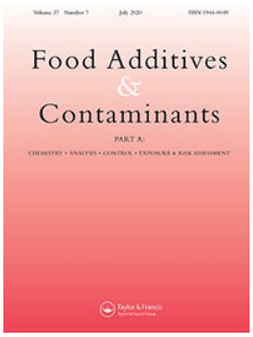

Food Additives & Contaminants: Part A

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/tfac20

In vitro biotransformation of OA-group and PTX-group toxins in visceral and non-visceral tissues of *Mytilus chilensis* and *Ameghinomya antiqua*

Javiera Oyaneder-Terrazas , Cassandra Polanco , Diego Figueroa , Andres Barriga & Carlos García

To cite this article: Javiera Oyaneder-Terrazas , Cassandra Polanco , Diego Figueroa , Andres Barriga & Carlos García (2020) *In vitro* biotransformation of OA-group and PTX-group toxins in visceral and non-visceral tissues of *Mytilus chilensis* and *Ameghinomya antiqua* , Food Additives & Contaminants: Part A, 37:7, 1216-1228, DOI: 10.1080/19440049.2020.1750710

To link to this article: https://doi.org/10.1080/19440049.2020.1750710

Published online: 09 Jun 2020.

FOOD ADDITIVES & CONTAMINANTS: PART A
2020, VOL. 37, NO. 7, 1216–1228
https://doi.org/10.1080/19440049.2020.1750710

Taylor & Francis
Taylor & Francis Group

In vitro biotransformation of OA-group and PTX-group toxins in visceral and non-visceral tissues of *Mytilus chilensis* and *Ameghinomya antiqua*

Javiera Oyaneder-Terrazas[a,b], Cassandra Polanco[a,b], Diego Figueroa[a], Andres Barriga[c] and Carlos García [a]

[a]Laboratory of Marine Toxins, Physiology and Biophysics Programme, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile; [b]Faculty of Technology, Universidad de Santiago, Santiago, Chile; [c]CEPEDEQ, Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, Santiago, Chile

ABSTRACT

Lipophilic marine toxins (LMTs) are made up of multiple groups of toxic analogues, which are characterised by different levels of cellular and toxic action. The most prevalent groups in the southern Pacific zone are: a) okadaic acid group (OA-group) which consists of okadaic acid (OA) and dinophysistoxin-1 (DTX-1); and, b) pectenotoxin-2 (PTX2) group which consists of pectenotoxin-2 (PTX-2). The main objective of our study was to examine *in vitro* biotransformation of OA-group and PTX-group in the tissues of two endemic species of bivalves from southern Chile; blue mussels (*Mytilus chilensis*) and clams (*Ameghinomya antiqua*). The biotransformation processes of both groups were only detected in the digestive glands of both species using LC-MS/MS. The most frequently detected analogues were acyl derivatives (≈2.0 ± 0.1 µg ml^{-1}) for OA-group and PTX-2SA (≈1.4 ± 0.1 µg ml^{-1}) for PTX-group, with a higher percentage of biotransformation for OA-group ($p < .001$). In addition, simultaneous incubations of the different analogues (OA/PTX-2; DTX-1/PTX-2 and OA/DTX-1/PTX-2) did not show any interaction between the biotransformation processes. These results show that the toxicological variability of endemic species leads to biotransformation of the profile of toxins, so that these new analogues may affect people's health.

ARTICLE HISTORY
Received 31 January 2020
Accepted 24 March 2020

KEYWORDS
Okadaic acid-group; acyl derivatives; pectenotoxin-group; pectenotoxin-2sa; transformation; Mytilus chilensis; Ameghinomya antiqua

Introduction

Marine biotoxins are categorised into different groups, which are characterised by different chemical structures. Okadaic acid group (OA-group) and pectenotoxin-group (PTX-group) are two of the groups that make up the so-called lipophilic marine toxins (LMTs) (Botana et al. 2017). Both groups have been identified worldwide in dinoflagellates of the genus Dinophysis and Prorocentrum (Wang et al. 2015; Park et al. 2019; Wu et al. 2019). The most prevalent analogues making up the OA-group are okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2) (Contreras and García 2019). This group is characterised by producing diarrhoetic shellfish poisoning (DSP), when shellfish contaminated with OA-group toxins are consumed (Swan et al. 2018). DSP is characterised by producing symptoms such as nausea, vomiting and diarrhoea, which disappear completely within a few days (3–5 days) in an otherwise healthy person (Blanco 2018). This group has been classified as a potent tumour promoter (Fujiki et al. 2018).

Pectenotoxin group (PTX-group) is composed of polyethers with lactone groups, among which pectenotoxin-2 (PTX-2) is noted (EFSA 2009). The toxic action of the PTX-2 analogue has been associated with the disruption of the actin cytoskeleton, which has also been determined as a hepatotoxic analogue using intraperitoneal administration (219 µg kg^{-1}). However, toxic symptoms in humans have not been documented from this group of toxins (Uchida et al. 2018; Alarcan et al. 2019).

Both groups of toxins have been identified worldwide in countries such as Spain, Portugal, Argentina, Australia, Chile and Japan (Blanco et al. 2018; García et al. 2018). The regulatory limit for DSP toxins (OA, DTXs and PTXs combined) is 160 µg kg^{-1} expressed as OA equivalents using LC-MS/MS to identify and classify toxic analogues (EFSA 2008; FAO/WHO 2016).

CONTACT Carlos García cgarcia@med.uchile.cl Laboratory of Marine Toxins Faculty of Medicine Universidad de Chile, Casilla 8380000, Correo 7, Santiago, Chile

Once the toxins are assimilated by shellfish, the toxic analogues produced by dinoflagellates which are chemically modified through oxidation, reduction and acylation reactions (Rossignoli et al. 2011). Thus, analogues associated with PTX-group such as pectenotoxin-1 (PTX-1), pectenotoxin-6 (PTX-6) and pectenotoxin-2sa (PTX-2sa) have been identified, while for the OA-group, the outcome of acylation reactions with fatty acids of varying lengths (C-14 – C22) may produce new toxic analogues, such as dinophysistoxin-3 (DTX-3) (Contreras and García 2019; Wu et al. 2019).

In Chile, in 1990, the OA-group was first detected using mouse bioassays, and in 2009 PTX-group was identified using LC-MS/MS. Both toxin groups show a variable prevalence along the coast of Chile (Krock et al. 2009; Díaz et al. 2016; García et al. 2018). The current health regulation in Chile establishes that toxins associated with DSP must be detected using the mouse bioassay (MBA) (ISP 2012; García et al. 2018). In recent years, Chile has been exposed to environmental variables that could relate the processes of algal bloom expansion associated with OA-group and PTX-group to the Regions of Los Lagos and Biobío. This has led the health authorities to develop a Health Programme for Bivalve Molluscs (Spanish acronym, PSMB) for products to be exported, and a health control programme for products to be domestically consumed, which is performed using the mouse bioassay (MBA) (Sernapesca 2014; Contreras and García 2019).

The chronic effects on people have not been unequivocally determined in either group, but poisoning symptoms associated with a tumour promoter such as OA-group may be a risk factor in populations with high incidence of digestive diseases, which could explain the high rates of gastric cancer in southern Chile (García et al. 2016; Fujiki et al. 2018; Jiménez-Cárcamo et al. 2020).

This study shows the biotransformation kinetics of OA-group and PTX-group toxins in visceral (hepatopancreas) and non-visceral (mantle and foot) tissues of two endemic species of high commercial value in south of Chile, *Mytilus chilensis* (hard rock) and *Ameghinomya antiqua* (sand bottom). Therefore, the dynamics of biotransformation that these species can exert on the toxic analogues associated with toxins produced by the most prevalent dinoflagellates in Chile can be determined.

Materials and methods

Materials

Certified reference materials containing DTX-1 (CRM-DTX-1), OA (CRM-OA-c) and pectenotoxins (CRM-PTX-2) were obtained from the National Research Council of Canada. Extraction and incubation reagents were purchased from Sigma-Aldrich. LC-grade solvents were purchased from Merck (MERCK, Darmstadt, Germany). Ultrapure water was obtained by elution through an ion exchange cartridge (NANOpure® Infinity, Barnstead, Thermo Fisher Scientific Inc.). Live non-toxic bivalves *Mytilus chilensis* and *Ameghinomya antiqua* were collected from the Islas Huichas, Region of Aysén (Regional Ministerial Secretary Health Authorisation No. 916 and 1238) and transported to the laboratory at 4°C to then be processed, according to the safety and bioethics protocol CBA 0862 FMUCH by the Universidad de Chile.

Preparation of the microsomal fractions from bivalves

The fresh specimen of *Mytilus chilensis* and *Ameghinomya antiqua* were dissected into four tissues of adductor muscle, foot, digestive gland, and mantle. Then, 30 g were homogenised with 30 ml of a buffer containing 50 mM Hepes-KOH (pH 7.6), 5.0 mM $MgCl_2$, 10 mM KCl, 5.0 mM sodium EDTA, 5.0 mM EGTA and protease inhibitor cocktail at 4°C. The crude suspension was successively centrifuged at 11,000 rpm for 15 min and the resulting supernatant was centrifuged at 40,000 rpm for 1 h at 4°C. The final centrifugation of microsomal fractions was resuspended in buffer 50 mM (Hepes-KOH, pH 7.6), 20 mM NaCl, 5.0 mM $MgCl_2$, 10% glycerol (v/v) and then stored at −80 °C until use. The protein concentration of these fractions were determined with protein assay (Bradford) (García et al. 2010; Konoki et al. 2013).

Vitro transformation

4.0 µg of OA/DTX-1 or 2.1 µg of PTX-2, 1.0 mM $MgCl_2$ and 10 mM of ATP were incubated with 1.0 mg of the microsomal fraction obtained from the shellfish tissues. ATP was used as a co-factor

for the esterification reaction between OA-group toxins and fatty acids (Rossignoli et al. 2011; Konoki et al. 2013). Each evaluation was pre-incubated for 3 minutes at 37° C. Then, the reaction was started with the addition of 5.5 µM NADPH, which provides the necessary electrons for cytochrome activation and catalyses the oxidation reactions (García et al. 2010). The reaction of each incubation was stopped with the addition of 10 µl of 90% methanol (v/v). Subsequently, each tube was centrifuged at 11,000 rpm for 15 minutes, and then analysed using LC-MS/MS (García et al. 2010; Contreras and García 2019).

LC-MS/MS analyses of lipophilic toxins

The LC system for measurements of OA- and PTX-group toxins was performed with an Agilent 1200 LC model coupled ion trap mass spectrometer (Esquire 4000 ESI-IT, Bruker Daltonik GmbH). The HPLC column was a Purospher STAR C-18, 50 × 2.1 mm, 3 µm particle sizes (Hibar HR Purospher STAR, Merck KGaA, Germany). The eluent flow consisted of an isocratic phase of 40% v/v mobile phase A (100% water with 2 mM ammonium formate/50 mM formic acid) and 60% v/v mobile phase B (95% acetonitrile with 2 mM ammonium formate/50 mM formic acid). Electron ionisation mass spectrometry was performed at 4000 V, assisted by nitrogen as the nebulisation gas, at a temperature of 300°C, 30 psi pressure and a 10 L min^{-1} flux. Chromatograms and mass spectra were obtained according to the following protocol: for OA, a time of 6 min; MRM, m/z 803 with the transitions: m/z 803→255, m/z 803→563 and m/z 803→741; DTX-1, a time of 15 min; 817 negative polarity with the transitions: m/z 817→255 and m/z 817→563; and for PTX-2, a time of 7–9.3 min; MRM, m/z 877 positive polarity with the transition: m/z 877→824 (EURLMB 2015; Contreras and García 2019). The LOD and LOQ for each analogue were: OA, 0.9 and 3.8; DTX-1, 0.8 and 3.5; PTX-2, 0.15 and 0.7 micrograms per kilogram of whole tissue (Zamorano et al. 2013). All the discarded material after the toxin extraction was properly stored, sealed, labelled in safety bags and picked up by the Biosafety Department of the Faculty of Medicine of the University of Chile.

Statistical analysis

Results were expressed as mean ± SEM (n = 5). Regression analyses were performed to obtain standard curves. Differences among groups were analysed using the one-way analysis of variance (ANOVA) test followed by the Bonferroni test. A significance level of $p < 0.05$ was used in all the assessments.

Results and discussion

Determination of the toxin percentage of recovery

Concentrations of 4.0 µg ml^{-1} of OA/DTX-1 and 2.1 µg ml^{-1} PTX-2 were incubated in each of the visceral (digestive glands) and non-visceral (foot and mantle) tissues of *Mytilus chilensis* and *Ameghinomya antiqua* to determine the percentage recovery. In *Mytilus chilensis*, the recovery averaged ≈ 94.6% in all tissues for OA and DTX-1 and ≈ 95.3% for PTX-2. Furthermore, in *Ameghinomya antiqua*, the recovery was ≈ 96.1 % for OA, DTX-1 and ≈ 98.1 % for PTX-2 (Table 1).

Variability of OA-group incubated in Mytilus chilensis and Ameghinomya antiqua tissues

Two species of bivalves, *Mytilus chilensis* (rock strata) and *Ameghinomya antiqua* (sand bottom), were used to determine the biotransformation of

Table 1. Percentage recovery of Okadaic acid (OA), dinophysistoxin-1 (DTX-1) and Pectenotoxin-2 (PTX-2) from matrices of *Mytilus chilensis* and *Ameghinomya antiqua* (n = 5).

			Mytilus chilensis			*Ameghinomya antiqua*	
Toxins			Certified values	Measured values	Recovery (%)	Measured values	Recovery (%)
Okadaic acid	OA	µg ml^{-1}	4.00 ± 0.50	3.83 ± 0.41	95.7	3.87 ± 0.50	96.8
Dinophysistoxin-1	DTX-1	µg ml^{-1}	4.00 ± 0.10	3.74 ± 0.21	93.5	3.81 ± 0.12	95.3
Pectenotoxin-2	PTX-2	µg ml^{-1}	2.11 ± 0.30	2.01 ± 0.11	95.3	2.07 ± 0.30	98.1

toxins of the OA-group (OA and DTX-1) in visceral (digestive glands) and non-visceral (foot and mantle) tissues during 180 min of incubation. An initial concentration of 4.0 μg ml^{-1} of OA and DTX-1 was used. The reactions were evaluated chromatographically for 180 min as well as the peak areas of OA, DTX-1, and products derived from the biotransformation (acyl derivatives) (Figure 1(a,b)).

The data obtained in the incubations of OA and DTX-1 in visceral and non-visceral tissues of *Mytilus chilensis*, showed that the biotransformation of incubated analogues (OA and DTX-1) did not take place in non-visceral tissues (Figure 2(a,b)), maintaining the percentage of recovery set up for each analogue (≈96%). However, the incubation performed on visceral tissues showed that OA and DTX-1 initiated an alteration in the toxic profile between 90 and 150 min for OA (≈ 25%, $p < 0.05$) and for DTX-1 (≈ 37%) (Figure 2(a,b)). Therefore, a more significant change towards DTX-1 ($p < 0.001$) was observed (Figure 2(b)).

In the time points of decreased concentration of the initial toxins (OA/DTX-1), new analogues (acyl derivatives) associated with biotransformation were identified (Figure 2(c,d)). The most frequently detected acyl derivative in the incubation was 7-O-palmytoil-OA, with a mass spectrum of m/z 1041, and ionic fragments in mass spectra such as m/z 254.9, m/z 430.9 and m/z 797.3 (Figure 3(d)), and the main biotransformed analogue was OA ($p < 0.001$) (Figure 2(d)).

In addition, analogues associated with biotransformation processes were also detected only in the digestive glands of *Ameghinomya antiqua* (Figure 4(a,b)). The biotransformation process did not show any preference for OA or DTX-1 in the tissue. The biotransformed analyte was detected between 90 and 180 min of the incubation and its representation was ≈ 35% in the toxin profile 1 ($p < 0.001$) (Figure 4(c,d)).

In Figure 3(c-d), spectra of MS/MS (negative polarity) are shown to identify acyl derivatives of

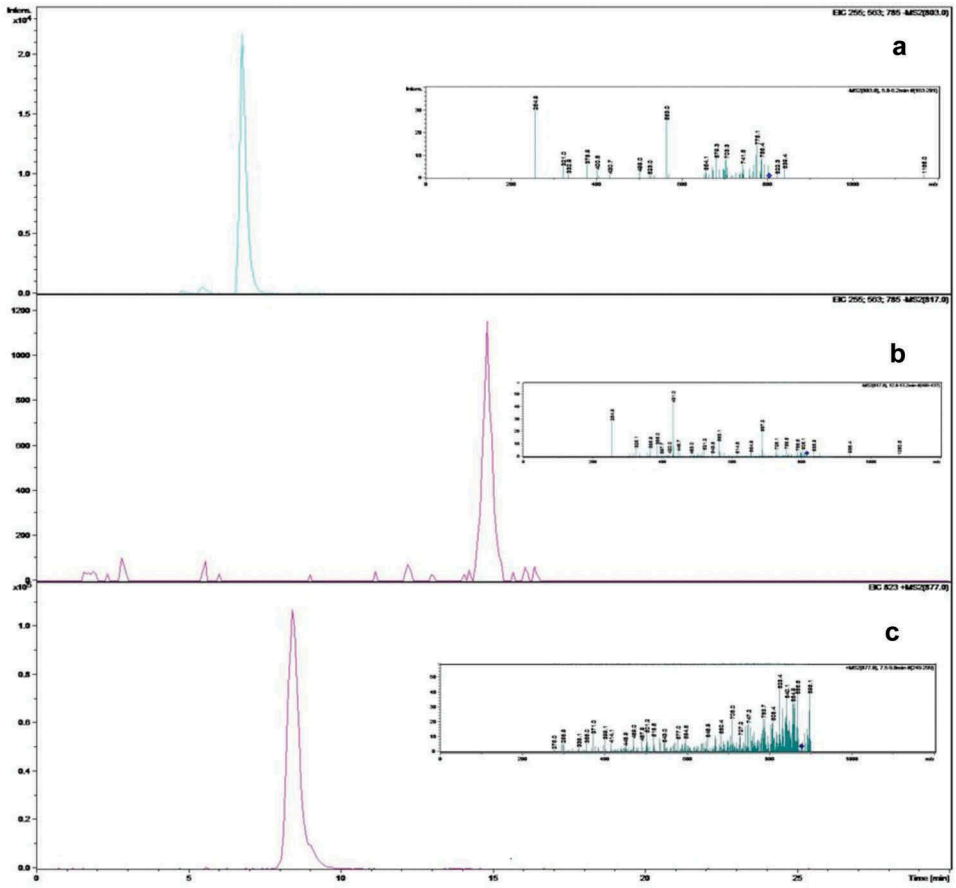

Figure 1. Results of LC-MS/MS analysis. (a) Chromatogram of okadaic acid (OA), *inset*: Mass spectrum and main fragments under negative mode; (b) dinophysistoxin-1(DTX-1), *inset*: Mass spectrum and main fragments under negative mode; and (c) pectenotoxin-2 (PTX-2), *inset*: Mass spectrum and main fragments under positive mode.

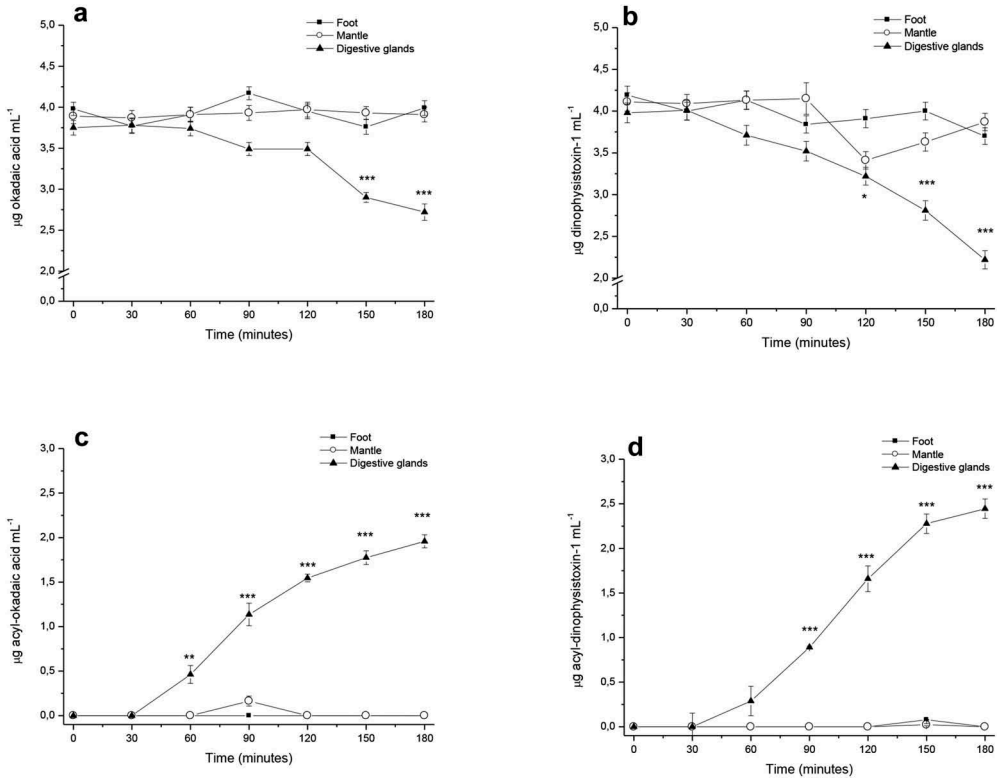

Figure 2. Variability of concentrations of (a) Okadaic acid (OA) and (b) dinophysistoxin-1 (DTX-1) and time course of Okadaic acid (c) and dinophysistoxin-1 (d) toxin metabolism during a 180 min reaction in tissues of *Mytilus chilensis* in foot (triangles), mantle (circles) and digestive glands (diamonds). The graph shows average toxin concentrations ± standard deviations (n = 5, SEM ± SD).

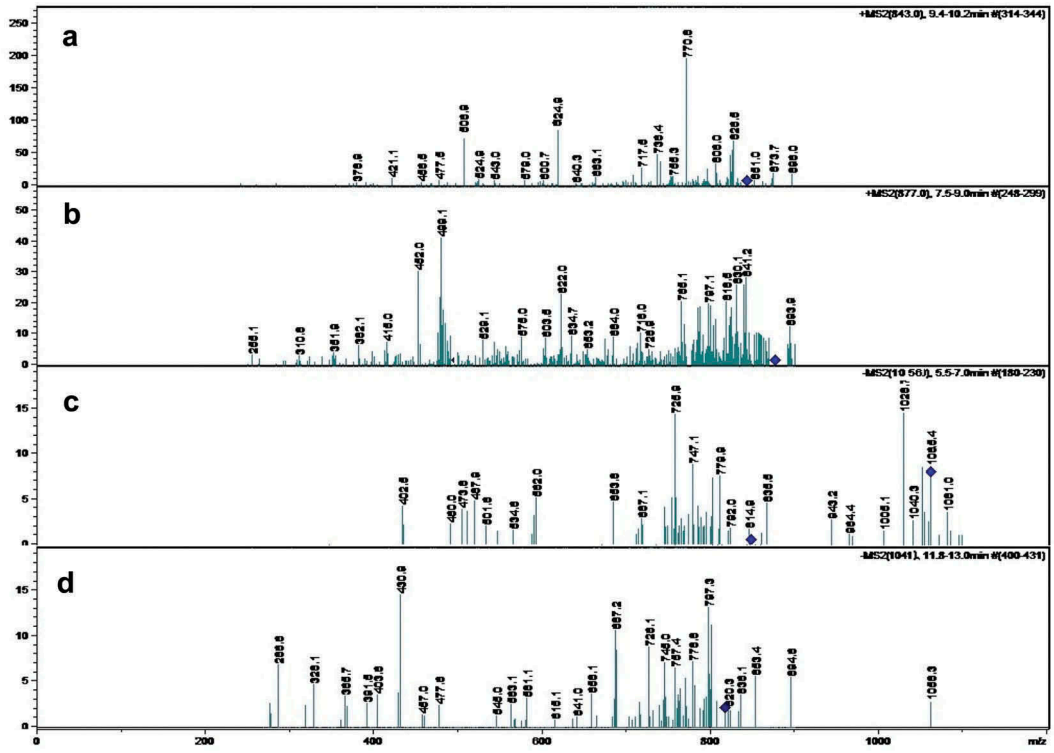

Figure 3. Mass spectrum results from full-scan monitoring of (a) 7-O-palmitoyl-dinophysistoxin-1 (7-O-acyl-DTX-1), (b) pectenotoxin-2 (PTX-2) and (c) pectenotoxin-2-secoacid (PTX-2sa).

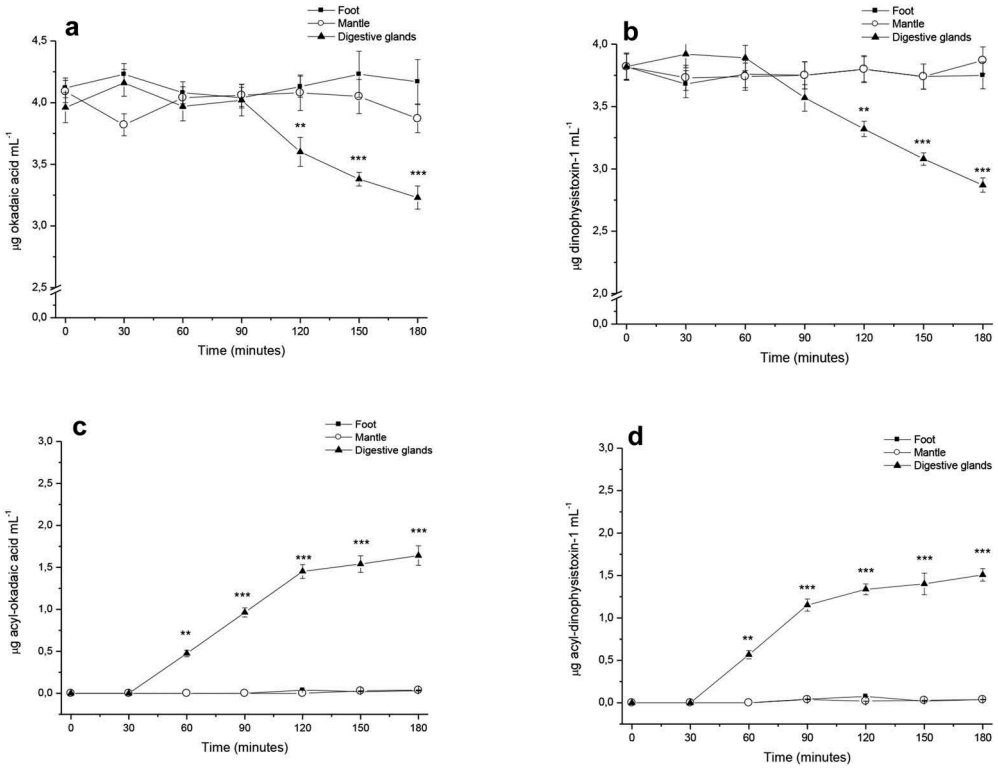

Figure 4. Variability of concentrations of (a) Okadaic acid (OA) and (b) dinophysistoxin-1 (DTX-1) and time course of Okadaic acid (c) and dinophysistoxin-1 (d) toxin metabolism during a 180 min reaction in tissues of *Ameghinomya antiqua* in foot (triangles), mantle (circles) and digestive glands (diamonds). The graph shows average toxin concentrations ± standard deviations (n = 5, SEM ± SD).

OA and DTX-1 obtained during *in vitro* incubation in the tissues of *Mytilus chilensis* and *Ameghinomya antiqua*. The analysis of analogues has identified a characteristic acylation in R-C_7 -O-acyl with fatty acids coming from the molluscs. A peak of greater intensity with a mass spectrum of m/z 1056, corresponding to 7-O-palmitoyl-DTX-1 (Figure 3(c)). The detection of ionic fragments in mass spectra such as m/z 402.0, m/z 725.0 and m/z 799.0 has confirmed the identification of DTX-1 (Figure 3(c)). Each fragment detected by LC-MS/MS was confirmed with the analysis of the standard solutions of OA and DTX-1.

OA-group is the most prevalent group of toxins in the south of Chile and blooms associated with *Dinophysis acuta* are detected in the austral fjords every year, reaching cell densities between 20 and ≈200 cell ml^{-1} (García et al. 2018). Contreras and García (2019) determined that the most prevalent analogues produced by *Dinophysis acuta* are OA and DTX-1 in a 3:7 ratio with a content toxin ≈18 pg OA equivalent $cell^{-1}$.

García et al. (2005), identified a shellfish poisoning incident associated with the OA-group due to consumption of *Mytilus chilensis* for the first time in Chile. In that event, the analogue DTX-3 derived from DTX-1 was detected as the responsible analogue for the poisoning. The identification of DTX-3 determined that biotransformation processes of analogues of the OA-group were involved and that they also produced shellfish poisoning symptoms in longer times (> 10 h) if compared to the more characteristic analogues of the OA-group toxins (OA and DTX-1), so shellfish poisoning symptoms are first associated with toxins produced by enteropathogens (García et al. 2005). That event evidenced that the determination of toxicities using the MBA is a risk to public health, since the analogues derived from biotransformation processes are not detected during the test procedure (>24 h) (Kameneva et al. 2015). Thus, when biotransformed analogues are consumed in acute doses, they can be hydrolysed into the initial toxic forms of OA-group toxins (DTX-3 → DTX-1) during the digestion process (EFSA 2008; Blanco 2018).

OA-group is characterised by causing specific levels of inhibition on PP2A, which is directly correlated with the toxic effects it can produce in people. Hence, the importance of determining toxic profiles including those produced by biotransformation processes (acyl-derivatives) in different shellfish species (FAO/WHO 2016).

Variability of PTX-group incubated in tissues of Mytilus chilensis and Ameghinomya antiqua

The incubation of PTX-2 in visceral and non-visceral tissues of *Mytilus chilensis* and *Ameghinomya antiqua* showed variability in their concentrations and in the toxic profile only in visceral tissues (digestive glands) from 120 min after the start of the incubation ($p < 0.01$), reaching a maximum point at 180 min ($p < 0.001$) (Figure 5).

The biotransformation process was more specific in *Mytilus chilensis* with a maximum point of 38% with respect to the control sample ($p < 0.001$), if compared to *Ameghinomya antiqua* (≈2.1 µg ml^{-1}, 180 min, $p < 0.01$). The only analogue detected as a biotransformation product via enzymatic catalysis was pectenotoxin-2 seco-acid (PTX-2sa) (Figure 3(b)) (Suzuki et al. 2001a, 2001b).

Spectra of LC-MS/MS in negative polarity for the detection of pectenotoxin-2-seco acid (PTX-2sa) formed during the incubation of toxins in the tissues of *Mytilus chilensis* and *Ameghinomya antiqua* are shown. The mass spectrum corresponding to the PTX-2sa + NH_4^+ ion is shown in Figure 3(b). The fragmentation pattern observed is similar to that of PTX-2, and it corresponded to the ions detected in the elimination of water molecules, such as m/z 841 [MNH_4 + -NH_4OH-H_2O] $^+$ and m/z 823 [MNH4 + -NH_4 OH-2H_2O] + and m/z 805 [MNH_4 + -NH_4 OH-3H_2O] + as well as to secondary ions at m/z 551, 457 and 439 corresponding to PTX-2sa (Figure 3(a,b)).

In Chile, PTX-2 is the most prevalent toxin associated with *Dinophysis acuminata* since the occurrence of HABs (Blanco et al. 2007a; Alves-de-Souza et al. 2014). The toxic variability of this analogue takes place through an oxidative reaction producing PTX-2sa, and this process may involve an interconversion to a thermodynamically more stable analogue, such as 7-epi-PTX-2sa (Vale and Sampayo 2002; García et al. 2016; Mackenzie 2019).

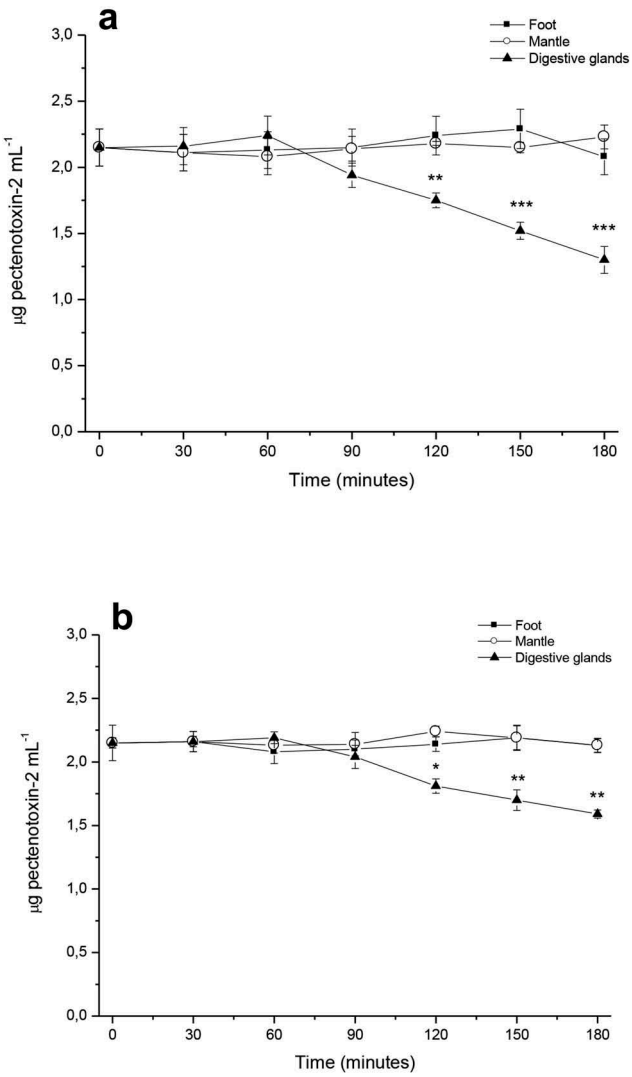

Figure 5. Variability of concentrations of pectenotoxin-2 (PTX-2) in *Mytilus chilensis* (a) and *Ameghinomya antiqua* (b) during a 180 min reaction in tissues of foot (triangles), mantle (circles) and digestive glands (diamonds) and control tissue. The graph shows average toxin concentrations ± standard deviations (n = 5, SEM ± SD).

The low levels of biotransformation detected (≤LoD) in the non-visceral tissues of both species demonstrates that the identification of PTX-2sa is more closely associated with stages of toxin distribution, this is to say, the stages following the assimilation process of dinoflagellates involved in HABs. In addition, the difference in the level of biotransformation shown in *Ameghinomya antiqua* is based on the habitat of the species; as it is a soft bottom dwelling species (not a stratified

bottom dwelling species), it is capable of filtrating and accumulating dinoflagellates and toxins from detritus, faeces and toxins from other species, which may imply a more efficient biotransformation capacity of toxins (Farrell et al. 2012; García et al. 2012). Likewise, as *Mytilus chilensis* shows greater interaction in the stratum of the water column, it is directly involved in the dynamics of the HABs, directly assimilating the toxins produced by toxic dinoflagellates (Krapivka et al. 2007; Zamorano et al. 2013)

Interaction of toxic groups in simultaneous incubation processes

On the coast of Chile, OA- and PTX-group toxins are produced by different species of dinoflagellates. Therefore, the assimilation of both toxin groups by filter-feeding bivalves necessarily involves the simultaneity of bloom processes in relation to the production of OA- and PTX-group toxins (Zamorano et al. 2013; Alves-de-Souza et al. 2014).

Incubations in the digestive glands at different rates of OA- and PTX-group toxins were performed simultaneously (OA+PTX-2; DTX-1 + PTX-2; OA+DTX-1+ PTX-2) at concentrations of 4.0 µg ml^{-1} of OA/DTX-1 and 2.1 µg ml^{-1} PTX-2 (Figure 6). The reactions assessed in both species showed significant differences in biotransformation kinetics for each group (Figure 6(a-e)), where derivatives of the OA-group were the most frequently detected analogues; mainly DTX-1 ($p < .001$), in *Mytilus chilensis* (Figure 6(b-c)) and *Ameghinomya antiqua* (Figure 6(e-f)). PTX-2 maintained toxic variability in all simultaneous incubation processes with toxins of the OA-group in both species, in which the oxidation of PTX-2 was not evidenced as a preferred reaction pathway ($p < 0.05$) (Figure 6(a-c)). Acyl-derivatives from PTX2sa (C-16-PTX2sa) were not detected in any of the tissues assessed.

Similar toxic processes has been detected in different species of bivalves in endemic species of southern Chile, which are characterised by different levels of nutrient assimilation. This is correlated with the levels of toxicity detected in different species (Zamorano et al. 2013). Biotransformation processes for analogues from different groups take place in parallel: acylation and oxidation pathways of OA-group and PTX-group, respectively (Blanco et al. 2007b, 2018). The tendency of the biological system by the acylation pathway of OA-group is directly related to the level of action of these toxins on the inhibition of complexes such as PP2A (IC_{50} for OA: 2.27 nM; DTX-1: 1.72 nM; DTX-2: 3.77 nM, Abal et al. 2017), so the acylation allows the inhibition ability

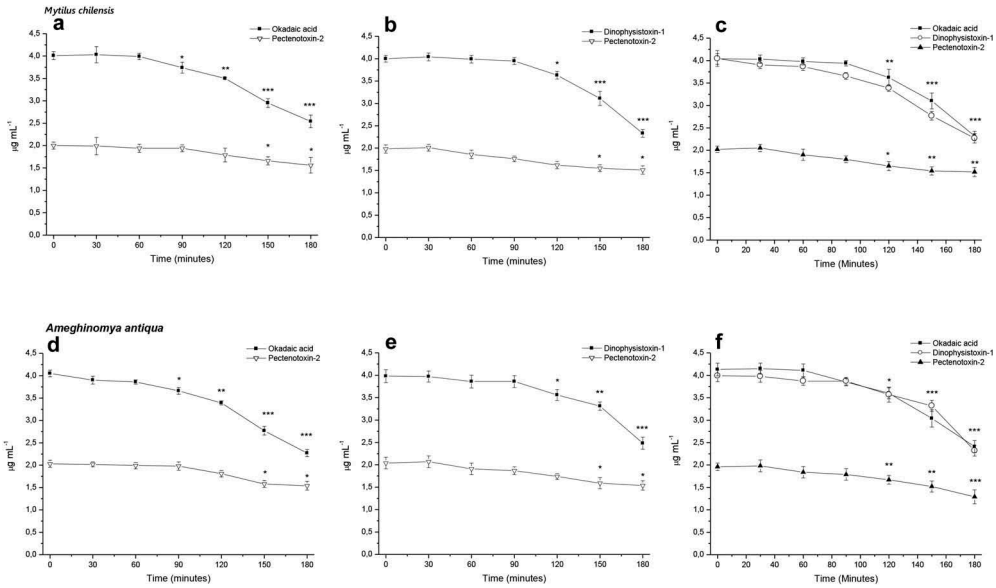

Figure 6. Variability in the concentration ratio in *Mytilus chilensis* of (a) OA/PTX-2, (b) DTX-1-PTX-2, (c) OA/DTX-1/PTX-2; and *Ameghinomya antiqua* of (d) OA/PTX-2, (e) DTX-1-PTX-2, (f) OA/DTX-1/PTX-2 during a 180 min reaction. The graph shows average toxin concentrations ± standard deviations (n = 5, SEM ± SD).

of PP2A to decrease by ~ 500 times (Furumochi et al. 2016; García et al. 2018), thus favouring a defence pathway against toxic analogues, such as OA and DTX-1 (Svensson and Förlin 2004).

Furthermore, the level of action and lipophilicity of PTX-2 makes the distribution percentage low, which is concomitant with a low biotransformation pathway carried out by the oxidative pathway (Lindegarth et al. 2009). Even though this process involves decreased toxicity of the species, this variability has a very low proportionality.

The main fatty acid detected in bivalves was palmitic acid (≈50%, C16:0), which has been defined as the most prevalent acid in filter-feeding species, such as *Mytilus chilensis* and *Ameghinomya antiqua*, if compared to other saturated fatty acids detected in this species (15%, C-14, C-18 and C22) (Contreras and García 2019). This demonstrates that despite the availability of compounds that can favour biotransformation processes, the involved pathways also depend on biotic-abiotic factors which allow for the maintenance of the appropriate physiological parameters of the species, transforming this pathway into species-specific processes.

Determination of the environmental risk associated with the toxicities of OA- and PTX-groups

Dinophysis sp. has been associated worldwide with the production of both groups of toxins (OA-group and PTX-group), this is the reason that the toxicities detected in shellfish are expressed in micrograms OA equivalents per kilogram of tissue (EFSA 2008; Farabegoli et al. 2018; Vilariño et al. 2018). However, along the coast of Chile (8,000 km), it has been reported that the species *Dinophysis acuminata* only produces PTX-2 (Krock et al. 2009; Alves-de-Souza et al. 2014). The prevalence of this species in southern Chile is relative (Reloncaví Sound, Region de los Lagos). Nevertheless, the PTX-group has been identified with a high prevalence in shellfish, but simultaneously to the OA-group, which demonstrates the co-occurrence of blooms in the austral Chilean fjords (Díaz et al. 2011; García et al. 2012).

In Chile, HABs associated with *Dinophysis acuta* have been identified in densities ~200 cel ml^{-1} with a toxicity ~18 pg cel^{-1}. It is mainly assimilated by filter-feeding species such as *Mytilus chilensis* and *Ameghinomya antiqua*, their average clearance rate is ~2.39 ± 0.4 L/h and ~0.79 ± 0.3 L/h, respectively (Velasco and Navarro 2002), being able to assimilate concentrations ≥ 160 µg OA equiv kg^{-1} in 2 to 3 days (Zamorano et al. 2013; García et al. 2015).

This study shows that both species of bivalves (*Mytilus chilensis* and *Ameghinomya antiqua*) produce biotransformed analogues of both groups of toxins only in their digestive glands, following a biotransformation preference of DTX-1 〉 OA 》 PTX-2. The transformation acylation (OA-group) and oxidation (PTX-group) processes occur in parallel according to the enzymatic pathways used, and no inhibition of the biotransformation pathways was produced when the groups of toxins were simultaneously incubated (Rossignoli and Blanco 2010; MacKenzie et al. 2012).

Data show that regardless of the zone of origin of sea products, the toxic profile of bivalves may involve biotransformed derivative analogues. Each toxic analogue has a toxic equivalency factor (TEF), which is defined as the *toxicity ratio of a compound from a chemical group that shares the same mode of action of a reference compound in the same group* (Botana et al. 2017; Alarcan et al. 2018). Thus, each analogue of lipophilic toxins has specific TEFs, where a TEF = 1.0 has been proposed for OA/DTX-1, based on a toxicity by i.p. injection and a relative inhibitory effect on PP2A (EFSA 2008; Botana et al. 2017). However, studies using a LD_{50} (oral toxicity) have proposed new TEF = 1 for OA, 1.5 for DTX-1, and 0.3 for DTX-2 (Abal et al. 2017). Furthermore, PTX-2 has a TEF = 1.0, highlighting that to this date, their toxicity has not been determined in animals (Botana et al. 2017; Vilariño et al. 2018).

In Chile, the official method for detecting DSP-toxins is the MBA, which has an approximate detection level ≥ 200 µg OA equiv kg^{-1}, and it only defines a health risk when shellfish show toxicities above the regulatory limits (Sernapesca 2014; García et al. 2018). In this way, this study demonstrates that biotransformation processes start quickly and that the toxins are assimilated after a few days, thus, the toxic profile in shellfish

will involve the presence of acyl-OA-group, which is not detected by the MBA. Previous studies have shown that consumed shellfish with acyl derivatives can be modified by a retro-conversion process mediated by esterases and digestive lipases towards their original analogues (acyl derivatives→OA/DTX-1) leading to DSP syndrome between 12 and 24 h post-consumption of shellfish (García et al. 2005; Doucet et al. 2007), which represents a serious risk to public health.

Likewise, due to the difficulty in determining blooms associated with *Dinophysis sp*., the toxicity determination is made directly in shellfish, where varying levels of OA and DTX-1 (3:7) and low levels of PTX-2 are shown. PTX-2 is characterised by a high prevalence in seafood from southern Chile; however, these levels do not exceed regulatory standards (≥ 160 µg OA equiv kg^{-1}). This could be explained due to micro-blooms of *Dinophysis acuminata* located in the austral area, where filter-feeding species show low concentrations of PTX (Contreras and García 2019).

Even when there are no indications of toxic symptoms associated with PTX-group and its toxic determination is only related to studies carried out in cells and in animals, the chronic consumption of PTX-2 and its biotransformed analogues (PTX-2sa) in the future may be linked to different digestive diseases which are not currently associated with the consumption of PTX-group traces.

Furthermore, the variability in the percentage of biotransformation detected in both groups of toxins (OA and PTX) demonstrates that the greatest contribution to the variability or alteration of the toxic profile comes from the reactions taking place in the digestive glands, so the contribution from other non-visceral tissues is negligible. This also means that the different analogues show different levels of distribution of these toxins (Blanco 2018). These processes also depend on both biotic and abiotic factors that, under optimal physiological conditions in bivalves allow for the clearance of the biotransformed analogues (Munday and Reeve 2013; Wells et al. 2015). In this way, previous studies have shown that DTX-1 tends to be more slowly cleared than OA, which is coincident with the most prevalent biotransformed analogue detected (DTX-3) from DTX-1 (Blanco 2018; Blanco et al. 2018). While for PTX-group, the following clearance level PTX-2 > PTX-2sa > PTX-2sa esters has been proposed (Blanco et al. 2007a). Therefore, compartmentalisation levels depend on the species involved in the bloom process, so the consumption of tissues with higher toxicity would favour the option of digesting mainly biotransformed toxins, such as PTX-2sa, whose chronic health effects on humans are still unknown.

Conclusions

In this study, biotransformation analogues of toxins associated with OA and PTX-groups have been detected and identified only in visceral tissues of *Mytilus chilensis* and *Ameghinomya antiqua*. The most frequently detected analogues in the incubations were acyl-derivatives of OA and DTX-1 (R-C_7-O-acyl) and PTX-2sa, which are produced by acylation and oxidation pathways, respectively. The data show that higher levels of biotransformation phases are detected in the visceral tissue (hepatopancreas), and then these new analogues are compartmentalised to non-visceral tissues. The detected biotransformation levels establish that the profile of toxins is modified by both species of bivalves which may be responsible for the failure to detect these toxins using the MBA. Therefore, this increases the risk of exposure in humans, due to the consumption of seafood with biotransformed analogues, which can also be subjected to a retro-conversion by enzymatic processes in the digestive system (DTX-3 → DTX-1) thus producing the syndrome characteristic of lipophilic toxins after ingestion of toxic analogues.

Acknowledgments

This study was performed with funds from Fondo Nacional de Ciencia y Tecnología ("Chilean National Science and Technology Fund") (FONDECYT) Number 1160168 (granted to C. García). The authors thank the Secretaría Regional Ministerial de Salud de la Región de Aysén ("Aysén Regional Health Ministry") and Capitanía de Puerto de Puerto Aguirre, Armada de Chile ("Port Authority of Puerto Aguirre, Chilean Navy").

Disclosure statement

No potential conflict of interest was reported by the author.

Funding

This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico [1160168].

ORCID

Carlos García http://orcid.org/0000-0001-7594-2156

References

Abal P, Louzao MC, Cifuentes JM, Vilarino N, Rodriguez I, Alfonso A, Vieytes MR, Botana LM. 2017. Characterization of the dinophysistoxin-2 acute oral toxicity in mice to define the toxicity equivalency factor. Food Chem Toxicol. 102:166–175. doi:10.1016/j.fct.2017.02.023.

Alarcan J, Barbé S, Kopp B, Hessel-Pras S, Braeuning A, Lampen A, Le Hégarat L, Fessard V. 2019. Combined effects of okadaic acid and pectenotoxin-2, 13-desmethylspirolide C or yessotoxin in human intestinal Caco-2 cells. Chemosphere. 228:139–148. doi:10.1016/j.chemosphere.2019.04.018.

Alarcan J, Biré R, Le Hégarat L, Fessard V. 2018. Mixtures of lipophilic phycotoxins: exposure data and toxicological assessment. Mar Drugs. 16:46. doi:10.3390/md16020046.

Alves-de-Souza C, Varela D, Contreras C, de La Iglesia PM, Fernández P, Hipp B, Hernández C, Riobó P, Reguera B, Franco JM, et al. 2014. Seasonal variability of *Dinophysis* spp. and *Protoceratium reticulatum* associated with lipophilic shellfish toxins in a strongly stratified Chilean Fjord. Deep Sea Res Part II Top Stud Oceanogr. 101:152–162. doi:10.1016/j.dsr2.2013.01.014.

Blanco J. 2018. Accumulation of dinophysis toxins in bivalve Molluscs. Toxins. 10:453. doi:10.3390/toxins10110453.

Blanco J, Álvarez G, Rengel J, Díaz R, Mariño C, Martín H, Uribe E. 2018. Accumulation and biotransformation of *Dinophysis* toxins by the surf clam *Mesodesma donacium*. Toxins. 10(8):pii: E314. doi:10.3390/toxins10080314.

Blanco J, Alvarez G, Uribe E. 2007a. Identification of pectenotoxins in plankton, filter feeders, and isolated cells of a *Dinophysis acuminata* with an atypical toxin profile, from Chile. Toxicon. 49:710–716. doi:10.1016/j.toxicon.2006.11.013.

Blanco J, Mariño C, Martín H, Acosta CP. 2007b. Anatomical distribution of diarrhetic shellfish poisoning (DSP) toxins in the mussel *Mytilus galloprovincialis*. Toxicon. 50:1011–1018. doi:10.1016/j.toxicon.2007.09.002.

Botana LM, Hess P, Munday R, Nathalie A, De Grasse SL, Feeley M, Suzuki T, van der Berg M, Fattori V, Garrido Gamarra E, et al. 2017. Derivation of toxicity equivalency factors for marine biotoxins associated with bivalve molluscs. Trends Food Sci Technol. 50:15–24. doi:10.1016/j.tifs.2016.09.015.

Contreras HR, García C. 2019. Inter-species variability of okadaic acid group toxicity in relation to the content of fatty acids detected in different marine vectors. Food Addit Contam Part A. 36:464–482. doi:10.1080/19440049.2019.1569265.

Díaz P, Molinet C, Cáceres M, Valle-Levinson A. 2011. Seasonal and intratidal distribution of *Dinophysis* spp. in a Chilean fjord. Harmful Algae. 10:155–164. doi:10.1016/j.hal.2010.09.001.

Díaz PA, Ruiz-Villarreal M, Pazos Y, Moita T, Reguera B. 2016. Climate variability and *Dinophysis acuta* blooms in an upwelling system. Harmful Algae. 53:145–159. doi:10.1016/j.hal.2015.11.007.

Doucet E, Ross N, Quilliam M. 2007. Enzymatic hydrolysis of esterified diarrhetic shellfish poisoning toxins and pectenotoxins. Anal Bioanal Chem. 389:335–342. doi:10.1007/s00216-007-1489-3.

[EFSA] European Food Safety Authority. 2008. Opinion of the scientific panel on contaminants in the food chain on a request from the European commission on marine biotoxins in shellfish – okadaic acid and analogues. EFSA J. 589:1–62.

[EFSA] European Food Safety Authority. 2009. Scientific opinion of the panel on contaminants in the food chain on a request from the European commission on marine biotoxins in shellfish – pectenotoxin group. EFSA J. 1109:1–47.

[EURLMB] European Union Reference Laboratory for Marine Biotoxins. 2015. EU-harmonised standard operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. Version. 5. [accessed 2016 Feb 4]. http://www.aecosan.msssi.gob.es/AECOSAN/docs/documentos/laboratorios/LNRBM/ARCHIVO2EUHarmonised-SOP-LIPO-LCMSMS_Version5.pdf.

[FAO/WHO] Food and Agriculture Organization/World Health Organization. 2016. Technical paper on toxicity equivalency factors for marine biotoxins associated with bivalve Molluscs. Rome (Italy): FAO/WHO; p. 108.

Farabegoli F, Blanco L, Rodríguez LP, Vieites JM, García Cabado A. 2018. Phycotoxins in marine shellfish: origin, occurrence and effects on humans. Mar Drugs. 16:188. doi:10.3390/md16060188.

Farrell H, Gentien P, Fernand L, Lunven M, Reguera B, González-Gil S, Raine R. 2012. Scales characterising a high density thin layer of *Dinophysis acuta* Ehrenberg and its transport within a coastal jet. Harmful Algae. 15:36–46. doi:10.1016/j.hal.2011.11.003.

Fujiki H, Sueoka E, Watanabe T, Suganuma M. 2018. The concept of the okadaic acid class of tumor promoters is revived in endogenous protein inhibitors of protein phosphatase 2A, SET and CIP2A, in human cancers. J Cancer Res Clin Oncol. 144(12):2339–2349. doi:10.1007/s00432-018-2765-7.

Furumochi S, Onoda T, Cho Y, Fuwa H, Sasaki M, Yotsu-Yamashita M, Konoki K. 2016. Effect of carbon chain length in acyl coenzyme A on the efficiency of enzymatic transformation of okadaic acid to 7-O-acyl okadaic acid. Bioorg Med Chem Lett. 26(13):2992–2996. doi:10.1016/j.bmcl.2016.05.027.

García C, Barriga A, Díaz JC, Lagos M, Lagos N. 2010. Route of metabolization and detoxication of paralytic shellfish toxins in humans. Toxicon. 55(1):135–144. doi:10.1016/j.toxicon.2009.07.018.

García C, Oyaneder-Terrazas J, Contreras C, Del Campo M, Torres R, Contreras HR. 2016. Determination of the toxic variability of lipophilic biotoxins in marine bivalve and gastropod tissues treated with an industrial canning process. Food Addit Contam Part A. 33(11):1711–1727. doi:10.1080/19440049.2016.1239032.

García C, Oyaneder-Terrazas J, Contreras HR. 2018. Instrumental methods for detection of lipophilic marine toxins in endemic species from Pacific Austral Fjords. In: Carmona EC, editor. London (UK): Endemic species. IntechOpen. doi:10.5772/intechopen.82438.

García C, Pérez F, Contreras C, Figueroa D, Barriga A, López-Rivera A, Araneda OF, Contreras HR. 2015. Saxitoxins and okadaic acid group: accumulation and distribution in invertebrate marine vectors from Southern Chile. Food Addit Contam Part A. 32(6):984–1002. doi:10.1080/19440049.2015.1028107.

García C, Rodriguez-Unda N, Contreras C, Barriga A, Lagos N. 2012. Lipophilic toxin profiles detected in farmed and benthic mussels populations from the most relevant production zones in Southern Chile. Food Addit Contam Part A. 29:1011–1020. doi:10.1080/19440049.2012.662704.

García C, Truan D, Lagos M, Santelices JP, Díaz JC, Lagos N. 2005. Metabolic transformation of dinophysistoxin-3 into dinophysistoxin-1 causes human intoxication by consumption of O-acyl-derivatives dinophysistoxins contaminated shellfish. J Toxicol Sci. 30:287–296. doi:10.2131/jts.30.287.

[ISP] Instituto de Salud Publica (Public Health Institute). 2012. Report on the results of the Programme for National Surveillance and Control of Intoxications due to harmful algal blooms Chile 2012. Santiago (Chile); p. 1–13.

Jiménez-Cárcamo D, García C, Contreras HR. 2020. Toxins of okadaic acid-group increase malignant properties in cells of colon cancer. Toxins. 12(3):179. doi:10.3390/toxins12030179.

Kameneva PA, Imbs AB, Orlova TY. 2015. Distribution of DTX-3 in edible and non-edible parts of *Crenomytilus grayanus* from the Sea of Japan. Toxicon. 98:1–3. doi:10.1016/j.toxicon.2015.02.011.

Konoki K, Onoda T, Watanabe R, Cho Y, Kaga S, Suzuki T, Yotsu-Yamashita M. 2013. In vitro acylation of okadaic acid in the presence of various bivalves' extracts. Mar Drugs. 11(12):300–315. doi:10.3390/md11020300.

Krapivka S, Toro JE, Alcap!an AC, Astorga M, Presa P, P! erez M, Guinez R. 2007. Shell-shape variation along the latitudinal range of the Chilean blue mussel *Mytilus chilensis* (Hupe 1854). Aquacult Res. 38:1770–1777. doi:10.1111/j.1365-2109.2007.01839.x.

Krock B, Seguel CG, Valderrama K, Tillmann U. 2009. Pectenotoxins and yessotoxin from Arica Bay, North Chile as determined by tandem mass spectrometry. Toxicon. 54(3):364–367. Epub 2009 Apr 23. doi:10.1016/j.toxicon.2009.04.013.

Lindegarth S, Torgersen T, Lundve B, Sandvik M. 2009. Differential retention of okadaic acid (OA) group toxins and pectenotoxins (PTX) in the blue mussel, *Mytilus edulis* (L.), and european flat oyster, *Ostrea edulis* (L.). J Shellfish Res. 28(2):313–323. doi:10.2983/035.028.0213.

Mackenzie LA. 2019. A long-term time series of *Dinophysis acuminata* blooms and associated shellfish toxin contamination in Port Underwood, Marlborough sounds, New Zealand. Toxins. 11(2):74. doi:10.3390/toxins11020074.

MacKenzie LM, Selwood AI, Marshall C. 2012. Isolation and characterization of an enzyme from the Greenshell mussel *Perna canaliculus* that hydrolyses pectenotoxins and esters of okadaic acid. Toxicon. 60(3):406–419. doi:10.1016/j.toxicon.2012.05.005.

Munday R, Reeve J. 2013. Risk assessment of shellfish toxins. Toxins. 5(11):2109–2137. doi:10.3390/toxins5112109.

Park JH, Kim M, Jeong HJ, Park MG. 2019. *Revisiting the taxonomy of the "Dinophysis acuminata* complex" (Dinophyta)'. Harmful Algae. 88:101657. doi:10.1016/j.hal.2019.101657.

Rossignoli AE, Blanco J. 2010. Subcellular distribution of okadaic acid in the digestive gland of *Mytilus galloprovincialis*: first evidences of lipoprotein binding to okadaic acid. Toxicon. 55(2–3):221–226. doi:10.1016/j.toxicon.2009.07.022.

Rossignoli AE, Fernández D, Regueiro J, Mariño C, Blanco J. 2011. Esterification of okadaic acid in the mussel *Mytilus galloprovincialis*. Toxicon. 57:712–720. doi:10.1016/j.toxicon.2011.02.003.

Sernapesca. 2014. Health program of bivalve molluscs for export. Santiago (Chile): LAB-MP1.

Suzuki T, Mackenzie L, Stirling D, Adamson J. 2001a. Pectenotoxin-2 seco acid: a toxin converted from pectenotoxin-2 by New Zealand Greenshell mussel, *Perna canaliculus*. Toxicon. 39:507–514. doi:10.1016/S0041-0101(00)00153-7.

Suzuki T, Mackenzie L, Stirling D, Adamson J. 2001b. Conversion of pectenotoxin-2 to pectenotoxin-2 seco acid in the New Zealand scallop, *Pecten novaezelandiae*. Fish Sci. 67:506–510. doi:10.1046/j.1444-2906.2001.00265.x.

Svensson S, Förlin L. 2004. Analysis of the importance of lipid breakdown for elimination of okadaic acid (diarrhetic shellfish toxin) in mussels, *Mytilus edulis*: results from a field study and a laboratory experiment. Aquatic Toxicology. 66(4):405–418. doi:10.1016/j.aquatox.2003.11.002.

Swan SC, Turner AD, Bresnan E, Whyte C, Paterson RF, McNeill S, Mitchell E, Davidson K. 2018. *Dinophysis acuta* in Scottish Coastal waters and its influence on diarrhetic shellfish toxin profiles. Toxins. 10(10):399. doi:10.3390/toxins10100399.

Uchida H, Watanabe R, Matsushima R, Oikawa H, Nagai S, Kamiyama T, Baba K, Miyazono A, Kosaka Y, Kaga S, et al. 2018. Toxin profiles of okadaic acid analogues and other lipophilic toxins in *Dinophysis* from Japanese Coastal waters. Toxins (Basel). 10(11):pii: E457. doi:10.3390/toxins10110457.

Vale P, Sampayo DMA. 2002. Pectenotoxin-2 seco acid, 7-epi-pectenotoxin-2 seco acid and pectenotoxin-2 in shellfish and plankton from Portugal. Toxicon. 40 (7):979–987. doi:10.1016/S0041-0101(02)00094-6.

Velasco LA, Navarro JM. 2002. Feeding physiology of infaunal (*Mulinia edulis*) and epifaunal (*Mytilus chilensis*) bivalves under a wide range of concentrations and qualities of seston. Mar Ecol Prog Ser. 240:143–155. doi:10.3354/meps240143.

Vilariño N, Louzao MC, Abal P, Cagide E, Carrera C, Vieytes MR, Botana L. 2018. Human poisoning from marine toxins: unknowns for optimal consumer protection. Toxins. 10(8):324. doi:10.3390/toxins10080324.

Wang S, Chen J, Li Z, Wang Y, Fu B, Han X, Zheng L. 2015. Cultivation of the benthic microalga *Prorocentrum lima* for the production of diarrhetic shellfish poisoning toxins in a vertical flat photobioreactor. Bioresour Technol. 179:243–248. doi:10.1016/j.biortech.2014.12.019.

Wells ML, Trainer VL, Smayda TJ, Karlson BSO, Trick CG, Kudela RM, Ishikawa A, Bernard S, Wulff A, Anderson DM, et al. 2015. Harmful algal blooms and climate change: learning from the past and present to forecast the future. Harmful Algae. 49:68–93. doi:10.1016/j.hal.2015.07.009.

Wu D, Chen J, He X, Wang J, Wang Z, Li X, Wang B. 2019. Distribution, partitioning, and seasonal variation of lipophilic marine algal toxins in aquatic environments of a typical semi-closed mariculture bay. Environ Pollut. 255(Pt 2):113299. doi:10.1016/j.envpol.2019.113299.

Zamorano R, Marín M, Cabrera F, Figueroa D, Contreras C, Barriga A, Lagos N, García C. 2013. Determination of the variability of both hydrophilic and lipophilic toxins in endemic wild bivalves and carnivorous gastropods from the Southern part of Chile. Food Addit Contam: Part A. 30 (9):1660–1677. doi:10.1080/19440049.2013.805438.