Marine Pollution Bulletin 136 (2018) 61–67

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Exploring alternative biomarkers of pesticide pollution in clams

Montserrat Solé[a,*], Martina Bonsignore[a], Georgina Rivera-Ingraham[b], Rosa Freitas[c]

[a] Institute of Marine Sciences (ICM-CSIC), Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain
[b] Laboratoire Environnement Petit Saut, Hydreco-Guyane, BP 823, 97388 Kourou, French Guiana
[c] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

ARTICLE INFO

Keywords:
Carboxylesterases
Organophosphorus pesticides
Chlorpyrifos oxon
Antioxidant enzymes
Ruditapes decussatus

ABSTRACT

Acetylcholinesterase (AChE) is a reliable biomarker of pesticide exposure although in clams this activity is often very low or undetectable. Carboxylesterases (CEs) exhort several physiological roles, but also respond to pesticides. Searching for an AChE alternative, baseline CE activities were characterised in *Ruditapes decussatus* gills and digestive glands using five substrates suggestive of different isozymes. The long chain *p*-nitrophenyl butyrate and 1-naphthyl butyrate were the most sensitive. In the digestive gland, their kinetic parameters (V_{max} and K_m) and *in vitro* sensitivity to the organophosphorus metabolite chlorpyrifos oxon (CPX) were calculated. IC50 values, in the pM–nM range, suggest a high protection efficiency of CE-related enzymes towards CPX neurotoxicity. Other targeted enzymes were: activities of glutathione reductase, glutathione peroxidase, catalase, glutathione *S*-transferases (GSTs) and lactate dehydrogenase in gills and digestive glands. The high GSTs activity and CE/AChE ratio suggests that *R. decussatus* has a great capacity for enduring pesticide exposure.

1. Introduction

Biomarkers of pollution can be classed as markers of exposure (and/or effect) and biomarkers of susceptibility, the latter being increasingly used as they indicate a higher potential to withstand chemical insults (Schlenk et al., 2008). So far, among the most frequently applied biomarkers of pesticide pollution in bivalves are i) cholinesterases (ChE), including acetylcholinesterase (AChE) activity, indicator of neurotoxic exposure, namely to organophosphorus pesticides (Assis et al., 2010; Fulton and Key, 2001; Matozzo et al., 2005); and ii) antioxidant defences as indicators of oxidative stress reactions (see review by Monserrat et al., 2007 and references therein). Some of the most common antioxidant defences comprise the activities of catalase (transforming hydrogen peroxide (H_2O_2) in water and oxygen), glutathione reductase (reducing oxidised glutathione (GSSG) into the antioxidant molecule glutathione (GSH)) and glutathione peroxidase (reducing H_2O_2 but also other organic peroxides). However, when animals are exposed to environmental stressors these defences can fail to counterbalance the deleterious effects of increased oxygen and nitrogen radical formation, and thus, oxidative markers such as the lipid peroxidation content arise as good indicators of oxidative stress damage (*e.g.* Regoli and Giuliani, 2014). Moreover, facing environmental challenges is an energetically expensive process (Rivera-Ingraham and Lignot, 2017); thus when aerobic metabolism cannot meet the required demands, anaerobic pathways take over, and thus, some biomarkers of such activities (*e.g.* lactate dehydrogenase (LDH)) can be also used to assess the impacts of stressful conditions imposed to bivalves.

Like ChEs, carboxylesterases (CEs) are also B-type esterases that can be measured using different substrates (naphthyl- and nitrophenyl-derivates), indicative of different isoforms (Sanchez-Hernandez and Wheelock, 2009). They have been more recently incorporated in monitoring measures as they seem to respond better than AChE to different chemicals, including pesticides, not only in bivalves but also in other invertebrate groups (Kristoff et al., 2010; Sanchez-Hernandez and Wheelock, 2009; Wheelock et al., 2008). In former studies, CE activities were used as markers, not only because they better respond to pesticide exposures but also because of their higher stoichiometric affinity for these chemicals. These properties confer them a protective role and can therefore be considered biomarkers of susceptibility.

Traditionally, ChE activity in bivalves has mostly been focused on AChE, but despite the use of detergents (*e.g.* Triton X100) in the homogenisation process (which facilitates measurements as it solubilizes membrane-bound enzymes) (Campillo et al., 2013) the activity of this enzyme in clams has been reported to be very low or even undetectable (Valbonesi et al., 2003). Nonetheless, recent *in vitro* studies with bivalves support the inclusion of CEs in the monitoring of not only pesticide pollution, but also the effects of pharmaceuticals and personal care products (Sole and Sanchez-Hernandez, 2018). The combined use

* Corresponding author at: CSIC, Recursos Marinos Renovables, Pg. Maritim de la Barceloneta 37-49, 08003 Barcelona, Spain.
E-mail address: msole@icm.csic.es (M. Solé).

https://doi.org/10.1016/j.marpolbul.2018.08.062
Received 25 June 2018; Received in revised form 27 August 2018; Accepted 28 August 2018

of AChE and CEs (*p*-nitrophenyl acetate; *p*NPA) as a measure of esterase activity has been adopted in *in vivo* studies addressing the impact of pollution on various mollusc species: such is the case of the Mediterranean mussel *Mytilus galloprovincialis* exposed to metals (Franco-Martinez et al., 2016) or the golden mussel *Limnoperna fortune* exposed to the insecticide glyphosate (Iummato et al., 2013). In the latter study, CE (*p*-nitrophenyl butyrate: pNPB) was shown to be more sensitive than AChE in the detection of glyphosate pollution (Iummato et al., 2013).

The Manila and European clams (*Ruditapes philippinarum* and *R. decussatus*, respectively) have been proposed as bioindicators of anthropogenic pollution and/or man-driven physical water modifications, such as those concerning climate change, under field and laboratory conditions (Aguirre-Martinez et al., 2016; Almeida et al., 2014; Almeida et al., 2015; Cruz et al., 2016; Matozzo et al., 2012; Velez et al., 2016a, 2016b, 2016c). Despite the fact that *R. philippinarum* is increasingly used as bioindicator given its wider spatial distribution (associated to the preference of the Manila clam for commercial exploitation), the value of *R. decussatus* as a good bioindicator has been singled out by many (Bebianno et al., 2004; Campillo et al., 2013; Cravo et al., 2013; Dellali et al., 2001; Nadji et al., 2010; Sellami et al., 2015).

Due to the influence of endogenous and physical variables in biomarker responses, it is necessary to characterise baseline activities in a selected species/organ before assessing their physically- and chemically-induced alterations. For the specific case of *R. decussatus,* the yearly variations in AChE activity has already been characterized in digestive glands of Atlantic clams (Cravo et al., 2013) and in whole animal homogenates from SW Mediterranean specimens (*i.e.* Bizerta lagoon, Tunisia) (Dellali et al., 2001). In clams, as well as in other bivalves, gills (Ochoa et al., 2013), whole tissue homogenates (De Marchi et al., 2017) or adduct muscles (Choi et al., 2011) are among the most preferred tissues for conducting such analyses. However, when targeting CEs, the digestive gland is the most relevant tissue given it often shows the highest CE activities (Sole and Sanchez-Hernandez, 2018; Sole et al., 2018).

Thus, the aim of the present study was the assessment of biochemical biomarker activities and partial characterisation of esterase activities (AChE and CEs) in gills and digestive glands from the autochthonous clam *R. decussatus* from a NW Mediterranean area devoted to shellfish production. In addition, *in vitro* responses to the organophosphorus (OP) pesticide metabolite chlorpyrifos oxon and the characterisation of kinetic parameters V_{max} (maximum velocity) and K_m (Michaelis constant for substrate affinity) of CEs using 5 substrates is provided for the first time in this species. The results of this baseline study may be of further application in laboratory and field conditions.

2. Materials and methods

2.1. Animal collection

Specimens of *R. decussatus* (with shell sizes ranging from 4.1 to 4.7 cm) were collected in February 2016 in Alfacs Bay (40° 35′ 20″N 0° 36′ 50″ E) in the Ebro Delta (NE Spain). Sampling was conducted before the regular application of pesticides in nearby rice and crop fields. After collection, organisms were transported under emerged and cold conditions to the laboratory for acclimation and depuration. Water temperature at the sampling site was 11.2 °C.

2.2. Animal acclimation and depuration

About 20 specimens were distributed in two tanks, each containing of about 20 L of seawater under continuous aeration, exposed to natural photoperiod (12:12 h, light:dark), constant temperature (15 ± 1 °C) and salinity (38 psu) at the aquaculture facilities from the ICM-CSIC in Barcelona, Spain. Every other day, clams were fed with a concentrated culture of lyophilized microalgae (Easyreefs®, Cádiz, Spain). The clams were kept in the laboratory for 2 weeks to ensure they were free from any potential chemical residues from their natural habitat. Samples were also confirmed as free from pesticides or other anthropogenic chemicals by GC-MS analysis (data not presented).

2.3. Sample preparation for biomarker determinations

Gills and digestive glands for each of the 20 clams were dissected to conduct the analyses described below. Gills were homogenized in a 1:5 ratio (w:v) using a 50 mM phosphate buffer pH 7.4 containing 1 mM ethylenediaminetetraacetic acid (EDTA). Digestive glands were homogenized in 10 mM Tris–HCl buffer pH 7.6 containing 0.15 M KCl and 0.25 M sucrose, in a 1:4 (w:v) ratio. Homogenization was carried out using an electrically driven Polytron® homogenizer. All analytical determinations were carried out on the supernatant resulting from a 10,000*g* centrifugation for 30 min at 4 °C, corresponding to the post-mitochondrial fraction and containing most of the cytosolic enzymes.

2.4. Esterase activities

Acetylcholinesterase (AChE; EC 3.1.1.7), the pseudocholinesterases (EC 3.1.1.8.) propionylcholinesterase (PrChE) and butyrycholinesterase (BuChE) activities were measured using a sample volume of 25 µL. Each sample was pre-incubated with 180 µM of 5,5 0-dithio-bis-2-nitrobenzoin acid (DTNB) for 2 min, time after which a substrate (which varied according to the targeted esterase, see below) was added. Absorbance was then monitored for 5 min using a Tecan Infinite M200 microplate reader (TECAN, Männedorf, Switzerland) at 412 nm ($\varepsilon = 13.6\ mM^{-1}\ cm^{-1}$) following Ellman et al. (1961) protocol. The substrates used were acetylthiocholine (ATC), propionylthiocholine (PTC) and butyrylthiocholine (BTC) for AChE, PrChE and BuChE activity measurements, respectively. All these were used at a 1 mM final concentration to allow comparison with other studies.

Carboxylesterase (CE; EC 3.1.1.1) activity was measured using the commercial colorimetric substrates *p*-nitrophenyl acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), 1-naphthyl acetate (1-NA), 1-naphthyl butyrate (1-NB) and 2-naphthyl acetate (2-NA). The selection of these substrates was based on a former characterisation with the mussel *M. galloprovincialis* (Sole and Sanchez-Hernandez, 2018) and also other bivalves such as *Solen marginatus* and *Cerastoderma edule* (Sole et al., 2018). Multiple substrates were used for these enzymatic determinations due to the occurrence of multiple isozymes co-existing in a single tissue homogenate, which display different substrate preference and sensitivity to potential inhibitors (Wheelock et al., 2005). The hydrolysis rate of *p*NPA and *p*NPB was determined by a continuous spectrophotometric enzyme assay according to Hosokawa and Satoh (2005). The kinetic assay was performed in a 50 mM phosphate buffer (pH = 7.4) containing 1 mM (final concentration) of substrate and 25 µL of sample (undiluted for gills and 1/5 diluted in the case of digestive gland). The formation of 4-nitrophenolate was monitored at 405 nm at 25 °C for 5 min. The extinction coefficient of $18\ mM^{-1}\ cm^{-1}$ was used to express the hydrolysis of these nitrophenyl esters. The hydrolysis rate of 1-NA, 2-NA and 1-NB was measured following the ultraviolet spectrophotometric method described by Mastropaolo and Yourno (1981). The reaction medium consisted of 50 mM buffer phosphate (pH = 7.4), 0.25 mM (final concentration) of substrate and 25 µL of sample (undiluted for gills and 1/5 diluted in digestive gland). The formation of 1-naphthol was monitored for 5 min at 25 °C. An extinction coefficient of $23.4\ mM^{-1}\ cm^{-1}$ was used for enzyme activity calculations.

2.5. Kinetic determinations

Kinetic parameters V_{max} and K_m were determined to ensure that the enzyme activity assay was at saturating substrate concentrations, allowing for a more accurate detection of differences in CE activity. Six serial concentrations of the substrates *p*NPA, *p*NPB, 1-NA, 2-NA and 1-