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Antioxidant responses in estuarine invertebrates exposed to repeated oil spills: Effects of frequency and dosage in a field manipulative experiment

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We have experimentally investigated the effects of repeated diesel spills on the bivalve *Anomalocardia brasiliana*, the gastropod *Neritina virginea* and the polychaete *Laeonereis culveri*, by monitoring the responses of oxidative stress biomarkers in a subtropical estuary. Three frequencies of exposure events were compared against two dosages of oil in a factorial experiment with asymmetrical controls. Hypotheses were tested to distinguish between (i) the overall effect of oil spills, (ii) the effect of diesel dosage via different exposure regimes, and (iii) the effect of time since last spill. Antioxidant defense responses and oxidative damage in the bivalve *A. brasiliana* and the polychaete *L. culveri* were overall significantly affected by frequent oil spills compared to undisturbed controls. The main effects of diesel spills on both species were the induction of SOD and GST activities, a significant increase in LPO levels and a decrease in GSH concentration. *N. virginea* was particularly tolerant to oil exposure, with the exception of a significant GSH depletion. Overall, enzymatic activities and oxidative damage in *A. brasiliana* and *L. culveri* were induced by frequent low-dosage spills compared to infrequent high-dosage spills, although the opposite pattern was observed for *N. virginea* antioxidant responses. Antioxidant responses in *A. brasiliana* and *L. culveri* were not affected by timing of exposure events. However, our results revealed that *N. virginea* might have a delayed response to acute high-dosage exposure. Experimental in situ simulations of oil exposure events with varying frequencies and intensities provide a useful tool for detecting and quantifying environmental impacts. In general, antioxidant biomarkers were induced by frequent low-dosage exposures compared to infrequent high-dosage ones. The bivalve *A. brasiliana* and the polychaete *L. culveri* are more suitable sentinels due to their greater responsiveness to oil and also to their wider geographical distribution.

1. Introduction

The production of reactive oxygen species (ROS) occurs naturally during cellular aerobic respiration processes (Vidal-Liñán and Bellas, 2013), but can also be highly affected by environmental factors, such as salinity and temperature (Lushchak, 2011), or exposure to contaminants (Monserrat et al., 2007; Lüchmann et al., 2011; Marques et al., 2014). Increased ROS levels may induce lipid, protein and DNA oxidation, leading to several deleterious effects at cellular level (Monserrat et al., 2007; Vidal-Liñán and Bellas, 2013). Cells are protected against the deleterious effects of oxyradical generation by maintaining ROS at low levels through several antioxidant defenses, which include both enzymatic and non-enzymatic antioxidants (Kaloyianni et al., 2009 Lüchmann et al., 2011; Turja et al., 2013; Zanette et al., 2015).

Changes in antioxidant defenses can be used as indicators of contaminant exposure. The antioxidant system involves enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Among the non-enzymatic defenses, glutathione (GSH) participates in many important biological processes including protection against toxic compounds (Lüchmann et al., 2011). Moreover, enzymes involved in the elimination of ROS byproducts, such as glutathione S-transferase (GST), play an important

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role as indirect antioxidant (Boutet et al., 2004; Lüchmann et al., 2011; Zanette et al., 2015). Eventually, deficiency in the antioxidant system of cells can increase the lipid peroxide levels (LPO), a major mechanism by which oxyradicals can damage the cellular membrane lipids (Turja et al., 2013; Zanette et al., 2015).

Polycyclic aromatic hydrocarbons (PAHs) are a common source of contamination in the aquatic environment, mostly as a result of petroleum-related activities (Lüchmann et al., 2014). PAHs are primarily associated with anthropogenic sources, particularly fossil fuels and their derivatives. The process of partial combustion, accidental oils spills and the disposal of domestic and industrial effluents are the major sources of PAHs to coastal systems (Martins et al., 2011; Abreu-Mota et al, 2014). PAHs may affect aquatic organisms in many ways and the oxidative stress is one of the key elements of their toxicity (Lushchak, 2011). PAHs are primarily metabolized via hydroxylation (phase-I reactions) and detoxified by enzymes in the cytochrome P450 system (Lushchak, 2011; Lüchmann et al., 2014).

Many studies have reported changes in oxidative stress biomarkers as a response to PAHs exposure in marine invertebrates, particularly in bivalves (Turja et al., 2013; Marques et al., 2014; Lüchmann et al., 2014; Turja et al., 2014; Vidal-Liñán et al., 2014; Won et al., 2013), but also in polychaetes (Nesto et al., 2010 Ramos-Gómez et al., 2011; Won et al., 2013). Filter-feeding mollusks are often used as sentinels in pollution monitoring due to their significant ability to bioaccumulate pollutants as well as to respond to their presence (Solé et al., 2009; Lüchmann et al., 2011). Polychaete worms are also good sentinels because they can adapt to stressful environmental conditions, are distributed worldwide and present a sedentary lifestyle (Solé et al., 2009; Díaz-Jaramillo et al., 2011). However, few studies have investigated the effects of PAHs on oxidative stress biomarkers in other marine invertebrates, such as crabs (Martín-Díaz et al., 2008; Morales-Caselles et al., 2008; Ricciardi et al., 2010) and gastropod mollusks (Reid and MacFarlane, 2003; Sarkar et al., 2006; Tim-Tim et al., 2009).

Experiments evaluating biomarker responses have often been done under laboratory conditions (Silva et al., 2005; Lüchmann et al., 2011; Luna-Acosta et al., 2011) to isolate the putative effects of PAH exposure from other factors. Such experiments, however, do not include the full set of naturally occurring abiotic and biotic variables (Goodsell et al., 2009), which can affect the persistence of contaminants and, ultimately, the response of selected biomarkers. Thus, results from laboratory studies should be compared to robust field experiments in order to generate ecologically relevant information (Reid and MacFarlane, 2003; Nesto et al., 2010; Díaz-Jaramillo et al., 2013; Marques et al., 2014). Field experiments can be conducted whether by transplanting organisms to polluted areas (e.g. Díaz-Jaramillo et al., 2013; Turja et al., 2014) or by experimentally adding contaminants to natural sites (e.g. Marques et al., 2014).

Particularly in coastal and estuarine habitats, the intense traffic of small and mid size ships, together with fishing and recreational boats are often responsible for the release of petroleum products at a range of frequencies and intensities. Most of these vessels use marine diesel oil as fuel, which is less persistent than crude oil although it is highly toxic (Lytle and Peckarsky, 2001). Nonetheless, biomarker responses to PAH exposure in marine invertebrates are often evaluated from acute, non-cumulative, single-dosage oil spills. Impact assessments are commonly carried out after accidents through descriptive approaches (Tim-Tim et al., 2009; Morales-Caselles et al., 2008; Sureda et al., 2011), but also by the use of field manipulative experiments (Marques et al., 2014). Consequently, little is known of how repeated oil spills at varying frequencies and intensities can affect biomarkers responses, especially in the field.

In this study, we examined the effects of the frequency and intensity of experimental diesel spills on enzyme activities (SOD, CAT, GST and GPx), levels of reduced glutathione (GSH) and lipid peroxidation (LPO) in three macrofaunal species: the bivalve *Anomalocardia brasiliana*, the gastropod *Neritina virginea* and the polychaete *Laeonereis culveri* (formerly identified as *Laeonereis acuta*). These species were chosen because they are adapted to stressful environmental conditions, relatively sessile, widely distributed and occupy different trophic levels. *A. brasiliana* is a filter feeder that feeds mostly on plankton; *N. virginea* is a grazer that feeds mainly on epiphytic algae, and *L. culveri* is a deposit feeder that forages within the sediment column.

By comparing the effects of three frequencies of exposure events against two dosages of oil in a factorial experiment with asymmetrical controls, we tested the following hypotheses: 1) if selected biomarkers are affected by repeated oil spill events, then biomarker responses in organisms exposed to frequent spills will be significantly different from those in the control treatment; 2) if different exposure regimes are determinant causes of variability, then biomarker responses in organisms exposed to frequent low-dosage spills will be significantly different from those exposed to infrequent high-dosage spills; 3) if the time elapsed since the last oil spill is determinant, then biomarker responses in organisms exposed to the same dosage of oil under the same frequency, but for which the timing of exposure differed, will vary significantly.

2. Materials and methods

2.1. Study area

Experimental oil spills were conducted on an intertidal flat at Papagaios Island in the polyhaline Cotinga sub-estuary (Fig. 1), a 20-km channel located in Paranaguá Bay (southern Brazil). Local tidal flats are mainly composed by moderately to well-sorted very fine sands (Souza et al., 2013) and are often covered with seaweeds such as *Ulva* and *Enteromorpha* (Ulvaceae, Chlorophyta) or diatom biofilms. The tidal regime is mainly semidiurnal, with diurnal inequalities, and may reach up to 1.7 m in the sub-estuary during spring tides (Lana et al., 2001; Marone et al., 2005).

The Cotinga sub-estuary receives a considerable amount of domestic effluents from the city of Paranaguá, where the municipal sewage is still released *in natura* (Leite et al., 2014). The waste of nearly 50% of the city's population of about 150,000 undergoes treatment, while the rest is directly discarded without any treatment (Souza et al., 2013). Other potential impact sources include the presence of an oil terminal, a grain port and tourism, which may contribute to a progressive increase in the disposal of domestic and industrial sewage, petroleum hydrocarbons, heavy metals and organic pollutants such as polychlorinated biphenyls (Barboza et al., 2013; Abreu-Mota et al., 2014).

Despite the existence of many man-induced impacts in the Cotinga sub-estuary, local sediments are not considered contaminated by oil (Abreu-Mota et al., 2014), although a gradient of fecal contamination from the vicinity of Paranaguá port towards the open sea is detected along the channel (Barboza et al., 2013).

2.2. Sampling of selected species

Selected species of macrofauna were collected at an intertidal flat located on the southwest margin of Mel Island (Fig. 1), at the entrance to Paranaguá Bay (25°33′25.5″S, 48°18′42.4″W). This area is approximately 12 km away from the Papagaios Island, where the experimental exposures took place, and it is also considered not contaminated by petroleum hydrocarbons (Martins et al., 2009). Sediments are predominantly composed of very fine sand with low silt–clay content; see Sandrini-Neto and Lana (2014) for details. This area was selected because large high-density patches of the