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

Montserrat Solé, Georgina Rivera-Ingraham, Rosa Freitas

PII: S1532-0456(18)30079-6
DOI: doi:10.1016/j.cbpc.2018.06.002
Reference: CBC 8427

To appear in: *Comparative Biochemistry and Physiology, Part C*

Received date: 22 March 2018
Revised date: 18 May 2018
Accepted date: 3 June 2018

Please cite this article as: Montserrat Solé, Georgina Rivera-Ingraham, Rosa Freitas , The use of carboxylesterases as biomarkers of pesticide exposure in bivalves: A methodological approach. Cbc (2018), doi:10.1016/j.cbpc.2018.06.002

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

Montserrat Solé[1]*, Georgina Rivera-Ingraham[2] and Rosa Freitas[3]

[1]Institute of Marine Sciences (ICM-CSIC), Pg. Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain.

[2] Laboratoire Environnement Petit Saut, Hydreco-Guyane, BP 823, 97388 Kourou, French Guiana.

[3]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal.

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 less than 2h after being retrieved from their natural habitat, and 24h after being transported in air under cold conditions (6-8ºC) 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 (Vmax and Km) and catalytic efficiency. The *in vitro* sensitivity to the organophosphorus metabolite chlorpyrifos oxon was also calculated. IC50 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.

***Keywords*:** pollution monitoring biomarkers, carboxylesterases, organophosphorus pesticides, cold transport, mussel, razor shell, cockle.

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 et al., 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, carboxylesterases (CEs) are hydrolytic enzymes involved in the detoxification of pesticides (e.g. pyrethroids and carbamates) and the metabolic transformation of some pharmaceutical drugs (Aceña 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 (2h max since they were withdrawn from the water) and 24h 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 40º 37.24'N, 0º 39.216'E, whereas sand buried razor shells and cockles were obtained by traditional fishing techniques from coordinates 40º36'10"N, 0º40'11"E and 40º37'35"N, 0º 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 2h while the same number of individuals was maintained in ice cold boxes for 24h. 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 ºC and further centrifuged (5,000g × 5 min at 4 ºC) 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 ºC 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, homogenization 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 1mM dithiothreitol (DTT) was used. The resulting homogenates were centrifuged at 10,000g for 30 min at 4 ºC, 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 ºC in 96-wellplates 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., 2005). 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 ºC for 5 min. An 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 by Mastropaolo and Yourno (1981). The reaction medium consisted of 50 mM phosphate buffer (pH=7.4), the substrate (0.25 mM, final concentration) and 25 μL of sample. The formation of 1-naphthol was monitored for 5 min at 25 ºC. An extinction coefficient of 23.4 mM^{-1} cm^{-1} was used for enzyme activity calculations. For CE measurements using PTA as substrate and AChE activity determinations, samples (25 μL) were pre-incubated with 180 μM dithiobisnitrobenzoate (DTNB) for 2 min. After this time, either the substrate (1 mM PTA) or 1mM acetylthiocholine (ATC) were added for measuring CE and AChE activities, respectively, and absorbance was recorded for 5 min at 412 nm (ε=13.6 $mM^{-1}cm^{-1}$) following Ellman's protocol (1961). All esterase activities were measured in triplicate and results were expressed as nmol/min/mg protein. AChE was only measured in haemolymph as it is the dominant esterase activity in this tissue while all CEs were measured in digestive glands. In gills, 2-NA was not included in the analysis

as it is not relevant in this tissue and PTA-CE activity is mostly reported in the digestive glands.

2.5. Kinetic determinations

Kinetic parameters K_m and V_{max} were determined to ensure that the enzyme activity assay was at saturating substrate concentrations, allowing for a more accurate detection of species-specific differences in CE activity. Six serial concentrations of the substrates pNPA, pNPB, 1-NA, 2-NA and 1-NB (0.03–1 mM) were assayed in digestive glands of the three bivalve species to estimate V_{max} and K_m values using the Michaelis-Menten equation, and the linearity transformation of Lineweaver-Burk plot. CE activity was measured in triplicate as described in 2.4.

2.6. Inhibition kinetic assays

An initial broader chlorpyrifos oxon (CPX) range concentration (10^{-15}-10^{-3} M) was screened but, due lack of changes at the highest and lowest concentrations, a narrower range (10^{-13}-10^{-7} M) displaying a sigmoidal curve was selected to calculate IC_{50} values for each CE-dependent activity in the three bivalve's digestive glands. Stock solutions of CPX were prepared in bi-distilled water and further serial 10-fold dilutions were made with the buffer used in the enzymatic assay. Inhibition kinetic assays were performed in 96-well bottom flat microplates. Homogenates (90 µL) were incubated individually in the presence of 10 µL of each CPX concentration for 30 min at 25 ºC. During incubation, inhibition was terminated by the addition of the selected substrates, and the residual CE activity was measured as described above in order to explore a dose-response relationship and, accordingly, obtain the associated IC_{50} value.

Total protein content of the samples was determined by the Bradford method (1976) adapted to microplate, using Bradford Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as standard (0.05-1 mg/mL). Absorbance was read at 595 nm.

2.8. Statistical analysis

The comparison between the biomarker activities measured 2h and 24h after sampling as well as the comparison between tissues (gills and digestive glands) in mussels was made using Student's *t*-test. Species statistical differences in digestive gland biomarkers were evaluated using one-way ANOVA after log transformation when data did not meet parametric requirements. Correlations among CE activities were performed using Spearman's correlation coefficients with the level of significance set at $p<0.05$. Statistical analyses were carried out using IBM SPSS package software v24.

For CE inhibition data, the non-parametric U-Mann Whitney test was used to compare residual CE activity values with and without incubations with CPX. Inhibitor concentration causing 50% decrease of controls (IC_{50} value) was obtained from the logistic curve using the GraphPad Prism software (ver. 7.00, GraphPad Software, La Jolla California USA) as described in more detailed by Sole and Sanchez-Hernandez (2018).

3. Results and discussion

3.1. Effect of time lapse between sampling and processing, tissue and species in CE activities

The main goal of this study was to confirm whether a 24h preservation of the bivalves under air exposure in cold conditions had no impact on CE activity measurements, here proposed as potential biomarkers of pesticide exposure.

Sample processing time did not influence CE-related activities in none of the three species (mussels, razor shells and cockles) or tissues (hemolymph, digestive gland and gills) considered in mussels (Table 1). Thus, in future studies aiming to use CE as biomarkers and using these bivalves as bioindicators, either sampling protocol could be adopted. In hemolymph, only AChE activity was considered as it was formerly revealed that it is the most significant B-esterase activity found in this fluid in mussels (Galloway et al., 2002; Sole and Sanchez-Hernandez, 2018). AChE results also confirmed no influence of the sampling protocol (37.19 ± 2.23 (2h) and 31.54 ± 3.26 (24h) in nmol/min/ mg prot; $p > 0.05$).

Table 1 further shows differences in mussel's tissues, being digestive gland CE activities significantly higher than in gills ($p < 0.05$) as well as their total protein content ($p < 0.05$). As far as species-specific differences concerns, only digestive gland activities were compared, being the organ where CEs are mainly expressed. In general, despite the substrate used, the hydrolytic activity followed the order: razor shells>mussels>cockles, coinciding with the total protein content in their respective digestive glands and after expressing the activity per mg protein.

The present results also demonstrated that, in general, activities recorded using pNPB and 1-NB were higher than with the reciprocal acetate forms (pNPA and 1-NA) and 2-NA had the lowest activity as formerly revealed in mussels (Sole and Sanchez-Hernandez, 2018). Nonetheless, in razor shell digestive glands 1-NA was the substrate that expressed the highest hydrolytic activity (Fig.1). In the digestive glands of other invertebrates such as the gastropods *Biomaphalaria glabrata* and *Planobarius corneus*,

1-NA and 2-NA were the preferred substrates followed by pNPA and pNPB (Kristoff et al., 2012; Otero and Kristoff, 2016). As compared to pNPB, CE activity measured using 1-NA and 2-NA as substrates was also about 20-fold higher in the worm *Lumbriculus variegatus* and 30-fold higher in the gastropod *B. glabrata* using whole body tissues (Kristoff et al., 2010). Other studies with the earthworm *Lumbricus terrestris* further support the interest of using multiple CE substrates and reported the most adequate substrate according to the tissue analyzed, which varied even within the digestive system (Gonzalez Vejares et al., 2010). All these observations demonstrate the need to test several substrates even within a given organ in order to find out the most adequate combination for pesticide monitoring.

3.2. Correlation among CE activities using different substrates

In mussel digestive glands, CE-related activities measured with all the six substrates pNPA, pNPB, 1-NA, 1-NB, 2-NA and PTA, were significantly correlated using Spearman's correlation coefficients (ρ = 0.614-0.889; $p < 0.05$; n = 20). In razor shell digestive glands these correlations were: ρ = 0.351-0.889; $p < 0.05$; n = 20 except between pNPB and PTA (ρ = 0.335; $p > 0.05$; n = 20). In the case of cockles correlations ranged between ρ = 0.800 and ρ = 0.967 and they were all significant.

Former studies considering aquatic species had used PTA as substrate (Bonacci et al., 2004; Galloway et al., 2002; Sole et al., 2009; Sole et al., 2010b; Vioque-Fernandez et al., 2007). Nonetheless, Wheelock et al. (2008) proposed the use of a battery of substrates as better alternatives to PTA as this substrate can also be hydrolyzed by paraoxonases and arylesterases. Despite the good correlation among the results obtained using different substrates in bivalves, which is mostly due to the overlapping substrate preference of the CE isoforms or their promiscuous nature, their

combined use should be considered when studying responses to chemical insults as they could differentially affect particular isoenzyme responses.

3.3. Kinetic parameters and response to CPX

To ensure that enzymatic assays were carried out at saturating substrate conditions, V_{max} and K_m as well as its catalytic efficiency (V_{max}/ K_m) were calculated for the five substrates assayed in the digestive glands of the 3 bivalve species (Table 2). The highest K_m obtained was 1 mM for pNPA and pNPB in mussels, supporting the suitability of using this concentration. 1 mM concentration of pNPA was also appropriate for measuring CE activity in other aquatic marine organisms, including several fish species and the shrimp *Aristeus antennatus* (Sole and Sanchez-Hernandez, 2015). For the naphthyl-substrates, 250 μM is here considered as adequate except for the CE measurements with 2-NA as a substrate, for which this concentration may be slightly limiting (K_m 390 μM). Nonetheless, as this substrate displayed the lowest V_{max} and its activity correlates well with the other naphthyl substrates, it could be discarded for further determinations in these bivalve species. For the three species used, the highest catalytic efficiency (V_{max}/K_m) was obtained using the 1-NB as substrate and the lowest with pNPA (Table 2).

We are not aware of any studies reporting the kinetic parameters for these CE-related activities in bivalves. The closest comparisons that could be made correspond to freshwater gastropods (Table 2). V_{max} and K_m values registered for *M. galloprovincialis*, *S. marginatus* and *C. edule* are within the range of those obtained in the digestive gland of the snails *B. glabrata* and *P. corneus*, although the V_{max} obtained using 1-NA and 2-NA was significantly higher in the case of the aforementioned gastropods (Kristoff et

al., 2012; Otero and Kristoff, 2016). Overall catalytic efficiency was also higher using the naphthyl substrates in the snails over the bivalves considered in this study.

In general, former measurements of bivalve CE activity involved the substrates PTA (Bonacci et al., 2004; Galloway et al., 2002; Ochoa et al., 2013), 1-NA or pNPA (Escartin and Porte, 1997; Franco et al., 2016). However, other studies with the freshwater gastropod *P. corneus* (Otero and Kristoff, 2016), earthworms (González Vejares et al., 2010) and mussels (Sole and Sanchez-Hernandez 2018) have demonstrated that, compared to the substrates pNPA and 1-NA, the hydrolysis rate of longer-chain carbon esters (e.g., pNPB and/or 1-NB) is more sensitive to OP inhibition. On four CEs measured in mussel digestive glands, the use of the oxon metabolite of the pesticide chlorpyrifos (CPX) showed two- to three-fold higher affinity (measured as IC50 values, present study) than formerly registered with dichlorvos (Sole and Sanchez-Hernandez, 2018), being pNPB and 1-NB associated CEs the most sensitive ones (Table 3). Similarly, in the terrestrial snail *Xeropicta derbentina*, IC_{50} values for CE using several substrates (1-NA, pNPA and p-nitrophenyl valerate-pPNV-) was between 10^2-10^3 times more sensitive to CPX than to dichlorvos (Laguerre et al., 2009). As for the other bivalves, 1-NB was the most sensitive substrate for razor shell CEs and for cockles 1-NA and 1-NB CEs were more equally sensitive to CPX (Table 3 and Fig. 2). The low IC_{50} (low pM–nM) values obtained for CPX in these bivalves are lower than those observed in several fish species (Koenig et al., 2013 and references therein), rats and humans (Pope et al., 2005) but are more in line with values recorded in selected tissues of the earthworm *L. terrestris* (Sanchez-Hernandez and Wheelock, 2009). Comparative *in vivo* studies with the parent pesticide chlorpyrifos and its oxon metabolite also confirmed that CPX was clearly a more potent CE inhibitor (IC_{50s} in the nM range) in earthworms (Sanchez-Hernandez et al., 2015).

4. Conclusions

The present study demonstrates that bivalves (mussel, razor shell and cockles) maintain their CE activities for at least 24h after sampling as long as animals are maintained in cold (6-8ºC) conditions. These results highlight the potential of using these species as bioindicators of agrochemical pollution, especially when sample collection is difficult in terms of long distances from the laboratory, allowing a significant reduction in sampling costs.

Baseline activities of pollution biomarkers are provided for the first time for *S. marginatus* and confirm that bivalve CEs have high affinity for pesticides such as CPX. This study also provides an array of CE-dependent measures obtained from hydrolysis rates and pesticide affinity (IC50 values) which support that CE values (with butyrate-derived forms as substrate) of mussel or razor shell digestive glands may be an optimal biomarker for pesticide pollution. Nonetheless, this should be further validated under field conditions and under pesticide exposures to demonstrate the usefulness of this approach in coastal marine pollution programs investigating agricultural impacts.

Acknowledgements

This work was financed by Spanish Ministry of Economy, Industry and Competitivity (ref CGL2016-76332-R MINECO/FEDER/UE). The authors are grateful to IRTA personnel for providing samples and lab facilities.

References

Acena, J., Perez, S., Eichhorn, P., Sole, M., Barcelo, D., 2017. Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry. Anal. Bioanal. Chem. 409, 5441-5450.

Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Brate, I.L.N., Schoyen, M., 2017. Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: A review. Mar. Environ. Res. 130, 338-365.

Bianco, K., Yusseppone, M.S., Otero, S., Luquet, C., de Molina, M.D.R., Kristoff, G., 2013. Cholinesterases and neurotoxicity as highly sensitive biomarkers for an organophosphate insecticide in a freshwater gastropod (*Chilina gibbosa*) with low sensitivity carboxylesterases. Aquat. Toxicol. 144, 26-35.

Bianco, K., Otero, S., Oliver, A.B., Nahabedian, D., Kristoff, G., 2014. Resistance in cholinesterase activity after an acute and subchronic exposure to azinphos-methyl in the freshwater gastropod *Biomphalaria straminea*. Ecotox. Environ. Safe. 109, 85-92.

Bonacci, S., Browne, M.A., Dissanayake, A., Hagger, J.A., Corsi, I., Focardi, S., Galloway, T.S., 2004. Esterase activities in the bivalve mollusc *Adamussium colbecki* as a biomarker for pollution monitoring in the Antarctic marine environment. Mar. Pollut. Bull. 49, 445-455.

Burgos-Aceves, M.A., Faggio, C., 2017. An approach to the study of the immunity functionsof bivalve haemocytes: physiology andmolecular aspects. Fish Shellfish Immunol. 67, 513–517.

Burgos-Aceves, M.A., Cohen, A., Smith, Y., Faggio, C., 2018. A potential microRNA regulation of immune-related genes in invertebrate haemocytes. Sci. Total Environ. 621, 302-307.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Total Environ. 247, 295-311.

Chiesa S., Chainho P., Figueira E., Soares A.M.V.M, Freitas R. (2018) Element bioaccumulation, biochemical responses and risk to human consumption of Manila clam *Ruditapes philippinarum* in the Tagus estuary (Portugal). Mar. Pollut. Bull. 126:, 281-292.

Ellman, G.L., Courtney, K.D., Andres Jr, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7.

Escartin, E., Porte, C., 1997. The use of cholinesterase and carboxylesterase activities from Mytilus galloprovincialis in pollution monitoring. Environ. Toxicol. Chem. 16, 2090-2095.

Faggio, C., Pagano, M., Alampi, R., Vazzana, I., Felice, M.R., 2016. Cytotoxicity, haemolymphatic parameters, and oxidative stress following exposure to sub-lethal concentrations of quaternium-15 in *Mytilus galloprovincialis*. Aquat. Toxicol. 180, 258-265.

Fernandez, N., Fernandez-Boan, M., Verisimo, P., Freire, J., 2013. Assessing the spatial variability, level and source of organic chemical contaminants in bivalve fishing grounds on the Galician coast (NW Spain). Mar. Pollut. Bull. 74, 291-301.

Ferrante, M.C., Clausi, M.T., Naccari, C., Fusco, G., Raso, G.M., Santoro, A., Meli, R., 2014. Does the Clam *Ensis siliqua* Provide Useful Information About Contamination by Polychlorinated Biphenyls and Organochlorine Pesticides

Beyond that of Mussel *Mytilus galloprovincialis*? Bull. Environ. Contam. Toxicol. 92, 636-641.

Franco, L., Romero, D., Garcia-Navarro, J.A., Teles, M., Tvarijonaviciute, A., 2016. 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. Mar. Pollut. Bull. 102, 30-35.

Galloway, T.S., Millward, N., Browne, M.A., Depledge, M.H., 2002. Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. Aquat. Toxicol. 61, 169-180.

Gonzalez-Fernandez, C., Albentosa, M., Campillo, J.A., Vinas, L., Fumega, J., Franco, A., Besada, V., Gonzalez-Quijano, A., Bellas, J., 2015. Influence of mussel biological variability on pollution biomarkers. Environ. Res. 137, 14-31.

Gonzalez Vejares, S., Sabat, P., Sanchez-Hernandez, J.C., 2010. Tissue-specific inhibition and recovery of esterase activities in Lumbricus terrestris experimentally exposed to chlorpyrifos. Comp. Biochem. Physiol. 151C, 351-359.

Hatfield, M.J., Umans, R.A., Hyatt, J.L., Edwards, C.C., Wierdl, M., Tsurkan, L., Taylor, M.R., Potter, P.M., 2016. Carboxylesterases: General detoxifying enzymes. Chem. Biol. Interact. 259, 327-331.

Hosokawa, M., Satoh, T., 2005. Measurement of carboxylesterase (CES) activities. In: Costa, L.G., Hodgson, E., Lawrence, D.A., Ozolins T.R., Reed D.J., Greenlee, W.F. (Eds.), Current Protocols in Toxicology, John Wiley & Sons, chapter 4, unit 4.7.

Ivanina, A.V., Sokolova, I.M., 2015. Interactive effects of metal pollution and ocean acidification on physiology of marine organisms. Current Zoology 61, 653-668.

Jebali, J., Ben-Khedher, S., Kamel, N., Ghedira, J., Bouraoui, Z., Boussetta, H., 2011. Characterization and evaluation of cholinesterase activity in the cockle Cerastoderma glaucum. Aquatic Biology 13, 243-250.

Koenig, S., Guillen, K., Sole, M., 2013. Comparative xenobiotic metabolism capacities and pesticide sensitivity in adults of *Solea solea* and *Solea senegalensis*. Comp. Biochem. Physiol. 157C, 329-336.

Kristoff, G., Guerrero, N.R.V., Cochon, A.C., 2010. Inhibition of cholinesterases and carboxylesterases of two invertebrate species, *Biomphalaria glabrata* and *Lumbriculus variegatus*, by the carbamate pesticide carbaryl. Aquat. Toxicol. 96, 115-123.

Kristoff, G., Barrionuevo, D.C., Cacciatore, L.C., Guerrero, N.R.V., Cochon, A.C., 2012. In vivo studies on inhibition and recovery of B-esterase activities in *Biomphalaria glabrata* exposed to "azinphos-methyl: Analysis of enzyme, substrate and tissue dependence. Aquat. Toxicol. 112, 19-26.

Laguerre, C., Sanchez-Hernandez, J.C., Kohler, H.R., Triebskorn, R., Capowiez, Y., Rault, M., Mazzia, C., 2009. B-type esterases in the snail *Xeropicta derbentina*: An enzymological analysis to evaluate their use as biomarkers of pesticide exposure. Environ. Pollut. 157, 199-207.

Manachini, B., Arizza, V., Rinaldi, A., Montalto, V., Sara, G., 2013. Eco-physiological response of two marine bivalves to acute exposition to commercial Bt-based pesticide. Mar. Environ. Res. 83, 29-37.

Manosa, S., Mateo, R., Guitart, R., 2001. A review of the effects of agricultural and industrial contamination on the Ebro delta biota and wildlife. Environmental Monitoring and Assessment 71, 187-205.

Mastropaolo, W., Yourno, J., 1981. An ultraviolet spectrophotometric assay for α-naphthyl acetate and α-naphthyl butyrate esterases. Anal. Biochem. 115, 188–193.

Nilin, J., Monteiro, M., Domingues, I., Loureiro, S., Costa-Lotufo, L.V., Soares, A., 2012. Bivalve Esterases as Biomarker: Identification and Characterization in European Cockles (*Cerastoderma edule*). Bull. Environ. Contam. Toxicol. 88, 707-711.

Nunes, B., Resende, S.T., 2017. Cholinesterase characterization of two autochthonous species of Ria de Aveiro (*Diopatra neapolitana* and *Solen marginatus*) and comparison of sensitivities towards a series of common contaminants. Environ. Sci. Pollut. Res, 24, 12155-12167.

Ochoa, V., Riva, C., Faria, M., Barata, C., 2013. Responses of B-esterase enzymes in oysters (*Crassostrea gigas*) transplanted to pesticide contaminated bays form the Ebro Delta (NE, Spain). Mar. Pollut. Bull. 66, 135-142.

Otero, S., Kristoff, G., 2016. In vitro and in vivo studies of cholinesterases and carboxylesterases in *Planorbarius corneus* exposed to a phosphorodithioate insecticide: Finding the most sensitive combination of enzymes, substrates, tissues and recovery capacity. Aquat. Toxicol. 180, 186-195.

Pagano, M., Porcino, C., Briglia, M., Fiorino, E., Vazzana, M., Silvestro, S., Faggio, C., 2017. The Influence of Exposure of Cadmium Chloride and Zinc Chloride on Haemolymph and Digestive Gland Cells from *Mytilus galloprovincialis*. Int. J. Environ. Res. 11, 207-216.

Pearce, N.J.G., Mann, V.L., 2006. Trace metal variations in the shells of *Ensis siliqua* record pollution and environmental conditions in the sea to the west of mainland Britain. Mar. Pollut. Bull. 52, 739-755.

Pope, C.N., Karanth, S., Liu, J., Yan, B.F., 2005. Comparative carboxylesterase activities in infant and adult liver and their in vitro sensitivity to chlorpyrifos oxon. Regul. Toxicol. Pharmacol. 42, 64-69.

Sanchez-Hernandez, J.C., Wheelock, C.E., 2009. Tissue distribution, isozyme abundance and sensitivity to chlorpyrifos-oxon of carboxylesterases in the earthworm Lumbricus terrestris. Environ. Pollut. 157, 264-272.

Sanchez-Hernandez, J.C., del Pino, J.N., Dominguez, J., 2015. Earthworm-induced carboxylesterase activity in soil: Assessing the potential for detoxification and monitoring organophosphorus pesticides. Ecotox. Environ. Safe. 122, 303-312.

Savorelli, F., Manfra, L., Croppo, M., Tornambe, A., Palazzi, D., Canepa, S., Trentini, P.L., Cicero, A.M., Faggio, C., 2017. Fitness Evaluation of *Ruditapes philippinarum* Exposed to Ni. Biol. Trace Elem. Res, 177, 384-393.

Sehonova, P., Svobodova, Z., Dolezelova, P., Vosmerova, P., Faggio, C., 2018. Effects of waterborne antidepressants on non-target animals living in the aquatic environment: A review. Sci. Total Environ. 631-632, 789-794.

Sole, M., Hambach, B., Cortijo, V., Huertas, D., Fernandez, P., Company, J.B., 2009. Muscular and Hepatic Pollution Biomarkers in the Fishes *Phycis blennoides* and *Micromesistius poutassou* and the Crustacean *Aristeus antennatus* in the Blanes Submarine Canyon (NW Mediterranean). Arch. Environ. Contam. Toxicol. 57, 123-132.

Sole, M., Shaw, J.P., Frickers, P.E., Readman, J.W., Hutchinson, T.H., 2010a. Effects on feeding rate and biomarker responses of marine mussels experimentally exposed to propranolol and acetaminophen. Anal. Bioanal. Chem. 396, 649-656.

Sole, M., Anto, M., Baena, M., Carrasson, M., Cartes, J.E., Maynou, F., 2010b. Hepatic biomarkers of xenobiotic metabolism in eighteen marine fish from NW Mediterranean shelf and slope waters in relation to some of their biological and ecological variables. Mar. Environ. Res. 70, 181-188.

Sole, M., Sanchez-Hernandez, J.C., 2018. Elucidating the importance of mussel carboxylesterase activity as exposure biomarker of environmental contaminants of current concern: An in vitro study. Ecol. Indic. 85, 432-439.

Velez, C., Galvao, P., Longo, R., Malm, O., Soares, A.M.V.M., Figueira, E., Freitas, R., 2015a. *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. Environ. Sci. Pollut. Res, 22, 11890-11904.

Velez, C., Leandro, S., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015b. Biochemical performance of native and introduced clam species living in sympatry: The role of elements accumulation and partitioning. Mar. Environ. Res. 109, 81-94.

Velez, C., Pires, A., Sampaio, L., Cardoso, P., Moreira, A., Leandro, S., Figueira, E., Soares, A., Freitas, R., 2016. The use of *Cerastoderma glaucum* as a sentinel and bioindicator species: Take-home message. Ecol. Indic. 62, 228-241.

Vioque-Fernandez, A., de Almeida, E.A., Lopez-Barea, J., 2007. Esterases as pesticide biomarkers in crayfish (*Procambarus clarkii*, Crustacea): Tissue distribution, sensitivity to model compounds and recovery from inactivation. Comp. Biochem. Physiol. 145C, 404-412.

Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D., 2008. Applications of carboxylesterase activity in environmental

monitoring and toxicity identification evaluations (TIEs). Rev. Environ. Contam. Toxicol. 195, 117-178.

Zuykov, M., Pelletier, E., Harper, D.A.T., 2013. Bivalve mollusks in metal pollution studies: From bioaccumulation to biomonitoring. Chemosphere 93, 201-208.

Figure 1. Percentage of contribution to the total carboxylesterases (CEs) of each CE-related activity using different substrates in the digestive gland of three bivalve species: mussel *Mytilus galloprovincialis*, razor shell *Solen margiatus* and cockle *Cerastoderma edule*.

Figure 2. Curve inhibition of remaining carboxylesterase (CE) activity (in % of control) using different substrates after *in vitro* incubation with different concentrations of chlorpyrifos oxon (CPX) in the digestive gland of three bivalve species: mussel *Mytilus galloprovincialis*, razor shell *Solen margiatus* and cockle *Cerastoderma edule*.

Table 1. Basal enzyme activities in gills and digestive gland of mussels and digestive gland of razor shell and cockle (expressed in nmol/min/mg prot) and protein content (mg/ml). Statistical comparisons in Carboxylesterase (CE-dependent) activities between sampling times and mussel tissues were carried out using Student's t-test. Comparisons between CE-dependent activities in digestive glands of the three bivalves were done using one-way ANOVAs. Significantly different groups are identified by different letters (p<0.05): capital and lower case letters indicate tissue and species comparisons, respectively. n.c. not calculated. Numbers in brackets indicate the number of animals tested.

Species/tissue	**Time after collection**	**Total Protein**	**pNPA-CE**	**pNPB-CE**	**1NA-CE**	**1NB-CE**	**2NA-CE**	***S*-PTA-CE**
Mussel *M. galloprovincialis* Gills	2h (10)	4.38 ± 0.17	24.92 ± 1.40	60.93± 4.28	39.97± 2.61	72.89± 4.97	n.c	n.c
	24 h (10)	4.19 ± 0.34	24.90 ± 1.96	62.33± 3.32	41.47 ± 3.09	77.44± 5.74		
	mean	**4.29 ± 0.19A**	**24.91 ± 1.17**	**61.63 ± 2.64**	**40.72 ± 1.98**	**75.16 ± 3.73**		
Mussel *M. galloprovincialis* Digestive gland	2h (10)	11.32 ± 0.59				115.1 ± 7.12	31.04 ±1.31	87.65 ± 3.03
	24 h (10)	10.14 ± 0.47				114.5 ± 7.06	29.82 ± 1.41	81.41 ± 3.96
			64.05 ± 3.37	132.41 ± 8.62	102.7 ± 4.81			
			61.61 ± 2.62	129.53 ± 8.95	103.8 ± 3.86			
	mean	**10.73 ± 0.39 Bb**				**114.8 ± 4.88a**	**30.43 ± 0.94b**	**84.53 ± 2.53b**
			62.83 ± 2.09a	**130.97 ± 6.06b**	**103.26 ± 3.01b**			
Razor shell *S. marginatus* Digestive gland	2h (10)	15.01±0.35				119.1±10.86	43.37±1.75	176.1 ± 7.90
	24 h (10)	15.60±0.32				133.3±7.838	47.55±1.46	
			99.04±4.86	95.07± 6.48	195.6±10.95			177.7 ± 8.25
			100.5±5.08	111.2 ± 7.58	203.6±7.96			
	mean	**15.31 ± 0.24 c**				**126.2 ± 6.72a**	**45.46 ± 1.21c**	**176.9 ± 5.56c**
			99.76 ± 3.42b	**103.1 ± 5.19a**	**199.3 ± 6.64c**			
Cockle *C. edule*	2h (10)	7.17± 0.30	52.92± 2.35	91.82±8.00	65.82±3.74	102.2±6.02	21.79±1.07	59.36

Digestive gland	(10)	7.45± 0.28	57.70±4 .07	100.5±1 0.1	76.61±5. 16	122.2±9. 66	25.56±2 .13	± 4.6 9 64. 60 ± 4.9 7
	mean	**7.31 ± 0.20 a**				**112.2 ± 2.60a**	**23.68 ± 1.24a**	**61. 98 ± 3.5 7a**
			55.31 ± 2.35a	**96.15 ± 6.33a**	**71.21 ± 3.34a**			

Table 2. Kinetic parameters V_{max} (in nmol/min/mg prot), K_m (in mM) and catalytic efficiency (V_{max}/K_m) corresponding to the digestive gland of the three bivalves selected in this study and digestive gland of another mollusc group: freshwater gastropods with comparable data. For the later, V_{max}/K_m values were estimated from the means obtained from the literature when this value was not explicitly provided. n.a. data not available.

Bivalves		pNPA-CE	pNPB-CE	1NA-CE	1NB-CE	2NA-CE	
Mytilus galloprovincilis	Vmax	164.6 ± 17.4	186.4 ± 8.4	289.1 ± 37.9	393.0 ± 76.9	92.5 ± 10.0	This study
	Km	1.00 ± 0.08	0.33 ± 0.01	0.18 ± 0.03	0.13 ± 0.03	0.39 ± 0.06	
	Vmax/Km	*164.2 ± 9.6*	*558.0 ± 20.8*	*1,631 ± 135*	*3,125 ± 208*	*244.4 ± 16.4*	
Solen marginatus	Vmax	211.1 ± 40.9	154.9 ± 26.1	298.5 ± 20.0	185 ± 14.8	67.44 ± 5.7	This study
	Km	0.72 ± 0.29	0.41 ± 0.06	0.18 ± 0.03	0.08 ± 0.02	0.12 ± 0.01	
	Vmax/Km	*374.6 ± 98.5*	*378.1 ± 41.0*	*1,795 ± 284*	*2,250 ± 144*	*555.8 ± 36.6*	
Cerastoderma edule	Vmax	50.2 ± 6.42	91.2 ± 15.0	92.79 ± 11.24	129.03 ± 19.0	35.93 ± 4.3	This study
	Km	0.09 ± 0.01	0.07 ± 0.013	0.06 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	
	Vmax/Km	*557.7 ± 101*	*1,270 ± 91.6*	*1,883 ± 545*	*3,750 ± 416*	*1,089 ± 122*	
Gastropods							
Biomphalaria glabrata	Vmax	143 ± 7	42 ± 2	2,809 ± 275	n.a	2,798 ± 305	Kristoff et al 2012
	Km	0.38 ± 0.10	0.21 ± 0.06	0.13 ± 0.04	n.a	0.14 ± 0.05	
	Vmax/Km	*376.3*	*200*	*21,608*		*19,986*	
Biomphalaria straminea	Vmax	621 ± 134	394 ± 135	n.a	n.a	n.a	Bianco et al 2014
	Km	0.91 ± 0.31	0.20 ± 0.09	n.a	n.a	n.a	
	Vmax/Km	*682.4*	*1,970*	n.a	n.a	n.a	
Planobarius corneus	Vmax	217 ± 40	299 ± 58	1,817 ± 96	n.a	1,690 ± 259	Kristoff and Otero 2016
	Km	0.284 ± 0.068	0.052 ± 0.010	0.052 ± 0.030	n.a	0.076 ± 0.008	

	Vmax/Km	*764.1*	*5,750*	*3,494*	n.a	*22,237*	
Chilina gibbosa	Vmax	56 ± 7	86 ± 11	n.a	n.a	n.a	Bianco et al 2013
	Km	0.47 ± 0.06	0.21 ± 0.01	n.a	n.a	n.a	
	Vmax/Km	*119*	*409.5*	n.a	n.a	n.a	

Table 3. IC50 (nM) values for carboxylesterases in digestive gland of the 3 bivalves studied using four substrates and after in vitro exposure to chlorpyrifos oxon (CPX). Mean values (and 95% confidence intervals indicated in brackets) after 4 independent measures.

	pNPA-CE	pNPB-CE	1NA-CE	1NB-CE
Mussel	0.0325 (0.0071-0.147)	0.0039 (0.0026-0.0057)	0.0625 (0.0114-0.341)	0.0025 (0.00121-0.00531
Razor shell	0.0395 (0.0295-0.0528)	0.0142 (0.0085-0.0238)	0.0142 (0.0074-0.0271)	0.0048 (0.0023-0.0099)
Cockle	0.0917 (0.0684-0.1229)	0.0850 (0.0637-0.1132)	0.0654 (0.0356-0.1203)	0.0642 (0.0289-0.1428)

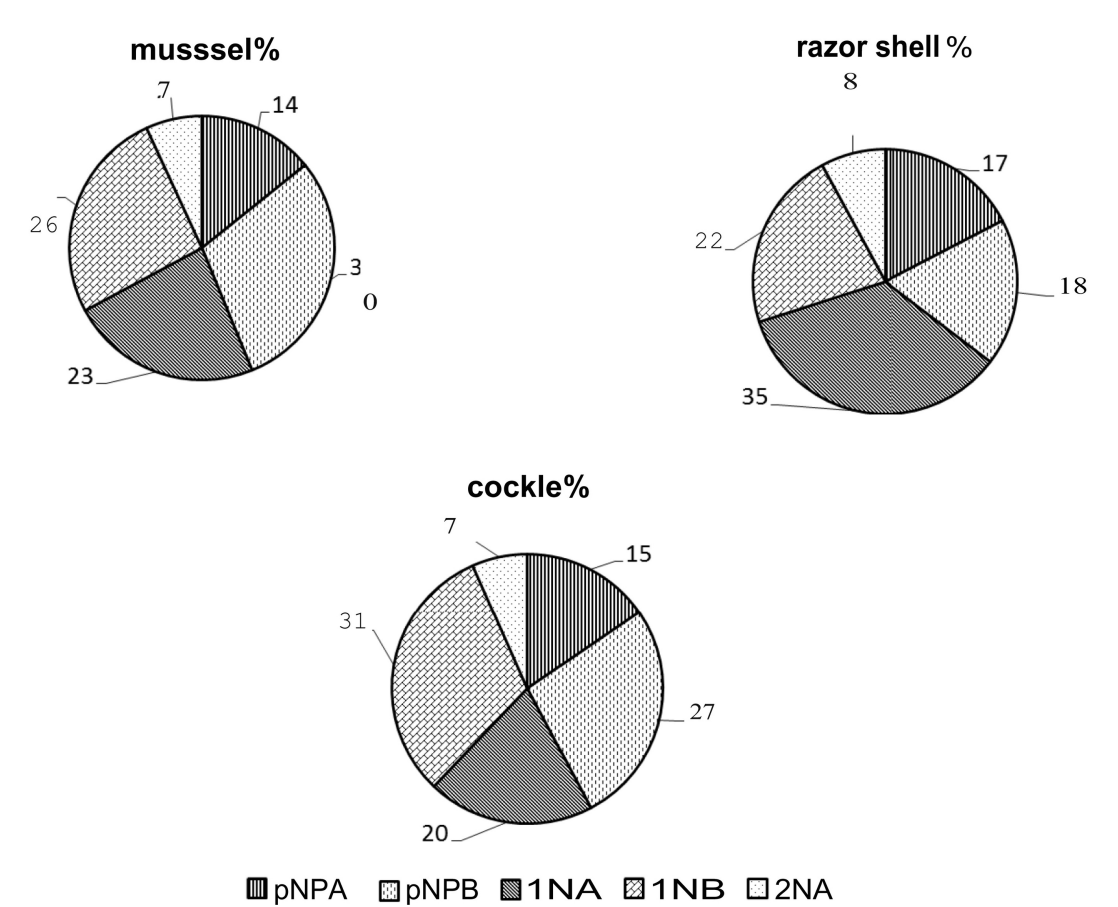
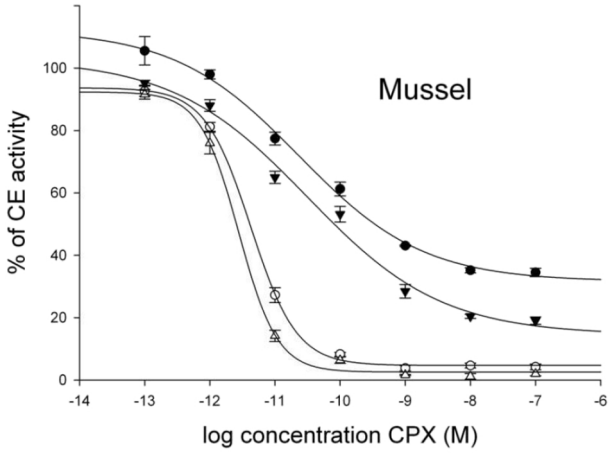
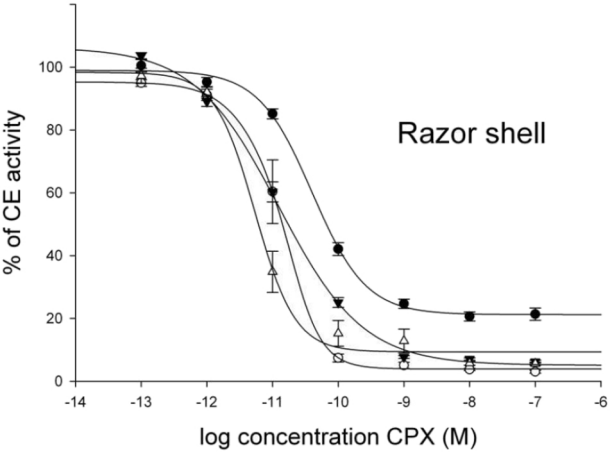

musssel%
7
14
26
3
0
23
razor shell %
8
17
22
18
35
cockle%
7
15
31
27
20
pNPA
pNPB
1NA
1NB
2NA

Figure 1R1


Mussel
% of CE activity
log concentration CPX (M)


Razor shell
% of CE activity
log concentration CPX (M)

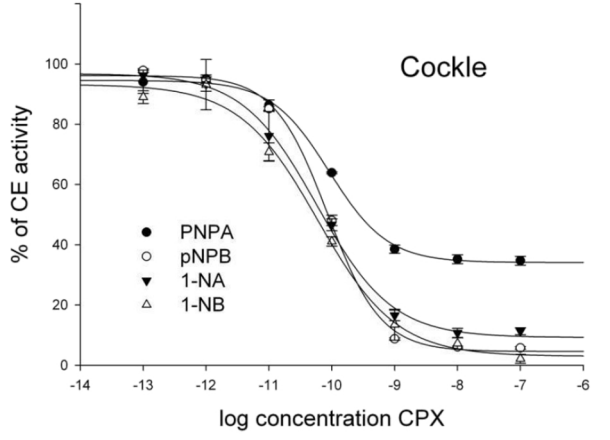

Cockle
% of CE activity
PNPA
pNPB
1-NA
1-NB
log concentration CPX

Figure 1R2