DEPARTAMENTO DE BIOLOGÍA CELULAR Y MOLECULAR

ÁREA DE GENÉTICA

Assessment of the Early Effects of the Diarrhetic Shellfish Toxins in the Mussel *Mytilus galloprovincialis* Using Cellular and Molecular Biomarkers

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Doctoral Thesis

2016

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Programa Oficial de Doutoramento en Bioloxía Celular e Molecular[1]

Universidade da Coruña

[1] Programa regulado polo RD 99/2011.

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INFORMAN

Que el trabajo titulado "**Assessment of the Early Effects of the Diarrhetic Shellfish Toxins in the Mussel *Mytilus galloprovincialis* Using Cellular and Molecular Biomarkers**" presentado por Dña. MARÍA VERÓNICA PREGO FARALDO para optar al Título de Doctora en Biología con Mención Internacional, ha sido realizado bajo nuestra dirección. Considerándolo finalizado, autorizamos la presentación y defensa de la tesis doctoral.

A Coruña, 26 de Julio de 2016

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Doctoranda

La autora de este trabajo ha disfrutado durante la realización de la tesis de un contrato temporal con cargo al proyecto de investigación “Evaluación citogenética y molecular de la respuesta temprana del mejillón *Mytilus galloprovincialis* al ácido ocadaico (OA) (EVALOA PLUS)” (AGL2012-30697), concedido por el Ministerio de Economía y Competitividad, Dirección General de Investigación Científica y Técnica del Gobierno de España (Diciembre 2013- Junio 2015). Parte del trabajo ha sido realizado en el *Interdisciplinary Centre of Marine and Environmental Research* (CIIMAR) (Universidade de Porto, Portugal) durante una estancia de tres meses en el año 2015, dentro del programa de becas para estancias predoctorales Inditex-UDC 2015.

La realización de esta tesis ha sido posible gracias a la financiación obtenida a través del proyecto AGL2012-30697, concedido por el Ministerio de Economía y Competitividad, Dirección General de Investigación Científica y Técnica, Gobierno de España, durante los años 2012 a 2015.

A mis padres

A Agustín

Agradecimientos

Quiero comenzar expresando mi más sincero agradecimiento a la Dra. Josefina Méndez Felpeto por darme la oportunidad de formar parte de su grupo de investigación, así como por la confianza depositada en mí durante todos estos años. Quisiera agradecer su cercanía y generosidad al transmitirme su experiencia. También quisiera agradecerle que me proporcionara libertad para investigar y que me confiara tareas que aunque en un principio me pudieran parecer complejas, me han permitido adquirir autonomía, empuje y decisión.

Quiero agradecer al Dr. Jose M. Eirin-Lopez su buena disposición, completa dedicación e incondicional apoyo durante la realización de esta tesis. Agradezco la confianza que depositó en mi al embarcarse en este proyecto como codirector, así como el mantenimiento de su apoyo a lo largo del mismo a pesar de los contratiempos surgidos. Gracias a su experiencia e ininterrumpida ayuda esta tesis se ha convertido en una realidad.

A la Dra. Lúcia Guilhermino, de la Universidade de Porto, quiero agradecerle el acogerme en su grupo de investigación ECOTOX durante tres meses. Gracias también al Dr. Luís Vieira por formarme en el apasionante mundo de las actividades enzimáticas y el estrés oxidativo. A él y a mi compañero Gabriel les agradezco el ambiente distendido durante el desarrollo del trabajo; de no ser por ellos este habría sido mucho más arduo. Al resto de compañeros les agradezco el buen trato recibido, en especial a Diego, Patricia y Ana. Gracias a todos vosotros mi estancia en Porto fue una experiencia altamente enriquecedora, tanto a nivel profesional como a nivel personal.

Quiero extender un sincero agradecimiento a la Dra. Blanca Laffon y a la Dra. Vanessa Valdiglesias por la excelente colaboración establecida con ambas durante el desarrollo de este proyecto. Su experiencia, entusiasmo y buena disposición se han contagiado a esta tesis, reflejándose en la creación de un conjunto de publicaciones que sin su implicación no habrían tenido el mismo éxito.

Por supuesto también agradezco a mis compañeros de laboratorio, los que continúan y los que ya se han ido, todo el apoyo prestado. En especial quisiera agradecer a Jose, quien me enseñó a trabajar en biología molecular, la paciencia que siempre ha tenido conmigo, así como la confianza depositada en mi y su buen sentido del humor. A Juan, por sus consejos sobre diseño experimental y expresión génica. A Fer, por enseñarme a "cometear". A Vicky, por sus lecciones de bioinformática. A Tamara, por ayudarme con la búsqueda de alojamiento en Porto. A Rosa, por auxiliarme durante el ensayo *in vivo*, sin su ayuda este hubiera resultado imposible. A Luisa, por los buenos momentos musicales. A Ciro, por mantener el optimismo y la tranquilidad en el grupo. A Rodri, Ruth, Alberto y Sandra, a los compañeros de Toxicología, Bioquímica y Gibe por los momentos compartidos. Y por último, pero no menos importante, a Ana y a Jeny, por estar siempre presentes, por los buenos y malos momentos, por las confidencias y las risas.

A todas las personas que me han ayudado durante la investigación o que de algún modo me han acompañado a lo largo de esta etapa predoctoral. Aunque no os haya citado específicamente, también a vosotros quiero dedicaros esta tesis.

Contents

Contents

Abbreviations & Symbols

7-AAD	7-amino-actinomycin D
$	Dollars
ºC	Degree Celsius
%	Percentage
%tDNA	Percentage of DNA in the tail
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
A	Absorbance
ALS	Alkali-labile sites
ANOVA	Analyses of variance
AP	Apurinic/apyrimidinic
ASP	Amnesic shellfish poisoning
AZA	Azaspiracid
AZP	Azaspiracid shellfish poisoning
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CAT	Catalase
cDNA	Complementary deoxyribonucleic acid
CFP	Ciguatera fish poisoning
cm	Centimeter
CMFS	Calcium-magnesium-free saline
CNRQ	Calibrated normalized relative quantities
DA	Domoic acid
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DSB	Double strand break
DSP	Diarrhetic shellfish poisoning
DTX	Dinophysistoxins
DTX1	Dinophysistoxin-1
DTX2	Dinophysistoxin-2
DTX3	Dinophysistoxin-3
EC	European Community
EDTA	Ethylenediaminetetraacetic acid
EF1	Elongation factor 1
EF2	Elongation factor 2
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FAO	Food and Agriculture Organization
FDA	Fluorescein diacetate
FL	Filter
FLD	Fluorimetric detecion
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
g	Gram
g	Relative centrifugal force
GC	Gas chromatography
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
GST-*pi*	Glutathione S-transferase *pi-1*
GTX	Gonyautoxin
h	Hour
H2A	Histone H2A

HAB	Harmful algal blooms
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
H_2O_2	Hydrogen peroxide
IAP	Inhibitors of apoptosis proteins
kg	Kilogram
KSS	Kenny´s salt solution
kV	Kilovolts
L	Liter
LC	Liquid chromatography
LPO	Lipid peroxidation
M	Mollar
M	Stability measures
MBA	Mouse bioassay
MDA	Malondialdehyde
MEKC	Micellar electrokinetic chromatography
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MN	Micronucleus
MRM	Multiple reaction monitoring
mRNA	Messeger ribonucleic acid
mS	Millisiemns
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	North
Nº	Number

n	The total number of individuals
NAC	N-acetyl cysteine
NADH	Nicotinamide adenine dinucleotide phosphate
NEO	Neosaxitoxin
ng	Nanogram
nM	Nanomolar
nm	Nanometer
N	North
NSP	Neurologic shellfish poisoning
O^{2-}	Superoxide anion radical
OA	Okadaic acid
OGG1	8-oxoguanine DNA glycosylase
OH	Hydroxy radical
P	P-value
PbTx	Brevetoxins
PC	Positive control
PI	Propidium iodide
PP	Protein phosphatase
PP2A	Protein phosphatase 2A
PSP	Paralytic shellfish poisoning
PTX	Pectenotoxin
PE	Phycoerythrin
pg	Picogram
PS	Phosphatidylserine
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
r	Spearman´s rank correlation coefficient
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute

rpS4	Ribosomal proteins S4
s	Second
SCGE	Single cell gel electrophoresis
SD	Standard deviation
Se-GPx	Selenium-dependent glutathione peroxidase
SEM	Standard error of the mean
SOD	Superoxide dismutase
SSB	Single strand break
STX	Saxitoxin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TMR	Tetramethylrhodamine
X	Magnification
U	Units
UVD	Ultraviolet detection
V	Volt
W	West
vs	*versus*

Short Abstracts

ABSTRACT

Diarrhetic shellfish poisoning (DSP) toxins constitute a group of marine toxins displaying wide geographic distribution and involved in frequent toxic episodes (Harmful algal blooms, HABs) in coastal areas. Okadaic acid (OA) and its derivatives the dinophysistoxins (DTXs) are the main toxic compounds of this group. Seafood contamination by DSP toxins, especially bivalve molluscs, constitutes the principal cause of diarrhetic shellfish poisoning syndrome (DSP) in human consumers. In order to reduce its prevalence, efficient pollution control programs are currently conducted in shellfish farming industries, causing collateral economic losses. Despite the toxicity caused by DSP toxins in human and mammalian cell lines, bivalve molluscs show an apparent resistance to these compounds. This thesis evaluates, for the first time, the early effects of DSP toxins in the mussel *Mytilus galloprovincialis* using cellular and molecular biomarkers. Accordingly, the genotoxic and cytotoxic effects of OA were evaluated in hemolymph and gill cells *in vitro*. This approach was supplemented with the *in vivo* assessment of the effects of DSP-toxin-producing dinoflagellate *Prorocentrum lima*. Additionally, the transcriptional and biochemical responses of several antioxidant enzymes were determined in mussels exposed *in vivo* to *P. lima*. The results presented in this thesis increase the general knowledge pertaining the genotoxic and cytotoxic potential of DSP toxins in marine organisms and, more especifically, in the case bivalve molluscs. In addition, this work helps elucidate the connection between the exposure to these toxins and the induction of oxidative stress. Similarly, the results obtained suggest that the resistance of these organisms to DSP toxins is associated with the ability of their antioxidant system to respond immediately to damage. In conclusion, this thesis lays the foundation for the future development of efficient monitoring programs.

RESUMEN

Las toxinas diarreicas (*Diarrhetic shellfish poisoning*, DSP) constituyen un grupo de toxinas marinas de amplia distribución geográfica responsable de frecuentes episodios tóxicos de contaminación en áreas costeras. Los principales compuestos tóxicos que conforman este grupo son el ácido ocadaico (*Okadaic acid*, OA) y sus derivados las dinofisitoxinas (*Dinophysistoxins*, DTXs). La contaminación por toxinas DSP en mariscos, especialmente en moluscos bivalvos, causa intoxicación diarreica (síndrome DSP) en consumidores humanos. Para reducir su prevalencia se llevan a cabo programas de control eficientes, pero que provocan pérdidas económicas colaterales en el cultivo de moluscos. A pesar de la toxicidad causada por las toxinas DSP en líneas celulares de humanos y mamíferos, los moluscos bivalvos muestran aparente resistencia a estos compuestos. En esta tesis se han evaluado, por primera vez, los efectos tempranos de las toxinas DSP en el mejillón *Mytilus galloprovincialis* mediante el uso de biomarcadores celulares y moleculares. Para ello se llevó a cabo una primera aproximación mediante el estudio *in vitro* de los efectos genotóxicos y citotóxicos del OA en células de hemolinfa y branquia de mejillón. Esta aproximación se complementó con el estudio *in vivo* de los efectos del dinoflagelado productor de toxinas DSP *Prorocentrum lima*. Además, se determinó la respuesta transcripcional y bioquímica de varias enzimas antioxidantes de mejillón a la exposición *in vivo* a *P. lima*. Los resultados obtenidos en la presente tesis incrementan el conocimiento del potencial genotóxico y citotóxico de las toxinas DSP en los moluscos bivalvos y lo relacionan con el estrés oxidativo. Del mismo modo, la resistencia de estos organismos a las toxinas DSP se asocia con la capacidad del sistema antioxidante para responder de forma inmediata a los primeros daños. En conclusión, la presente tesis sienta las bases para el desarrollo futuro de mecanismos eficientes de monitorización.

RESUMO

As toxinas diarreicas (*Diarrhetic shellfish poisoning*, DSP) constitúen un grupo de toxinas mariñas de ampla distribución xeográfica responsable de frecuentes episodios tóxicos de contaminación en áreas costeiras. Os principais compostos tóxicos que conforman este grupo son o ácido ocadaico (*Okadaic acid*, OA) e os seus derivados as dinofisitoxinas (*Dinophysistoxins*, DTXs). A contaminación por toxinas DSP en mariscos, especialmente en moluscos bivalvos, causa intoxicación diarreica (síndrome DSP) en consumidores humanos. Para reducir a súa prevalencia lévanse a cabo programas de control eficientes, pero que provocan perdas económicas colaterais no cultivo de moluscos. A pesar da toxicidade causada polas toxinas DSP en liñas celulares de humanos e mamíferos, os moluscos bivalvos mostran aparente resistencia a estes compostos. Nesta tese avaliáronse, por primeira vez, os efectos temperáns das toxinas DSP no mexillón *Mytilus galloprovincialis* mediante o uso de biomarcadores celulares e moleculares. Para iso levouse a cabo unha primeira aproximación mediante o estudo *in vitro* dos efectos xenotóxicos e citotóxicos do OA en células de hemolinfa e branquia de mexillón. Esta aproximación complementouse co estudo *in vivo* dos efectos do dinoflaxelado produtor de toxinas DSP *Prorocentrum lima*. Ademais, determinouse a resposta transcricional e bioquímica de varias enzimas antioxidantes de mexillón á exposición *in vivo* a *P. lima*. Os resultados obtidos na presente tese incrementan o coñecemento do potencial xenotóxico e citotóxico das toxinas DSP nos moluscos bivalvos e relaciónano co estrés oxidativo. Do mesmo xeito, a resistencia destes organismos ás toxinas DSP asóciase coa capacidade do sistema antioxidante para responder de forma inmediata aos primeiros danos. En xeral, nesta tese séntanse as bases para o desenvolvemento futuro de mecanismos eficientes de monitoraxe.

Introduction

Harmful Algal Blooms

Harmful algal blooms (HABs), commonly known as “red tides”, constitute one of the most important sources of contamination in the marine environment. These natural phenomena are characterized by the proliferation and occasional dominance of particular species of toxic or harmful algae (Anderson, 2007).

In recent years, the occurrence of HABs has experienced a marked increase as a consequence of ballast water discharge, shellfish translocations, natural dispersal, eutrophication and climate change (Anderson, et al., 2012). This is really important considering that many HABs are responsible for the production of marine toxins, which can be accumulated by filter organisms as bivalve molluscs and transferred throughout the food chain, causing severe human intoxications (Prego-Faraldo, et al., 2013; Van Dolah, 2000).

With the purpose to reduce notably the number of intoxications recorded, efficient prevention programs have been adopted to prohibit the selling of contaminated shellfish (Van Egmond, et al., 2004). However, these actions have caused a negative impact on the economy of many coastal regions. In particular, the economic impact of HABs in the aquaculture of the Europe during the 2000s, according to Food and Agriculture Organization (FAO) shellfish aquaculture data, was estimated to be about $757 millions (Ferreira, et al., 2014). Nowadays, despite the important progress achieved in the methods for detecting marine toxins focused on reducing costs and time of analysis (Penna and Galluzzi, 2014; Prego-Faraldo, et al., 2013; Reverté, et al., 2014; Zhang and Zhang, 2015), no efficient way to reduce pre- and post-harvest annual losses has been established so far.

Marine Toxins

Marine toxins produced during the HABs are usually classified according to their chemical properties and the symptoms they cause in humans in diarrhetic shellfish poisoning (DSP) toxins, paralytic shellfish poisoning (PSP) toxins, amnesic shellfish poisoning (ASP) toxins, neurologic shellfish poisoning (NSP) toxins, azaspiracid shellfish poisoning (AZP) toxins and ciguatera fish poisoning (CFP) toxins (Daranas, et al., 2001; Suresh, et al., 2014).

Each of these categories includes several toxins that can cause different syndromes in humans after consumption of contaminated seafood. Thus, DSP syndrome is caused by okadaic acid (OA), dinophysistoxins (DTXs) and pectenotoxins (PTXs), PSP is caused by saxitoxin (STX), neosaxitoxin (NEO), gonyautoxin (GTX) and other analogues, ASP is caused by domoic acid (DA) and its analogues, NSP is caused by brevetoxins (PbTx), AZP is caused by azaspiracids (AZA) and its analogues, and CFP is caused by ciguatoxin or maitotoxin (James, et al., 2010; Manfrin, et al., 2012).

Diarrhetic Shellfish Poisoning Toxins

Marine toxins are not equally relevant in terms of geographical distribution and frequency (Manfrin, et al., 2012). DSP and PSP toxins are globally distributed unlike the limited occurrence of toxins as NSP or CFP, whose distribution is restricted to coastal waters of the United States or New Zealand, respectively (Gerssen, et al., 2010) (Figure 1). As a result, DSP toxins, such as OA, have received much more attention in the literature compared with other marine toxins (Figure 2).

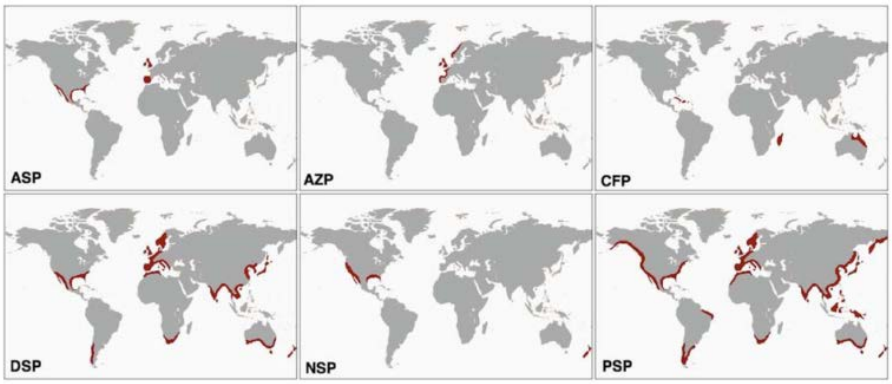


Figure 1: Geographical occurrence of shellfish poisoning syndromes. ASP: Amnesic shellfish poisoning, AZP: Azaspiracid shellfish poisoning, CFP: Ciguatera shellfish poisoning, DSP: Diarrhetic shellfish poisoning, NSP: Neurotoxic shellfish poisoning, PSP: Paralytic shellfish poisoning (Gerssen, et al., 2010).

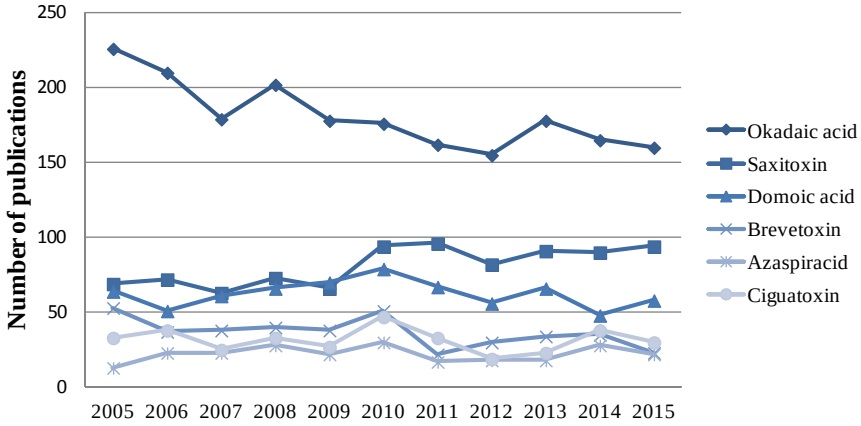
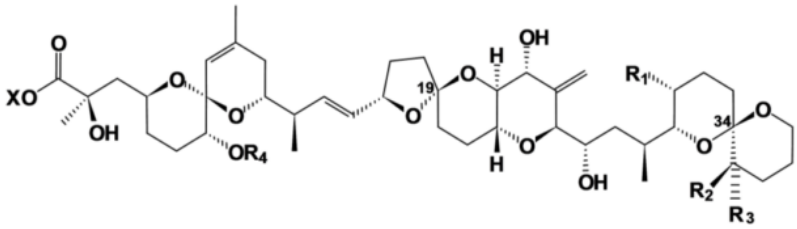


Figure 2: Number of published studies on the different marine toxins (Scopus database), limiting the search to "toxin name" in the title, abstract or keywords, from 2005 to 2015.

The relevance of these toxins in the literature is also associated with their potential to cause DSP syndrome in human consumers of contaminated shellfish (Yasumoto, et al., 1984). Although first documented in Japan (Yasumoto, et al., 1978), the DSP syndrome is now a global disease (Van Dolah, 2000) characterizes by diarrhea, nauseas, vomiting and abdominal

pains. Symptoms generally occur 0.5-3 h after consuming contaminated shellfish and disappear after 3 days (Valdiglesias, et al., 2013).

The main diarrhetic toxins causing DSP syndrome are OA (Sellner, et al., 2003) and its analogs dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) and their acyl-derivatives, generally known as dinophysistoxin-3 (DTX3). These toxins are heat-stable long-chain compounds containing polyether rings, with hydroxyl and carboxyl functions, and methyl groups differing in number or position (Sosa and Tubaro, 2015). OA and DTX2 structure only differ in the position of one methyl group in the molecule. However, DTX1 has one additional methyl group and DTX3 includes a wide range of derivatives of OA, DTX1 and DTX2, esterified with saturated and unsaturated fatty acids, products of metabolic transformations that occurs in some bivalve molluscs (Reguera, et al., 2014) (Figure 3).



Name	R1	R2	R3	R4	C-19*	C-34*
OA	CH_3	H	H	H	S	S
DTX1	CH_3	CH_3	H	H	S	R
DTX2	H	H	CH_3	H	S	R

Figure 3: Chemical structure of OA and its analogues. (This figure is a modified version of Regera et al., 2014). * Relative stereochemistry. X = H.

Okadaic Acid

In addition to being considered the active principle of DSP syndrome, OA is the most predominant DSP toxins. OA and DTX1 have a similar toxicity, while DTX2 and DTX3 have less toxic potential (Munday, 2013). For this reason, OA properties and its harmful effects have been studied in greater depth (if compared to the rest of DSP toxins), developing different approaches to detect its accumulation in shellfishs.

OA was firstly isolated from the marine sponge *Halichondria okadai* (Tachibana, et al., 1981) and usually produced by dinoflagellates of the *Dinophysis* and *Prorocentrum* genera (Lee, et al., 1989; Reguera, et al., 2012). However, the *Prorocentrum* species are benthic, therefore these dinoflagellates are frequently not available for filter-feeding organisms. Thus, *Dinophysis* species are considered the main source of OA and DSP toxins for marine invertebrates (Nielsen, et al., 2016). When the filtering organisms are feeding with these toxic dinoflagellates, the OA and DTXs are released and accumulated on their fatty tissues rapidly spreading to their predators in the food chain and eventually reaching human consumers (Figure 4).

The worldwide predominance of OA, together with the symptoms associated with the DSP syndrome in human consumers of contaminated shellfish have motivated numerous studies aimed to evaluate the negative effects of this toxin at cellular and molecular levels. Bialojan and Takai et al. (1988) demonstrated the ability of OA to inhibit several types of serine/threonine protein phosphatases (PP). This property is shared by the OA analogues, except for DTX3, which due to structural differences does not have the same affinity to PP (Munday, 2013). Since then, a large number of studies have been conducted in order to understand the mechanisms of action of OA and its effects on different model systems (Prego-Faraldo, et al., 2013; Valdiglesias, et al., 2013).

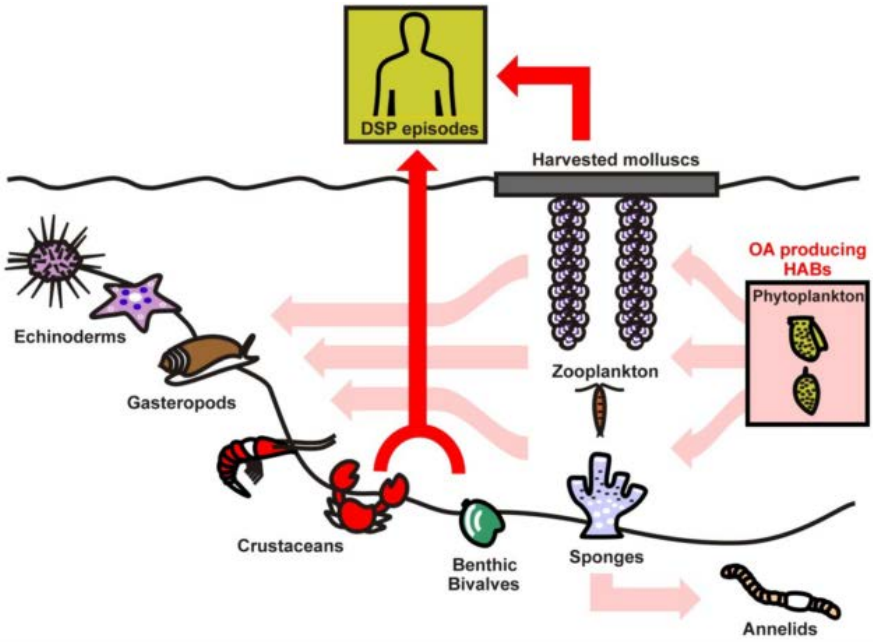


Figure 4: Schematic diagram depicting the transmission of OA across invertebrates in a typical marine food chain. The toxins produced by harmful algal blooms (HABs) are initially accumulated by herbivorous consumers including zooplankton, annelids, bivalves and other invertebrates (light pink arrows). OA is subsequently transmitted and further accumulated by their predators, including crustaceans, gasteropods and echinoderms. Bivalves (either harvested or benthic) and crabs (to a lesser extent) are the commonest vectors transmitting OA to human consumers (red arrows) causing diarrhetic shellfish poisoning (DSP) syndrome (Prego-Faraldo, et al., 2013).

Methods Used for the Detection of Okadaic Acid

Commercial shellfish harvesting is subject to extensive *in situ* monitoring of the concentrations accumulated of algal toxins. Indeed, the great diversity of toxic compounds originated during HAB episodes requires complex detection and quantification strategies. During the last 40 years, the development of such strategies walked hand in hand with the technological progress in life sciences, resulting in a wide range of detection and quantification approaches that can be globally classified into analytical and

non-analytical methods, depending on whether or not they are able to unequivocally identify and quantify the toxins in a give sample (Vilariño, et al., 2010). Nevertheless, given that different detection methods rely on either biological or chemical (or a combination of both) parameters, this introduction addresses them following this classification of biological, chemical and biochemical methods (Figure 5).

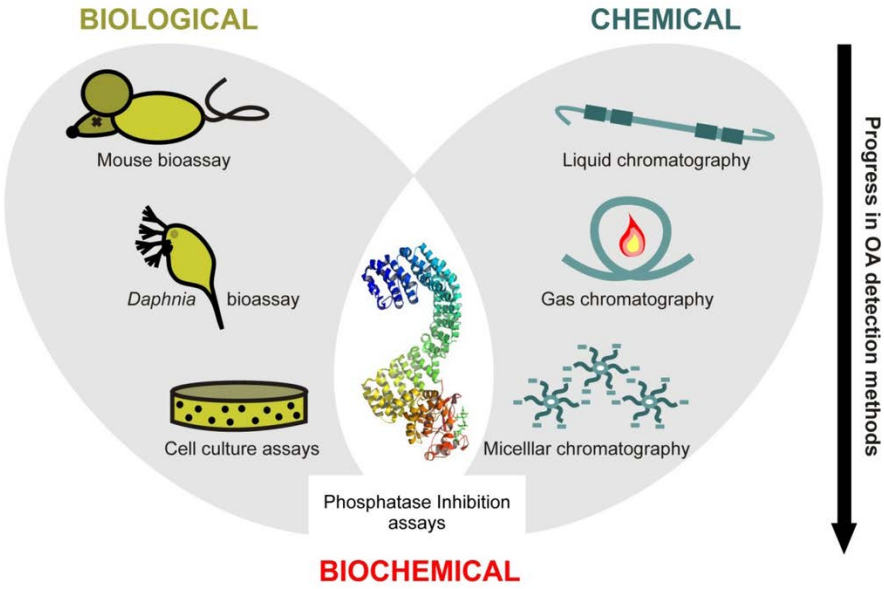


Figure 5: Methods most commonly used for okadaic acid (OA) detection and quantification in marine environmental samples (Prego-Faraldo, et al., 2013).

Biological Methods

Among the different approaches for detecting marine biotoxins, those based on biological parameters were the first to be developed and are currently the most widely used. The biological detection of OA is based on the study of its toxicological effect on either animals, or tissues or cells. The mouse bioassay (MBA) stands out among biological methods because of its wide application (Yasumoto, et al., 1978) and for constituting the standard

operating procedure for the detection of OA in food samples (European Union regulation EC Nº 2074/2005). Yet, the application of the MBA is hampered by its low specificity and sensitivity as well as relying on the use of test laboratory animals, raising ethical and technical drawbacks (Vilariño, et al., 2010).

Consequently, the development of alternative methods of improving or replacing the MBA has been fostered by authorities (EC Nº 15/2011), including the development of biological detection methods using alternative test organisms such as the planktonic crustacean *Daphnia magna* (Daphnia bioassay), which constitutes an inexpensive tool able to measure OA levels up to 10 times below the threshold of the MBA (Vernoux, et al., 1993). Nonetheless, this method still lacks sufficient sensitivity to completely replace the MBA (Garthwaite, 2000).

Similarly, alternative detection strategies based on molecular methodologies have been put forward, including cytotoxic assays based on the study of morphological changes of cultured cell lines exposed to OA (Amzil, et al., 1992; Croci, et al., 1997; Tubaro, et al., 1996a). Such approaches provide increased levels of sensitivity in the detection of OA while abolishing the use of test laboratory animals. Altogether, the progress in the development and optimization of biological methods for OA detection opens up the door to a very promising future of new developments.

Chemical Methods

Although biological methods constitute the preferred approach for detecting marine biotoxins they are unable to provide a quantitative measure of the studied compounds. Such inconvenience has led to the development of chemical detection and quantification methods based on the chromatographic properties of biotoxins (Gerssen, et al., 2010; Gerssen, et al., 2011). The chemical methods most frequently used for detection of OA

are based on liquid chromatography (LC) or high performance liquid chromatography (HPLC) separation strategies, coupled with several detection methods including mass spectrometry (LC-MS), tandem mass spectrometry (LC-MS/MS), fluorimetric detection (HPLC-FLD) and ultraviolet detection (HPLC-UVD) (Christian and Luckas, 2008; Lee, et al., 1987; Vilariño, et al., 2010). In addition, alternative chromatography-based chemical methods are also available for the detection of OA (though much less used) including gas chromatography (GC) (Hungerford and Wekell, 1992) and micellar electrokinetic chromatography (MEKC) (Bouaïcha, et al., 1997).

Biochemical Methods

For quite some time, the development of simple, rapid, sensitive, reproductive and inexpensive detection methods for OA has become a major goal, given the critical relevance of this toxin during DSP episodes on the European coasts (Armi, et al., 2012; Aune and Yndestad, 1993; Gerssen, et al., 2010; Nincevic Gladan, et al., 2011). Within this scenario, the combination of biological and chemical methods has provided the basis for the development of very powerful biochemical strategies currently being applied in the detection and quantification of OA. Among them, the inhibitory effect of this toxin on protein phosphatases is the most widely used target in detection routines (Vieytes, et al., 1997). This is the case of the protein phosphatase 2A (PP2A) inhibition assay, a biochemical method able to accurately detect and quantify OA (Tubaro, et al., 1996b). Overall, the effectiveness of different methods to detect OA has been widely documented during the last 20 years, with most of them suggesting that both chemical and biochemical strategies could eventually replace the MBA as the standard method for OA detection (Baut, et al., 1994; González, et al., 2002; Louppis, et al., 2010; Morton and Donald, 1996; Mouratidou, et al., 2006; Turrell and Stobo, 2007; Vale and Sampayo, Maria Antonia De M,

1999; Vieites, et al., 1996) (Figure 5). Nevertheless, the MBA method will still be preferred as long as some biochemical methods keep underestimating the total amount of toxin present in the samples (Morton and Donald, 1996). However, enzyme-linked immunosorbent assay (ELISA) based on direct labeling, which are more sensitive to OA than tests based on indirect labeling, are currently being developed (Sassolas, et al., 2013).

Despite their effectiveness to detect OA and to reduce the number of human intoxications registered, the biological, chemical and biochemical methods presented do not yet have the enough potential to reduce the economic losses generated by these toxins in the aquaculture industry. With the aim of obtaining more efficient detection methods, the effects of DSP toxins in their main vectors, the bivalve molluscs, have acquired a special interest in the last few decades.

Bivalve Molluscs

The harmful effects of OA have been characterized, to a greater or less extent, in different human cell lines (Le Hégarat et al., 2006; Souid-Mensi et al., 2008; Dogliotti et al., 2010; Valdiglesias et al., 2011a,b), as well as in different organisms, including rodents (Le Hégarat et al., 2004), birds (Shumway et al., 2003) or annelids (Franchini et al., 2006). However, study of the toxicity of these compounds on their principal vectors, the bivalve molluscs, has received less attention in comparison with humans and mammals.

Response Strategies to Okadaic Acid

In bivalve molluscs, OA is mainly absorbed and accumulated in the digestive gland either in a free form or (in the most part) associated with high density soluble lipoproteins (Rossignoli and Blanco, 2008). This

association results in the sequestration of OA, preventing its transportation to other tissues and hindering its elimination from the organism. On the contrary, free OA is easily transported and quickly removed by means of different passive detoxification mechanisms such as direct OA excretion through the gill or the digestive system (Blanco, et al., 2007; Svensson, 2003). In addition, active depuration of OA in bivalves has also been investigated, although it was eventually ruled out by independent studies based on environmental and endogenous factors. On the one hand, it was demonstrated that regulation of OA depuration is insensitive to immediate environmental changes (Svensson and Förlin, 2004). Furthermore, additional reports indicated that neither organism size nor age play a decisive role in the depuration rate of OA, suggesting that depuration rates cannot be accelerated, even in artificial systems, as a cost-effective way to solve the problem with toxic mussels for the industry (Duinker, et al., 2007).

Resistance to Okadaic Acid

The scarce recent studies on the effects of marine toxins on bivalve molluscs seem to indicate that certain species of this *phylum* have acquired a mechanism of resistance to avoid the harmful effects of HAB toxins. Despite their ongoing contact with some marine toxins, bivalves such as mussels have not shown increases in their mortality rates, what has been corroborated experimentally in mussels fed with toxic algae (Pinto-Silva, et al., 2005; Svensson and Förlin, 1998).

Bivalve molluscs seem to be able to use toxin-producing marine microalgae as a nutrition source by blocking their toxicity (Shumway and Cucci, 1987). Indeed, although their response is variable (Shumway and Gainey, 1992), it seems that the longer bivalve molluscs are exposed to HABs, the greater the resistance (Shumway and Cucci, 1987). Svensson and Förlin (1998) suggested the existence of a specific and powerful mechanism against

harmful effects of OA marine toxin in mussels. Since then, the number of studies aimed to elucidate how bivalve molluscs avoid the damage potentially induced by marine toxins and minimize their harmful effects has progressively increased (Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2005; Prado-Alvarez, et al., 2012; Prado-Alvarez, et al., 2013). These studies generally report that harmful effects of marine toxins are limited to low concentrations or short exposure times, being reduced or even disappearing after longer exposures. For instance, Pinto-Silva et al. (2005) measured the frequency of micronucleus (MN) in mussels (*Perna perna*) exposed to *Prorocentrum lima*, an OA-producer dinoflagellate, and only obtained increases in this parameter at low concentrations of the toxin. Similarly, Prado-Alvarez et al. (2012) also observed high percentage of damaged hemocytes, positive for Annexin V staining, at low concentrations of exposure to OA when the mussel *Mytilus galloprovincialis* was exposed *in vitro* and *in vivo*.

Cell and Molecular Biomarkers

Knowledge of the responses of bivalve molluscs to DSP toxins has become a key objective to determine the harmful effects of these compounds and to allow the development of the new detection methods, which will enable the efficient reduction of economic losses associated with HAB episodes. This monitoring may be carried out by using of tools as the biomarkers.

Biomarkers are defined as a biochemical, cellular, physiological or behavioral variations that can be measured in body fluids, cells, tissues or whole organisms, and that provide evidence of exposure to contaminants (Livingstone, 1993; Monserrat, et al., 2003). Biomarkers are usually classified as biomarkers of exposure, which give information on the characteristics of the exposure, biomarkers of effect, quantifiable changes in

the organism as consequence of the exposure, and biomarkers of susceptibility, indicators of such specific characteristics of an organism that make it more susceptible to the effects of the exposure.

The biomarkers of effect commonly employed at cellular and molecular levels to assess the harmful effects of marine toxins on bivalve molluscs are represented in the Figure 6. Cellular biomarkers, such as DNA breaks, chromosome alterations or cell viability rates, are useful tools to assess genotoxicity and cytotoxicity, providing information about toxic effects before they are observed at higher levels of biological organization (Cajaraville, et al., 2000). Molecular biomarkers, including gene expression levels, protein levels and enzymatic activities of antioxidant enzymes, heat-shock proteins, esterases or multi-xenobiotic-resistance proteins, contribute to study the effects and mechanisms of action of DSP toxins in the bivalve molluscs, evaluating the toxicity by recognizing primary as well as secondary molecular targets. They are proposed as complementary biomarkers to assess the response to DSP toxins in bivalve molluscs. Together with this cellular and molecular biomarkers, recent technological advances in "-omics", including genomics, transcriptomics and proteomics, currently have a positive impact on the discovery of new biomarkers and molecular targets for DSP toxins (Suárez-Ulloa, et al., 2013).

To finish, this introduction is intended to provide an overview of the current status of the use of cellular biomarkers such as DNA breaks and cell viability rates, and molecular biomarkers of oxidative stress (gene expression levels and enzymatic activities of antioxidant enzymes) in the assessment of the effects of marine toxins in bivalve molluscs. This final section focuses in the main studies dealing with the application of these biomakers for monitoring and assessing the different effects induced by exposure of bivalve molluscs to marine toxins.

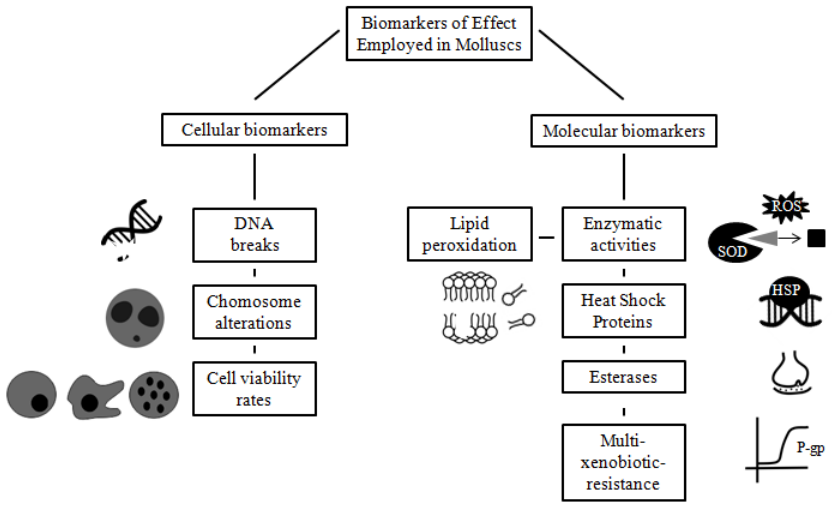


Figure 6: Schematic diagram indicating the different types of biomakers used to study the harmful effects of marine toxins in the bivalve molluscs (Prego-Faraldo, et al., 2016a).

DNA Breaks

It has been found that some marine toxins can alter, directly or indirectly, the integrity of the DNA structure of bivalve molluscs. DNA alterations induced by some of these toxins include single (SSBs) and double strand breaks (DSBs) or modified bases. DNA strand breaks may also be induced indirectly by interaction with reactive oxygen species (ROS), by the action of excision repair enzymes, or as a consequence of apoptosis or necrosis processes (Viarengo, et al., 2007). Thus, DNA strand breaks are considered as a sensitive indicators of genotoxicity and the approach most commonly used for their assessment is the single cell gel electrophoresis (SCGE) assay, also known as comet assay. The alkaline version of this assay, developed by Singh et al. (1988), allows the detection of both SSBs and DSBs. Briefly, this technique consists of embedding individual cells in an agarose gel prepared on a microscope slide, where they are lysed by

detergents, electrophoresed under alkaline conditions and stained with a florescent DNA binding dye. The result is a collection of “comets” in which the distribution of DNA throughout the comet tail reflects the level of DNA breaks (Figure 7). Parameters such as tail length, percentage of DNA in the tail or DNA tail moment are used to quantify this damage.

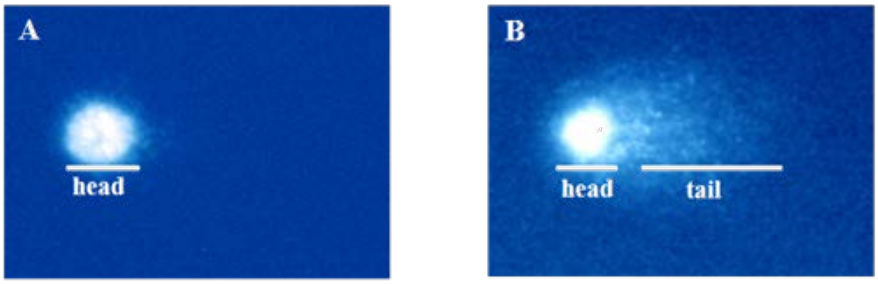


Figure 7: Evaluation of DNA breaks using comet assay. The exposure of isolated cells to an electric field results in the migration of DNA fragments creating the “tail” of the “comet”. Intensity of the comet tail relative to the head reflects the level of DNA damage: **A**) undamaged cell, **B**) damage cell.

In recent years, the comet assay has been successfully applied to assess the harmful effects of marine toxins to bivalve molluscs. Flórez-Barrós et al. (2011) used this technique to study DNA damage in hemolymph and gill cells of clams (*Ruditapes decussatus*) exposed *in vitro* to different concentrations of OA. Also in this study, the possible genotoxic damage associated with the *in vivo* exposure to this toxin was assessed. The results revealed a rapid effect of OA on hemolymph as well as increase in DNA damage only in gill cells at low concentrations. Using the same technique, Juhel et al. (2007) identified genotoxicity in zebra mussels (*Dreissena polymorpha*) exposed to microcystins, and McCarthy et al. (2014) applied the comet assay to mussel (*M. edulis*) and oyster (*Crassostrea gigas*) hemolymph and digestive gland cells exposed *in vivo* to OA, finding a significant increase in DNA damage, higher in oysters.

DNA damage can also be the consequence of indirect effects, such as oxidative damage, apurinic/apyrimidinic (AP) sites or DNA repair processes (Smith, et al., 2006). Together with strand breaks, comet assay can be also employed to evaluate oxidative DNA damage by using specific repair enzymes. However, enzyme-modified comet assay to detect oxidative DNA damage is just starting to be applied to bivalve molluscs with still too few studies employing this technique. Michel and Vincent-Hubert (2012) suggested that 8-oxoguanine DNA glycosylase (OGG1) is the most effective enzyme for the detection of oxidative damage in bivalve cells exposed *in vitro* and *in vivo* to benzo[*a*]pyrene, although other repair enzymes have been successfully used to assess DNA damage in mussels exposed to different chemical contaminants (Dallas, et al., 2013; Martins, et al., 2013). This assay has not been applied yet to evaluate the potential oxidative DNA damage caused by marine toxins in bivalve molluscs cells, becoming a promising future field of study.

Cell Viability Rates

Apoptosis is a program of genetic and biochemical changes used by the cell to control its own death. Under non standard conditions (e.g., exposure to marine toxins), the cell can cause its own death by this self-defence mechanism. Exposure to toxic compounds at low concentrations or during short exposure times tends to induce apoptosis, while more severe exposure conditions can cause necrosis (Klaassen, 2013). Thus, after exposure to a toxic agent, the decrease in cell viability is one of the first physiologically measurable effects, which can be used as a biomarker of effect. Currently, there are several approaches to assess cell viability such as the MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay or the Annexin V assay (Figure 8), and different vital dyes as fluorescein diacetate (FDA), propidium iodide (PI) or 7-amino-actinomycin D (7-AAD) and tetramethylrhodamine (TMR) red.

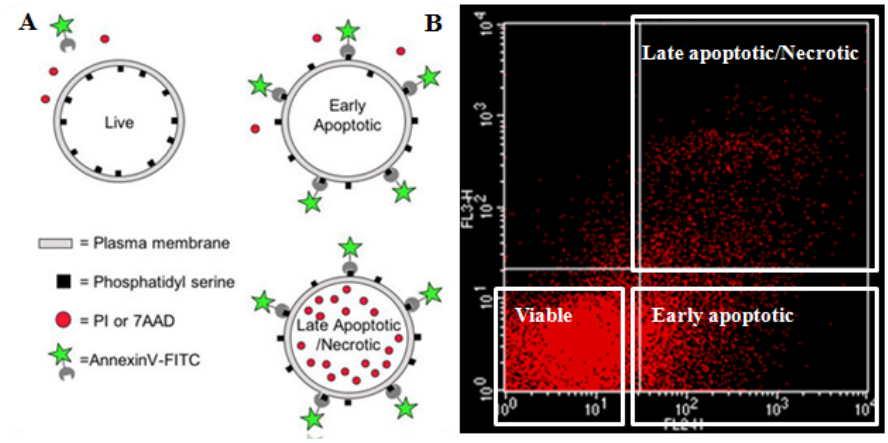


Figure 8: Detection of apoptotic cells by Annexin V staining. **A**) Since PI or 7AAD can not enter the live cells, phosphatidylserine (PS) is not exposed. In early stages of apoptosis PS is exposed, while in later stages of apoptosis PS exposed and 7-AAD can enter in the cell. Retrieved September 4, 2014 from: http://www.lifesci.dundee.ac.uk/cast/flow-cytometry-corfacility/techniques-3. **B**) The combination of Annexin V-7-AAD allows for the distinction between early apoptotic/late apoptotic/necrotic cells and viable cells.

Change in cell viability is one of the few cellular biomarkers used to assess the harmful effects on bivalve molluscs of all four major marine toxin categories (DSP, PSP, ASP and NSP). Firstly, Dizer et al. (2001) evaluated the toxicity of DA in mussel (*M. edulis*) hemolymph by means of FDA. Their results exhibited a significant increase in hemolymph viability after incubation with this toxin, supporting the activation of metabolism and resistance mechanisms as a consequence of exposure. Subsequently, Da Silva et al. (2008) and Mello et al. (2012) studied the hemolymph cell viability in clams (*R. philippinarum*) and oysters *(C. gigas*), respectively, exposed to brevetoxin. Da Silva et al. (2008) did not observe any alterations of the cell viability in clam using PI as vital dye. Similarly, Mello et al.

(2012) did not obtain significant differences in the viability of oyster hemocytes evaluated by MTT assay. Hégaret et al. (2011) suggested that, although some marine toxins act as immunostimulants, others can act as immunosuppressive agents, causing depression of immune functions, decrease in phagocytosis, production of oxidative stress, or decrease in cell viability. That is the case of the STX, since oyster (*C. virginica*) hemocytes decreased their viability after exposure to *Alexandrium fundyense* (Hégaret, et al., 2011). Similar results were found in the same work when clam (*Mya arenaria*) hemolymph was exposed to the OA-producer *P. minimum*. Medhioub et al. (2013) also observed an immunosuppression when oyster (*C. gigas*) hemocytes were exposed to PSP; however, effects disappeared after 48 h of exposure, showing an efficient mechanism to control apoptosis. This viability increase with time was also observed by Prado-Alvarez et al. (2013) when Annexin V assay was applied to study the effect of *P. lima* on clam (*R. decussatus*) hemocytes. These authors had previously found a decrease of cell viability, both *in vitro* and *in vivo*, in hemocytes of mussels (*M. galloprovincialis*) contaminated with high concentrations of OA (Prado-Álvarez et al., 2012). Besides, Estrada et al. (2010) found a correlation between the increase in the apoptotic rate and the activation of caspases in scallop (*Nodipecten subnodosus*) hemocytes exposed *in vitro* to STX**.**

Oxidative Stress

The antioxidant system of bivalve molluscs may in part explain their sensitivity to xenobiotic compounds (Livingstone, et al., 1990). Toxic effects of contaminants often depend on their capacity to increase the cellular levels of toxic ROS (Viarengo, et al., 2007). A wide range of contaminants elicit the production of ROS, including superoxide anion radicals (O^{2-}), and hydroxyl radicals ($OH^{\cdot}$).

In the absence of antioxidant activity and DNA repair mechanisms, the overproduction of ROS can trigger oxidative damage. Indeed, marine biotoxins produced during algal blooms can induce pro-oxidant activity through ROS generation as well as an increase of the antioxidant defense mechanisms, which may be used as a key tool in biomonitoring processes (Contardo-Jara, et al., 2008; Žegura, et al., 2004). The antioxidant systems are the first line of defense against ROS. These systems are composed of several molecules that work together in a sequential manner, destroying and preventing the formation of free radicals. Components of antioxidant systems can be categorized into two broad groups: antioxidant enzymes (including superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx] and glutathione reductase [GR]) and molecules without enzymatic activity (such as glutathione [GSH] and *N*-acetylcysteine [NAC]).

SOD, CAT and GPx are the three main antioxidant enzymes involved in the destruction of ROS in the cell (Manduzio, et al., 2005). SOD catalyzes the dismutation of the superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2). A range of SOD isoenzymes are known, which differ in their metal cofactor, including copper/zinc (Cu/Zn-SOD), manganese (Mn-SOD), iron (Fe-SOD), etc. These isoenzymes are found in several compartments of the cell. Although they are all supporting parts of antioxidant systems, Mn-SOD is less expressed than Cu/Zn-SOD in the tissues of some bivalves (Livingstone and Pipe, 1992; Manduzio, et al., 2005). CAT is an antioxidant enzyme which contains a heme group and nicotinamide adenine dinucleotide phosphate (NADPH) in its active center; it has been used as biomarker of oxidative stress in multiple species of bivalve molluscs (Lima, et al., 2007). This enzyme, like SOD, is present in peroxisomes, where catalyzes the conversion of H_2O_2 generated by SOD to H_2O and O_2. CAT activity is linked and complementary to GPx activity, since it also catalyses the

metabolism of H_2O_2 to water. Further, GPx activity is coupled to GSH oxidation and generates alcohols. Thus, GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), which is necessary for the enzymatic activity of GPx. On the other hand, glutathione *S*-transferase (GST) catalyzes the conjugation of xenobiotics with GSH.

Only a few studies have examined the antioxidant response of bivalve molluscs to toxin-producing algae (Estrada, et al., 2007; Qiu, et al., 2013; Sabatini, et al., 2011). Sabatini et al. (2011) documented a significant increase in the activity of SOD, CAT and GST, as well as in GSH in the freshwater clam *Diplodon chilensis* fed with toxic *Mycrocistis aeruginosa*. Similarly, Estrada et al. (2007) and Qiu et al. (2013) obtained an increase in the activity of SOD, CAT and GPx when scallops (*N. subnodosus*) and mussels (*M. galloprovincialis*) were fed with *Gymnodinium catenatum* and *Alexandrium tamarense*, respectively. However, decreases in the activity of SOD and CAT were observed in muscle, mantle and hemocytes when scallops (*N. subnodosus*) were injected with PSP toxin (Estrada, et al., 2010). A recent preliminary work studied the response of some antioxidant enzymes (CAT, GR, GPx and GST) in the digestive gland of *M. galloprovincialis* exposed to DSP toxins (Vidal, et al., 2014), corroborating a modulation of GPx and GST enzyme activity in relation to the OA accumulation during different toxic blooms. These authors also observed a different behavior of GR activity in relation to OA accumulation, which was attributed to the sex of the mussels. Thus, although GR activity increased in both sex after exposure to OA, males showed a positive correlation between the toxin accumulated and the activity of GR, while females showed a negative correlation between GR and OA accumulated at the beginning of the toxic episodes.

Lipid Peroxidation

When the antioxidant defense mechanisms are not able to neutralize ROS, oxidative stress may produce DNA damage, enzymatic inactivation and peroxidation of cell constituents, especially lipids of the cell membrane (Gutteridge and Halliwell, 1990). The oxidation of membrane lipids is considered one of the primary events in the oxidative cellular damage. A single oxidative event caused by a free radical can induce the oxidation of many molecules, providing an amplification of damage (Niki, et al., 1991). Lipid peroxidation is often related to antioxidant enzyme activities; under normal conditions, these enzymes detoxify oxidized lipids and preventing the spread of the lipidic peroxidation chain. The methods most widely used to quantify lipid peroxidation are those relating to the production of secondary lipid peroxidation products such as malondialdehyde (MDA) (Valavanidis, et al., 2006). MDA levels are usually measured by high performance liquid chromatography (HPLC) or by spectrophotometry. Overall, lipid peroxidation represents a particularly cytotoxic event with multiple effects on enzyme activity as well as on the initiation of apoptosis (Lesser, 2006). This is not an isolated process; hence, it is often assessed in combination with the analysis of antioxidant enzymes.

Various biomarkers of oxidative stress, including lipid peroxidation, were investigated in digestive gland, gills, mantle and adductor muscle of the scallop *Nodipecten subnodosus* exposed to PSP toxins (Estrada, et al., 2010). Results of this study showed an increase of lipid peroxidation together with alterations in the activity of some enzymes in gills, including increase of GPx, accompanied by reduction of SOD, which relates these events to oxidative damage. It has also been previously reported that peroxidation end-products are accumulated in lysosomes (Viarengo, et al., 2007). Accordingly, Svensson et al. (2003) observed a significant increase in the volume of the lysosomal compartment in cells of mussels (*M. edulis*)

after their exposure *in vitro* to different OA concentrations (10 nM – 1 µM), associated with storage of this toxin. Nevertheless, lack of significant accumulation of lipid peroxidation products was observed when mussels (*M. galloprovincialis*) were exposed to *Ostreopsis* cf. *ovate*, producer of ovatoxin and palytoxin (Gorbi, et al., 2012; Gorbi, et al., 2013).

Finally, although all these cellular and molecular biomarkers have been used to assess the effects of marine toxins in bivalve molluscs, they have not yet been employed to determine the immediate-early effects of DSP toxins in this group of marine organisms. This approximation is considered necessary for the knowledge of the function response of bivalve molluscs to DSP toxins, as well as developing new strategies for early detection of algal blooms associated with DSP toxins, which could reduce economic losses originated in the marine aquaculture. To this end, the following chapters of this thesis collected a set of *in vitro* and *in vivo* assays aimed at the assessment of the harmful effects of these toxins in bivalve molluscs, taking into account their absortion, distribution, metabolisms and depuration, as well as the synergistic effects between DSP toxins.

Objectives

The aim of this doctoral thesis is to investigate different aspects of the early responses of bivalve molluscs to diarrhetic shellfish poisoning toxins. For this purpose, this work addresses the following specific objectives:

1. To analyze the *in vitro* genotoxic and cytotoxic effects attributed to okadaic acid in different cell types of the mussel *Mytilus galloprovincialis.*

2. To study the *in vivo* genetoxic and cytotoxic effects of the exposure to DSP-toxin producing dinoflagellate *Prorocentrum lima* during the simulation of the an early harmful algal bloom (HAB) episode in different cell types of the mussel *M. galloprovincialis*.

3. To determine the transcriptional and biochemical response of different antioxidant enzymes in the most relevant tissues of the mussel *M. galloprovincialis* exposed to the toxic dinoflagellate *P. lima*.

Outline of the Thesis

Okadaic acid (OA) and its derivatives, the dinophysistoxins (DTXs), constitute the main diarrhetic shellfish toxins producing during harmful algal blooms (HABs) in the European coasts. Studies carried out on mammalian cell lines have demonstrated the potential of OA to induce cytogenotoxic damage. On the contrary, it seems that different marine organisms (notably bivalve molluscs) are resistant to the harmful effects associated with constant exposure to these toxins in the oceans. Given the little knowledge about the early effects and their potential relevance for toxicology and biomonitoring environmental, the present thesis deals with the analysis of immediate-early response of bivalve molluscs to DSP toxins. To achieve this goal, the thesis is divided into three chapters.

Chapter 1 of this thesis analyzes the *in vitro* genotoxic and cytotoxic effects of OA in the mussel *Mytilus galloprovincialis*, which is considered not only the main vector of DSP toxins, but also a sentinel organism. Two cellular biomarkers, DNA breaks and cell viability rates, are determined using the comet assay and flow cytometry, respectively. Both techniques are applied to hemolymph and gill cells based on their role during the early response to toxins. In that way, these *in vitro* assays establish the bases for the analysis of *in vivo* DSP effects that are studied in the following chapter.

Chapter 2 simulates experimentally an early HAB episode through the exposure of mussels to DSP-toxin-producing dinoflagellate *Prorocentrum lima* to achieve a better knowledge about the combined cytogenotoxic response to these toxins, taking into account their absorption, distribution, metabolisms and depuration in the mussel. *In vivo* assays are developed using the biomarkers and techniques employed in the previous chapter. Additionally, this chapter considers the use of the modified comet assay with the OGG1 enzyme to assess the oxidative DNA damage caused by

DSP toxins in hemolymph of mussels and discusses the correlation between *in vitro* and *in vivo* assays.

Chapter 3 considers the findings obtained in the previous chapter and concentrates on the early response of the antioxidant system of the bivalve molluscs to DSP toxins. To this end, an integrated transcriptomic and biochemical approaches are carried out in digestive glands and gills of mussels exposed to *P. lima*, providing a basis for developing molecular biomarkers of exposure to DSP toxins in bivalve molluscs.

Chapter 1

IN VITRO ANALYSIS OF EARLY GENOTOXIC AND CYTOTOXIC EFFECTS OF OKADAIC ACID IN DIFFERENT CELL TYPES OF THE MUSSEL *Mytilus galloprovincialis*

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(Journal of Toxicology and Environmental Health Part A 2015, 78:814-824)

SUMMARY

Okadaic acid (OA) is the predominant toxin responsible for diarrhetic shellfish poisoning (DSP) syndrome in humans. While its harmful effects have been extensively studied in mammalian cell lines, the impact on marine organisms routinely exposed to OA is still not fully known. Few investigations available on bivalve molluscs suggest less genotoxic and cytotoxic effects of OA at high concentrations during long exposure times. In contrast, no apparent information is available on how sublethal concentrations of OA affect these organisms over short exposure times.

The present chapter addressed for the first time *in vitro* analysis of early genotoxic and cytotoxic effects attributed to OA in two cell types of the mussel *Mytilus galloprovincialis*. Accordingly, hemocytes and gill cells were exposed to low OA concentrations (10, 50, 100, 200 or 500 nM) for short periods of time (1 or 2 h). The resulting DNA damage, apoptosis and necrosis were subsequently quantified using comet assay and flow cytometry, respectively.

Data demonstrated that (1) mussel hemocytes seem to display a resistance mechanism against early genotoxic and cytotoxic OA-induced effects, (2) mussel gill cells display higher sensitivity to early OA-mediated genotoxicity than hemocytes, and (3) mussel gill cells constitute more suitable systems to evaluate the genotoxic effect of low OA concentrations in short exposure studies. Taken together, this investigation provides evidence supporting the more reliable suitability of mussel gill cells compared to hemocytes to evaluate the genotoxic effect of low short duration exposure to OA.

INTRODUCTION

Harmful algal blooms (HAB) are oceanographic phenomena characterized by the proliferation and occasional dominance of particular species of toxic or harmful algae (Anderson, 2007). Examples of HAB species include *Dynophysis* and *Prorocentrum* dinoflagellates, which are the predominant producers of the toxin Okadaic Acid (OA) (Yasumoto, et al., 1985), responsible for diarrhetic shellfish poisoning (DSP). OA is generally accumulated by marine organisms (especially shellfish) due to its thermostable lipophilic nature, entering the human food chain and producing the DSP syndrome, which is characterized by nausea, abdominal pain and diarrhea (Dominguez, et al., 2010).

The frequency, geographical area, intensity and magnitude of HAB have increased during the last few decades (Díaz, et al., 2013; Van Dolah, 2000), especially in coastal areas dependent upon the economic input of aquaculture and fisheries (Rodríguez, et al., 2011). Altogether, the economic impact of HAB on European coasts is in the order of 177 million Euro per year (Eriksson, 2011).

The molecular basis underlying the harmful effects of OA was discovered by Bialojan and Takai et al. (1988), demonstrating the toxin ability to inhibit several types of serine/threonine protein phosphatases. Since then, a large number of studies contributed to better understand the influence of OA (Valdiglesias, et al., 2012a; Valdiglesias, et al., 2012b), showing the role played in promoting apoptosis, cytoskeleton disruption and cell cycle alterations in mammalian cell lines (Valdiglesias, et al., 2013). Unfortunately, the assessment of OA-induced toxicity in marine organisms, which constitute an important economic and ecological relevance, is still limited (Prego-Faraldo, et al., 2013). However, the few studies that are available on bivalve molluscs indicated that OA produces the most dramatic

genotoxic and cytotoxic effects occur at low concentrations after short exposure duration, whereas high concentrations and longer exposures initiate resistance mechanisms (Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2003). Such resistance behavior was adscribed to frequent and ongoing contact of bivalves with this toxin in the marine environment (Flórez-Barrós, et al., 2011; Svensson and Förlin, 1998).

Among all marine species affected by OA, the mussel *Mytilus galloprovincialis* offers a dual benefit for assessing the harmful effects of this toxin: (1) this species is the aquaculture resource most severely affected by HAB, leading to drastic economic losses in coastal areas; and (2) the wide geographical distribution of mussels combined with their sessile and filter-feeding lifestyle (Viarengo and Canesi, 1991) make this organism an appropriate sentinel organisms widely used in pollution biomonitoring studies (Goldberg, 1986). The choice of mussels as sentinel organisms is further supported by the suitability of their tissues for experimental procedures. Hemolymph cells are easily extracted and separated to readily examine critical roles in the immune response to marine toxins (Haberkorn, et al., 2010; Hégaret, et al., 2007; Hégaret, et al., 2011). Gill cells are the first part of the organism to enter into contact with marine toxins and other compounds dissolved in the water, thus often used as model systems in bivalve ecotoxicological studies (Akcha, et al., 2004; Flórez-Barrós, et al., 2011; Hanana, et al., 2012; Rank and Jensen, 2003; Talarmin, et al., 2008; Venier, et al., 1997).

The aim of this study was to further our understanding of the harmful effects of OA on marine invertebrates using mussels as model organisms. Indeed, for the first time the early effects of this toxin was determined at genetic and cytological levels in different tissues.

MATERIAL AND METHODS

Sample Collection

Mussels (*M. galloprovincialis*) were collected in 2014 from a commercial mussel raft from Lorbé in the Ria of Ares-Betanzos (Galicia, NW Spain, Figure 1). This location was selected based upon the presence of low OA levels as reported by the Galician aquaculture administration (www.intecmar.org). Mussels were acclimated to lab conditions for 48 h in highly aerated tanks with filtered sea water in a chamber of photoperiod (18 ºC, 12 h light-dark cycle), and fed daily with a 1:1 mixture of two microalgae species (*Isochrysis galbana* and *Tetraselmis suecica*).

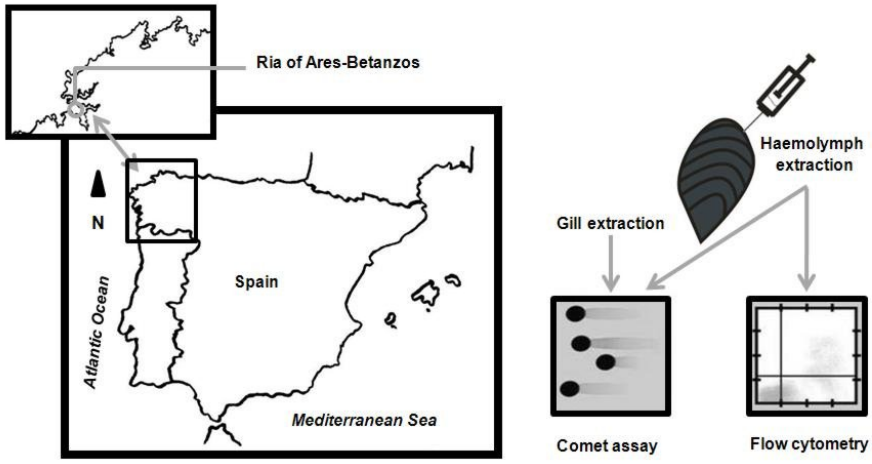

Figure 1: Sampling location of mussel specimens (*M. galloprovincialis*) in the Ria of Ares-Betanzos (Galicia, northwestern Spain). Hemolymph and gill cells were extracted, individualized and subsequently exposed to short pulses of low OA concentrations. The resulting DNA damage and apoptosis/necrosis levels were quantified using the comet assay and flow cytometry, respectively.

Sample Preparation: Isolation of Haemolymph and Gill Cells

H*emolymph* was extracted from the posterior adductor muscle of each mussel with a sterilized syringe. Samples (1.5 mL) were mixed simultaneously with precooled anticoagulant solution (modified Alsever´s: NaCl 382 mM, glucose 115 mM, sodium citrate 27 mM, ethylenediamine tetraacetic acid [EDTA] 11.5 mM), 1:5 (hemolymph:Alsever). Samples (20 mussels) were pooled to eliminate inter-individual variations and the resulting pool was filtered using a nylon mesh (55 µm diameter). The number of hemocytes was determined post-filtering by counting in a Thoma chamber (Marienfeld, Lauda-Königshofen, Germany) under the microscope.

Gill cells were isolated from the same 20 mussel individuals following a modified protocol described by Pérez-Cadahía et al. (2004). After dissection at room temperature, gills were washed three times in 2 mL ice-cold calcium magnesium-free saline solution (CMFS: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl, 5mM EDTA in RPMI medium, pH 7.5). Gills were shredded after the last wash and cell suspension was added to 6 mL CMFS and shaken gently for 1h at 4°C in the dark. The entire suspensions were subsequently distributed (1 mL per tube), filtered through a nylon mesh (55 µm diameter) and centrifuged at 500 *g* for 5 min. The resulting pellet was re-suspended in 1 mL Kenny´s salt solution (KSS: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$, pH 7.5) and kept on ice. Gill cell number was determined using microscopy.

In both tissues, cell viability was determined by trypan blue exclusion method obtaining viability values above 80% in all samples.

***In vitro* Exposure to Okadaic Acid**

Hemolymph and gill cells were incubated *in vitro* with OA (Sigma, CAS No. 78111-17-8) for 1 or 2 h. Temperature was kept between 15-18 ºC

during each exposure. OA was diluted in DMSO to obtain the final concentrations of 10, 50, 100, 200 or 500 nM. Final volumes of 10 µL of each OA solution were added to cell suspension. DMSO was used as negative control solution, whereas hydrogen peroxide (100 µM, 10 min) or camptothecin (4 µM, 4 h) were used as positive controls in comet assay and flow cytometry experiments, respectively.

Assessment of DNA Damage Using Comet Assay

Comet assay, also known as single-cell gel electrophoresis (SCGE) assay, is a sensitive, easy, rapid and quantitative technique providing detection of DNA damage in individual cells. The comet assay capacity to detect damage in cells of marine organisms was previously established (Lee and Steinert, 2003; Mitchelmore and Chipman, 1998; Wilson, et al., 1998) and its application for the study of marine toxin effects has increased dramatically in recent years (da Silva, et al., 2011; Flórez-Barrós, et al., 2011; Juhel, et al., 2007; McCarthy, et al., 2014).

In this investigation, the comet technique used was based on the alkaline assay described by Wilson et al. (1998), with minor modifications. Accordingly, hemolymph and gill cells were centrifuged for 5 min at 250 *g* and 3 min at 1000 *g*, respectively. The resulting pellet was re-suspended in 90 µL 0.5% low-melting-point agarose (Invitrogen) in KSS; each sample was divided in two and placed on a slide pre-coated with a layer of 0.5% normal-melting-point agarose (Intron Biotechnology, Seoul, Korea). After 25 min at 4 °C, the slides were placed in a Coplin jar with lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 250 mM NaOH, 10 mM Tris–HCl, 1% sarcosyl, pH 10 with 1% Triton X-100 added just before use) for 1 h at 4 °C. From this point on, all steps were conducted in dark to prevent additional DNA damage. After lysis, slides were placed in alkaline solution

(0.3 M NaOH, 1 mM Na_2EDTA, pH >13) for 20 min for DNA unwinding, followed by electrophoresis in the same solution for 20 min (0.83 V/cm).

After electrophoresis, slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.5) and stained with 4,6-diamidino-2-phenylindole (DAPI). Image capture and analysis were performed using the Comet IV Software (Perceptive Instruments, Bury St Edmunds, UK). Fifty cells were scored from each replicate slide (100 cells in total) and the percentage of DNA in the tail (%tDNA) was used as DNA damage parameter.

Assessment of Cytotoxicity Using Flow Cytometry

Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) staining were used in flow cytometry experiments to evaluate apoptosis/necrosis resulting from OA exposure. BD PharmingenTM Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used according to the manufacturer´s instructions with minor modifications.

After OA exposure, hemolymph samples were centrifuged at 250 *g* for 5 min at 4º C. Cell pellets were re-suspended in 200 µL Annexin binding buffer (0.5X) previously diluted in saline solution (NaCl, 500 nM), yielding a final concentration ranging between 4 x 10^5 and 6 x 10^5 cells per mL. Samples were incubated with Annexin V-PE and 7-AAD (1 mg/mL) for 15 min at room temperature in the dark. Cells were then analyzed by flow cytometry.

Hemocytes population was fixed in the dot-plot according to Prado-Alvarez et al. (2012). Flow cytometry analyses were performed in a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A minimum of 20,000 events were acquired in each case and fluorescence signal for Annexin V-PE and 7-AAD were measured using the FL-2 and

FL-3 detectors, respectively. The % apoptotic and dead cells were analyzed using Cell Quest Pro software (Becton Dickinson, Mountain View, CA, USA). Early apoptosis and late apoptosis/necrosis were expressed as % Annexin V+/7-AAD- and Annexin V+/7-AAD+ cells, respectively.

Statistical Analyses

Three independent experiments were performed for each OA exposure condition tested. Experimental data were expressed as mean ± standard error and tested for normality using the Kolmogorov-Smirnov test. Since data obtained did not fit a normal distribution, analyses were performed using non-parametric tests. Differences between groups were tested using Kruskal-Wallis test and the Mann-Whitney's *U*-test. Dose-response relationships for apoptotic/necrotic rates were initially analyzed using Pearson´s correlation coefficient and Spearman's rank correlation coefficient (*r*). Since both methods yielded similar results, Spearman´s coefficient was selected as the most appropriate approach for testing monotonic relationships between variables that are not normally distributed. *P*-values < 0.05 were considered significant. Statistical analyses were performed using the IBM SPSS software package V. 20 (IBM, Armon, NY, USA).

RESULTS

The early effects of OA exposure on mussel DNA integrity were studied by exposing cells to low concentrations of OA (10, 50, 100, 200 or 500 nM) for short periods of time (1 or 2 h). DNA damage and cytotoxicity were evaluated using the comet assay and flow cytometry, respectively (Figure 1). Experiments were developed in two different tissues based on their role during early responses to toxins: a) hemolymph which is in close contact with toxins in the open circulatory system of mussels, b) gills which

constitute the first tissue to enter in contact with OA-producing dinoflagellates during HAB episodes.

Evaluation of DNA Damage After *in vitro* Exposure to OA

The quantification of DNA damage in hemolymph cells resulting from OA exposure is shown in Figure 2. No significant differences were observed between OA-treated hemocytes for 1 *vs* 2 h with the exception of 10 nM OA at 2 h. In contrast, incubation of gill cells with OA for 1 h (Figure 3) produced significant increases in DNA damage at 50, 100, 200 or 500 nM, with a marked concentration-response correlation.

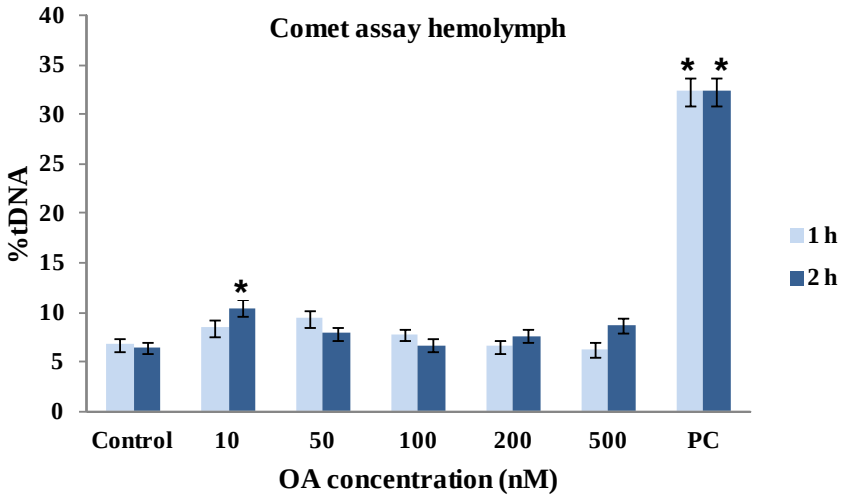

Figure 2. DNA damage quantified using comet assay in mussel's hemolymph cells after *in vitro* exposure to different OA concentrations for 1 and 2 h. Control and PC represent negative and positive controls, respectively. %tDNA represent percentage of DNA in the comet tail. **P* < 0.05 indicate significant differences from negative control in Mann-Whitney's *U*-test.

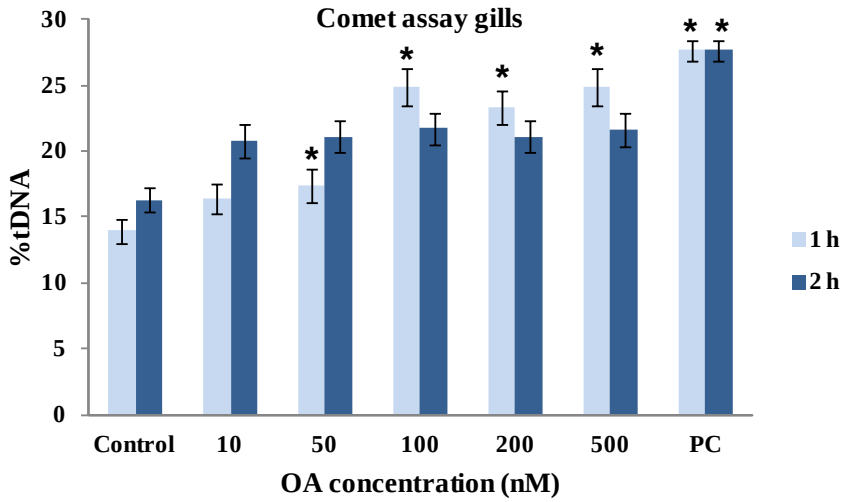

Figure 3. DNA damage quantified using comet assay in gill cells after *in vitro* exposure to OA. %tDNA represent percentage of DNA in the comet tail. Control and PC represent negative and positive controls, respectively. *$P < 0.05$ indicate significant differences from negative control in Mann-Whitney's *U*-test.

A possible explanation for the lack of significant damage after 2 h might reside in the progressive rise in basal DNA damage over time. In addition, it is important to bear in mind that the mechanical procedures employed in the isolation of gill cells, together with tissue-specific features, may be contributing to enhanced basal DNA damage. To test this hypothesis, basal DNA damage levels were compared between negative controls in hemolymph and gill cells. Our results seem to refute this hypothesis, as no significant DNA damage differences were found between gill controls at 1 and 2 h (Figure 4). However, significant differences between controls from different tissues were observed, with gill cells displaying higher basal damage levels. Taken together, our comet results suggest that: a) OA exerts different genotoxic potential in different cell types, and b) gill cells constitute a more appropriate tissue to assess DNA damage in mussel after 1 h exposure.

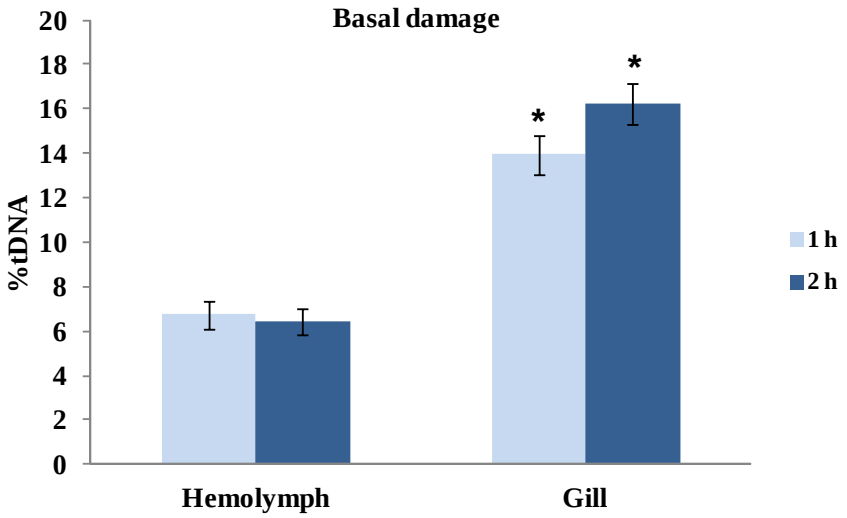

Figure 4. Comparison between basal DNA damage levels in negative controls from hemolymph and gill cells after 1 h and 2 h exposure of OA. %tDNA represent percentage of DNA in the comet tail. *P < 0.05 indicate significant differences using Mann-Whitney *U*-test.

Evaluation of Apoptosis/Necrosis After *in vitro* Exposure to OA

The cytotoxic effect of early OA exposure was studied in mussels in parallel to DNA damage experiments using the same toxin concentrations and exposure times. Apoptosis and necrosis rates were measured using flow cytometry analyses of Annexin V/7-AAD staining. While this approach has multiple advantages (high efficiency, clear distinction between apoptotic and necrotic cells, high degree of automation) it is also conditioned by the specific characteristics of the tissue under study, limiting the present analyses to hemolymph cells. In that specific tissue, our results showed that OA lacks cytotoxic potential (Figure 5A), with the exception of high levels of necrosis at 500 nM OA after 2 h (Figure 5B). Still, significant concentration-response relationships were obtained for necrosis after 1 and 2 h (Figures 5A and 5B). In summary, flow cytometry results suggest that: a) although OA exposure did not produce significant cytotoxic damage at

low concentrations, the contribution of apoptosis to the overall cytotoxicity is substantially higher than of necrosis; b) flow cytometry may only be useful to study OA-mediated cytotoxicity in hempolymph after long exposure periods.

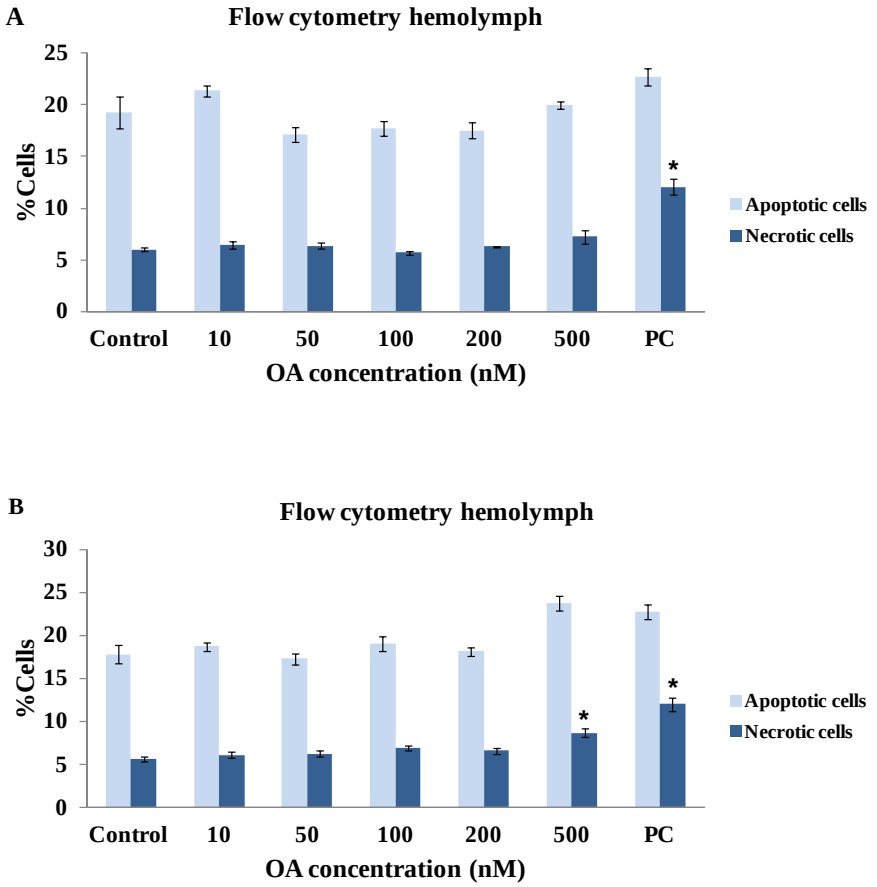

Figure 5. Flow cytometry evaluation of cytotoxicity in hemolymph cells treated with OA for 1 (**A**) and 2 h (**B**). Control and PC represent negative and positive controls, respectively. **P* < 0.05 indicate significant differences from negative control in Mann-Whitney's *U*-test.

DISCUSSION

Early Genotoxic Effect of OA in Hemolymph and Gill Cells in Mussels

Comet assay results revealed an overall absence of significant DNA damage in hemolymph cells after exposure to OA. However a significant increase in DNA damage was noted at 10 nM OA after 2 h exposure. These results are in agreement with the lack of DNA damage observed in hemocytes from the clam *Ruditapes decussatus* after *in vivo* exposure to different concentrations of the OA-producing dinoflagellate *Prorocentrum lima* (Flórez-Barrós, et al., 2011). Similarly, low levels of micronucleus formation were also reported for mussel hemocytes after exposure to OA (Pinto-Silva, et al., 2003). Flórez-Barrós et al. (2011) noted the absence of DNA damage to exposure to less active metabolite of OA in circulating cells which might be attributed to hydrolysis and acylation of parent OA. These metabolic pathways were proposed as the main mechanisms by which *M. galloprovincialis* might metabolize OA and diminish toxicity (Rossignoli, et al., 2011). Pinto-Silva et al. (2003) suggested that the apparent lack of damage might have resulted from either exposure to excessive quantities of OA, the short life span of hemocytes or a chronic OA effect.

Our results support a rapid genotoxic effect mediated by OA on hemocytes, suggesting the presence of an immediate cellular protective response against OA in those organisms frequently exposed to this toxin (Svensson and Förlin, 1998; Svensson, et al., 2003). These results need to be considered with caution, as at least one study has demonstrated increased damage levels at high OA concentrations in mussels and oysters (McCarthy, et al., 2014).

In contrast to hemocytes, gill cells displayed a significant increase in DNA damage at all OA concentrations studied with the exception of the lowest,

10 nM. In addition, a clear positive concentration-response correlation was found after 1 h exposure, disappearing at 2 h. As in the previous case, these results are in agreement with those described for gill cells in the clam *R. decussates* (Flórez-Barrós, et al., 2011), where higher levels of DNA damage occurred at low OA concentrations and short duration exposure.

The observed stability in DNA damage after 2 h is likely due to greater basal damage at these specific conditions. Consequently, this can may account for differences detected with regard to control at 1 h fall after 2 h OA treatment, thus leading to a false negative response under this second condition. A similar picture emerges when comparing digestive gland and hemolymph with higher levels of basal damage in the former (McCarthy, et al., 2014).

An alternative explanation for the maintenance of genetic damage might be induction of apoptosis by OA (Flórez-Barrós, et al., 2011). Accordingly, only cells more resistant to OA (and therefore less damaged) would prevail, producing false results in comet assays. Within this scenario, mussel gill cells would be only useful for the assessment of OA-induced genotoxicity at short exposure duration. However, usefulness of gill cells continues to be hampered by damage increase created during the cell isolation procedure.

Overall, our results suggest that, in the case of mussels, hemolymph is more resistant than gill cells to OA-mediated genotoxicity. This notion is consistent with earlier reports showing heterogeneous levels of OA-induced genotoxicity depending on the specific cell type in mammals (Souid-Mensi, et al., 2008; Valdiglesias, et al., 2010) as well as in oysters (Hanana, et al., 2012; Talarmin, et al., 2008) and clams (Coughlan, et al., 2002; Flórez-Barrós, et al., 2011). Recently, McCarthy et al (2014) described similar OA-mediated genotoxic effects in hemolymph and digestive gland cells from blue mussel and pacific oyster. This behavior may be attributed to the roles

played by these tissues during immune and detoxification responses which are different from the respiratory role of gills. Further, gills constitute the first tissue to enter into contact with marine toxins in the external environment, thus experiencing higher genotoxic potential. Finally, it was suggested that differential resistance to DNA damage across different tissues might in fact be dependent on the specific type of genotoxic compounds (Venier, et al., 1997). Given the considerable resistance of mussel hemolymph to OA-induced genotoxicity, gill cells seem more appropriate to assess genotoxicity at low OA concentrations and short exposure times.

Early Cytotoxic Effect of OA in Hemolymph Cells

Flow cytometry experiments did not reveal marked differences in OA-mediated cytotoxicity in hemolymph cells exposed to different OA concentrations after 1 h. However, a significant positive concentration-response relationship was found after 2 h both for apoptosis and necrosis, supporting previous results obtained in mussels (Prado-Alvarez, et al., 2012). An absence of cytotoxic effects after short exposure times (1 h) to different OA concentrations was also reported by Talarmin et al. (2008) in cultured oyster heart cells. Indeed, data suggested that lack of cytotoxicity might be due to the presence of caspase inhibitors (Flórez-Barrós, et al., 2011; Rossini, et al., 2001). In contrast, Prado-Álvarez et al. (2013) found a significant rise in % apoptotic/necrotic cells when hemolymph cells of the clam *R. decussatus* were exposed to OA for 2 and 4 h. To a lesser degree, this elevation was also observed in our results. Therefore, consistent with our DNA damage results, our cytotoxicity analyses suggest that hemolymph cells appear more resistant to OA compared to other cells in bivalve molluscs, as suggested by Hégaret et al. (2011).

Findings reported in the present study resemble those obtained by Mello et al. (2010), showing that mussels are more immunologically active than other bivalves when exposed to a natural bloom of *Dinophysis acuminate*, a prominent producer of OA. The stress induced by OA may also influence the mussel´s immune system as well as phagocytic activity (Malagoli, et al., 2008), since hemocytes are mainly responsible for the phagocytic process in bivalve molluscs. However, the significant concentration-response correlation findings suggests that hemolymph cytotoxic resistance to OA is reduced over time, which might be due to the role hemocytes play in early response to OA-mediated cytotoxicity. Overall, our observations suggest that the effectiveness of hemocytes might not be sufficient at the time of response to a natural HAB episode that lasts for a long period of time.

CONCLUSIONS

This study constitutes the first research effort assessing the early effects of OA in different mussel cell types using comet assay and flow cytometry. Our results significantly contribute to expanding our current understanding regarding genotoxic and cytotoxic consequences following exposure to sublethal levels of this toxin. Several key conclusions may be drawn from this study: (1) our results suggest the presence of a resistance mechanism against early genotoxic and cytotoxic OA-induced effects in mussel hemocytes, (2) mussel gill cells display higher sensitivity to early OA-mediated genotoxicity than hemocytes, and (3) mussel gill cells constitute more suitable systems to evaluate genotoxic effect of low OA concentrations for short exposure durations. Overall, the present investigation provides a framework for future molecular cytogenetic studies aimed at developing rapid, sensible and efficient biomonitoring mechanisms to assess sublethal effects of OA in marine invertebrates.

Chapter 2

EARLY GENOTOXIC AND CYTOTOXIC EFFECTS OF THE TOXIC DINOFLAGELLATE *Prorocentrum lima* IN THE MUSSEL *Mytilus galloprovincialis*

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(Toxins 2016, 8:159)

SUMMARY

Okadaic acid (OA) and dinophysistoxins (DTXs) are the main toxins responsible for diarrhetic shellfish poisoning (DSP) intoxications during harmful algal blooms (HABs). Although the genotoxic and cytotoxic responses to OA have been evaluated *in vitro*, the *in vivo* effects of these toxins have not yet been fully explored. The present chapter fills this gap by evaluating the *in vivo* effects of the exposure to the DSP-toxin-producing dinoflagellate *Prorocentrum lima* during the simulation of an early HAB episode in the mussel *Mytilus galloprovincialis*. The obtained results revealed that *in vivo* exposure to this toxic microalgae induced early genotoxicity in hemocytes, as a consequence of oxidative DNA damage. In addition, the DNA damage observed in gill cells seems to be mainly influenced by exposure time and *P. lima* concentration, similarly to the case of the oxidative damage found in hemocytes exposed *in vitro* to OA. In both cell types, the absence of DNA damage at low toxin concentrations is consistent with the notion suggesting that this level of toxicity does not disturb the antioxidant balance. Lastly, *in vivo* exposure to growing *P. lima* cell densities increased apoptosis but not necrosis, probably due to the presence of a high number of protein apoptosis inhibitors in molluscs. Overall, this work sheds light into the *in vivo* genotoxic and cytotoxic effects of *P. lima*. In doing so, it also demonstrates for the first time the potential of the modified (OGG1) comet assay for assessing oxidative DNA damage caused by marine toxins in marine invertebrates.

INTRODUCTION

Harmful algal blooms (HABs) constitute a major environmental threat for marine organisms and human consumers of shellfish. During the last few years, HABs have been displaying increased frequencies and intensities

(Díaz, et al., 2013; Reguera, et al., 2014). Okadaic acid (OA) and its derivatives, the dinophysistoxins (DTXs), constitute the main lipophilic toxins produced during HABs in the Atlantic coast of Europe (Rodríguez, et al., 2015). During these episodes, large amounts of these toxins are produced by dinoflagellates from the genera *Dinophysis* and *Prorocentrum*. Their subsequent accumulation by marine organisms is responsible for the Diarrhetic shellfish poisoning (DSP) syndrome (Tubaro, et al., 2008), a disorder causing vomiting, diarrhea, and abdominal pains, among other symptoms, in human consumers of contaminated shellfish (Gestal-Otero, 2014). In order to prevent intoxications, the European Union has limited the harvesting and sale of shellfish with OA levels above 160 µg of OA equivalent/kg dry weight (EC 853/2004). Overall, HABs impact aquaculture (Smaal, 2002) and the economy of coastal areas, especially those that are heavily dependent on this industry (Rodríguez, et al., 2011; Rodríguez, et al., 2015).

The harmful effects of DSP toxins on human health have motivated studies addressing the mechanisms of action of these molecules, notably in the case of OA and its inhibitory activity on serine/threonine protein phosphatases (Bialojan and Takai, 1988). Additionally, it has been reported that OA derivatives (*i.e.*, acyl derivatives) may lead to toxicity without actually binding protein phosphatases, although little is known about their effects and synergistic interactions (Konoki, et al., 2013). OA causes alterations on the DNA molecule, on cellular components, on the immune and nervous systems, as well as on the embryonic development of mammalian cells (Valdiglesias, et al., 2013). Studies conducted in invertebrates, more specifically in bivalve molluscs, have suggested that these organisms are able to accumulate high concentrations of DSP toxins thanks to dedicated resistance and detoxification mechanisms. Indeed, while low levels of OA appear to produce an early cytogenotoxic response, the cytogenetic integrity

of some cell types recovers rapidly after exposure to high and persistent concentrations of this toxin (Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2005; Prego-Faraldo, et al., 2015).

The number of studies addressing the biological responses of marine invertebrates to DSP toxins has increased dramatically over the last 10 years, however, most of them rely on *in vitro* approaches for toxicity assessment [*i.e.*, exposure of organisms to purified individual toxins (Flórez-Barrós, et al., 2011; Prego-Faraldo, et al., 2015)], as these often increase the speed, precision, and reproducibility of analyses while reducing costs (Bravo, et al., 2001; Wernersson, et al., 2015). On the contrary, the number of *in vivo* studies using complex toxin mixtures are still scarce, including studies considering *Prorocentrum lima*, the dinoflagellate most commonly used as a source of DSP toxins (Bravo, et al., 2001; Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2005; Prego-Faraldo, et al., 2015; Wernersson, et al., 2015). These are particularly interesting because they allow the potential to determine the indirect effects of toxins, improving the assessment of the different types of organismal responses including absorption, distribution, metabolism, and depuration (Kilemade and Quinn, 2003). Although certain studies have observed a correlation between both assays (Taju, et al., 2012), the extrapolation of *in vitro* data to *in vivo* situations is often problematic (Garle, et al., 1994). Thus, the most realistic way to evaluate the synergistic effects of all toxins involved in HABs episodes would optimally involve the combination of both approaches.

The present chapter builds on this knowledge to investigate the genotoxic and cytotoxic responses of the mussel *Mytilus galloprovincialis* to low densities of the toxic dinoflagellate *P. lima*, a producer of DSP toxins. To this end, DNA damage was evaluated on hemolymph and gill cells using alkaline comet assay. Hemocytes (based on their low basal damage and easy

individualization) were also used to assess the oxidative DNA damage and cytotoxic effects of these toxins using the modified (OGG1) comet assay and flow cytometry, respectively. The obtained results revealed, for the first time, the dynamics of the genotoxic and cytotoxic damage resulting from the *in vivo* exposure to low densities of *P. lima* in marine invertebrates. In doing so, this study pioneers the use of the modified (OGG1) comet assay as a valid experimental approach improving the evaluation of the oxidative DNA damage caused by marine toxins in the hemolymph of marine invertebrates.

MATERIAL AND METHODS

Specimen Collection and Microalgae Cultures

M. galloprovincialis individuals (5–7 cm shell length) were obtained from a commercial mussels raft from Lorbe in the Ria of Ares-Betanzos (Galicia, NW Spain) in April 2015 (Figure 1). The invertebrate animals experiment was assessed by the Spanish Ministry of Economy and Competitivity (project AGL2012-30897 and approved on 28 December 2012). These rafts [previously used in our research (Prego-Faraldo, et al., 2015)] were chosen as sampling sites based on the low density of toxic microalgae (www.intecmar.org). Mussels were acclimated to laboratory conditions (18 °C, 12 h light-dark cycle) for a week in vigorously aerated tanks with filtered sea water, and fed two times a day with a 1:1 mixture of two nontoxic microalgae species (*Isochrysis galbana* and *Tetraselmis suecica*).

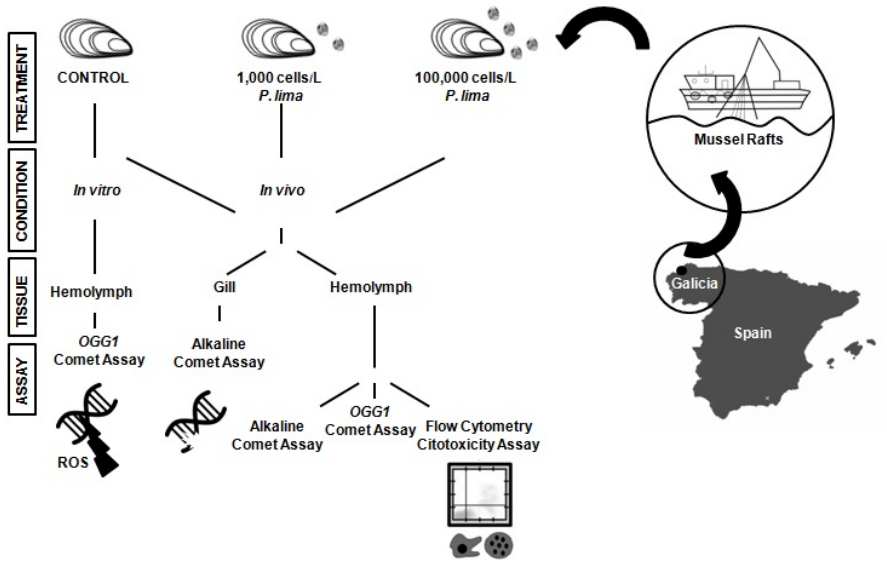

Figure 1: Schematic diagram describing the experimental design followed in the present chapter. Mussel specimens were collected and acclimated to laboratory conditions before exposing them *in vivo* to different cellular densities of the diarrhetic shellfish poisoning (DSP)-producing dinoflagellate *P. lima* for 24 h and 48 h. The genotoxic and cytotoxic effects of the exposure to *P. lima* were evaluated by means of the comet assay (alkaline and OGG1-modified) and flow cytometry in different cell types. A group of mussels was also exposed in vitro to okadaic acid (OA) to quantify oxidative DNA damage in hemolymph cells using the OGG1-modified comet assay.

The culture of the DSP-toxin-producing dinoflagellate *P. lima* (strain AND-A0605) was obtained from the Quality Control Laboratory of Fishery Resources (Huelva, Spain). The production of OA (the main DSP toxin) in *P. lima cultures* was quantified as 0.4 pg OA/cell using high performance liquid chromatography/mass spectrometry (HPLC/MS). Based on this data, mussels were exposed to two different cell densities (1,000 and 100,000 cells/L) of toxic dinoflagellate for 24 h and 48 h, simulating OA concentrations observed during the early stages of the development of an algal bloom (Diaz, et al., 2013). Cell concentrations in the *P. lima* culture were determined by cell count in Sedgwick-Rafter counting slides (Pyser-Sgi, Edenbridge, UK) after fixation with Lugol´s solution.

Sample Preparation and HPLC/MS Analysis

HPLC/MS analyses were carried out by the chromatography unit at SAI-University of A Coruña, following the protocol of the European Union Reference Laboratory for Marine Biotoxins (www.aesan.msssi.gob.es). Toxin extractions were performed without alkaline hydrolysis before MS, consequently, these included complete extraction of OA, plus partial extraction of DTXs. For that purpose, certified Reference Material Mussel Tissues with certified values of 10.1 ± 0.8 µg OA/g and 1.3 ± 0.2 µg DTX1/g (NRC CRM-DSP-Mus-b; Institute for Marine Biosciences, National Research Council of Canada, Halifax, NS, Canada) were used for recovery determination in the HPLC/MS method. Accordingly, mussel tissues (20 g per sample) were lyophilized (Christ LMC-2, model beta 2–16, Christ, Osterode, Germany) and 2 g of the liophilizate were extracted three times with 15 mL of 100% methanol and homogenized for 1 min. The methanolic phase was subsequently centrifuged (5000 *g* for 25 min) and the supernatant was filtered (0.45 µm pore size) and transferred to a 50 mL volumetric flask.

HPLC/MS analyses were performed on a Thermo LTQ Orbitrap instrument (Thermo Fisher Scientific, Bremen, Germany), using a C18 column (5 mm, 150 mm × 4.6 mm) at 30 °C (Phenomenex, Aschaffenburg, Germany). The mobile phase A was 100% water with 16 mM ammonium formate, and the mobile phase B was 100% acetonitrile. Gradient elution from 30% to 90% B was performed over 6 min; then, 90% B and 10% A were held for 6 min, decreased to 30% B over 4 min, which was held again for 10 min until the next run. The flow rate was set at 0.5 mL/min using an injection volume of 20 µL. Detection was performed using electrospray ionization (ESI) coupled with multiple reaction-monitoring (MRM). The electrospray capillary was set at 4 kV, the nebulizer at 50 arbitrary units, dry gas at 50

arbitrary units, and dry temperature at 350 °C. Data analyses and peak integration were accomplished through the ThermoXcalibur™ software (Thermo Fisher Scientific, San José, CA, USA).

In vivo Exposure to *P. lima*

After acclimation, mussels were randomly divided into three groups (Figure 1) including: a control group fed with a 1:1 mixture of the microalgae *I. galbana* and *T. suecica*, and two experimental groups fed with the DSP toxin-producing *P. lima* (1,000 and 100,000 cells/L, respectively, four times a day). Total OA body burden was determined in tissue homogenates after exposures (indirect measure of *P. lima* intake by mussels) according to the procedure described above. Mussel specimens were exposed to *P. lima* cultures in groups of n = 25 individuals (three replicates). Experimental samples were randomly sampled from tanks to complete a total of 20 g of pooled mussel tissue (using approximately n = 5–8 individuals), which was subsequently lyophilized. Analyses were performed from a sample (2 g) of each lyophilized fraction. Complementary to *in vivo* exposures, the assessment of oxidative DNA damage was completed using the modified comet assay on hemolymph pools from 20 control individuals exposed *in vitro* to OA (Sigma-Aldrich, St. Louis, MO, USA) for 1 h and 2 h [these conditions were selected as representative of early genotoxic effects of OA based on our previous analyses (Prego-Faraldo, et al., 2015)]. For that purpose, OA was diluted in dimethyl sulfoxide (10, 50, 100, 200, and 500 nM final concentration) and 10 µL of each OA solution was added to cell suspensions (15 °C to 18 °C). Exposures were completed by pelleting cells (825 *g* for 3 min) before proceeding with the comet assay. *In vitro* incubations with hydrogen peroxide (100 µM for 10 min and 100 µM for 2 min) or camptothecin (4 µM for 4 h) were used as positive control in the comet assay, modified comet assay, and flow cytometry experiments, respectively.

Isolation of Hemocytes and Gill Cells

Hemolymph and gill cells were used to quantify DNA damage using the alkaline comet assay. Additionally, hemolymph cells were used to assess oxidative DNA damage and cytotoxicity by means of the modified comet assay and flow cytometry, respectively (Figure 1). Hemolymph samples from each mussel (1.5 mL) were withdrawn from the posterior adductor muscle with a sterilized syringe and were mixed simultaneously (1:5) with a precooled modified Alsever´s anticoagulant solution (NaCl 382 mM, glucose 115 mM, sodium citrate 27 mM, EDTA 11.5 mM). The hemolymph from five mussels was pooled to eliminate interindividual variation and was subsequently filtered using a 55 µm nylon mesh, counting the number of hemocytes in a Thoma chamber (Marienfeld, Lauda-Königshofen, Germany) under the microscope. Gill cells from the same five mussels were isolated as described elsewhere (Pérez-Cadahía, et al., 2004; Prego-Faraldo, et al., 2015), dissected at room temperature, and washed three times in 2 mL of ice-cold calcium magnesium-free saline solution (CMFS: 20 nM HEPES, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA in RPMI medium). Gills were then meticulously shredded and the resulting suspension was placed in a tube containing 4 mL of CMFS and shaken gently for 1 h at 4 °C in the dark. The entire suspensions were then filtered gently (55 µm nylon mesh) and centrifuged at 500 *g* for 5 min. The resulting pellet was re-suspended in 1 mL of Kenny´s salt solution (KSS: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$) and kept on ice until required. Gill cell number was determined microscopically. Cell viability was determined by trypan blue exclusion method, setting the viability threshold at 80% in all samples used.

In vitro Exposure to OA

The oxidative DNA damage produced by direct *in vitro* exposure to OA was evaluated in hemolymph cells using the modified (OGG1) comet assay. Accordingly, hemolymph was pooled from 20 mussels belonging to the control group and subsequently exposed *in vitro* to OA (Sigma-Aldrich, St. Louis, MO, USA) for 1 and 2 h (the range of OA concentrations and exposure times were selected based on previous reports (Prego-Faraldo, et al., 2015). OA was diluted in DMSO to final concentrations of 10 nM, 50 nM, 100 nM, 200 nM, and 500 nM, adding 10 µL to the cell suspensions (15 °C to 18 °C). Exposures were stopped by centrifugation (825 *g* for 3 min), collecting the pelleted cells for comet assay. *In vitro* incubations with hydrogen peroxide (100 µM, 10 min; 100 µM, 2 min) or camptothecin (4 µM, 4 h) were used as positive control in the comet assay, the modified comet assay and flow cytometry experiments, respectively.

Alkaline Comet Assay

The alkaline comet assay was performed as described elsewhere (Prego-Faraldo, et al., 2015; Wilson, et al., 1998) with slight modifications as follows. Hemolymph cells were centrifuged for 5 min at 250 *g*, and gill cells were centrifuged for 3 min at 1,000 *g*. The resulting pellets were re-suspended in 90 µL of 0.5% low-melting-point agarose (Invitrogen, Carlsbad, CA, USA) in KSS. Each sample was divided in two, placed on a slide pre-coated with a layer of 0.5% normal-melting-point agarose (Intron Biotechnology, Gyeonggi-do, Korea), and incubated at 4 °C for 25 min. The slides were subsequently placed in a Coplin jar with lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 250 mM NaOH, 10 mM Tris-HCl, 1% sarcosyl, pH 10 with 1% Triton X-100 added just before use) for 1 h at 4 °C. From this point on, all steps were conducted in the dark to prevent additional DNA damage. After lysis, slides were placed in an alkaline solution (0.3 M

NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min for DNA unwinding, and subjected to electrophoresis for 20 min (0.83 V/cm). Slides were subsequently washed with cold neutralization buffer (0.4 M Tris-HCl, pH 7.5), stained with 4,6-diamidino-2-phenylindole (DAPI), and stored in the dark at 4 °C. Image capture and analysis was performed using the Comet IV Software (Perceptive Instruments, Bury St Edmunds, UK). Fifty cells were scored from each replicate slide (100 cells total) and the percentage of DNA in the tail (%tDNA) was used as DNA damage parameter.

Modified Comet Assay with OGG1 Incubation

A modified version of the comet assay incorporating incubation with the 8-oxoguanine DNA glycosylase (OGG1) repair enzyme was used to assess the oxidative DNA damage in hemolymph cells (the excessive basal damage in gill cells precluded its inclusion in this analysis), according to the procedure described in (Smith, et al., 2006). After the lysis treatment, slides reserved for the modified comet assay were washed three times for 5 min in a Coplin jar with OGG1 buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). Fifty microliters of OGG1 enzyme (0.0016 U/µL, New England Biolabs, Beverly, MA, USA) were added to each slide and covered with a slip (controls were treated with 50 µL of buffer without enzyme). After incubation at 37 °C in a dark, humidified chamber for 10 min, the cover slips were removed and the slides were placed on an electrophoresis platform with the rest of slides, following subsequent steps similar to the alkaline comet assay.

Flow Cytometry Cytotoxicity Assay

The apoptosis/necrosis induced by OA (used as indicator of the effect of *P. lima* exposure) was evaluated by means of flow cytometry using Annexin V-phycoerythrin (PE) and 7-amino-actinomycin D (7-AAD) staining. The Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes,

NJ, USA) was used according to the manufacturer´s guideline with minor modifications. Gills cannot be used in this approach due to the presence of aggregated cells and debris. After OA exposure, hemolymph samples were centrifuged at 250 *g* for 5 min at 4 °C. The cell pellet was re-suspended in 200 µL of annexin binding buffer (0.5X) previously diluted in saline solution (NaCl, 500 nM) to a final concentration ranging between 4×10^5 and 6×10^5 cells. Annexin V-PE and 7-AAD (1 mg/mL) were added and incubated for 15 min at room temperature in the dark before analyzing cells by flow cytometry. The hemocyte population was fixed in the dot-plot following (Prado-Alvarez, et al., 2012) and analyses were carried out in a FACScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A minimum of 20,000 events were acquired in each case and fluorescence signals for Annexin V-PE and 7-AAD were measured using the FL-2 and FL-3 detectors, respectively. The percent of apoptotic and necrotic cells were analyzed using Cell Quest Pro software (BD Biosciences, Franklin Lakes, NJ, USA). Early apoptosis and late apoptosis/necrosis were expressed as the mean ± SE percentages of annexin V+/7-AAD- and annexin V+/7-AAD+ cells, respectively.

Statistical Analyses

At least three replicates were performed for each experimental condition tested. Experimental data were expressed as mean ± standard error and were tested for normality using the Kolmogorov-Smirnov test. As the data obtained showed a violation of the assumption of normality, non-parametric testing was deemed adequate. Differences between groups were therefore tested using Kruskal-Wallis and Mann-Whitney *U*-tests. *P*-values < 0.05 were considered significant. Statistical analyses were performed using the IBM SPSS software package V. 20 (IBM, Armon, NY, USA)

RESULTS

Toxin Accumulation and DNA Damage Resulting from *in vivo* Exposure to *P. lima*

Mussels were experimentally exposed to two cellular densities of the dinoflagellate *P. lima* (1,000 cells/L and 100,000 cells/L, for 24 h and 48 h, Figure 1). The subsequent accumulation of OA (the main DSP toxin) was used as an indicator of *P. lima* intake and the accumulation of DSP toxins by mussels, with results ranging between 21.67 ng/g and 112.12 ng/g dry weight (Table 1). Since these levels are well below the limit allowed by the European Commission Regulation for harvesting and sale (160 µg of OA equivalent/kg dry weight), mussel specimens were considered as being exposed to an early HAB stage and were used to evaluate the resulting genotoxic and cytotoxic effects.

DNA damage was quantified in hemolymph and gill cells using the alkaline comet assay (Figure 1), with results showing a lack of significant genotoxic effects in both cell types at extremely low dinoflagellate concentrations (1,000 cells/L, after 24 h and 48 h exposure, Figures 2 and 3). On the contrary, genotoxicity appeared to be dependent on the cell density of *P. lima* cultures, given the significant amount of DNA damage detected in hemolymph after a 24 h exposure to 100,000 cells/L ($P < 0.05$). These results are supported by the increase in the accumulation of OA by mussels exposed to higher *P. lima* concentrations (Table 1). On the contrary, no significant DNA damage was detected in gill cells after a 48 h exposure to 100,000 cells/L of *P. lima* ($P < 0.05$).

Table 1. The intake of *P. lima* by mussels after *in vivo* exposures was estimated by quantifying the accumulation of okadaic acid. Data resulting from three independent experimental replicates.

***P. lima* (cells/L)**	**Exposure Time (h)**	**Mean OA (ng/g Dry Weight) ± Standard Error**
1,000	24	28.35 ± 3.07
1,000	48	21.67 ± 2.02
100,000	24	64.77 ± 5.77
100,000	48	112.12 ± 7.78

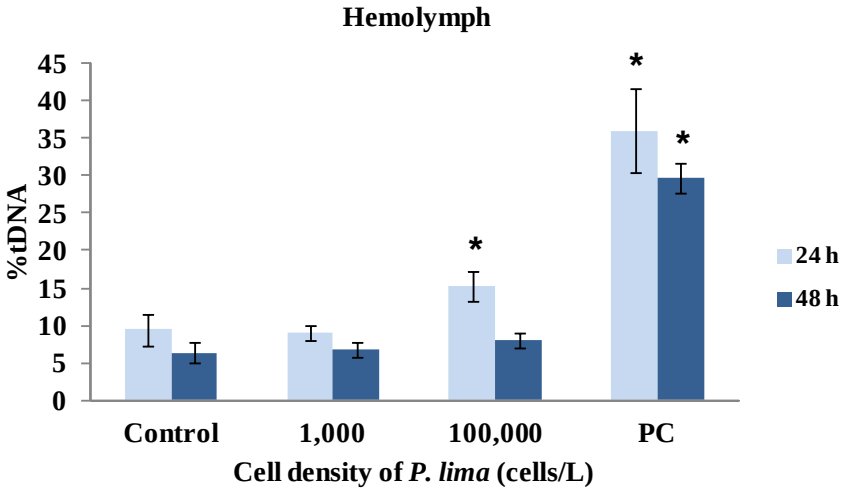

Figure 2. Quantification of DNA damage using the alkaline comet assay in mussel hemocytes after in vivo exposure to different cellular densities of *P. lima* for 24 h and 48 h. Control and PC represent negative and positive controls, respectively. The percentage of DNA in the comet tail is indicated by %tDNA. * indicates significant differences with respect to negative control in Mann-Whitney's U-test ($P < 0.05$).

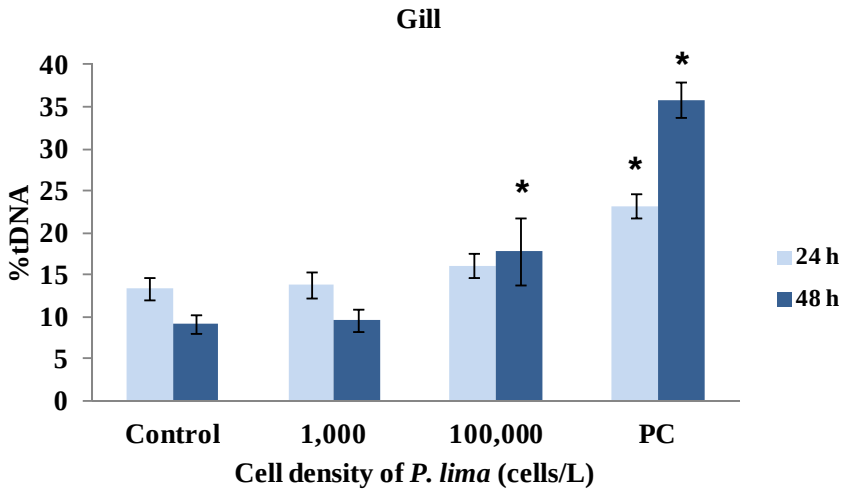

Figure 3. Quantification of DNA damage using the alkaline comet assay in mussel gill cells. Treatments and statistical analyses are as in Figure 2. * indicates significant differences with respect to negative control in Mann-Whitney's *U*-test ($P < 0.05$).

***In vivo* vs. *in vitro* Oxidative DNA Damage**

Although the comet assay constitutes a useful method to assess the genotoxicity of the toxins produced by *P. lima*, the standard alkaline method provides limited information about the nature of the DNA damage. Therefore, this assay was modified with the enzyme OGG1 (a DNA-glycosysale responsible for the excision of 8-oxoGua) in order to study oxidative DNA damage, thereby allowing to specifically detect oxidized bases in the DNA (Smith, et al., 2006). For that purpose, the amount of DNA damage observed in the buffer treatment represents the overall damage resulting from single-stranded breaks (SSB), double-stranded breaks (DSB), and alkali-labile sites (ALS). On the other hand, the DNA damage observed in the presence of the OGG1 enzyme, corresponds to the combination of SSB, DSB, and ALS plus oxidative DNA damage.

In the present chapter the modified comet assay was used to evaluate oxidative damage in hemolymph cells exposed *in vivo* to *P. lima* cultures. In addition, given the lack of *in vitro* data in this regard, the oxidative damage was also evaluated in cells directly exposed to purified OA. The obtained results showed that oxidative DNA damage remained constant in hemolymph cells exposed *in vitro* to low OA concentrations (10 to 100 nM) independently of exposure time, experiencing a significant increase at 200 nM and 500 nM (Figure 4, $P < 0.05$).

The *in vivo* exposure to *P. lima* cultures revealed similar results, namely a significant increase in the percentage of oxidative DNA damage after exposure to high cell densities for 24 h (100,000 cells/L, Figure 5). However, the amount of oxidative damage seems to be dependent on exposure time in this case, as no significant effects were observed after 48 h exposure. Lastly, it must be noted that the results obtained without OGG1 are quite similar to those for the alkaline comet assay, supporting the complementarity of both approaches.

In vivo Cytotoxic Damage After Exposure to *P. lima*

The study of the genotoxic effects resulting from the *in vivo* exposure of mussel cells to the DSP-toxin producer *P. lima* was complemented with the characterization of cytotoxic damage using the Annexin assay [detection of phosphatidylserine in the outer leaflet of the plasma membrane (Flórez-Barrós, et al., 2011; Martin, et al., 1995)]. This approach provides information about the apoptosis/necrosis rates, which can be subsequently compared with the DNA damage levels obtained in comet assays.

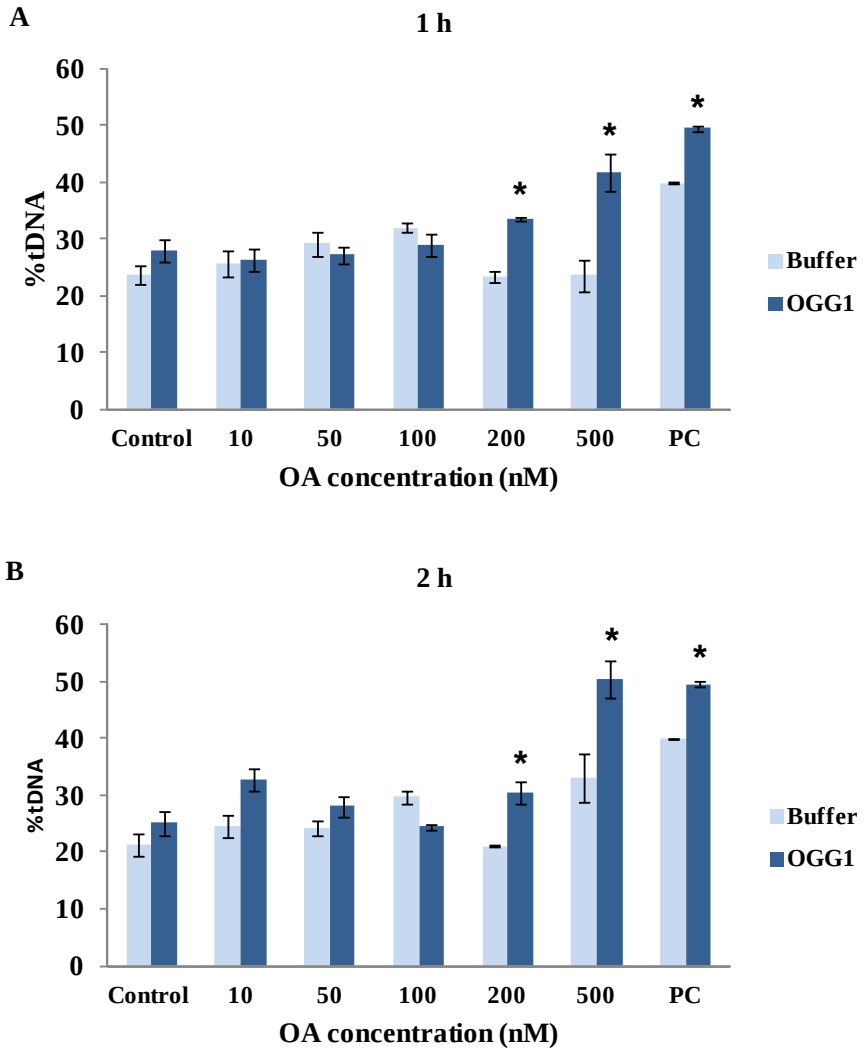

Figure 4. Quantification of oxidative DNA damage using the OGG1-modified comet assay in mussel hemocytes after *in vitro* exposure to different OA concentrations for 1 h (**A**) and 2 h (**B**). Control and PC represent negative and positive controls, respectively. The difference between buffer and OGG1 treatments specifically represents oxidative damage. The percentage of DNA in the comet tail is indicated by %tDNA. * indicates significant differences in respect to buffer in Mann-Whitney's *U*-test ($P < 0.05$).

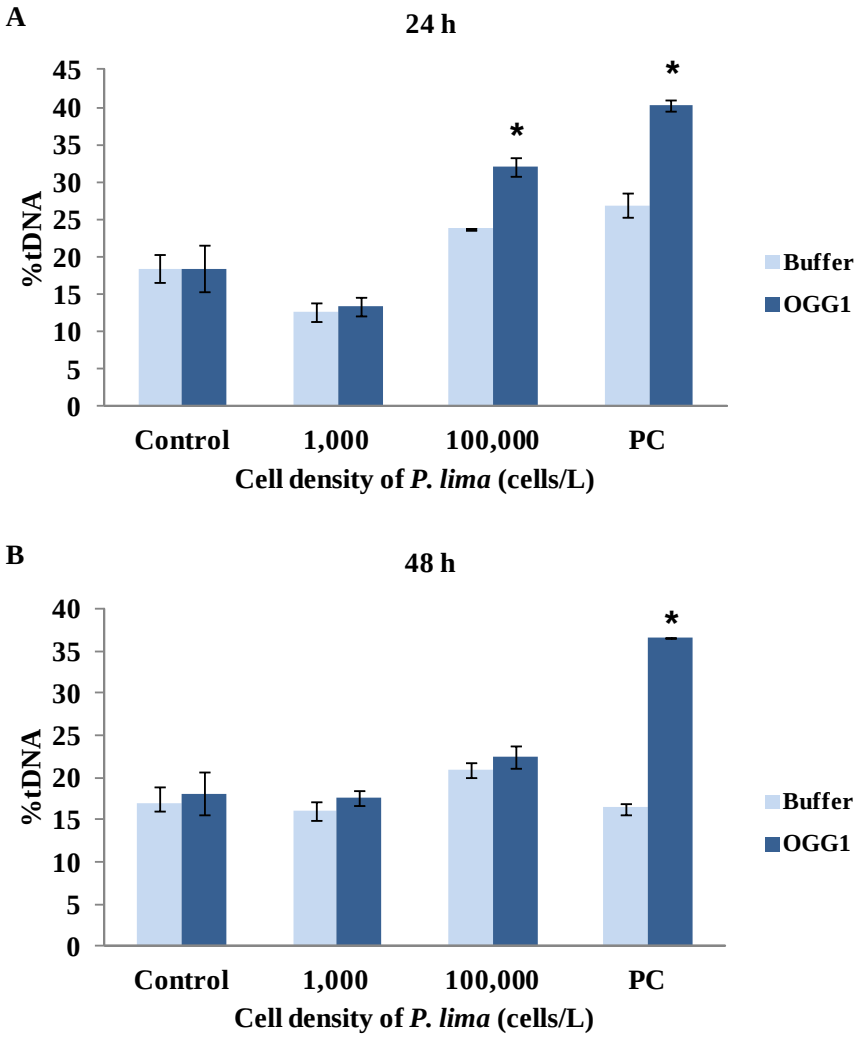

Figure 5. Quantification of oxidative DNA damage using the OGG1-modified comet assay in mussel hemocytes after *in vivo* exposure to different cellular densities of *P. lima* for 24 h (**A**) and 48 h (**B**). Controls and statistical analyses are as in Figure 4. * indicates significant differences in respect to buffer in Mann-Whitney's *U*-test ($P < 0.05$).

Cell viability was evaluated on mussel hemocytes, revealing an absence of significant levels of cytotoxicity after 24 h exposure to low and high *P. lima* cell densities (Figure 6A). On the contrary, a significant increase in cytotoxicity was found after 48 h exposure (Figure 6B), underscoring the dependence of this type of damage mainly on the amount of exposure time. The differences between 24 h and 48 h negative apoptosis controls are probably the result of small variations during the preparation of samples for flow cytometry, which might include variation in the extraction of the cells, slight fluctuations in incubation temperatures, *etc.* In any case, these variations do not modify the major conclusions of the present analysis (Lee and Steinert, 2003).

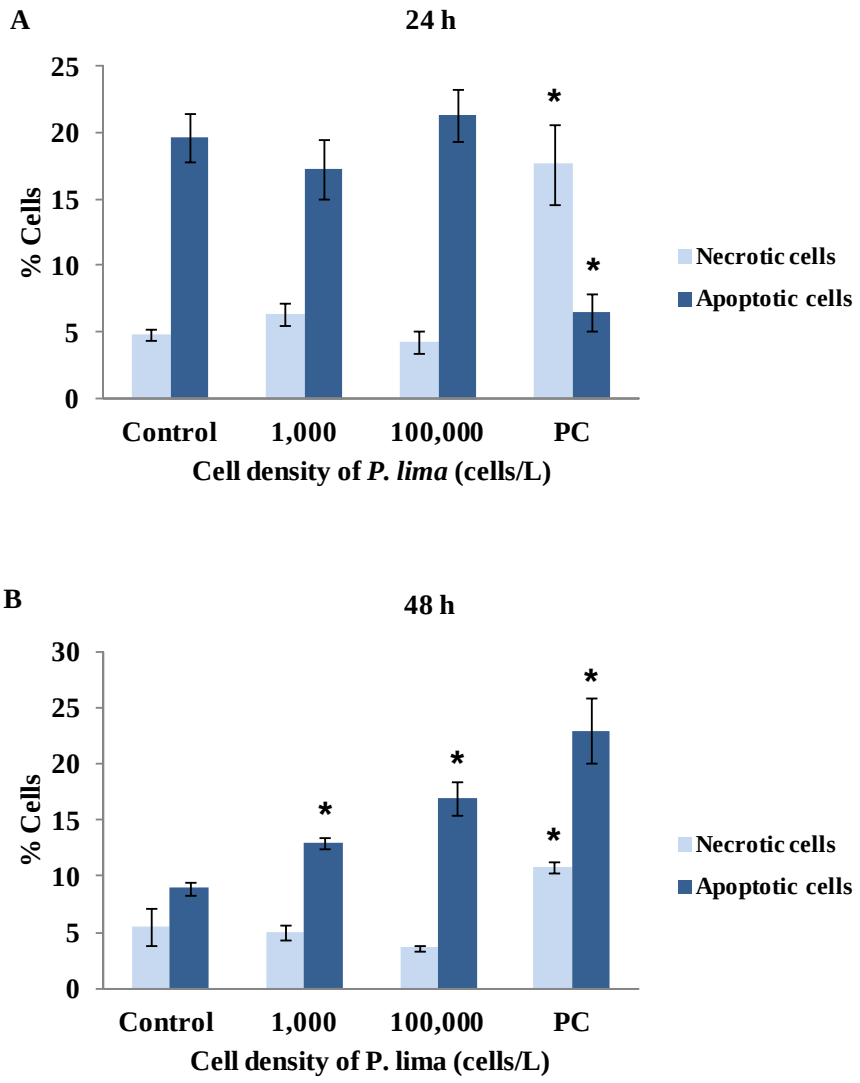

Figure 6. Flow cytometry evaluation of cytotoxicity in mussel hemocytes after *in vivo* exposure to different cellular densities of *P. lima* for 24 h (**A**) and 48 h (**B**). Control and PC represent negative and positive controls, respectively. The percentage of cells classified as necrotic or apoptotic is indicated by % Cells. * indicates significant differences in respect to negative control in Mann-Whitney's *U*-test ($P < 0.05$).

DISCUSSION

In the present work, mussels were exposed *in vivo* to different cell densities of the DSP-producing dinoflagellate *P. lima*. With that in mind, OA concentration in tissue was used as an indicator of *P. lima* intake and accumulation of DSP toxins by experimental mussels. OA accumulation was subsequently compared with that present in mussels during natural HAB episodes, corroborating that the conditions used in this work mirror early stages of a HAB episode (Prado-Alvarez, et al., 2012). Although seafood is still fit for human consumption at this point, low toxin concentrations might encompass sublethal effects. The present work reveals that OA accumulation increases with time and, specially, with high cellular densities of *P. lima* (Table 1). Yet, the reduction found after a 48 h exposure to low *P. lima* concentrations (1,000 cells/L) might be mirroring an increase in the rate of depuration in mussels. On the contrary, exposure to higher *P. lima* concentrations would saturate depuration, accounting for the higher accumulation of OA observed at 100,000 cells/L. This is the first time that such response pattern has been detected in mussels, although steady OA concentration levels throughout time having been also described in the clam *Ruditapes decussatus* and in the mussel *Perna perna* after exposure to low cellular densities of *P. lima* (Pinto-Silva, et al., 2005; Prado-Alvarez, et al., 2013). Further studies will be required in order to clearly determine how depuration mechanisms affect toxin accumulation at low concentrations. It will be similarly interesting to ascertain how DSP estimation based on OA quantification from whole mussels (instead of independent tissues) might affect the results obtained here, especially as to how the effect of OA derivatives (notably 7-*O*-acyl derivatives or DTX3) might influence genotoxicity. Since toxin extractions were performed in the present work without an alkaline hydrolysis step before mass spectrometry (MS) quantification, the toxins analyzed included OA plus a partial extraction of

DTXs. That raises the possibility that at least part of the genotoxic and cytotoxic effects observed in the present work could be attributed to contamination by the effect of DTX3 and OA acyl derivates (in addition to OA). That being said, it has been previously suggested that these derivates are formed at a very low rate during biotoxin exposure (at least for the case of *M. galloprovincialis*), with the bulk of their toxicity happening mostly at latter stages of prolonged DSP episodes (Moroño, et al., 2003). Therefore, while the unaccounted effect of DTX3 and OA acyl derivates could be contributing to the genotoxicity/cytotoxicity determined in the present work, we suggest that such effects might be less important than previously anticipated, as the present work is focused on the early genotoxic and cytotoxic effects of *P. lima* exposure (*i.e.*, not enough time for the production of DTX3 and OA acyl derivates of significant amounts). Further studies will help elucidate the specific contribution of these compounds over time.

Mussel Responses to the Toxic Dinoflagellate *P. lima*

The results obtained in the present work revealed a conspicuous lack of genotoxic damage in mussel hemocytes, suggesting an absence of genotoxic stress at extremely low densities of *P. lima* (Flórez-Barrós, et al., 2011; McCarthy, et al., 2014; Prego-Faraldo, et al., 2015). Additionally, the resemblance in the DNA damage observed during *in vivo* exposure to *P. lima* and during *in vitro* exposure to OA might be indicative of a similar mode of toxin action in both cases (Flórez-Barrós, et al., 2011; McCarthy, et al., 2014; Prego-Faraldo, et al., 2015). Although previous studies have proposed that OA might have limited genotoxic potential (Dallas, et al., 2013) and that the chronic exposure to low-medium OA levels can lead to adaptation (Rocher, et al., 2006), our results support the ability of the toxins produced by *P.* lima (notably OA) to cause oxidative stress, similarly to what it has been described for mammalian cells exposed to low levels of

other marine toxins (Fracasso, et al., 2006; Valdiglesias, et al., 2011b). Furthermore, it seems that mussels are able to repair DNA damage after short exposures (24 h) to low DSP and OA concentrations.

The analysis of the DNA damage induced indirectly by the exposure to *P. lima* in gill cells revealed a positive correlation with time and dinoflagellate concentration. However, while genotoxic effects were not observed after the first 24 h, an increase in DNA damage was evident after 48 h. Additional studies covering longer periods of time will be required to elucidate if resistance mechanisms are reinstated in mussel gills after longer exposure times, similarly to the case of clam gills after *in vivo* exposure to DSP toxins (Flórez-Barrós, et al., 2011). Altogether, similar amounts of DNA damage were found in hemocytes and gill cells, in agreement with *in vitro* results obtained for hemolymph and digestive glands of mussels and oysters exposed to OA (McCarthy, et al., 2014). However, hemocytes seem to experience genotoxic effects faster than gill cells, contrasting with previous *in vitro* results by our research group (Prego-Faraldo, et al., 2015). A discordance between the *in vivo* and *in vitro* effects of DSP toxins was also observed in gill cells, in agreement with previous results obtained in clams exposed to DSP toxins (Flórez-Barrós, et al., 2011).

Oxidative DNA Damage in Mussel Hemolymph

The OGG1-modified comet assay was used in the present work to determine the amount of oxidative damage caused by *P. lima* on mussel DNA. Given that this assay cannot be implemented in gill cells due to the excessive basal DNA damage caused by OGG1 buffer, the results presented here refer to hemocytes. The comparison between *in vivo* exposure to *P. lima* and *in vitro* exposure to OA revealed that this biotoxin causes oxidative damage both directly and indirectly on this cell type. More specifically, DNA damage appears to be time-independent after 2 h exposure under *in vitro*

conditions. On the contrary, a significant decrease in the level of oxidative damage was observed after a 48 h *in vivo* exposure. A similar reduction in damage after longer exposure times was previously described in molluscs (de Lapuente, et al., 2015; Martins, et al., 2013; Noventa, et al., 2011; Vidal, et al., 2014). While these results are consistent with a higher oxidative genotoxic potential of OA *in vitro*, it is also evident that exposure to *P. lima* can induce oxidative DNA damage in hemocytes mainly as a consequence of reactive oxygen species (ROS) formation, although that is most likely alleviated by antioxidant mechanisms *in vivo*.

The results from modified comet experiments revealed a significant increase in oxidative DNA damage at high densities of *P. lima*, very early (24 h exposure) during HAB simulation. This effect is concurrent with closure periods in commercial mussel rafts, probably due to saturation in depuration mechanisms and lower activity of cellular antioxidant defenses. Different studies have provided evidences of oxidative stress caused by DSP toxins in marine organisms, notably by evaluating transcriptional levels of genes encoding detoxifying enzymes and biochemical markers (Romero-Geraldo, et al., 2014; Vidal, et al., 2014). In those cases, an increase in the effect of antioxidant mechanisms was observed, concomitantly with the accumulation of DSP toxins (Vidal, et al., 2014). Additional transcriptomic analyses (together with other omic approaches) of mussels exposed to DSP toxins will be critical to elucidate the mechanisms by which these toxins affect marine invertebrates *in vivo*. Overall, the differences observed between *in vitro* and *in vivo* studies are in agreement with previous studies (Levin, et al., 2008), where the synergistic interactions between OA and other DSP toxins might be responsible for higher levels of toxicity (Rodríguez, et al., 2015; Traoré, et al., 1999) and/or different toxicological effects (Rubiolo, et al., 2011).

Cytotoxic Damage in Mussel Hemocytes

Flow cytometry results did not show significant differences between necrosis and apoptosis levels in mussel hemocytes exposed *in vivo* to *P. lima* for 24 h. Apoptosis, on the contrary, increased significantly after 48 h, supporting results from our previous work studying the *in vitro* effect of OA in mussels (Prego-Faraldo, et al., 2015). By comparing cytotoxicity data, OA accumulation at different exposure times and *P. lima* concentrations (Table 1), it appears that exposure to *P. lima* does not cause significant cytotoxic damage at intermediate concentrations, supporting previous results obtained in mussels exposed *in vitro* to OA (Prado-Alvarez, et al., 2012). However, the opposite situation is observed at extreme *P. lima* densities, encompassing a significant increase in the percentage of apoptotic cells. The discordance between this observation and the aforementioned *in vitro* reports (Prado-Alvarez, et al., 2012) might be determined by the use of different types of controls. Accordingly, while *in vitro* exposures used control specimens with very low concentrations of OA (48 ng/g dry weight), the present work used control mussels virtually lacking any OA (<0.10 ng/g). Indeed, a similar increase in the number of apoptotic cells over time was found on hemocytes from carpet shell clams exposed to OA, using controls completely lacking OA (Prado-Alvarez, et al., 2013). Lastly, the increase in the number of apoptotic cells observed in the present work is negatively correlated with the number of necrotic cells, displaying a stable number of viable cells. This result is similar to those reported by several other papers, suggesting that OA does not have a negative influence in the viability of mussel cells (Galimany, et al., 2008; Louzao, et al., 2010; Prado-Alvarez, et al., 2013; Prego-Faraldo, et al., 2015; Simões, et al., 2015) probably due to a higher number of protein apoptosis inhibitors (IAPs) in bivalve molluscs (Simões, et al., 2015) compared with other species.

CONCLUSIONS

This study is the first describing the early genotoxic and cytotoxic effects resulting from the *in vivo* exposure of the mussel *M. galloprovincialis* to the DSP-toxin-producing dinoflagellate *P. lima*. The obtained results revealed that: (1) low *P. lima* cell densities can be used to recreate early stages of HAB episodes in laboratory conditions; (2) the *in vivo* exposure to extremely low dinoflagellate concentrations (1,000 cells/L) did not produce significant genotoxic effects in hemocytes or gill cells; (3) the DNA damage observed in gill cells exposed *in vivo* to *P. lima* was predominantly influenced by exposure time and *P. lima* cell density; (4) the oxidative DNA damage of hemocytes exposed *in vitro* to OA was dependent on toxin concentration, while in the case of hemocytes exposed *in vivo* to *P. lima* damage was only observed after 24 h exposure to the highest dinoflagellate concentration studied (100,000 cells/L). This suggests a more relevant role of the antioxidant system in this latter case; (5) the absence of significant levels of DNA damage at low *P. lima* and OA concentrations underscores the inability of these toxin concentrations to disturb the antioxidative balance in mussel cells; (6) *in vivo* exposure to growing *P. lima* densities increased apoptosis but not necrosis, probably due to a high number of protein apoptosis inhibitors (IAPs) in molluscs.

Overall, these conclusions increase the knowledge regarding the *in vivo* genotoxic and cytotoxic potential of the marine biotoxins produced by the dinoflagellate *P. lima* in bivalve molluscs. In doing so, this work demonstrates for the first time the suitability of the modified (OGG1) comet assay as a valid experimental approach improving the evaluation of the oxidative DNA damage caused by marine toxins in the hemolymph of marine invertebrates. Furthermore, the present chapter corroborates the value of the comet assay and flow cytometry as a means to evaluate early

genotoxic and cytotoxic responses, laying the foundations for future experiments aimed at identifying exposure biomarkers and mitigating economic losses associated with HABs in coastal areas.

Chapter 3

TRANSCRIPTIONAL AND BIOCHEMICAL RESPONSES OF DIFFERENT ANTIOXIDANT ENZYMES IN MUSSELS EXPOSED TO THE TOXIC DINOFLAGELLATE *Prorocentrum lima*

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(In preparation)

SUMMARY

The genotoxic and cytotoxic effects of diarrhetic shellfish poisoning (DSP) toxins have been widely addressed in bivalve molluscs, the main vectors of these compounds in European Atlantic coastal areas during harmful algal blooms (HABs). During the development of such toxic episodes, bivalves display an apparently increased resistance to the harmful effects of these toxins. Although previous reports have suggested that the antioxidant system may be responsible for that resilience, very little is known about the precise mechanisms medianting this protective effect. In order to fill that gap, the present study combined transcriptomic and biochemical approaches to analyze the enzymatic responses of bivalve molluscs to DSP toxins. Accordingly, digestive glands and gills of the mussel *Mytilus galloprovincialis* were exposed to 100,000 and 1,000 cells/L of the DSP-producing dinoflagellate *Prorocentrum lima*, for 24 and 48 h. The obtained results revealed significant transcriptional changes in genes encoding glutathione S-transferase *pi-1* (GST-*pi*), catalase (CAT), selenium-dependent glutathione peroxidase (Se-GPx) and superoxide dismutase (SOD) enzymes in both tissues, suggesting compensatory gene expression among them. Such observation was further supported by the identification of significant changes in the activity of these enzymes. The modifications in transcriptional activity identified in the present work provide a basis for developing biomarkers of exposure to DSP toxins. In addition, the differences observed in enzymatic activity constitute complementary exposure and/or effect biomarkers. Nonetheless, the lack of significant correlations between transcription and enzymatic activity prevents the application of transcription patterns as predictors of functional responses. Overall, the results provided by this work underscore the importance of the antioxidant system during early protective responses to DSP toxins and identifies new targets to develop biomarkers.

INTRODUCTION

The okadaic acid (OA) and its analogues, the dinophysistoxins (DTXs), constitute the main diarrhetic shellfish poisoning (DSP) toxins found in the Atlantic coast of Europe, with OA being the predominant one. These toxins are produced during harmful algal blooms (HABs) by dinoflagellates of the genera *Dinophysis* and *Prorocentrum* (Reguera, et al., 2014). The increased frequency and duration of these episodes in recent years attracted significant attention due to the negative impacts of DSP toxins on human consumers of contaminated shellfish (Gestal-Otero, et al., 2014), as well as economic losses associated with the toxic episodes caused by these toxins (Rodríguez, et al., 2011). This is particularly relevant in the case of OA, whose harmful effects have been associated with its inhibitory effect on several types of serine/threonine protein phosphatases (Bialojan and Takai, 1988). Numerous studies carried out in mammalian cell lines demonstrated the ability of this toxin to cause cytotoxicity, genotoxicity and oxidative DNA damage (Valdiglesias, et al., 2010; Valdiglesias, et al., 2011a; Valdiglesias, et al., 2013).

Importantly, the number of studies assessing the harmful effects of OA on their principal vectors, the bivalve molluscs, has increased consistently for the last few years (McCarthy, et al., 2014; Pinto-Silva, et al., 2003; Prego-Faraldo, et al., 2013; Prego-Faraldo, et al., 2015). However, the evaluation of the combined effects of DSP toxins and their synergies still remains incomplete. The few studies developed so far on this regard suggest that DSP toxins (including OA) produced by toxic dinoflagellates such as *Prorocentrum lima* cause the most dramatic genotoxic and cytotoxic effects on bivalve molluscs at low concentrations and short exposure times. On the contrary, high concentrations and long exposure times seem to be consistent with the onset of resistance mechanisms in bivalves (Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2005; Prado-Alvarez, et al., 2012; Prego-Faraldo,

et al., 2015). Additionally, a recent study carried out by our research group has determined the presence of early oxidative DNA damage in hemolymph cells from the mussel *Mytilus galloprovincialis* exposed to DSP toxins (Prego-Faraldo, et al., 2016b). Interestingly, such oxidative damage seems to be rapidly repaired, suggesting that the antioxidant system could play a main role in the defence against these toxins.

The ability of antioxidant defense system from bivalve molluscs to effectively respond to a wide variety of environmental stressors has been reported in numerous studies (Lima, et al., 2007; Oliveira, et al., 2015; Regoli and Giuliani, 2014). This defense system is able to mitigate the effects of genotoxic pollutants [inducing those producting reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2)] thanks to the combined and sequential activity of different antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST) and non-enzymatic molecules. Consequently, these enzymes have been routinely used as biomarkers to assess the genotoxic effects of diverse pollutants including saxitoxins (STX), DSP toxins and paralytic shellfish toxins (PST) (Astuya, et al., 2015; Fabioux, et al., 2015; Núñez-Acuña, et al., 2013; Romero-Geraldo and Hernández-Saavedra, 2014; Romero-Geraldo, et al., 2014; Suarez-Ulloa, et al., 2015). On the other hand, although the biochemical response of bivalves to metals and organic contaminants has been widely studied (Verlecar, et al., 2007; Verlecar, et al., 2008), the details of the response to toxin producing-algae have received less attention. Accordingly, a significant increase in the activity of SOD, CAT, GPx or GST was reported in different bivalve species (*Diplodon chilensis*, *Nodipecten subnodosus* and *M. galloprovincialis*) fed with toxin-producing microalgae (*Microcystis aeruginosa*, *Gymnodinium catenatum* and *Alexandrium tamarense*) (Estrada, et al., 2007; Qiu, et al., 2013;

Sabatini, et al., 2011). However, only one study evaluated the response of antioxidant enzymes to DSP toxins in the mussel *M. galloprovincialis*, showing a modulation of GPx activity in relation to the OA accumulation during different algal blooms (Vidal, et al., 2014).

Although both approaches, transcriptional and biochemical, are often considered as alternatives the relationships between them are still not clear. Indeed, only a handful of studies have combined transcriptional and biochemical responses of antioxidant enzymes to assess the effect of pollutants in marine invertebrates (Banni, et al., 2014; Canesi, et al., 2007; Canesi, et al., 2008; Giuliani, et al., 2013). The discrepancies obtained between both methods have been associated to differences in transcriptional and translational mechanisms, metabolic capability of tissues, post-transcriptional modifications of proteins, bi-phasic response of antioxidant enzymes and interactions between chemical mixtures (Regoli and Giuliani, 2014). Within this scenario, the present work assessed the transcriptional and enzymatic activity responses of different antioxidant enzymes in different tissues of the mussel *M. galloprovincialis* upon exposure to the toxin-producing dinoflagellate *P. lima*. To our knowledge, this is the first work combining both approaches to elucidate the oxidative stress caused by marine toxins in a marine invertebrate.

MATERIAL AND METHODS

Specimens Sampling

Adult *M. galloprovincialis* specimens (mean ± SD anterior-posterior shell length = 3.39 ± 0.06 cm) were collected from a natural population in the rocky shores of O Rañal beach (Galicia, NW Spain, 43°19'40.1"N 8°30'45.1"W) in April 2015, placed in thermally insulated boxes with seawater from the sampling site and immediately transported to the laboratory. The salinity, conductivity and pH of the seawater were measured

within the following 12 hours using a Mettler-Toledo Seven Easy S33K (Mettler-Toledo, Schwarzenbach, Switzerland) and a Crison MicropH 2001 (Crison Instruments, Barcelona, Spain), respectively. The levels of OA were evaluated in whole mussel samples using High-Performance Liquid Chromatography Mass Spectroscopy (HPLC/MS) carried out by the unit of chromatography techniques at the Servicios de Apoyo a la Investigación of the University of A Coruña (Prego-Faraldo, et al., 2016b). Mussels were acclimated to laboratory conditions for a week in highly aerated tanks, with filtered seawater in a photoperiod chamber (17 ºC, 12 h light-dark cycle) and fed two times a day with a 1:1 mixture of two nontoxic microalgae species (*Isochrysis galbana* and *Tetraselmis suecica*).

Experimental Design

After acclimation, the mussels were randomly divided into three groups (Figure 1) including: a control group fed with a 1:1 mixture of *I. galbana* and *T. suecica*, and two treatment groups additionally fed with the DSP toxin-producing *P. lima* (1,000 and 100,000 cells/L, respectively, 4 times per day) for 24 h and 48 h. The *P. lima* culture (strain AND-A0605) was obtained from the Quality Control Laboratory of Fishery Resources (Huelva, Spain) and cell concentrations in the culture were determined by cell count in Sedgwick-Rafter counting slides (Pyser-Sgi, Edenbridge, UK) after fixation with Lugol´s solution. Subsequently, calculations and dilutions were made to obtained densities of 1,000 and 100,000 cells/L, which were previously chosen according to OA concentrations observed during the early stages of the development of an algal bloom (Díaz, et al., 2013). Upon exposure, digestive gland and gill tissue were randomly sampled from 5 individuals at each exposure treatment (1,000 cells/L for 24h, 1,000 cells/L for 48h, 100,000 cells/L for 24h, 100,000 cells/L for 48h) and pooled [reducing sample variability (Suarez-Ulloa, et al., 2015)] for gene expression analyses. Complementarily, gland and gill tissue were

randomly sampled from twelve individuals per treatment for biochemical analyses. All samples were frozen in liquid nitrogen and stored at -80 ºC until further analyses.

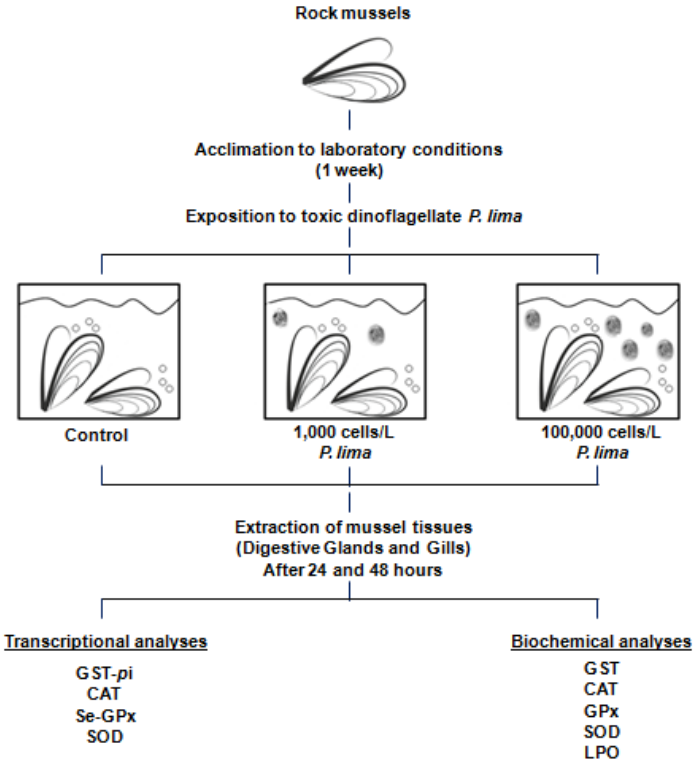


Figure 1: Schematic diagram describing the experimental design followed in the present study. Mussel specimens were collected, acclimated to laboratory conditions and subsequently exposed to different cellular densities of the DSP-producing dinoflagellate *P. lima* (1,000 and 100,000 cells/L) for 24 h and 48 h. Digestive gland and gill tissues were dissected from experimental mussels and used in the evaluation of modifications in transcription and activity of antioxidant enzymes involved in the response to DSP toxins. Lipid peroxidation levels (LPO) were also examined.

RNA Isolation, Experimental and Reference Gene Selection

Genes encoding antioxidant proteins were selected as indicators of mussel's early response to the harmful effects of DSP toxins, including glutathione S-transferase *pi-1* (GST-*pi*), catalase (CAT), selenium-dependent glutathione peroxidase (Se-GPx) and superoxide dismutase (SOD). This choice is further supported by the differential expression of these genes in a digestive gland microarray exposed to low concentrations of OA (Suarez-Ulloa, et al., 2015). RNA was extracted from pooled tissues (digestive gland and gill separately) using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA concentration and quality were verified using a NanoDrop spectrophotometer (A260/280 and A260/230, Thermo Scientific, Waltham, MA). RNA integrity was further confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). cDNA was synthesized from 1 µg of total RNA, using First Strand cDNA Synthesis kit, according to the manufacturer´s instructions (Roche Diagnostics, Mannheim, Germany). Reference genes for expression quantification were selected among, six potential candidate housekeeping genes including 18S ribosomal RNA (18S) (Cubero-Leon, et al., 2012), glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) and ribosomal proteins S4 (rpS4) (Lozano, et al., 2015), and elongation factor 1 (EF1), elongation factor 2 (EF2), histone H2A (H2A). The primers for the latter three genes designed in the present work from the sequences of the selected genes (Table 1) using the Universal Probe Library program (www.universalprobelibrary.com). Two different algorithms, Normfinder and GeNorm PLUS implemented in Biogazelle´s qbasePLUS software (Biogazelle, Ghent, Belgium), were conducted to ranking these candidate genes according to their stability and to decide on the optimal number of reference genes required for accurate normalization. Normfinder was used with R version 3.0.1 (Team, 2015).

Table 1: Primers used for qPCR analyses.

Gene Name Abbreviation	Reference	Primers 5´→3´
18S	L33452	F-CCTGGAAAGGTCGGGTAAC R-AATTACAAGCCCCAATCCCTA
EF1	Suarez-Ulloa et al., 2013	F-CCTCCCACCATCAAGACCTA R-GGCTGGAGCAAAGGTAACAA
EF2	Suarez-Ulloa et al., 2013	F-ACCACGACGCTTGTTGAGA R-TTCTTGGTAGAAATTCAGTGTCCA
H2A	AY267755.1	F- CGGAGCACCAGTCTACCTTG R- GATGACGGGGGATGATTCTGC
GAPDH	Lozano et al., 2015	F-AGGAATGGCCTTCAGGG R-TCAGATGCTGCTTTAATGGCTG
rpS4	Lozano et al., 2015	F-TGGGTTATCGAGGGCGTAG R-TCCCTTAGTTTGTTGAGGACCTG
GST-*pi*	AF527010.1	F-TCACCTGGATGTCTTGATGC R-TGGTCTAGCTAACACTCGCTCA
CAT	AY743716.2	F-TGCTCTGGGATTTCATTACACTT R-CCACGGTCAGAGAACAGGA
Se-GPx	HQ891311.1	F-TTCACAATCATGGAAGACATCAG R-AAGGCCGAAAATTGATGAAA
SOD	FM177867.1	F-CAGCAGTGACAGTGACAGGAG R-AACTCGTGAACGTGGAAACC

Quantitative PCR Analyses

The qPCR analyses were performed using four biological replicates per treatment and two technical replicates per biological replicate. Specific qPCR primers were designed from sequences retrieved from the GenBank database (Table 1) using the Universal Probe Library program (www.universalprobelibrary.com), according to qPCR restrictions. The primer specificity was verified by agarose gel electrophoresis, yielding a single product with the desired length. qPCR amplifications were carried out using the FastStart Essential DNA Green Master kit (Roche Diagnostics, Mannheim, Germany) on a LightCycler 96 instrument (Roche Diagnostics, Mannheim, Germany) following the manufacturer´s instructions with modifications. All reactions were performed in a final volume of 20 µL of master mix containing 6.4 µL H_2O, 0.8 µL of each primer (10 µM), 10 µL of the SYBR Green Mix (Roche Diagnostics,

Mannheim, Germany) and 2 µL of each reverse transcribed RNA. Reactions consisted of an initial denaturation step of 10 min at 95 ºC followed by an amplification of the target cDNA for 40 cycles, each cycle consisting of a denaturation at 95 ºC for 10 s, anneling at 60 ºC for 10 s, elongation at 72 ºC for 10 s, melting curve analysis (1 cycle at 95 ºC for 5 s, 65º C for 60 s and 95º C for 1s), and cooling at 40 ºC for 20 s. Specificity of the qPCR product was analyzed by melting curve analysis. The dynamic range for the genes under study was conducted to obtain their efficiencies. The relative expression levels of the genes were normalized using rpS4, GAPDH and EF2 as references genes. For data analyses, Cq values were extracted with the qPCR instrument software LightCycler Software 1.5.0 (Roche) and subsequently analysed using qbasePLUS software (Biogazelle). Calibrated normalized relative quantities (CNRQ) of each gene were represented in bar plots (treatment *vs.* control). Two-way ANOVA was not used in gene expression data analysis because the RNA extraction and cDNA synthesis from the groups exposed at different treatments during different times of exposure were carried out in different days. In addition, the qPCR runs to quantify the different genes were grouped in such way that each plate contained the treatment samples and their respective controls (sample maximization).

Sample Preparation for Biochemical Analyses

The enzymatic activities of GST, CAT, GPx and SOD were quantified as indicators of oxidative stress. Also, the levels of lipid peroxidation (LPO) were analyzed to be associated with the amount of ROS produced. Twelve biological replicates per treatment were defined for biochemical analyses, with four technical replicates analyzed per biological replicate. Before biochemical analyses, gills and digestive glands were weighed and homogenized in phosphate buffer (0.1 M, pH 7.4) in a proportion of 1:10 (weight/volume), using an Ystral GmbH d-7801 Doltingen homogenizer

(Ystral GmBH, Ballrechten-Dottingen, Germany). The homogenate was used for LPO analyses and to determine the activity of antioxidant enzymes (GST, CAT, GPx and SOD). All enzyme activities were calibrated to protein concentrations measured at 600 nm and 25 ºC in the SpectraMax M2 (Molecular Devices, Sunnyvale, CA) following the method described by (Guilhermino, et al., 1996) using bovine γ-globuline (Sigma G5009) as standard protein. Sample protein content was standardized to 0.3 mg/mL for GST analyses, and to 1 mg/mL for antioxidant enzymes (CAT, GPx and SOD).

Quantification of Enzymatic Activities

GST activity was determined using the method described by Gerssen et al. (2011) and Habig et al. (1974), with adaptation to microplates (Frasco and Guilhermino, 2002). The reaction mixture consisted of 150 µL CDNB 50 mM in methanol, 900 µL GSH 10 nM in phosphate buffer (0.1 M, pH 6.6) and 4,000 µL phosphate buffer were combined and the reaction was monitored spectrophotometrically at 340 nm and 25 ºC each 36 s for 5 min, using SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as nM GSH-CDNB produced per min per mg protein. CAT activity was determined according to Aebi (1984). The reaction mixture contained 0.95 mL of phosphate buffer (0.05 M, pH 7.0), 0.5 mL of H_2O_2 and 0.05 mL of gill or digestive gland supernatant, measuring H_2O_2 consumption spectrophotometrically at 240 nm and 25 ºC, for 1 min. GPx activity was determined indirectly in accordance with the method described by Flohé and Günzler (1984), through a coupled reaction to glutathione reductase (GR). The reaction mixture consisted of 0,1 M potassium phosphate buffer with 2mM Na_2-EDTA, 1 mM DTT, 1mM of sodium azide, 1mM NADPH, 0,2 mM GSH and 30 U/mL GR. GSH oxidation was measured at 340 nm and 25 ºC each 36 s for 2 min, using a microplate reader (Bio-Tek, model

Power Wave 340, Winooski, USA). Results were expressed as nmol of NADPH oxidized per min and per mg protein. SOD activity was determined according to McCord and Fridovich (1969) adapted to microplate. Briefly, 50 nM sodium phosphate buffer with 1 mM Na_2-EDTA (pH 7.8), 0,7 mM xanthine (Sigma-Aldrich, St. Louis, MO), 0.03 mM cytochrome c (Sigma-Aldrich, St. Louis, MO) and 0.3 U/mL xanthine oxidase (Sigma-Aldrich, St. Louis, MO) were combined. The reduction of the cytochrome c was measured at 550 nm and 25 ºC, using a microplate reader (Bio-Tek, model Power Wave 340, Winooski, USA). The SOD activity was calculated as relative to its ability to inhibit 50% reduction of cytochrome c per min and expressed as units (U) per mg protein. Analyses of LPO were determined by generation of thiobarbituric acid reactive substances (TBARS), according to Ohkawa et al. (1979), preventing the artificial lipid oxidation by the addition of butylated hydroxytoluene (BHT) 4% in methanol. Briefly, in a tube of 15 mL, 1 mL of 12% trichloroacetic acid (TCA), 0.9 mL of Tris-HCl (60 mM, pH 7.4) and 1 mL of 0.73 % thiobarbituric acid (TBA) were added to 0.1 mL of homogenate. The tubes were incubated at 100 ºC for 60 min, and 2 m of these samples was removed and placed in a tube of 2 mL and centrifuged at 12,000 *g* during 5 min. LPO levels were then measured reading the absorbance at 535 nm and expressed in nM TBARS per mg protein using SpectraMax M2 cuvette reader (Molecular Devices, Sunnyvale, CA).

Statistical Analyses

The results of the biochemical analyses are presented as mean ± standard error of the mean (SEM). All data was tested for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Barlett's test) (Zar, 1996). Strong deviations from normality and homoscedasticity were corrected using square root and log (x) transformations. Each biomarker data set was analyzed by a two-way analysis of variance (ANOVA) with

interaction using treatment and time of exposure as fixed factors, followed by the *post hoc* Tukey's test to discriminate significantly different groups (Zar, 1996). The significance level was 0.05. Statistical analyses of data were performed using the Sigmaplot package V. 12 (Systat Software, Richmond, CA, USA).

RESULTS AND DISCUSSION

The genotoxic and cytotoxic effects of DSP-producing dinoflagellates on marine invertebrates have been previously studied by different groups, including our own (Flórez-Barrós, et al., 2011; Prado-Alvarez, et al., 2012), suggesting the ability of these toxins to produce transient oxidative DNA damage at low concentrations (Prego-Faraldo, et al., 2016b). However, the effects of toxin exposure on gene transcription and antioxidant enzymatic activity have received less attention (Romero-Geraldo and Hernández-Saavedra, 2014; Vidal, et al., 2014). The present work fills this gap by characterizing the modifications in transcriptional and enzymatic activity of genes encoding antioxidant enzymes in different tissues of the mussel *M. galloprovincialis*. Analyses were carried out after 24 h and 48 h exposure to low densities of the toxic dinoflagellate *P. lima*.

Experimental Conditions and OA Concentration

Mussels were experimentally exposed to two cellular densities of the dinoflagellate *P. lima* (1,000 cells/L and 100,000 cells/L, for 24 h and 48 h). The subsequent accumulation of OA (the main DSP toxin) was used as an indicator of *P. lima* intake and the accumulation of DSP toxins by mussels The water conductivity values ranged from 48.87 mS/cm ± 0.66 (at sampling site) to 47.69 mS/cm ± 0.03 (at laboratory conditions), while salinity values ranged from 31.83 ppm ± 0.49 (at sampling site) to 30.95 ppm ± 0.03 (at laboratory conditions). pH values ranged from 8.33 ± 0.01 (at sampling site) to 8.14 ± 0.01 (at laboratory conditions). OA body burden

in contaminated mussels ranged between 21.67 and 112.12 ng/g dry weight. Control mussels, fed with the mix of *I. galbana* and *T. suecica*, did not accumulate OA at any the sampling times. Mussels fed with 1,000 cells/L of *P. lima* accumulated 28.35 ± 3.07 and 21.67 ± 2.02 ng/g after 24 and 48 h of exposure, respectively. On the other hand, mussels fed with 100,000 cells/L of *P. lima* accumulated 64.77 ± 5.77 and 112.12 ± 7.78 ng/g after 24 and 48 h of exposure respectively. These OA accumulations are consistent with the patterns obtained in a other experiment carried out in pararel by our research group (Prego-Faraldo, et al., 2016b).

Selection of References Genes

The suitability of reference genes was analyzed using the Normfinder and GeNorm algorithms as indicated in Figure 2. The Normfinder analysis produced a ranking with GAPDH, EF2 and rpS4 as the most stables genes (Figure 2A). The wort stability value was obtained for 18S gene. The GeNorm PLUS algorithm implemented in Biogazelle´s qbasePLUS software calculates the pairwise variation among all tested genes and assigns stability measures (*M*). Complementarily, GeNorm produced rather similar results, defining GAPDH, rpS4 and EF1 as most stable (Figure 2B). Both algorithms were additionally employed to define the most appropriate number of reference genes for normalization, resulting in the selection of GAPDH, rpS4 and EF2 as reference genes in all subsequent qPCR analyses. These results are not consistent with a previous report in which EF and 18S are considered two of the most appopiated genes for normalizing the variation in molluscan studies (Cubero-Leon, et al., 2012), although recently Lozano et al. 2015 also identified GAPDH and rpS4 as optimal reference genes to be used in digestive glands and gills of the mussel *M. galloprovincialis*.

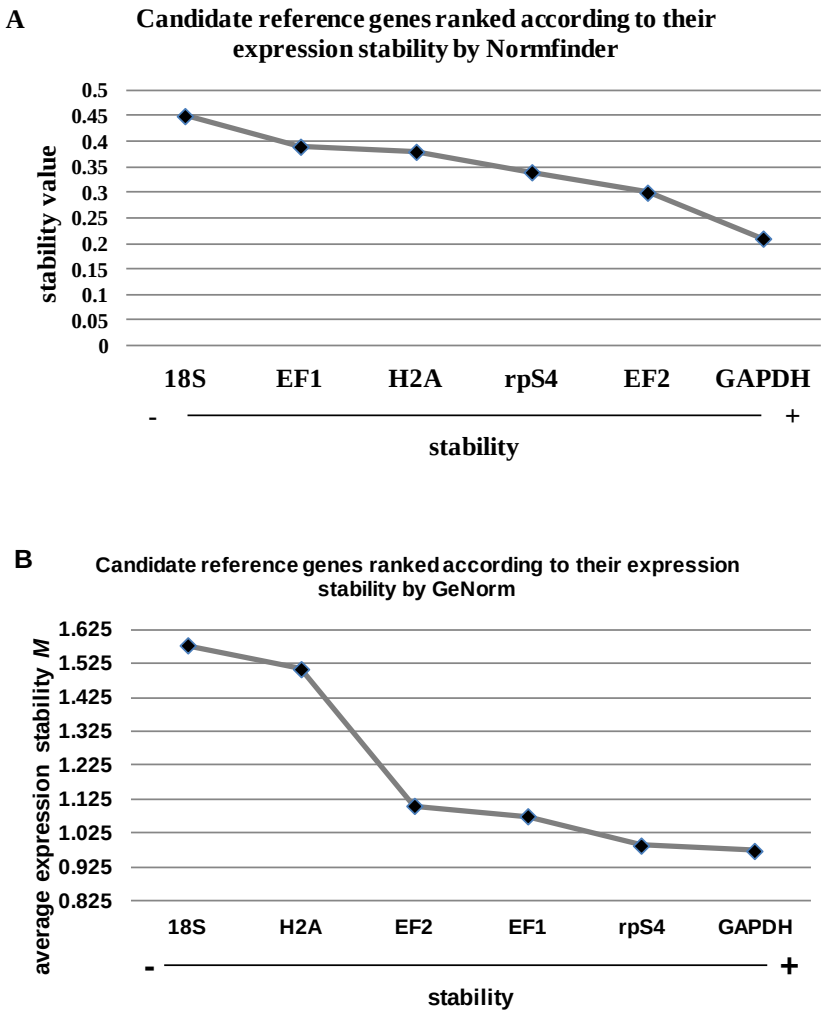
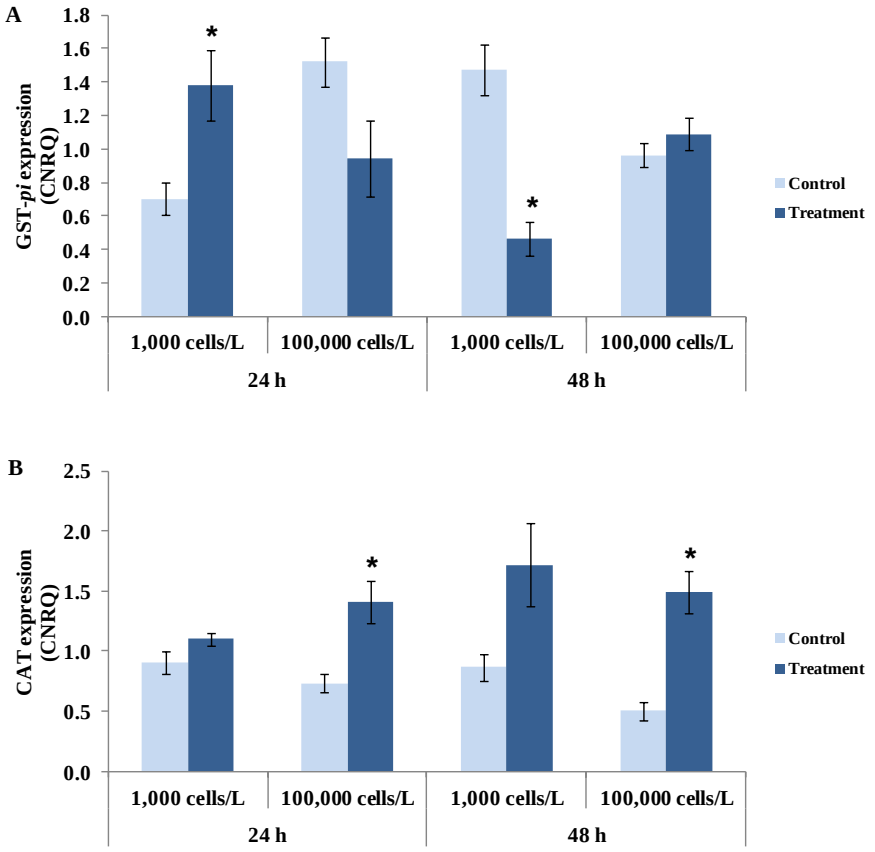


Figure 2: Evaluation of six candidate reference genes for transcriptional analyses. Candidates were ranked according to their expression stability based on two different approaches: **A**, "stability values" as determined by the Normfinder algorithm; **B**, "average expression stability, *M*" as defined by the GeNorm algorithm.

Transcriptional Responses to *P. lima* Exposure in Mussel Tissues

The transcriptional levels of GST-*pi*, CAT, Se-GPx and SOD genes were evaluated using mRNA samples obtained from digestive glands and gills from mussels fed with the DSP-producing dinoflagellate *P. lima*. qPCR reactions were carried out using the primers indicated in Table 1 (efficiencies for each qPCR primer pair were > 1.8). The results obtained in this work revealed significant transcriptional differences in GST-*pi*, CAT and SOD genes in mussel digestive gland tissue after exposure to *P. lima* (Figure 3). These results showed a significant increase (24 h) and decrease (48 h) in GST-*pi* transcription at 1,000 cells/L, as well as a significant down-regulation of SOD after 24 h at 1,000 cells/L. Indeed, exposure to 1,000 cells/L for 24 h is enough to alter SOD and GST-pi transcript levels. Consistent with this observation, an initial decrease in SOD mRNA expression has been also observed in the oyster *Crassostrea gigas* after exposure to *P. lima* (Romero-Geraldo and Hernández-Saavedra, 2014). While this result might mirror the inability of this tissue to neutralize the oxidative damage caused by extremely low concentrations of *P. lima*, the significant increase in GST-*pi* mRNA expression observed at similar conditions suggest a more complex scenario. Similar compensatory responses have been previously observed in digestive gland of mussels exposed to natural estrogen and estrogenic chemicals over short periods of time (Canesi, et al., 2008). Additionally to SOD and GST-*pi*, a significant increase in CAT mRNA levels were also found in mussel's digestive gland after exposure to 100,000 cells/L for 48 h. This result agrees with the presence of high CAT mRNA levels in mussel digestive gland during the first response to nickel and heat stress (Banni, et al., 2014), in gills of mussels exposed to sublethal chromium concentrations (Astuya, et al., 2015; Ciacci, et al., 2012) as well as in hemocytes of the mussel *M. chilensis* injected and exposed *in vitro* to saxitoxins (Núñez-Acuña, et al.,

2013). On the contrary, Se-GPx transcription levels remained unchanged in digestive gland after exposure to *P. lima*, in agreement with previous results from gills of the Pacific oyster *Crassostrea gigas* exposed to paralytic shellfish toxins (PST) (Fabioux, et al., 2015).



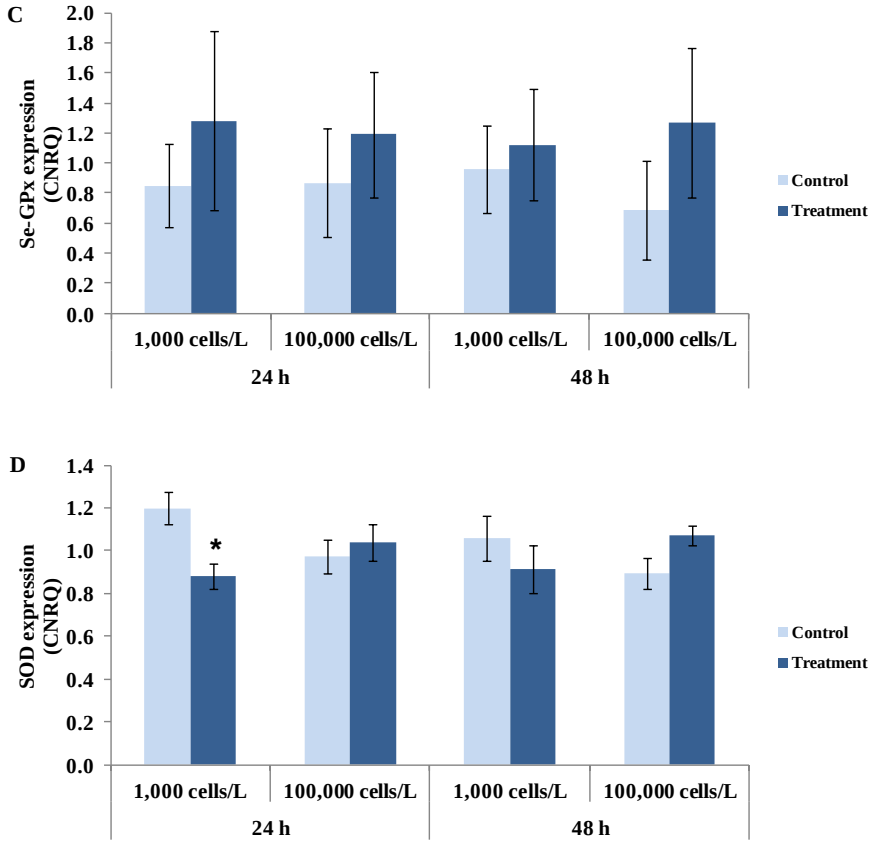


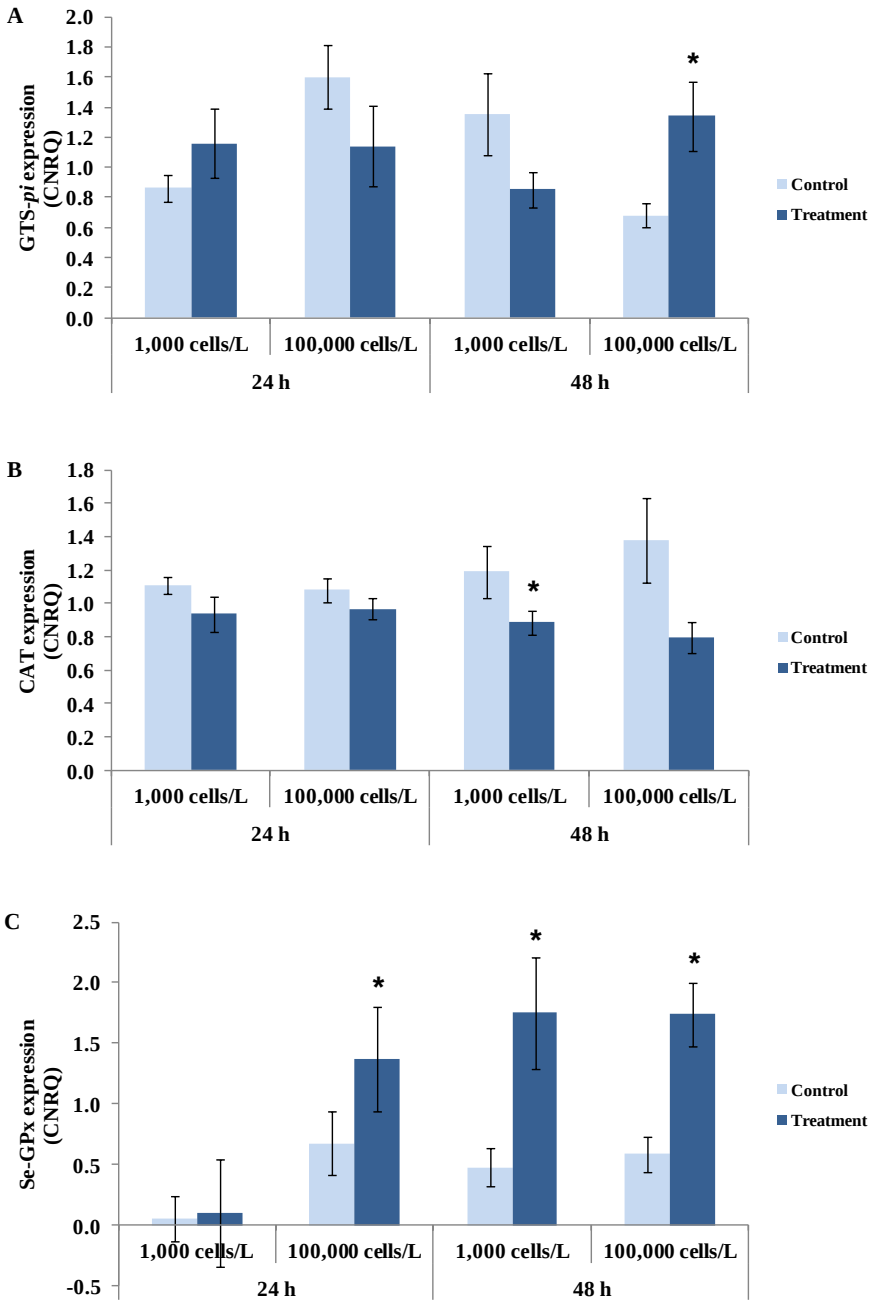
Figure 3: Transcriptional modifications in genes encoding antioxidant enzymes from digestive gland of *M. galloprovincialis* after exposure to the DSP-producing dinoflagellate *P. lima*. **A**, GST-*pi*; **B**, CAT; **C**, Se-GPx; **D**, SOD. Relative transcript expression levels were calculated respect to non-exposed control mussels. Data was averaged from 4 biological replicates, each deriving from a pool of 5 mussels in duplicates, and were analyzed using qbasePLUS software.

On the contrary, significant differences were found in all genes in the case of gill (Figure 4). Also, the response was different to that observed in digestive gland, corroborating the differential expression of antioxidant enzymes across bivalve molluscs tissues (Jo, et al., 2008).

In this tissue, a 48 h exposure to 100,000 cells/L elicited the strongest response, increasing GST-*pi* and Se-GPx mRNA expression and decreasing CAT and SOD mRNA expression. The induction of GST-*pi* is consistent

with previous reports studying gills of the clam *Corbicula fluminea* in response to copper and cadmium exposure (Bigot, et al., 2010). Overall, such responses agree with the increase in genotoxicity previously described by our research using comet assay (Prego-Faraldo, et al., 2016b), suggesting a potential role of ROS production on oxidative DNA damage. Additionally, it might be plausible that the down-regulation of CAT is partially compensated by up-regultation of Se-GPx, since both enzymes use the same substrate (Regoli, et al., 2011a; Regoli, et al., 2011b). Lastly, high levels of Se-GPx were observed in all treatments except for 24 h at 1,000 cells/L. The up-regulation of this gene was also reported in gills from the mollusc *Haliotis discus* in response to thermal, low-salinity and hypoxic stress (De Zoysa, et al., 2009).

The early increase in the mRNA levels observed for antioxidant enzymes might be consistent with the subsequent capacity of mussels to eliminate potential ROS induced by the exposure to DSP-producing dinoflagellates. In addition, the obtained results suggest that this process will take place faster in digestive glands compared with gills. This observation might seem counterintuitive, as gills constitute the primary absorption route for pollutants and toxic substances [e.g., antioxidant genes display greater expression levels in gills than in digestive gland after exposure to cadmium (Jo, et al., 2008)]. However, the observed results are still in agreement with previous reports revealing higher genotoxicity in gill cells from mussels exposed to the toxin OA (Prego-Faraldo, et al., 2015), as well as with other studies documenting the elimination and intracellular storage of DSP toxins (including OA) in the digestive gland of molluscs (Blanco, et al., 1999; Rossignoli and Blanco, 2008; Rossignoli and Blanco, 2010).


A
GTS-pi expression (CNRQ)
2.0
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*
1,000 cells/L
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1,000 cells/L
100,000 cells/L
24 h
48 h
Control
Treatment
B
CAT expression (CNRQ)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
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1,000 cells/L
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1,000 cells/L
100,000 cells/L
24 h
48 h
Control
Treatment
C
Se-GPx expression (CNRQ)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
*
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1,000 cells/L
100,000 cells/L
1,000 cells/L
100,000 cells/L
24 h
48 h
Control
Treatment

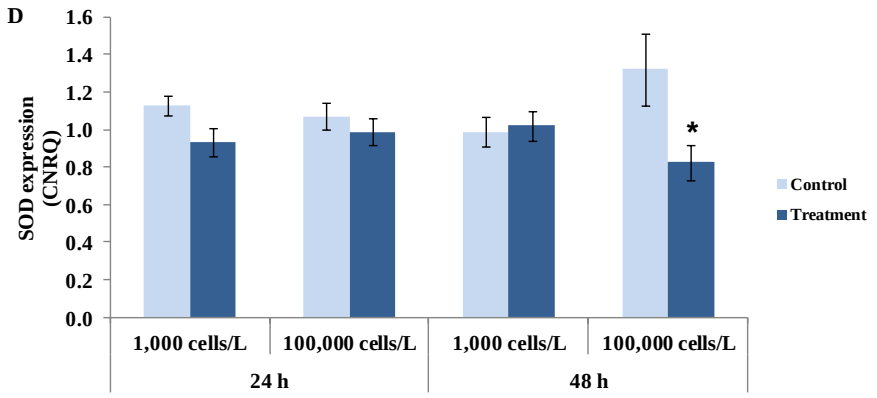


Figure 4: Transcriptional modifications in genes encoding antioxidant enzymes from gill of *M. galloprovincialis* after exposure to the DSP-producing dinoflagellate *P. lima*. **A**, GST-*pi*; **B**, CAT; **C**, Se-GPx; **D**, SOD. Data are represented as in Figure 3.

Effects of DSP Toxins on Antioxidant Enzyme Activities

The eftects of stressors on selected biomarkers in the mussels digestive gland and gills are shown in Figures 5 and 6, respectively. The results of the statistical analysis are indicated in Table 2.

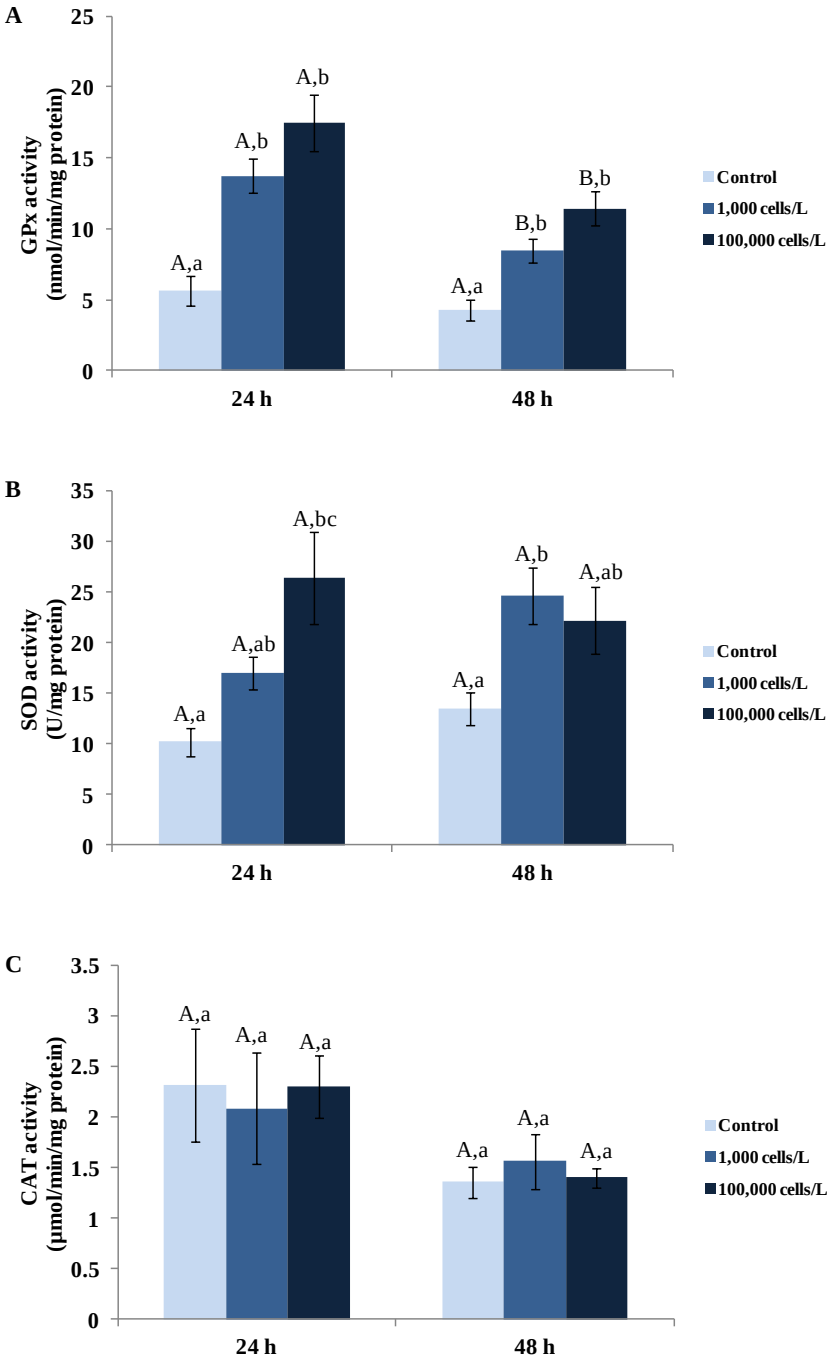
Regarding digestive gland, the values of GPx quantified in mussels exposed to 1,000 and 100,000 cells/L for 24 h were significant higher than 48 h. Also these values were significant higher in digestive glands exposed to 1,000 and 100,000 cells/L for 24 and 48 h than in control mussels. When GPx activity of digestive glands exposed to 1,000 and 100,000 cells/L was compared between treatments no significant differences were found at any exposure time (Figure 5A). These results were in good agreement with Gorbi et al. (2012), who also observed an increase of digestive gland GPx activity in the *M. galloprovincialis* exposed to dinoflagellates. The values of SOD measured in digestive glands of mussel followed a similar pattern for 24 and 48 h, although significant higher SOD activity values were found in

mussel´s digestive glands exposed to 100,000 cells/L of the toxic dinoflagellate for 24 h. However, the values of SOD activity recorded in digestive glands of mussels exposed to 100,000 cells/L for 24 h were not significantly different than those exposed to 1,000 cells/L, while the values of SOD activities quantified after 48 h were only significant different between 1,000 cells/L and the control (Figure 5B).

In terms of biochemical response, the activity of GPx and SOD enzymes in digestive gland of mussels exposed to DSP toxins was significantly higher for conditions tested, which supports that both enzymes play a main role in the digestive gland response to oxidative stress produced by DSP toxins. Qui et al. (2013) found high levels of SOD activity when digestive glands of mussels *M. galloprovincialis* were exposed to paralytic shellfish toxin (PST). Present results indicated that SOD provided the first line of defence through dismutation of O_2 radicals into H_2O_2, which is can be further scavenged by GPx or CAT. This is consistent with previous research showing a similar enzymatic activity in digestive glands and gills of the mussel *Perna viridis* exposed to seasonal variation (Verlecar, et al., 2008). However, no significant differences were found for CAT activity measured in digestive glands of mussels, when compared between exposure times and treatments (Figure 5C). Considering that CAT and GPx enzymes work cooperatively and taking into account that CAT activity remained unchanged during all experience, GPx enzyme seems to be the main responsible of the H_2O_2 neutralization under the experimental conditions reported. The observed results are in good accordance with Regoli et al. (2011a,b), who suggested that the absence of an increase in the CAT activity can be due to a compensation based on the increase of GPx activity. Estrada et al. (2007) also observed no significant differences in CAT activity measured in the digestive gland of *Nodipecten subnodosus* exposed to paralytic shellfish poisoning (PSP). Digestive glands of mussels exposed

for 48 h to 1,000 cells/L of *P. lima* had significantly lower GST activity than those exposed for 24 h to the same concentration of toxic cells. On the contrary, mussels exposed for 48 h to 100,000 cells/L had higher GST activity than those exposed to the same concentration for 24 h only. Regarding the treatment (cell density of *P. lima* chosen), the values of GST activity obtained in digestive glands of mussels exposed to 1,000 cells/L for 24 h were significant lower than in mussels exposed to 100,000 cells/L during the same period of time; however no significant differences were found regarding control mussels (Figure 5D). Thus, GST activity only seems to be responding favorably at the most extreme treatment (100,000 cells/L, 48 h). In fact, Barata et al. (2005) suggested that the high GST activity may also compensate the low CAT activity, since GST also present peroxidase activity.

These results were associated with a significant decrease in LPO levels over the time, which was significative when the digestive glands of mussels were exposed at 100,000 cells/L, indicating lipid peroxidation damage, at these concentrations. The levels of LPO quantified in digestive glands of mussels exposed to 100,000 cells/L for 48 h were significant lower than 24 h. Although there were no significant differences in the levels of LPO measured in digestive glands of mussels exposed for 24 h, mussels exposed to 100,000 cells/L for 48 h obtained LPO levels significant lower than control, resulting in significant differences between both treatments (1,000 and 100,000 cells/L) (Figure 5E). High GST, GPx and SOD activities could have contributed to the low LPO levels, suggesting that these exposure conditions to DSP toxins provide a suitable protection for digestive glands against damage caused by ROS.


A
GPx activity (nmol/min/mg protein)
25
20
15
10
5
0
A,a
A,b
A,b
A,a
B,b
B,b
24 h
48 h
Control
1,000 cells/L
100,000 cells/L
B
SOD activity (U/mg protein)
35
30
25
20
15
10
5
0
A,a
A,ab
A,bc
A,a
A,b
A,ab
24 h
48 h
Control
1,000 cells/L
100,000 cells/L
C
CAT activity (µmol/min/mg protein)
3.5
3
2.5
2
1.5
1
0.5
0
A,a
A,a
A,a
A,a
A,a
A,a
24 h
48 h
Control
1,000 cells/L
100,000 cells/L

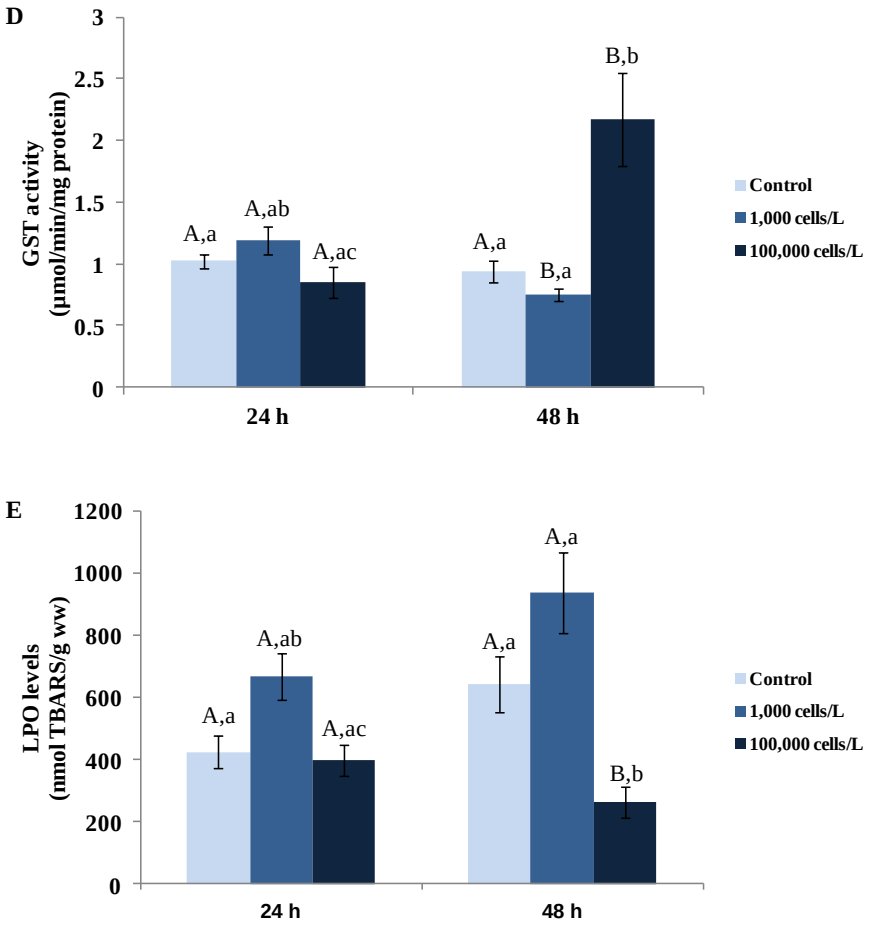


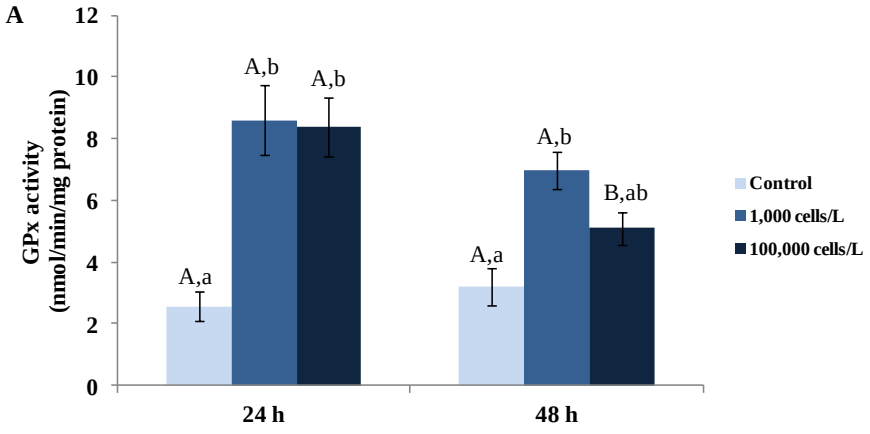
Figure 5: Lipid peroxidation levels and response of antioxidant enzymes to DSP toxins measured in the digestive gland of *M. galloprovincialis* exposed for 24 and 48 h to 1,000 and 100,000 cells/L. **A**, GPx; **B**, SOD; **C**, CAT; **D**, GST; **E**, LPO. The values are the mean with the corresponding SEM. For each enzyme, significant differences among time (capital letters) and treatments (lower case letters) are identified (two-way ANOVA and Tukey test, $P < 0.05$).

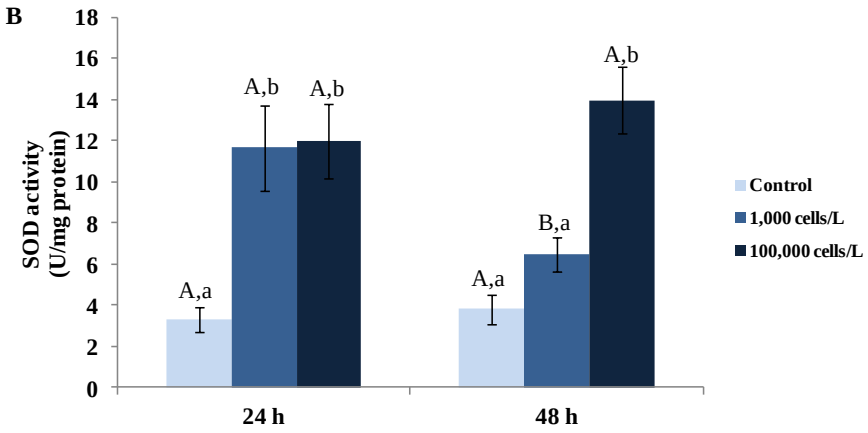
Considering the results observed in the mussel gills, the activity of all antioxidant enzymes tested (GST, CAT, GPx and SOD) was significantly higher for any of the treatments evaluated, indicating that in the range of tested concentrations, DSP toxins induces oxidative stress in this mussel species. In good agreement with the biomarker responses obtained in the

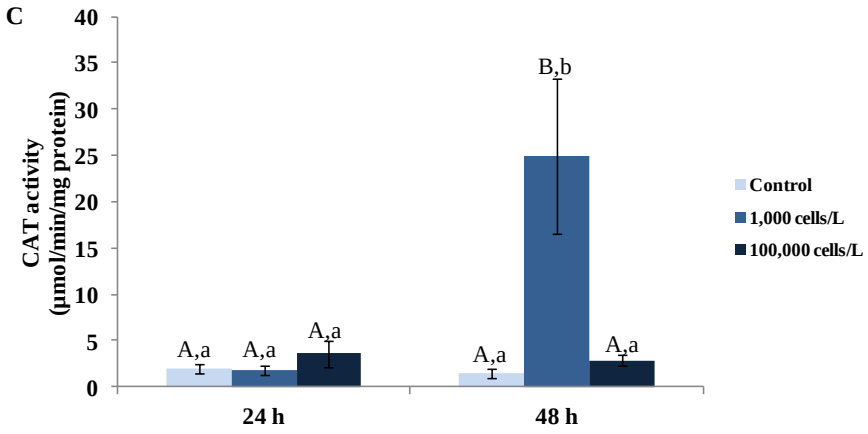
digestive gland, the GPx and SOD activity in gills was significantly higher for the majority of exposure conditions. The values of GPx obtained in gills of mussels exposed to 100,000 cells/L for 24 h were significant higher than 48 h. However, the values of GPx activities measured in gills of mussels exposed to 100,000 cells/L for 48 h were not significant different than control (Figure 6A). On the contrary, the SOD activity in gills of mussels exposed to 1,000 cells/L for 48 h were significant lower than 24 h. However, the values of SOD activity measured in gills of mussels exposed to 1,000 and 100,000 cells/L for 24 h were significantly higher than in those not exposed (control treatment); while no significant differences were found when two treatments were compared. Mussel exposed to 100,000 cells/L for 48 h had significantly higher values of SOD activities in gills than those not exposed (control treatment) or exposed to low densities (1,000 cells/L) (Figure 6B). Estrada et al. (2007) also reported high activities of these two enzymes (GPx and SOD) in gills of the scallop *N. subnodosus* exposed to PSP. Parallel changes in GPx and SOD activities were observed in both tissues, which suggest that these two enzymes may be act interdependently to neutralize ROS. On the other hand, the values of CAT activity in gills of mussels exposed to 1,000 cells/L for 48 h were also significant higher than 24 h. Additionally, the values of CAT activity obtained in gills of mussels exposed to 1,000 cells/L for 48 h were significantly higher than those exposed to the other experimental treatments (control and 100,000 cells/L) for 48 h (Figure 6C). A similar behavior between SOD and CAT, was previously found in digestive glands and gills of the mussel *Perna perna* exposed to mercury and thermal stress (Verlecar, et al., 2007). No significant differences in gill GST activity were found between mussels exposed for 24 and 48 h. In a similar way, no significant differences in the values of GST activity quantified in gills exposed to toxic dinoflagellate for 24 h; however, there were significant differences in the values of GST activity obtained in gills exposed to 100,000 cells/L for 48 h with regarding

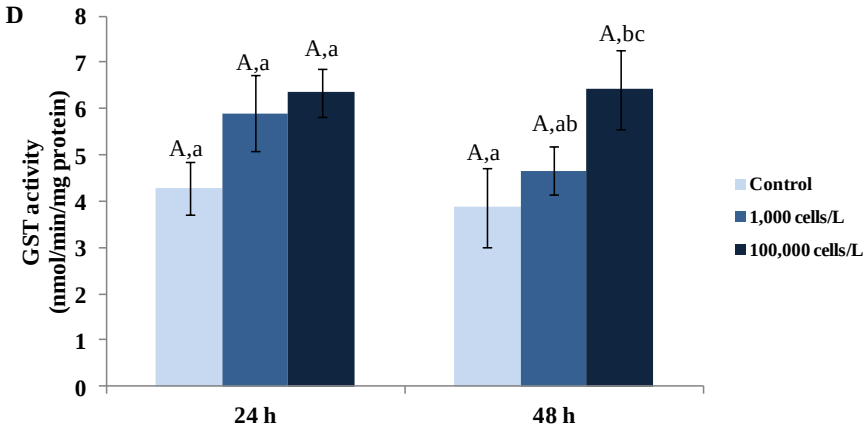
to control (Figure 6D). Thus, GST activity only results significantly increased at 48 h of exposure to DSP toxins, while CAT activity showed a significant increase in gills of mussels exposed to 1,000 cells/L for 48 h. These results were also associated with a significant decrease in LPO levels over the time, which as remarkable when gills of mussels exposed to 1,000 and 100,000 cells/L for 48 h were compared to controls (Figure 6E).

Our results confirm in part a protective role of DSP toxins in digestive glands and gills of mussel. The absence of parallel global response between tissues can be supported by differences in the bioavailability of the DSP toxins, in the same way that this pattern was detected in the scallop *Chlamys farreri* exposed to benzo(k)fluoranthene (Pan, et al., 2005). High antioxidant enzyme activities obtained in gills of mussels exposed to DSP toxins seem to involve in keeping low LPO levels, as well as decreasing. Finally, as might be expected, there were no significant differences in digestive glands and gills of mussels not exposed (control treatment) to the toxic dinoflagellate when the values of all antioxidant enzymes assessed in this work were compared between 24 and 48 h; with the only exception of LPO levels, which were significant higher in gills measured at 48 than 24 h.




B
SOD activity (U/mg protein)
18
16
14
12
10
8
6
4
2
0
A,a
A,b
A,b
A,a
B,a
A,b
24 h
48 h
Control
1,000 cells/L
100,000 cells/L


C
CAT activity (µmol/min/mg protein)
40
35
30
25
20
15
10
5
0
A,a
A,a
A,a
A,a
B,b
A,a
24 h
48 h
Control
1,000 cells/L
100,000 cells/L


D
GST activity (nmol/min/mg protein)
8
7
6
5
4
3
2
1
0
A,a
A,a
A,a
A,a
A,ab
A,bc
24 h
48 h
Control
1,000 cells/L
100,000 cells/L

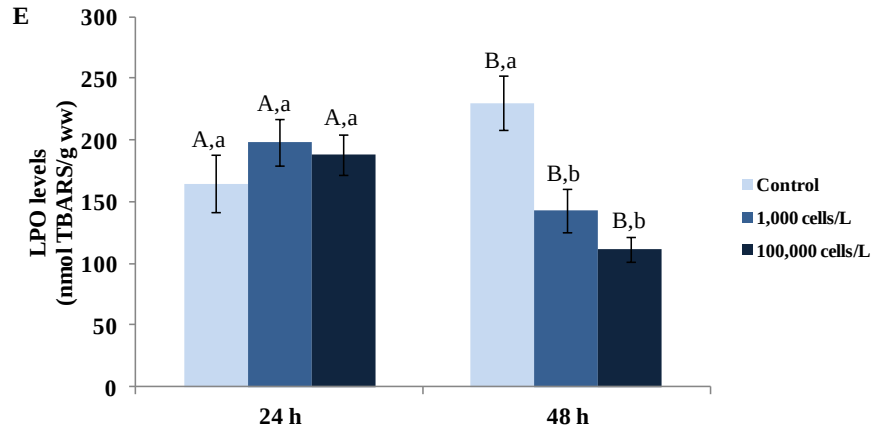


Figure 6: Lipid peroxidation levels and response of antioxidant enzymes to DSP toxins measured in the gills of *M. galloprovincialis* exposed for 24 and 48 h to 1,000 and 100,000 cells/L. **A**, GPx; **B**, SOD; **C**, CAT; **D**, GST; **E**, LPO. The values are the mean with the corresponding SEM. For each enzyme, significant differences among time (capital letters) and treatments (lower case letters) are identified (two-way ANOVA and Tukey test, $P < 0.05$).

Significant differences in the activity among treatments were found for GPx, SOD, and GST (in both tissues), and for LPO and CAT measured in the digestive glands and gills, respectively (Table 2A). Regarding the time of exposure, significant differences were observed for CAT (in both tissues) and for GPx measured in the digestive gland (Table 2A). Statistical significant effects of DSP toxins concentrations along time (interactions) were found for LPO (measured in both tissues), GST (measured in digestive gland) and for CAT and SOD (measured in the gills). On the other hand, significant differences in the activity among tissues (digestive gland and gills) were found for all enzymes assessed (Table 2B).

Table 2: Results of two-way analysis of variance (ANOVA) with interaction using treatment, time and tissuses as fixed factors: Biomarker responses within each tissue (**A**) and between tissues (**B**). The significance level used for all tests was 0.05.

A

Digestive Gland

	GPx		**SOD**		**CAT**		**GST**		**LPO**	
Source of Variation	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Treatment	33.88	**<0.001**	8.489	**<0.001**	0.155	0.857	3.567	**0.035**	19.50	**<0.001**
Time	15.54	**<0.001**	1.357	0.249	5.565	**0.024**	1.726	0.195	0.191	0.664
Treatment x Time	1.120	0.334	1.328	0.273	0.393	0.678	19.34	**<0.01**	5.060	**0.010**
Gills										
Treatment	25.87	**<0.001**	28.670	**<0.001**	7.868	**0.000**	5.773	**0.006**	2.656	0.080
Time	2.977	0.090	0.415	0.523	6.805	**0.020**	1.199	0.279	2.609	0.113
Treatment x Time	2.879	0.070	3.382	**0.042**	12.490	**<0.001**	0.333	0.719	2.261	**<0.001**

B

	GPx		**SOD**		**CAT**		**GST**		**LPO**	
Source of Variation	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Tissue	49.900	**<0.001**	65.150	**<0.001**	9.176	**0.004**	379.7	**<0.001**	184.9	**<0.001**
Treatment x Time	3.842	**0.025**	0.444	0.643	9.007	**<0.001**	8.769	**<0.001**	10.06	**<0.001**
Treatment x Tissue	3.882	**0.024**	0.607	0.547	4.628	**0.014**	1.859	0.162	9.138	**<0.001**
Time x Tissue	3.913	0.051	1.368	0.245	9.671	**0.003**	3.459	0.066	1.660	0.201
Treatment x Time x Tissue	0.429	0.653	3.185	**0.046**	5.663	**0.006**	5.991	**0.004**	1.814	0.168

Overall results indicate, an increase in antioxidant enzyme activities following short-term exposure of the mussel *M. galloprovincialis* to low cell densities of the DSP-toxin-producing dinoflagellate *P. lima*. This is not in agreement with the decrease previously observed by some authors when, under laboratory conditions, these mussels are exposed to resin acids (Gravato, et al., 2005) and to metals (Canesi, et al., 1999) for short periods of time. Thus, the early increase in the activity of antioxidant enzymes may reflect a rapid adaptation to the exposure to the DSP toxins and an increase in the protection against oxidative stress. From an immunological perspective, it is really interesting given that these organisms may also be less vulnerable to parasitic infections during the development of an HAB episode. However, further studies need to be performed to confirm this hypothesis.

Enzymatic Activity: Digestive Glands *vs* Gills

Significant differences between selected tissues were obtained for all measured biomarkers, and significant interactions between tissues, tested concentrations and exposure time were observed for GST and CAT activities (Table 2B). When the overall results obtained to digestive glands and gills are compared, similar detoxification rates are observed. This is in contrast to previous works in which one of these tissues showed higher detoxification rates than the other. Thus, while some works carried out in mussels considered that gills showed higher detoxification rates than digestive glands given that this tissue experience higher exposure to environmental contaminants than digestive gland (Cheung, et al., 2001; Lima, et al., 2007), other works conduced in fishs have observed that digestive glands, considered the metabolic center of these organisms, are more efficient than gills in converting an transcriptional signal into protein synthesis (Ortiz-Delgado, et al., 2008; Regoli, et al., 2011b). The similarity in the antioxidant response between tissues observed in this work can be

explained by the fact that gills are the first tissue come into contact with toxic dinoflagellate during the filtration, while the toxins are may be released in high concentrations during digestion of dinoflagellates in the digestive glands (Contardo-Jara, et al., 2008).

Gene Transcription *vs* Enzymatic Activity

A functional response can only be inferred from transcriptional data when the correlation between mRNA levels and enzymatic activities is confirmed (Giuliani, et al., 2013; Regoli, et al., 2011a). Accordingly, although a significant increase in CAT mRNA levels was observed in digestive gland after exposure to maximum *P. lima* densities (100,000 cells/L), these results are not in accordance with those obtained for enzymatic activity. Conflicting results between mRNA levels and enzymatic acivity have been reported for CAT as well other antioxidant enzymes (GPx and SOD) in zebrafish exposed to atrazine (Jin, et al., 2010). Similarly, the absence of significant Se-GPx transcriptional differences between treatments might be indicative of the lack of implication of this gene in the digestive gland's response to *P. lima* exposure in the mussel *M. galloprovincialis*. However, the higher Se-GPx levels observed in gills support the role of this enzyme balancing the antioxidant system in this tissue. A similar pattern of behavoiur was also obtained for SOD. On the other hand, there is the possibility that the discrepancy obtained between GST transcription and biochemical activity, in digestive gland and gill, might be due to the transcript analysis of a specific isoform (GST-*pi*), oppositely to biochemical analyses evaluating total GST activity including different isoforms (Giuliani, et al., 2013).

In the specific case of gill gills, although CAT and SOD mRNA levels seem to be decrease in response to *P. lima* exposure, a considerable increase of activity of both enzymes was subsequently observed. This result supports their implication in the response to oxidative stress caused by DSP toxins. Similarly, the higher Se-GPx mRNA levels observed in gills are consistent with an active antioxidant role for this enzyme in response to *P. lima* toxins. In contrast to digestive gland, this pattern was partially consistent with the results obtained for enzymatic activity. Lastly, it is important to note that differences in male/female ratio in mussel samples could be contributing to discrepancies between gene expression and enzymatic activity obtained in this work.

CONCLUSIONS

This study is the first describing the early transcriptomic and biochemical responses of the mussel *M. galloprovincialis* to the DSP-toxin-producing dinoflagellate *P. lima*. The results obtained revealed that: (1) GAPDH, rpS4 and EF2 may be employed as reference genes to normalize the gene expression in qPCR experiments carried out in digestive glands and gills of mussels exposed to DSP toxins; (2) significant transcriptional changes in genes encoding GST-*pi*, CAT, Se-GPx and SOD enzymes in both tissues, suggesting compensatory gene expression among them; (3) significant changes in the activity of GST, CAT, GPx and SOD enzymes in both tissues, revealing the implication of antioxidant system in the early response to DSP toxins in mussels; (4) an important reduction in the LPO levels in both tissues, suggesting the role of DSP toxins to increase the protection against oxidative stress in general; (5) absence of parallel global response between tissues and (6) absence of correlation between transcriptomic and bioquemical response. This last conclusion discourages the prediction of functional responses though data of gene expression. Overall, the results provided by this work underscore the importance of the antioxidant system

during early protective responses to DSP toxins and identifies new targets to develop biomarkers. In terms of coastal management, this work involves a substantial progress in the risk assessment and public health.

Concluding Remarks

The main conclusions from the studies reported in this thesis can be summarized as follows:

1. We used cell biomarkers of DNA breaks and cell viability rates (apoptosis and necrosis) to analyze the early genotoxic and cytotoxic response of hemolymph and gill cells of the mussel *M. galloprovincialis* to *in vitro* OA exposition. To this end, the comet assay and flow cytometry techniques were successfully applied, providing the following specific conclusions:

   1.1 Mussel hemolymph cells offer resistance to early genotoxic and cytotoxic effects induced *in vitro* by OA.

   1.2 Mussel gill cells present high susceptibility to early genotoxic effects induced *in vitro* by OA.

   1.3 Mussel gill cells constitute more suitable cell type to assess the early genotoxic effects of OA.

2. We simulated the early stages of an harmful algal bloom (HAB) in laboratory conditions by exposing mussels to the DSP-toxin-producing dinoflagellate *P. lima*. That made possible the study of the *in vivo* genotoxic and cytotoxic effects of DSP toxins. Aditionally, we determined the potential of the modified (OGG1) comet assay for assessing oxidative DNA damage caused by marine toxins in bivalve molluscs cells. The specific conclusions extracted from these studies are as follows:

   2.1 Low *P. lima* cell densities can be used to simulate early stages of HAB episodes in laboratory conditions.

   2.2 *In vivo* exposure to extremely low dinoflagellates densities did not produce significant genotoxic effects in hemolymph or gill cells.

2.3 The observed DNA damage in gill cells exposed *in vivo* to *P. lima* is predominantly influenced by exposure time and *P. lima* cell density.

2.4 The oxidative DNA damage observed in hemolymph exposed *in vitro* to OA is dependent on toxin concentration.

2.5 The oxidative DNA damage observed in hemolymph exposed *in vivo* to *P. lima* is only observed after 24 h exposure to the highest dinoflagellate concentration tested (100,000 cells/L).

2.6 The absence of significant levels of oxidative DNA damage at low OA and *P. lima* concentrations underscores the inability of these toxin concentrations to disturb the antioxidant balance in mussel cells.

2.7 *In vivo* exposure to low *P. lima* densities increased apoptosis but not necrosis.

3. We have analyzed the transcriptional and biochemical responses of several mussel antioxidant enzymes to determine early response of antioxidant system of mussels to the DSP-toxin-producing dinoflagellate *P. lima*. The specific conclusions extracted from these analyses are as follows:

   3.1 GAPDH, rpS4 and EF2 may be used as reference genes to normalize gene expression in qPCR experiments carried out in digestive glands and gills from mussels exposed to *P. lima*.

   3.2 The genes encoding GST-*pi*, CAT, Se-GPx and SOD enzymes experienced significant transcriptional changes in digestive glands and gills exposed to *P. lima*, suggesting compensatory gene expression among them.

   3.3 The presence of significant changes in the activity of GST, CAT, GPx and SOD enzymes in both tissues, is consistent with the

implication of the antioxidant system in the early response to DSP toxins in mussels.

3.4 The reduction in LPO levels observed in both tissues, suggests a role of DSP toxins increasing the protection against oxidative stress in general.

3.5 Absence of parallel global response between tissues and absence of correlation between transcriptomic and biochemical response.

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Extended Abstract

INTRODUCCIÓN

Las mareas rojas, técnicamente denominadas *Harmful algal blooms* (HABs), son fenómenos de contaminación natural caracterizados por la proliferación y dominio ocasional de especies de algas tóxicas o nocivas (Anderson, 2007). Su frecuencia, intensidad y distribución geográfica se ha incrementado de forma significativa en los últimos años (Anderson, et al., 2012). Esto ha afectado negativamente al sector marisquero, debido a que durante estos episodios se pueden producir toxinas capaces de ser acumuladas por organismos marinos filtradores, especialmente moluscos bivalvos, a través de los cuales se transfieren por la cadena trófica, pudiendo causar graves intoxicaciones en consumidores humanos (Prego-Faraldo, et al., 2013; Rodríguez, et al., 2011; Rodríguez, et al., 2015).

Las toxinas marinas producidas durante los HABs pueden ser clasificadas en diferentes grupos, atendiendo a su estructura química y a los síntomas de intoxicación que causan en humanos (Daranas, et al., 2001). De entre todos ellos, el grupo de las toxinas diarreicas o *diarrhetic shellfish toxins* (DSP) es el que presenta mayor distribución geográfica y mayor frecuencia de aparición (Gerssen, et al., 2010). Además, este grupo de toxinas tiene potencial para causar el síndrome DSP en consumidores humanos de marisco contaminado. Un síndrome caracterizado por diarrea, nauseas, vómitos y fuertes dolores abdominales (Valdiglesias, et al., 2013). Todo ello origina que las toxinas marinas que conforman este grupo hayan recibido importante atención en la literatura.

Las principales toxinas DSP son el ácido ocadaico o *okadaic acid* (OA) y sus derivados las dinofisitoxina-1 (DTX1), dinofisitoxina-2 (DTX2) y sus acil derivados, generalmente conocidos como dinofisitoxina-3 (DTX3) (Reguera, et al., 2014). El OA, aislado por primera vez de la esponja marina *Halichondria okadai* (Tachibana, et al., 1981) y producido por

dinoflagelados de los géneros *Dynophysis* y *Prorocentrum* (Lee, et al., 1989; Reguera, et al., 2014), es la toxina DSP más persistente en las costas Europeas, considerándose el principio activo del síndrome DSP.

La implicación del OA en el síndrome DSP motivó el desarrollo de estudios inciales cuyo objetivo principal era dilucidar los efectos nocivos de este compuesto en humanos. De esto modo, los mecanismos de acción del OA y sus efectos a nivel celular y molecular se asociaron con su capacidad para inhibir varios tipos de proteínas serina/treonina fosfatasas (Bialojan and Takai, 1988). Desde entonces, numerosos estudios con diversas líneas celulares de humanos y otros mamíferos han demostrando la capacidad del OA para inducir daños genotóxicos y citotóxicos (Valdiglesias, et al., 2013).

Como consecuencia de lo anteriormente expuesto, el cultivo comercial de moluscos bivalvos está sujeto a exhaustivos programas de monitorización para la determinación de concentraciones nocivas de algas tóxicas. La gran complejidad de los compuestos producidos durante los HABs ha dado lugar a una amplia variedad de métodos de detección de toxinas marinas, los cuales pueden ser clasificados en biológicos, químicos y bioquímicos (Prego-Faraldo, et al., 2013; Vilariño, et al., 2010). Sin embargo, aunque los actuales métodos de detección de toxinas DSP son efectivos, parecen no ser suficientemente resolutivos, pues no consiguen reducir las pérdidas económicas asociadas a estos eventos. En vista de ello, el sector del marisqueo, especialmente el miticultor, solicita el desarrollo de nuevos métodos de vigilancia destinados a reducir las actuales pérdidas económicas. Un mayor conocimiento sobre los efectos de toxinas DSP en sus principales vectores, los moluscos bivalvos, podría promover el desarrollo de nuevos métodos de detección más eficientes.

Sin embargo, a pesar de los efectos adversos que las toxinas DSP causan en humanos, los moluscos bivalvos no ven incrementadas sus tasas de

mortalidad tras su exposición a frecuentes y continuos episodios tóxicos. Estos organismos parecen poder bloquear la toxicidad de las microalgas productoras de toxinas DSP, empleándolas como recurso nutritivo. Aunque la respuesta es variable, parece que cuanto más prolongada es la exposición de los bivalvos a los episodios tóxicos, mayor es su resistencia (Shumway and Cucci, 1987). Estos indicios motivaron el dasarollo de estudios posteriores que determinaron que los efectos nocivos de las toxinas DSP en estos organismos son más intensos a concentraciones bajas y tiempos de exposición cortos (Flórez-Barrós, et al., 2011; Pinto-Silva, et al., 2005; Prado-Alvarez, et al., 2012; Prado-Alvarez, et al., 2013).

Para evaluar los efectos de las toxinas marinas en los moluscos bivalvos los biomarcadores constituyen una herramienta útil. Los biomarcadores se definen como variaciones bioquímicas, celulares, fisiológicas o comportamentales que pueden ser medidas en fluídos, células, tejidos u organismos enteros, y que proporcionan evidencias de la exposición a contaminantes (Livingston 1993, Monserrat 2003). Los biomarcadores son clasificados como biomarcadores de exposición, cuando aportan información sobre las características de la exposición; biomarcadores de efecto, cuando cuantifican cambios en el organismos como consecuencia de la exposición, y biomarcadores de susceptibilidad, cuando indican las características específicas de un organisms que son más susceptibles a los efectos de la exposición. Varios biomarcadores de efecto han sido empleados a nivel celular y molecular para evaluar los efectos nocivos de toxinas marinas en los moluscos bivalvos. Así, biomarcadores celulares como las roturas en el ADN, alteraciones cromosómicas o tasas de viabilidad celular constituyen herramientas útiles para evaluar los efectos genotóxicos y citotóxicos de las toxinas marinas, proporcionando información sobre ellos antes de que sean observados a mayores niveles de organización biológica. Adicionalmente, biomarcadores moleculares como

los niveles de expresión génica o proteica y actividades enzimáticas contribuyen a evaluar la toxicidad, a través del reconocimiento y empleo de dianas moleculares.

OBJETIVOS

Dada la escasez de estudios sobre los efectos inmediatos de las toxinas DSP en los moluscos bivalvos, esta tesis persiguió como objetivo general investigar la respuesta temprana del mejillón *Mytilus galloprovincialis* a las toxinas DSP. Los datos obtenidos permiten comprender mejor la primera respuesta funcional de los moluscos bivalvos a estos compuestos tóxicos, además de facilitar el desarrollo futuro de nuevos métodos de detección de toxinas DSP que favorezcan la reducción de las actuales pérdidas económicas asociadas a estos episodios tóxicos.

Para alcanzar el objetivo general propuesto se plantearon los siguientes objetivos específicos: (1) análisis *in vitro* de los efectos genotóxicos y citotóxicos del OA en diferentes tipos celulares de mejillón *M. galloprovincialis*, (2) estudio *in vivo* de los efectos genotóxicos y citotóxicos de la exposición de varios tejidos de mejillón al dinoflagelado productor de toxinas DSP *Prorocentrum lima* y (3) determinación de la respuesta transcripcional y bioquímica de varias enzimas antioxidantes de mejillón a los efectos causados por exposiciones *in vivo* a *P. lima*. Los detalles de cada objetivo específico se recogieron en tres capítulos independientes. A continuación se resumen la información más relevante contenida de cada uno de ellos.

CAPÍTULO 1

El capítulo 1, con el propósito de realizar una primera aproximación al conocimiento de los primeros efectos genotóxicos y citotóxicos del OA (la toxina DSP más persistente en las costas Europeas), recoge la exposición *in*

vitro de dos tipos celulares del mejillón *M. galloprovincialis* a concentraciones bajas (10, 50, 100, 200 y 500 nM), durante tiempos de exposición cortos (1 y 2 h).

El mejillón *M. galloprovincialis* ofrece una doble ventaja para evaluar *in vitro* los efectos del OA, ser uno de los moluscos bivalvos más afectado por los episodios HAB y ser un reconocido organismo centinela de la contaminación (Goldberg, 1986; Viarengo and Canesi, 1991). Para completar el primer objetivo se seleccionaron dos de sus tipos celulares, hemocitos y células branquiales. Los hemocitos o células de hemolinfa se seleccionaron por ser fáciles de extraer y permir estudiar respuestas asociadas al sistema inmune (Haberkorn, et al., 2010; Hégaret, et al., 2007; Hégaret, et al., 2011), mientras que las células de branquia se seleccionaron por ser la primera barrera del organismo en entrar en contacto con los dinoflagelados productores de OA, además de ser consideradas un sistema modelo para estudios ecotoxicológicos en moluscos bivalvos (Akcha, et al., 2004; Flórez-Barrós, et al., 2011; Hanana, et al., 2012; Rank and Jensen, 2003; Talarmin, et al., 2008; Venier, et al., 1997).

Dos biomarcadores celulares, roturas en el ADN y tasas de viabilidad celular, se emplearon para evaluar los efectos genotóxicos y citotóxicos del OA. Las roturas en el ADN se evaluaron en células de hemolinfa y branquia mediante la técnica del ensayo del cometa, mientras que las tasas de viabilidad celular se determinaron en células de hemolinfa usando la técnica de la Anexina V mediante citometría de flujo.

El ensayo del cometa o electroforesis en gel de células individuales, es una técnica sensible, fácil , rápida y cuantitativa que permite detectar daño en el ADN de células individuales. La capacidad de esta técnica para detectar daños en células de organismos marinos ha sido ampliamente demostrada (Lee and Steinert, 2003; Mitchelmore and Chipman, 1998; Wilson, et al.,

1998). En el capítulo 1 se llevó a cabo la versión alcalina del ensayo del cometa, la cúal permite detectar roturas de cadena simple y cadena doble en el ADN, sitios álcali-lábiles y procesos incompletos de reparación por escisión (Singh, et al., 1988). Esta técnica consiste en embeber las células individuales en un gel de agarosa localizado sobre un portaobjetos, posteriormente las células se lisan y se someten a electroforesis bajo condiciones alcalinas. Al finalizar la electroforesis, los núcleos celulares se tiñen con un colorante fluorescente y se visualizan bajo un microscopio de fluorescencia. El resultado es un conjunto de núcleos con forma de "cometa", en los que los fragmentos de ADN se han distribuído a lo largo de la cola del cometa reflejando las roturas presentes en el ADN.

El ensayo de la Anexina V se empleó para evaluar las tasas de apoptosis/necrosis resultantes de la exposición *in vitro* de células de hemolinfa a OA. Este ensayo se basa en la detección de la localización de la fosfatidilserina en el membrana plasmática. La fosfatidilserina es un tipo de fosfolípido que en células viables se mantiene en la monocapa interior de la membrana celular. Cuando se empieza a desencadenar el proceso de apoptosis, la fostatidilserina migra a la capa externa de dicha membrana. La localización de la fosfatidilserina puede ser detectada específicamente mediante la Anexina V, ya que esta se une específicamente a la fostatidilserina en una reacción dependiente de calcio. Las moléculas de Anexina V se pueden marcan con diferentes fluoróforos para detectar apoptosis mediante citometría de flujo. Además, en estadíos tardíos de apoptosis, cuando la membrana plasmática ha perdido su integridad, el ADN se hace accesible. De esta manera, si se emplean moléculas fluorescentes que actúan como intercalantes en los ácidos nucleicos de doble cadena (como 7-amino-actinomicina D) se pueden detectar también las células apoptóticas tardías.

Los resultados obtenidos mediante el ensayo del cometa revelaron ausencia general de daño significativo en el ADN de células de hemolinfa después de ser expuestas *in vitro* a OA. Sin embargo, se observó un incremento significativo en el ADN de hemocitos expuestos a 10 nM de OA después de 2 h de exposición. Así, el OA parece ejercer un efecto genotóxico rápido en células de hemolinfa, sugiriendo la presencia inmediata de respuestas celulares protectoras contra esta toxina. En contraste, las células de branquia incrementaron significativamente el daño en el ADN a todas las concentraciones de OA evaluadas, excepto a las más baja (10 nM). Además, después de 1 h de exposición se observó una correlación positiva entre la concentración y la respuesta, aunque esta desapareció después de 2 h de exposición. Sin embargo, la estabilidad del daño obtenida tras las 2 h de exposición puede ser consecuencia de excesivo daño basal acumulado o consecuencia de una inducción de la apoptosis por el OA. Por todo ello, las células branquiales parecen ser más útiles que las células de hemolinfa para determinar los efectos inmediatos del OA en mejillón, aunque únicamente deben emplearse durante tiempos exposición cortos.

Los ensayos de Anexina V realizados mediante citometría de flujo no revelaron citotoxicidad en células de hemolinfa expuestas a diferentes concentraciones de OA durante 1 h. Sin embargo, después de 2 h de exposición se observó una correlación positiva entre la concentración y la respuesta, tanto para la apoptosis como para la necrosis.

En conclusión, el capítulo 1 determinó la presencia de un mecanismo de resistencia contra los efectos genotóxicos y citotóxicos inducidos por OA en hemocitos de mejillón, así como la gran sensibilidad de las células de branquia a la genotoxicidad temprana causada por OA con respecto a las células de hemolinfa y la constitución por las células de branquia de un sistema adecuado para evaluar los primeros efectos genotóxicos causado por OA en mejillón expuestos a concentraciones bajas y tiempos de exposición

extremadamente cortos. El conjunto de este capítulo contribuye a incrementar el actual conocimiento sobre las consecuencias genotóxicas y citotóxicas relacionadas con la exposición a concentraciones bajas de OA.

CAPÍTULO 2

La evaluación de la toxicidad del OA a partir de estudios *in vitro*, tales como los recogidos en el capítulo 1, ofrecen ventajas como la rapided, precisión, y reproducibilidad, así como el bajo coste (Bravo, et al., 2001; Wernersson, et al., 2015). Por todo ello, constituyeren una herramienta apropiada para la realización de una primera aproximación a los efectos directos del OA. Sin embargo, los resultados obtenidos mediante ensayos *in vitro* necesitan ser validados mediante ensayos *in vivo* complementarios.

A diferencia de los ensayos *in vitro*, los ensayos *in vivo* permiten determinar el efecto indirecto de las toxinas, considerando un abanico más amplido de respuestas, tales como la absorción, distribución, metabolización y depuración del tóxico (Kilemade and Quinn, 2003). Además, los ensayos *in vivo* posibilitan la determinación de los efectos sinérgicos entre las diferentes toxinas DSP (OA y DTXs) producidas por los dinoflagelados.

El capítulo 2 de estas tesis se centra en la evaluación *in vivo* de la primera respuesta genotóxica y citotóxica del mejillón *M. galloprovincialis* a densidades bajas del dinoflagelado productor de toxinas DSP *P. lima* (1.000 y 100.000 células/L), durante tiempos de exposición cortos (24 y 48 h). Para ello, el daño en el ADN se evaluó mediante ensayo del cometa alcalino en células de hemolinfa y de branquia. Las células de hemolinfa también se emplearon para evaluar el daño oxidativo causado en el ADN (*in vitro* por el OA e *in vivo* por las toxinas DSP) mediante ensayo del cometa modificado con la enzima OGG1. Asimismo, esta tesis empleó por primera

vez el ensayo del cometa modificado con la enzima OGG1 para determinar el daño oxidativo causado por toxinas marinas en células de bivalvos.

Los primeros efectos citotoxicos manifestados en célula de hemolinfa también se evaluaron mediante el ensayo de la Anexina V utilizando citometría de flujo.

Tras la exposición de los mejillones al dinoflagelado productor de toxinas DSP, la concentración de OA acumulada en sus tejidos se empleó como indicador de la ingesta de *P. lima*, y de la consecuente incorporación de toxinas DSP a los mejillones. Los valores de OA acumulados se compararon con los presentados en mejillones contaminados de forma natural durante episodios HAB, corroborandose que las condiciones empleadas en el capítulo 2 simulan las presentadas durante los primeros estados de un episodio HAB (Prado-Alvarez, et al., 2012).

Los resultados de genotoxicidad obtenidos en el capítulo 2 revelaron ausencia de daño genotóxico en células de hemolinfa y branquias expuestas a densidades extremadamente bajas (1.000 células/L) de *P. lima*. El daño genotoxico analizado en células de branquia estuvo predominantemente influenciado por la densidad celular de *P. lima* y el tiempo de exposición, diferenciándose notablemente de los resultados obtenidos en el capítulo 1. Sin embargo, en células de hemolinfa se observó un incremento significativo del daño genotóxico después de 24 h de exposición a 100.000 células/L de *P. lima*, que desapareció rapidamente en el tiempo. Además, los resultados obtenidos mediante ensayo del cometa modificado con la enzima OGG1 sugieren que el OA ejerce daño oxidativo directa e indirectamente y que los daños genotóxicos observados en células de hemolinfa expuestas *in vivo* a *P. lima* pueden ser consecuencia de estés oxidativo. Sin embargo, tales daños parecen neutralizarse en el tiempo como consecuencia de un sistema antioxidante eficiente.

Por otra parte, los daños citotóxicos causados por la exposición temprana de células de hemolinfa a *P. lima* se revelaron mediante un incremento en los niveles de apoptosis pero no de los de necrosis, sugiriendo que estas toxinas no presentan una influencia negativa en las viabilidad. Este hecho probablemente se deba al elevado número de proteínas inhibidoreas de apoptosis presentes en los moluscos bivalvos (Simões, et al., 2015).

En conclusión, en el capítulo 2 se simularon en el laboratorio estados tempranos de un episodio HAB. Bajo estas condiciones, las células de hemolinfa y branquia no parecen experimentar efectos genotóxicos a densidades celulares extremadamente bajas de dinoflagelado tóxico (1.000 células/L). Además, el daño observado en el ADN de células de branquia estuvo predominantemente influenciado por el tiempo de exposición y la densidad célular de *P. lima*. Así, las células de hemolinfa parecen experimentar daño en el ADN más pronto que las células de branquia, aunque parecen ser capaces de neutralizarlo con mayor rapidez. Respecto al daño oxidativo en el ADN, los hemocitos expuestos *in vivo* a *P. lima* presentaron daño únicamente después de 24 h de exposición a la densidad más alta empleada (100.000 células/L), sugiriendo la implicación del sistema antioxidante. Por otra parte, densidades crecientes de *P. lima* incrementaron la citotoxicidad en células de hemolinfa mediante aumentos significativos de la apoptosis.

CAPÍTULO 3

El daño oxidativo detectado en el ADN de células de hemolinfa y su rápida desaparición en el tiempo (Capítulo 2), provocó sospechas sobre la posible implicación del sistema antioxidante en la resistencia de los moluscos bivalvos a las toxinas DSP, motivando el desarrollo del capítulo 3. En el tercer capítulo se determinó la respuesta transcripcional y bioquímica de varias enzimas antioxidantes de mejillones *M. galloprovincialis*, expuestos

in vivo a densidades celulares bajas del dinoflagelado tóxico *P. lima* (1.000 y 100.000 células/L), durante tiempos de exposición cortos (24 y 48 h); condiciones experimentales coincidentes con las empleadas en el capítulo anterior. En esta ocasión, las glándula digestivas y branquias de mejillón se seleccionaron como tejidos diana debido a sus importantes implicaciones metabólicas. La respuesta transcripcional se determinó mediante ensayos de PCR (*Polymerase Chain Reaction*) cuantitativa en tiempo real, mientras que la respuesta bioquímica se determinó mediante el empleo de diversos ensayos específicos de actividades enzimáticas.

En determinadas ocasiones la toxicidad de los contaminantes se puede asociar con su capacidad para incrementar los niveles celulares de especies reactivas de oxígeno (ROS). De manera general, los organismos regulan estos niveles mediante la actividad de enzimas antioxidantes y mecanismos de reparación del ADN. Teniendo esto en cuenta, una inducción del sistema antioxidante de mejillones debido a la exposición a toxinas DSP podría aclarar, en parte, la aparente resistencia que experimentan estos organismos frente a estas toxinas, además de poder emplearse como herramienta útil en procesos de biomonitorización (Contardo-Jara, et al., 2008; Žegura, et al., 2004).

El sistema antioxidante de los moluscos bivalvos está compuesto por varias moléculas que trabajan cooperativamente y de forma secuencial. La superóxido dismutasa (SOD), catalasa (CAT) y glutatión peroxidasa (GPx) son las principales enzimas antioxidantes que participan en la destrucción de ROS en la célula. SOD cataliza la dismutación del anión superóxido (O^{2-}) a peróxido de hidrógeno (H_2O_2). CAT está presente en los peroxisomas donde cataliza la conversión del H_2O_2 generado por SOD a H_2O y O_2. La actividad CAT está complementada con la actividad GPx, quien también metaboliza el H_2O_2 a H_2O. Por otra parte, la glutatión S transferasa (GST)

cataliza la conjugación de xenobióticos con glutatión reducido (GSH) (Manduzio, et al., 2005).

En el capítulo 3 se determinó la expresión de los genes que codifican para la glutatión S transferasa *pi-1* (GST-*pi*), la CAT, la SOD, y la GPx dependiente de selenio (Se-GPx). Para poder normalizar los datos de expresión génica obtenidos mediante PCR cuantitativa en tiempo real se evaluó la estabilidad de varios genes susceptibles de ser usados como genes de referencia [ARN ribosomal 18S (18S), gliceraldehido 3-fosfato-dehidrogrenasa (GAPDH), proteínas ribosomales (rpS4), factor de elongación 1 (EF1), factor de elongación 2 (EF2) e histona H2A (H2A)]. Los resultados obtenidos determinarón a GADPH, rpS4 y EF2 como los genes de referencia más estables. Además, revelaron diferencias trasncripcionales significativas en todos los genes de las enzimas antioxidantes evaluadas (GST-*pi*, CAT, SOD y GPx), intuyéndose una respuesta compensatoria entre ellas. Sin embargo, las respuestas observadas en glándula digestiva y blanquia fueron diversas, corroborando diferencias en la expresión génica de las enzimas antioxidantes entre tejidos.

La posible relación entre las variaciones de expresión génica de las enzimas antioxidantes evaluadas y su respuesta frente a toxinas DSP se vio confirmada tras el análisis de la actividad enzimática. En glándula digestiva la actividad de SOD y GPx se vió incrementada para todas las condiciones evaluadas. Sin embargo, la actividad de CAT no se vió alterada, pudiendo esta estar suplida por la actividad GPx (Regoli, et al., 2011a; Regoli, et al., 2011b). Por otra parte, la actividad GST solo respondió favorablemente al tratamiento experimental más extremo (100.000 células/L, 48 h). Además, estas actividades se asociaron con una reducción en los niveles de LPO a lo largo del tiempo. En branquia, la actividad de todas las enzimas antioxidantes evaluadas (GST, CAT, GPx and SOD) fue significativamente mayor para alguno de los tratamiento empleados. De manera similar a la

glándula digestiva, la actividad SOD y GPx fue significativamente mayor para la mayoría de las condiciones experimentales utilizadas, sugiriendo que estas dos enzimas actúan interdependientemente para neutralizas los ROS producidos por las toxinas DSP en los moluscos bivalvos. Por otra parte, la actividad GST solo se incrementó significativamente después de 48 h de exposición a *P. lima*. Estos resultados también se asocian con reducción de los niveles de LPO, sugiriendo una gran capacidad del sistema antioxidante de mejillón para neutralizar el daño oxidativo causado por las toxinas DSP.

Sin embargo, los niveles de expresión génica obtenidos no coinciden con los niveles de actividad enzimática. Basándose en esta discrepancia, los estudios transcriptómicos deben ser empleados cuidadosamente, y no deben utilizarse en solitario para extraer respuestas funcionales. Así, este capítulo pone de manifiesto la importancia de realizar estudios proteicos para comprender el modo de acción de estas toxinas, considerando además que las proteínas son efectores moleculares eventuales de procesos biológicos y su modulación puede estar relacionada con los posibles efectos nocivos de estas toxinas en la fisiología de los moluscos bivalvos. Además, este último capítulo revela la implicación del sistema antioxidante de los moluscos bivalvos en la primera respuesta contra las toxinas DSP e identifica nuevas dianas para el desarrollo de biomarcadores de detección temprana de la contaminación.

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Research Fellowships

2015 Visiting Researcher Fellowship (Inditex Fundation and University of A Coruña) in the Ecotoxicology group of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) (University of Porto, Portugal) (October-December 2015).

2013 Collaboration Fellowship in the Department of Cellular and Molecular Biology, Science Faculty, University of A Coruña,

Spain (September 2012- Junne 2013).

2012 Master Fellowship. International Campus of Excellence Campus Do*Mar, University of Vigo, Spain. Study of differential gene expression in the mussel *Mytilus galloprovincialis* in response to okadaic acid exposure (October 2011- July 2012)

2011 Undergraduate Fellow in the Department of Cellular and Molecular Biology, University of A Coruña. (December 2010-July 2011)

2010 Undergraduate Fellow in the Scientific Museums of A Coruña, Spain. Program: Guide Tour (December 2009-July 2010).

Contracts

2015 Contract associated to the project Cytogenetic and Molecular Assessment of the Early Response of the Mussel *Mytilus galloprovincialis* to Okadaic Acid (EVALOA-PLUS). Funded by the Spanish Ministery of Economy and Competitiveness. (01/01/2015- 30/06/2015)

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Principal Investigator: Josefina Méndez