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Exposure to phenanthrene and depuration: Changes on gene transcription, enzymatic activity and lipid peroxidation in gill of scallops *Nodipecten nodosus*

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ABSTRACT

Understanding the mechanism of phenanthrene (PHE) biotransformation and related cellular responses in bivalves can be an important tool to elucidate the risks of polycyclic aromatic hydrocarbons (PAHs) to aquatic organisms. In the present study it was analyzed the transcriptional levels of 13 biotransformation genes related to cytochrome P450 (CYP), glutathione S-transferase (GST), sulfotransferase (SULT), flavin-containing monooxygenase and fatty acid-binding proteins by qPCR in gill of scallops *Nodipecten nodosus* exposed for 24 or 96 h to 50 or 200 μg L^{-1} PHE (equivalent to 0.28 and 1.12 μM, respectively), followed by depuration in clean water for 96 h (DEP). Likewise, it was quantified the activity of catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PDH), GST and levels of lipid peroxidation. Increased transcriptional levels of *CYP2UI-like, CYP2D20-like, CYP3A11-like, GSTomega-like, SULT1B1-like* genes were detected in organisms exposed to PHE for 24 or 96 h. In parallel, GR and GPX activities increased after 96 h exposure to 200 μg L^{-1} PHE and G6PDH activity increased after 24 h exposure to 50 μg L^{-1} PHE. This enhancement of antioxidant and phase I and II biotransformation systems may be related to the 2.7 and 12.5 fold increases in PHE bioaccumulation after 96 h exposure to 50 and 200 μg L^{-1} PHE, respectively. Interestingly, DEP caused reestablishment of GPX and GR activity, as well as to the transcript levels of all upregulated biotransformation genes (except for *SULT1B1-like*). Bioaccumulated PHE levels decreased 2.5–2.9 fold after depuration, although some biochemical and molecular modifications were still present. Lipid peroxidation levels remained lower in animals exposed to 200 μg L^{-1} PHE for 24 h and DEP. These data indicate that *N. nodosus* is able to induce an antioxidant and biotransformation-related response to PHE exposure, counteracting its toxicity, and DEP can be an effective protocol for bivalve depuration after PHE exposure.
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1. Introduction

Aquatic environment is considered quite vulnerable to anthropogenic pressure as a final recipient of a variety of pollutants. Oil spills are among the major factors causing marine contamination, as about 87 million barrels of oil are globally produced every day. Spilled oil consists primarily of aliphatic/aromatic hydrocarbons and polar compounds, and leakage may occur during transportation and pyrolytic processes plus domestic and industrial wastewater discharges (Martins et al., 2013; Wei et al., 2015). Polycyclic aromatic hydrocarbons (PAH), main components of crude oil, are ubiquitously found in the marine environment. Phenanthrene

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(PHE) is a low molecular weight PAH with three fused benzene (aromatic) rings and is a representative component of PAH compounds present in the marine ecosystem (De Luca-Abbott et al., 2005; Schäfer and Köhler, 2009).

PHE is often used as a model for the study on PAHs metabolism, it is readily bioavailable (Hannam et al., 2010a; Wootton et al., 2003) and the smallest PAH to have a bay and a K-region that represent a highly reactive region where the main carcinogenic species can be formed (Zhang et al., 2014). Owing to its high lipophilic nature, PHE can easily penetrate biological membranes; bioaccumulation tends to be fast and, as a global persistent organic pollutant, it may raise health concerns even several decades after exposure (Jin et al., 2015; Noh et al., 2015; Xiu et al., 2015). PHE has been included in "Priority Pollutants" list by the United States Environment Protection Agency (US, EPA), and although not carcinogenic and mutagenic, it has been shown to be toxic to several marine animals (Zhang et al., 2014). Studies have shown that PHE induces oxidative stress, immunosuppression and genotoxicity in mollusks, besides molecular alterations at transcriptional gene levels (Einsporn and Koehler, 2008; Hannam et al., 2010a). PAHs toxicity is associated to its cellular detoxification mechanisms, with some of the produced metabolites being highly reactive to DNA to adduct formation (Akcha et al., 2000). Different mechanisms of PHE toxicity have been proposed, but the most convincing evidence is related to its metabolic activation by members of the cytochrome P450 (CYP) superfamily during the phase I biotransformation (Schlenk, 1998; Zanette et al., 2008). Phase I by-products can conjugate to endogenous substrates during the phase II biotransformation, catalyzed by enzymes like glutathione-S-transferase (GSTs) and sulfotransferases (SULTs), leading to the formation of hydrophilic conjugates for further excretion.

PAH biotransformation can lead to the production of reactive oxygen species (ROS), which may oxidizes proteins, DNA and cause lipid peroxidation (Grintzalis et al., 2012; Noh et al., 2015). Consequently, a complex array of antioxidant systems acts preventing cumulative oxidative damage by neutralizing excess of ROS (Halliwell and Gutteridge, 1985). In addition, animals subjected to stress have an increased energy demand, mobilizing metabolic fuel (*e.g.* fatty acids) for survival. Fatty acid-binding proteins (FABP) are highly active in fatty acid metabolism, participate in lipid uptake, transport and homeostasis (Bayır et al., 2015; Travers et al., 2010).

The use of bivalves in pollution-related studies has increased over the last decade, especially regarding PAH, crude oil, pesticide and metal toxicity (Hannam et al., 2010a). The scallop *Nodipecten nodosus* is considered a good sentinel bivalve, with wide geographic distribution (from USA to Brazil), because of its susceptibility to the toxic effects of a large number of contaminants, besides their important economic value (Cranford, 2006; Freites et al., 2010). Considering its filter-feeding behavior and its capacity to bioaccumulate xenobiotics, the legislation in some countries request the use of scallops depuration protocols in order to minimize the risk of human contamination. In this context, it is clear that depuration procedures may help to increase bivalve health and production. It can be an effective tool (at different extents) in removing many thermo-tolerant bacteria, viral contaminants, vibrios, biotoxins, trace metals or organic chemicals (FAO, 2008).

Encompassing these questionings and in order to get answers to clarify the mechanisms of PHE biotransformation, the aim of this study was to investigate the changes in activities of biotransformation and antioxidants enzymes, lipid peroxidation levels, as well as the levels of transcriptional genes with important role in the biotransformation mechanisms of PHAs in gill of *N. nodosus*. A depuration protocol was developed and tested in order to estimate the applicability of this technique to allow decontamination of animals exposed to PAH.

2. Materials and methods

2.1. Scallops and exposure conditions

Scallops *N. nodosus* of similar shell length (6.0–8.0 cm) were collected from a scallop farm at Enseada do Brito beach, (SC-Brazil). After collection, animals were cleaned and transported to the Marine Mollusks Laboratory (LMM, UFSC, Brazil) for an acclimation period of 7 days before the exposure. Animals were placed in aerated fiberglass tanks with filtered seawater (0.45 μm) on a net support to avoid contact with the bottom. A renewal water system (1.4 L min^{-1}, 18 °C and 30 PSU) was applied and scallops were fed with a maintenance diet (a mix of *Isochrysis galbana* and *Chaetoceros muelleri).*

PHE (P1140-9, 98% purity, Sigma – Aldrich) was dissolved in dimethylsulfoxide (DMSO) and added to filtered seawater to achieve final nominal PHE concentrations of 50 and 200 μg L^{-1}, equivalent to 0.28 and 1.12 μM, and a final maximum DMSO concentration of 0.01% (v/v). PHE concentrations were chosen based on previous data reported in studies with bivalves (Hannam et al., 2010a, 2010b; Wootton et al., 2003; Zhang et al., 2014). Scallops were allocated in three static tanks with the same water conditions after acclimation period (control group, 50 and 200 μg L^{-1} PHE), each one maintaining the ratio of 8 L of water per animal. Seawater was renewed (100%) and spiked with PHE stock solution every 24 h to maintain water quality and reestablish PHE levels during 96 h of exposure. Control group was subjected to the same conditions, except for the addition of 0.01% DMSO instead PHE. Animals were not fed during this period in order to prevent potential bioaccumulation of PHE by food. After 24 h and 96 h, 10 scallops from each group were collected, the gill was immediately excised, frozen in liquid nitrogen and stored at −80 °C for biochemical and molecular assays.

After 96 h of PHE exposure, a depuration system was established (DEP). Animals were fed with the maintenance diet and seawater was renewed once a day (100%). DMSO and PHE were not added to the tanks in this period. After DEP, 10 scallops per group were dissected and the gill was stored as described.

2.2. Tissue PHE concentration

To confirm PHE bioaccumulation and the detoxification capacity of scallops, whole soft tissue samples (pools of 10 individuals per group after 96 h and DEP periods) were analyzed for PHE by the method of McLeod et al. (1985) with minor modification. Briefly, samples were frozen-dried, homogenized and 1 g was Soxhlet-extracted with a 50% mixture of residue grade n-hexane and dichloromethane for 8 h. Before extraction, d8-naphthalene, d10-acenaphthene, d10-phenanthrene, d12-chrysene and d12-perylene were added as surrogates in the blank, samples and standard reference material (SRM 2974a – Organics in freeze-dried mussels tissue *Mytilus edulis*) from National Institute of Standards and Trends (NIST). Hydrocarbon extracts were cleaned up by silica gel–alumina column chromatography and were quantitatively analyzed by an Agilent 6890 gas chromatograph coupled to a 5973 N mass spectrometer (GC/MS) in a selected ion mode (SIM). A 25m, 0.32 mm i.d., 0.25-μm HP5MS film, capillary column from Agilent was temperature programmed from 40 °C to 60 °C at 20 °C min^{-1} and 60 °C to 300∘C at 4 °C min^{-1}, and held at 300 °C for 10 min in GC/MS. Phenanthrene was quantitatively analyzed based on reten-

Table 1
Primer sequences used for the qPCR for the amplification of each target and endogenous reference gene with putative gene name and amplicon size (bp).

Gene name	Primer sequence 5′- 3′	Amplicon size (bp)
–		
40s-like	F – GCTTAGATGTGCTTGCCCTGAAAGAG	131
	R – GTGGACACCATCAGCTTTGCGTTTA	
Ef1-like	F – CAAGGTCAGAGTATTGCCCATCAC	96
	R – CGGTCAACACAGGATAAGGATGTC	
CYP1A1-*like*	F – AAGGAAAGAAGCCGAGGAGGAAGATG	111
	R – CTGGAGGTATCAAATCCCGCCAAGAA	
CYP1A5-*like*	F – CCGCTTTCTTCCTACAACTTCATCC	100
	R – GCCTGCCTTACTGACTTGGTTATTG	
CYP2D20-*like*	F – CCACAACAAACCCGTCCAAGACAAAC	135
	R – CCCATCCGAAGAACCTCCATGATGA	
CYP2J6-*like*	F – GCCTCATCTAACAGGGACACTAACT	141
	R – GGAAACAGCAGGTCAACCATTTGATA	
CYP2UI-*like*	F – CGATCCGTTCCGTAAACTTTGCCATC	99
	R – CAACTCTGTCCACACCGATCCTGAAATA	
CYP3A11-*like*	F – CAAGGAGACAGGTCGCAGTGATTT	95
	R – TGTGATCTGAGGAACCGTTGCTTTC	
CYP3A25-*like*	F – CCAGAGAAGTATGACCCAGAAAGA	138
	R – AATTGAAATCTTGAGTTCCAGGAGAG	
CYP3A29-*like*	F – ACACTCTCAAGAAGACAGCCCAGAAC	120
	R – GGATACCGAAAGCCGTGGAACTGATTA	
FABP-*like*	F – GATTGGAGAGGAGTTTGATGAAGT	86
	R – AGTGAACAAGTGAACCATCTTCTAT	
GST_omega-*like*	F – CTCTATCTTCTTGTGTGCCAATACC	120
	R – TCATTCTCTAAGGGTTCGGAGTTT	
SULT1B1-*like*	F – GGATTGCTGCTGCCATCTAACGAAATC	132
	R – AGGAACTGACCATGACTCTGCTTTGT	
SULT1C1-*like*	F – AGCAGGTACACATTGGGTATCGGAAAT	124
	R – GATGGCATCAAGACATTCAGGTGTTCG	
SULT4A1-*like*	F – CGTACTGGCACATTGAACTATCACG	148
	R – TCCCTTTACCGTTCCTCATCTCTTC	
FMO-*like*	F – GTGACACTCTGACGAGAGGAATGGAAAG	85
	R – AAATGACGAAGAAGTGACGGTGGACA	

tion time of certified standard and mass to charge ratio (m/z) of the quantitation ion. The method detection limit for phenanthrene was 3.48 ng g^{-1} and its recovery ranged from 95 to 105%.

2.3. Enzyme assays

Gill samples from all groups (n = 10) were weighed and individually homogenized in 1:4 (w:v) chilled buffer (50 mM Tris-HCl, pH 7.6, containing 0.5 M sucrose, 1 mM DTT, 1 mM EDTA, 0.15 M KCl, 0.1 mM PMSF) using a Tissue-Tearor™ homogenizer. Homogenates were centrifuged at 9000*g* for 30 min at 4 °C, followed by another centrifugation of the supernatant at 100,000*g* for 1 h at 4 °C to obtain the cytosolic fraction (supernatant).

Catalase (CAT) activity was measured through the decomposition of H_2O_2 by the decrease in absorbance at 240 nm, according to Beutler (1975). Superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome c reduction in the presence of an $O_2^{\bullet -}$ generator system (xanthine/xanthine oxidase) at 550 nm (McCord and Fridovich, 1969). Glutathione peroxidase (GPx) was measured by the NADPH oxidation rate at 340 nm using cumene hydroperoxide as substrate according to Wendel (1981). Glutathione reductase (GR) was measured by the NADPH oxidation rate at 340 nm in the presence of oxidized glutathione (Carlberg and Mannervik, 1985). Glucose 6-phosphate dehydrogenase (G6PDH) activity was determined by the increase in absorbance at 340 nm caused by reduction of NADP+ to NADPH in the presence of glucose 6-phosphate (Glock and McLean 1953). GST activity was determined according to Keen et al. (1976); the method is based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to glutathione, monitored at 340 nm. Total protein levels were quantified according to Bradford (1976) using bovine serum albumin as standard.

2.4. Lipid peroxidation

The levels of lipid peroxidation in gills were assessed by the quantification of the product formed by the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA). For this analysis 100 mg of samples were homogenized in 0.3 mL of Tris buffer 0.1 M (pH 8.0). Then, 40 mg of TBA was dissolved in 10 mL of 0.2 M HCl and 0.3 mL of this solution was added to each sample. This mixture was heated at 90 °C for 40 min. Next, 1 mL of *n*-butanol was added and samples were centrifuged at 3500 rpm for 3 min. The supernatant was collected and quantified by high performance liquid chromatography coupled at 532 nm, in terms of a malondialdehyde (MDA) standard calibration curve that had been previously prepared using the same sample procedure (Arantes Felício et al., 2015).

2.5. Gene selection and transcriptional levels

The following genes of interest were selected from *N. nodosus* transcriptome database (manuscript in preparation): *CYP1A1-like*, *CYP1A5-like*, *CYP2D20-like*, *CYP2J6-like*, *CYP2UI-like*, *CYP3A11-like*, *CYP3A25-like*, *CYP3A29-like*, *FABP-like*, *GST_omega-like*, *SULT1B1-like*, *SULT1C1-like*, *SULT4A1-like* and *FMO-like*. Primers were designed with OligoAnalyzer® and PrimerQuest® (IDT, http://www.idtdna.com) software based on the complete and/or partial messenger RNA (mRNA) sequences. The primer sequences are described in Table 1.

Total RNA was extracted from tissues (100 mg) using QIAzol Lysis Reagent (Qiagen) according to the manufacturer's protocol (Qiagen, 2009a). RNA concentration and purity were measured using a NanoDrop®ND-1000 Spectrophotometer (Thermo Scien-

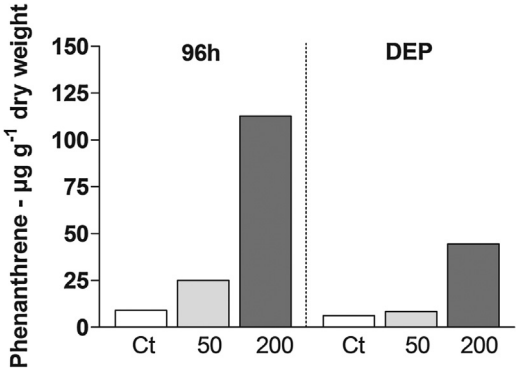

Fig. 1. Phenanthrene levels ($\mu g\,g^{-1}$) on a dry weight basis detected in whole soft tissues of scallops from both control and exposed groups (50 and 200 $\mu g L^{-1}$) after 96 h of exposure and 96 h depuration (n = 10 animals per group).

tific). The reverse transcription was performed with QuantiTect® Reverse Trancription Kit (Qiagen 2009b), using 1 μg of total RNA. The complementary DNA (cDNA) quantification from each sample was measured using NanoDrop® at 260 nm and diluted with nuclease-free water (Sigma) before use. The aliquots of diluted cDNA were stored at −20 °C.

Real-time reactions were performed with 100 ng of template cDNA per reaction following QuantiFast® SYBR® Green PCR kit methodology (Qiagen, 2011) using Real-time cycler Rotor Gene Q Qiagen® and Rotor Gene 6000 Series software. Cycling conditions were set as: PCR initial heat activation (95 °C for 5 min), denaturation (95 °C for 10 s), and combined annealing/extension (60 °C for 30 s). For all reactions, 40 cycles of denaturation and annealing/extension were performed. Samples were verified by gel electrophoresis to check for single amplicons and primer dimers. Relative mRNA expression ratio for each gene was analyzed using an efficiency corrected $2^{-\Delta Ct}$ method, normalized by the geometric mean of two endogenous reference gene, *Elongation factor-1-like* (*Ef1-like*) and 40*s ribosomal protein-like* (40*s-like*) (Hellemans et al., 2007; Schmittgen and Livak, 2008).

2.6. Statistical analysis

For all parameters, normality (Shapiro–Wilks test) and homogeneity of variances assumptions were previously checked (Bartlett's test) and outliers were excluded according to the Grubbs test. When necessary, data were normalized using the logarithmic transformation (Y = log (Y)) (Zar, 1999). Parametric data was analyzed by one-way ANOVA followed by Tukey's post hoc test to evaluate any significant differences ($p < 0.05$) among groups. For non-parametric data, Kruskal-Wallis test was performed, followed by Dunn's test. Results are presented as mean ± standard deviation (SD).

3. Results

3.1. Tissue PHE concentration

The levels of PHE detected in tissues of scallops ($ng\,g^{-1}$ dry weight) exposed to nominal concentrations of 50 and 200 $\mu g L^{-1}$ PHE after 96 h were 2.7 fold and 12.5 fold higher when compared with control group (9.00 $ng\,g^{-1}$ dry weight), respectively (Fig. 1). After DEP, PHE levels decreased 2.9 and 2.5 fold in 50 and 200 $\mu g L^{-1}$ PHE treatments, respectively, in relation to the respective 96 h groups (Fig. 1). Organisms exposed to 50 $\mu g L^{-1}$ and submitted to depuration for 96 h showed PHE values close to control group levels, while animals exposed to PHE 200 $\mu g L^{-1}$ followed by DEP still presented PHE levels 4.9 fold higher than control group (Fig. 1).

3.2. PHE effects on gene transcription levels in gills

Three genes involved in phase I biotransformation showed a marked upregulation in the gill of scallops expose to 200 $\mu g L^{-1}$ PHE after 24 h and 96 h when compared to control group (Ct): *CYP2D20-like* levels of transcription increased 2.95 fold and 5.6 fold (Fig. 2A), respectively, as well as *CYP2UI-like* levels increased 2.05 and 2.4 fold (Fig. 2B). *CYP3A11-like* levels only showed an increased following exposure of 200 $\mu g L^{-1}$ PHE after 96 h (Fig. 2C), while other analyzed CYP isoforms remained unchanged (Supplementary Fig. S1A–E in the online version at DOI: 10.1016/j.aquatox.2016.05.025).

For phase II biotransformation genes, *GST_omega-like* transcript levels increased 1.6 and 2.3 fold after 24 h and 96 h of exposure to 200 $\mu g L^{-1}$ PHE, respectively (Fig. 2D). Similar response was observed in the transcript levels of *SULT1B1*-like which increased 7.8 and 11.4 fold in the same exposure conditions (Fig. 2E).

This gene was even up-regulated in the group exposed for 96 h to 50 $\mu g L^{-1}$ PHE (Fig. 2E). Two other sulfotransferases isoforms, SULT1C1-like (Supplementary Fig. S1H in the online version at DOI: 10.1016/j.aquatox.2016.05.025) and SULT4A1-like (Fig. 2F) remained unchanged after PHE exposure, as well as FMO and FABP (Supplementary Fig. S1 F and G in the online version at DOI: 10.1016/j.aquatox.2016.05.025, respectively).

DEP caused a reestablishment of transcript levels for all upregulated genes, except for SULT1B1, which remained higher (5.95 fold, Fig. 2E) than control group. In addition, *SULT4A1-like* levels showed a down-regulation after DEP in the 200 $\mu g L^{-1}$ PHE exposure (3 fold, Fig. 2F).

3.3. PAH effects on biochemical responses: enzymes and lipid peroxidation

Scallops exposed to 200 $\mu g L^{-1}$ PHE for 96 h presented higher GPx (1.34 fold, Fig. 3A) and GR (1.7 fold, Fig. 3B) activities. Enhanced G6PDH activity was observed in the organisms exposed to 50 $\mu g L^{-1}$ PHE for 24 h and in the organisms depurated for 96 h (1.24 fold, Fig. 3E). DEP was able to restore GR and GPx activities to control levels (Fig. 3A and B). Interestingly, the clams exposed to 200 $\mu g L^{-1}$ PHE for 96 h and depurated for 96 h showed higher CAT activity (1.66 fold, Fig. 3C) and lower SOD activity (1.2 fold, Fig. 3D). G6PDH activity was also significantly increased (1.2 fold, Fig. 3E) in the group depurated for 96 h which has been previously exposed to 50 $\mu g L^{-1}$ PHE. No significant changes among groups were observed in GST activity (Fig. 3F).

Scallops exposed to 200 $\mu g L^{-1}$ PHE for 24 h and 96 h, as well as the group depurated for 96 h showed lower levels of lipid peroxidation than the respective control groups (Fig. 3G).

4. Discussion

Considering that bivalves are sessile (e.g. oysters, mussels) or low-movement organisms (e.g. scallops), they show low ability to escape from a point source of contaminants. Because of that, they have been widely used as sentinel organisms, since they tend to bioconcentrate contaminants. Many authors found evidences that bivalves show low PAH metabolism capacity, which reinforce the use of these organisms as important sentinel species for the evaluation of chemical contamination in coastal regions (Livingstone, 1998; Snyder, 2000).

However, the present study shows interesting data evidencing that scallops *N. nodosus* possess molecular and biochemical strate-

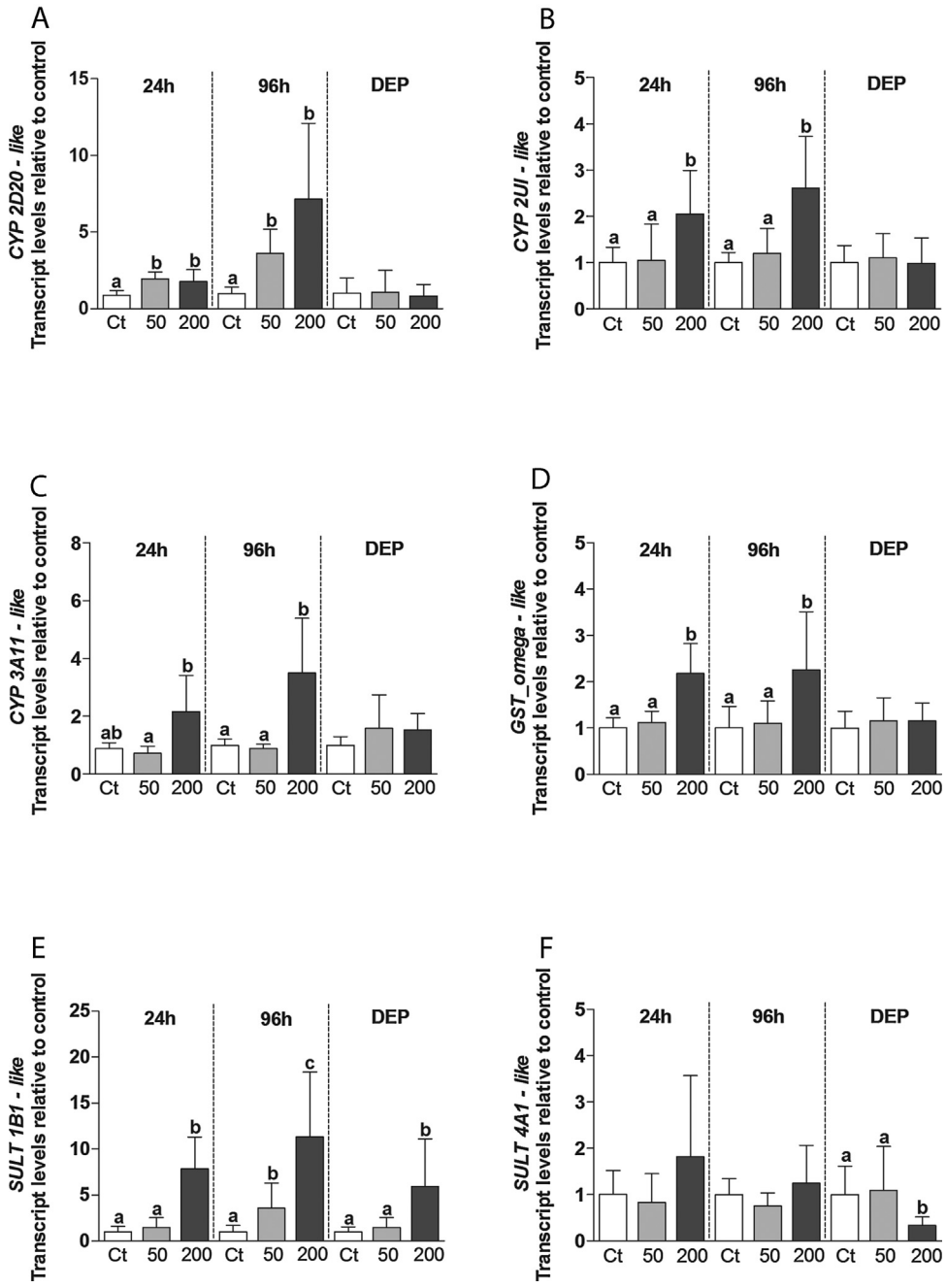

Fig. 2. Normalized relative transcription ratio of biotransformation genes A – *CYP2D20-like*, B – *CYP2UI-like*, C – *CYP3A11-like*, D – *GST_omega-like*, E – *SULT1B1-like*, F – *SULT4A1-like* in gill of scallops *N. nodosus* exposed to phenanthrene for 24 h, 96 h and depurated for 96 h (DEP). The gene transcript levels were assessed by qRT-PCR and data are expressed as relative fold induction of exposure treatments – concentration 50 μg L^{-1} (50) and 200 μg L^{-1} (200) – in relation to the control group (Ct). For each exposure period (24 h, 96 h or DEP) columns not sharing letters are significant different ($p < 0.05$), n = 10 animals per group.

gies which are possibly involved in the adaptation to PHE exposure, PHE metabolism and depuration.

N. nodosus bioconcentrated PHE in a time- and concentration-dependent manner, similarly to what has been observed previously in oysters *Crassostrea brasiliana* (Lüchmann et al., 2014). Increased PAHs levels have been described in many bivalves, such as scallops *Pecten maximus* (Hannam et al., 2010a), *Perna viridis* (Cheung et al., 2001), *Ruditapes philippinarum* (De Luca-Abbott et al., 2005; Liu et al., 2014), *Mytilus galloprovincialis* (Bocchetti et al., 2008) and *Chlamys islandica* (Hannam et al., 2009).

An important route for uptake, bioconcentration, metabolism and excretion of PAHs are the gills because of their wide surface area in contact with seawater. In this context, it is expected that this tissue possesses molecular and biochemical modulation systems more rapidly responsive than digestive gland. This has been demonstrated by Pan et al. (2009) in scallops exposed to benzo[a]pyrene and by Trevisan et al. (2012) in oysters exposed to CDNB. Therefore, this tissue can be target by several pollutants in aquatic organisms, probably affecting different metabolic and cellular pathways.

In the present study, the transcription of eight different *CYP* genes were analyzed in gills of scallops exposed to PHE. Three of them, *CYP2D20-like*, *CYP2UI-like* and *CYP3A11*-like, showed significantly higher transcript levels in the organisms exposed both, for 24 and 96 h period, to 200 μg L^{-1} PHE, comparing to the control group. Similar effect was observed in the transcription of *CYP2D10-like*

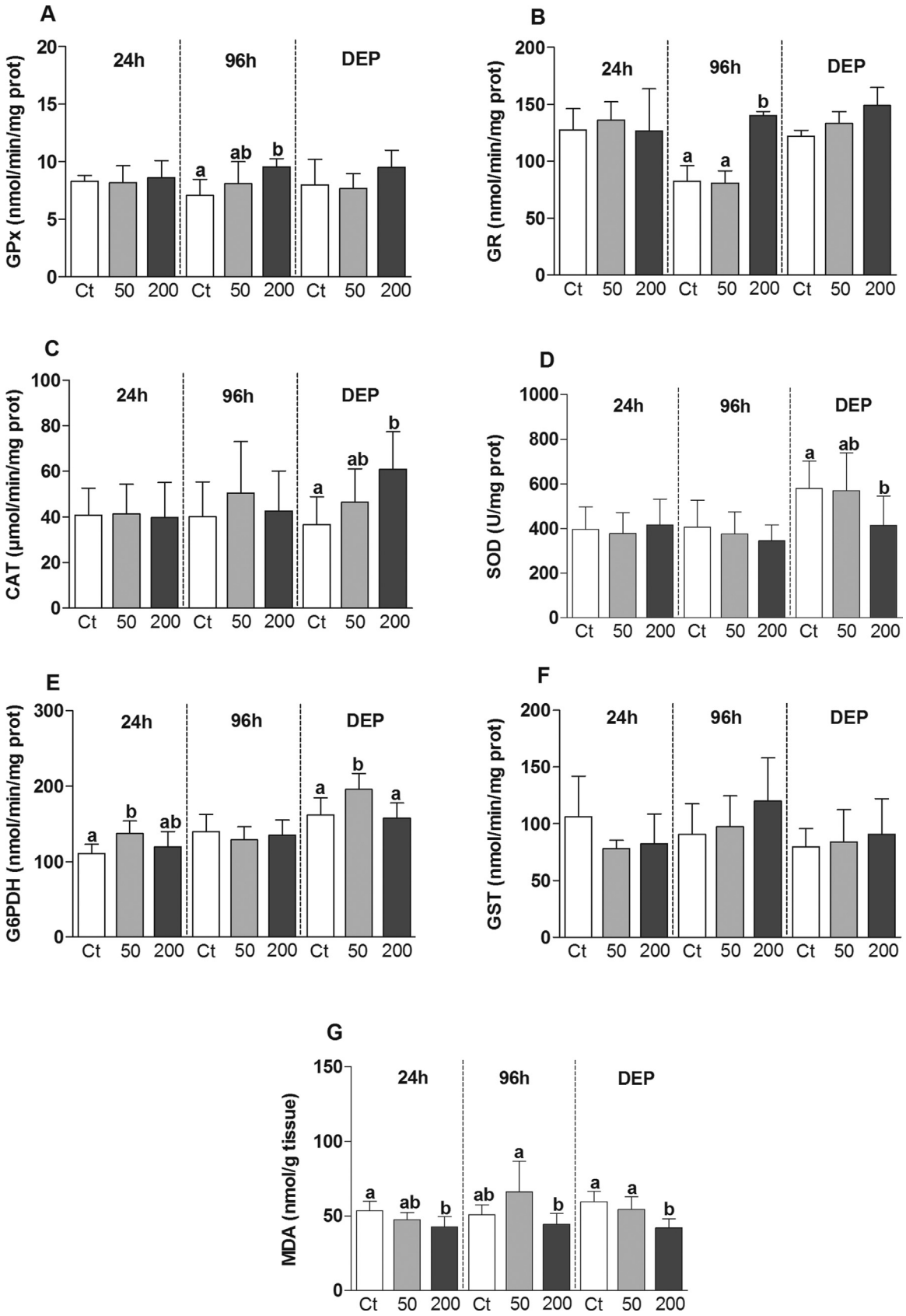

Fig. 3. Enzymatic activities (mean ± SD) of A, glutathione peroxidase (GPx), B, glutathione reductase (GR), C, catalase (CAT), D, superoxide dismutase (SOD), E, glucose 6 phosphate dehydrogenase (G6PDH), F, glutathione S- transferase (GST) and G, malondialdehyde (MDA) levels in gill of scallop *N. nodosus* exposed to phenanthrene for 24 h, 96 h and depurated for 96 h (DEP). The data are expressed as relative fold induction of exposure treatments – concentration 50 µg L^{-1} (50) and 200 µg L^{-1} (200) – in relation to the control group (Ct). For each exposure period (24 h, 96 h or DEP) columns not sharing letters are significant different ($p<0.05$), n = 10 animals per group.

even in the group exposed to the lower PHE concentration. These data indicate that PHE is activating the transcription of different phase I enzymes possibly to enhance its metabolism and depuration. Corroborating this hypothesis, increased transcript levels of *GST omega-like* and *SULT 1B1-like* were also up-regulated in the scallops exposed for 24 and 96 h to 200 $\mu g\,L^{-1}$ PHE (Fig. 2D and E).

The biotransformation of such compounds can also produces ROS, which are also generated through aerobic metabolism, electron transport chain, enzymatic activities or immunological reaction (Regoli and Giuliani, 2013).

Interestingly, MDA levels decreased after exposure (24 h) to the highest PHE concentration, remaining low even after DEP. This pattern has already been reported with other aquatic species exposed to PAH mixtures (Arantes Felício et al., 2015; Nogueira et al., 2015) and on a time-dependent oxidative damage (Ansaldo et al., 2005; Lüchmann et al., 2011, 2014). At the same time, the absence of increased MDA levels along with PHE bioaccumulation could indicate a low acute toxicity of this compound to *N. nodosus*. On the other hand, it is known that different aldehyde dehydrogenases are capable of reducing MDA and some other aldehyde products of lipid peroxidation to the corresponding alcohols, suggesting a potential physiological role for these enzymes in detoxifying cytotoxic aldehydes (Janero, 1990). Previous studies showed that a possible biochemical pathway for MDA degradation and excretion involves its oxidation by mitochondrial aldehyde dehydrogenase followed by decarboxylation to produce acetaldehyde, which is oxidized by aldehyde dehydrogenase to acetate and further to CO_2 and H_2O (Siu and Draper, 1982; Esterbauer et al., 1991). Based on that, we could suggest that exposure to PHE could induce aldehyde dehydrogenase which, in turns, could decompose MDA to more soluble alcohols to be excreted in the gills. This hypothesis is corroborated by the fact that aldehyde dehydrogenase genes have been identified in the list of up-regulated genes in oysters *Crassostrea brasiliana* exposed to PHE (K.H.Luchmann, personal communication). Likewise, aldehyde dehydrogenase mitochondrial type-coding genes have been identified in the group of the up-regulated genes in scallops *Nodipecten nodosus* exposed *in situ* to produced water discharges at petroleum exploration platforms (Wendt et al., manuscript in preparation).

PHE caused an increase in activity of three antioxidant enzymes related to the glutathione system, namely GR, GPx and G6PDH. These points to a scenario with increased reduced glutathione turnover, peroxide detoxification and NADPH consumption, probably related to an increased ROS production during PHE metabolism. It is known that the GPx system is essential for protection against peroxide in bivalves, and impairment on its ancillary systems (such as the GR cycle) can decrease the *in vivo* organic peroxide detoxification and increase animal susceptibility to oxidative stress in mussels (Trevisan et al., 2014a,b). GST, another enzyme that confers cellular protection during PHE exposure, did not show changes in activity after PHE exposure, agreeing with previously studies done with *Nacella concinna*, *Mytilus galloprovincialis* and *Crassostrea brasiliana* (Ansaldo et al., 2005; Lüchmann et al., 2011; Trisciani et al., 2012). GST activity has been reported to increase and decrease in marine bivalves expose to pollutants. These opposed results could be explained by inhibitory effect caused by high concentration of pollutants, or alternatively, an adaptive response of GST detoxification pathway that would need to be further studied (Pan et al., 2009). Moreover, GST activity was analyzed using CDNB as substrate, which quantifies total GST activity, and this protocol does not allow the identification of activation of inactivation of GST isoforms specifically (Trisciani et al., 2012). Therefore, the lack of enzymatic response of GST after PHE exposure does not mean that specific GST isoforms were not responsive to PHE. This could be further addressed by the use of additional and more specific GST substrates. In fact, increased GST-*omega-like* mRNA levels (~2 fold) were observed in organisms exposed for 24 h and 96 h to the higher PHE concentration.

Interestingly, enzymatic activity was mainly modulated after 96 h of PHE exposure, suggesting that 24 h might not have been long enough to activate the antioxidant defense system, except for G6PDH activity. Although responsible for the NADPH generation, this enzyme is related to glucose metabolism, and probably, to feeding/starvation periods. Feeding was not supplied during PHE exposure, returning during the depuration period. Although this scenario may affect energy-related metabolism, it should not be considered as a general rule since no-effect and reduction of G6PDH activity was reported during food deprivation in oyster gill and fish liver (Lüchmann et al., 2011; Morales et al., 2004).

In this study, it was analyzed mRNA expression of PAH detoxification genes in gill of scallops. Despite CYP1A plays an important role in this mechanism and was the most important and studied gene related by chemical detoxification via the aryl hydrocarbon receptor (AHR) (Toledo-Silva et al., 2008; Goldstone and Stegeman, 2006; Zanette et al., 2013), the present transcriptional results showed non-significant changes in the CYP1A1-*like* and CYP1A5-*like* genes. The interpretation of results related to CYP1As has been hindered by the low catalytic activity of microsomal enzymes and the lack of specific cross-reactivity of antibodies for these proteins in bivalves (Zanette et al., 2013). Our results are in agreement with Akcha et al. (2000) that showed no differences on the level of CYP1A-*like* immunopositive proteins in digestive gland of *Mytilus galloprovincialis* exposed to benzo[a]pyrene. Zanette et al. (2013) also verified no significant changes in the levels of mRNA expression of the CYP1-*like* and CYP3- *like* genes in digestive gland and gill of *Mytilus edulis* after injections of AHR agonists.

Other and equally important subfamily is known such CYP2 and performs an important function in the biotransformation of organic compounds (Kim et al., 2014). CYP2 also has been previously reported in mammals, fish and mollusks (Goldstone et al., 2010; Kubota et al., 2013; Lüchmann et al., 2014; Peters et al., 1998). Agreeing with CYP2 role in detoxification of PAHs, mRNA levels of CYP2D20-*like* and CYP2UI-*like* increased significantly in the gill of scallops both, after 24 h and 96 h exposure to PHE. Important to note that after depuration treatment the transcriptional levels both CYP2 s were nearly identical to the control, not presenting significant differences. These results further confirm that CYP2 transcriptional changes could be suggested as biomarkers of exposure to PAH in bivalves. The CYP3 subfamily is known to metabolize a several range of substrates such as xenobiotics, bile acids, endogenous sex hormones and prevent bioaccumulation of lipophilic substances (Tian et al., 2014). Notably, we found an increase in the CYP3A11-*like* after 96 h of exposure to higher PHE treatment returning to basal levels after DEP. Compared to previous studies we can observe a discrepancy in results such as moderate upregulation or absence of change in CYP3 isoforms for fishes and bivalves exposed to organic contaminants (Della Torre et al., 2010; Han et al., 2014; Lüchmann et al., 2014; Zanette et al., 2013). A possible explanation for these different outcomes could be due to tissue distribution, exposure time and concentration. While recent studies have shown increases as well as decreases or blocked induction in CYP expression (Toledo-Silva et al., 2008; Lacroix et al., 2014; Tian et al., 2014), such divergences still lack a mechanistic explanation that would support inference and prediction (Zanette et al., 2013). Another phase I gene analyzed in this study was FMO-*like*. This gene did not present any significant variation to PHE exposure, as already verified by Boutet et al. (2004a) in gills of oyster *Crassostrea gigas* following pesticide and hydrocarbon exposure. Schlenk and Buhler, (1989) detected FMO enzyme activity in *C. gigas* gill, while Boutet et al. (2004a) showed a rise of FMO transcripts levels in digestive gland of *C. gigas* exposed to PAHs and suggested that FMO levels

was normally greater in digestive gland, liver or hepatopancreas than in others tissues.

After phase I, metabolites can be conjugated to endogenous compounds by phase II biotransformation enzymes such as GST isoforms which is known to promote differential regulation (Lacroix et al., 2014). As stated before, an increment in mRNA levels of GST-*omega-like* (~2 fold) was observed in organisms exposed for 24 h and 96 h to higher levels of PHE. GST-*omega* class also has been identified of up-regulated genes in oysters exposed to hydrocarbon (Boutet et al., 2004b; Lüchmann et al., 2014), domestic sewage (Medeiros et al., 2008a) and in transplanted zebra mussels (Châtel et al., 2015). This GST isoform is also suggested to be involved in the antioxidant defense system (Medeiros et al., 2008a; Rola et al., 2012), that can be important during PAH exposures.

Cytosolic SULT belong to a superfamily of multifunctional phase II enzymes that catalyze sulfate conjugation in the metabolism of xenobiotics and endogenous compounds (Michio and Hiroshi, 1994). There are few studies with these proteins in bivalves. (Wang et al., 2012) suggested *SULT* might be a candidate gene related to growth in clams and Jia et al. (2009) related it to stress responses in abalone. *SULT1C1-like* levels did not present significantly changes in our study, as in oyster *Crassostrea brasiliana* from a polluted site at southern Brazil (unpublished data). However, *SULT1B1-like* presented a major increase by PHE exposure, a result previously observed in bivalves exposed to benzo[a]pyrene (Liu et al., 2014; Milan et al., 2013; Tian et al., 2013). This gene remained elevated even after the depuration period in the organisms exposed to the higher PHE concentration, which suggests a possible key role on PHE detoxification in gills.

Another gene investigated in our study was FABP, a small (14–15 kDa) cytosolic protein which belongs to the large multigene family of intracellular lipid-binding proteins. Vertebrate FABPs are involved in energy pathways, growth and development, fatty acid transport between membranes but other biological roles are being investigated, such as participation on signal transduction, regulation of gene expression and involvement in mediating the absorption and metabolism of xenobiotics (Medeiros et al., 2008b). The FABP mRNA levels did not show significant differences in our study, nonetheless, showed an increase in FABP levels in gill oyster *C. brasiliana* (unpublished data) and *C. gigas* (Medeiros et al., 2008b), contaminated by sewage. Conversely Bain (2002) reported an inhibition of FABP levels after pyrene exposure in fish heart. It is still unclear the role of FABP in bivalves exposed to xenobiotics.

Like other bivalves, scallops are able to eliminate PAHs from its tissues when in contact to clean seawater under conditions able to maximize their filtering activity. The present study used a flow-through water systems depuration to evaluate the detoxification capacity of scallops. Depuration caused a decrease in PHE concentration by 2.9- and 2.5 fold after 96 h of exposure to PHE 50 and 200 $\mu g\,L^{-1}$, respectively, as well as the restoration to basal levels for most of the parameters affected by PHE exposure. Decrease of PHE levels can lead to animal recovery, with a probable lower signaling input for gene transcription and lower ROS production due to decreased PHE biotransformation. Even though, at the end of DEP scallops still presented modulation of key cellular defense mechanisms, such as lower *SULT4A1* transcript levels and SOD activity, as well as increased CAT and G6PDH activities. Some of the sulfate conjugates are known to become toxic or pharmacologically active (Michio and Hiroshi, 1994), which may lead to reduction of SULT mRNA levels (Chapman et al., 2004) and enzymatic activity (Janer et al., 2005). Decreased SOD activity in parallel to increased CAT activity suggests an effort to stabilize H_2O_2 concentration, minimizing oxidative stress and lipid peroxidation (Ameur et al., 2015). In addition, at low H_2O_2 concentrations, CAT modulates the detoxification of other substrates, such as phenols and alcohols, over reactions coupled to H_2O_2 reduction (Regoli and Giuliani, 2013).

In conclusion, data suggests that scallops can adopt some strategies to metabolize, eliminate and survive in the presence of PHE. The transcription genes associated to cellular parameters develop an important role in the characterization of the detoxification mechanisms in bivalves. PHE was responsible by alterations in membrane stability and important genes related to PHE biotransformation. Induction of phase I and II biotransformation genes during the exposure period is possibly associated to decreased levels of PHE in the tissues of scallops submitted to 96 h depuration. Depuration in clean water associated to feeding could be an effective method to be used in order to minimize the effects induced by PAHs and promoting their excretion, possibly by the gill in scallop *N. nodosus*.

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