PAPER II

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RESEARCH ARTICLE

Oxidative stress in two tropical species after exposure to diesel oil

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Abstract

Recent offshore petroleum exploration has increased the risks of oil spills worldwide. We investigated biomarker responses to diesel oil exposure in two tropical and subtropical species, the clam *Anomalocardia flexuosa* and the polychaete *Laeonereis culveri*. Animals were exposed to oil-spiked sediment at two different concentrations (0.5 L and 1.0 L m^{-2}). Activities of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx); glutathione transferase (GST); and lipid peroxides (LPO) were assessed in bivalve digestive glands and polychaete whole-body homogenates at 36 and 60 h of exposure. Significant variation in enzymatic antioxidant activity depended on the sampling time after exposure. No similar response patterns, either increases or decreases, were detected for the two target species, and biomarker responses were species-specific. *L. culveri* showed clearer patterns in its antioxidant response and should be prioritized over other species in biomonitoring studies involving oil exposure. Understanding the temporal variability of these biomarkers is a necessary action before implementing them as indicators measures in oil contamination biomonitoring programs. Our results provide a better understanding of biomarker responses in subtropical species, evidencing their potential use as sentinels of oil contamination.

Keywords: Antioxidant enzymes, Diesel oil, Estuaries, Clams, Polychaete, Oil biomonitoring.

Introduction

Crude or light oil and their by-products can enter the marine environment through riverine discharges, shipping activities, sewage disposal, offshore production, and transport, or oil spills. The negative effects of oil on biota, including changes in abundance and composition at various levels of biological organization, are well known (Peterson et al. 2003; Sanchez et al. 2006; Serrano et al. 2006; Sandrini-Neto et al. 2016a, b).

Diesel oil is a complex mixture of aliphatic (AHs) and polycyclic aromatic hydrocarbons (PAHs), with light volatile n-alkanes and alkylated PAHs as principal components (Wang et al. 1999; Wang and Fingas 2003). Freshly spilled diesel will remain in the water or sediment interface and spread with wind action and waves. Given its low specific gravity, low viscosity, and moderately volatile characteristics, diesel oil spills will disperse and evaporate naturally within a day or less. Assessments of the effects of the non-soluble fraction of diesel oil on benthic fauna are still scarce, due to its highly volatile nature and associated methodological issues. However, diesel can persist after adhering to fine-grained suspended sediments, or by direct contact with tidal flat sediments (Gong et al. 2014). Contaminated sediments are a risk to both pelagic and benthic fauna, since contaminated sediments can yield toxic effects following a resuspension event (Edge et al. 2014).

The term biomarker describes physiological responses to environmental stressors (Goodsell et al. 2009). Biomarkers were first developed as tools to detect sublethal effects of pollution in exposed organisms. Once in the water and contact with animals, petroleum and its metabolic products induce a broad range of such biochemical responses, related to oil biotransformation and excretion (Luchmman et al. 2011). Among these, the activity of phase II enzymes like glutathione sulfotransferase (GST) is characterized by the addition of an endogenous polar compound to either a hydrophobic xenobiotics compound or processed products from phase-I biotransformation reactions (Ribalta et al. 2015). During biotransformation, the production of reactive oxygen species (ROS) is enhanced, ultimately promoting cellular damage through protein oxidation, DNA damage, and lipid peroxidation (LPO) (Livingstone 2001). LPO is considered a primary mechanism by which oxyradicals can cause injury, impairing cellular function and resulting in the failure of normal cell function (Livingstone 2001). Therefore, to cope with increased ROS formation, enzymatic and non-enzymatic antioxidant defenses are activated. Among the enzymatic antioxidants, superoxide dismutase (SOD) dismutases the superoxide anion radical (O_2) into hydrogen peroxide (H_2O_2), which is degraded by catalase (CAT) and glutathione peroxidase (GPx) (Luchmman et al. 2011).

Altogether, these mechanisms are expected to maintain the redox homeostasis, which is essential for the physiological health of organisms (Livingstone 2003; Valavanidis et al. 2006). Such biochemical responses after a contamination event have been long used as indicators of pollution.

Polychaetes and bivalve mollusks from temperate regions have been widely used as sentinel organisms for estuarine and marine contaminants monitoring (Viarengo and Canesi 1991; Reish and Gelinger 1997; Rittschof and McClellan-Green 2005; Kimbrough et al. 2008). However, information on the biochemical responses of tropical and subtropical species remain scarce (Monserrat et al. 2007; Egres et al. 2012; Marques et al. 2014; Sandrini-Neto et al. 2016b).

In this study, we assess the antioxidant responses of the polychaete *Laeonereis culveri* (also known as *Laeonereis acuta*) and the bivalve *Anomalocardia flexuosa* (also known as *Anomalocardia brasiliana*) in diesel oil-spiked aquaria under laboratory conditions. Antioxidant responses were expressed by the activity of the enzymes CAT, GPx, SOD, and GST and levels of lipid peroxidation. We hypothesize that biomarker responses in *L. culveri* and *A. flexuosa* will significantly differ between control and oil-exposed treatments and will depend on the concentration of oil and time of exposure. The two selected species are numerically dominant benthic species in impacted and non-impacted estuarine areas all along the Brazilian coast. *L. culveri* is a detritus-feeding infaunal polychaete and, as such, potentially more vulnerable to the diesel oil fraction adsorbed to the sediment. As a filter feeder, the also infaunal *A. flexuosa*, a commercially important food source (Silva-Cavalcanti and Costa 2011), may provide a better indication of water-soluble toxic compounds, following desorption of diesel from the sediment. *L. culveri* shows antioxidant stress when exposed to copper (Geracitano et al. 2004a, b), arsenic (Lima et al. 2007), hydrogen peroxide (da Rosa et al. 2008), and nanomaterials (Marques et al. 2013). Less information is available concerning the antioxidant and biomarker responses of *A. flexuosa* when exposed to pollutants, as PAHs (Martins et al. 2005; Sandrini-Neto et al. 2016b). With this experimental protocol, we aim to assess the efficacy of biomarker measures as monitoring tools for diesel oil contamination in benthic habitats from subtropical estuaries, such as the Paranaguá Estuarine System (PES).

Materials and methods

Sampling and bioassay conditions

The subtropical Bay of Paranaguá, located in southern Brazil, is a large and multihabitat estuarine system. The bay sustains artisanal fisheries, urban and touristic activities, industries, fuel terminals, and host the main South American grain shipping port (Combi et al. 2013). Although sewage discharge represent the main source of contamination in the bay (Souza et al. 2013), the Paranaguá harbor hosts the Transportation Terminal of Paranaguá (TEPAR), which operates refining, storing, and transporting of oil and its derivatives (Egres et al. 2012). Field sampling was done in austral summer 2013. Only adult specimens were collected. *A. flexuosa* individuals ranging from 20 to 30 mm length were collected at Papagaio Island, Bay of Paranaguá, Brazil (25° 32′ 55″ S; 48° 26′ 03″ W). *L. culveri* specimens, ranging between 30 and 50 mm (500 μm), were collected from the saltmarsh Saco do Limoeiro at Mel Island (25° 33′ 36″ S; 48° 18′ 52″ W). Both locations are considered as non-contaminated by petroleum or oil by-products (de Abreu- Mota et al. 2014). Sediment samples were also taken from both collection sites, and it is predominantly composed of fine and very fine sand with low organic matter content (1.2–4.7 %) (Sandrini-Neto 2015).

Individuals of *A. flexuosa* were directly handpicked from the sediment. *L. culveri* specimens were obtained after sieving sediment samples through a 0.5-μm sieve. After collection, animals were transported to the lab on cold seawater (4 °C, 30 ‰) and kept in acclimation aquaria. Lab temperature and photoperiod were fixed at 20 °C and 12 light-12 dark regime, respectively. Salinity, pH, and water temperature were recorded daily during 96 h of acclimation. Average and standard deviation values during acclimation for salinity, temperature, and pH for *A. flexuosa* acclimation aquaria were 31.6 ± 1.5, 21.4 ± 0.9, and 7.76 ± 0.25, respectively. Similarly, average and standard deviation values for *L. culveri* salinity were 31.3 ± 4.6, temperature 21.6 ± 1, and pH 7.98 ± 0.05. Water was changed every day. Animals were not fed during acclimation neither during the experiment.

A two-factor experimental design was conducted to assess biomarker responses to diesel oil, with oil concentration and time of exposure as fixed factors. Diesel oil was purchased from a local fuel station, where it is commercialized under the name of marine diesel oil (MDO). MDO results from a mix of heavy oil fractions obtained by atmospheric distillation together with fractions from secondary crude oil processing. MDO is used by

small and medium vessels as also within the auxiliary engines of large ships (Leite et al. 2014).

Each treatment combination included three replicated aquaria, making a total of 36 aquaria. For *L. culveri*, glass beakers of 600 mL capacity were employed, and each aquaria consisted of 200 g of sediment (around 5 cm of sediment deep) and 400 mL of filtered seawater. Up to seven polychaetes were added at each aquaria (making about 1 g wet weight of *L. culveri*). For the clam *A. flexuosa*, 1 L capacity glass beakers included 400 g of sediment (around 5–7 cm deep), 600 mL of filtered seawater, and 5– 6 bivalves. The average weight and length of each of the clams employed was around 8 g (including the shell) and 25 mm. Diesel oil concentration in aquaria (treatments C1 and C2) was equivalent to 0.5 and 1 L m^{-2} oil spills, respectively. Concentrations were chosen based on a pilot experiment that evaluated mortality on both species exposed to diesel oil under laboratory conditions. Before mounting the aquaria, sediments were diesel oil-spiked by spilling diesel over the sediment surface and mixing it briefly. With this procedure, we intended to simulate putative real-world conditions where the non-soluble fraction of diesel oil originated from chronic vessels leakages is adhered to suspended matter and settle into the sediment. Water and animals were added to each beaker. A control treatment, using non-exposed sediment from the sampling site, was simultaneously done. All control beakers were covered to avoid cross-contamination. In each aquarium, bubbling was included for aeration and water was only changed after 24 h. At 36 and 60 h, whole *L. culveri* individuals or pieces of digestive glands from three individuals of *A. flexuosa* were dissected, and tissue samples freeze in liquid nitrogen. Samples remained stored at −80 °C until further analysis. The time length for each sampling was deliberately short, to simulate short-term biomarker responses after a hypothetical oil spill. A sediment sample was collected for each experimental treatment to determine the concentration of PAHs. The top 2 cm of surface sediment was collected with a spoon placed in pre-cleaned aluminum foil and stored at −20 °C. Only one replicate of sediment per treatment was collected. The material was freeze-dried, carefully homogenized with a mortar, and stored in clean glass bottles at room temperature prior to PAHs analysis.

Polycyclic aromatic hydrocarbons analysis in sediment samples

The analytical procedures for PAH analysis were based on the United Nations Environment Program method (UNEP 1992) with minor modifications described in Bícego et al. (2006). Ten to 15 g of freeze-dried sediment was extracted using 80 mL of a (1:1) dichloromethane (DCM) and n-hexane mixture over 8 h. The surrogates acenaphthene-d_{10},

phenanthrene- d_{10}, chrysene-d_{12}, and perylene-d_{12} were added before each blank or sample extraction. The organic extract was purified by column chromatography using 5 % deactivated alumina and silica. The elution was performed with 10 mL of n-hexane (fraction 1—aliphatic hydrocarbons, not analyzed) and 15 mL of a (3:7) DCM/n-hexane mixture (fraction 2— PAHs). A 1-mL aliquot of each concentrated extract from fraction 2 was injected in an Agilent GC (Model 7890A) coupled to a mass spectrometer (Agilent 5973N inert MSD with Triple-Axis Detector). Detailed instrumental analyses are described in Dauner et al. (2016). PAHs were identified by matching the retention times and ion mass fragments for a standard mixture (NIST 2260) at concentrations from 0.10 to 1.50 µg L^{-1}. Procedural blanks analyzed with each series of 11 extractions showed that no peaks interfered with the analyses of the target compounds. The measured target PAH concentrations in the reference material provided by the International Atomic Energy Agency were in agreement with the certified values, within 85–110 %.

Laboratory procedures

For each replicate, a pool of two to three *L. culveri* individuals or pieces of digestive glands from three individuals of *A. flexuosa* (~100 mg) were homogenized. Regarding *L. culveri*, Ferreira-Cravo et al. (2007 and 2009) have described differences in the antioxidant activity regarding to the polychaete body section in a similar species. Based in our objective, we decided to not include body sections as another source of variation within the experimental design, which as a fixed and orthogonal factor would make harder the interpretation of the effects of diesel oil concentration and length of exposure. Homogenization buffer consisted of cold 0.1 mol L^{-1} phosphate-buffered saline solution (PBS) at pH 7 (1:10 w/v). Samples were then centrifuged at 15,000 g for 30 min at 4 °C in a Heraeus Biofuge fresco microcentrifuge. The supernatant of each sample was collected and stored at −80 °C until further analysis. Total protein content was measured at 595 nm following Bradford's method (Bradford, 1976) with bovine serum albumin as standard.

Catalase activity was measured following the decrease in absorbance at 240 nm due to H_2O_2 consumption (Aebi 1984). Samples were analyzed by quadruplet for 1 min at intervals of 15 s in a multimode microplate reader Biotek Synergy HT. We use UV-star microplates (Greiner Bio REF. 655801). A linearity curve of absorbance vs. time was constructed to obtain the best range of kinetics. Final concentrations in a volume of 300 µL were 1 mol L^{-1} Tris/5 mmol L^{-1} EDTA buffer (pH 8) and 20 mmol L^{-1} H_2O_2. Molar extinction coefficient used was 40 M^{-1} cm^{-1}.

Superoxide dismutase activity was determined following the inhibition of the autoxidation of pyrogallol as described by Gao et al. (1998). Room temperature was 23 °C and SOD activity was obtained by comparing pyrogallol autoxidation with control, which contained 1 mol L^{-1} HCl as an oxidation inhibitor. The reaction medium contained 40 µL of sample diluted 1:10 in 0.1 mol L^{-1} PBS buffer pH 7, 885 µL of buffer 1 mol L^{-1} Tris acid buffer containing 5 mmol L^{-1} EDTA pH 8, and 50 µL of 15 mmol L^{-1} pyrogallol (NEON, REF.: 00,311). Twenty-five microliters of 1 mol L^{-1} HCl were added to test tubes after 30 min of incubation in the dark while, for control tubes, hydrochloric acid was added following the addition of pyrogallol. Then, 300 µL of this reaction medium were pipetted by triplicate in a 96-well microplate, and absorbance was read at 440 nm in a TECAN Sunrise absorbance microplate reader.

Glutathione peroxidase activity was assayed following the rate of NADPH oxidation at 340 nm by the coupled reaction with glutathione reductase (GR). In this case, the velocity of the oxidation of NADPH is proportional to the velocity of GSSH production from GSH in the presence of H_2O_2 catalyzed by GPx (Hafeman et al. 1974). Final concentrations in a final volume of 130 µL were 0.1 mol L^{-1} PBS buffer pH 7,2 mmol L^{-1} sodium azide, 0.2 mmol L^{-1} NADPH, 0.2 mmol L^{-1} GSH, 1 U mL^{-1} glutathione reductase, and 1.5 mmol L^{-1} H_2O_2. Samples were analyzed in triplicate during 3 min at intervals of 10 s in a TECAN Sunrise absorbance microplate reader. An extinction coefficient for $NADP^+$ of 6.22 mM^{-1} cm^{-1} was used.

Glutathione S-transferase (GST) activity was measured following increases in absorbance at 340 nm due to conjugation of this enzyme with chloro-2,4-dinitrobenzene (CDNB) as a substrate (Keen et al. 1976). Final concentrations in a volume of 200 µL were 3 mmol L^{-1} GSH, 3 mmol L^{-1} CDNB, and 1.1 mol L^{-1} potassium phosphate buffer, pH 6.5. Samples were analyzed for 3 min at intervals of 15 s in a TECAN Sunrise absorbance microplate reader. The extinction coefficient of GS-DNB conjugate was 9.6 mM^{-1} cm^{-1}.

Lipid peroxidation (LPO) was measured as described by Jiang et al. (1991) using the ferric/xylenol orange reaction. Sample aliquots of 100 µL were diluted (1:2) in methanol and centrifuged at 5000 g for 5 min at 4 °C. The resulting supernatant (100 µL) was transferred to clean tubes and mixed with 900 µL of a reaction solution containing 0.1 mmol L^{-1} xylenol orange, 25 mmol L^{-1} sulfuric acid, 2.5 mmol L^{-1} ammonium ferrous sulfate, and 4 mmol L^{-1} BHT and incubated for 30 min at room temperature. Then, 300-µL aliquots were placed in a 96-well microplate by triplicate and absorbance was measured at 560 nm in a TECAN Sunrise absorbance microplate reader. For the hydroperoxide quantification, an extinction coefficient of 4.3×10^{-4} mol^{-1} cm^{-1} was used. 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.

Data processing

Enzyme activity for each treatment combination was standardized regarding the activity recorded for their corresponding control and presented as the mean percentage change in activity from control. A similar standardization was done by Limaye et al. (2003) and Persichetti et al. (2014), being a conventional practice in medicine tests that want to identify departures from normal or basal status. Before such standardization, the variation of the control treatment between sampling times was also tested. For each enzyme, significant differences among treatments were tested by permutational analysis of variance. The significance level adopted was 5 % ($\alpha = 0.05$). For those terms that were found to be significant at $P < 0.05$, means were compared using pairwise *t* test using the Bonferroni correction. Analyses and figures were performed within the R environment (R Development Core Team, 2009) using *vegan* (Oksanen et al. 2013) and *ggplot2* (Wickham 2009) packages. Raw unstandardized enzyme activities expressed as mean ± standard deviation are available in supplementary material (Table S1).

Results

Polycyclic aromatic hydrocarbons

Total concentration of PAHs and related parameters are summarized in Table 1. Higher ΣPAHs concentrations, including the 16 USEPA PAHs, alkyl naphthalenes and phenanthrenes, were detected in impacted sediments (4738–5034 ng g^{-1}; mean = 4886 ± 208.8) than in control sediments (1325– 1514 ng g^{-1}; mean = 1420 ± 134). ΣPAHs levels were compared to the effect concentrations (TEL) (MacDonald et al., 1996), and the effects range low (ERL) (Long et al., 1995) thresholds. Both thresholds are specified by the National Oceanic and Atmospheric Administration under the National Status & Trends Program (NS&T) sediment quality guideline as indicators of biological effects. Reported mean values from impacted sediments have higher total PAHs values than TEL (1684 ng g^{-1}) and ERL (4022 ng g^{-1}) thresholds.

The ratio between low molecular weight (LMW, 2 and 3 rings) and high molecular weight PAHs (4–6 rings) was high in impacted sediments (19.4–47, mean = 33.2 ± 15.9), indicating diesel oil contamination. The petrogenic source of PAHs for the impacted

sediments was confirmed by the evaluation of the following isomer pair ratios (Yunker et al., 2002): (a) anthracene/anthracene + phenanthrene (An/178) and (b) fluoranthene/ fluoranthene + pyrene (Fl/Fl + Py). For interpretation, the former ratios indicate dominance of petroleum source when (a) An/178 < 0.10 and (b) Fl/Fl + Py < 0.40. The mean values of these ratios in impacted samples were ± 0.01 and 0.19 ± 0.05, respectively. The PAH isomer pair ratios values in control sediments were higher, mainly to the Fl/Fl + Py (0.30 ± 0.10) and $C_0/C_0 + C_1$ P (0.51 ± 0.05), confirming the changes in PAH distribution between control and impacted sediments.

Table 1. Concentrations of polycyclic aromatic hydrocarbons (PAHs), and related parameters from control and oil-exposed sediments. ΣPAHs, total polycyclic aromatic hydrocarbons (ng g^{-1} dry weight); 2–3 rings, total PAHs with two to three aromatic rings (ng g^{-1} dry weight); 4–6 rings, total PAHs with four to six aromatic rings (ng g^{-1} dry weight).

	Control		Low dosage spill (0.5 L m^{-2})		High dosage spill (1 L m^{-2})	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**
Σ PAHs	1514.45	1324.83	4950.12	5029.74	5033.5	4738.24
16 EPA PAHs	398.60	371.65	1136.81	862.25	903.78	663.97
Alkyl-PAHs	1497.01	1306.58	3793.30	4155.93	4113.22	4043.17
2-3 rings with alkylated	1115.85	952.66	914.70	769.10	723.97	495.25
2-3 rings without alkylated	381.16	353.92	4708.00	4925.03	4837.19	4538.42
4-6 rings	17.44	18.25	242.12	104.71	196.31	199.82

Biomarker responses in the clam A. flexuosa

The relative percentage change in enzymes activities in the species *A. flexuosa* is summarized in Fig. 1. No significant variation was detected between times in the control treatment. None of the enzyme biomarkers assessed was sensitive enough to distinguish the concentration treatment, and significant differences were observed between times of exposure only (Table 2). CAT activity was increased after 36 h of exposure (T1) followed by a decrease 24 h later (T2). This response was significant and consistent for both concentration treatments (Table 2). A similar pattern was observed for GST (Fig. 1). In addition, the interaction between time and concentration was also significant (Table 2) with lower GST activity in individuals from C1 and higher activity in C2 (Fig. 1).

The activities of SOD and GPx (Fig. 1) were not significantly different neither between concentration and time of exposure treatment. However, SOD activity was higher in all impacted treatments compared to control (from ~6 to 27 %). On the contrary, GPx activity decreased in exposed treatments from control's activity (from −7 to −13 %).

Changes in lipid peroxides levels in *A. flexuosa* were in all cases not significant (Table 2, Fig. 3). At 36 h, LPOs levels decreased in both concentrations. However, this pattern inversed after 60 h.

Biomarker responses in the polychaete L. culveri

Enzymes activity was not significantly different between concentration treatments, and differences in the relative change of antioxidant enzymes in the polychaete *L. culveri* were mostly explained by the factor time of exposure (Table 2). SOD and CAT activities at 36 and 60 h of exposure were clearly decreased by diesel oil treatments (Fig. 2). On the other hand, GPx activity at 36 h after spiking sediments was similar to the control, which contrasted with results at 60 h, when there was a significant reduction (Table 2, Fig. 2). The activity of GST significantly increased with time (Table 2, Fig. 2).

Lipid peroxides levels in *L. culveri* consistently increased from T1 to T2. There was an initial reduction of ~30 % from control observed at T1 that after 24 h went up to 50 % relative to the control, suggesting oxidative deterioration of lipids (Fig. 3).

Figure 1. Percentage changes from control in activity of SOD, CAT, GPx and GST enzymes in *Anomalocardia flexuosa*, 36 h (T1) and 60 h (T2) after spiking sediment with diesel oil. Diesel oil concentration treatments were equivalent to 0.5 L m^{-2} (C1) and 1 L m^{-2} (C2) spills, respectively. Plots represent the mean in percentage change (n=3) plus standard errors. A posteriori comparisons for time after exposure treatment are indicated with asterisks whereas letters indicate significant differences among combination of treatments ($P < 0.05$).

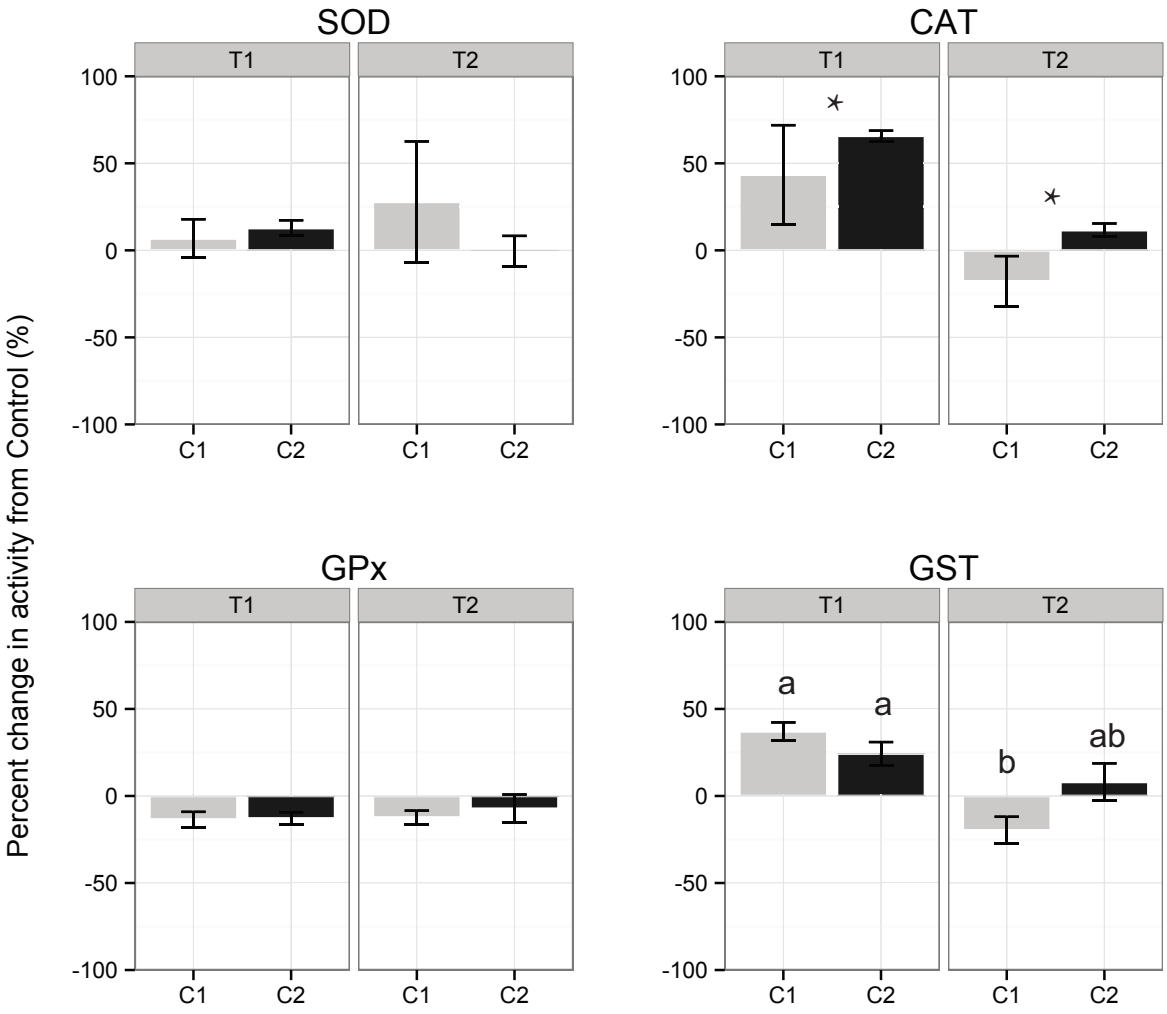


Table 2. Permutational ANOVA for mean percentage change in activity from control of enzymatic activities and lipid peroxide levels after exposure to diesel oil. Abbreviations stand for df: degree of freedom; MS: mean squares; *F:* F-ratio; *P:* probability of *F*.

		(i) *Anomalocardia flexuosa*			(ii) *Laeonereis culveri*		
	df	MS	*F*	*P*	MS	*F*	*P*
(a) SOD							
Concentration	1	370.79	0.35	0.72	619.23	2.26	0.17
Time	1	45.78	0.04	0.94	104.66	0.38	0.55
C x T	1	879.29	0.82	0.49	298.71	1.09	0.32
Residual	8	1072.34			258.81		
(b) CAT							
Concentration	1	2002.60	2.56	0.16	98.886	0.38207	0.56
Time	1	9994.70	12.77	**<0.05**	2.932	0.01133	0.92
C x T	1	36.80	0.05	0.80	196.322	0.75855	0.41
Residual	8	782.70			273.92		
(c) GPx							
Concentration	1	23.91	0.28	0.65	46.58	1.3058	0.26
Time	1	33.80	0.40	0.58	912.48	25.5813	**<0.01**
C x T	1	14.48	0.17	0.73	30.33	0.8504	0.37
Residual	8	85.04			35.67		
(d) GST							
Concentration	1	168.70	0.91	0.37	400.13	1.3358	0.27
Time	1	4010	21.72	<0.01	2147.59	7.1697	**<0.05**
C x T	1	1229.60	6.66	**<0.05**	9.57	0.0319	0.88
Residual	8	184.70			299.54		
(e) LPO							
Concentration	1	220.33	0.13	0.79	80	0.07	0.79
Time	1	1676.35	0.98	0.39	31899	27.59	**<0.01**
C x T	1	21.37	0.01	0.94	116	0.10	0.79
Residual	8	1715.59			1156		

Figure 2. Percentage changes from control in activity of SOD, CAT, GPx and GST enzymes in *Laeonereis culveri*, 36 h (T1) and 60 h (T2) after spiking sediment with diesel oil. Diesel oil concentration treatments were equivalent to 0.5 L m^{-2} (C1) and 1 L m^{-2} (C2) spills, respectively. Plots represent the mean in percentage change (n=3) plus standard errors. Asterisks indicate significant differences among times of exposure groups ($p < 0.05$).

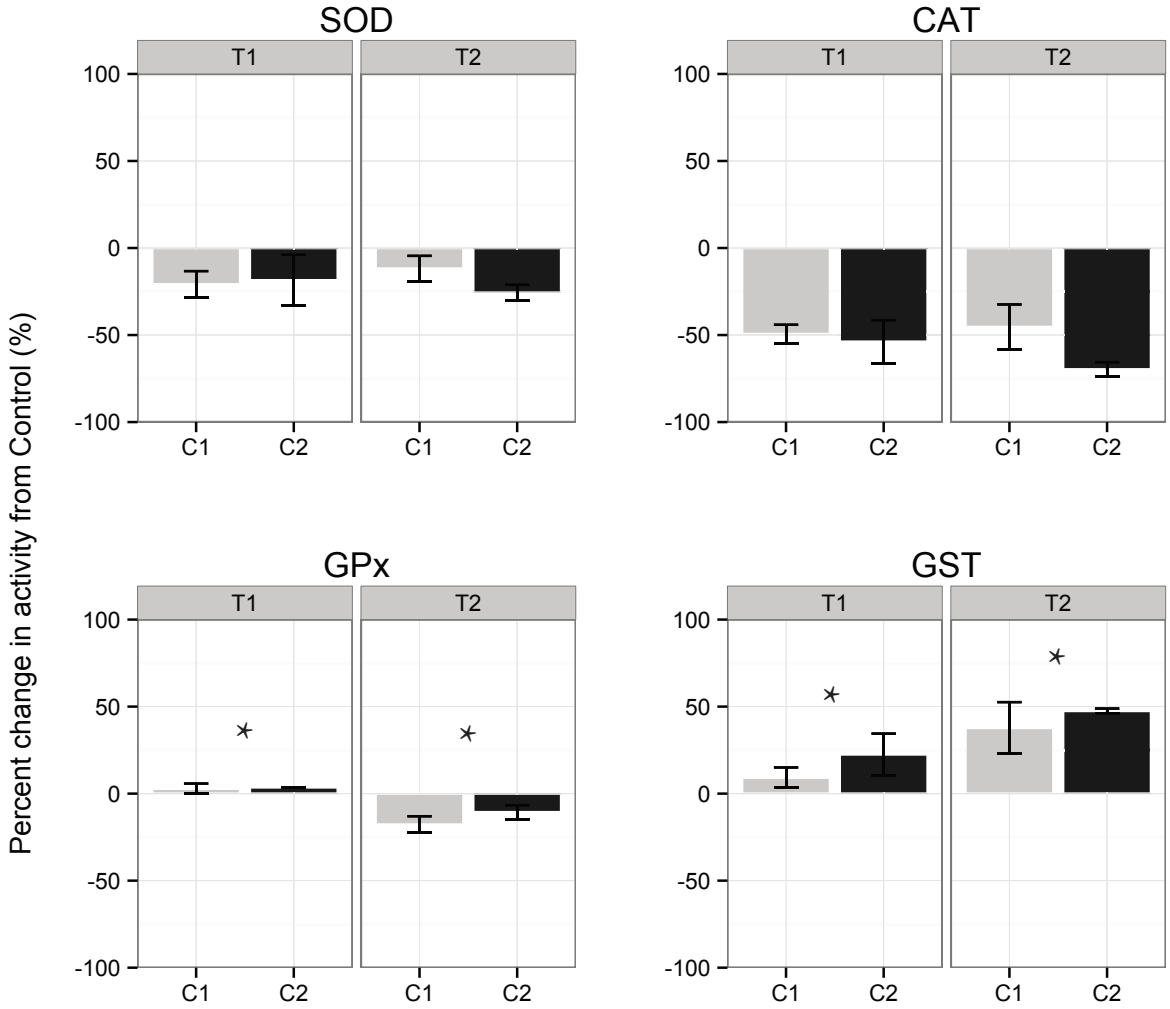
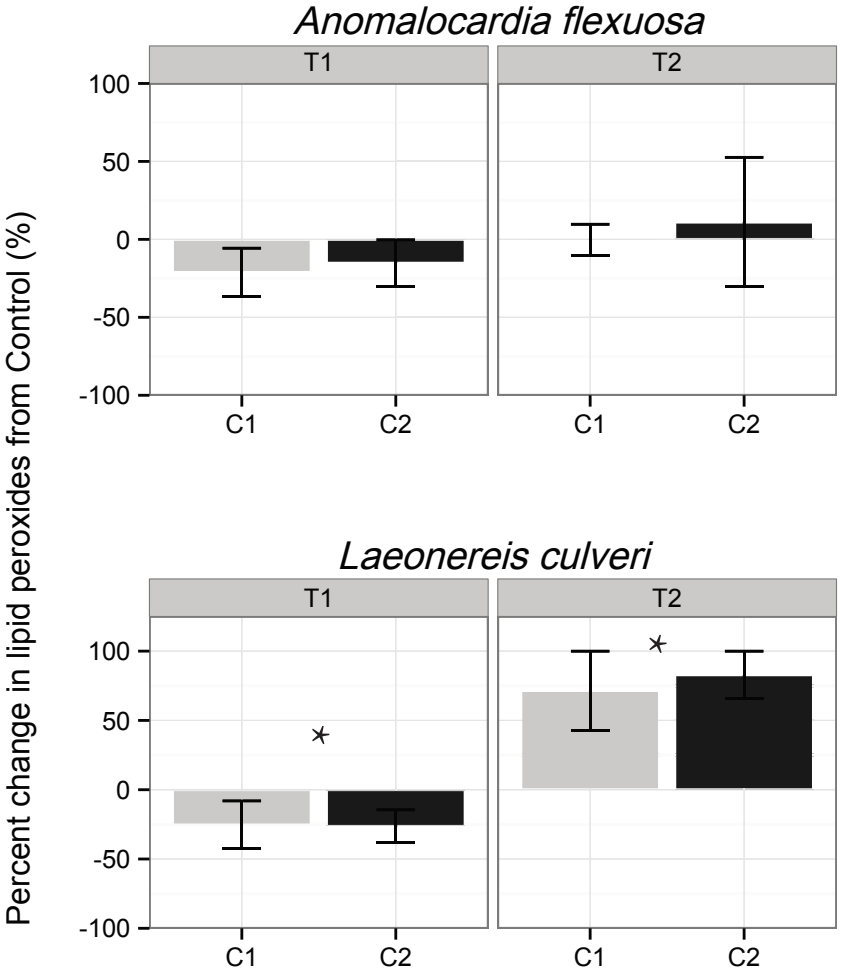


Figure 3. Percentage changes from control in lipid peroxides from *Anomalocardia flexuosa* and *Laeonereis culveri* 36 h (T1) and 60 h (T2) after spiking sediment with diesel oil. Diesel oil concentration treatments were equivalent to 0.5 L m^{-2} (C1) and 1 L m^{-2} (C2) spills, respectively. Plots represent the mean in percentage change (n =3) plus standard errors. Asterisks indicate significant differences among times of exposure groups ($p < 0.05$).



Discussion

The hypothesis that the activity of antioxidant enzymes in *A. flexuosa* and *L. culveri* exposed to diesel oil would significantly differ from the control treatment was not refuted. Most of the measured antioxidant enzymes varied significantly after exposure to diesel oil.

Results were presented as percentage changes from control, which can be interpreted as increase or decrease of the activity of the measured enzymes following a particular event of diesel oil exposure.

Under the assumption of biomarkers being fast and early signs of pollution, the most significant difference from control levels was expected to occur within 36 h of exposure (T1) at the highest concentration (C2). Nonetheless, PAHs levels did not differ significantly between treatments, indicating that nominal concentration does not represent a real exposure scenario, and justifying the little variability in enzyme activities explained by the concentration treatment. The difference between nominal and recorded PAHs concentration in sediments may be potentially related to (i) sediment samples being only collected at the end of the experiment when the more volatile fraction of diesel could have already evaporated, and (ii) turbulence created from bubbling the aquaria, which could have enhanced the evaporation rate by altering the air boundary layer (Fingas 2013). However, the fast evaporation of volatiles is the common scenario in the field, where tidal regimes, UV exposition, waves, elevated temperature, and winds facilitate the evaporation of the volatile fraction of diesel oil. Previous studies on PAHs concentration in sediments from the same control area have reported contradictory results regarding the PAHs concentration found in sediments. In this context, Sandrini-Neto (2015) reported total PAHs 3.72 ng g^{-1} dry weight, while Gilbert (2011) reported total PAHs of 1410 ng g^{-1} dry weight. For both studies, sediment samples were collected directly in the field. Such variance may indicate patched contamination in field sediments and justify the high levels of PAHs encountered in control sediments. Another possible explanation for the differences between nominal and sediment concentration of PAHs would be the rate PAHs are internalized and accumulated by the animals. Unfortunately, we did not conduct bioaccumulation analysis in animal tissues, which could have provided a hint in the results. Given the disagreement between nominal and actual PAHs concentration in the treatments, the biggest the change from control was expected to occur shortly after exposure (T1) regardless of the concentration treatment, with a putative recovery to normal control levels for the second sampling time.

The rationale for using relative change from control values was to determine if these enzymatic measures could be employed as early signs and tools for monitoring diesel oil contamination in the selected species, taking the behavior of control samples as the unexposed departure status. Most measured endpoints varied significantly between sampling times (36 vs. 60 h after spiking sediments), even after standardizing treatment results with their respective control. This result indicates that the time scale at which biomarkers show significant variation is as short as 24 h and further highlights the need for reducing the sampling intervals after oil spills for impact assessment purposes. Moreover, the velocity in which the enzymatic response adjusts to the environmental conditions is species-specific, as observed for CAT values for *A. flexuosa* that showed a fast recovery, not evident for *L. culveri*.

The overall pattern of the enzymatic response is also species dependent, indicating that none of the measured enzymes can be expected to respond in the same way (increasing or decreasing) for both species. In general, there was a reduction in the antioxidant capacity of *L. culveri*, whereas diesel exposure induced the activity of *A. flexuosa* enzymes. Acute sensitivity assessments showed that different taxonomic group and functional or feeding groups respond differently to contaminants (Brix et al. 2001). However, comparisons of biomarker response in various species belonging to different feeding guilds are still scarce. Brown et al. (2004) conducted a laboratory exposure to copper using the omnivorous shore crab *Carcinus maenas*, the grazer limpet *Patella vulgata*, and the filter-feeder mussel *Mytilus edulis*. Each species presented a different concentration threshold at which sublethal biomarker responses were significantly different from control. The authors attributed such variation in sensitivity to differences in the exposure route to copper, also dependent on feeding mechanism of each species. Moreover, the mussel *M. edulis*, a common sentinel species, was found as the less sensitive of the tested species. The authors related the past result with a behavioral trait; *M. edulis* individuals avoided toxic exposure by remaining closed for most of the exposure (Brown et al. 2004).

Different bioavailability of pollutants for both species may also explain the species-specific biomarker responses. Bioavailability depends on partitioning properties of diesel oil between the sediment, pore water, and overlying water (Di Toro et al. 1991; Gong et al. 2014). Clams are mostly exposed to the water-soluble fraction from diesel oil. Polychaetes as infaunal and detritivorous organisms are exposed to the fraction of diesel attached to the particulate matter. *A. flexuosa* as an intertidal species has a natural defense

against the exposure, being able to close their valves for extended periods of time, which allows them to avoid, to a certain extent, the toxic effects of diesel oil.

The conducted bioassay did not result in individual or mass mortality, indicating that both species can stand short-term exposures to diesel oil. Nevertheless, the mean total PAHs concentration in the impacted samples exceeded the TEL and ERL, suggesting potential toxicological effects of chronic exposure. Under acute exposure, sublethal endpoints such as antioxidant enzymatic activities are considered as relevant indicators of PAHs pollution in marine organisms (Orbea et al. 2002; De Luca-Abbott et al. 2005; Sureda et al. 2011; Bebianno et al. 2015). These non-specific biomarkers have frequently been used for environmental monitoring assessments sometimes with results that are difficult to interpret (Vlahogianni et al., 2007; Valavanidis et al. 2006; Brooks et al. 2011; Nahrgang et al. 2013). The induction of antioxidant enzymes is suggested to be a response of exposed organisms, but the same antioxidants can be reduced if pollution levels are extreme (Regoli et al. 2011). Therefore, either increases or decreases in the enzymatic activity of antioxidant biomarkers can result from oxidative stress, with duration and intensity of the pro-oxidant stressor driving such opposite responses (Regoli et al. 2011).

An *in situ* exposure to diesel oil at concentrations comparable to the ones used in the present study was conducted in Paranaguá Bay using *L. culveri* and *A. flexuosa* as study species (Sandrini-Neto et al. 2016b). The comparison was made using exclusively the results from treatment 4d 250 mL 0.25 m^{-2} (Sandrini-Neto et al. 2016b), which correspond to the same exposure concentration from C2 treatment. Our results for *L. culveri* were at least 3 times higher (approximate values for treatment 4d 250 mL 0.25 m^{-2}: 250 U SOD mg $prot^{-1}$) for SOD activity, 2 times greater for GST and GPx activities (~25 µmol min^{-1} mg $prot^{-1}$ and ~140 µmol min^{-1} mg $prot^{-1}$, respectively) and LPO levels up to 15 times higher (~1 nmol mg prot−1). On the other hand, CAT activity was similar to field and laboratory exposure (~13 – 15 µmol min^{-1} mg $prot^{-1}$) (Sandrini-Neto et al. 2016b).

Antioxidant biomarkers studies in the clam *A. flexuosa* are rare. Sandrini-Neto et al. (2016b) also exposed this species under field conditions and reported lower values for all the measured endpoints. SOD, CAT, and GPx activities under lab conditions were approximately 4, 3, and 1.3 times higher than the values reported by Sandrini-Neto (SOD 175 U SOD mg $prot^{-1}$, CAT 24 µmol min^{-1} mg $prot^{-1}$, and GPx 180 µmol min^{-1} mg $prot^{-1}$). GST activity was slightly higher (~160 µmol min^{-1} mg $prot^{-1}$) and LPO levels from our experiment were at least 16 times greater than those reported by Sandrini-Neto's work. Summarizing, in all

cases, except for CAT in *L. culveri*, the activity of enzymes was higher during lab versus field exposure. Such discrepancy between results from field and laboratory studies is expected given the many mechanics and physical forces that favor the dilution and dispersion of diesel under field conditions; ultimately interfering with the exposure.

L. culveri is tolerant to low oxygen levels, and often a numerically dominant species in organically enriched locations (Souza et al. 2013). High levels of basal antioxidant enzymes, such as that observed in CAT (see supplementary material), could be an adaptive response to this highly variable environment as also a response to the constant oxygenation employed during the bioassay. Nonetheless, additional exposure to pollutants may in the long term overwhelm their redox homeostasis, as indicated by the 50 % reduction in its activity for both concentration treatments and times of exposure.

There was an evident induction in GST activity in *L. culveri* from T1 to T2 and a progressive increase regarding the concentration treatment. This increase indicates the elimination of xenobiotics and ROS by-products and corroborates the potential use of GST as a marker of PAH exposure. Similarly, GPx activity and LPO levels were significantly different between T1 and T2. There was an inverse relationship between GPx activity and the concentration of lipid hydroperoxides, suggesting that GPx is reducing lipid hydroperoxides to alcohol, with the concomitant oxidation of GSH to GSSG (Regoli et al. 2011).

The constant levels of lipid peroxides in *A. flexuosa* indicated that the overall antioxidant response was not overwhelmed and that there was no oxidative lipid deterioration. Moreover, the induction of antioxidant defenses in response to increases in organic contamination could reverse lipid peroxidation, converting lipid hydroperoxides (LOOH) back to lipids and alcohol by the activities of GPx and GST enzymes (Regoli et al. 2011).

Conclusions

Our experimental design allowed for the assessment of the combined effects of diesel concentration and time after spiking diesel oil in experimental sediments. However, for most of the endpoints measured, the interaction of diesel concentration and time of exposure was not significant. Observed responses were primarily dependent on exposure time rather than to diesel concentration. This result is likely related to the small differences in measured PAHs between experimental replicates. CAT and GST enzymes activities responded towards diesel exposure in both species; therefore, we recommend its use for future biomonitoring

involving the two target species. Our results also shed light into biomarkers temporal variation within an experimental laboratory exposure. However, it is still necessary to test biomarker responsiveness and temporal variability under field conditions.

Our study is a first step to characterize two potential sentinel species and to validate the use of oxidative stress parameters as tools for monitoring oil impacts in tropical and subtropical regions. Both *L. culveri* and *A. flexuosa* are suitable for impact assessment and monitoring. *L. culveri* should be prioritized since it showed clearer patterns in its antioxidant response.

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Supplementary material

Table S1. Mean enzyme activity and standard deviation values for the clam species *Anomalocardia flexuosa* and the nereid polychaete *Laeonereis culvari* obtained after spiking experimental sediments with diesel oil at two different concentrations equivalent to 0.5 L m^{-2} (C1) and 1 L m^{-2}. T1, T2 and T2 correspond to 36 h 60 h and 84 h post spiking sediments.

Species	Concentration treatment	Time	SOD (U mg protein^{-1})			CAT (µmol min^{-1} mg protein^{-1})			GPx (µmol min^{-1} mg protein^{-1})			GST (µmol min^{-1} mg protein^{-1})			LPO (nmol mg protein^{-1})		
Anomalocardia flexuosa	CONTROL	T1	657.54	±	15.90	55.85	±	16.70	278.11	±	8.30	147.45	±	15.10	18.11	±	3.01
		T2	747.89	±	18.43	62.80	±	10.81	239.59	±	6.67	159.70	±	33.84	19.11	±	7.21
	C1	T1	702.01	±	126.43	80.12	±	27.53	240.56	±	21.04	202.13	±	13.22	14.28	±	4.87
		T2	955.72	±	450.57	51.64	±	15.75	210.02	±	16.26	128.21	±	21.50	19.08	±	3.27
	C2	T1	741.48	±	51.32	92.59	±	2.89	242.30	±	16.97	183.34	±	17.00	15.35	±	4.68
		T2	744.53	±	113.24	70.06	±	4.20	222.05	±	33.85	172.52	±	29.62	21.22	±	13.75
Laeonereis culveri	CONTROL	T1	889.69	±	203.05	32.25	±	12.56	260.36	±	18.38	28.06	±	1.15	32.21	±	6.98
		T2	1228.11	±	360.97	44.78	±	23.30	320.75	±	61.97	32.54	±	11.36	7.84	±	0.50
	C1	T1	703.85	±	117.65	16.31	±	2.99	268.30	±	13.33	30.63	±	4.01	24.03	±	9.62
		T2	1083.08	±	155.02	24.47	±	10.03	263.73	±	26.55	44.82	±	8.37	16.47	±	7.15
	C2	T1	724.75	±	225.25	14.89	±	6.90	289.87	±	34.98	34.38	±	5.81	23.70	±	6.63
		T2	913.22	±	95.18	13.56	±	3.17	286.56	±	22.27	47.99	±	0.78	18.37	±	5.81