UNIVERSIDADE FEDERAL DO PARANÁ

MARIANA ARAKI BRAGA

MARCADORES ENZIMÁTICOS E DE CITOXICIDADE SÃO INDICADORES PRECOCES DE RESPOSTAS DO BENTOS À FRAÇÃO HIDROSSOLÚVEL DE ÓLEO DIESEL

CYTOTOXICITY AND ENZYMATIC BIOMARKERS AS EARLY INDICATORS OF BENTHIC RESPONSES TO THE SOLUBLE-FRACTION OF DIESEL OIL

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Zoologia, setor de Ci^ncias Biológicas da Universidade Federal do Paraná, como requisito parcial para obtenção do grau de Mestre em Zoologia.

Orientador: Dr. Paulo da Cunha Lana

Co-orientadora: Drª. Kalina Manabe Brauko

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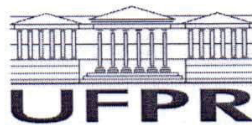

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Curitiba, 26 de Fevereiro de 2018.

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“Não é na ciência que está a felicidade, mas na aquisição da ciência”.

Edgar Allan Poe

Dedico este trabalho para toda população brasileira.

Por mais valorização da nossa fauna local e mais incentivo para a ciência no Brasil.

RESUMO

Os xenobióticos derivados de vazamentos de petroleiros e de descargas industriais estão entre os principais impactos antropogênicos em áreas costeiras confinadas. Investigamos a resposta genotóxica de hidrocarbonetos policíclicos aromáticos (HPAs) provenientes da fração hidrossolúvel de óleo diesel (FHD) no poliqueta *Laeonereis culveri* e no bivalve *Anomalocardia flexuosa*, duas espécies bênticas comumente encontradas em estuários subtropicais do Atlântico Sudoeste. Hipotetizamos que a maior sensibilidade será expressa por respostas de biomarcadores significativamente diferentes entre o controle e os tratamentos afetados pelo óleo, dependendo das concentrações de contaminantes e dos tempos de exposição. A sensibilidade ao óleo diesel foi investigada experimentalmente usando um projeto experimental com dois fatores fixos (porcentagens de contaminantes e tempos de exposição). Após a exposição, verificamos as respostas das enzimas de estresse oxidativo e realizamos testes de micronúcleos. Os resultados foram congruentes para as duas espécies. A defesa antioxidante da glutationa S-transferase (GST) e a indução de micronúcleos e brotos nucleares, o último apenas para o bivalve, foram significativamente afetados pelos HPAs, com aumentos significativos no sétimo dia de exposição e nas maiores concentrações, em comparação com os grupos controles. Avaliamos os benefícios e desvantagens de usar cada biomarcador em experimentos laboratoriais, no qual ambas as espécies mostraram ser indicadores de respostas rápidas a contaminantes genotóxicos de estuários subtropicais. Sugerimos que o teste de micronúcleos em *A. flexuosa* é simples, rápido e barato para genotoxicidade em áreas afetadas pelo óleo diesel. Estes biomarcadores iniciais são necessários para desenvolver melhores protocolos para avaliação de impacto e monitoramento em condições de campo reais.

Palavras-chave: Micronúcleos; Estresse oxidativo; Biomarcadores; Bioensaios; Hidrocarbonetos policíclicos aromáticos; Fração hidrossolúvel de óleo diesel.

ABSTRACT

Xenobiotics from oil tanker leaks and industrial discharges are among the main human impacts to confined coastal areas. We assessed the genotoxic response to polycyclic aromatic hydrocarbons (PAHs) from the water-soluble fraction of diesel oil (WSFD) in the polychaete *Laeonereis culveri* and the bivalve *Anomalocardia flexuosa*, two widespread benthic species in subtropical estuaries from the Southwestern Atlantic. We hypothesized that the highest sensitivity would be expressed by significantly different biomarkers responses between control and oil-impacted treatments, depending on pollutant concentrations and times of exposure. Sensitivity to diesel oil was experimentally investigated using an experimental design with two fixed factors (contaminant percentages and times of exposure). After exposure, we monitored the responses of the oxidative stress enzymes and performed micronuclei tests. Results were congruent for both species. Antioxidant defense of glutathione S-transferase (GST) and the induction of micronuclei and nuclear buds, the latter just for the bivalve, were significantly affected by PAHs, with significant increases on the seventh day of exposure and in the higher concentrations, compared to controls groups. We assessed the benefits and drawbacks of using each biomarker in laboratory experiments. Both species are indicators of early, and rapid responses to genotoxic contaminants in subtropical estuarine habitats. We suggest that the micronuclei frequency in *A. flexuosa* is a simple, fast and cheap test for genotoxicity in oil-impacted areas. Such early biomarkers are needed to develop better protocols for impact assessment and monitoring under real field conditions.

Key words: Micronucleus; Oxidative stress; Biomarkers; Bioassays; Polycyclic aromatic hydrocarbons; Diesel water soluble fraction.

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Cytotoxicity and enzymatic biomarkers as early indicators of benthic responses to the soluble-fraction of diesel oil

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Keywords: Micronucleus; Oxidative stress; Biomarkers; Bioassays; Polycyclic aromatic hydrocarbons; Diesel water soluble fraction.

A B S T R A C T

Xenobiotics from oil tanker leaks and industrial discharges are among the main human impacts to confined coastal areas. We assessed the genotoxic response to polycyclic aromatic hydrocarbons (PAHs) from the water-soluble fraction of diesel oil (WSFD) in the polychaete *Laeonereis culveri* and the bivalve *Anomalocardia flexuosa*, two widespread benthic species in subtropical estuaries from the Southwestern Atlantic. We hypothesized that the highest sensitivity would be expressed by significantly different biomarkers responses between control and oil-impacted treatments, depending on pollutant concentrations and times of exposure. Sensitivity to diesel oil was experimentally investigated using an experimental design with two fixed factors (contaminant percentages and times of exposure). After exposure, we monitored the responses of the oxidative stress enzymes and performed micronuclei tests. Results were congruent for both species. Antioxidant defense of glutathione S-transferase (GST) and the induction of micronuclei and nuclear buds, the latter just for the bivalve, were significantly affected by PAHs, with significant increases on the seventh day of exposure and in the higher concentrations, compared to controls groups. We assessed the benefits and drawbacks of using each biomarker in laboratory experiments. Both species are indicators of early, and rapid responses to genotoxic contaminants in subtropical estuarine habitats. We suggest that the micronuclei frequency in *A. flexuosa* is a simple, fast and cheap test for genotoxicity in oil-impacted areas. Such early biomarkers are needed to develop better protocols for impact assessment and monitoring under real field conditions.

1. Introduction

Marine confined habitats, such as estuaries, are among the most impacted by xenobiotics derived from coastal human activities. The acute or chronic release of pollutants like diesel oil into the water requires monitoring and impact assessment

strategies (Farrington, 2014). The crude oil-related contaminants of primary concern, because of their potential carcinogenic, genotoxic and teratogenic effects on the marine organisms, are the polycyclic aromatic hydrocarbons, hereafter PAHs (Boehm and Page, 2007). PAHs are among the most stable, persistent, and harmful xenobiotics in aquatic environments due to their low water solubility and particulate apprehension deposited in sediments (Bacosa and Inoue, 2015). Once released into the sea, oil suffers subsequent chemical transformations after weathering processes. Marine diesel oil carries volatile compounds highly dispersible due to its low viscosity, which results in faster evaporation, dispersion and dissolution processes (Hansen et al., 2013). Most of these compounds evaporate fastly after spills, but remaining fractions can still harm organisms (Neff et al., 2000).

Estuaries are transitional areas between seawater and freshwater and may act as terrestrial pollutants reservoirs and sources of pollutants to the ocean (Jiang et al., 2013). Estuarine-coastal regions are affected by PAHs contamination from the atmosphere and land, by sedimentary processes and by river discharge (Wang et al., 2016). Benthic estuarine animals may accumulate and retain organic and inorganic contaminants due to their bottom-living strategies. They are frequently used to assess the damage caused by oil spills, mainly because they respond to changes in physical and chemical parameters very precisely and quickly (Dauvin et al., 2010).

Polychaetes numerically dominate the estuarine communities and are considered potential sentinels of xenobiotic impacts, due to their physiological plasticity and sensitivity (Bat, 2005). Populations of the nereidid polychaete *Laeonereis culveri*, a common detritivore species found in tropical and subtropical estuaries in South America, are often exposed to oil pollution, absorbing several types of pollutants through the epidermis and digestive tube (Durou et al., 2005).

Filter-feeding bivalves, on the other hand, are more sessile and may accumulate chemical compounds present in the water column (Gowland et al., 2002). They display limited abilities to metabolize PAHs and may accumulate high levels of these compounds in their tissues (Dyrynda et al., 1997). *Anomalocardia flexuosa* is a common bivalve species present along the subtropical and tropical Atlantic coasts of South America (Colonese et al., 2017). Their populations can withstand significant contamination caused by diesel oil due to the protection conferred by their shells (Sandrini-Neto et al., 2016). They are suitable for impact assessment and monitoring (Sandrini-Neto et al., 2016; Sardi et al., 2017, 2016), but still need to be tested in ecotoxicological experiments.

Molecular and cellular responses are in general the earliest signals after environmental disturbance, and they are increasingly used as biomarkers in ecotoxicological investigations (Regoli et al., 2004). The use of a multi-biomarker approach can provide a consistent combination of evidence on the mechanisms that define the appearance of biological alterations (Guidi et al., 2010). In general, genotoxic responses are driven by multiple factors such as time of exposure, uptake, metabolic activation, defense mechanisms and repair efficiency, and may differ significantly for different cell types (Lewis and Galloway, 2008). At sub-organism levels, one of the most investigated pathways after exposure to xenobiotics is the induction of oxidative stress. Oxidative stress has received increasing attention from aquatic toxicologists because it reflects perturbations of oxyradical metabolism, in which environmental pollutants can change the natural balance between prooxidant forces and antioxidant defenses (Benedetti et al., 2015). The measurement of marine invertebrates is extensively used as a pollution index (Regoli and Giuliani, 2014).

PAHs can also affect the integrity of DNA due to DNA strand breaks, loss of methylation and the formation of DNA adducts (Pisoni et al., 2004). Micronuclei appear when cells fail to incorporate complete or fragmented chromosomes into the daughter nuclei during cell division. Genetic fragments are instead incorporated in small additional nuclei, where they remain throughout the life of the cell. The presence of micronuclei is an indicator of chromatin breakage which may be caused by clastogens or spindle dysfunctions, ultimately caused by toxic compounds (Carrano and Natarajan, 1988). The micronucleus test has been widely applied in the field and cultivated marine invertebrates (Bolognesi et al., 2004; Siu et al., 2004; Viarengo et al., 2007). It is a cytogenetic technique commonly used for the assessment of genotoxic effects caused by environmental stressors.

Recent studies have been integrating MN tests in bivalves to evaluate the presence of xenobiotics in the environment (D'Agata et al., 2014; D'costa et al., 2018; Falfushynska et al., 2018) and also attributed as the biomarker of PAHs' toxicity (Baršienė et al., 2010; Farhadi et al., 2011; Vincent-Hubert et al., 2011). The MN frequency detected in these bivalves is a guide to accumulated genetic damage during the cell lifespan, providing a time-integrated response of an organism's exposure to contaminant mixtures (Gomiero et al., 2015).

Experimental evaluations of the response of subtropical benthic species combined with the use of multi-biomarker approaches are still scant. We carried out

ecotoxicological tests to assess the responsiveness of the polychaete *L. culveri* and the bivalve *A. flexuosa* to the soluble fraction of diesel oil. We evaluated: i) the activity of oxidative stress enzymes (i.e., superoxide dismutase, SOD; catalase, CAT, and glutathione S-transferase, GST) along with levels of lipid peroxides (LPO), and ii) the frequency of cytogenetic abnormalities. Considering that both species display different life strategies and external body protection, we hypothesized that the highest responsiveness will be associated with the most vulnerable species and will express by significantly different biomarkers responses between control and oil-impacted treatments as a function of concentrations and times of exposure to the contaminant.

Comparisons among different species and biomarkers allied to the use of robust experimental designs are much needed to develop cheap and fast protocols to evaluate impacts produced by diesel oil in subtropical estuaries.

2. Materials and methods

2.1. Experimental design

The experiments followed the sampling design shown in Figure 1. For the first experiment, 1,040 individuals of *L. culveri* were collected in tidal flats from Cotinga Island, located in Paranaguá Bay, Southern Brazil (25° 30′ 30.2 S; 48° 28′ 9.7 W), in the fall of 2017. The island is located in an area minimally impacted by PAHs, compared to other regions from the Paranaguá Bay (Cardoso et al., 2016, de Abreu-Mota et al., 2014). Approximately 20 specimens are ranging between 30 and 60 mm each (adult size) were allocated in 1,000 ml sterile beakers containing 1 cm of defaunated sediment and filtered seawater, following the methodology of Sandrini-Neto and Lana (2014). Animals were acclimated for 5 days under temperature, salinity, and photoperiod conditions of 20 °C, 25 ‰, and 12 light-12 dark regimes, respectively, to simulate the environmental conditions and were fed with the dry extract of *Spirulina*.

The experimental controls of sampling, transport and acclimation effects were carried out in three sequential stages (Figure 1). For the field control, 30 polychaetes were sacrificed *in situ* in dry ice; three samples of 4 individuals each were fixed in methanol for the micronucleus test, while three samples of 6 individuals each were kept on dry ice for enzymatic analyses. 30 individuals were sacrificed to evaluate the potential effects of stress due to transport, once arriving at the laboratory and kept in an ultrafreezer -80 ºC (Ultrafreezer Cold Lab, Piracicaba, Brazil) instead of dry ice for subsequent analyses. To assess potential consequences of acclimation, 30 individuals were analyzed after 5 days

following the same routines. The experiment itself included five treatments of increasing concentrations (5%, 25%, 50% and 75%) of a water-soluble fraction of diesel oil. The effects of different times of exposure were evaluated in the individuals of three replicates per treatment after 1, 2 and 7 days of exposure (1d, 2d, and 7d). At the end of each exposure period, 6 individuals were fixed in an ultrafreezer for enzymatic analyses, and other 4 were fixed in absolute methanol for the micronucleus test in each replicate.

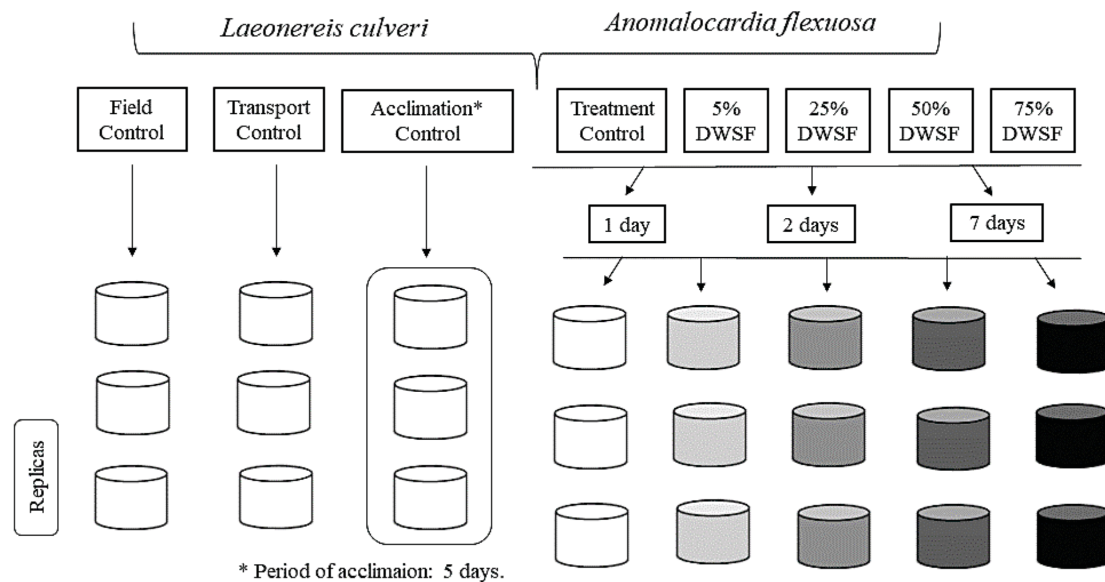

Figure 1: Experimental design. The three preliminary controls, with three replicates each, **are** displayed on the left side. The experimental treatments, with the corresponding concentrations of diesel water soluble fraction (DWSF) (Control, 5%, 25%, 50% and 75%) and time of exposure (1d, 2d, and 7d) are shown on the right side. For each exposure time and treatment, three replicates were distributed, totaling 45. For each time, specimens of three beakers from each treatment were sacrificed.

For the second experiment, 354 individuals of *A. flexuosa* were collected in Papagaios Island (25° 32′ 51.39 S; 48° 26′ 14.41 W), Paranaguá Bay, Brazil, at the end of fall 2017. Papagaios Island is non-contaminated by petroleum or oil by-products (Abreu-Mota et al., 2014). Individuals had in average 23 mm height, 27.5 mm length and 19.5 mm width. In the laboratory, 7 bivalves were allocated in beakers (replicates), similarly to the previous experiment. Each beaker contained 3 cm of defaunated sediment. The acclimation process followed the same test conditions from above, except that the bivalves were fed with the diatom *Chaetoceros muelleri*. The pre-experiment controls were also carried out in three sequential stages. Nine bivalves were sacrificed in the field, nine in the laboratory after transport and three bivalves from three beakers after the five days of acclimation.

2.2 Laboratory procedures

2.2.1. Enzymatic analysis

Each replicate consisted of a pool of four individuals of *L. culveri* (~ 200 g) and the digestive gland of three individuals of *A. flexuosa* (~ 100 g). The samples were homogenized in a potassium phosphate buffer (0.1 M at pH 7.0) in a 1:10 V/V ratio. They were then centrifuged at 15,000 g for 30 min at 4°C, and the supernatant was kept refrigerated at -80 °C until analysis.

For glutathione S-transferase (GST) activity, the samples were diluted to 1 mg/ml protein and 3 mM of chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) using the method of Keen et al. (1976). For superoxide dismutase (SOD), the *L. culveri* samples were diluted at 1:5 V/V and the *A. flexuosa* samples at 1:10 V/V. SOD activity was measured using Tris/EDTA buffer (1 M / 5 mM; pH 8.0), pyrogallic acid (15 mM) and 1 N HCl, according to Gao et al. (1998). The analysis of catalase (CAT) activity was based on Aebi (1984), using 20 mM reaction solution (1M Tris Buffer / 5mM EDTA pH 8.0, 30% hydrogen peroxide and deionized water). Lipid peroxidation (LPO) was measured according to the method of Jiang et al. (1992), using a FOX reaction solution (100 µM xylenol orange, 25 mM H 2 SO 4,4 µM BHT, 250 µM ammoniacal ferrous sulfate and 90% methanol). The concentration of total proteins followed the Bradford (1976) method. For each step of quantification, three blanks were put together in the microplate and analyzed in the same step of the samples. Blank values were obtained following analysis of the reagent mixture. Unfortunately, aliquots to verify CAT levels for *L. culveri* were not sufficient.

2.2.2. Micronucleus analysis (MN)

For the polychaetes, each sample consisted of four individuals after removal of parapodia and prostomium. The remaining tissues were added to a 2 ml microtube in acetic acid at 99.5% (1 ml total) and were mechanically broken down using a glass stirring rod. The microtube content was then adjusted to the final dilution of 3:1 acetic acid, methyl alcohol. Macerated tissue was centrifuged for 4 min at 900 rpm, and after discarding the supernatant (~ 0.5 ml), the remaining content was re-macerated to suspend the cells. All tissue and cell debris were eliminated, absolute methanol was added to complete the microtube content (2 ml) and then centrifuged for 4 min at 900 rpm. The supernatant containing disaggregated cells was collected with a Pasteur pipette and

dispersed on glass slides by trickling, 3 drops of the mixture per slide. Cytological preparations were stained using a solution of 9:1 distilled water and Giemsa for 5 min. Finally, slides were washed in running water and mounted in Permount.

For the bivalves, each sample consisted of a hemolymph extracted from the adductor muscle mixture of three individuals using a 1 ml sterile syringe containing modified anticoagulant solution ALSEVER (MAS) anticoagulant (following the methodology of Schleder et al., 2008) and then stored at -18 ºC. A subsample of 40 µl was then collected with a micropipette for the preparation of hemolymph smears, and the material was dispersed in a glass slide. After quickly drying under a ventilator, the smears were immersed in May grünvald's stain for 3 min and were then diluted with a few drops of distilled water for 15 min. Smears were then rinsed in distilled water and slides were mounted in Permount.

The micronucleated cells were observed under 1000x magnification on an optical microscope based on generally accepted criteria (Scarpato et al., 1990): well-preserved cell cytoplasm and membrane, micronuclei of similar or weaker staining and ≤1/3 of the size of the main nucleus. A thousand cells (in approximately four glass slides for polychaetes and two glass slides for bivalves) were scored to determine the frequency of micronuclei per sample. Additionally, other cytogenetic abnormalities were identified and counted only in bivalves' slides: binucleated (BN) and nuclear buds (NB) cells, that are such as nuclear buds, notched and lobed nuclei.

2.3. PAHs analysis

2.3.1. Water-Soluble Fraction of Diesel Oil (WSFD)

The diesel oil used in the experiments was stored in amber glass flasks, protected from light and maintained under 20 ºC. The soluble fraction was prepared using five 2 l graduated flasks and, in each one, a 3:1 solution of 25 ‰ filtered marine water with diesel oil was added. The solutions were mixed in a magnetic stirrer for 16 h in the dark (the flasks were wrapped with aluminum foil) and then exposed to sunlight for 6 h. The soluble fraction was removed from the flasks after a 1h standby. The hydrophilic portion was extracted with the aid of a hose and macro pipette and then homogenized in a previously sterilized 14 l-aquarium (protocol adapted from Bettim et al., 2015 and Vanzella et al., 2007 work).

To characterize and assess the increasing toxicity associated with each of the five different concentrations of the WSFD, we analyzed the concentrations of 16 priority polycyclic aromatic hydrocarbons (PAHs). Each water sample was represented by a mixture of subsamples collected from three experimental replicates, (details are shown in Table 1), the latter two were included for detecting the source of contamination.

2.3.2 Reagents, solvents and analytical standards

In this study, high purity analytical standards were used, higher than 99.0% of the 16 priority PAHs, according to USA (2001) [naphthalene (NA); acenaphthylene (ACY); acenaphthene (AC); fluorene (FL); phenanthrene (PHE); anthracene (AN); fluoranthene (FA); pyrene (PY); benzo(a)anthracene (BaA); chrysene (CH); benzo(b)fluoranthene (BbF); benzo(k)fluoranthene (BkF); benzo(a)pyrene (BaP); indeno(1,2,3-cd); dibenz(a,h)anthracene (DahA); benzo(g,h,i) perylene (Bghi)], 5 internal deuterated standards [naphthalene (NAD8); acenaphthene (ACD10); phenanthrene (PHED10); chrysene (CHD12) and perylene (BghiD12)] and 1 standard surrogate *p*-terphenyl deuterated (p-Terphenyl-d14), all of the brand Accustandart (New Haven, USA).

To extract the PAH from water samples, a Vortex-assisted (VA-DLLME) liquid-liquid dispersion microextraction method was used which was adapted from the work of Rezaee et al. (2006) and Zhang and Lee (2012). Glass tapered tubes (15 ml) were used for centrifugation, where an aliquot of 5.00 ml standard solution of a sample containing the PAH was transferred. To this solution, 1.00 ml of chloroform or extractor solution (75 μl) solubilized in acetone or dispersing solvent (925 μl) was rapidly injected with a micropipette. In order to increase the extraction efficiency, vortexing was carried out for 1.00 min, then centrifugation for 10 min, favoring the formation of a sedimented (drop) phase, which was quantitatively removed (50 μl) and finally transferred to a chromatographic insert containing 10 μl deuterated PAH internal standard mix solution, providing a concentration of 100 $\mu g\ l^{-1}$ of the IS. The same procedure was applied to obtain the analytical curves, which were prepared according to the internal standardization method for analytical curves was used to evaluate the 16 PAH and a subrogated standard, all in 7 concentration levels: 0.25; 0.50 0.80; 1.20; 1.50; 2.00; 4.00 $\mu g\ l^{-1}$, containing the 5 internal standards in concentration of 100 $\mu g\ l^{-1}$.

The determinations were performed on a Shimadzu QP2010 gas chromatograph coupled to Tandem mass spectrometer TQ8040 (Kyoto, JAPAN). To separation of the

PAHs, an analytical capillary column crossbond diphenyl dimethyl polysiloxane (30 m × 0.25 mm × 25 μm) and the following temperature programming was used: Initial 40 ° C for 2 min, followed by ramp of 50 °C min^{-1} up to 80 °C, ramp from 10 °C min^{-1} to 240 °C for 2 min, ramp from 4 °C min^{-1} to 260 °C for 5 min and finally ramp from 20 °C min^{-1} to 300 °C for 7 min. Chromatograms were obtained via selective ion monitoring (SIM), using the following m/z ratios for quantification and confirmation: 6 min (128, 136, 152, 154, 162 and 164); 13 min (165, 166, 178, 188, 202 and 244); 24 min (228, 236, 240, 252, 253, 260, 264) and 32 min (276, 277, 278 and 279). Also, the other determination conditions were the injection of 1 μl in splitless mode, injector, transfer line and source of ions maintained at 270, 280 and 230 ºC respectively and helium 99.99% at 1.2 ml min^{-1}.

2. 4. Data analysis

Differences in enzyme response rates of SOD, CAT, GST and levels of LPO were individually tested by mixed linear models with the following design: Concentration (Co, Fixed, 5 levels: Control, 5%, 25%, 50% and 75%), Time (Ti, Fixed, 3 levels: 1, 2 and 7 days) and interaction between Concentration & Time. As the data showed to be extremely heteroscedastic, even after transformations, the models were constructed with different variance structures for Concentration, Time and their interaction. After defining the best variance structure, the fixed effects structure was defined. The entire selection process was based on the significance of the terms and the Akaike Information Criterion (AIC) according to the protocol described by (Zuur et al., 2009). Significant terms of the fixed structure ($\alpha = 0.05$) were tested by post-hoc comparisons of least squares means. We used the same fixed structure model for the frequency of micronucleus and the other cytogenetic abnormalities, following the same selection criterion applying generalized linear models (GLM) with Poisson distribution. Normality of the residuals was verified by the Shapiro and Wilk (1965) test. All statistical and graphical analyzes were generated in the R language (R Core Team, 2017) with the aid of nlme (Pinheiro et al., 2017) and lsmeans (Lenth, 2016) packages.

The means and standard deviations were calculated for all biomarkers data from the experiment samples field, transport and acclimation controls. As low values of each biomarker appeared, the means were only recorded for a priori comparisons against the experimental controls.

3. Results

3.1. PAHs

The PAHs were measured in water samples taken from each concentration and time of exposure of both the polychaete and the bivalve experiment. Total PAHs were not detectable for most controls, excepting the polychaete experimental control with low values ranging from 4.45 to 0.56 µg/l (Table 1). Total PAHs values for exposure treatments of both species ranged from 0.26 to 71.0 µg/l (Table 1). Higher total PAHs concentrations were observed in the polychaetes experiment, at the 75% concentration of the first day (Table 1). The PAHs concentrations decreased according to the time of exposure during the experiments (Table 1).

Table 1: Concentrations (µg/l) of polycyclic aromatic hydrocarbons (PAH) in aqueous analysis of *A. flexuosa* and *L. culveri*, related parameters in control and oil-exposed water. ΣPAHs, total polycyclic aromatic hydrocarbons (µg/l); 2–3 rings, total PAHs with two to three aromatic rings (µg/l); 4–6 rings, total PAHs with four to six aromatic rings (µg/l).

A. flexuosa				***L. culveri***			
	ΣPAHs	2–3 rings	4–6 rings		ΣPAHs	2–3 rings	4–6 rings
C				**C**			
1d:	n.d.	n.d.	n.d.	1d:	4.45	n.d.	4.45
2d:	n.d.	n.d.	n.d.	2d:	n.d.	n.d.	n.d.
7d:	n.d.	n.d.	n.d.	7d:	0.56	n.d.	0.56
5%				**5%**			
1d:	0.26	0.26	n.d.	1d:	0.28	0.28	n.d.
2d:	2.24	n.d.	2.24	2d:	n.d.	n.d.	n.d.
7d:	n.d.	n.d.	n.d.	7d:	n.d.	n.d.	n.d.
25%				**25%**			
1d:	1.62	0.99	0.63	1d:	4.50	2.33	2.17
2d:	0.27	0.27	n.d.	2d:	2.08	1.06	1.02
7d:	-	-	-	7d:	0.76	0.76	n.d.
50%				**50%**			
1d:	5.4	5.4	n.d.	1d:	28.34	28.34	n.d.
2d:	4.2	4.2	n.d.	2d:	7.4	7.4	n.d.
7d:	5.59	3.7	1.89	7d:	2.7	2.7	n.d.
75%				**75%**			
1d:	24.72	10.12	14.60	1d:	71.0	39.2	31.80
2d:	9.29	4.49	4.80	2d:	22.2	18.8	3.40
7d:	4.24	2.9	1.34	7d:	9.5	5.6	3.90

n.d. = not detected;

Concentrations of low molecular weight (LMW - 2–3 rings) PAHs were not detected in the control treatments of both experiments and varied from 0.26 to 39.2 µg/l in the treatments exposed to the WSFD. Only High molecular weight (HMW - 4–6 rings) PAHs were detected in the control of the polychaetes experiment, ranging from 0.56 to 4.45 µg/l. HMW PAHs showed highest values on 1d, ranging from 0.63 to 31.8 µg/l in exposure treatments. The clear dominance of LMW PAHs in all samples of both experiments indicates a petrogenic source. However, the appearance of HMW PAHs in the polychaete control treatment may be indicative of pyrolytic sources, derived from the combustion of wood and not necessarily from diesel contamination.

3.2. Biomarkers responses in the polychaete L. culveri

Significant variation of the enzymatic activities among the different concentrations with diesel oil was verified, but none differed considerably from the control treatment, except the GST activity (Figure 2). SOD and GST activities were significantly different in the interaction between concentration and time of exposure, but only GST was significantly different from the control. The only significantly different response for SOD occurred on 1d when the activity in the 75% concentration was lower than the 25% (Figure 2, Table S1). Conversely, GST activity was only significantly different on d7, with a lower value in control compared to the 75% treatment (Figure 2, Table S1). Lipid peroxide levels (LPO) did not respond to the different concentrations but showed significantly lower values after 2d, compared to the other sampling dates (Figure 2, Table S1).

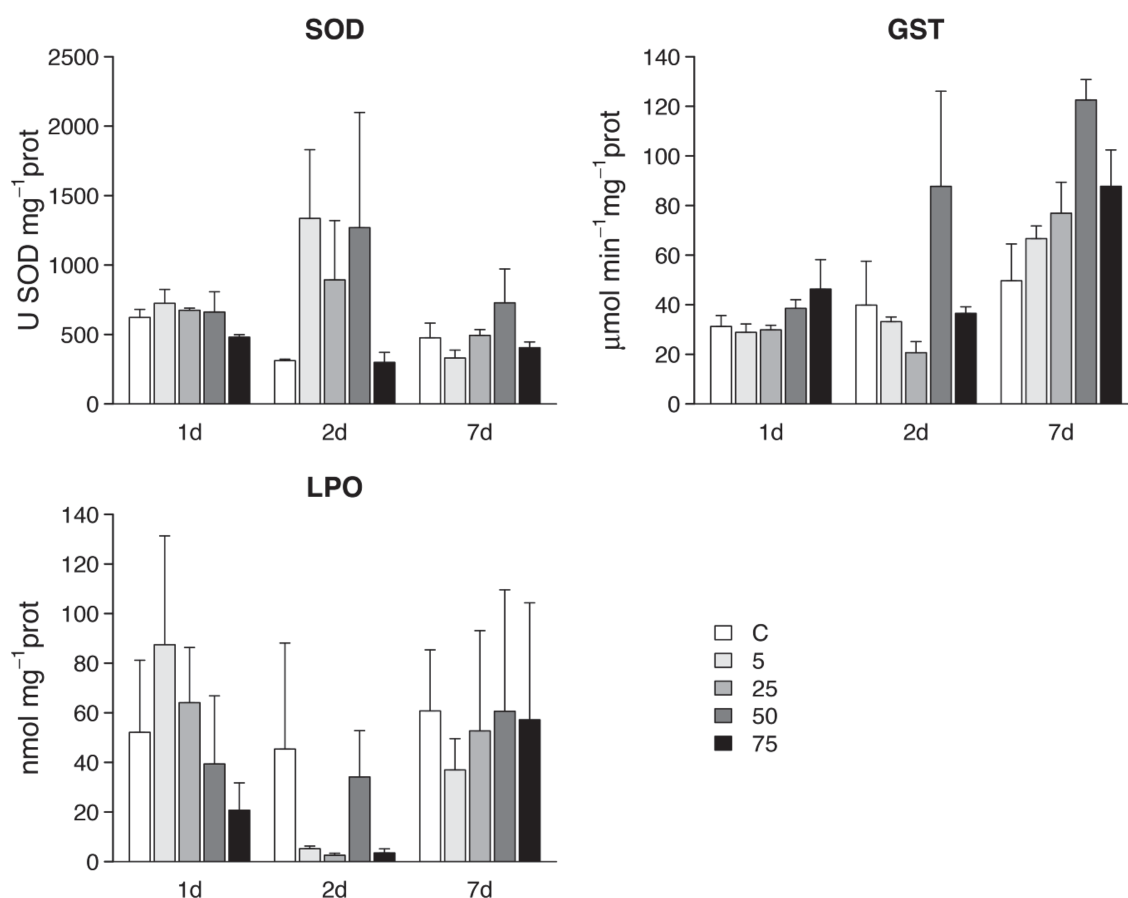

Figure 2: Activity of the antioxidant enzymes in polychaete *L. culveri* (mean ± SE): results of SOD activity analysis were expressed as µmg protein1 (where µ ¼ mmol substrate hydrolyzed per min), GST activities expressed as mol of thioether formed/milligram protein/minute and LPO levels (nmol mg prot−1). Undisturbed controls (C) are shown in white, low-concentration (5% and 25%) treatments are shown in light grays and high-concentration (50% and 75%) treatments are shown in dark grays.

There was significant variation in the frequency of MN between concentrations and times taken separately, but not in their interaction (Figure 3, Table S2). The frequency of MN was lower in the control than in the 50% and 75% concentrations, and the latter was higher than the 5% concentration (Figure 3, Table S2). Considering exposure times, 1d showed a smaller frequency of MN comparing to 7d, which presented the highest frequency of 5.66% MN.

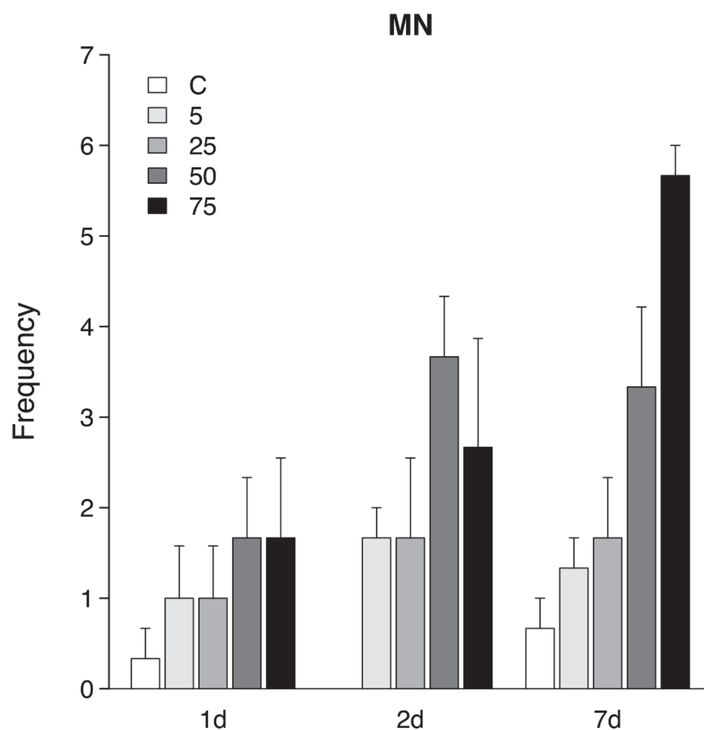

Figure 3: Comparison of the frequencies in one thousand cells of MN (mean ± SE) in the polychaete *L. culveri* in Undisturbed controls (C, white bars), low-dosage treatments (5% and 25%, light grays) and high-dosage treatments (50% and 75%, dark grays) subjected to three times of exposure (1d, 2d and 7d) to diesel oil.

The values for the pre-experiment controls are shown in Figure S1. Values are similar to those found in the control groups on the first day of exposure, especially those of acclimation (Figure 2, Figure S1). Values of the MN counts before exposure to diesel were lower (0.003) than the control groups at all experiment times.

3.3. Biomarkers responses in the bivalve A. flexuosa

The SOD activity in *A. flexuosa* varied significantly amongst concentrations and over time. The activity was consistently higher in the 5% concentration than in the 50%, but none of them differed from the control. SOD activity was higher on 1d of exposure and significantly decreased on 2d and 7d (Figure 4, Table S3). CAT activity was only significantly responsive over time, with higher values on 1d about 7d (Figure 4, Table S3). GST activity, on the other hand, was more responsive to the diesel exposure as showed by the significant interaction in the a posteriori comparisons, with significantly increased activity in the control in contrast to the 50% treatment of 7d (Figure 4, Table S3). LPOs levels also differed only over time, with the highest value on 1d in comparison to the others, and with a higher level on 7d comparing to 2d (Figure 4, Table S3).

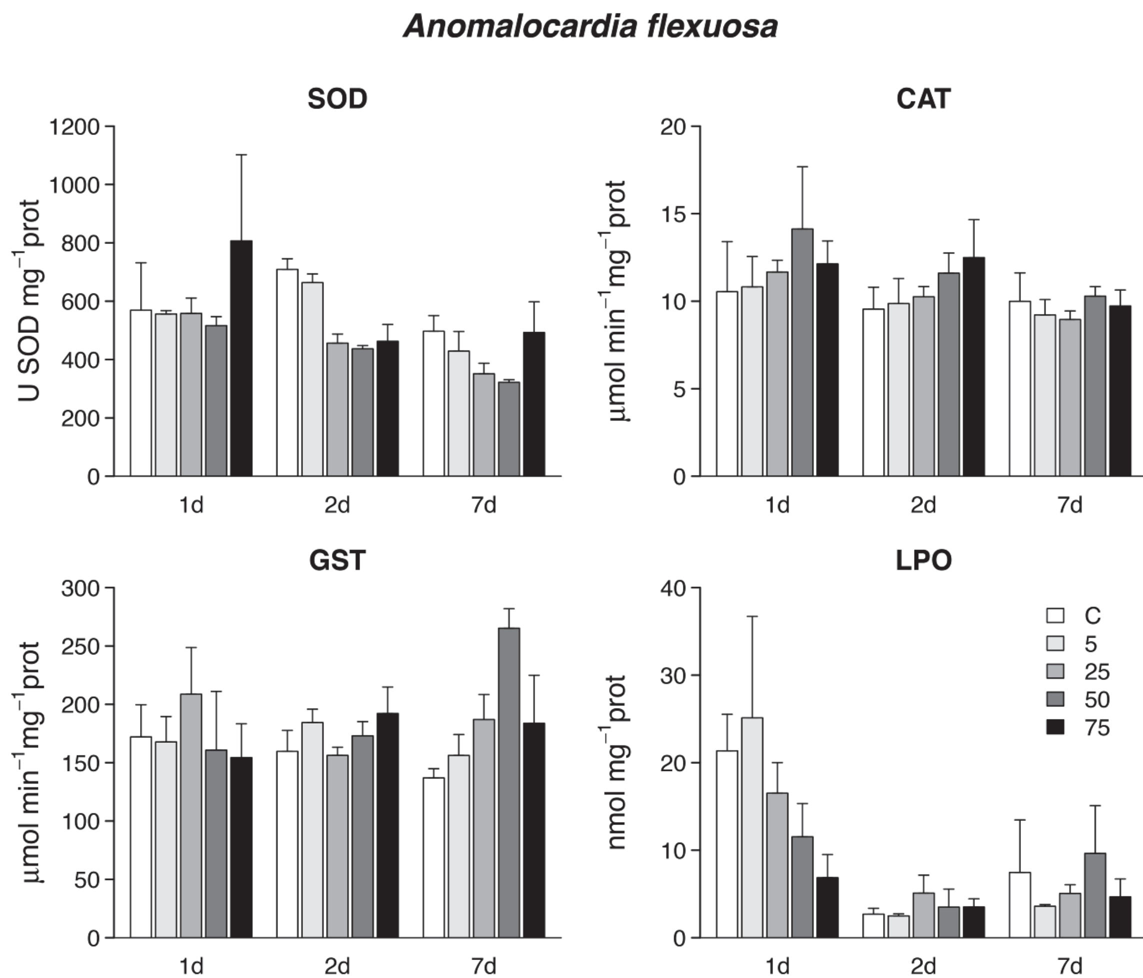

Figure 5: Activity of the antioxidant enzymes in the bivalve *A. flexuosa* (mean ± SE): results of SOD and CAT activities were expressed as µmg protein1 (where µ ¼ mmol substrate hydrolyzed per min), GST activity expressed as mol of thioether formed/milligram protein/minute and LPO levels as nmol mg prot−1.

The frequency of micronuclei and binucleated and bud cells differed significantly only between concentrations. All control groups of MN, NB and BN (BN slightly significant *P*: 0.086) presented significantly lower frequencies than the 75% treatment (Figure 5, Table S4).

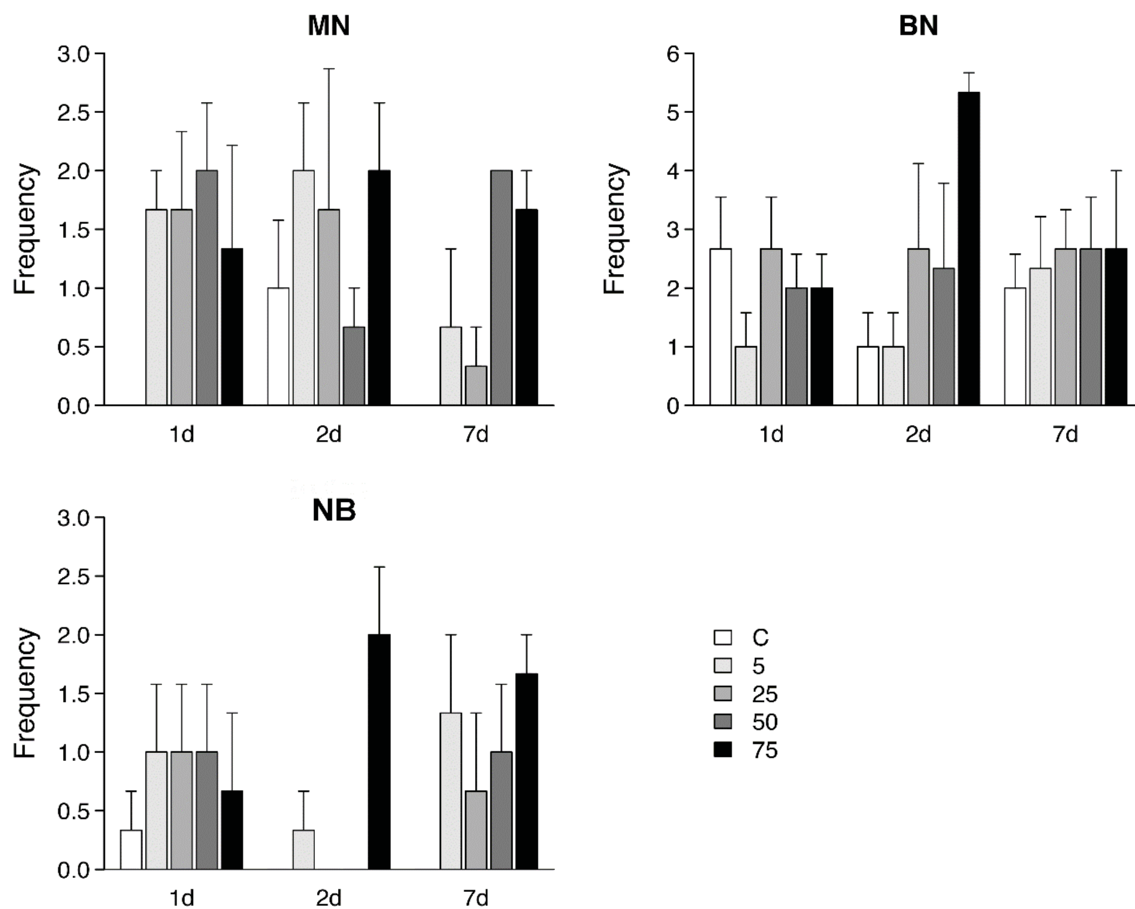

Figure 6: Frequencies of nuclear abnormalities in one thousand cells in the bivalve *A. flexuosa*, comparison of the MN, binucleated (BN) and nuclear buds (NB) frequencies in hemocytes of *A. flexuosa* in control and different treatment groups of diesel oil (means ± SE).

The results of the pre-experiment controls of the enzymatic responses are shown in Figure S2. The values obtained in the acclimation were close to those found in the control groups of 1d, except for LPO which was close to the control group in the field (Figure 4, Figure S2). As in the control groups in the MN count, no BN and NB were found, excepting a small binucleate cell count (0.009%).

4. Discussion

4.1. PAHs

The concentration of PAHs in the water indicated that the WSFD effectively contaminated the experimental treatments in accordance to their increasing concentrations, reaching levels close to those reported for polluted regions (Countway et al., 2003; Ross and Oros, 2004). In water, ΣPAH concentrations higher than 10 µm/l are

indicative of highly contaminated water (Chen et al. 2004), mainly due to industrial sources and shipyards, atmospheric deposition and urban runoff. The concentrations of PAHs in the 75% and 50% treatments for the *L. culveri* experiment and in the 75% for *A. flexuosa* exceeded 10 μm/L, suggesting that the water was heavily contaminated by PAHs. In general, diesel oil leakages display moderate concentrations of monocyclic aromatic hydrocarbons (BTEX), as benzene, toluene, ethylbenzene and xylenes, and low concentrations of PAHs of low molecular weight, as naphthalene, fluorene and phenanthrene (Neff et al., 2000; Simonato et al., 2008), although these LMW were predominant in our both experiments (tables with all the PAHs in Table S5), also found by Pacheco and Santos, 2001.

The threshold value of ΣPAH 10 μm/l was exceeded only in the treatments exposed to the highest diesel concentrations. Moreover, none of the controls were contaminated by diesel oil, as indicated by the low ΣPAH concentrations (between 0.56 to 4.45 μm/l). The composition of individual PAHs is known to indicate the petrogenic or pyrogenic sources in aquatic systems, usually by the use of several diagnostic ratios (Martins et al., 2011). Natural petrogenic hydrocarbon sources include crude oil seeps and coal and shale deposits, while anthropogenic sources include oil spills, chronic discharges and coal (Harris et al., 2011).

PAHs have low water solubility, and although contamination was detected, they were present at very low concentrations in the WSFD. For these compounds, water solubility is related to both the number of benzene rings and the angularity of their molecules, and high molecular weight compounds tend to be less soluble (Bettim et al., 2015). For example, phenanthrene, with only two aromatic rings and low molecular weight, displays the highest solubility in water, and phenanthrene was the PAH with the highest concentration and remained quantifiable throughout the sampling period for all treatments, although the concentrations decreased over time.

There was significant variation in concentrations at different exposure times; 1d displayed the highest concentrations since most of these compounds are volatile. Likewise, other authors have demonstrated a temporal reduction of PAHs compounds during exposure experiments (Akaishi et al., 2004; Fedato et al., 2010) similar to those observed in our study on 2d. Similar situations may occur in real conditions after spill accidents, which can reinforce the environmental relevance of this study.

The soluble fraction of oil can be highly toxic to marine life even in low concentrations, and generally, the toxicity is greater than that of the originally spilled

insoluble portion because of the increase in concentrations of chemical compounds that remain in the water and that are more rapidly ingested or absorbed by the biota (Beyer et al., 2016). Even when exposure levels are not high enough to cause lethality, organisms may be affected by toxic available dissolved hydrocarbons.

4.2. Biomarkers responses to diesel oil exposure

Both *L. culveri* and *A. flexuosa* were equally sensitive or responsive to the diesel oil experiment, at similar concentrations and times of exposure. However, not all biomarkers were efficient in showing stress responses. Our statistical analyses showed that, for both species, glutathione S-transferase (GST) activity and the frequency of micronuclei increased significantly at the higher oil concentrations (of 50% and/or 75%) compared the control treatments after seven days of exposure. Thus, these two biomarkers are the most efficient in showing early sub-cellular effects.

The stress responses were similar, even though the two target-species display different external morphology for protection with possible consequences to their vulnerability against xenobiotics. *L. culveri* has an epidermis directly exposed to the sediment and water column. Bivalves, on the other hand, can close their valves to protect themselves, as previously observed in ecotoxicological experiments (Cope et al., 2008; Redmond et al., 2017). Moreover, most bivalve species are recognized as more tolerant to pollution than other invertebrates (Smolders et al., 2003). Faster responses were expected in *L. culveri* because this species was more likely to absorb contaminants directly through the skin and intestine (Sun and Zhou, 2007), but the responses of both species only occurred on the last day of exposure. It is possible that the two species, which have evolved in tidal flats from subtropical estuarine intertidal flats, have developed similar antioxidant and other defense mechanisms.

Among the antioxidant responses, GST was the best biomarker for both species, with a relatively late significant activity at the last day of exposure. The induction of GST activity is prominently related to the detoxification of organic contaminants, as it represents the phase II enzyme involved in the elimination of lipophilic contaminants from the cells, such as PAHs (Vidal-Liñán et al., 2014). GST has considered the best antioxidant response among the others tested for PAHs contamination (Bhagat et al., 2016; Delunardo et al., 2015; Sandrini-Neto et al., 2016, Sardi et al., 2016), and real-life conditions (Durou et al., 2007). The GST family of enzymes is involved in the transport and the biosynthesis of endogenous compounds and act as cellular defense mechanisms

against xenobiotics and oxidative damage by catalyzing the conjugation of reduced glutathione through its cysteine thiol (Sheehan et al., 2001). GSTs are also specifically involved in the metabolic activation and deactivation of PAH metabolites (Bhagat et al., 2016). The successful response to PAH contamination of this enzyme led to two possible mechanisms of action for GST: by either promoting the conjugation with glutathione in phase II reactions, resulting in induction of enzymatic activity; or by binding to the PAH and its metabolites, reducing the concentration of toxic compounds and thus causing enzymatic inhibition (Almeida et al., 2012). GST activities were studied as potential biomarkers for pollution monitoring in other bivalves (Hoarau et al., 2002; Won et al., 2011) and polychaete species (Durou et al., 2007; Geracitano et al., 2004).

SOD and CAT activities, the latter only for bivalves' responses, were activated in the first moments of exposure to the contaminant, but with no significant differences between treatments and control. They are in general fast activated in a short period in the aftermath of exposure to contamination (Lüchmann et al., 2011). SOD acts as a primary defense against oxidative damages, eliminating hydrogen peroxide (H_2O_2) and oxygen (O_2) and thus increasing tolerance and persistence of organisms in polluted environments (Livingstone, 2001). CAT acts firstly on the protection against hydroxyl radical toxicity through the removal of H_2O_2 (Regoli et al., 2004). LPO is considered an important biomarker of cell damage resulting from the interaction of free radicals with membrane lipids (Barata et al., 2005). However, the general increases in the LPO levels over time likely were not related to the experimental diesel spill, since it presented high levels in the control groups.

These enzymes were virtually activated in oil-impacted treatments, but not sufficient to properly assess the damage caused to the test species. This inconclusive absence of significant differences between control and impacted oil treatments has already been shown for other bivalve species (Freitas et al., 2012, Zhang et al., 2010). Antioxidant defenses and repair capabilities of cells are not well-understood in polychaetes, as well as in other invertebrates (Lewis and Galloway, 2008). Other assessments of the responses of these enzymes under stress oil contamination were also inconclusive (Cheung et al., 2001; Lüchmann et al., 2011; Niyogi et al., 2001; Pichaud et al., 2008). Pollutant-induced responses are complex since they depend on the enzymes, contaminants, time of exposure and species (Van der Oost et al., 2003).

Oxidative damage assessed by an enzymatic response in association with MN frequency is often reported as an indirect induction agent in the genotoxicity mechanism

(Valavanidis et al., 2006). The impairment of oxidative defenses is associated with the accumulation of lipid peroxidation products, lysosomal membrane destabilization, DNA fragmentation and chromosomal disturbances (Winston and Di Giulio, 1991). Thus, simultaneous micronuclei and other abnormalities induction may often be a result of this oxidative stress (Sacchi et al., 2013). However, the MN frequency produced by exposure to the diesel oil did not seem modulated by the antioxidant enzymes and their inhibitors, so maybe it was not induced by oxidative stress. Conversely, the induction of MN has been attributed to the activation of lipid peroxidation and inhibition of GST and CAT activities in the fish *Mugil cephalus* (Tsangaris et al., 2011). Other studies reported the induction of MN by heavy metals, polychlorinated biphenyls (PCBs) and organochlorine pesticides (DDTs), being modulated by lipid peroxidation and antioxidant enzymes (Ünyayar et al., 2006). In general, the enzymatic activity of CAT, SOD, and levels of LPO in our experiments did not vary significantly among control groups and treatments, suggesting that they do not act directly as mutagenic agents to induce chromosomal defects.

The water-soluble fraction of diesel oil showed clastogenic effects on *L. culveri* and *A. flexuosa*, with an increase in micronuclei frequency in the highest concentrations compared to the controls. MN frequencies were also investigated in the nereid *Hediste diversicolor* exposed to PAHs, which also showed increases in the most concentrated treatment with benzo(a)pyrene B(a)P revealing DNA damage and a strong dose-dependent relationship (Catalano et al., 2012). This biomarker was also measured on *L. acuta* (also known as *L. culveri,* our test-species) from subtropical Brazilian estuaries, with higher MN frequencies in urbanized than in non-urbanized sites, mostly related to genotoxicity to metals (Weis et al., 2017). The toxicity to metals was also assessed in the polychaete *Perinereis aibuhitensis*, which displayed increased MN frequency with exposure times and concentrations of mercury (Zhang et al., 2017). Our results corroborate these previous findings, as the MN test was effective in assessing the levels of chromosomal damage in *L. culveri* exposed to experimental oil contamination.

MN frequencies and bud cells increased under high oil concentrations in *A. flexuosa*. The detection of buds and BN, which are usually formed in the bi- or multinucleated interphase cells, is also indicative of chromosomal damage caused by increasing concentrations of xenobiotics (Tsangaris et al., 2011). The abnormalities were measured in the hemolymph of the bivalves, and could, therefore, be present in hemocytes that play an essential physiological role in immune defense, phagocytosis, transport,

excretion and detoxification of xenobiotics (Cheng, 1975). Hemocytes are quickly recruited during defense and immune reactions, and may rapidly divide mitotically under chemical stress (Venier et al., 1997), evidencing an excellent resource for toxicological evaluations.

In resume, polycyclic aromatic hydrocarbons activated antioxidant responses of GST and caused DNA damage indicated by micronuclei and other chromosomal aberrations in both *A. flexuosa* and *L. culveri*. The two-target species and the two types of biomarkers may be assigned to future protocols for assessing and monitoring diesel oil impacts in subtropical estuaries. We indicate that micronuclei induction in *A. flexuosa* is a simpler, faster and cheaper test for genotoxicity. Its combination with antioxidant biomarkers could help in establishing more consistent causal relationships after oil exposure.

5. Practical implications

The usage of two different types of biomarkers in two distinct species has advantages and disadvantages for practical purposes. Stress biomarkers may detect early oxidative damage, which acts as a primary defense against toxic compounds (Livingstone, 2001). Among the four oxidative stress biomarkers used in our experiments, only GST responded to the WSDF, as an early biomarker of PAH exposition. However, enzymatic analyses tend to be more expensive than others because they require more expensive equipment and consumables. It is also time-consuming and requires precise lab routines and more expertise in interpreting results.

The frequency of micronuclei and other DNA damage also proved to be an efficient biomarker of oil exposition. Micronuclei assays are easily done and demand less expertise and shorter times for laboratory procedures. However, the time of preparation of MN slides depends on the target species. Micronuclei and other nuclear abnormalities were much easier to detect in *A. flexuosa* than in *L. culveri* since tissue maceration was not required because of the direct use of hemolymph. Because of the higher cellular integrity, cells with MN were of easier identification and counting was faster. Another advantage of using the MN test in *A. flexuosa* is that just a few individuals were sufficient to perform the test, and the hemolymph was directly extracted from the adductor muscle without sacrificing the animals.

The MN test was also satisfactory for *L. culveri* but showed more deteriorated cells due to the structure of the cytoplasmic membrane being affected. Polychaetes are

more difficult to manipulate, and many individuals are needed to get enough macerated tissue for slides preparation. However, we suggest that despite these drawbacks, measurements of MN in polychaetes can still represent a valuable tool for field impact assessments.

There are also marked differences in the experimental manipulation of both species. Bivalves are easier to sample and to manipulate during experiments for being relatively more resistant, even if they demand more attention to water exchange and for feeding. Polychaetes were easier to control since they live buried in the sediment but were of difficult handling because of their body fragility and the easy loss of the pygidium under stressful conditions.

The Resolution Issue 357 of CONAMA (2005), which regulates water quality in Brazil, still needs to be adequately validated by ecotoxicological tests both in the field and in labs. Target organisms designated for both marine and brackish environments with procedure standards are echinoderms (ABNT, 2012), amphipods (ABNT, 2016a), crustaceans (ABNT, 2011) and bivalve embryos (ABNT, 2016b). There are no species of polychaetes indicated to evaluate impacts in estuarine environments, despite their vulnerability as receptors of diffuse pollution sources. The potential interest of our work is thus greatly enhanced for practical purposes.

6. References

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Supplementary Results

TABLES

Table S1: Mixed linear models adjusted for the different enzymes activities in *Laeonereis culveri*. For each enzyme, the variance structure of the selected models is presented in the first panel. The fixed structure of the selected models is presented in the second panel, followed by the analysis of variance and post-hoc tests of least square means (C = Control; 5%, 25%, 50% and 75% refers to diesel oil concentrations; 1d, 2d, and 7d correspond to exposure days). Significant fixed structure terms used in post-hoc tests are highlighted in bold. In post-hoc comparisons, letters (A and B) indicate significant differences at the significance level of 5%; in case of interaction, the analyzed levels are separated by Ti and Co.

SOD

Structure of variance: ~ 1|Co*Ti

Ti/Co	C	5%	25%	50%	75%
1d:	3.95	6.81	1.01	9.93	1.00
2d:	0.70	33.72	29.09	56.54	4.92
7d:	7.20	3.89	2.84	16.63	2.77

Structure of fixed effects: SOD ~ Co*Ti

Font	df_{num}/df_{den}	F	*P*
Concentration	4/30	92.868	<.0001
Time	2/30	26.911	<.0001
Co*Ti	8/30	**2.111**	**0.067**

Post-hoc

Between levels of **Co*Ti**

Ti/Co	C	5%	25%	50%	75%
1d:	AB	AB	B	AB	A
2d:	A	A	A	A	A
7d:	A	A	A	A	A

GST

Structure of variance: ~ 1|Co*Ti

Ti/Co	C	5%	25%	50%	75%
1d:	0.45	0.34	0.19	0.36	1.00
2d:	1.82	0.19	0.45	3.94	0.27
7d:	1.52	0.52	1.28	0.69	1.51

Structure of fixed effects: GST ~ Co*Ti

Font	df_{num}/df_{den}	F	*P*
Concentration	4/30	9.935	<.0001
Time	2/30	65.283	<.0001
Co*Ti	8/30	**4.103**	**0.0024**

Post-hoc

Between levels of **Co*Ti**

Ti/Co	C	5%	25%	50%	75%
1d:	A	A	A	A	A
2d:	A	AB	AB	AB	B
7d:	A	A	A	B	AB

LPO

Structure of variance: ~ 1|Co*Ti

	C	5%	25%	50%	75%
1d:	2.34	4.55	2.66	2.28	1.00
2d:	3.39	0.09	0.07	1.60	0.14
7d:	2.04	1.08	3.17	3.75	3.70

Structure of fixed effects: LPO ~ Co + Ti

Font	df_{num}/df_{den}	F	*P*
Concentration	4/30	5.572	0.001
Time	2/30	**8.265**	**0.001**

Post-hoc

Between levels of **Ti**		
1d	2d	7d
B	A	B

Table S2: Generalized linear models (GLM) with Poisson distribution for the frequency of MN in *Laeonereis culveri*. The fixed structure of the selected models is presented in the first panel followed by the analysis of variance, and post-hoc tests of least square means (C = Control; 5%, 25%, 50% and 75% refers to diesel oil concentrations; 1d, 2d, and 7d correspond to exposure days). Significant fixed structure terms used in post-hoc tests are highlighted in bold. In post-hoc comparisons, letters (A, B and C) indicate significant differences at the significance level of 5%; in case of interaction, the analyzed levels are separated by Ti and Co.

MN				
Structure of fixed effects: MN ~ Co + Ti				
Font	df_{num}/df_{den}	F	*P*	
Concentration	4/30	**41.198**	**<.0001**	
Time	2/30	**32.919**	**0.01593**	
Post-hoc				
Between levels of **Co**				
C	5%	25%	50%	75%
A	AB	ABC	BC	C
Between levels of **Ti**				
1d	2d	7d		
A	AB	B		

Table S3: Mixed linear models adjusted for the different enzymes activities in the bivalve *Anomalocardia flexuosa*. For each enzyme, the variance structure of the selected models is presented in the first panel. The fixed structure of the selected models is presented in the second panel, followed by the analysis of variance and post-hoc tests of least squares mean (C = Control; 5%, 25%, 50% and 75% refers to diesel oil concentrations; 1d, 2d and 7d corresponding to exposure days). Significant fixed structure terms used in post-hoc tests are highlighted in bold. In post-hoc comparisons, letters (A, B and C) indicate significant differences at the significance level of 5%; in case of interaction, the analyzed levels are separated by Ti and Co.

SOD				
Structure of variance: ~ 1| Co				
C	5%	25%	50%	75%
0.54	0.23	0.22	0.07	1.00
Structure of fixed effects: SOD ~ Co + Ti				
Font	df_{num}/df_{den}	F	*P*	

CAT				
Structure of variance: ~ 1|Co				
C	5%	25%	50%	75%
1.30	0.89	0.37	1.40	1.00
Structure of fixed effects: CAT ~ Co + Ti				
Font	df_{num}/df_{den}	F	*P*	

Concentration	4/30	**6.835**	**<.0001**
Time	2/30	**50.515**	**<.0001**
Post-hoc			
Between levels of **Co**			

C	5%	25%	50%	75%
AB	B	AB	A	AB

Between levels of **Ti**

1d	2d	7d
A	B	C

Concentration	4/30	1.057	0.390
Time	2/30	**7.424**	**0.001**
Post-hoc			
Between levels of **Ti**			

1d	2d	7d
A	AB	B

GST

Structure of variance: ~ 1|Ti

1d	2d	7d
1.00	0.43	0.67

Structure of fixed effects: GST ~ Co*Ti

Font	df_{num}/df_{den}	F	*P*
Concentration	4/30	1.600	0.2000
Time	2/30	0.539	0.5892
Co*Ti	8/30	**2.022**	**0.0779**

Post-hoc

Between levels of **Co*Ti**

Ti/Co	C	5%	25%	50%	75%
1d:	A	A	A	A	A
2d:	A	A	A	A	A
7d:	A	A	AB	B	AB

LPO

Structure of variance: ~ 1|Co*Ti

Ti/Co	C	5%	25%	50%	75%
1d:	1.29	4.39	1.32	1.43	1.00
2d:	0.25	0.08	0.77	0.78	0.35
7d:	0.77	2.06	0.37	0.07	2.27

Structure of fixed effects: LPO ~ Co + Ti

Font	df_{num}/df_{den}	F	*P*
Concentration	4/30	3.161	0.024
Time	2/30	**16.754**	**<.0001**

Post-hoc

Between levels of **Ti**

1d	2d	7d
A	B	C

Table S4: Generalized linear models (GLM) with Poisson distribution. For each cytogenetic damage in *Anomalocardia flexuosa*, the fixed structure of the selected models is presented in the first panel followed by the analysis of variance and post-hoc tests of least square means (C = Control; 5%, 25%, 50% and 75% refers to diesel oil concentrations; 1d, 2d and 7d corresponding to exposure days). Significant fixed structure terms used in post-hoc tests are highlighted in bold. In post-hoc comparisons, letters (A and B) indicate significant differences at the significance level of 5%; in case of interaction, the analyzed levels are separated by Ti and Co.

MN

Structure of fixed effects: MN ~ Co

Binucleated

Structure of fixed effects: Binucleated ~ Co

Font	df_{num}/df_{den}	F	*P*		Font	df_{num}/df_{den}	F	*P*	
Concentration	4/30	**42.240**	**0.0316**		Concentration	4/30	**51.290**	**0.086**	
Post-hoc					Post-hoc				
Between levels of **Co**					Between levels of **Co**				
C	5%	25%	50%	75%	C	5%	25%	50%	75%
A	AB	AB	AB	B	A	A	A	A	A

Nuclear buds				
Structure of fixed effects: NB ~ Co				
Font	df_{num}/df_{den}	F	*P*	
Concentration	4/30	**44.552**	**0.0112**	
Post-hoc				
Between levels of **Co**				
C	5%	25%	50%	75%
A	AB	AB	AB	B

Table S5: Concentrations (µg/l) of 16 priority polycyclic aromatic hydrocarbons (PAHs) in the aqueous analysis of *Laeonereis culveri* and *Anomalocardia flexuosa*, related parameters in control and oil-exposed water. Values above the detection level are highlighted in bold. * It was not possible to verify the analysis.

Laeonereis culveri

	Control			**5%**			**25%**			**50%**			**75%**		
	1d	2d	7d	1d	2d	7d	1d	2d	7d	1d	2d	7d	1d	2d	7d
Naphthalene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.39**	**0.26**	**0.33**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acenaphthylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.31**	**0.43**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acenaphthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluorene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.50**	n.d.	n.d.	**6.64**	**2.20**	**1.30**	**9.40**	**3.00**	**1.30**
Phenanthrene	n.d.	n.d.	n.d.	**0.28**	n.d.	n.d.	**1.44**	**0.49**	n.d.	**21.7**	**5.20**	**1.40**	**29.8**	**8.30**	**2.20**
Anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**7.50**	**2.10**
Fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**3.00**	**1.00**	**0.90**
Benz[a]anthracene	**0.61**	n.d.	0.28	n.d.	n.d.	n.d.	**0.42**	**0.28**	n.d.	n.d.	n.d.	n.d.	**4.90**	**1.40**	**1.40**
Chrysene	**0.58**	n.d.	n.d.	n.d.	n.d.	n.d.	**0.32**	n.d.	n.d.	n.d.	n.d.	n.d.	**5.90**	n.d.	**0.60**
Benzo[b]fluoranthene	**0.86**	n.d.	n.d.	n.d.	n.d.	n.d.	**0.58**	**0.38**	n.d.	n.d.	n.d.	n.d.	**5.70**	**1.00**	**1.00**
Benzo[k]fluoranthene	**0.82**	n.d.	**0.28**	n.d.	n.d.	n.d.	**0.44**	**0.36**	n.d.	n.d.	n.d.	n.d.	**5.30**	n.d.	n.d.
Benzo[a]pyrene	**0.66**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**2.80**	n.d.	n.d.
Indeno[1,2,3-cd]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Dibenz[a,h]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[ghi]perylene	**0.92**	n.d.	n.d.	n.d.	n.d.	n.d.	**0.41**	n.d.	n.d.	n.d.	n.d.	n.d.	**4.20**	n.d.	n.d.

n.d. = not detected;

Anomalocardia flexuosa

	Control			5%			25%			50%			75%		
	1d	2d	7d	1d	2d	7d	1d	2d	7d	1d	2d	7d	1d	2d	7d
Naphthalene	n.d.	n.d.	n.d.	**0.26**	n.d.	n.d.	**0.27**	n.d.	*	**2.60**	**2.20**	**2.10**	n.d.	n.d.	n.d.
Acenaphthylene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.28**	**0.27**	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Acenaphthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluorene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	**2.14**	**1.01**	**1.30**
Phenanthrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	**0.44**	n.d.	*	**2.80**	**2.00**	**1.60**	**7.98**	**3.48**	**1.60**
Anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Fluoranthene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	**4.80**	**2.70**	n.d.
Benz[a]anthracene	n.d.	n.d.	n.d.	n.d.	**0.40**	n.d.	**0.30**	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Chrysene	n.d.	n.d.	n.d.	n.d.	**0.34**	n.d.	n.d.	n.d.	*	n.d.	n.d.	**0.45**	**4.10**	n.d.	n.d.
Benzo[b]fluoranthene	n.d.	n.d.	n.d.	n.d.	**0.53**	n.d.	n.d.	n.d.	*	n.d.	n.d.	**0.59**	n.d.	n.d.	n.d.
Benzo[k]fluoranthene	n.d.	n.d.	n.d.	n.d.	**0.47**	n.d.	**0.33**	n.d.	*	n.d.	n.d.	**0.55**	**3.00**	**1.30**	**1.34**
Benzo[a]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Indeno[1,2,3-cd]pyrene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenz[a,h]anthracene	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo[ghi]perylene	n.d.	n.d.	n.d.	n.d.	**0.50**	n.d.	n.d.	n.d.	*	n.d.	n.d.	**0.30**	**2.70**	**0.80**	n.d.

n.d. = not detected;

Supplementary Results

FIGURES

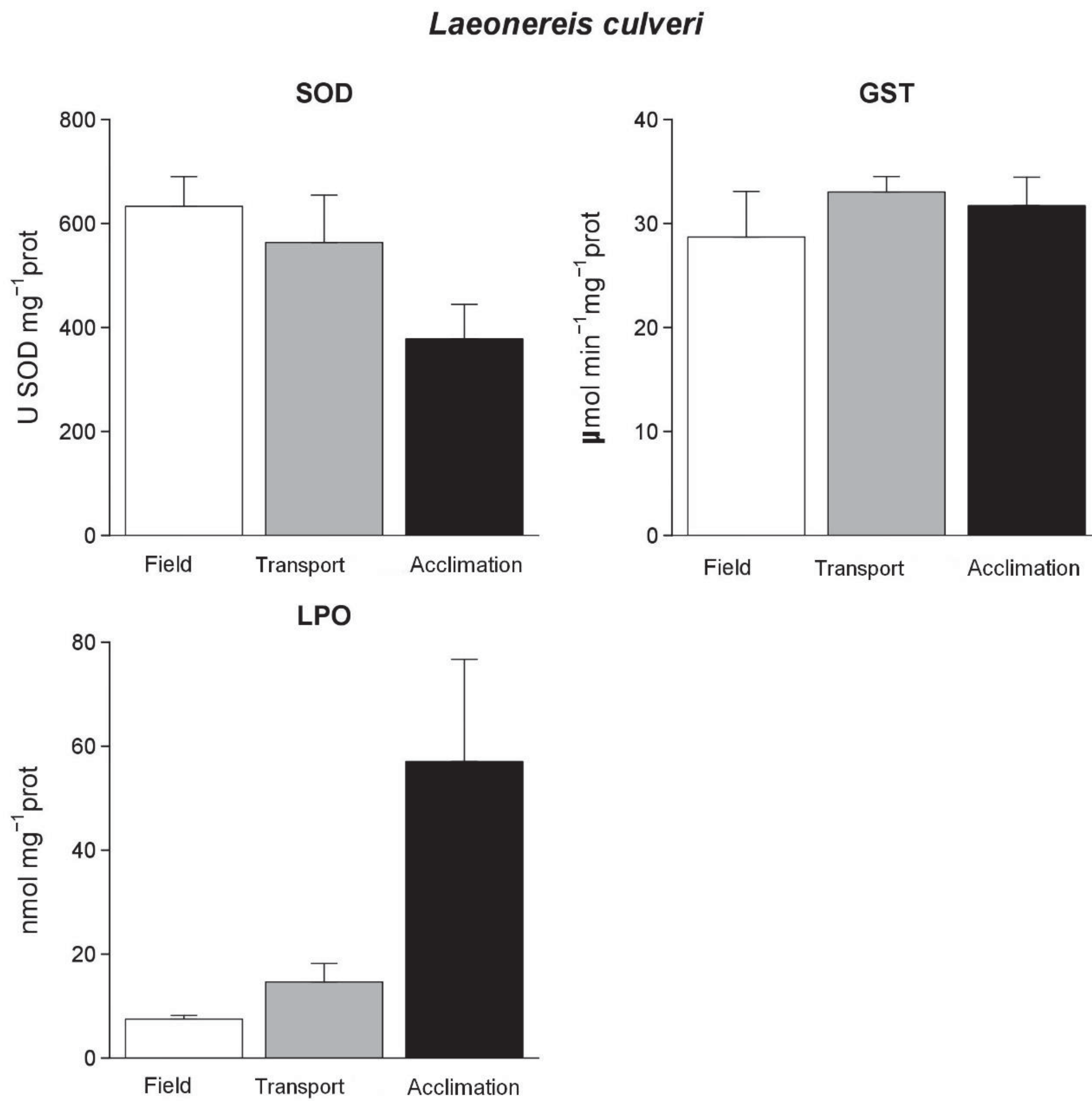

Figure S4: Mean and standard deviation (± SD) of the enzymatic activities in the polychaete *Laeonereis culveri*. The values presented are for preliminary controls: field (white box), transport (gray box) and acclimation (dark box).

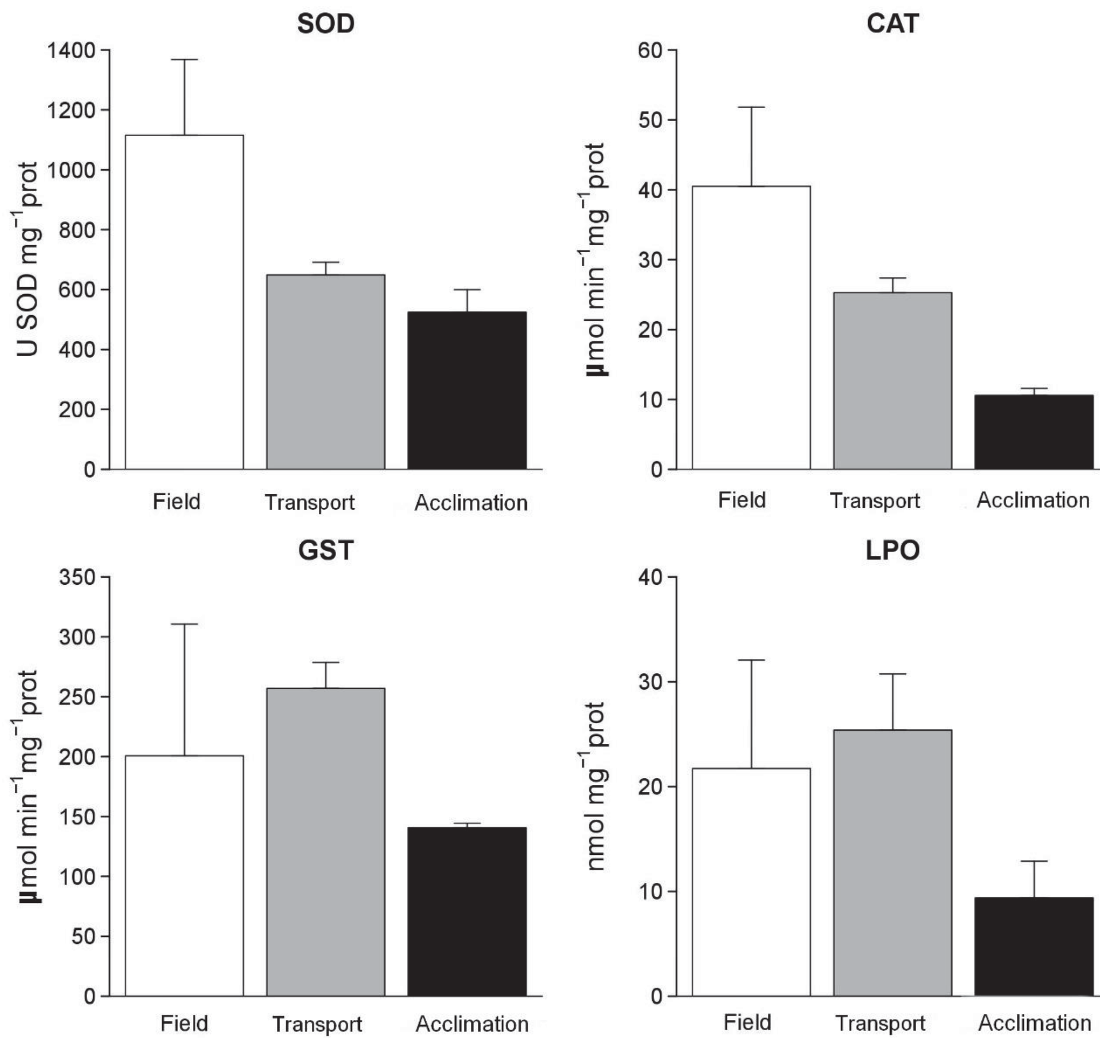

Figure S5: Mean and standard deviation (± SD) of the enzymatic activities in the bivalve *Anomalocardia flexuosa*. The values presented are for preliminary controls: field (white box), transport (gray box) and acclimation (dark box).