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The use of carboxylesterases as biomarkers of pesticide exposure in bivalves: A methodological approach

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ABSTRACT

Bivalves are worldwide sentinels of anthropogenic pollution. The inclusion of biomarker responses in chemical monitoring is a recommended practise that has to overcome some difficulties. One of them is the time frame between sample collection and sample processing in order to ensure the preservation of enzymatic activities. In the present study, three bivalve species of commercial interest (mussel, *Mytilus galloprovincialis*, razor shell, *Solen marginatus*, and cockle, *Cerastoderma edule*) were processed within < 2 h after being retrieved from their natural habitat, and 24 h after being transported in air under cold conditions (6–8 °C) to laboratory facilities. The enzymatic activities were compared in the three species submitted to both conditions revealing no differences in terms of carboxylesterase dependent activities (CEs) using different substrates: *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB) and 2-naphthyl acetate (2-NA). In mussels, three tissues were selected (haemolymph, gills and digestive gland). For comparative purposes, in razor shell and cockle only digestive gland was considered as it is the main metabolic organ. Baseline enzymatic activities for CEs were characterised in the digestive gland of the three bivalves using four out of the five selected CE substrates as well as the kinetic parameters (V_{max} and K_m) and catalytic efficiency. The *in vitro* sensitivity to the organophosphorus metabolite chlorpyrifos oxon was also calculated. IC_{50} values (pM-nM range) were lower than those obtained for vertebrate groups which suggest that bivalves have high protection efficiency against this pesticide as well as species dependent particularities.

1. Introduction

Marine coastal habitats (e.g. lagoons, estuaries, deltas, tidal rivers) are areas with high productivity that tend to be devoted to aquaculture activities. In addition, they experience important physical fluctuations in water parameters and low rates of water renewal. Being often near urban/agricultural sites, these areas are also subjected to the input of anthropogenic discharges that can alter their ecological balance with detrimental effects on inhabiting wildlife. Therefore, it is of major relevance to assess the impacts of natural changes (e.g. temperature and salinity shifts resulting from extreme weather events) but also the effects caused by human actions (e.g. use of pesticides in agriculture) on inhabiting marine fauna, including shellfish populations.

In marine coastal systems, bivalves are considered good sentinel and bioindicator species of pollution due to their sedentary lifestyle and bioaccumulation ability, as well as their capacity to provide information on environmental health and changes, by identifiable biochemical, physiological, and/or behavioural responses to anthropogenic chemical insults (Beyer et al., 2017; Gonzalez-Fernandez et al., 2015; Ivanina and Sokolova, 2015; Manachini et al., 2013). Among the marine bivalves of economic interest, such as mussels, oysters, clams, cockles and razor shells, there is evidence of differences in terms of bioaccumulation capacity and tolerance to pollutants (Burgos-Aceves and Faggio, 2017; Faggio et al., 2016; Fernandez et al., 2013; Pagano et al., 2017; Zuykov et al., 2013). Mussels are by far the most selected bivalve species in pollution monitoring studies (Burgos-Aceves et al., 2018), but clams such as *Ruditapes decussatus* and *Ruditapes philippinarum* have also been largely selected as bioindicator and sentinel species (Chiesa et al., 2018; Savorelli et al., 2017; Sehonova et al., 2018; Velez et al., 2015a; Velez et al., 2015b). Meanwhile, cockles (Jebali et al., 2011; Nilin et al., 2012; Velez et al., 2016) and razor shells (Ferrante et al., 2014; Nunes and Resende, 2017; Pearce and Mann, 2006) have only been marginally used.

Several biomarkers have been used to evaluate the impacts of pollutants in bivalves, including measurements at a cellular level (Cajaraville et al., 2000). Among the biochemical responses,

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carboxylesterases (CEs) are hydrolytic enzymes involved in the detoxification of pesticides (e.g. pyrethroids and carbamates) and the metabolic transformation of some pharmaceutical drugs (Acena et al., 2017). Furthermore, CEs also have a role in hydrolysing endogenous esters (Hatfield et al., 2016). Due to their high affinity to organophosphorus pesticides (OP) by stoichiometric binding, CEs can exhort a protective role of the enzyme acetylcholinesterase (AChE) involved in neural transmission in the cholinergic synapses. For this reason, CEs have been comprehensively recommended in terms of monitoring pollution by neurotoxic chemicals (Wheelock et al., 2008). In fact, in many bivalve species, CEs are reported to be more abundant and/or sensitive to OPs and other anthropogenic chemicals than AChE (Escartin and Porte, 1997; Galloway et al., 2002; Sole et al., 2010a). Thus, CEs are a good alternative to the neurotoxic marker AChE for pesticide monitoring when using bivalves as bioindicators. Several substrates with different lipophilicity can be used to quantify CE activities, suggestive of different CE isozymes. Recently, the quantification of the most abundant of these isoforms for a given species and tissue has been proposed as an adequate tool to reveal the impact of the chemical of concern (Bianco et al., 2014; Kristoff et al., 2012; Otero and Kristoff, 2016; Sanchez-Hernandez and Wheelock, 2009). For the specific case of the mussel *Mytilus galloprovincialis*, Sole and Sanchez-Hernandez (2018) revealed that in digestive glands and gills, pNPB and 1-NB were the most suitable reporters of CE inhibition by OP and other drugs impacting the environment.

The rationale of this work comes from the fact that one of the main constraints of monitoring pollution using biomarkers that rely on enzymatic activities is the time lapse between sample collection and processing. From a practical methodological perspective, sampling sites and laboratories, where to process the samples, can be geographically distant and, moreover, laboratory processing cannot always be readily organized due to sampling limitations resulting for example from unfavourable weather conditions. Thus, it is of great importance to know the stability of the targeted biomarkers, namely enzymes, in order to validate sampling protocols and define alternatives in sample processing. Additionally, for monitoring purposes in the particular case of OP and other anthropogenic drugs in a defined area of concern, it is essential to identify the most appropriate species, tissue and substrate for the characterization of enzyme activities. All these factors can determine differential results in terms of bioaccumulation patterns and chemical susceptibilities.

Therefore, the goal of the present study was to confirm the suitability of ice-cold preservation under air exposure of bivalves used as bioindicators of pesticide exposure. To this end, CE activities using different substrates were measured in three tissues of mussel (haemolymph, gills and digestive glands) and for comparative purposes also in digestive gland of razor shells and cockles soon after being collected from their habitat (2 h max since they were withdrawn from the water) and 24 h after sampling. Characterization of CE enzymatic activities with different substrates, calculation of their respective V_{max} and K_m and in vitro sensitivity to a metabolite of the pesticide chlorpyrifos (chlorpyrifos oxon) was carried out in the digestive gland of the three bivalve species. The suitability of both sampling procedures on CE measurements and the identification of the most adequate substrate for each species will be valuable in pesticide monitoring as an alternative to other cholinesterases (e.g. AChE) which are less represented/sensitive in some bivalves.

2. Material and methods

2.1. Animal collection

Three species of economic interest were considered in this study: mussels (*Mytilus galloprovincialis*), razor shells (*Solen marginatus*) and cockles (*Cerastoderma edule*). Twenty mussels (4.97 ± 0.23 cm, total shell length), twenty razor shell specimens (10.65 ± 1.40 cm) and forty cockles (2.39 ± 0.36 cm) were collected in April 2017 (mussels) and May 2017 (razor shells and cockles) from Alfacs Bay (Ebro Delta, NW Spain), an area of high ecological importance in which agriculture, aquaculture, fisheries and tourism coexist (Manosa et al., 2001). The interest of this site relies additionally on the fact that the Ebro Delta is the main Mediterranean shellfish production area of Spain. Mussels were collected from suspended cords at 40° 37' 14.4"N, 0° 39' 12.96"E, whereas sand buried razor shells and cockles were obtained by traditional fishing techniques from coordinates 40°36′10″N, 0°40′11″E and 40°37′35″N, 0° 39′ 40″ E, respectively, all being neighbouring sites.

2.2. Sample processing

The effect that the time lapse between animal collection and dissection may have on CEs activities was tested on the three selected bivalve species. To do this, ten individuals of each species were collected and dissected within a maximum period of 2 h while the same number of individuals was maintained in ice cold boxes for 24 h. From mussels, haemolymph was collected from the adduct muscle using a 1 mL syringe with a 0.21 gauge needle. Haemolymph was frozen at −80 °C and further centrifuged (5000 *g* × 5 min at 4 °C) to obtain a cell-free supernatant just before further analysis. Gills and digestive glands were dissected avoiding contamination by other tissues and frozen immediately in liquid nitrogen and stored at −80 °C until analysis. In the case of razor shells and cockles, only digestive glands were considered given they are the main detoxification organ, and these were preserved following the same procedure as for the mussels digestive glands.

2.3. Tissue preparation

About 0.2 g of tissue was used from each animal for the analyses described further below, except for cockles where samples consisted in two pooled individuals due to the small size of their tissue. Tissues were homogenised (1:5, *w/v*) in ice-cold homogenisation buffer using a Polytron® blender. In the case of gills, homogenisation was carried out in a phosphate buffer (50 mM, pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), while for digestive glands a phosphate buffer (100 mM, pH 7.4) containing 150 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) was used. The resulting homogenates were centrifuged at 10,000 *g* for 30 min at 4 °C, and the post-mitochondrial supernatants were used for the enzymatic determinations. Assay conditions were kept similar and only the sample volume was changed in order to maintain linearity in the enzymatic measurements during the recording. All assays were carried out in triplicate at 25 °C in 96-well-plates using a TECAN Infinite M200 microplate reader.

2.4. Carboxylesterase (CE) and acetylcholinesterase (AChE) activity measures

The activity of CE (EC 3.1.1.1) was measured using the commercial colorimetric substrates *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), 1-naphthyl acetate (1-NA), 1-napthyl butyrate (1-NB), 2-naphthyl acetate (2-NA) and *S*-phenyl thioacetate (PTA). The selection of these substrates was based on a former characterization of CEs in mussel *M. galloprovincialis* (Sole and Sanchez-Hernandez, 2018). Multiple substrates were used for enzymatic determinations due to the occurrence of multiple isozymes generally co-existing in a single tissue homogenate, which display different substrate preference and sensitivity to potential inhibitors (Wheelock et al., 2008). The hydrolysis rate of pNPA and pNPB was determined by a spectrophotometric continuous enzyme assay according to Hosokawa and Satoh (2005). The kinetic assay was performed in a 50 mM phosphate buffer (pH = 7.4), containing the substrate (1 mM, final concentration) and 25 µL of sample. The formation of 4-nitrophenolate was monitored at 405 nm and 25 °C for 5 min. An extinction coefficient of 18 $mM^{-1}\,cm^{-1}$ was