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Shell alterations in limpets as putative biomarkers for multi-impacted coastal areas☆

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ABSTRACT

During the last years, shell alterations in gastropods have been proposed as tools to be used in monitoring programs. However, no studies were so far performed investigating the relationships among shell parameters and classical biomarkers of damage. The relationship between shell alterations (biometrics, shape and elemental composition) and biomarkers (LPO and DNA strand break) was evaluated in the limpet *L. subrugosa* sampled along a contamination gradient in a multi-impacted coastal zone from southeastern Brazil. Statistically significant differences were detected among sites under different pollution levels. The occurrence of shell malformations was consistent with environmental levels of several hazardous substances reported for the studied area and related to lipid peroxidation and DNA damage. In addition, considering the low mobility, wide geographic distribution, ease of collection and abundance of limpets in coastal zones, this putative tool may be a cost-effective alternative to traditional biomarkers. Thus, shell alterations in limpets seem to be good proxies for assessing biological adverse effects in multi-impacted coastal zones.

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1. Introduction

Molluscs are widely used as model in studies of environmental contamination and toxicology due to their ecological and economic relevance (Laitano et al., 2013). Several gastropod and bivalve species have been proposed and used as tools in monitoring of environmental quality (Sericano et al., 1995) and ecotoxicology (Rittschof and Clellan-Green, 2005). Their wide geographic distribution, abundance, sedentary behaviour, ease of sampling and capacity to accumulate contaminants make molluscs suitable organisms for monitoring pollution (Oberdorster and Clellan-Green, 2003). In addition, several mollusc species are sensitive to anthropogenic impacts, exhibiting morphological (Nuñez et al., 2012), reproductive (Castro et al., 2012), biochemical (Márquez et al., 2011) and/or behavioural (Phelps et al., 1983) alterations when exposed to xenobiotics.

The structure of mollusc's shells preserves information about their life histories and environmental pressure related to morphology and chemical composition (Nuñez et al., 2012). Environmental parameters such as hydrodynamics (e.g., wave action), temperature, sun exposure (e.g., desiccation), type of substrate and salinity can lead to inter- and intraspecific changes in the shape, thickness, composition and sculpture of mollusc shells (Underwood, 1979; Vermeij, 1973). Studies using gastropods (Avaca et al., 2013; Laitano et al., 2013; Márquez et al., 2011; Nuñez et al., 2012) and bivalves (Alzieu, 2000; Alzieu et al., 1986) have shown that morphological and chemical composition changes in shells can also be induced by the exposure to hazardous chemicals in laboratory assays and along pollution gradients in the environment.

Limpets are patelliform gastropods numerically dominant in the macrobenthos of many intertidal rocky shore communities

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worldwide (Niu et al., 1992). *Lottia subrugosa* is a limpet species, presenting low mobility and broad geographic distribution along South American coastal zones (Rosenberg, 2016). In addition, it is easily identified and caught in rock shore substrates where it is usually abundant even on highly polluted sites. *L. subrugosa* presents a conical shell from which several biometric parameters can be readily assessed using biometric and morphometric analysis. These features favour the use of patelliform gastropods as suitable tools for evaluating environmental contamination. For instance, Nuñez et al. (2012) successfully identified distinct pollution levels in Argentina based on shell malformation of the limpet *Siphonaria lessoni*.

To the best of our knowledge, no studies were so far performed investigating the relationships among shell shape parameters, elemental composition and classical biomarkers used in marine pollution monitoring, such as lipid peroxidation (LPO) and DNA damage. These biomarkers are not specific to a particular group of contaminants, exhibiting a common response for either environmental parameters or multiple xenobiotics (Mayer et al., 1992). LPO and DNA damage have been extensively used as early proxies for deleterious effects produced by exposure to hazardous substances in marine species (Franzellitti et al., 2015; Pavlaki et al., 2016; Wen and Pan, 2016), including gastropods (Bhagat et al., 2016).

The use of limpet biometric parameters for monitoring marine chemical contamination might serve as a simple, cost-effective alternative for time-consuming, expensive methods currently available. Simultaneous assessment of traditional biomarkers and shell alterations in patelliform gastropods should be employed for validating biometrics as a tool for coastal biomonitoring. This study evaluated the relationship between shell alterations (biometrics, shape and elemental composition) and damage biomarkers (LPO and DNA strand break) in *L. subrugosa* sampled along a contamination gradient in a multi-impacted coastal zone from southeastern Brazil.

2. Material and methods

2.1. Study area and sampling

Santos is the major city of Baixada Santista metropolitan area (southeastern Brazil) with an urban population density of 1497 inhabitants per square kilometre. It hosts the largest industrial complex along the coast of Brazil and a major commercial port in Latin America (Fig. 1). Wastewaters from urban, port and industrial activities are released in Santos Estuarine System (SES) that flows into Santos Bay (Harari and Camargo, 1998). Several studies pointed out SES as a hot spot of hazardous chemicals, presenting estuary to bay gradient of trace metals (Kim et al., 2017), organic contaminants and toxicity (Abessa et al., 2005; Buruaem et al., 2013; Cesar et al., 2014; Torres et al., 2015).

At least 100 adult individuals (9.0–15.5 mm) of *L. subrugosa* were manually caught from rocky substrates during low tides at three sampling sites (P1, P2 and P3) located across a contamination gradient from estuary to bay (Fig. 1). Sites were chosen based on the occurrence of limpets as well as similarity of local hydrodynamics and salinity influence. Surface sediments (upper top 2 cm) were collected next to each site using a stainless-steel Ekman grabber. In the laboratory, sediment samples were homogenized, frozen, freeze-dried and stored at −20 °C until subsequent analysis. Organisms were identified and dissected for separation of soft tissues and shells.

2.2. Chemical analyses in sediments

Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) in surface sediments were determined for confirming chemical contamination gradients across sampling sites (P1, P2 and P3). Extraction and clean up were carried out according to Burns et al. (1992). Briefly, 5 g of sediment was extracted in a Soxhlet apparatus with a mixture of n-hexane and dichloromethane (1:1, v/v). The extract was split into two fractions for column chromatography clean up. The first fraction was cleaned up with silica gel and alumina (both 5% deactivated) for determination of PAHs. The second fraction was cleaned up with alumina (5% deactivated) for determination of PCBs and OCPs. Extracts were eluted with a mixture of n-hexane and dichloromethane (7:3, v/v). The purified extracts were injected into a gas chromatograph coupled to a mass spectrometer (GC–MS, Agilent Technologies, model 7820A/5975C).

Quality control criteria were based on limits typically used in marine pollution monitoring programs (e.g., Lauenstein and Cantillo, 1998; Wade and Cantillo, 1994). Limits of quantification (LQ) were 0.20, 0.99 and 0.99 ng g^{-1} dry weight (dw) for individual PAHs, PCBs and OCPs, respectively. Concentration of target analytes was calculated using internal standards (IS) whose recoveries were 83 ± 17% (mean ± standard deviation). Standard reference material (SRM 1944) from the National Institute of Standards and Technology (NIST), USA was extracted in duplicate for checking analytical accuracy and precision. Recovery of analytes in the SRM was 107 ± 30% (mean ± standard deviation) while the coefficient of variation (CV) between duplicates was 7 ± 4% (mean ± standard deviation).

2.3. Lipid peroxidation and DNA damage analyses in tissues

Soft tissues from 10 organisms randomly selected were immediately removed upon sampling. Thereafter, all samples were homogenized in buffer solution (NaCl 100 mM, Hepes NaOH 25 mM, EDTA 0.1 mM, DTT 0.1 mM, pH 7.5) following procedures developed by Lafontaine et al. (2000). The homogenate was analysed for determination of DNA damage, lipid peroxidation (LPO) and total protein content. Total protein content of the homogenate was analysed according to the dye-binding principle (Bradford, 1976). DNA strand breaks was assessed by alkaline precipitation assay (Olive, 1988) based on the K-SDS precipitation of DNA-protein crosslink, followed by fluorometric detection of DNA strands (Gagné et al., 1995). DNA quantitation was achieved using Hoescht dye at a concentration of 100 nM in 200 mM TrisHCl, pH 8.5, containing 300 mM NaCl and 4 mM sodium cholate. Salmon sperm DNA standards were used for calibration, and fluorescence readings were done at 360 nm (excitation) and 460 nm (emission). The results were expressed as μg of DNA per milligram of total protein. LPO was measured according to Wills (1987), being determined in tissue homogenates by thiobarbituric acid. Thiobarbituric acid reactants (TBARS) were determined by fluorescence at 530 nm for excitation and 630 nm for emission using a fluorescence microplate reader. The results were expressed as μg of TBARS per milligram of total protein.

2.4. Biometric and morphometric analyses in shells

After complete drying in an oven (60 °C), 100 shells from each sampling site were randomly selected and weighed on analytical balance (0.01 ± 0.003 mg). Biometric parameters (length, width, height) were measured using a digital calliper (0.01 ± 0.005 mm) and thickness of shell apex was obtained using a digital micrometer (0.001 ± 0.0005 mm). Measurements of shell specific gravity (relative density) were made by pycnometry according to DiResta et al. (1991).

Geometric morphometric analysis was performed using the

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