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Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure

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ABSTRACT

The aims of the present study were to optimize and validate methods for esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) determination in mussel' gills, and to establish the relationships between these biomarkers and Pb, Cd and Cu pollution, in single form and ternary mixture. Two different buffers for sample homogenization, the need of ultracentrifugation, and analytical validation were evaluated. Coefficients of variation, when buffer without additives and ultracentrifugation were used, were <15%, and recovery were 97%–109% in all cases. The EA response tends to decrease with treatments, TOS decreased significantly in Cd and ternary groups, while TAC tended to increase in treatments with Pb, Cd and ternary groups. In conclusion, the methods for EA, TOS and TAC measurements in gills of mussel were precise and accurate and could be interesting resources in biomonitoring programmes.

1. Introduction

The effects of warming, acidification and deoxygenation in oceans are major causes of species extinctions. The impacts of these effects are increased by pollution and other human activities such as eutrophication and overfishing (Bijma et al., 2013). In order to preserve marine ecosystems, the attempts of restoring the oceans quality have to be urgent, focused, innovative and global (Hutchinson et al., 2013).

In aquatic ecosystems, a coherent and effective water policy must consider the vulnerability of them. In this sense, concentration of pollutants and their derivate effects in the biota are one of the most important elements in the evaluation of aquatic environment, and their measurement is an imperative in national action plans. Also, the protection of water's state can bring economic benefits derivate of the protection of fish population, including those who have their habitat close to coasts. The European Union's Water Framework Directive (WFD, Directive 2000/60/CE), one of the most important pieces of environmental legislation, is focused on the water quality monitoring across all member states. However successful implementation of this Directive across EU member states would require the establishment and use of emerging and low-cost tools as part of monitoring programmes, to complement monitoring already in use by providing additional information with the aim to obtain a more representative picture of the quality of a water body (Allan et al., 2006).

Through monitoring, possible changes in the characteristics of many water quality elements, such as physico-chemical, biological, chemical or hydro morphological parameters are detected. Biomonitoring is based on sampling and analysis of tissues and fluids (Zhou et al., 2008), but to assess the biological risks in biota it is necessary to use biomarkers, which can be studied in wild organisms or in whole-organism bioassays. Biomarkers were described by Allan et al. (2006) as changes in a biological response which can be related to exposure to or toxic effects of environmental chemicals. Biomarkers aim to give a quick response to a risk of pollution allowing rapid decision making meanwhile, a toxic effect is apparent at the subcellular level before it is noticeable at higher biologicals levels (Allan et al., 2006; Zuykov et al., 2013). Biomarkers are measured in bioindicator organisms, which are organisms that contain information on the quality of the environment (Markert, 2007). Those organisms can be bred in laboratories in a standardized form, or taken from natural ecosystems.

For evaluation of water quality in costal zones, the main bioindicator used is mussel (*Mytilus galloprovincialis*). Mussels are considered ideal as bioindicators of coastal pollution because of their features, such as

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they are widespread across the world, are abundant, sedentary, easy to sampling and have a relative long life (Kimbrough et al., 2008; Sericano et al., 2014; Widdows et al., 2002). Wild mussels are filter-feeding organism whereby accumulate high concentrations of pollutants (Zhou et al., 2008) and they are very resistant to adverse conditions such as hypoxia, environmental stress or pollution (Benedicto et al., 2012). Furthermore, these bivalves are eating by higher trophic levels organism, and are commercial products all around the world, posing a risk to human health. For those reasons, the use of mussels in coastal monitoring is widely accepted and supported by many international organizations, and numerous bioassays were developed with these animals in order to evaluate biological response on various biomarkers such as SOD, catalase, glutation S transferase in biomonitoring programmes with mussels (Canesi et al., 1999; Cooper et al., 2009; Di Salvatore et al., 2013; Fernández et al., 2012; Najimi et al., 1997; Pellerin-Massicotte, 1994; Regoli and Principato, 1995; Sparks et al., 2014).

Pollutants, such as heavy metals, promote the generation of reactive oxygen species (ROS), which induce antioxidant defence mechanisms in exposed organism to prevent oxidative damage to biomolecules such as DNS, proteins, and lipids among others (Livingstone, 2001; Manduzio et al., 2005). The measurement of the biological response methods such as oxidative stress can indicate links between contaminants and ecological responses and can be used to indicate the presence of harmful substances in the marine environment (Thain et al., 2008). EA, TOS and TAC are biomarkers of oxidative stress that are measured easily in automatic analysers, fast and economic. In this sense, EA has received increasing attention in human and veterinary medicine, as alterations in its activity were associated with various pathologies that involve oxidative stress (Cifici et al., 2010; Dong, 2006; Marsillach et al., 2009; Tvarijonaviciute et al., 2012). TOS is the measurement of different oxidant species in an organism (Aslan et al., 2014; Barbosa et al., 2014), that was shown to be of help in the diagnosis of different pathologies (Erel, 2005). Finally, TAC determinations are of help to study the capacity of known and unknown antioxidants and their additive, synergistic and/or antagonistic actions, in chemical and biological systems (Fraga et al., 2014). Currently TAC assays have applications in human and veterinary medicine to evaluate overall defence status against oxidative stress and biomedical sciences (Bartosz et al., 1998; Erhola et al., 1997; Goldberg et al., 1999; Langley et al., 1993; Opara et al., 1999). Nonetheless, these biomarkers have not been widely used in biomonitoring programmes, even when their utilities are demonstrated in the above-mentioned fields.

The main objectives of the present study were to optimize and to validate methods of EA, TOS and TAC determination in gills of *M. galloprovincialis* for use in marine environmental assessments. Also, to evaluate the behaviour of these biomarkers to metal exposure, so the mussels were exposed to single and mixed heavy metal concentrations (Pb, Cd and Cu) and the relationships between biomarkers and these pollutants were discussed.

2. Materials and methods

2.1. Mussels collection and conditioning period

Wild mussels (*M. galloprovincialis*) (4–5 cm length, 9–10 g weight) were collected from Cabo Home (geographic reference LA 5 42°15.007 LO 08°52.333 5) Galicia (NW Spain). Cabo Home (Pontevedra) is considered uncontaminated area without any nearby sources of pollution (Albentosa et al., 2012), with a rate of chemical pollution below 1 (Besada et al., 2014; González-Fernández et al., 2015) and with a high nutritional and reproductive status (González-Fernández et al., 2015). Mussels were transported in cold and air exposed, via overnight express delivery services (less than 24 h) to Toxicology laboratories (University of Murcia). Mussels were acclimated (2 weeks) in twelve litre tanks under laboratory controlled conditions: water pH (8.03 ± 0.07), osmolarity (1086.3 ± 28.39 mmol kg^{-1}), temperature (24 °C), continuous aeration, and natural photoperiod. Mussels were fed with microalgae *Isochrysis galbana*, clon t-ISO (0.1% of microalgal organic matter per mussel live weight).

2.2. Heavy metal exposure

Mussels were exposed to single metal (Pb -Panreac, Cd -Sigma and Cu -Panreac) and ternary mixture at dose of 1000 μg L^{-1} (Pb) and 100 μg L^{-1} (Cd and Cu). The exposure experiment was carried out in 3 tanks by treatment with 12 mussels each one. Heavy metals stock solutions were prepared in milliQ water and 3 control tanks were included. Exposure conditions were maintained for a period of 7 days. During this period temperature, pH, osmolarity, photoperiod, aeration and feed conditions were as described above. The toxicant was distributed daily within each tank.

2.3. Sample collection and processing

Gills were extracted from each mussel, weighed and frozen at −80 °C until analysis. On the day of analysis all samples were thawed and mechanically homogenized with automatic homogenizer (Precellys Evolution, Bertin Technologies, Saint-Quentin, Yvelines, France) in 1:4 (weight/volume, w/v) buffer. Homogenization was performed at 6500 rpm in three cycles of ten seconds, with a 10 s pause between cycles. After 30 min incubation at room temperature samples were centrifuged at 3500 rpm for ten minutes at 4 °C. Supernatant was harvested in Eppendorf tubes and analysed.

2.4. Biochemical measurement

EA was analysed by measuring the hydrolysis of p-nitrophenyl acetate to p-nitrophenol as described elsewhere (Haagen and Brock, 1992), with some modifications (Tvarijonaviciute et al., 2012). The reaction was monitored at 405 nm. The nonenzymatic hydrolysis of phenyl acetate, which was based on the hydrolysis rate in the absence of sample, was subtracted from the total hydrolysis rate. The activity was expressed as units per millilitre of sample, in which 1 U equals 1 μmol of phenyl acetate hydrolysed min^{-1}. The molar extinction coefficient used to calculate the rate of hydrolysis was 14,000 M^{-1} cm^{-1} (Tvarijonaviciute et al., 2012).

TOS was measured as previously described (Erel, 2005), with some modifications (Barbosa et al., 2014). The method is based on the reaction that the ferric ion makes a coloured complex with xylenol orange in an acidic medium. The colour intensity, which was measured spectrophotometrically at 560 nm using 800 nm as the reference, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of micromolar hydrogen peroxide equivalent per litre (μmol H_2O_2 Equiv L^{-1}).

TAC was determined as described elsewhere (Erel, 2004), with some modifications (Tvarijonaviciute et al., 2012). The method used was based on 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) decolourization by antioxidants according to their concentrations and antioxidant capacities. The colour change was measured as a change in light absorbance at 660 nm. For the process, the assay was calibrated with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ((R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma-Aldrich Co, St. Louis, Mo) (Erel, 2004), and the activity was expressed as mmol L^{-1}.

EA, TOS and TAC were performed in automatic analyser (AU 600 automated biochemical analyser, Olympus, Minneapolis, Minn).

2.5. Buffer selection

To select optimal buffer for EA, TAC and TOS extraction from mussels and their subsequent measurement, two different buffers were evaluated: