ARTICLE IN PRESS

Marine Pollution Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Effects of metal contamination on the gene expression profile of two benthic species: *Cerastoderma edule* and *Ruditapes philippinarum*

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ARTICLE INFO

Keywords:
Suppressive Subtractive Hybridization (SSH)
qPCR
Tagus estuary
Biomarkers
Environmental pollution
Risk assessment

ABSTRACT

This study aimed to identify new biomarkers for metal exposure in two bivalve species. Suppressive Subtractive Hybridization (SSH) was employed to evaluate the transcriptomic response of *Cerastoderma edule* and *Ruditapes philippinarum* to metal pollution. Protein synthesis and catalytic activity were the most affected metabolic processes in *C. edule* and *R. philippinarum*, respectively. Also, different genes responded to the effect of contamination in each species. The different response observed in both species reinforces the importance of including more than one bioindicator species in risk assessment studies. These results provide the basis for new studies, which are necessary for further validation of the use of the identified genes as molecular biomarkers for metal exposure.

1. Introduction

Environmental pollution has been raising concern worldwide, which leads to the need of developing methods to monitor the health of ecosystems. Sub-lethal and lethal effects are often used as biomarkers to assess the impact of environmental contamination on local biota. However, molecular biomarkers can provide an early response even before those sub-lethal effects are manifested (Cajaraville et al., 2000). Biomarker analysis provides responses even if the contaminant has been degraded and is no longer detectable in the chemical analysis (Depledge and Galloway, 2005). In fact, it is now widely accepted that chemical analysis of environmental matrices (such as water and soil) are not sufficient for risk assessment purposes, as they provide no information on bioavailability (Torres et al., 2008) or on the deleterious effects of contaminants on the local biota (Bayne, 1989; Cajaraville et al., 2000). More reliable results can be achieved when a battery of biomarkers is used in combination with other chemical and biological measurements (Cajaraville et al., 2000). By combining these different assessment methods, a relation between commonly used risk assessment endpoints (such as reproduction and growth) and molecular responses can be established (Galay-burgos et al., 2003).

Biomarkers can be particularly useful to assess metal contamination on estuarine environments, as metals can either be dissolved in the water column or, more often, associate with particles and accumulate in the sediment layer (DelValls et al., 1998; Pan and Wang, 2012). Estuaries are environmentally and ecologically relevant, as they constitute the transition between marine and freshwater environments (Meire et al., 2005). These unique ecosystems are usually located near metropolis and industrialised areas, which increases the risk of contamination (Canário et al., 2003; Chainho et al., 2008).

The present study aims to identify a battery of molecular biomarkers for the assessment of metal contamination in two widespread estuarine species (*Cerastoderma edule* and *Ruditapes philippinarum*) using Suppression Subtractive Hybridization (SSH) (Diatchenko et al., 1996). The main advantage of the SSH technique is that it allows the simultaneous normalisation of cDNA (to ensure that highly abundant transcripts do not mask the results) and subtraction of transcripts that are present in both libraries (one for each condition being tested) (Diatchenko et al., 1996). This allows the enrichment and identification of genes that are differentially expressed. In the present study, SSH was used to compare areas impacted by different contamination pressures, providing an overview of the molecular processes altered in response to metal exposure. The transcriptional response analysis is a sensitive tool to assess early-stage effects of pollution (at the molecular level), before other biological damages can be detected (Schirmer et al., 2010). Transcriptomic approaches have been used in several studies, showing that exposure to contaminants affects the gene expression profile of organisms (Milan et al., 2011; Milan et al., 2013; Cruz et al., 2015).

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http://dx.doi.org/10.1016/j.marpolbul.2017.08.014
Received 21 February 2017; Received in revised form 3 August 2017; Accepted 5 August 2017

Please cite this article as: Rodrigues, R., Marine Pollution Bulletin (2017), http://dx.doi.org/10.1016/j.marpolbul.2017.08.014

Furthermore, it has been shown that the genetic profile of benthic organisms is affected by metal contamination (Li et al., 2011; Paul-Pont et al., 2012; Chalghmi et al., 2015), reinforcing the validity of the SSH approach to identify molecular biomarkers for metal exposure in bioindicator species.

Bivalves are good candidates as bioindicator species, as they provide a rapid response to stress conditions, have a sedentary lifestyle and, due to their feeding habits, accumulate high concentrations of metals (Cheggour et al., 2001; Griscom et al., 2002; Hédouin et al., 2007; Anajjar et al., 2008; Metian et al., 2009). These metals may be biomagnified across the food chain (Wang, 2002) and, due to their mutagenic and carcinogenic properties (Hartwig and Schwerdtle, 2002; Leonard et al., 2004), affect populations and consequently the entire ecosystem. Bivalves are commonly consumed by humans (Wang, 2002) and, if contaminated, may pose a threat to human health.

2. Materials and methods

2.1. Sampling area and target species

The Tagus estuary is one of the largest in Europe, with a total area of approx. 300 km^2 (Caçador et al., 1996). This estuary has several sources of contamination due to its proximity to Lisbon, the main urban centre in Portugal (Vale et al., 2008). Contaminant input is very common due the discharge of residues from industry (Canário et al., 2003; Duarte et al., 2008) and agriculture activities (Ferreira et al., 2003), as well as sewage effluents (Chainho et al., 2010). Due to all these contaminant sources, certain areas located near industrial sites (e.g. Barreiro) are highly contaminated with metals. Contrastingly, part of the estuary is located in a natural reserve area, where the input of contaminants is minimal (e.g. Alcochete) (Fig. 1).

The cockle *C. edule* and the clam *R. philippinarum* were selected as bioindicator species, due to their economic and ecological relevance (de Montaudouin et al., 2010; Paul-Pont et al., 2010a, 2010b; Oliveira et al., 2011). *C. edule* lives buried in the sediment, a few centimetres below the surface, and it is a suspension and occasionally deposit feeder (Anajjar et al., 2008; Van Colen et al., 2013). *R. Philippinarum* is a filter feeder, which means it feeds on organic and inorganic materials suspended in the water column (Chong and Wang, 2000). The feeding habits of these bivalves increase the chances of metal bioaccumulation since, as previously mentioned, metals are often associated with sediments and particles suspended in the water column (DelValls et al., 1998; Pan and Wang, 2012), which can be ingested by cockles and clams.

2.1.1. Sampling procedures

C. edule and *R. philippinarum* specimens were collected from the Tagus estuary (Fig. 1), namely from Barreiro (BAR – near industrial sites; contaminated) and Alcochete (ALC – natural reserve; reference). The samples herein analysed were those collected and described by Piló et al. (2015), but only animals collected in October 2013 were used for the transcriptional response analysis.

The specimens presented lengths of 1.4–2.1 cm for *C. edule* and 2.0–4.3 cm for *R. philippinarum*. The digestive gland of the clam was selected for further analysis because it is known to be involved in metal accumulation and detoxification (Nott and Nicolaidou, 1989; Marigómez et al., 2002; Amiard et al., 2006). As for the cockle, due to size limitations, the whole soft tissue was analysed. The tissues of the samples to be used in the SSH experiments were submerged in RNAlater (Qiagen) immediately after collection and kept at 2–8 °C overnight. Samples were then transferred to − 80 °C for storage.

2.2. Determination of metal concentrations in sediments and biological samples

Metal concentrations in the sediments and tissues were determined by Piló et al. (2015, 2017), respectively.

Metal concentration in the sediments was evaluated by comparison with defined thresholds: ERM – Effect range-low and ERM – Effect range-medium (Long et al., 1995). Metal concentration values below ERL are unlikely to exert toxicity, whereas values above ERM indicate that biological effects are likely to be observed. Values between ERL and ERM indicate that the local biota may be affected, but with lower probability than values above ERM.

The determination of metal biota-sediment accumulation factors (BSAF) was only possible for *R. philippinarum* due to the limited number of available *C. edule* specimens. In *R. philippinarum*, metal concentrations in the gills have been previously determined (Piló et al., 2017).

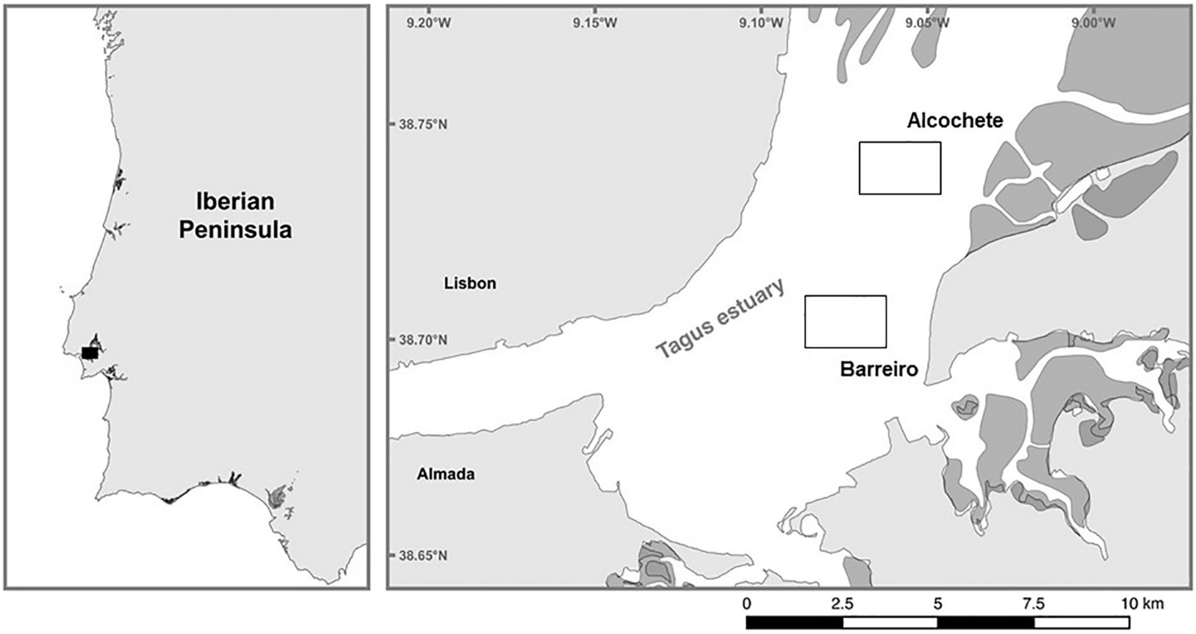

Fig. 1. Schematic representation of the Tagus estuary showing the locations of the sampling areas (adapted from (Marques et al., 2016)).