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Transcriptional and biochemical analysis of antioxidant enzymes in the mussel *Mytilus galloprovincialis* during experimental exposures to the toxic dinoflagellate *Prorocentrum lima*

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ABSTRACT

The genotoxic and cytotoxic effects of Diarrhetic Shellfish Poisoning (DSP) toxins have been widely investigated in bivalve molluscs, representing the main vectors of these compounds in the Atlantic coast of Europe. DSP toxins are produced by Harmful Algal Blooms (HABs) of *Dinophysis* and *Prorocentrum* dinoflagellates, being subsequently accumulated by marine organisms and biomagnified throughout trophic webs. Yet, bivalves display increased resistance to the harmful effects of these toxins during HAB episodes. While previous reports have suggested that such resilience might be the result of an increased activity in the bivalve antioxidant system, very little is still known about the specific mechanism underlying the protective effect observed in these organisms. The present work aims to fill this gap by studying transcriptional expression levels and biochemical activities of antioxidant enzymes in different tissues the mussel *Mytilus galloprovincialis* during experimental exposures to DSP toxins produced by the dinoflagellate *Prorocentrum lima*. Results are consistent with the presence of a compensatory mechanism involving a down-regulation in the expression of specific genes encoding antioxidant enzymes [i.e., SuperOxide Dismutase (SOD) and CATalase (CAT)] which is counterbalanced by the up-regulation of other antioxidant genes such as Glutathione S-Transferase *pi-1* (GST-*pi*) and Selenium-dependent Glutathione PeroXidase (Se-GPx), respectively. Enzymatic activity analyses mirror gene expression results, revealing high antioxidant activity levels (consistent with a protective role for the antioxidant system) along with reduced lipid peroxidation (increasing the defense against oxidative stress).

1. Introduction

Okadaic Acid (OA) and its analogs, the DinophysisToXins (DTXs), constitute the main toxins responsible for the Diarrhetic Shellfish Poisoning (DSP) syndrome in humans, displaying the highest incidence along the Atlantic coast of Europe (Gerssen et al., 2010). This syndrome is characterized by diarrhea, nauseas, vomiting and abdominal pain in human consumers of contaminated shellfish (Valdiglesias et al., 2013). The toxins responsible for the DSP syndrome (DSP toxins) are produced during Harmful Algal Blooms (HABs) of *Dinophysis* and *Prorocentrum* dinoflagellates (Reguera et al., 2014), and their harmful effects on human health and environment have been mainly associated with their ability to inhibit protein phosphatases involved in critical cellular processes (Bialojan and Takai, 1988) The increasing frequency and duration of HABs worldwide have attracted significant attention in recent years, due to their negative impact on human health (Gestal-Otero, 2014) and aquaculture industries (Rodríguez-Rodríguez et al., 2011). This is particularly relevant in the case of OA, a toxin responsible for the inhibition of several types of Serine/Threonine protein phosphatases (Bialojan and Takai, 1988), and whose toxic effects include cytotoxicity and genotoxicity in mammalian cell lines (Valdiglesias et al., 2011, 2010, 2013).

The number of studies evaluating the harmful effects of OA on

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List of abbreviations

CAT	CATalase
DSP	Diarrhetic Shellfish Poisoning
GPx	Glutathione Peroxidase
GST	Gluthatione S-Transferase
HPLC/MS	High-Performance Liquid Chromatography Mass Spectroscopy
LPO	Lipid PerOxidation
OA	Okadaic Acid
PSP	Paralytic Shellfish Poisoning
SOD	SuperOxide Dismutase

their principal vectors (i.e., bivalve molluscs) has increased consistently in the last few years (McCarthy et al., 2014; Pinto-Silva et al., 2003; Prego-Faraldo et al., 2013, 2015). On the other hand, the effects resulting from simultaneous exposures to several DSP toxins remains unexplored in these organisms. These type of studies are progressively gaining interest, as toxic dinoflagellates can produce different types of DSP toxins in addition to OA (Reguera et al., 2014). Compared with other organisms, DSP toxins (including OA) cause the most severe genotoxic and cytotoxic effects on bivalve molluscs, even after exposure to low cell densities for short periods of time (e.g., 200 cells/L for 24 h) (Prado-Alvarez et al., 2012). Such observation has been further corroborated by previous experiments from our own group, describing DNA damage in mussel hemocytes exposed to low concentrations of DSP toxins, followed by a rapid DNA repair process. (Prego-Faraldo et al., 2016). On the contrary, longer exposures to higher OA concentrations (e.g., 20,000 cells/mL for 4 days) seem to trigger the onset of resistance mechanisms in bivalves, notably involving the antioxidant defense system (Flórez-Barrós et al., 2011; Pinto-Silva et al., 2005; Prado-Alvarez et al., 2012; Prego-Faraldo et al., 2015).

The ability of the bivalve antioxidant defense system to effectively respond to a broad variety of environmental stressors has been widely reported (Lima et al., 2007; Oliveira et al., 2015; Regoli and Giuliani, 2014). Accordingly, this system is capable to partially mitigate the effects of genotoxic pollutants thanks to the combined action of different antioxidant enzymes, including SuperOxide Dismutase (SOD), CATalase (CAT), Glutathione Peroxidase (GPx), Gluthatione S-Transferase (GST), and non-enzymatic molecules. Those, along with other antioxidant enzymes, have been used to assess the genotoxic effects of diverse pollutants including saxitoxins, DSP toxins and paralytic shellfish toxins (Astuya et al., 2015; Fabioux et al., 2015; Núñez-Acuña et al., 2013; Romero-Geraldo and Hernández-Saavedra, 2014; Suarez-Ulloa et al., 2015). Accordingly, some studies have revealed a significant increase in the activity of SOD, CAT, GPx or GST in different bivalve species (*Diplodon chilensis*, *Nodipecten subnodosus* and *M. galloprovincialis*) fed with the toxin-producing microalgae *Microcystis aeruginosa, Gymnodinium catenatum* and *Alexandrium tamarense* (Estrada et al., 2007; Qiu et al., 2013; Sabatini et al., 2011). On the contrary, a repression in the genes encoding CAT, SOD and GPx was reported in oysters exposed to the algae *Alexandrium minutum* (Mat et al., 2013). However, only one study has evaluated the activity of antioxidant enzymes during exposure to DSP toxins in the mussel *M. galloprovincialis*, revealing an increase in GPx activity in response to OA accumulation (Vidal et al., 2014).

With the advent of omics technologies, new approaches are now available for studying pollution in the ocean, notably gene expression and function. Accordingly, numerous reports have addressed the potential of transcriptional profiles to identify genes involved in specific responses to marine toxins in different bivalve cell types (Suarez-Ulloa et al., 2015; Venier et al., 2006). Nonetheless, the links between gene transcription profiles and subsequent cell responses remain elusive, requiring additional biochemical analyses in order to ascertain their functional role. So far, only a handful of studies have combined transcriptomic and biochemical approaches to assess the effect of pollutants in marine invertebrates, reaching conflicting results (Banni et al., 2014; Canesi et al., 2007, 2008; Giuliani et al., 2013). Such discrepancies result from differences in transcriptional and translational mechanisms, in tissue-specific metabolic rates, in the post-transcriptional modifications of proteins, in the bi-phasic response of antioxidant enzymes as well as in the interactions between chemical mixtures (Regoli and Giuliani, 2014). The present work builds on these results to study the transcriptional expression levels and biochemical activities of antioxidant enzymes in the mussel *M. galloprovinciallis* during experimental exposures to the DSP-producing dinoflagellate *P. lima*. For that purpose, the present work combined quantitative PCR gene expression analyses with biochemical assays. Overall, the obtained results underscore the importance of the antioxidant system during early protective responses to DSP toxins.

2. Material and methods

2.1. Specimen sampling

Adult *M. galloprovincialis* specimens (33.9 ± 0.6 mm anterior-posterior shell length) 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. They were placed in thermally insulated boxes with seawater from the sampling site and immediately transported to the laboratory. Seawater salinity, conductivity and pH were measured within the following 12 h using a Mettler-Toledo Seven Easy S33K (Mettler-Toledo, Schwarzenbach, Switzerland) and a Crison MicropH 2001 (Crison Instruments, Barcelona, Spain), respectively. Water conductivity ranged between 48.87 mS/cm ± 0.66 (at sampling site) and 47.69 mS/cm ± 0.03 (at laboratory conditions), salinity ranged between 31.83 ppm ± 0.49 (at sampling site) and 30.95 ppm ± 0.03 (at laboratory conditions), and pH ranged between 8.33 ± 0.01 (at sampling site) and 8.14 ± 0.01 (at laboratory conditions). OA content was evaluated in whole mussel samples (20 g) 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. Samples were stored at −20 °C until HPLC/MS analyses. A total of 240 mussel individuals were acclimatized to laboratory conditions for a week in a filtered seawater aerated tank (45 L, 5.3 mussels/L) in a photoperiod chamber (17 °C, 12 h light-dark cycle). Specimens were fed two times a day with a 1:1 mixture of two nontoxic microalgae species (*Isochrysis galbana* and *Tetraselmis suecica*). Water was replaced in experimental tanks 2 h before of each feeding cycle.

2.2. Experimental design and exposure to DSP-producing microalgae

Upon acclimatization, mussels were randomly divided into three groups and introduced in tanks (15 L) containing filtered aerated seawater. Each tank was assigned to a different treatment as follows (Fig. 1): a control group fed daily with a 1:1 v/v mixture of *I. galbana* and *T. suecica* (3×10^6 and 12×10^6 cells/L, respectively), and two treatment groups additionally fed with the DSP-producing dinoflagellate *P. lima* (1000 and 100,000 cells/L,

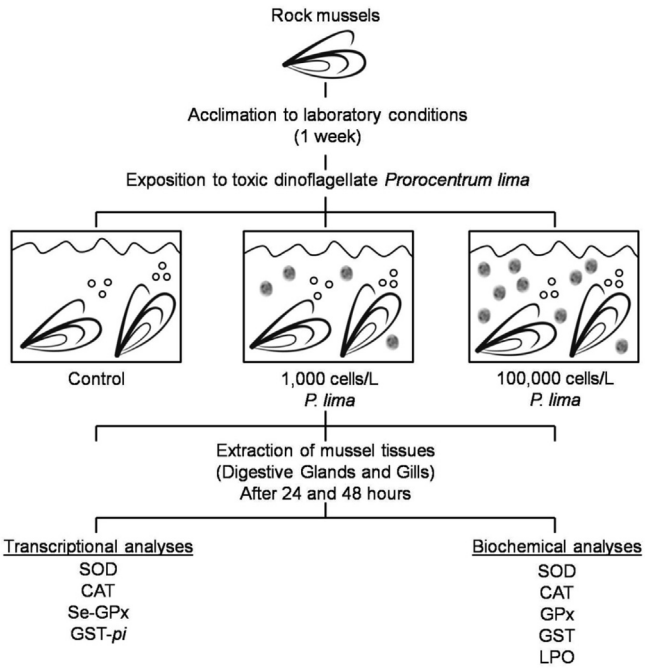

Fig. 1. Experimental design of mussel exposures to DSP-producing dinoflagellates. Mussels were collected in the field, acclimated to laboratory conditions and subsequently exposed to different cellular densities of the DSP-producing dinoflagellate *P. lima* (1000 and 100,000 cells/L) for 24 h and 48 h. Digestive gland and gill tissues were dissected from experimental mussels and used to evaluate modifications in gene transcription and antioxidant enzyme activity. Lipid peroxidation levels were additionally examined in both tissues.

respectively, 4 times/day) for 24 h and 48 h. Algae concentrations were monitored through the experiment. The experiment was replicated twice. Each treatment consisted of 80 mussel specimens (24 for biochemical analyses, 40 for transcriptomic analyses and 16 for OA quantification). Since two different exposure times were assayed, half specimens were collected at 24 h and the remaining half at 48 h. The *P. lima* culture (strain AND-A0605) was obtained from the Quality Control Laboratory of Fishery Resources (Huelva, Spain). Cell concentrations were determined by performing cell counts in Sedgwick-Rafter counting slides (Pyser-Sgi, Edenbridge, UK) after fixation with Lugol's solution. Experimental concentrations (1000 and 100,000 cells/L) were subsequently prepared from the original culture. The choice of these cell densities was based on previous reports describing OA concentrations during early stages of HABs (Díaz et al., 2013). Upon exposure, digestive gland and gill tissues were randomly sampled from 5 individuals at each treatment (1000 cells/L for 24 h, 1000 cells/L for 48 h, 100,000 cells/L for 24 h, 100,000 cells/L for 48 h) and pooled [thus reducing sample variability (Suarez-Ulloa et al., 2015)] for gene expression analyses. A total of four biological replicates were performed (4 pools per treatment) in order to account for potential sources of variability in the different parameters studied. Additionally, digestive gland and gill tissues were randomly sampled from twelve individuals per treatment for biochemical analyses. All samples were frozen in liquid nitrogen and stored at −80 °C until further analyses.

2.3. RNA isolation, experimental and reference gene selection

Genes encoding antioxidant enzymes were selected as indicators of early responses to the harmful effects of DSP toxins in mussels, including Glutathione S-Transferase *pi-1* (GST-*pi*), CATalase (CAT), selenium-dependent Glutathione Peroxidase (Se-GPx) and SuperOxide Dismutase (SOD). The choice of these genes was further supported by a previous report from our laboratory showing their differential expression in a microarray from digestive gland of mussels exposed to low OA concentrations (Suarez-Ulloa et al., 2015). RNA was extracted from pooled digestive gland and gill tissues 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) and RNA integrity was further confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN values were obtained in the present work but are not shown because they are not informative in the specific case of bivalves. In this group 28S rRNA runs differently from mammalian rRNA in gels (Barcia et al., 1997), which is the marker used by Agilent as reference in estimations. Still, the absence of basal noise in signal along with the consistency of the peak observed for 28S in all samples, suggests that this is not a result or consequence of RNA degradation. Spectrometry results shown that all samples had a 260/280 ratio >1.9 and a 260/230 ratio >1.7, safely discarding simple contamination. cDNA was subsequently 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 EF1, EF2 and H2A were designed as part of the present work (Table 1) using the Universal Probe Library program (www.universalprobelibrary.com). Two different algorithms, Normfinder [R version 3.0.1 (Team, 2014)] and GeNorm PLUS as implemented in Biogazelle's qbasePLUS software version 3.0 (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, 2014).

2.4. Quantitative PCR analyses (qPCR)

qPCR analyses were performed using four biological replicates per treatment (parallel measurements of biologically distinct samples, 4 pools of 5 mussels each, 20 mussels total, capturing random biological variation) and two technical replicates per biological replicate. Standard deviation of replicates for each individual was calculated and threshold was applied to verify if variation was acceptable. Since a difference of 1 Ct is equivalent to a 2-fold difference in quantity, a standard deviation less than 0.250 constitutes a 95% confidence interval. A “compromise” cut-off of 0.200 was thus chosen for the present analyses. Specific qPCR primers were designed from sequences retrieved from GenBank (Table 1) using the Universal Probe Library program (universalprobelibrary.com), according to qPCR restrictions. Primer specificities were verified using agarose gel electrophoresis, yielding a single DNA product of the expected 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 the following 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 °C, followed by an amplification of the target cDNA for 40 cycles, each cycle consisting of a denaturation at

Table 1
qPCR primers employed in the amplification of genes studied in the present work.

Gene name	Reference	Abbreviation	Efficiencies ± Standard Error	Amplicon Size (nt)	Melting Temperature	Primers 5′→3′(Lenght nt)
18S ribosomal RNA	L33452	18S	–	114	F - 58.3 R - 55.9	F-CCTGGAAAGGTCGGGTAAC (19) R-AATTACAAGCCCCAATCCCTA (21)
Elongation Factor 1	#Suarez-Ulloa et al., 2015	EF1	–	106	F - 55.8 R - 57	F-CCTCCCACCATCAAGACCTA (20) R-GGCTGGAGCAAAGGTAACAA (20)
Elongation Factor 2	#Suarez-Ulloa et al., 2015	EF2	1.87 ± 0.28	103	F - 59.1 R - 55.8	F-ACCACGACGCTTGTTGAGA (19) R-TTCTTGGTAGAAATTCAGTGTCCA (24)
Histone H2A	AY267755.1	H2A	–	112	F - 60.1 R - 59.9	F- CGGAGCACCAGTCTACCTTG (20) R-GATGACGGGGGATGATTCTGC (21)
GlycerAldehyde 3-Phosphate-DeHydrogenase	Lozano et al., 2015	GAPDH	1.89 ± 0.11	114	F - 59.4 R - 58.4	F-AGGAATGGCCTTCAGGG (17) R-TCAGATGCTGCTTTAATGGCTG (22)
Ribosomal proteins S4	Lozano et al., 2015	rpS4	1.87 ± 0.30	138	F - 58.8 R - 60.3	F-TGGGTTATCGAGGGCGTAG (19) R-TCCCTTAGTTTGTTGAGGACCTG (23)
Glutathione S-Transferase pi1	AF527010.1	GST-pi	2,00 ± 0.37	66	F - 59 R - 60	F-TCACCTGGATGTCTTGATGC (20) R-TGGTCTAGCTAACACTCGCTCA (22)
CATalase	AY743716.2	CAT	1.97 ± 0.20	70	F - 60 R - 59	F-TGCTCTGGGATTTCATTACACTT (23) R-CCACGGTCAGAGAACAGGA (19)
Selenium-dependent Glutathione Peroxidase	HQ891311.1	Se-GPx	1.92 ± 1.15	71	F - 59 R - 59	F-TTCACAATCATGGAAGACATCAG (23) R-AAGGCCGAAAATTGATGAAA (20)
SuperOxide Dismutase	FM177867.1	SOD	1.97 ± 0.15	70	F - 59 R - 60	F-CAGCAGTGACAGTGACAGGAG (21) R-AACTCGTGAACGTGGAAACC (20)

primers designed from sequences described in the cited paper.
- Efficiencies not shown as these primers were not used to amplify reference genes.

95 °C for 10 s, annealing at 60 °C for 10 s, elongation at 72 °C for 10 s, melting curve analysis (1 cycle at 95 °C for 5 s, 65° C for 60 s and 95° C for 1s), and cooling at 40 °C for 20 s.

The specificity of qPCR products was analyzed using 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 and GADPH as references genes. For data analyses, Cq values were extracted with the qPCR instrument software LightCycler Software 1.5.0 (Roche) and subsequently analyzed using qBasePLUS software (Biogazelle). Requirements implemented in the software qBasePLUS were used as follows. Quality Control Criteria: replicate variability (difference in Cq) > 0.5, negative control threshold (difference in Cq between sample with highest Cq value and control) < 5. Data Exclusion Criteria: well with Cq value > 35, difference to negative control sample <5. Calibrated normalized relative quantities (CNRQ) of each gene were represented in bar plots (treatment *vs.* control). In addition, each qPCR run contained the treatment samples and their respective controls (sample maximization).

2.5. Sample preparation for biochemical analyses

Enzymatic activities were quantified as indicators of oxidative stress. Lipid PerOxidation (LPO) was additionally analyzed to account for Reactive Oxygen Species (ROS) produced. Oppositely to qPCR analyses, samples were analyzed individually for enzymatic activity (given the feasibility of these experiments), controlling the potential effect of inter-individual variability through subsequent statistical analyses as detailed below. Twelve biological replicates per treatment were defined for biochemical analyses, including four technical replicates per biological replicate. Gills and digestive glands were weighed and homogenized in 1:10 (w:v) cold phosphate buffer (0.1 M, pH 7.4) with EDTA-free protease inhibitor tablets, using anYstral GmbH d-7801 Doltingen homogenizer (YstralGmBH, Ballrechten-Dottingen, Germany).

Different fractions were obtained by decanting the homogenate. The supernatant was used for Lipid PerOxidation (LPO) analyses and to determine the activity of antioxidant enzymes (SOD, CAT, GPx and GST). Lipid peroxidation levels were determined in 0.2 mL of the homogenates, preventing the oxidation by the addition of butylated hydroxytoluene (BHT) 4% in methanol. For the quantification of antioxidant enzymes activity, the homogenates were centrifuged at 15,000 g (15 min at 4 °C) for the SOD, CAT and GPx, and at 9000 g (30 min at 4 °C) for the GST. All enzyme activities were calibrated to protein concentrations measured at 600 nm and 25 °C in a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, CA) using the Bradford method (Bradford, 1976) adapted to microplate (Guilhermino et al., 1996) and using bovine γ-globuline (Sigma G5009) as standard protein. Assays followed the original techniques, adapted to the microplate (GST, SOD GPx) and cuvette (CAT). All enzymatic activities were determined in specific buffers: SOD (phosphate buffer 0.05, pH = 7.8, with 1 mM Na2-EDTA), CAT (phosphate buffer 0.05 M, pH = 7.0); GST (phosphate buffer 0.1 M, pH 6.5) and GPx (phosphate buffer 100 mM, pH = 7.5, with 1 mM Na-EDTA and 1 mM NaN3). The slope of the linear part of the reaction curve was used in all assays. All enzymatic activities were expressed as substrate hydrolysed per time per protein weight (nmol/min/mg protein) with the exception of CAT, where

activity was expressed as μmol/min/mg protein. Sample protein content was standardized to 0.3 mg/mL for GST analyses, and to 1 mg/mL for antioxidant enzymes (SOD, CAT and GPx). Standardization is required in the present case to account for different protein contents resulting from the application of biochemical assays with different specificities.

2.6. Quantification of enzymatic activities

GST activity was determined as described elsewhere (Habig et al., 1974a, 1974b) with adaptation to microplates (Frasco and Guilhermino, 2002). Reaction solution (200 μl per microplate well with 100 μl of supernatant per microplate) consisted of CDNB solution (60 mM in ethanol), GSH solution (10 mM in phosphate buffer) and phosphate buffer (0.1 M, pH = 6.5). The enzymatic reaction was monitored in a spectrophotometer at 340 nm and 25 °C each 36 s for 5 min, using SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as nmol GSH-CDNB produced per min per mg protein. SOD activity was determined according to (McCord and Fridovich, 1969) adapted to microplate. Reaction solution consisted of 200 μl per microplate well including xanthine solution (0.7 mM in 0.001 N NaOH, Sigma-Aldrich, St. Louis, MO), cytochrome *c* solution (0.03 mM phosphate buffer), with 50 μl supernatant and 50 μl xanthine oxidase solution (0.3 U/mL in Na_2EDTA solution, Sigma-Aldrich, St. Louis, MO). The reduction of the cytochrome *c* was measured at 550 nm and 25 °C, using a microplate reader (Bio-Tek, model Power Wave 340, Winooski, USA). The activity of SOD was calculated as relative to its ability to inhibit 50% reduction of cytochrome *c* per min and expressed as units (U) per mg protein. CAT activity was determined according to (Aebi, 1984). The reaction solution (in cuvette) consisted of 200 μl including H_2O_2 solution with 400 μl of supernatant (30 mM in phosphate buffer 0.05 M, pH = 7.0). Consumption of H_2O_2 was measured in spectrophotometer at 240 nm and 25 °C, 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 130 μl phosphate buffer, 50 μl supernatant, 30 μl NADPH solution 1 mM in Tris buffer, 30 μl GSH solution 0.2 mM in phosphate buffer, 30 μl GR solution 3 U/ml in phosphate buffer, 30 μl H_2O_2 solution 6 mM in phosphate buffer.

0.1 M potassium phosphate buffer with 2 mM Na_2-EDTA, 1 mM DTT, 1 mM of sodium azide, 1 mM NADPH, 0,2 mM GSH and 30 U/mL GR.

GSH oxidation was measured at 340 nm and 25 °C 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. 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 °C for 60 min, and 2 mL 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 nmol TBARS per mg protein using SpectraMax M2 cuvette reader (Molecular Devices, Sunnyvale, CA).

2.7. Statistical analyses

Transcriptional results were statistically analyzed using qBasePLUS (version 3.0) with the default settings, using reference targets as normalization strategy. This software calculates RQs (Relative Quantitie) for each gene in each sample, taking into account differences in PCR amplification efficiencies. RQ is normalized using the references genes, providing the results in CNRQs (Calibrated Normalized Relative Quantity) represented as means and standard error of the mean (SEM) in the form of bar charts. The obtained results followed a normal distribution displaying variance homogeneity, and the differences of gene expression between treatments and control groups were tested by an unpaired *t*-test. For all statistical analyses, p-value < 0.05 was considered significant. Results from biochemical analyses are presented as mean ± SE. All data was tested for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Barlett's test) (Zar, 1996). Deviations from normality and/or homoscedasticity were corrected using the square root or the log (x) transformations. Each 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). A three-way ANOVA was similarly used to evaluate the contribution of tissue, treatment and time on enzyme activity. The significance level was 0.05. Statistical analyses of data were performed using the Sigmaplot package V. 12 (Systat Software, Richmond, CA, USA).

3. 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; Prego-Faraldo et al., 2015, 2016), revealing the ability of these toxins to produce transient oxidative DNA damage at low concentrations (Prego-Faraldo et al., 2016). On the contrary, the effects of toxin exposure on gene transcription and antioxidant enzymatic activity potentially involved in defense and protection mechanisms 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 two concentrations of the DSP-producing dinoflagellate *P. lima*.

3.1. Exposure to DSP-producing dinoflagellates and accumulation of OA in mussels

Mussels were experimentally exposed to two cellular densities of *P. lima* (1000 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 accumulation of DSP toxins by mussels. Physicochemical parameters were measured during experiments, including water conductivity, salinity, and pH values. The observed stability allows to safely discard a significant effect on the observed results. The OA body burden was estimated from 20 g of mussel tissue at each condition, following the protocol established by the European Union Reference Laboratory for Marine Biotoxins (EU, 2010) and expressed as mean ± standard error. OA content ranged between 21.67 and 112.12 ng/g dry weight.

Mussels fed with 1000 cells/L of *P. lima* accumulated 28.35 ± 3.07 ng/g dry weight after 24 h and 21.67 ± 2.02 ng/g dry weight after 48 h exposure. 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 dry weight after 24 h and 48 h, respectively. Control mussels did not show OA at any sampling time. Overall, the observed levels of OA content are consistent with previous experiments developed by our groups, validating the exposure

conditions used in the present work (Prego-Faraldo et al., 2016). Additionally, the OA accumulation registered is consistent with that observed in mussels during natural HAB episodes (Díaz et al., 2013), corroborating that the experimental conditions used in this study faithfully simulate early stages of a toxic natural HAB episode. Similarly, the observed OA accumulation are similar to those observed in mussels exposed to *P. lima* in laboratory conditions (Prado-Alvarez et al., 2013).

3.2. Transcriptional responses to P. lima exposure in mussel tissues

Optimal normalization (reference) genes were analyzed using the Normfinder and GeNorm algorithms as indicated in Fig. 2. Normfinder ranked GAPDH, EF2 and rpS4 as the most stable genes (Fig. 2A), with 18S on the opposite side of the spectrum. The GeNorm PLUS algorithm implemented in Biogazelle's qbasePLUS software calculates the pairwise variation among all tested genes and assigns stability measures (M) produced similar results, defining GAPDH, rpS4 and EF1 as the most stable genes (Fig. 2B). By combining both algorithms it was possible to define GAPDH, rpS4 and EF2 as the most appropriate reference genes for normalization in all subsequent qPCR analyses. These results contrast with a previous report in which EF and 18S were considered two of the most suitable genes for normalizing the variation in molluscan studies (Cubero-Leon et al., 2012). Nonetheless, the choice of GAPDH and rpS4 as reference genes in digestive gland and gill from *M. galloprovincialis* is further supported by recent reports (Lozano et al., 2015).

The transcriptional levels of GST-*pi*, CAT, Se-GPx and SOD genes were evaluated based on their value to assess the effects of pollution in mussels (Doyen et al., 2008). qPCR reactions were carried out using the primers indicated in Table 1 (qPCR primer efficiencies >1.8), revealing significant transcriptional differences among GST-*pi*, CAT and SOD genes in digestive gland (Fig. 3). Results showed a significant increase (24 h) and subsequent decrease (48 h) in GST-*pi* transcription at 1000 cells/L, along with a significant down-regulation of SOD after 24 h at 1000 cells/L. Indeed, exposure to 1000 cells/L for 24 h is enough to alter SOD and GST-*pi* transcript levels. This observation is consistent with reports also describing an initial decrease in SOD mRNA expression 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 might suggest a more complex scenario. Indeed, 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 GST-*pi* (down-regulated for 48 h at 1000 cells/L), a significant increase in CAT mRNA levels was found in all treatments (except for 24 h at 1000 cells/L), matching similar results previously found 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). Given the similar behavior displayed by CAT across species in response to different types of stressors, it is unlikely that this antioxidant enzyme may constitutes a uselful candidate assess the harmful effect of toxins produced during HABs. On the other hand, 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 (Fabioux et al., 2015).

Oppositely to the case of digestive gland, significant differences were found among all genes in the case of gills (Fig. 4). Futhermore, the response was different from that observed in digestive gland, corroborating the differential expression of antioxidant enzymes across bivalve tissues (Jo et al., 2008). In gills, 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., 2016), 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-regulation of Se-GPx, since both enzymes use the same substrate (Regoli et al., 2011a, 2011b). Lastly, high levels of Se-GPx were observed in all treatments, being significant at 100,000 cells/L for 24 h and 1000 cells/L for 48 h. The up-regulation of this gene was also reported in gills from the mollusc *Haliotis discus* in response to three physical stress conditions, thermal, low-salinity and hypoxic (De Zoysa et al., 2009).

The early increase observed in the mRNA levels of the studied antioxidant enzymes might be consistent with the subsequent ability of mussels to eliminate potential ROS induced by exposure to DSP-producing dinoflagellates. Previous reports indicate that OA exposure increases ROS levels human monocytic U-937 cells (Ravindran et al., 2011) as well as in bivalves (Chi et al., 2016). In this latter case, ROS production constitutes a very important immune mechanism enhancing internal defense against pathogens, however, serious damage to lipids, proteins, and DNA occurs when the generation of ROS becomes excessive, probably requiring dedicated responses in antioxidant enzymes. In addition, the early increase of

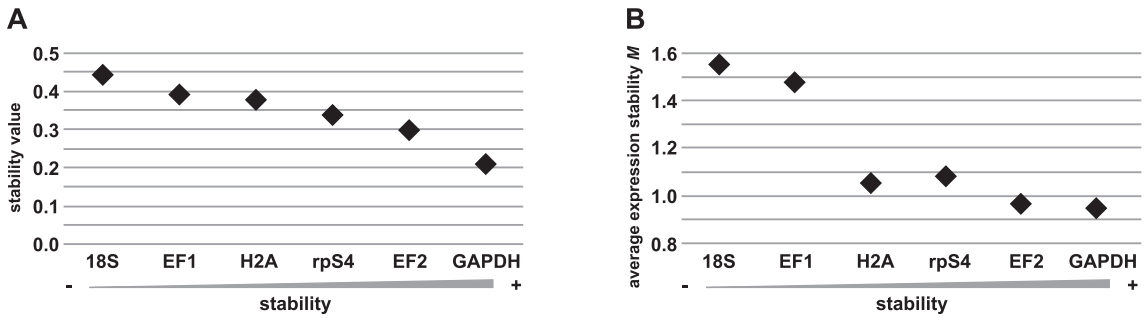

Fig. 2. Evaluation of expression stability in reference genes for transcriptional analyses. Genes were ranked according to their expression stability based on two different approaches: A, stability values as determined by the Normfinder algorithm; B, average expression stability (M) as defined by the GeNorm algorithm.

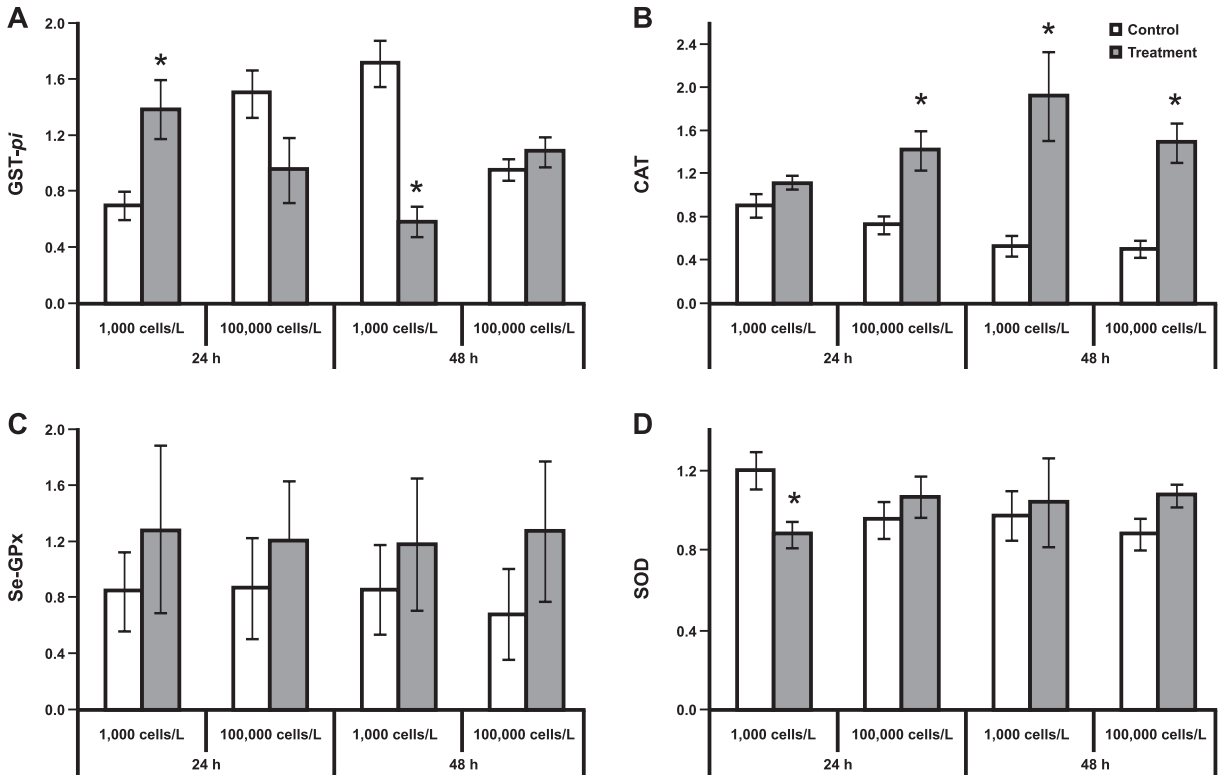

Fig. 3. Transcriptional activity of genes encoding antioxidant enzymes in digestive gland from *M. galloprovincialis* after exposure to DSP toxins (24 h and 48 h). Expression levels are represented as calibrated normalized relative quantities (CNRQ) for genes encoding: A, Selenium-dependent Glutathione Peroxidase (Se-GPx); B, SuperOxide Dismutase (SOD); C, CATalase (CAT); and D, Glutathione S-Transferase *pi-1* (GST-*pi*). Relative transcript expression levels were calculated compared with control (non-exposed) mussels. Data were averaged from 4 biological replicates, each deriving from a pool of 5 mussels in duplicates, and were analyzed using qbasePLUS software. * indicates significant differences with respect to the corresponding control in unpaired *t*-test ($p < 0.05$). Error bars represent SE of the mean.

GST-*pi* expression observed in digestive gland at low cell densities (1000 cells/L, 24h) suggests that this process would take place faster in this tissue (as compared to gills). This observation is consistent with the absorptive role of the digestive gland after digestion of toxic microalgae (Prego-Faraldo et al., 2013), with gills constituting the primary line of contact with elements being filtered in the water column [e.g., antioxidant genes display greater expression levels in gills than in digestive gland after exposure to cadmium (Jo et al., 2008)].

3.3. Effects of DSP toxins on antioxidant enzymatic activities in digestive gland

The amount of GPx activity quantified in mussels exposed to 1000 cells/L and 100,000 cells/L for 24 h was significantly higher compared with 48 h exposure and controls. Yet, GPx activity in digestive gland from mussels exposed to 1000 cells/L and 100,000 cells/L did not show significant differences acroos exposure time (Fig. 5A). These results are consistent with previous reports describing an increase of GPx activity in the digestive gland from *M. galloprovincialis* exposed to the toxic dinoflagellate *Ostreopsis* cf. *ovate* (Gorbi et al., 2012). The values of SOD activity measured in digestive glands of mussel followed a similar pattern for 24 h and 48 h exposures, although significantly higher SOD activity values were found in the digestive gland of mussels exposed to 100,000 cells/L of *P. lima* for 24 h. On the other hand, the values of SOD activities quantified after 48 h were only significant different between 1000 cells/L and the control (Fig. 5B).

The activity of GPx and SOD in digestive gland was significantly higher for the conditions tested (1000 and 100,000 cells/L, for 24 and 48 h), supporting a principal role for both enzymes during the response of digestive gland to oxidative stress produced by DSP toxins (Qiu et al., 2013). The present results suggest that SOD might provide the first line of defense through dismutation of O_2 radicals into H_2O_2, which is can be further scavenged by GPx or CAT. Nonetheless, CAT activity did not display significant differences in digestive gland of mussels in comparisons between exposure times and treatments (Fig. 5C). Considering that CAT and GPx enzymes work cooperatively, and taking into account that CAT activity remained constant during the assayed exposures, it seems plausible that GPx enzyme constitutes the main responsible for H_2O_2 neutralization under the reported experimental conditions. These results are in agreement with those reported by (Regoli, 2011; Regoli et al., 2011a), suggesting that the apparent lack of responsiveness in CAT activity in response to chemical stress might be compensated by the increase of GPx activity. Such observation is further supported by previous reports describing an absence of significant changes in CAT activity in the digestive gland of *Nodipecten subnodosus* exposed to Paralytic Shellfish Poisoning (Estrada et al., 2007).

The digestive gland of mussels exposed for 48 h to 100,000 cells/L displayed higher GST activity compared with 24 h exposure. Regarding the treatment, the values of GST activity obtained in digestive glands of mussels exposed to 1000 cells/L for 24 h were significant lower than in mussels exposed to 100,000 cells/L during the same period of time (Fig. 5D). Thus, GST activity only seems to be responding to the most extreme treatment (100,000 cells/L, 48 h). In fact (Barata et al., 2005), suggested that the high GST activity might be also compensating for the low CAT activity observed, as GST also presents peroxidase activity. Along with these

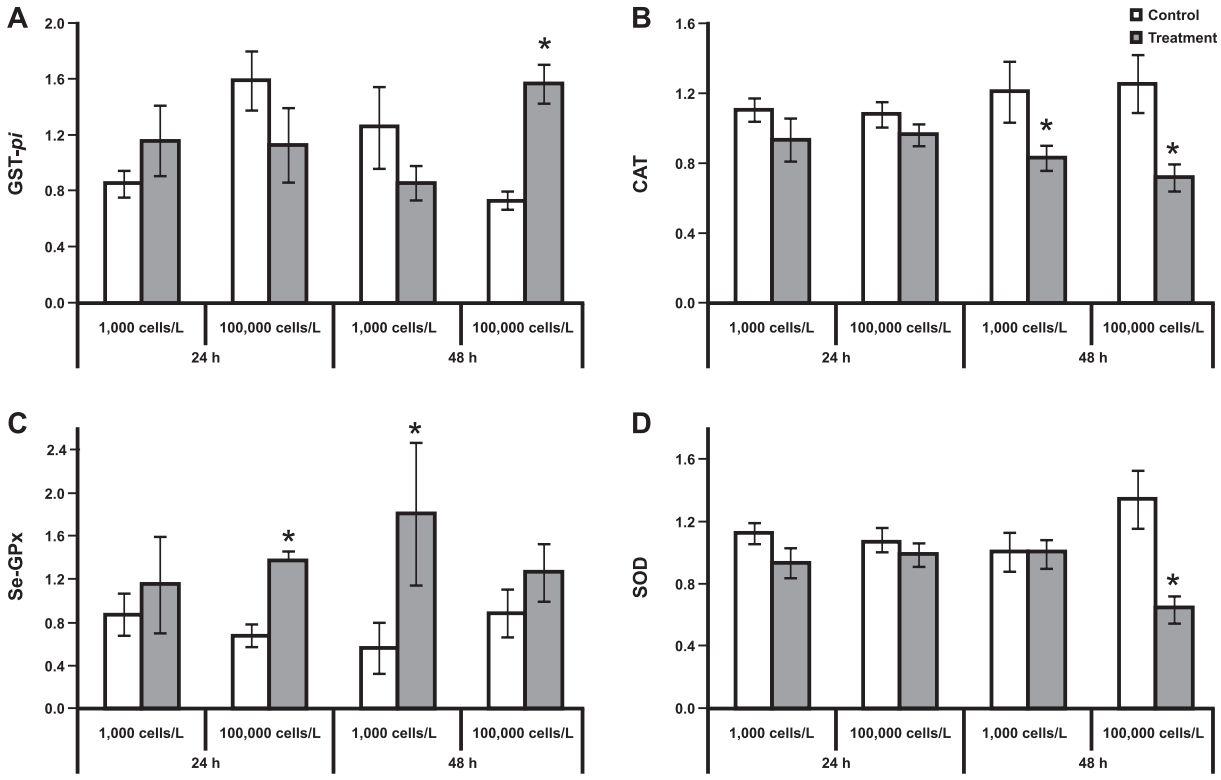

Fig. 4. Transcriptional activity of genes encoding antioxidant enzymes in gill from *M. galloprovincialis* after exposure to DSP toxins (24 h and 48 h). Expression levels are represented as in Fig. 3 for genes encoding: A, Selenium-dependent Glutathione Peroxidase (Se-GPx); B, SuperOxide Dismutase (SOD); C, CATalase (CAT); and D, Glutathione S-Transferase *pi-1* (GST-pi). Relative transcript expression levels were calculated and compared with control (non-exposed) mussels. Data was averaged from 4 biological replicates, each resulting from a duplicated pool of 5 mussels, and were analyzed using qbasePLUS software. * indicates significant differences with respect to the corresponding control in unpaired *t*-test ($p < 0.05$). Error bars represent SE of the mean.

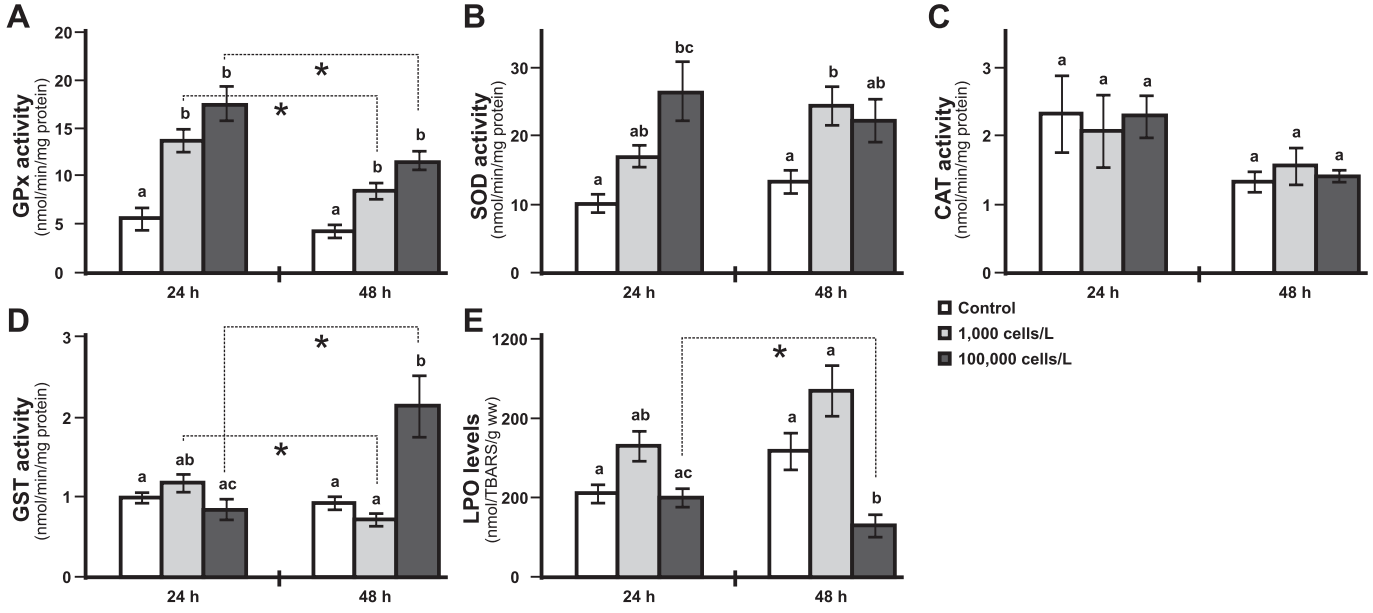

Fig. 5. Lipid peroxidation and antioxidant enzyme activity in digestive gland from *M. galloprovincialis*. Individuals were exposed to 1000 cells/L and 100,000 cells/L of *P. lima* for 24 h and 48 h. Significant statistical differences among groups are represented by different lowercase letters (a, b, c), while differences between exposure time for each treatment are indicated by an asterisk (*), in both cases using two-way ANOVA and Tukey's test ($p < 0.05$).

results, a significant decrease in LPO levels is observed over time in digestive gland from mussels exposed to 100,000 cells/L. Accordingly, mussels exposed to 100,000 cells/L for 48 h displayed LPO levels significantly lower than controls, resulting in significant differences between both treatments (1000 cells/L and 100,000 cells/L, Fig. 5E). Lastly, it is possible that low LPO levels

might have been influenced by the interaction of GST, GPx and SOD activities, thus providing protection against damage caused by ROS.

3.4. Effects of DSP toxins on antioxidant enzymatic activities in gill tissue

In the case of gills, the activity of all antioxidant enzymes tested (GPx, SOD, CAT and GST) was significantly higher than in controls in most of the treatments evaluated, with certain exceptions such as SOD activity in gills exposed to 1000 cells/L for 48 h, CAT activity in gills exposed to 1000 cells/L for 24 h and 100,000 cells/L, as well as GST activity in gills exposed to both cell densities at 24 h. Increased activities could be indicative of oxidative stress caused by DSP toxins in *M. galloprovincialis* at all concentrations studied. As in the case of digestive gland, gill GPx and SOD activities were also significantly higher than controls for the majority of conditions studied (Fig. 6A). Similarly, SOD activity in mussels exposed to 1000 cells/L for 48 h was significantly lower than the activity determined for individuals exposed to these conditions for 24 h. However, SOD activity in mussels exposed to 1000 cells/L and 100,000 cells/L for 24 h was significantly higher than in the case of controls (Fig. 6B). High activities of these two enzymes (GPx and SOD) were also reported in gill from the scallop *N. subnodosus* exposed to PSP. Similar changes in GPx and SOD activities were observed in both tissues, which suggest that these two enzymes may be act interdependently to neutralize ROS.

The activity determined for CAT was significantly higher in gills from mussels exposed to 1000 cells/L for 48 h when compared with 24 h exposure. Additionally, CAT activity in gills from mussels exposed to 1000 cells/L for 48 h was significantly higher than the activity determined for mussels exposed to other experimental treatments (control and 100,000 cells/L) for 48 h (Fig. 6C). A similar SOD and CAT response pattern was previously found in digestive glands and gills of the mussel *Perna perna* exposed to mercury and thermal stress (Verlecar et al., 2007), supporting the potential role of these enzymes during responses to different types of stress in addition to marine toxins. On the contrary, no significant differences in gill GST activity were found between different treatments or exposure times. Yet, significant differences in GST activity values were observed between gills exposed to 100,000 cells/L for 48 h and controls (Fig. 6D). Consequently, GST activity only displayed a significant increase respect to control after 48 h exposure to DSP toxins, while CAT activity also showed a significant increase in gills of mussels exposed to 1000 cells/L for 48 h. Lastly, as in the case of digestive gland, a decrease in LPO levels was observed over time, which was statistically significant when gills of mussels exposed to 1000 and 100,000 cells/L for 48 h were compared to controls (Fig. 6E).

3.5. Comparison of enzymatic activities between digestive gland and gills

The high activity levels observed in the different antioxidant enzymes could be linked to the existence of a resistance mechanism against DSP toxins in digestive gland and gills. Since it has been suggested that DSP toxins are heterogeneously distributed across mussel tissues, with toxins being predominantly accumulated in digestive gland (Blanco et al., 2007), a possible explanation for the absence of parallel global responses in both tissues could be the existence of differences in the bioavailability of DSP toxins. While speculative, that hypothesis constitute a promising direction for future studies. Indeed, this idea is indirectly connected with the absence of parallel response between the gills and digestive glands of the scallop *Chlamys farreri* after exposure to benzo(k)fluoranthene (Pan et al., 2005), The pattern observed in the present work matches previous reports in the scallop *Chlamys farreri* exposed to benzo(k)fluoranthene (Pan et al., 2005). High antioxidant enzyme activities obtained in gills from mussels exposed to DSP toxins seem to be involved in keeping low LPO levels, providing a protective effect. Significant differences in enzyme activity among treatments were found for GPx, SOD, and GST, when comparing digestive gland and gill tissues (Table 2). Regarding exposure time, significant differences were observed for CAT (in both tissues) and for GPx measured in the digestive gland (Table 2). Statistical significant effects of DSP toxins concentrations along time (interactions) were found for LPO (measured in both tissues), GST

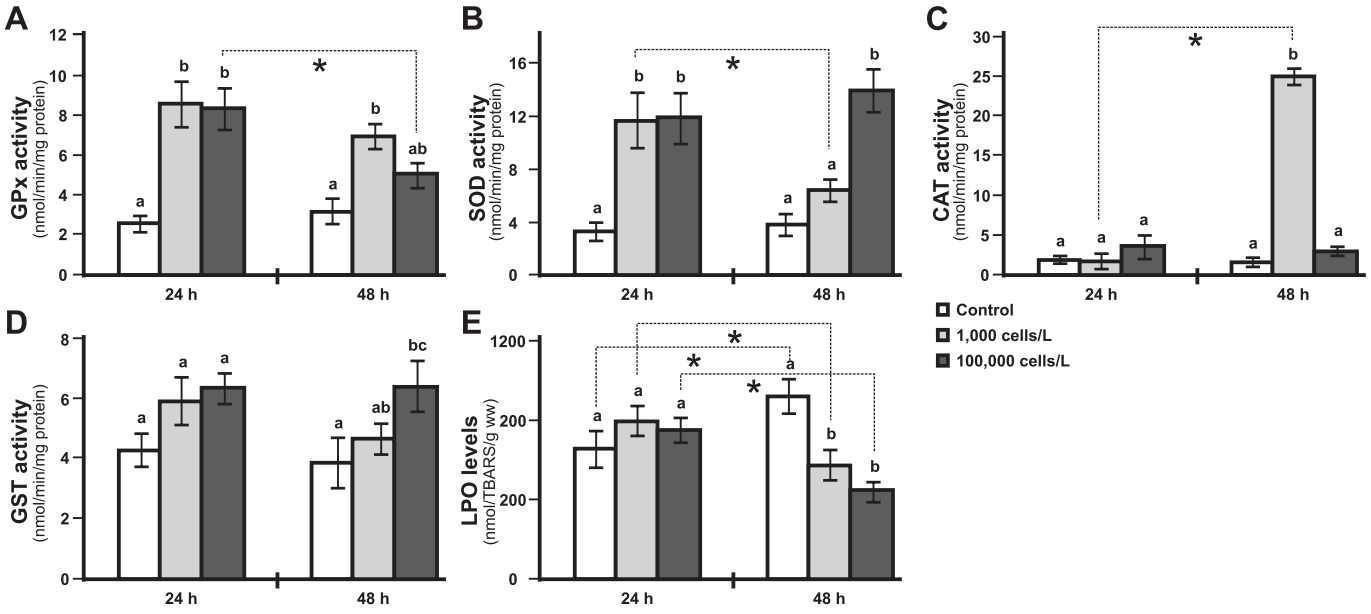

Fig. 6. Lipid peroxidation and antioxidant enzyme activity in digestive gland from *M. galloprovincialis*. Individuals were exposed to 1000 cells/L and 100,000 cells/L of *P. lima* for 24 h and 48 h. Significant statistical differences among groups are represented by different lowercase letters (a, b, c), while differences between exposure time for each treatment are indicated by an asterisk (*), in both cases using two-way ANOVA and Tukey's test ($p < 0.05$).

Table 2
Effect of *P. lima* treatment (1000 cells/L, 100,000 cells/L) and exposure time (24 h, 48 h) on antioxidant enzyme activity at each tissue using two-way analysis of variance (ANOVA).

	Source of Variation	GPx		SOD		CAT		GST		LPO	
		F	p	F	p	F	p	F	p	F	p
Digestive Gland	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*
Gill	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

Asterisk (*) indicates significance level of $p < 0.05$.

(measured in digestive gland) and for CAT and SOD (measured in the gills). Overall, the obtained results are consistent with an increase in antioxidant enzyme activities following short-term exposure to low cell densities of *P. lima*. This observation is in contrast with the decrease in enzymatic activity during mussel exposure to resin acids (Gravato et al., 2005) and to metals (Canesi et al., 1999) for short periods of time. Consequently, the early increase in antioxidant enzyme activity might be indicative of a rapid response to DSP toxin exposure, increasing the protection against oxidative stress in mussels.

Significant differences in activities were observed in all enzymes between digestive gland and gills (Table 3). Results also revealed that time constituted a relevant parameter influencing the effect of DSP toxins across treatments, displaying significant effects in all cases except for SOD. In the case of the effect of tissue across treatments, significant effects were observed in GPx, CAT and LPO. The combination of effects from time and tissue resulted significant only in the case of CAT. Lastly, significant interactions between tissues, experimental *P. lima* concentrations and exposure times were observed for GST, CAT and SOD enzymatic activities (Table 3). Overall, CAT resulted the most sensible enzyme under the conditions studied, responding significantly in all cases. On the contrary, SOD only displayed significant differences in activity based on tissue and on the interaction among all factors studied. When the overall results obtained in digestive gland and gill are compared, similar responses are observed. That contrasts with previous reports suggesting that both tissues differ in their detoxification rates., including studies in mussels reporting higher detoxification rates in gill (Cheung et al., 2001; Lima et al., 2007), and works in fish pointing towards digestive gland as the metabolic center of these organisms (Ortiz-Delgado et al., 2008). The similarity in the antioxidant response between tissues observed in this work could be explained by the fact that gills are the first tissue come into contact with toxic dinoflagellates during filtration, while toxins may be released in high concentrations during digestion of dinoflagellates in the digestive gland (Contardo-Jara et al., 2008).

3.6. Comparison of gene transcription levels with enzymatic activities

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., 2011b). 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 agreement with enzymatic activity analyses. Conflicting results between mRNA levels and enzymatic activities 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 during the response to *P. lima* exposure in the digestive gland of *M. galloprovincialis*. However, the high Se-GPx levels observed in gills support the role of this enzyme balancing the antioxidant system in this tissue. A similar response pattern was also obtained in the case of 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). Similarly, post-transcriptional modifications or mRNA degradation might also contribute to discrepancies between gene transcription and biochemical activity (Regoli and Giuliani, 2014).

In the specific case of gills, although CAT and SOD mRNA levels seem to 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.

Table 3
Effect of *P. lima* treatment (1000 cells/L, 100,000 cells/L) and exposure time (24 h, 48 h) and tissue (digestive gland and gill) on antioxidant enzyme activity at each tissue using analysis of variance (ANOVA).

Source of Variation	GPx		SOD		CAT		GST		LPO	
	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

Asterisk (*) indicates significance level of $p < 0.05$.
F and p values for treatment and time are indicated in Table 2.

4. Conclusions

The present work constitutes the first characterization of the response of the mussel *M. galloprovincialis* to the DSP-toxin-producing dinoflagellate *P. lima* using a combination of transcriptomic and enzymatic approaches. The major conclusions can be summarized as follows: (1) GAPDH, rpS4 and EF2 may be employed as reference genes to normalize the gene expression in qPCR experiments carried out in digestive gland and gill tissues of mussels exposed to DSP-producing dinoflagellates; (2) the significant transcriptional changes observed in genes encoding SOD, CAT and GST-*pi* enzymes in both tissues suggest the presence of compensatory gene expression mechanisms; (3) the significant changes observed in the activities of SOD, CAT, GPx and GST enzymes in both tissues are consistent with the implication of antioxidant system during early responses to dinoflagellate exposure; (4) the substantial reduction in the LPO levels in both tissues supports the role of DSP toxins to increase the protection against oxidative stress in general; (5) an absence of parallel global responses between tissues (digestive gland and gill) along with a lack of correlation between transcriptomic and biochemical responses are evident during responses to DSP-producing dinoflagellate exposure. 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.

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