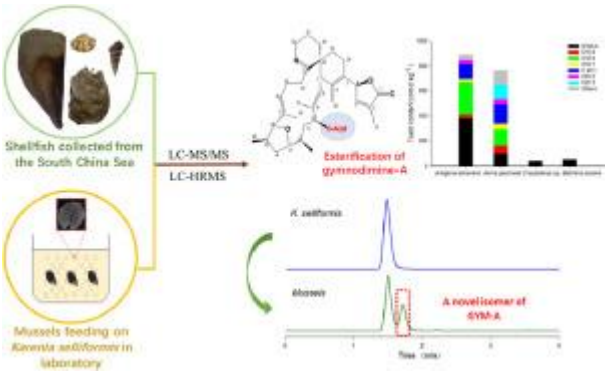
Abstract :

Marine shellfish exposed to the microalgae Karenia selliformis can accumulate gymnodimines (GYM). Shellfish samples collected from Beihai City in Guangxi Autonomous Region, and Ningde City in Fujian Province, in the South China Sea, as well as mussels Mytilus galloprovincialis fed on K. selliformis under laboratory conditions were analyzed. Gymnodimines and various fatty acid ester metabolites were detected in the clam Antigona lamellaris and pen shell Atrina pectinata, while no esters were found in the oyster Crassostrea sp. and the gastropod Batillaria zonalis despite positive detection of free GYM in both species. When present, the predominant acyl esters observed were 18:0-GYM-A and 20:1-GYM-A. Under laboratory conditions GYM-A was accumulated and metabolized to fatty acid esters in mussels exposed to K. selliformis, with 16:0-GYM-A and 20:1-GYM-A as the major variants. A novel compound with the same accurate mass as GYM-A and its 16:0 fatty acid ester were observed in the experimental mussels but was not present in the microalgal strain to which mussels were exposed. No significant differences of reactive oxygen species (ROS) levels and antioxidant enzymes were found between mussels fed on K. selliformis or GYM-free microalgae Isochrysis galbana. This suggests the accumulation of GYM and its metabolites does not significantly impact the physiological status of mussels. While it is currently not proven that GYM affects human health, risk assessments should consider the presence of GYM esters in naturally contaminated shellfish as part of exposure analysis.

Graphical abstract



Highlights

► GYM-A was detected in four species of field marine mollusks from Chinese coast. ► Diverse fatty acid esters of GYM-A were found in clam and pen shell, but not in oyster and gastropod. ► GYM-A and its different fatty acid esters accumulated in mussels fed with *Karenia selliformis*. ► The esters 16:0-GYM-A and 20:1-GYM-A were the major components in the experimental mussels. ► A novel compound with the same accurate mass as GYM-A and its esters were also observed in mussels.

Keywords : Gymnodimines (GYM), Fatty acid ester, *Karenia selliformis*, Toxin profiles, Antioxidant enzymes

1. Introduction

Marine bivalves have the capacity to accumulate phycotoxins produced by harmful algae and generate a diverse range of secondary metabolites, collectively known as shellfish toxins (Salas and Clarke, 2019). These toxins can be divided into eight groups, including saxitoxins, azaspiracids, brevetoxins, cyclic imines, domoic acids, okadaic acids, pectenotoxins, and yessotoxins (Toyofuku, 2006). Gymnodimines (GYM) (Fig. 1) are a group of fast-acting cyclic imine toxins initially identified in oysters collected in New Zealand (Seki et al., 1995; Stewart et al., 1997). GYM-A, GYM-B and GYM-C are produced by the dinoflagellate *Karenia selliformis* (Miles et al., 2000, 2003; Seki et al., 1995). 12-methyl-gymnodimine was subsequently verified as natural product in the dinoflagellate *Alexandrium ostenfeldii* from estuaries on the U.S. East Coast (Van Wagoner et al., 2011) and the Netherlands (Van de Waal et al., 2015). Additionally, *A. ostenfeldii* from the northern Baltic Sea was shown to produce a new analogue, GYM-D (Harju et al., 2016). Gymnodimines have also been documented in shellfish from coastal regions of Tunisia (Biré et al., 2002; Marrouchi et al., 2010), Australia (Takahashi et al., 2007), Europe & North America (Kharrat et al., 2008), South Africa (Krock et al., 2009) and China (Jiang et al., 2017; Li et al., 2015; Liu et al., 2011a). A recent study demonstrated that fatty acid ester metabolites of GYM-A constitute the majority (>90%) of GYM toxins present in clam samples (*Ruditapes decussatus*) from Tunisia (de la Iglesia et al., 2013). Several unknown GYM-like compounds have been detected in shellfish (McCarron et al., 2014) and in *A. ostenfeldii* by liquid chromatography-mass spectrometry (LC-MS) (Harju et al., 2016; Qiu et al., 2018). Considering the high intraperitoneal toxicity (LD_{50} of 80-96 mg kg^{-1}) of GYM-A to mice, and albeit lower relative oral toxicity (Munday et al., 2004), these metabolites should be considered when evaluating the risks of GYMs in shellfish for human consumption.

Antioxidant enzyme activity in bivalves is influenced by reactive oxygen species (ROS) and may be an indication of organism health and oxidative stress (Box et al., 2007; Fernández et al., 2012; Gillis et al., 2014; Hu et al., 2015a; Regoli and Principato,

1995). Antioxidant responses are enhanced when the production of ROS increases, resulting in oxidative stress (Hu et al., 2015b; Pan et al., 2006). The antioxidant and immune systems of shellfish, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GR), glutathione (GSH) and lysozyme (LZM), are responsible for eliminating ROS and foreign particles. Paralytic shellfish toxins accumulated from toxic microalgae induced oxidative stress in mussels (*Mytilus galloprovincialis*) and scallops (*Patinopecten yessoensis*), where ROS were reduced by antioxidant enzymes under laboratory conditions (Qiu et al., 2013).

The objective of this work was to analyze the profiles of GYM and its fatty acid esters in various species of shellfish relevant to the coastal regions of China, with a specific focus on the South China Sea. Laboratory studies were conducted on mussels (*M. galloprovincialis*) fed with the GYM-producing microalgae *K. selliformis* to study the uptake and metabolism of GYM-A, and to monitor the response of antioxidant enzymes (SOD, CAT, GSH).

2. Materials and Methods

2.1 Reagents

Ammonium formate and formic acid were from Fisher Scientific (Fair Lawn, NJ, USA) or Honeywell-Fluka (Oakville, ON, Canada). Acetonitrile, methanol, monopotassium phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were from Merck Ltd. (White-house Station, NJ, USA). A certified reference material (CRM) of GYM-A was obtained from the National Research Council of Canada (Halifax, NS, Canada). Milli-Q water was supplied by a Milli-Q water purification system from Millipore Ltd. (Bedford, MA, USA) to 18.2 MΩ quality or better.

2.2 Culture of microalgae and sample preparation

K. selliformis (strain GM94GAB) used in the feeding experiment was isolated from the Gulf of Gabes, Tunisia (Medhioub et al., 2009). The strain was maintained in sterilized seawater filtered with 0.45 μm mixed fiber membrane (Jinjing Ltd., China) in photo-bioreactors (120 L) without aeration. The culture was enriched with f/2 medium without silicate (Guillard and Ryther, 1962) at 18±2°C under 6000 lx light intensity

with a 12 h: 12 h illumination cycle. Algal cells were counted by optical microscopy at a magnification of 100 on a Sedgewick Rafter Counting Chamber and collected at the stable growth stage to feed mussels.

Isochrysis galbana 3011 kept at Ocean University of China was used as a control diet in the feeding experiment. The culture conditions of *I. galbana* were as above for *K. selliformis*, without aeration. The batch of *I. galbana* was collected when the algal density approached 10^8 cells mL^{-1}.

A freeze-dried pellet (2.6×10^6 cells) of the cultured *K. selliformis* was extracted using a 1/16'' microtip ultrasonicator probe with coupler (QSonica LLC (CT, USA) in 1 mL methanol using a cycle with 40% amplitude pulse mode for a total of 3 min. The sample was held in an ice bath to prevent the solution from heating during extraction. The sample was then filtered using a 0.45 μm PVDF centrifugal filter (Merck Millipore Ltd.) prior to LC-MS analysis.

2.3 Shellfish samples

Multiple species of commercially grown shellfish were collected at shellfish harvesting regions in the South China Sea in March 2016. This included clams (*Antigona lamellaris*), pen shells (*Atrina pectinata*) and oysters (*Crassostrea* sp.) from Beihai City, Guangxi Autonomous Region, and gastropod (*Batillaria zonalis*) from Ningde City, Fujian Province. Whole tissue samples (~1 g) were weighed and extracted with 3 mL methanol on an ice using a superfine homogenizer (F6/10, Fluko) at speeds ranging from 5000 to 32000 rpm in three 30 s cycles. The supernatant was carefully transferred to a 10 mL volumetric flask after centrifugation (2400 ×*g* for 10 min at 4ºC). The remaining pellet was re-extracted twice more as above and the combined supernatants were adjusted to a final volume of 10 mL and passed through 0.22 μm polyamide nylon filters into HPLC vials and stored at -20ºC until LC-MS analysis.

2.4 Feeding experiment and sample preparation

Mussels (*M. galloprovincialis*) were obtained from a marine aquaculture zone along the coast of Qingdao, China, in October 2016. They were transported alive to the laboratory and washed to remove epibionts. Mussels were randomly divided into two

tanks (60 L) and acclimated in filtered seawater with continuous aeration at 16 ± 2°C for 3 days. During this period, seawater (without any feed) was renewed twice daily at 08:00 and 20:00.

After acclimation, mussels in the first tank (treatment group) were fed with *K. selliformis* for four days and then starved for three days. They were maintained in a 60 L tank with fresh seawater containing the toxic microalgae at an initial density of 1000 cells mL^{-1}. The *K. selliformis* culture and fresh seawater were continuously supplied for four days using a peristaltic pump to maintain the initial algal density. The volume of seawater in the tank was kept constant. Fresh seawater alone was added for the three days of starvation. Acclimated mussels in the second tank were used as a blank control. Experimental settings for the control group were the same as for the treatment group, with the exception of the diet. The non-toxic microalga *I. galbana* was pumped and maintained at a density of 10 000 cells mL^{-1} for the first four days. Both tanks were continuously aerated during the entire experiment. Mussels (n=10) were selected randomly and dissected at various time points (0, 12, 24, 48, 72, 96, 120, 144, and 168 h). The muscle (containing foot and adductor muscle) and digestive gland of mussels were separated. Tissue samples (1 ± 0.1 g) of both treatment groups were prepared as described in section 2.3.

For ROS determinations, tissue samples (0.5 g) were homogenized in 10 mL phosphate buffer (0.1 M, pH=7.0) containing 2.6 mM KH_2PO_4 and 4.1 mM Na_2HPO_4 for 3 mins on an ice-water bath. The supernatant from centrifugation (2400 ×*g*, 4ºC, 20 min) was collected. To analyze activity of antioxidant enzymes (CAT, SOD, GSH) additional tissue samples (1 ± 0.1 g) were homogenized and centrifuged in 9 mL physiological saline (0.9%) following the conditions as described above. The supernatant was collected.

2.5 LC-MS analysis for GYM and esters

The LC-MS method was modified from a previous study (Li et al., 2016). An HPLC (Agilent 1290, Palo Alta, CA, USA) was coupled with a triple quadrupole mass spectrometer (Agilent 6430, Palo Alta, CA, USA) equipped with an ESI interface. A

Luna C_{18} column (50 × 2.1 mm i.d., 3 µm, Phenomenex) was used at 35°C. Mobile phases A (water) and B (95% acetonitrile) contained with 50 mM formic acid and 2 mM ammonium formate. A gradient at 300 µL min^{-1} was run from 25% to 100% B over 7 min, held for 3 min and returned to 25%B over 1 min before re-equilibration for 3 min. Injection volume was set at 5 µL. Selected reaction monitoring (SRM) transitions for all the acyl ester derivatives of GYM-A were described previously (de la Iglesia et al., 2013). Product ion *m/z* 490.3, fragmentor energy 150 V, collision energy 30 V, and dwell time 15 ms were used to scan fatty acid esters of GYM-A. Concentrations of GYM and metabolites of GYM-A were roughly estimated using CRM-GYM-A as a reference material assuming equal molar responses.

2.6 LC-HRMS analysis for GYM and esters

An HPLC (Agilent 1200, Palo Alto, CA, USA), was coupled with high resolution mass spectrometer (Q-Exactive HF, Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization probe (HESI-II). A Luna C_{18} column (50 × 2 mm i.d., 2.5 µm, Phenomenex) was used with mobile phase, flow rate, and temperature the same as described in section 2.5. The gradient ran from 15% to 100% B over 10 min, held for 15 min, and then returned to 15%B over 0.1 min before re-equilibration for 7 min. Injection volumes were 1 µL. Mobile phase was diverted to waste for the first 1.5 minutes of the run. Source conditions were spray voltage 3000, capillary temperature 350°C, sheath gas and auxillary gas at 35 and 10 (arbitrary units) respectively with heat set to 300°C, and S-Lens RF Level at 50. Full scan data was acquired in positive mode from *m/z* 400-900 using the 60 k resolution setting with an automatic gain control (AGC) target 1e6 ions, 100 ms maximum injection time. MS/MS spectra were collected through 'TopN' experiments. These TopN experiments used data-dependent acquisition with the top 10 most intense ions (not on an exclusion list of background ions) selected for fragmentation from the full scan survey, with preferential fragmentation given to ions above an intensity threshold of 1.6e5 ions from an inclusion list of known GYM and acyl ester variants. Apex trigger was set from 2-6 s with dynamic exclusion for 4 s in efforts to capture spectra near the apex of the

chromatographic peak. Spectra were collected at the 15 k resolution setting, 50 ms maximum injection time, an isolation window of 1 *m/z* about the parent mass with a collision energy spread of 30, 65 eV. Focused collection of free GYM spectra was done using parallel reaction monitoring (PRM) mode at the 30 k resolution setting, 2e5 AGC target, 100 ms maximum injection time, isolation window 0.4 *m/z*, and collision energy spread set at 40, 75 eV.

2.7 Analysis of antioxidant enzymes

The level of ROS and activities of antioxidant enzymes (SOD, CAT, GSH) in the digestive gland and muscle of mussels from both groups were analyzed using test kits for antioxidant enzyme biomarkers acquired from Nanjing Jiancheng Bioengineering Institute Ltd. (Nanjing, China) as described by (Liu, et al., 2011b; Ji et al., 2018a). The measuring principles are in accordance with specifications in kits.

3. Results

3.1 Profiles of GYM and esters in field shellfish collected from China

Varying concentrations of GYM-A was present in the four shellfish species tested. Concentrations of GYM-A as well as the relative molar percentages of predominant acyl esters in field mollusk samples are shown in Table 1. Acyl esters of GYM-A were found in the clam (*Antigona lamellaris*) and the pen shell (*Atrina pectinata*), while no fatty acid esters were found in the oyster (*Crassostrea* sp.) and the gastropod (*Batillaria zonalis*). The profile of GYM-A acyl esters observed varied between clams and pen shell samples. Octadecanoic acid (18:0) ester was the most abundant (50%), followed by the eicosenoic acid (20:1) (23%) in the clam. While 20:1-GYM ester (23%), 18:0-GYM ester (20%), and the docosadienoic acid (22:2) (18%) ester dominated the profile in the pen shell sample. The extent of GYM-A esterification was estimated for clam and pen shell samples assuming equimolar response for the esters against GYM-A standard, and were approximately 58% and 87% respectively.

3.2 GYM and esters in mussels from feeding experiment

LC-MS/MS SRM chromatograms for esters of GYM-A in digestive gland tissue of

mussels (*M. galloprovincialis*) fed with *K. selliformis* after 96 h are shown in Fig. 2. Twenty-eight fatty acid esters (odd- and even-chains) were identified in the experimental mussels. The *K. selliformis* used as feed microalga for mussels in this study primarily produces only free GYM-A and a trace amount of GYM-B (Fig. S1A). The mussels were free of GYM toxins before feeding on *K. selliformis* (Fig. S1C and D). LC-HRMS full scan chromatograms for GYM-A CRM, 16:0 GYM-A positive control tissue which was previously confirmed in using synthetic 16:0 GYM-A (Iglesia et al., 2013), *K. selliformis* culture and experimental mussels are shown in Fig. 3. Product-ion spectra (MS/MS) (Fig. 4) of GYM-A in the extract of digestive gland sample (treatment group-96 h), were acquired to confirm the accumulation and esterification of GYM-A in mussels fed with *K. selliformis* in laboratory. An additional GYM peak (4.66 min), not present in the microalgae, was also observed in the shellfish with the same measured accurate mass as GYM (Fig. 3 and 4). This new GYM analogue also formed esters (14.84 min) in the mussels (Fig. 3).

The concentrations of GYM-A and the relative amounts of acyl ester metabolites varied in the muscle and digestive gland tissues of mussels (Fig. 5). The acyl esters accounted for an estimated 98% of the GYM total in the muscle and digestive glands. Toxin concentrations increased during the algae-feeding period and stabilized during the starvation period. The highest concentration in the muscle tissues was at 120 h and 96 h for the digestive gland tissues. The sum concentration of GYM and GYM esters reached a maximum of ~760 nmol kg^{-1} in the muscle tissues, while it was four times higher in the digestive gland samples. The relative proportion of GYM-A and the major ester metabolites (16:0, 18:0, 18:1, 20:1, 20:2, 22:2) differed in both tissues (Fig. 5). In the muscle samples, the relative abundance of 16:0-GYM-A was dominant (33%) in the first 48 h but gradually declined to 14%, while the relative abundance of 20:1-GYM-A increased from 12% to 34%, and reached the highest level among ester metabolites after 48 h. The relative proportions of acyl esters in the digestive gland samples was consistent over the duration of the experiment with 16:0-GYM-A constituting approximately 30% esters detected.

Molar concentrations of GYM-A in the muscle and digestive gland tissues are

shown in Fig. S2A. The esters 22:2-GYM-A, 20:1-GYM-A, 18:0-GYM-A and 16:0-GYM-A were most abundant for their respective carbon chain lengths (Fig. S2B-E). The nutritionally important ω-3-fatty acids, EPA (C20:5) and DHA (C22:6) abundant in mussels (Fernández-Reiriz et al., 1996), formed much less acyl esters compared to the four derivatives above. Trends of each metabolite over time in these four groups were similar to the variations of GYM-A in the digestive gland tissues.

3.3 Responses of Antioxidant System

The ROS levels from the control and treatment groups followed similar trends (Fig. S3). ROS levels reduced slightly when feeding on toxic or non-toxic microalgae at 12 h, and did not change significantly until 144 h. Variations of CAT activity, SOD activity and GSH level in muscle and digestive glands are also shown in Fig. S3. These enzyme activity levels exhibited similar trends, showing minor fluctuation without significant changes over the course of the study.

4. Discussion

Accumulation of marine phycotoxins in shellfish is an important seafood safety issue due to frequent occurrence of toxic algal blooms in coastal areas worldwide (James et al., 2010). To properly assess the toxicity of shellfish exposed to harmful algal species, esterification of lipophilic toxins such as okadaic acid, pectenotoxins, spirolides and pinnatoxins has been studied (JØrgensen et al., 2005; Aasen et al., 2006; McCarron et al., 2012). The occurrence of GYM esters in shellfish highlights the need for methods to quantitate total profiles of GYM. Gymnodimines degrade under alkaline hydrolysis conditions and the enzymatic hydrolysis method still needs modification to be applied to cyclic imines (de la Iglesia et al., 2013; Doucet et al., 2007). Currently direct analysis of acyl esters by LC-MS is the most practical option for GYM ester measurement, however this is limited by availability of standards for GYM esters. In this work, individual ester concentrations were estimated assuming equal response of the esters and GYM-A. While total GYM esters measured were significant, these numbers can only be considered indicative. Monitoring lipophilic toxins in cultured mussels (*Mytilus coruscus*) during a previous study (Li et al., 2015) showed low

amounts of GYM-A are common throughout the year in the mariculture zone of Gouqi Island in the East China Sea. A subsequent study on lipophilic shellfish toxins showed higher concentrations of GYM-A were frequently detected in shellfish collected from the South China Sea (Ji et al., 2018b). GYM-A was present in the four field mollusk samples tested here. GYM-B, an analog of GYM-A, was first isolated and elucidated from *K. selliformis* (Miles et al., 2000). No GYM-B was detected in field samples. Trace amounts of GYM-B were present in the *K. selliformis*, and both the experimental mussels and seawater collected at 96 h during the feeding experiment (Fig. S1A and B). A positive detection of GYM-B in clams also occurred in a previous study (Naila et al., 2012).

Acyl ester metabolites have been observed for a variety of marine phycotoxins in a range of shellfish species (Comesaña Losada et al., 1999; García et al., 2004; JØrgensen et al., 2005; Torgersen et al., 2008a; 2008b; Wilkins et al., 2006). Based on previous reports of acyl ester metabolites for other classes of cyclic imines including spirolides (Aasen et al., 2006) and pinnatoxins (McCarron et al., 2012), and because of the previous study showing GYM esters in Tunisian clams (*Ruditapes decussatus*) (de al Iglesia et al., 2013), the presence of GYM esters in Chinese shellfish is not unexpected. In this study, concentration and profile of ester metabolites varied in clam (*Antigona lamellaris*) and pen shell (*Atrina pectinata*) samples, but weren't detected in oyster (*Crassostrea* sp.) or gastropod (*B. zonalis*) samples (Table 1), indicating that GYM esterification in mollusks may be species-specific. Possibly some key enzymes govern the metabolism of GYM esters in shellfish. The species specific profiles observed in GYM esterification should be studied further.

The feeding experiment demonstrated the acylation process under controlled laboratory conditions. GYM-A and GYM derived esters analyzed in this work showed parent masses < 5 ppm, and 490-fragment masses < 3.5 ppm. The 392-fragment observed for GYM-A and its esters, was not present for GYM-like species (*m/z* 508) (Fig. 4). Various ester metabolites were confirmed in the experimental mussels based on characterized transitions and relative retention times of esters published before (Fig. 2). The high relative abundance (90%-98%) of esterified GYM at each sampling-time

point (Fig. 5), including the first time point at 12 h, indicates that these Phase-II metabolites of GYM-A are formed very rapidly and that esterification constitutes a major metabolic process for these toxins. Relatively static distribution of acyl esters and GYM-A in digestive gland samples indicates GYM-A and its metabolites are readily retained in shellfish, possibly explaining the persistence of GYM-A in oysters (Seki et al., 1995) and clams (Naila et al., 2012). Considering the relative abundance of different GYM-A esters in the digestive gland tissue, percentages of free GYM-A and 16:0 ester slightly decreased in the depuration period after 96 h, which demonstrated that they were excreted or transformed faster than 20:1 ester, 22:2 ester, and 18:0 ester. This tendency is also observed for GYM-A and the 16:0 ester in muscle tissue in the depuration period (data not shown).

It is interesting to note that the unidentified GYM analog observed in the mussels after feeding, with the same accurate mass as GYM-A, also formed ester metabolites (Fig. 3 and 4). The origin of this GYM analog was not confirmed in this study, but it was not observed in direct analysis of the toxin-producing *K. selliformis* by LC-HRMS. The typical GYM-A product ions *m/z* 392 and 446 were absent in the MS/MS spectra of this new compound (Fig. 4), indicating a possible structural variation between C1-4 (Fig. 1). This compound may have been a product of metabolism in the mussels, or may be an artefact from sample preparation or processing. LC-HRMS experiments did not reveal the presence of any other significant GYM related compounds in the microalgae (data not shown). The formation of this compound will be considered in further studies.

All mussels were alive during the feeding experiment. Similar to the response of scallops (*Chlamys farreri*) and mussels (*M. galloprovincialis*) exposed to azaspiracids in our previous study (Ji et al., 2018), no significant difference of ROS levels and antioxidant enzymes were found in mussels exposed to the GYM-producing microalgae. This indicates there is no measurable impact of GYM accumulation on the antioxidant enzyme system of mussels based on this study.

5. Conclusions

GYM-A and ester metabolites were confirmed in the clam (*Antigona lamellaris*)

and pen shell (*Atrina pectinata*) samples collected from the South China Sea and in experimental mussels (*M. galloprovincialis*) fed with *K. selliformis* under laboratory conditions. While GYM-A was detected in the oyster (*Crassostrea* sp.) and gastropod (*B. zonalis*) samples, no GYM-A esters were detected. Various acyl esters were detected by SRM analysis and the predominant esters were confirmed by LC-HRMS. Esterification and ester profiles appear to be species-specific for mollusks. In mussels, ester metabolites constituted the majority of total GYMs (>90% for laboratory fed samples), with the 18:0-GYM-A and 20:1-GYM-A formed as the most abundant ester derivatives in the field samples analyzed. *K. selliformis* feeding demonstrated no toxic effects on mussels based on responses of antioxidant enzymes. A novel GYM analogue with the same accurate mass as GYM-A, which also formed fatty acid ester, was found in the experimental mussels. Risks posed by GYMs and their acyl esters should be assessed in future toxicological studies and risk assessments.

Conflict of interest

All authors declare that there are no conflicts of interest.

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Tables

Table 1. Concentrations of GYM-A (nmol/kg) and molar percentages of predominant GYM-A esters (to the sum esters in each sample) in the field samples collected from Chinese coasts.

Sample	GYM-A	percentage of predominant esters (%)						ER*
	(nmol/kg)	C16:0	C18:0	C18:1	C20:1	C20:2	C22:2	（%）
clam (*Antigono lamellaris*)	382	4.9	50	4.3	23	5.9	4.2	58
pen shell (*Atrina pectinata*)	99	11	20	7.6	23	7.1	18	87
oyster (*Crassostrea* sp.)	42	-	-	-	-	-	-	-
gastropod (*Batillaria zonalis*)	55	-	-	-	-	-	-	-

Esters of GYM-A were estimated using GYM-A as calibration standards.

* ER means the esterification rate of GYM-A roughly estimated by GYM-A reference material; ER(%) = esters/(esters + GYM-A) ×100.

- means not detected in samples.

Legends of Figures

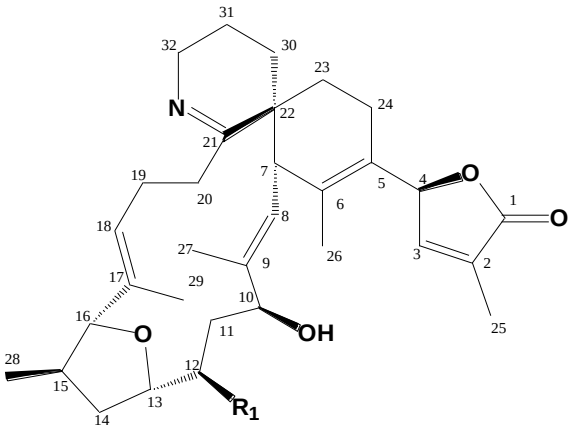
Figure 1. Chemical structures of select gymnodimine analogs.

Figure 2. LC-MS/MS chromatograms for fatty acid esters of GYM-A in digestive gland tissue of mussels (*M. galloprovincialis*) fed with *K. selliformis* after 96 h.

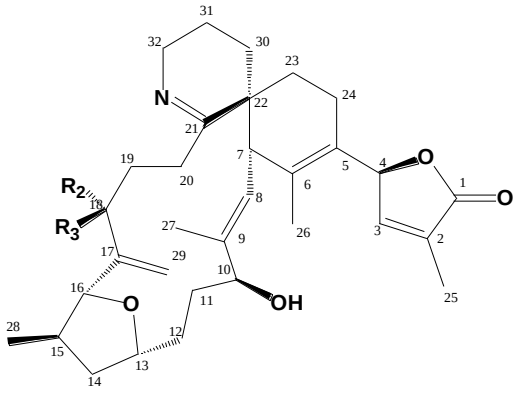
Figure 3. LC-HRMS full scan chromatograms for GYM-A (left) and 16:0 GYM-A (right) using a 1.5 ppm mass tolerance, normalized to 100% intensity for *m/z* 508.3421 in each sample. Samples shown are NRC-CRM gymnodimine (A), NRC positive control tissue (B), microalgal feed culture (*K. selliformis*) (C), and muscle tissues (D) and digestive gland tissues (E) of mussels (*M. galloprovincialis*) after 96 h feeding time point of this study.

Figure 4. High resolution MS/MS spectra for GYM-A in certified reference material, and GYM-A and a second peak bearing GYM-A mass in mussel digestive gland (MDG) sample (LEFT). Spectra for 16:0 GYM-A acyl ester in positive control tissue, and the two 16:0 acyl esters derived from the GYM-A and the second GYM-like compound in MDG sample (RIGHT).

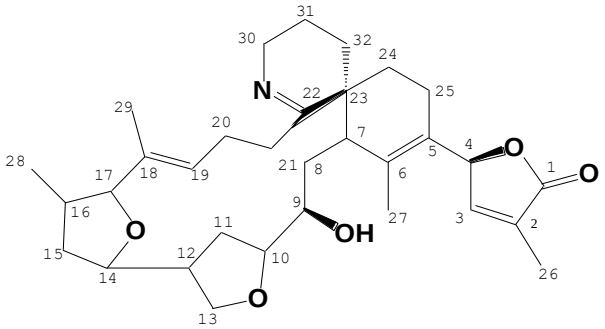
Figure 5. Fatty acid esters of GYM-A in muscle (A) and digestive gland (B) tissues of mussels (*M. galloprovincialis*) collected at each sampling time point. The bars show the relative distribution of GYM-A including free form, selected individual ester and other esters (total free form and esters of GYM-A =100). The lines show the sum of the relative amounts of esters to the total toxin burden in % (filled triangle), the level of esters (filled squares) and esters + toxin (filled circles), both in nmol kg^{-1} tissue (right hand side axis).



	Toxin	$[M+H]^+$	R_1
1	Gymnodimine (GYM-A)	508	H
2	12-Methylgymnodimine	524	CH_3



	Toxin	$[M+H]^+$	R_2	R_3
3	Gymnodimine B	524	H	OH
4	Gymnodimine C	524	OH	H



	Toxin	$[M+H]^+$
5	Gymnodimine D	524

Fig. 1

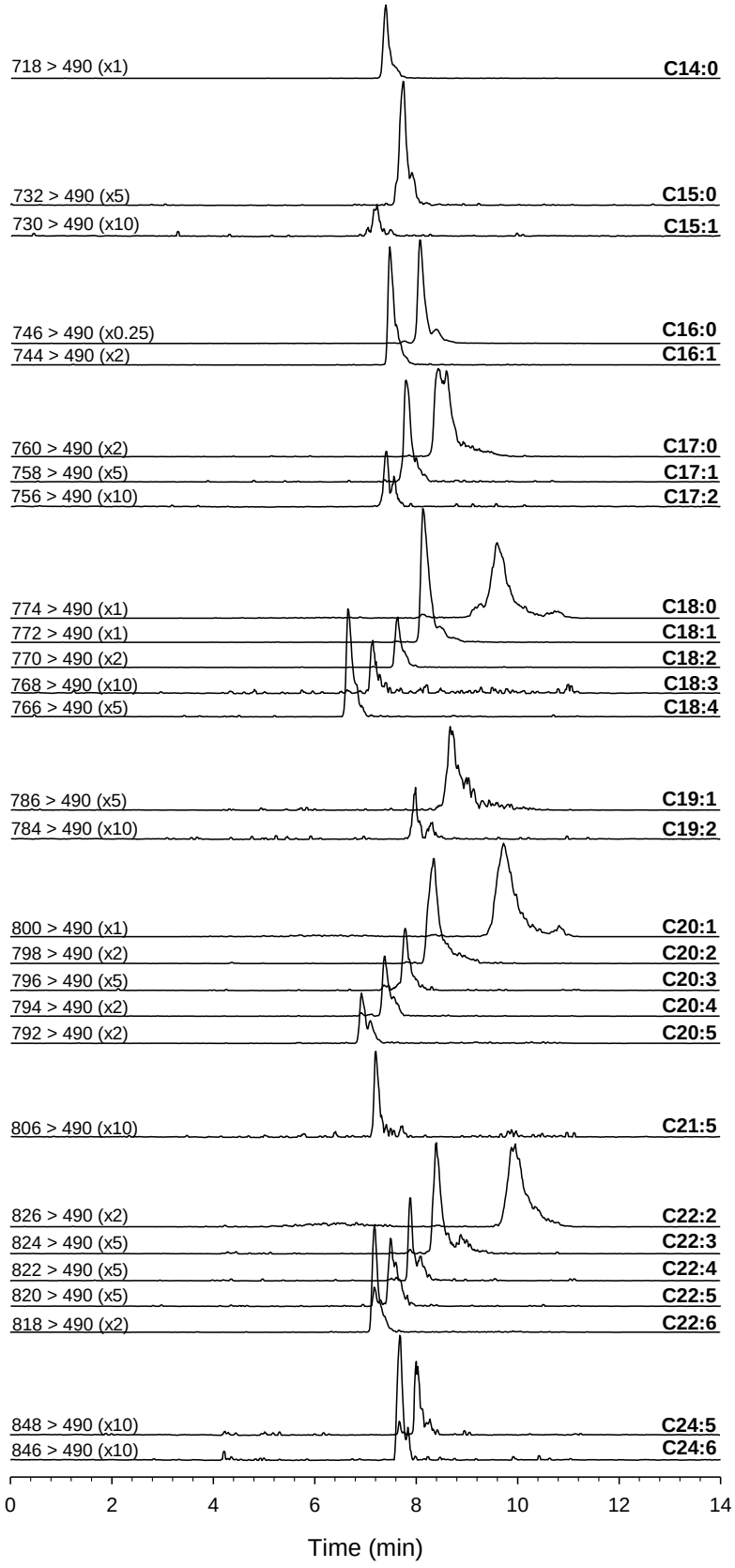

718 > 490 (x1)
C14:0
732 > 490 (x5)
C15:0
730 > 490 (x10)
C15:1
746 > 490 (x0.25)
C16:0
744 > 490 (x2)
C16:1
760 > 490 (x2)
C17:0
758 > 490 (x5)
C17:1
756 > 490 (x10)
C17:2
774 > 490 (x1)
C18:0
772 > 490 (x1)
C18:1
770 > 490 (x2)
C18:2
768 > 490 (x10)
C18:3
766 > 490 (x5)
C18:4
786 > 490 (x5)
C19:1
784 > 490 (x10)
C19:2
800 > 490 (x1)
C20:1
798 > 490 (x2)
C20:2
796 > 490 (x5)
C20:3
794 > 490 (x2)
C20:4
792 > 490 (x2)
C20:5
806 > 490 (x10)
C21:5
826 > 490 (x2)
C22:2
824 > 490 (x5)
C22:3
822 > 490 (x5)
C22:4
820 > 490 (x5)
C22:5
818 > 490 (x2)
C22:6
848 > 490 (x10)
C24:5
846 > 490 (x10)
C24:6
0
2
4
6
8
10
12
14
Time (min)

Fig.2

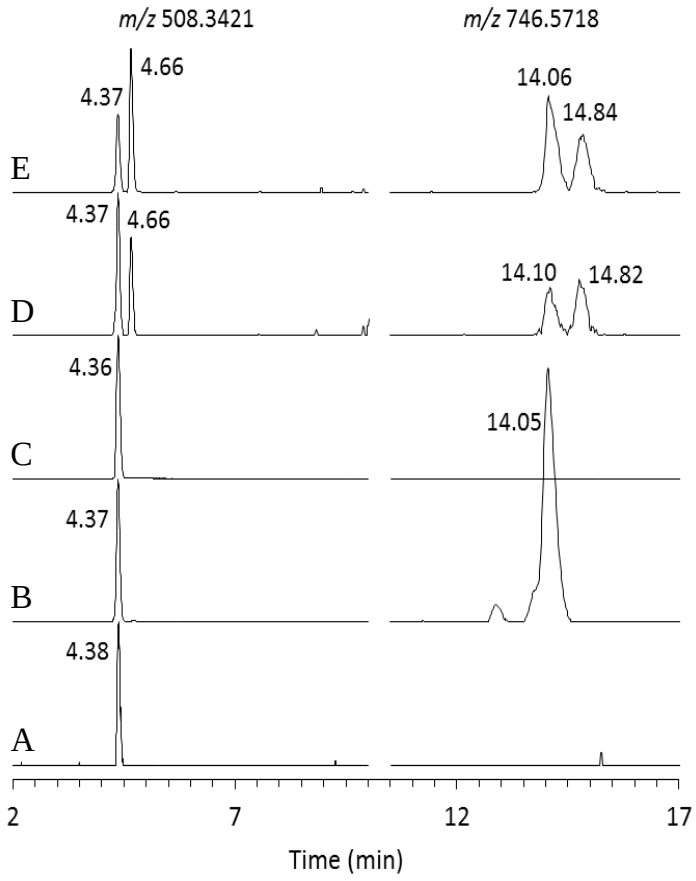

m/z 508.3421
m/z 746.5718
4.66
4.37
14.06
14.84
E
4.37
4.66
14.10
14.82
D
4.36
14.05
C
4.37
B
4.38
A
2
7
12
17
Time (min)

Fig. 3

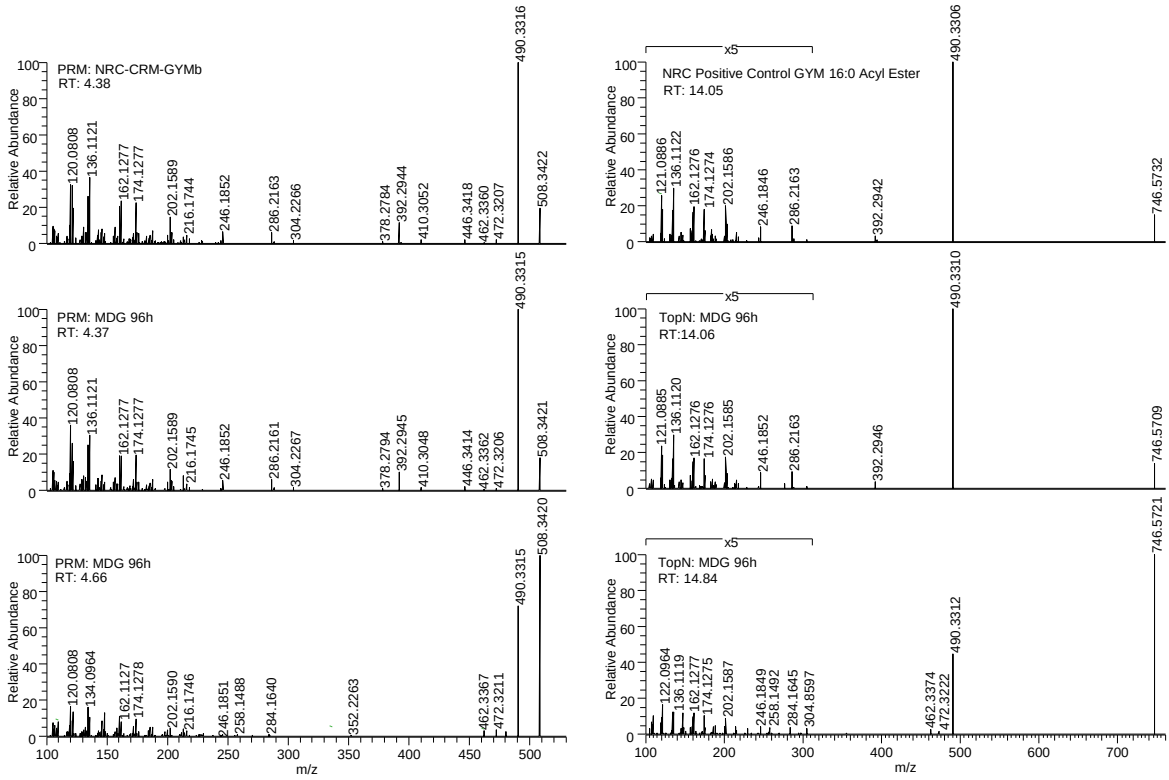

PRM: NRC-CRM-GYMb
RT: 4.38
PRM: MDG 96h
RT: 4.37
PRM: MDG 96h
RT: 4.66
NRC Positive Control GYM 16:0 Acyl Ester
RT: 14.05
TopN: MDG 96h
RT:14.06
TopN: MDG 96h
RT: 14.84
Relative Abundance
m/z
x5

Fig. 4

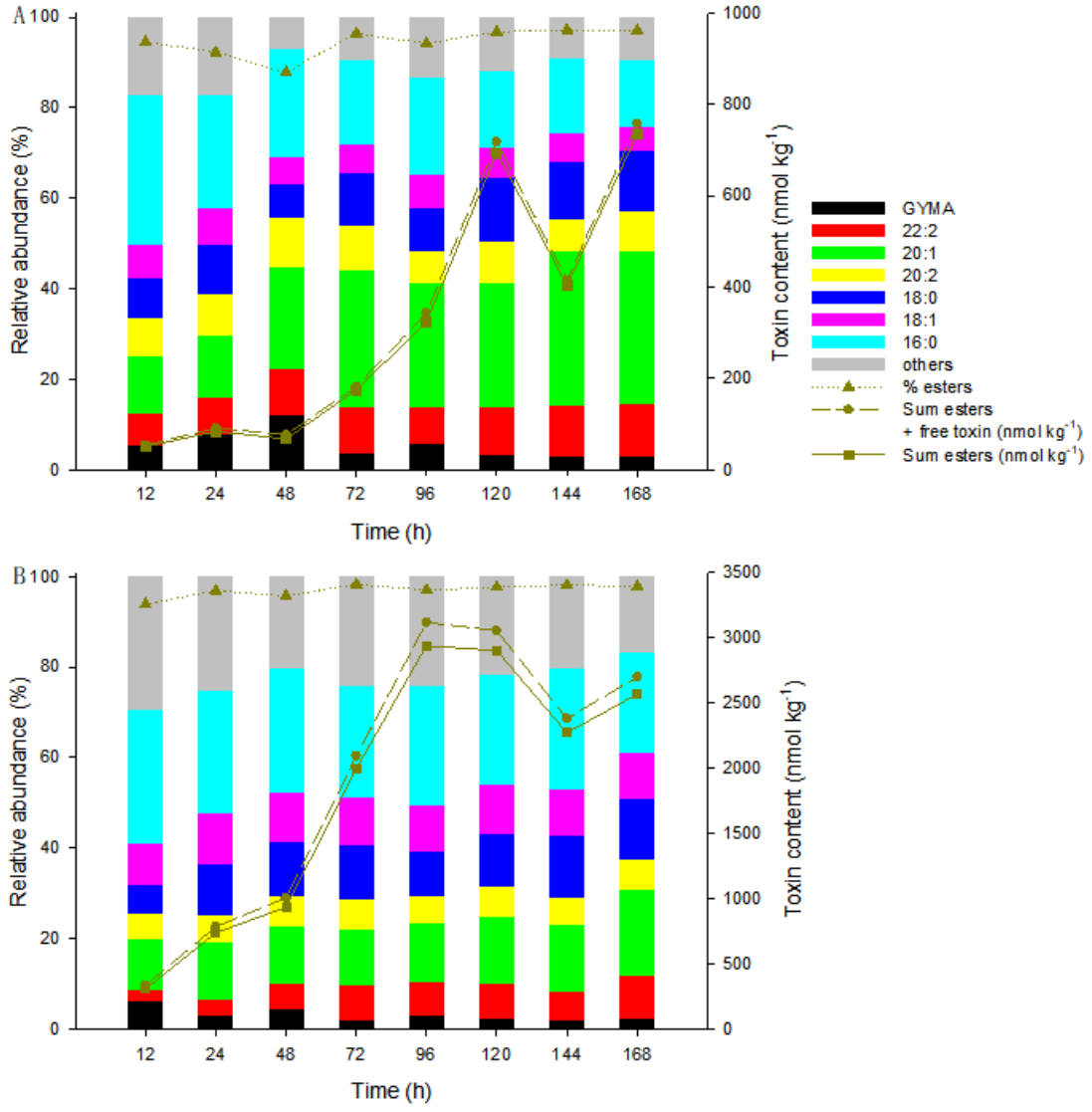

A
B
Relative abundance (%)
Toxin content (nmol kg-1)
Time (h)
12
24
48
72
96
120
144
168
GYMA
22:2
20:1
20:2
18:0
18:1
16:0
others
% esters
Sum esters
+ free toxin (nmol kg-1)
Sum esters (nmol kg-1)

Fig. 5