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Are Mussels Always the Best Bioindicators? Comparative Study on Biochemical Responses of Three Marine Invertebrate Species to Chronic Port Pollution

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Abstract Bivalves have traditionally been considered good bioindicators due to their sensitivity to pollution, among other features. This characteristic is shared by several other non-bivalve species as well, though studies in this respect remain scarce. This work aims to compare biomarker sensitivity to chronic port pollution among three intertidal invertebrate species with good bioindicator characteristics. Mussels' immunological (phenoloxidase and peroxidases) and biotransformation (glutathione-S-transferase) responses were contrasted against those of limpets and barnacles. The three species under study evidenced activity of all the enzymes measured, although with differences. Barnacle *Balanus glandula* was the most sensitive species showing pollution modulation of the three enzymes, which suggests that mussels would not always be the best bioindicator species among marine invertebrates depending on the responses that are assessed.

Keywords Bioindicators · Marine invertebrates · Port pollution · Biochemical biomarker

In general, biochemical biomarkers are considered useful tools to monitor pollution, and are studied as signs of early warning. However, due to their relatively recent application in ecotoxicological studies, their validation as such a tool remains to be demonstrated, and several investigations are being conducted to improve their applications. Most of them focus on the effects of abiotic factors on biomarkers responses which act as confounding factors (Vidal et al. 2002; Múgica et al. 2015). On the other hand, the variability in the response of biomarkers to contaminants in different indicator organisms has not been established yet (Cotou et al. 2013). Additionally, several potential bioindicator species have never been studied from a biochemical perspective in response to pollution.

Bivalves are the sentinel species of choice because they present some features which render them good for such purpose. Among said features are: wide distribution, ease to sample, high abundance, low mobility, and ecological and economic importance. The sensitivity of these mollusks to pollutants, together with their capacity to accumulate them, has also been established (e.g., Luna-Acosta et al. 2015). On the other hand, there are several invertebrate species which also present these characteristics; however, comparative biochemical studies on their sensitivity to pollutants are scant. In this way, most comparative studies have been developed among different bivalve species (Wootton et al. 2003; Cotou et al. 2013). In order to fill this gap, in the present study, the biochemical biomarker responses to port pollution of three marine intertidal invertebrate species, including a mussel, were compared. The activity of two immunological (phenolxidase: PO and peroxidases: Pe) and one phase II-biotransformation enzymes (glutathione-S-transferase: GST) were considered as biochemical responses. The species selected were: the mussel *Brachidontes rodriguezii*, the limpet *Siphonaria lessoni* and the barnacle *Balanus glandula*. They all belong to different major taxa and thus may have different biological, physiological and ecological characteristics. Despite this, they all have many characteristics of good bioindicators such as widely distributed, sessile, abundant, easy to sample and they are available all year round. PCB

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accumulation has been established in *B. rodriguezii* and *S. lessoni* (Laitano et al. 2016) whilst Cohen (2005) reported metal bioaccumulation in *Balanus glandula* from British Columbia, Canada. Moreover, these species are dominant in the rocky marine intertidal of the Southwest Atlantic, though representatives of their genus and/or their family can be found in such environments all around the world.

Materials and Methods

The specimens were collected at two sites of the South Atlantic coast: Mar del Plata port (MDPP; 38°02′S, 57°31′W) and Punta Cantera (PC; 38°04′S, 57°30′W). MDPP is a relatively small port (1400,000 m^2), although it holds about 60 % of the Argentinian fishing fleet and has one of the most important shipyard facility in Argentina. This port can be considered chronic contaminated; many studies report high levels of different contaminants (such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), tributyl tin, etc.) in its sediments over many years (Goldberg et al. 2004; Cledón et al. 2006; Bigatti et al. 2009; Waisbaum et al. 2010; Albano et al. 2013; Laitano et al. 2015). On the other hand, PC, located 3.5 km south from the port, is a quartzite outcrop emerging on a beach that can be considered free of such pollution. Moreover, its usefulness as a reference site when assessing port pollution effects has been demonstrated in a previous work (Nuñez et al. 2012).

At each site, ten replicates of water samples were taken to measure pH, temperature and salinity in situ through a pHmeter Adwa AD12, and a Bio-Marine Aquafauna refractometer, respectively. During low tide, specimens of *B. rodriguezii* (mussels), *S. lessoni* (limpets) and *B. glandula* (barnacles) (n = 30 of each species), were collected by hand using a spatula to remove the organisms. Then, they were carried to laboratory in cool conditions, immediately dissected and pooled (five individuals per pool). Whole soft tissues were homogenized with distilled water on ice and then samples were centrifuged 30 min at 10,000 rpm and 4°C and the supernatant (protein extract) was carefully removed and stored at −20°C (PO and Pe) or −80°C (GST). Soluble protein was measured by the method described by Bradford (1976), using serum bovine albumin (Sigma, A9647) as the standard.

Phenoloxidase activity measurement was adapted from Palmer et al. (2011). Briefly, 25 µL of each protein extract, 20 µL of distilled water (dH_2O) and 40 µL of phosphate buffer (100 mM, pH 6) were added to a 96-well microtiter plate and incubated 20 min at room temperature. Then, 30 µL of L-DOPA (3 mg ml^{-1}) (Aldrich, 333786) as substrate were added and after incubation (40 min dark condition), absorbance at 490 nm was recorded (Biotek EPOCH spectrophotometer). Control wells were prepared with 45 µL of dH_2O, 40 µL of phosphate buffer and 30 µL of L-DOPA.

Peroxidases activity was determined using 20 µL of protein extract, 64 µL of phosphate buffer (100 mM, pH 6), 420 µL of dH_2O, 64 µL of pyrogallol (Sigma P0381) as substrate and 32 µL of hydrogen peroxide 1.6 volumes to activate the assay, according to Lamela et al. (2005). The reaction mixture was placed in a micro quartz cell of 0.7 ml and after 20 s, absorbance was registered at 420 nm (Shimadzu UV-2102 PC, UV–visible Scanning Spectrophotometer). Two control trails were done for these enzymes: one containing 20 µL of extract and 580 µL phosphate buffer (100 mM, pH 6) and other containing all the reagents but without the protein extract.

Glutathione-S-transferase activity was measured following Habig et al. (1974) modified to microplate lecture; 10 µL of extract, 200 µL of phosphate buffer (100 mM, pH 6.5), 10 µL of reduced glutathione (Sigma G4251) and 8 µL of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma 138630) were added to a 96-well microtiter plate. Absorbance at 340 nm was recorded every minute during 10 min. Control wells contained the reagents without the protein extract. All assays were done in triplicate. PO and Pe activities are expressed as the change of absorbance per minute per mg protein (Abs min^{-1} mg $protein^{-1}$). GST activity is expressed as units per mg protein (U mg $protein^{-1}$). One unit of enzyme is the quantity of enzyme that catalyzes the formation of 1 µmol of product per minute.

Results are presented as mean ± standard error. Enzymatic activities were compared among species (only with data of the reference site) and between sites per species, through generalized linear models (GLM). Models with enzymatic activity as depend variable and species or site as factors were developed, specifying Gaussian or Gamma family, as appropriate, according to the distribution of each data set. In order to determine the significance of such factors on the enzymatic activities, models were contrasted with a null model (without any independent variable) through the Akaike's Information Criteria. When the Akaike's number of a model with a factor (species or site) is lower than that of the null model, indicate significant differences in enzyme activity due to such factor. When significant differences among species were found, Tukey's tests were applied to the constructed model in order to make post hoc multiple comparisons and detect which species differ in the activity of each enzyme. All statistical analyses were conducted in R 2.13.0 (R Development Core Team 2011).

Results and Discussion

A comparative study of biochemical responses to port pollution was developed among three species with suitable bioindicator characteristics in order to determine whether there are differences on their sensitivity at the sub-

individual level. When studying biochemical biomarkers in invertebrate species, the activity of a diverse group of enzymes is not always found in all the investigated species. For instance, only three of the eight immune enzymes analyzed by Wootton et al. (2003) presented activity in three bivalve species studied by such authors. Notably, they found Pe activity only in *Cerastoderma edule* and *Mytilus edulis* and PO activity only in *M. edulis*. Also, Smith and Söderhäll (1991) searched for PO activity in 25 marine invertebrate species, and although most, not all the species showed activity of that enzyme. In this study, however, the three species analyzed presented activity of all measured enzymes, though showing some differences (Fig. 1). Mean soluble protein content was in general similar among species and/or locations: 1.97 and 2.1 mussels of PC and MDPP, respectively; 1.55 limpets from PC and 2.46 those from MDPP and 2.31 and 2.43 barnacles form PC and MDPP, respectively. PO mean activity varied between 0.029 and 0.044 Abs min^{-1} mg $protein^{-1}$, Pe means ranged from 0.18 to 0.59 Abs min^{-1} mg $protein^{-1}$, while GST varied between 1.12 and 12.36 U mg $protein^{-1}$. PO activity was comparable among species (Fig. 1a), whereas Pe activity was significantly lower in *B. glandula* as compared to *S. lessoni* and *B. rodriguezii* (Fig. 1b) and GST activity differed among the three species, being highest in limpets and lowest in barnacles (Fig. 1c). Such differences among species were expected since the specific variation of biomarkers has been well documented, even among species of the same family (Smith and Söderhäll 1991; Cotou et al. 2013; Fernández-Gimenez et al. 2014).

Several factors can affect the biochemical processes of organisms: abiotic environmental parameters, season, reproduction period, which are natural factors and some non-natural factors such as habitat degradation and pollution. The natural variation of biomarkers (e.g., seasonal) has been a research topic of great interest in recent years (Monserrat et al. 2007); and, in fact, the sensitivity of some biomarkers to different pH and temperatures, among other environmental and biological conditions has been demonstrated (Vidal et al. 2002; Robillard et al. 2003). Even though in this study sampling was simultaneously carried out at polluted and non-polluted sites, abiotic factors were measured to ensure the presence of the same main environmental conditions. In fact, pH, salinity and temperature were similar between sites. Salinity was 35 ‰ at both sites, water temperature was 12.04 ± 0.3°C, and 12.24 ± 0.2°C, at PC and MDPP, respectively, while mean pH was 8.11 ± 0.04 at PC and 8.06 ± 0.04 at MDPP. Other characteristics which could affect biochemical responses like chlorophyll-a, particulate organic matter and wave energy among others, can be considered very similar between the study sites due to the proximity of them and the lack of sources of variation of such conditions (Nuñez

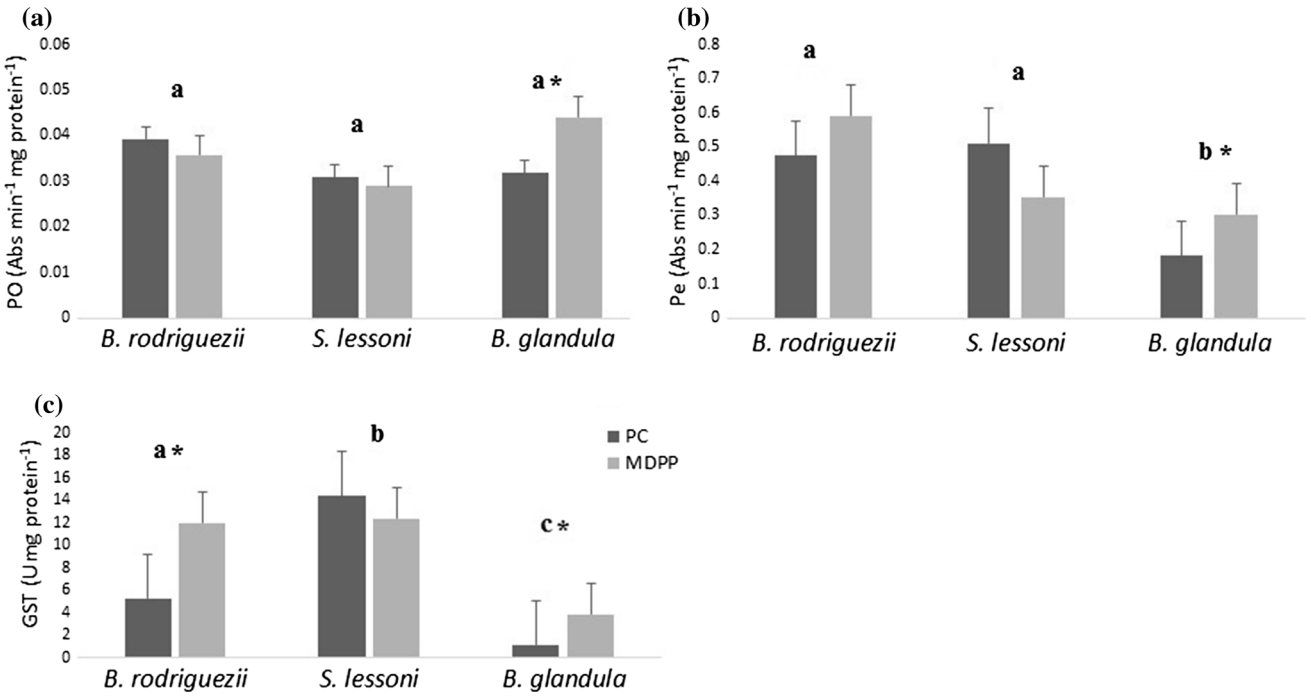

Fig. 1 Phenoloxidase (**a**), Peroxidases (**b**) and GST (**c**) mean activities with corresponding *SE bars*, of the studied species at Punta Cantera (PC; reference site) and Mar del Plata port (MDPP). *Different letters indicate* significant differences among species and *asterisk* indicates significant differences between sites in the same species

et al. 2012). On the other hand, the anthropic influences over the sampling sites are highly contrasting. While MDPP is located within a port, a highly boat transited zone in which chronic pollution is well reported, PC is located in a farther area off Mar del Plata coast where the influence of the city port does not reach (Laitano et al. 2015).

As regards the effects of MDPP chronic pollution on enzyme activities, *B. glandula* showed the greatest difference between sites (Table 1), yielding higher enzyme activity in MDPP with respect to PC in the three enzymes measured (Fig. 1a, b, c). Mussel *B. rodriguezii* only exhibited differences in GST activity, being also higher in MDPP (Table 1; Fig. 1c) whereas limpet *S. lessoni* did not show any difference between sites. Due to the complexity and the interdependence of its components, the immune system is particularly sensitive to environmental stressors, including exposure to contaminants (Pipe and Coles 1995). Both inhibition and induction of different components of the invertebrate immune system have been associated with pollution (Coles et al. 1994; Fournier et al. 2001; Jing et al. 2006). In the case of this study, the immune enzyme activities (PO and Pe) were enhanced compared to the reference site. Phenoloxidase is involved in melanin formation, which constitutes a common immune response to pathogens in invertebrates (Söderhäll and Cerenius 1998). The activation of PO from its precursor pro-phenoloxidase, can be induced as a consequence of pathogen (e.g.: bacteria) entry in the organism or even just in the presence of minute amounts of compounds of microbial origins (Cerenius and Söderhäll 2004). Further, it is widely reported that bacteria are natural decomposers of organic contaminants such as PAHs, BTs, PCBs, etc. (Jong-Rok et al. 2016); hence it is expected to find high bacterial load associated with such contaminants. In fact, in MDPP the above contaminants were historically found in high concentrations (Goldberg et al. 2004; Cledón et al. 2006; Bigatti et al. 2009; De Waisbaum et al. 2010; Albano et al. 2013; Laitano et al. 2015) as well as bacterial load (Pérez Guzzi et al. 2006). Thus, it is probable that PO activity is stimulated in barnacles of the port due to pathogens abundance. However, the increase of PO activity in different marine invertebrates' species has been associated with a variety of contaminants such as metals (Tujula et al. 2001), certain PAHs (Coles et al. 1994), herbicides and pharmaceuticals (Luna-Acosta et al. 2012), not only organic contaminants. Further, Bado-Nilles et al. (2008) found increase in PO activity in Pacific oysters exposed to benzo[*b*]fluoranthene, but did not find modulation of other haemocyte parameters and thus suggest that the increment is produced by a direct influence of the contaminant on the enzyme activity. On the other hand, the peroxidase superfamily is made up of enzymes which scavenge H_2O_2 previously generated by different metabolic pathways. Little information about pollution effects on peroxidases activity of marine invertebrates is available; it deals exclusively with mussels and mainly reports no pollution effect on Pe activity (Pipe et al. 1995; Dyrynda et al. 1998; Pipe et al. 1999). However, we found an induction of Pe activity in barnacles from MDPP. The reactive oxygen species are released as sub-products of the reactions to the presence of both pathogens and contaminants (Pipe and Coles 1995); therefore, in the case of MDPP barnacles, both factors could be inducing Pe activity as occurs with PO.

Both barnacles and mussels evaluated in this study showed induction of GST activity in Mar del Plata port as compared to the reference site. The increase in the activity of this enzyme as response to environmental pollution of the habitat has been previously accounted for by several authors in different coastal invertebrates' species, such as the mud crab *Scylla serrate* (van Oosterom et al. 2010), the mussels *Perna perna* and *Mytilus galloprovincialis* (Kaaya et al. 1999) and the barnacles *Pollicipes pollicipes* (Ramos et al. 2014) and *Balanus improvisus* (Zanette et al. 2015). Glutathione-S-transferase takes place in the phase II of the xenobiotic biotransformation processes, using reduced glutathione as co-substrate. Despite the fact that this function has been traditionally ascribed to this enzyme, its role in other processes, such as lipid peroxidation prevention, has also been demonstrated. Therefore, the induction of its activity is generally consider coherent with the rationale of using this biochemical response as biomarker of environmental pollution (Ramos et al. 2014). Moreover, there has been also reported an enhanced expression of GST genes upon exposure to many contaminants (Hoarau et al. 2006; Won et al. 2011). Thus, the results show a consistent pattern of GST response in barnacles and mussels with respect to the contamination status of the study sites. Furthermore, two out of the three species studied in this work showed induction of GST activity, so that the

Table 1 AICc for both, the null model (without independent variable; m0) and that with site (*B. rodriguezii, S. lessoni, B. glandula*) or species (Among species) as independent factor (m1)

	PO	Pe	GST
B. rodriguezii	m_0: −52.2	m_0: 0.74	m_0: 62.82
	m_1: −48.04	m_1: 4	m_1: 59.22*
S. lessoni	m_0: −7.06	m_0: −50.6	m_0: 57.18
	m_1: −1.39	m_1: −46.7	m_1: 60.43
B. glandula	m_0: −63.84	$m_0 = -13.47$	$m_0 = 40.15$
	$m_1 = -69.92$*	$m_1 = -14.93$*	$m_1 = 26.64$*
Among species	$m_0 = -80.75$	$m_0 = 0.49$	$m_0 = 93.04$
	$m_1 = -74.4$	$m_1 = -3.03$*	$m_1 = 62.15$*

The lower AICc indicates that is the best fitted model compared with the corresponding null model

* Significant differences between sites or among species

activity of this enzyme would be a sensitive biomarker of in situ port pollution, although further study would confirm this.

The species selected in this study provided different biochemical responses to chronic port pollution: *B. glandula* featured alterations in the activity of the three analyzed enzymes, while *B. rodriguezii* did so only in GST, and *S. lessoni*, in turn, did not show any variation in enzymes activity due to pollution. Part of this variation would be explained by the feeding habit of the species which would lead to different degree of exposure to contaminants (Martín-Díaz et al. 2008). *S. lessoni* grazes on micro and macroalgae whereas the other two species are filter-feeders. Thus, it is probable that mussels and barnacles are more exposed to contaminants than limpets, reflecting this in the biochemical responses assessed here. Different sensitivity and/or other defense mechanism upon pollution among the studied species, should not be discarded as a cause of the differences in such responses. As a consequence, although the three species under study presented good bioindicator features, barnacles would be the most sensitive one regarding the biomarkers analyzed in this study. Several authors have postulated the importance of analyzing a battery of biomarkers in pollution impact studies (Farcy et al. 2013). The present results highlight the importance of studying a set of bioindicator species (at least as a preliminary biomonitoring study) as a platform to reach more accurate results. Moreover, it has been demonstrated that mussels would not always be the best bioindicator species among marine invertebrates, depending on the responses that are assessed.

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