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Biomonitoring of environmental stress in *Pollicipes pollicipes* from the northern coast of Portugal: a non-destructive approach using haemolymph

A.S. Ramos [a], S.C. Antunes [a,b,*], B. Nunes [c,d]

[a] Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, Edifício FC4, 4169-007 Porto, Portugal
[b] CIIMAR-Centro Interdisciplinar de Investigação Marinha e Ambiental, Rua dos Bragas 289, 450-123 Porto, Portugal
[c] CESAM-Centro de Estudos do Ambiente e do Mar, Campus Universitário de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal
[d] Departamento de Biologia, Campus Universitário de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

In the intertidal area, the interactions between anthropogenic contaminants and natural variations (biotic and abiotic factors) are poorly understood. Consequently, there is a great need for new assessment procedures to characterize the biological responses occurring in organisms from this extreme environment. Considering the intrinsic inter-individual variations among organisms from a single population, it is important to propose new methods that address this variability, by validating a sampling strategy in target groups of organisms, encompassing seasonal fluctuations. This strategy must however be less invasive than traditional methods, avoiding the mandatory sacrifice of the sampled organisms. By doing so, it is also possible to increase the ecological relevance of obtainable data, and contribute to minimize damage to endangered species. The main purpose of the present study was to assess the influence of seasonal variations in the responses elicited by anthropogenic compounds on a marine crustacean species, by using a biomarker-based approach. According to this purpose, the seasonal variations in key physiological responses (biomarkers) were investigated in the crustacean *Pollicipes pollicipes* from the Northern coast of Portugal. Biomarkers used for this purpose were the activity of the phase II biotransformation isoenzymes glutathione-S-transferases (GSTs), the activity of cholinesterases (ChEs), and the levels of lipid peroxidation (TBARS). All biomarkers were quantified in distinct tissues (such as cirri, and peduncle) and haemolymph (a non-destructive source of biological samples). The glycogen content in peduncle tissue, and the variation in haemocyte number in haemolymph were also analyzed. Samples were collected monthly, during a year, in Lavadores, located in the proximity of an estuarine area (Douro River). The results showed a seasonal pattern in all tested biomarkers. The results also showed a significant increase in GSTs activities, and in peroxidative damage, especially during warmer months. The lowest ChEs values were recorded during the rainy season. The results also showed a similar pattern among all tested tissues, validating the proposed use of the haemolymph as a source tissue for non-lethal sampling strategy for biomarker determinations. Glycogen content was apparently related to the reproductive cycle, with lower values being observed in spring and summer. Total haemocyte count (THC) increased during summer months. The results pointed to an influence of natural variations in the levels of biomarkers in *P. pollicipes*, highly dependent upon abiotic factors, a factor that must be considered when interpreting biological responses elicited by anthropogenic contaminants from the marine coastal environment. The validation of haemolymph as a non-lethal source tissue for the quantification of biomarkers was successfully attained, opening the possibility of less invasive and almost unlimited sampling in a small number of organisms.

1. Introduction

Coastal zones are contaminated by a large number of contaminants of diverse anthropogenic origin (Venturini et al., 2008), acting simultaneously with alterations in abiotic factors such as pH, dissolved oxygen and temperature (Montserrat et al., 2007).

* Corresponding author at: Departamento de Biologia, Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre s/n, Edifício FC4, 4169-007 Porto, Portugal.
E-mail address: scantunes@fc.up.pt (S.C. Antunes).

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This transforms the intertidal area into an extreme and challenging environment for marine organisms. The major sources of contamination in coastal waters include agriculture and urban runoff, released directly in the coastal area or into the adjacent environment (Mora et al., 1999). Oporto coastal area (located in the north of Portugal) is under the influence of Douro River, where several types of anthropogenic pollutants in river water have already been described (Mucha et al., 2003; Ferreira et al., 2005; Carvalho et al., 2009; Reis et al., 2012). Furthermore, these compounds were also found in seawater and sediments dispersed along the adjacent coastal area. In these classes of substances, one can find metals, hydrocarbons, pesticides and several other anthropogenic contaminants, which result mainly from domestic sewage, but also from industrial effluents being discharged directly in the estuary (and its tributaries), in some cases without treatment (Ferreira et al., 2005). However, and despite the characterization of such compounds, more studies are necessary to assess the ecotoxicological outcomes of anthropogenic contaminants on aquatic organisms in the northern coastal areas of Portugal.

Aquatic pollutants are commonly found in very complex mixtures, especially in marine environments, and the alterations that these mixtures can cause in biota difficult (and even confound) the evaluation of their effect (van der Oost et al., 2003; Costa et al., 2009). The biological/physiological alterations elicited by xenobiotics can be assessed by the use of biomarkers, which is an adequate solution to overcome the difficulties and complexities of interpreting complex biological responses to diffuse pollution. This biomarker-based strategy allows the performance of a global estimation of the simultaneous effects of a large set of substances on wildlife. However, biomarkers alone may not provide the complete information required for the development of a comprehensive set of data in a contamination scenario. To obtain ecologically relevant data from monitoring studies, the incorporation of wild resident species in monitoring programmes is of fundamental significance, especially in the marine coastal environment. Wild species, being part of ecotoxicological studies, can be adopted as sentinel species (Basu et al., 2007), reflecting the impact of biological/physiological changes in biota caused by pollutants or environmental changes (Livingstone, 1993).

One of the most commonly used biomarkers for the evaluation of chemical impacts by anthropogenic stressors is the activity of glutathione-S transferases (GSTs), a group of isoenzymes involved in phase II metabolism; this group of enzymes acts to enhance detoxification metabolism through the conjugation pathway of electrophilic xenobiotics with glutathione. The quantification of lipid peroxidation levels, by the TBARS assay, is also a reliable biomarker to quantify the oxidative effects of pollutants in the aquatic ecosystem, being one of the most widely used estimators of oxidative damage (Oakes and Van Der Kraak, 2003; Nunes et al., 2006). The inhibition of cholinesterasic (ChEs) activity is also described as a sensitive biomarker of neurotoxicity, suitable to diagnose exposure to specific pollutants, namely organophosphates, carbamates and other pollutants, as recently demonstrated, such as metals and contaminants from domestic sewage (Payne et al., 1996; Bonacci et al., 2009). Chemical stressors can unbalance normal energy metabolic processes, causing variations in glycogen levels, a major energetic reserve in many species. An important method for evaluating the impact of biological changes in biota caused by pollutants can be the quantification of glycogen levels in the organisms (Becker et al., 2009). Glycogen is a carbohydrate essentially found in liver and muscle tissue, which can be substantially decreased in contamination scenarios (e.g., Becker et al., 2009).

Among the multiplicity of tissues that can be used for the assessment of biological responses, haemolymph seems suitable as a non-lethal source tissue for the quantification of markers of ecotoxicity (Fossi, 1994; Fossi et al., 2000; van Oosterom et al., 2010). The use of haemolymph in biomonitoring studies can be also a valuable tool since it allows repeated individual evaluation over time, allowing a compilation of historical data. In fact, changes in the biochemical composition of haemolymph has been used as an evaluation criteria of physiological and pathological conditions in crustaceans (Jayasree, 1999), being a useful way to evaluate the alterations in marine ecosystems. Changes in haemolymph are not limited to the normal fluctuations in an organism's physiology (e.g. due to developmental phases) but can reflect the enhancement of defence mechanisms including responses to high pollution levels. Haemolymph analysis can thus be used as an indicator of environmental alterations, including exposure to chemical pollution (Jayasree, 1999). In fact, several antioxidant enzymes occur in haemolymph, namely for protection against the presence of reactive oxygen species (ROS) (Pipe et al., 1993), and their activity may be also affected by pollutants or by abiotic parameters (eg. salinity, pH; Gagnaire et al., 2006a). The use of haemolymph as a non-lethal source tissue for the quantification of biomarkers has been proposed and validated by Fossi (1994) and Fossi et al. (2000), in the littoral crab species *Carcinus aestuarii*, and by van Oosterom et al. (2010) in the crab *Scylla serrata*. Variations in the count of haemolymph cells, the haemocytes, are considered to be a good indicator of stress in crustaceans (Le Moullac and Haffiner, 2000), being a useful methodology for the determination of effects caused by aquatic pollution (Sami et al., 1993; Pipe and Coles, 1995). The use of a non-lethal sampling approach is not limited to issues of feasibility and practicability. In fact, the use of non-lethal sampling strategies has been proposed as a solution to increase the ecological relevance of a large number of biomarkers used to quantify the ecotoxicological effects of anthropogenic contaminants, especially in biomonitoring studies. According to Hopkin (1993), biomarkers must comply with a series of mandatory characteristics, such as relevance, reliability, robustness, responsiveness, and reproducibility. By sampling and analyzing distinct organisms (a common procedure adopted in most biomonitoring programmes, with or without the use of biomarkers), the reproducibility of obtained results may be questioned, due to the influence of interindividual variations among sampled organisms. The variability among organisms (even those collected from a single population), is natural and unavoidable, but may act as an insurmountable confounding factor. The use of a repeated, non-lethal sampling strategy (with the collection of biological material always from the same organisms) is an adequate response to this need. However, and despite the validity of such considerations, the use of non-lethal sampling strategies is nowadays an uncommon practice. The review by Colin et al. (2016) shows that only a small number of such approaches has been so far presented and validated, not only for invertebrates, but also for fish species. Nevertheless, the here-proposed strategy, which combines non-lethal sampling with the determination of unspecific biomarkers, may be considered promising. By being exposed to complex chemical mixtures, wild organisms may trigger generic responses that may be quantified through the use of unspecific biomarkers (e.g. oxidative stress, haemocyte count) that signal the onset of deleterious processes and effects (Maceda-Veiga et al., 2015; Colin et al., 2016)

According to this rationale, the main objective of the present biomonitoring study was to assess the influence of seasonal variations in the toxic response elicited by anthropogenic compounds in the barnacle *Pollicipes pollicipes*. To attain this purpose, several biochemical parameters were quantified in different tissues: oxidative stress, by measuring the activity of glutathione-S-transferases (GSTs); peroxidative damage, analyzed by means of lipid peroxidation levels (TBARS); neurotoxicity, by measuring the cholinesterase activity (ChE); and general fitness markers, such as