B-esterases characterisation in the digestive tract of the common octopus and the European cuttlefish and their in vitro responses to contaminants of environmental concern

S. Omedes a, M. Andrade b, O. Escolar a, R. Villanueva a, R. Freitas b, M. Solé a,*

a Institut de Ciències del Mar ICM-CSIC, E-08003, Barcelona, Spain
b Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193, Aveiro, Portugal

ARTICLE INFO

Keywords:
Carboxylesterases
Cholinesterases
Octopus vulgaris
Sepia officinalis
Chemicals of environmental concern
Plastic additives

ABSTRACT

Cephalopods are a group of marine invertebrates that have received little attention as sentinel species in comparison to other molluscs, such as bivalves. Consequently, their physiological and biochemical xenobiotic metabolism responses are poorly understood. Here we undertake a comparative analysis of the enzymatic activities involved in detoxification reactions and neural transmission in the digestive tract of two commercial cephalopods: the Common octopus, Octopus vulgaris, and the European cuttlefish, Sepia officinalis. For methodological purposes, several common B-esterases (five carboxylesterase (CE) substrates and three cholinesterase (ChE) determinations) were assayed as a proxy of metabolic and neuronal activities, respectively. Four components of the digestive tract in each species were considered: salivary glands, the stomach, the digestive gland and the caecum. The in vitro responses of digestive gland homogenates to model chemicals and contaminants of environmental concern were contrasted between both cephalopod species. The baseline biochemical activities in the four digestive tract components were also determined. Moreover, in order to validate the protocol, purified proteins, recombinant human CE (CE1 and CE2) and purified eel acetylcholinesterase (AChE) were included in the analysis. Overall, carboxylesterase activities were higher in octopus than in cuttlefish, with the activity quantified in the digestive tract components in the following order: digestive gland ≈ caecum > stomach ≈ salivary glands, with higher hydrolysis rates reached with naphthyl-derived substrates. In contrast, cuttlefish hydrolysis rates with ChE substrates were higher than in octopus. This trend was also reflected in a higher sensitivity to CE inhibitors in octopus and to AChE inhibitors in cuttlefish. Given the detoxification character of CEs and its protective role preventing AChE inhibition, octopus could be regarded as more efficiently protected than cuttlefish from neurotoxic exposures. A full characterisation of B-esterases in the digestive tract of the two common cephalopods is also provided.

1. Introduction

Biomarkers are biochemical and other higher tissue-level changes in organisms in response to environmental abiotic stressors and/or contaminant exposures (Hook et al., 2014). Cephalopods have already been utilized as sentinel species for coastal habitat quality monitoring, either by using physiological biomarkers (Sillero-Ríos et al., 2018) or fatty acid composition at the biochemical level (Arechavala-Lopez et al., 2019). Physiological stress in Octopodidae species related to fishing techniques has also been observed (Barragán-Méndez et al., 2019). In most cases, the target organ for biomarker determinations is the digestive gland, as it is the main metabolic organ in molluscs (including cephalopods) (Barragán-Méndez et al., 2019; Rodrigo and Costa, 2017; Semedo et al., 2012, 2014; Sillero-Ríos et al., 2018). However, because haemocytes are involved in the cephalopods’ internal defence against pathogens as well as other biological and physiological functions, haemolymph is a potential candidate for a conservative target for acute-stress assessment (Locatello et al., 2013). Among some commonly used biochemical biomarkers, carboxylesterases (CEs) belonging to the serine hydrolase family are important enzymes involved in the detoxification of pesticides and other foreign chemicals, as well as being involved in endogenous physiological processes (Hosokawa, 2008; Ross and Crow, 2007; Sanghani et al., 2009; Satoh and Hosokawa, 1998, 2006). These enzymes mainly catalyse the ester bond of multiple

* Corresponding author.
E-mail address: msole@icm.csic.es (M. Solé).

https://doi.org/10.1016/j.envres.2022.112961
Received 26 October 2021; Received in revised form 11 February 2022; Accepted 12 February 2022
Available online 16 February 2022

0013-9351/© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
substrates, releasing the corresponding alcohol and carboxylic acid. So far, only a few studies on cephalopods have focussed on this family of enzymes in relation to their lipase activities (Hoskin, 1996; Mancuso et al., 2014). Together with CEs, cholinesterases (ChEs) are B-esterases which degrade choline-based esters. Their well-recognised role in most organisms, including cephalopods, is to act as neurotransmitters in the nervous system, controlling the excitatory stimulus mostly through acetylcholinesterase (AChE) (Hook et al., 2014). Nonetheless, AChE and the pseudocholinesterases butyrylcholinesterase (BuChE) and propionylcholinesterase (PrChE) may have other physiological roles, such as cell differentiation (Lockridge, 2015), apoptosis, and arm regeneration in cephalopods (Fossati et al., 2013). Another interesting association between both B-esterases is the high affinity expressed by CEs for organophosphorus pesticides (OPs); thus preventing OPs action with the neural transmitter AChE (Wheelock et al., 2008). This protective role conferred by CEs has been regarded as a biomarker of susceptibility to neurotoxic substances (Kristoff et al., 2012).

Digestion in cephalopods takes place along all digestive tract organs: salivary glands, the stomach, the digestive gland and the caecum (Bastos et al., 2020; Boucaud-Camou and Boucher-Rodoni, 1983; Saï et al., 2018). In the Common octopus, Octopus vulgaris, food digestion is initiated externally with the aid of enzymes secreted by the salivary glands, whereas in the European cuttlefish, Sepia officinalis, the digestion process is mainly internal. The enzyme composition of the cephalopods digestive tract is made of many hydrolytic enzymes, including some with esterase-like activities (Balti et al., 2009). During digestion, chemicals within the food can be metabolised and excreted, or generate more reactive metabolites that interact with endogenous molecules, thereby altering key physiological processes.

Both cephalopod species share ecological similarities. They have a similar depth distribution along littoral and coastal waters of the Mediterranean Sea and NE Atlantic, and compete for similar prey, mostly crustaceans, bony fishes and molluscs (Pierce et al., 2010). Although cuttlefish preferentially occupy sandy areas and octopus favour rocky bottoms, both species can be in close contact and, in fact may even prey on each other (Alves et al., 2006; Escolar, unpublished data). Feeding activity in octopus and cuttlefish appears to be higher during crepuscular and dark periods (Guerra, 2006).

Recently, adults of cephalopod species have been used as sentinels of chronic exposure to metal and organic pollution, including emerging contaminants as microplastics (Ariano et al., 2019; Lischka et al., 2020; Martinez-Morillo et al., 2020; Oliveira et al., 2020; Penicaud et al., 2017; Roldán-Wong et al., 2018; Wu et al., 2019). However, cephalopods susceptibility to this group of emerging chemicals, including plastic additives, has not yet been investigated. The use of in vitro approaches to mimic potential in vivo disturbances caused by chemicals has been proposed (Testai, 2001). Recently, in vitro enzyme-substrate interactions using digestive gland homogenates of several marine invertebrates has revealed that some emerging chemicals could lead to toxic consequences in metabolic enzymes such as CEs (Solé et al., 2021a). Metabolic enzymes currently used in vertebrates (CYP450-related enzymes) are not suitable for cephalopods (Rodrigo and Costa, 2017). However, CE measures have been used in other mollusc species, and could be candidate biomarkers for cephalopods as well (Solé and Sanchez-Hernández, 2018).

The aim of this study is to fully characterise B-esterases hydrolytic activities composition on elements of the digestive tract of two common cephalopods. Two species with high commercial interest, the Common octopus, O. vulgaris and the European cuttlefish, S. officinalis, are analysed. The in vitro susceptibility of digestive gland homogenates to chemicals of environmental concern is contrasted between both species, and the potential use of haemolymph from octopus as a conservative biomarker is also investigated. The results of this study provide information on the baseline activities of B-esteras in two cephalopod species, as well as investigate their potential as biochemical biomarkers in pollution monitoring studies. By contrasting the response to in vitro exposures to emerging contaminants in digestive gland homogenates of the two species, we also establish which species is more vulnerable to anthropogenic stressors.

2. Material and methods

2.1. Sample collection

Eight Common octopuses (O. vulgaris) (mean ± SD) of 3036 ± 456 g total weight and 209 ± 12 mm mantle length at an identified sex ratio (M:F) 3:5, and seven European cuttlefishes (S. officinalis) 514 ± 60 g total weight and 162 ± 7 mm mantle length of sex ratio (M:F) 4:3 were captured during April 2019 in the NW Mediterranean, off Vilanova i la Geltrú, Barcelona, Spain (Fig. 1) by the artisanal fishing fleet using pots for octopus and basket traps for cuttlefish. Individuals were transported to the open seawater system of ICM’s Aquaria and Experimental Chambers facility and maintained at least for 1 month in 3000 L circular (octopuses) and in 500 L rectangular (cuttlefish) tanks at the natural seasonal temperature of 14–20 °C and the natural photoperiod. Since the wild specimens sampling was not simultaneous and within a limited geographical area, all cephalopods were given about a 1 week period in laboratory controlled conditions to reduce potential individual variability at the time of sampling. The octopuses were fed daily with live or frozen crabs (Carcinus mediterraneus) and frozen fish (Boga boga, Sardina pilchardus) and cuttlefish with live crabs (C. mediterraneus). The animals were fasted 24 h before sacrifice by using anaesthesia (immersion in 3.5% MgCl2) and subsequent brain destruction. The capture, transport, and maintenance of these individuals in the laboratory as well as the sacrifice followed the protocols of Fiorito et al. (2015). An additional set of octopus individuals captured in the same zone (11M and 6F) with a total weight of 1466 ± 475 g were utilized for haemolymph extraction, using a 5 mL syringe from the systemic heart or branchial vessel. The extracted haemolymph was immediately frozen in dry ice for further biochemical analysis.

The laboratory procedures were approved under the project “Practice of handling, behaviour, haemolymph extraction and euthanasia in cephalopods: Sepia officinalis (cuttlefish) and Octopus vulgaris (octopus)” Ref. 10,243, authorized by the Animal ethics committee of the ICM and the local government, in accordance with the European legislation (2010/63/EU) for the protection of animals used for scientific purposes.

2.2. Sample processing

Components from the main digestive tract were dissected immediately after sacrifice: the salivary glands, the stomach, and the digestive gland. Due to its reduced size in S. officinalis, the anterior salivary gland was not sampled for this species. All tissues were immediately and independently stored at −80 °C until analysis. Each individual sample was homogenized in a 1/5 (w/v) ratio with a phosphate buffer (100 mM, pH 7.4) with 150 mM KCl and 1 mM ethylenediaminetetraacetic acid (EDTA). Afterwards, the homogenates were centrifuged at 10,000 g × 30 min with the temperature kept at 4 °C throughout all processes. The obtained supernatant (S9) was divided into identical aliquots which were stored at −80 °C until analysis.

2.3. B-esterase activities determination

For basal CE activity determinations, 5 different commercial substrates were used: p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), α-naphthyl acetate (αNA), β-naphthyl acetate (βNA) and α-naphthyl butyrate (αNB). In each well, 25 μL of diluted sample and 200 μL of reaction mixture for each substrate were loaded into the microplate wells. The final substrate concentration in each well was either 1 mM (pNPA and pNPB) or 0.25 mM (αNA, βNA and αNB). The dilution of the sample was adapted for each determination to ensure kinetic linearity during the 5 min timeframe of the spectrophotometric
measure. For haemolymph, the sample remained undiluted. The substrates pNPA and pNPB yield p-nitrophenol that was analysed at 405 nm (Hosokawa and Satoh, 2001); whereas naphthol was the metabolite produced when using αNA, βNA and αNB as substrates and it was measured at 235 nm (Mastropaolo and Yourno, 1981).

For ChE measures, the substrates acetylthiocholine iodide (ASCh), butyryl thiocholine iodide (BuSCh) and propionyl thiocholine iodide (PrSCh) were used following the adapted protocol of Ellman et al. (1961) to microplate conditions. The samples (25 μL) were diluted to ensure kinetic linearity and incubated with 150 μL of DTNB (5, 5′-dithio-bis- 2-nitrobenzoate) for 2 min, to eliminate nonspecific hydrolysis, and after that, 50 μL of each substrate (1 mM final concentration) was added and the reaction monitored for 5 min at 412 nm.

### 2.4. Protein content determination

The total protein content of the samples, to which enzyme activities were referred, was determined with the Bradford (1976) method adapted to microplate using the Bio-Rad Protein Assay reagent. In parallel to the samples, a standard was made with bovine serum albumin (0.05–0.5 mg/mL) with readings performed at 495 nm.

### 2.5. In vitro inhibitory effect by chemicals of concern

For the in vitro study, model chemicals and other chemicals of environmental concern (CECs) were selected (Table 1). Recombinant human CE1 isoforms 1 and 2 (CE1, ref. E0162 and CE2, ref. E0412 from Sigma-Aldrich) and electric eel purified AChE (CAS 9000-81-1) were used to validate the protocol and for comparative purposes. The chosen substrates were: pNPB for CE and ASCh for ChE determinations. Contrasting in vitro tests were done only with the digestive gland S9 extracts of both octopus and cuttlefish. The chemicals were diluted in a phosphate buffer and ethanol solution 1:1 (v/v) after it was verified that the buffer and ethanol solution did not affect the enzymatic measures. Since the pollutants used had different solubility and lipophilic properties, a concentration of 50 μM was used as a compromise for all pollutants analysed (Solé et al., 2021a). The samples were incubated with each targeted contaminant and the carrier (control) at room temperature and with gentle shaking during 15 min. Subsequently, the substrates (pNPB or ASCh) were added to 25 μL of the incubated sample, and the residual activity was measured at 405 nm (for CE) or 412 nm (for AChE) during 5 min, as previously described. For the haemolymph samples, the undiluted samples were also incubated for 15 min but a narrower selection of
chemicals were used (see Table 1). All the spectrophotometric analyses were carried out in 96-well plates, in triplicate and at 25 °C in the Infinite Pro200 plate reader (Tecan, Austria).

2.6. Statistical analyses

Normality and equality of variance were verified with the Shapiro-Wilk and Levene tests, respectively. In cases where the data presented a normal distribution and equal variances, a Two Sample t-test was performed. Data that did not present a normal distribution or/and equal variances were analysed with a Wilcoxon test in the two-group comparisons. In more than two-groups comparisons, ANOVA with Post-hoc Tukey was performed for normal distributed data and Kruskal-Wallis with Mann-Whitney test was used for non-normal distributed data. These statistical analyses were carried out using R software (R Core Team, 2020) and car packages (Fox and Weisberg, 2019).

The biochemical measures (aNB-CE, βNA-CE, αNA-CE, pNPA-CE, pNPB-CE, AChE, PrChE, BuChE) corresponding to the 4 targeted tissues of the 2 species (independently) were used to produce an Euclidean distance similarity matrix using PRIMER v6 (Anderson et al., 2008). This matrix was then simplified by calculating the distance among centroids based on the tissue for each species, and then submitted to ordination analysis using Principal Coordinates (PCO) Analysis. Vectors corresponding to the biochemical descriptors were added as supplementary variables superimposed on the PCO graph by using the Spearman test with a correlation >75%.

3. Results

3.1. Baseline B-esterase activities and its particular contribution

Baseline activities corresponding to all B-esterase measured, 5 CE5 and 3 CH3s, in four tissues of octopus and cuttlefish digestive tracts are presented in Tables 2 and 3, respectively. In octopus, the hydrolysis rates for CE measures generally followed the substrate preference order: αNA > αNB ≈ pNPA ≈ pNPB > βNA in a tissue dependent manner. This trend was also confirmed by the percentage of contribution of each individual measure to the total CEs (Table 2). Higher hydrolysis rates were recorded in the digestive gland and caecum, followed by the stomach and salivary glands, which displayed similar rates. In cuttlefish, although substrate preference was similar to that seen in octopus, the CE hydrolysis rates were significantly lower. The CH3s for both species had a high enzymatic activity, with AChE measuring the highest, followed by PrChE and BuChE, with slight variation among tissues: AChE > PrChE > BuChE in the digestive gland, and AChE > PrChE > BuChE in the caecum and posterior salivary glands. In the stomach tissues of cuttlefish this last trend was maintained, while in the stomach tissue of octopus, all CH3s were co-dominant.

Only octopus haemolymph was available for B-esterase analysis of activity data (mean ± SEM) and units expressed per volume of haemolymph (nmol/min/mL). In this matrix, CEs activities were: 124.8 ± 23.7 (pNPA) and 97.3 ± 18.6 (pNPB). CH3s activities were 217.6 ± 9.0 (AChE) and 183.7 ± 11.4 (PrChE). Hydrolysis rates with BuChE were not reliably measurable.

3.2. Sex related differences in B-esterase activities

Despite sample size constraints, sex-related contrasts were attempted, and some statistical differences were able to be identified. In O. vulgaris, female CE activities were greater than in males in both the stomach and anterior salivary gland, reaching significance (p < 0.05) for the substrates αNA, αNB, and βNA. By contrast, in the digestive gland, the CE activities were greater in males, reaching statistical significance for the substrates pNPB and βNA (p < 0.05). In S. officinalis, sex-related trends were also seen as higher activities in the stomach of females with the substrates αNA, βNA, pNPA and pNPB as well as in the digestive gland with the substrate pNPB. Male S. officinalis displayed higher αNA-CE activity in caecum samples.

For O. vulgaris samples, AChE and PrChE were higher in the female posterior salivary gland and anterior salivary gland, respectively. By contrast, BuChE was higher in S. officinalis posterior salivary gland and particularly in the stomach of O. vulgaris males. In cuttlefish, females
had higher BuChE and PrChE activity in the salivary gland than males, whereas males had higher ChEs activities than females in the stomach. The complete set of data for the two species is presented as Figures S1 and S2 supplementary material. Despite some sex-related differences, no conclusive trends could be derived and a larger number of individuals of each sex would be needed to reach sound conclusions.

3.3. Correlation among B-esterase activities

A significant (\( p = 0.05 \)) correlation between all CE measures in the digestive tract of \( O. vulgaris \) (\( n = 40 \)) was obtained. The Pearson correlation coefficient ranged from \( r = 0.688 \) to \( r = 0.950 \) (with the most moderate values corresponding to measures that include the substrate \( \alpha NB \)). Among ChEs, ASCh and PrSCh related measures show a moderately positive correlation (\( r = 0.616 \)) as well as those with BuSCh and

---

**Table 2**

Activity (in nmol/min/mg prot) of carboxylesterases (CE) using different substrates. Results as mean ± SEM of the enzymatic activities in the different tissues of the digestive tract of the two cephalopods: octopus, Octopus vulgaris, (\( n = 8 \)) and cuttlefish, Sepia officinalis, (\( n = 7 \)). In brackets after the activity, the percentage of contribution of each enzymatic measure in respect to the total hydrolysis rate of CEs in each tissue is indicated. Different low case letters indicate biomarker differences per tissue and capital letters between tissues per each substrate. n.a. not analysed. N.S. not significant p > 0.05

| Tissue                  | Octopus Activity (%) | Cuttlefish Activity (%) | t-test p value |
|-------------------------|----------------------|-------------------------|---------------|
| Digestive gland (DG)    |                      |                         |               |
| PNPB-CE                 | 63.92 ± 4.52 (19.8) b| 26.07 ± 1.43 (13.5) c A | <0.05         |
| CE                      | AB                   |                         |               |
| PNPB-CE                 | 63.44 ± 4.71 (19.6) b| 39.93 ± 2.15 (20.6) b A | <0.05         |
| aNCE                   | 95.13 ± 7.48 (29.4) a A| 72.02 ± 2.68 (37.2) a A | <0.05         |
| aNB-CE                 | 82.53 ± 6.12 (25.5) ab | 42.43 ± 2.43 (21.9) b A | <0.05         |
| sNA-CE                 | 18.16 ± 1.67 (5.6) c AB | 13.38 ± 0.43 (6.9) d B | <0.05         |
| Carnec (CA)             |                      |                         |               |
| PNPB-CE                 | 74.84 ± 2.68 (20.6) b A| 19.03 ± 1.37 (10.0) b B | <0.05         |
| CE                      | 80.77 ± 2.92 (22.3) b A | 55.73 ± 5.15 (29.4) a A | <0.05         |
| aNCE                   | 113.69 ± 3.91 (31.3) a A | 75.80 ± 6.39 (40.0) A a | <0.05         |
| aNB-CE                 | 72.18 ± 6.10 (19.9) b | 20.33 ± 2.24 (10.7) b B | <0.05         |
| sNA-CE                 | 21.62 ± 0.86 (6.0) c A | 18.74 ± 1.47 (9.9) b A N. S. | <0.05        |
| Stomach (ST)            |                      |                         |               |
| PNPB-CE                 | 26.80 ± 2.20 (13.6) b C | 11.48 ± 1.25 (13.2) bc C | <0.05         |
| CE                      | 33.87 ± 2.15 (17.2) b D | 17.38 ± 2.19 (20.1) b B | <0.05         |
| aNCE                   | 67.39 ± 6.96 (34.2) a C | 33.97 ± 3.35 (39.2) a B | <0.05         |
| aNB-CE                 | 54.49 ± 5.07 (26.7) b A | 15.18 ± 1.67 (17.5) b B | <0.05         |
| sNA-CE                 | 14.67 ± 1.50 (7.4) b BC | 8.70 ± 1.19 (10.0) b C | <0.05         |
| Posterior salivary gland (PSG) |              |                         |               |
| PNPB-CE                 | 51.15 ± 3.05 (23.4) b A | 9.10 ± 1.52 (15.3) ab C | <0.05         |
| CE                      | 44.54 ± 2.44 (20.3) a C | 13.01 ± 3.12 (21.9) a B | <0.05         |
| aNCE                   | 52.97 ± 4.76 (24.2) a A | 21.11 ± 4.84 (35.5) ab C | <0.05         |
| aNB-CE                 | 58.42 ± 4.97 (26.7) b A | 12.17 ± 2.69 (20.5) A b | <0.05         |
| sNA-CE                 | 11.95 ± 0.99 (5.5) b C | 4.12 ± 1.52 (6.9) b C | <0.05         |
| Anterior salivary gland (ASG) |              |                         |               |
| PNPB-CE                 | 26.83 ± 1.96 (17.7) c C | n. a.                 | 0.05          |
| CE                      | 33.39 ± 2.34 (22.0) bc | n. a.                 | 0.05          |
| aNCE                   | 44.08 ± 3.25 (29.1) a D | n. a.                 | 0.05          |
| aNB-CE                 | 39.42 ± 2.94 (26.0) ab | n. a.                 | 0.05          |
| sNA-CE                 | 8.01 ± 0.72 (5.3) d D | n. a.                 | 0.05          |

PrChE (\( r = 0.535 \)). Between both types of B-estersases, CEs and ChEs, only two significant negative correlations were found: BuChE with pNPB-CE (\( r = -0.387 \)) and BuChE with pNPB-CE (\( r = -0.432 \)) measures. In octopus haemolymph (\( n = 7 \)) pNPB-CE and pNPB-CE measures show a strong positive correlation (\( r = 0.935 \)) as well as AChE and PrChE measures (\( r = 0.866 \)).

In \( S. officinalis \) (\( n = 25 \)), the activities measured in their digestive tract had a lower correlation between CEs compared to ChE-related measures. Among CEs, the most moderate correlation value was seen between the substrates \( \alpha NA \) and \( \alpha NB \) (\( r = 0.475 \)); and the strongest correlation was between pNPB and \( \alpha NA \) (\( r = 0.932 \)). By contrast, the 3 ChE activities were highly positively correlated (\( r = 0.916–0.970 \)). Between both type of B-estersases, ASCh was moderately correlated to pNPB-CE (\( r = 0.535 \)) and to \( pNCE \) activities (\( r = 0.411 \)).

3.4. In vitro inhibitory effect by chemicals of concern on purified enzymes

The inclusion of recombinant human CE1 and CE2 and purified electric eel AChE confirmed the suitability of this in vitro approach to reveal enzyme interactions (Fig. 2). The substrate pNPB was selected for CE and ASCh for AChE related measures. The use of a positive controls for CE (BNPP) and AChE (BW284c51) determinations confirmed almost full inhibition of 98.0 ± 0.5% (CE1), 102.5 ± 0.2% (CE2) and 99.6 ± 0.1% (AChE). The degree of inhibition was expressed in percentage (%) in respect to the controls (0% inhibition and 100% residual activity) after 15 min incubation with a unique 50 μM concentration of the chemicals. The inhibition percentages resulting from the incubation with selected flame retardants on the isomeric CE1 were: TBBPA (99.0 ± 1.7%), DCP (92.9 ± 0.2%), TPhP (91.5 ± 0.4%), EHDHP (79.0 ± 0.4%), and TCP (71.4 ± 0.4%). They were stronger than those reached when using the CE2 isomeric: TBBPA (30.9 ± 1.4%), DCP (31.7 ± 0.7%) and TPhP (42.0 ± 1.5%). Other plastic additives such as BPA inhibited the isomeric CE1 (44.8 ± 1.3%) and CE2 (45.7 ± 1.2%) at a similar extent but for BPAF the inhibition was stronger on CE2 (61.1 ± 0.3%) than on CE1 (36.3 ± 2.6%). Other plastic additives such as TCS and NP acted mostly on CE1 with inhibitions of the 71.4 ± 0.4% and 37.6 ± 2.5%.
respectively. The lipid regulator drugs acted mainly on CE1 (SIM) with an inhibition of 80.0 ± 0.5% and of 15.6 ± 1.2% on CE2 (Feno). The inhibitory action on AChE was also confirmed for incubations with TBBPA (61.6 ± 5.3%), TCS (32.7 ± 4.6%) and NP (34.1 ± 6.4%) and for the bisphenol monomers and OPFRs of about 20% (15.7 ± 5.8% - 26.4 ± 2.7%).

3.5. In vitro inhibitory effect by chemicals of concern on cephalopod digestive gland extracts

The results of the inhibition by a range of CECs at a single 50 μM concentration in octopus and cuttlefish digestive gland extracts are represented in Fig. 3 a for CE1 (using pNPB as substrate) and Fig. 3 b for AChE measures (with ASCh). As expected, the percentage of inhibition in the sample extracts was lower than the reported when using recombinant CE isoforms or purified eel AChE, even when using their

Fig. 2. Percentage of residual carboxylesterase (pNPB-CE) and acetylcholinesterase (AChE) activities in respect to controls after 15 min incubation with the chemicals of environmental concern (full names in Table 1). Data are mean ± SEM (n = 3) of the measures using human recombinant CE1 and CE2 and electric eel purified AChE. The model CE inhibitor BNPP caused an inhibition >98% in CE1 and CE2 and the specific AChE inhibitor BW284c51 caused a reduction of the 99.6% of the basal AChE activity. All activities 20% over and bellow the control line are considered significant.

Fig. 3. Percentage of residual carboxylesterase (CE; a) and acetylcholinesterase (AChE; b) activities in respect to controls after 15 min incubation with the chemicals of environmental concern with digestive gland extracts of Octopus vulgaris and Sepia officinalis. Data are mean ± SEM (n = 4) of the measures. The model inhibitors BNPP and BW284c51 are included as positive control for the CE and AChE measures. *p < 0.05 Indicates significantly different from control activity. Full name of abbreviations can be obtained in Table 1.
respective specific model inhibitors: BNPP and BW284c51. In this case, significant \( p < 0.05 \) species differences were identified. That is, the pesticide BNPP showed greater CE1 inhibition in octopus (41.5 ± 3.6%) than in cuttlefish (17.1 ± 2.6%). However, TBBPA and BPA-E showed a similar degree of inhibition in both species, about 60% and 24%, respectively. Incubations with the plastic additive BPAF caused an inhibitory effect of 35% and 22.5% in octopus and cuttlefish, respectively. TCS caused a significant inhibition of 24.2 ± 0.9% only in cuttlefish. The neonicotinoid, BW284c51 (used as diagnostic control) was responsible for the highest inhibitions: 95 ± 1.0% in cuttlefish and 82 ± 3.0% in octopus. For CECs, no inhibitory effects were observed in octopus AChE activity \( p > 0.05 \) but in cuttlefish extracts TCP caused a 20% inhibition.

3.6. In vitro inhibitory effect by chemicals of concern on octopus haemolymph

Incubation with CECs was narrowed to TBBPA, BPA, BPA-E, BPA-F, TCP and DCP and with the model inhibitors BNPP and BW284c51, all at the adopted 50 \( \mu \)M concentration. In this case, residual CE measures \( n = 4 \) pooled samples suggested an inhibition by TBBPA (24 ± 1.1%) which was even stronger than with the model pesticide BNPP (18 ± 3%). BPA-E (14 ± 1.7%) and BPAF (11 ± 1.1%) also were shown to cause a significant inhibition. The AChE measures in haemolymph had an inhibition of 51 ± 2.4%, though this was only achieved with the diagnostic AChE inhibitor: BW284c51. No other significant inhibitions were revealed with incubations of the selected CECs in this conservative matrix.

**Fig. 4.** Ordination analysis by Principal Coordinates (PCO) using a Euclidian distance similarity matrix of the biochemical data (or enzymatic activity) obtained from the digestive tissues extracts of octopus, Octopus vulgaris (A) and cuttlefish, Sepia officinalis (B). ASG: anterior salivary gland; PSG: posterior salivary gland; CA: caecum; ST: stomach; DG: digestive gland.
3.7. Multidimensional PCO of biomarkers in octopus and cuttlefish

In Fig. 4, the integrative responses of B-estersases in the digestive tract of the two cephalopod species are presented separately. For *O. vulgaris* the first principal component (PCO1) explained 62.5% of total variation among organs, mainly separating the anterior and posterior salivary glands (ASG, PSG) as well as the stomach (ST) on the negative side, from the digestive gland and caecum (DG, CA) located on the positive side. PCO2 explained 30.5% of total variation, strongly distinguishing the ASG on the negative side from the ST in the positive side. The biochemical descriptors superimposed on the PCO showed a strong positive correlation (*p* > 0.75) between all CEs measures and the DG and CA (PCO1 positive side) since the highest activities were attained in these two organs. All ChEs were strongly correlated with the ST on the positive side of PCO2 and negatively correlated with ASG, since lower values of ChEs activities corresponded to this last organ.

Results from cuttlefish indicated that PCO1 explained 78.5% of the total variation among organs, primarily differentiating the PSG and ST on the negative side from the DG and CA on the positive side, similar to what was observed in octopus. PCO2 explained 19.9% of total variation, distinguishing the CA on the negative side from the DG on the positive side. The biochemical descriptors superimposed on the PCO showed a strong positive correlation (*p* > 0.75) between all CEs and ChEs substrates with DG and CA, whereas PSG and ST were negatively correlated with those, particularly ωNB-CE and pNPB-CE measures, indicating low basal values with these two substrates. On the other hand, ChEs were positively correlated with the DG on the positive side of PCO2 whereas CE measures with the substrates ωNPA, pNPA and ωNA were strongly correlated with CA on the negative side of the axis.

4. Discussion

A mandatory step before applying a biomarker approach to environmental monitoring is to identify the most adequate tissue and substrate for enzymatic measures. Tissue selection is usually based on enzymatic hydrolysis rates and biomarker sensitivity to chemicals of concern. In this study, we focused on metabolic enzymes from the digestive tract, as it corresponds to the main site where foreign metabolism (food and xenobiotics) takes place. Thus, several components of the digestive system of two cephalopod species, *S. officinalis* and *O. vulgaris*, were screened for basal activities characterisation of selected B-estersases (CEs and ChEs) and their respective substrate preferences.

This is the first study to comprehensively characterise all the hydrolyse measures in the digestive tract of two commercially important cephalopod species.

Due to the commercial importance of cuttlefish and octopus as well as ongoing efforts to rear these species in aquaculture settings, digestive enzymes involved in food assimilation have been the most studied enzymes thus far. The study by Mancuso et al. (2014) demonstrates there are a wide variety of enzymes in the posterior salivary gland, with decreasing activity values of amylase from this gland to the caecum, and the preferential localisation of lipase (an esterase enzyme closely related CE) in the digestive gland of both cephalopods used in the present study (Mancuso et al., 2014). Other enzymatic measures (lipase, amylase, trypsin and chymotrypsin) were found to be optimised in the digestive gland and posterior salivary gland (and gastric juice from the crop) in the Californian two-spot octopus, *Octopus bimaculoides* (Ibarra-García et al., 2018). However, B-esterase measures have not yet been described in the digestive system with any of the CE and ChE substrates selected here (with the exception of an early study on squid by Hoskin (1990)).

The use of several substrates in CE activity measures is considered a proxy of potentially diverse isoforms of CEs, although there is a high degree of overlapping substrate specificity. Within the total CE activity, measures with the substrate ωNPA were dominant while those with ωNA were the lowest in all components of the digestive tract of both cephalopods. A particular high contribution of the butyrate-derived substrates was noticed in octopus. The relative contribution of particular CE activities (according to substrates assayed) to the total activity load in digestive glands extracts is similar to that seen for other molluscs (see Table 4).

The use of multidimensional tools (in the case of a large set of biological parameters in several tissues/organs) facilitates an integrative picture of the present results. In this case, a graphical output (PCO) was built for each species, considering the dissimilarities among tissues based on the biomarker measures and using the individual correlations between each variable and the PCO axes to compare the relationship between tissues and biomarkers. The PCO analysis for each cephalopod species independently confirmed that CE enzymes were positively correlated in the caecum and digestive gland in both species, coincident with the higher hydrolysis rates recorded, particularly in octopus. Nonetheless, the stomach and especially the salivary glands of both species were the most negatively correlated with CE enzymes, particularly the measures with butyrate-derived substrates in cuttlefish.

PCO also showed that ChE enzymes were better correlated in the digestive gland of cuttlefish and the stomach of octopus. This last result relates to the co-dominance of all ChEs in this organ. In fact, ChE measures in all tissues of cuttlefish were equally or more dominant than in octopus. Thus, the contribution of AChE to ChE measurements was always better represented in all components of cuttlefish digestive tract, and the hydrolysis rates of ChEs in their digestive gland were double those seen in octopus samples. Although AChE is best known for its function at synapses of cholinergic neurons, it is also involved in important non-cholinergic functions such as cell proliferation, differentiation, and apoptosis (Jiang and Zhang, 2008; Soreq and Seidman, 2001), explaining its presence in organs other than the nervous system. Moreover, AChE has been shown to participate in arm development and regeneration of *O. vulgaris* (Fossati et al., 2013) and has been associated with a particular organophosphorus anhydrase in squid giant axons which is thought to act as an AChE protector (Hoskin, 1990). Since no reports are available on B-esterase measures in cephalopods, comparisons can only be made with other invertebrates and mollusc species (Table 4). In terms of non-destructive markers, the use of enzymatic measures in haemolymph is promising. These measures were only possible on octopus and revealed an equal contribution of the substrates ωNPA and pNPB towards CE while ASCh and PrSCh equally contributed to ChE measures, although hydrolysis rates of ChEs doubled that of CEs in haemolymph. Further research is needed before validating its use as a less invasive matrix.

B-esterase’s distribution in other invertebrates, such as the earthworm (*Lumbricus terrestris*), indicate a parallel distribution of ωNA-CE and pNPB-CE activities in 7 tissues, including some involved in digestion (Sanchez-Hernandez and Wheelock, 2009). The gastropod, *Biomphalaria glabrata* presented higher CE activity in the digestive gland than in the other tissues analysed, particularly when the activity was assayed with ωNA, ωNA or pNPA. Using pNPB, the pulmonary region presented higher CE activity while the head-foot region was rich in ChEs and poor in CEs (Kristoff et al., 2012). Overall, in bivalves, ChE activity was found to be lower in digestive glands when compared with other tissues. For instance, Brown et al. (2004) reported that ChE activity in the bivalve *Mytilus edulis* was higher in the foot and gills than in the posterior adductor muscle and the digestive gland. Similarly, Bonacci et al. (2009) reported that ChE activity in the Antarctic bivalve *Adamussium colbecki* was higher in gills than muscle. In the digestive gland. On the other hand, Mora et al. (2009) found that *Prunella vulgaris* and *Mytilus galloprovincialis* CE activity in gills was about four times higher than in muscles, digestive glands and mantle tissues whereas in the bivalve *Corbicula fluminea*, the highest ChE activity was measured in the mantle. In the crustacean *Procambarus clarkii*, ChE activity prevailed in nervous tissue, whereas muscle and digestive gland had very low activity (Vinue-Fernández et al., 2007).

In addition to modifications on baseline activities in healthy specimens by the action of environmental chemicals, alterations on enzymatic correlations patterns can also be indicative of an isof orm
modulation by stressors as seen in some mussel species (Solé et al., 2020).
Thus, the strong correlation between CE measures and ChE substrates suggested in our study conducted with healthy wild specimens, kept over 1 week at laboratory-controlled conditions, could be regarded as a reference point for application in field studies using B-esters as biomarkers of chemical exposures.

The rational for proposing B-esters as markers of exposure to CEC comes from in vitro evidence from purified proteins and digestive gland tissue homogenates. In order to compare our results with other reported studies, the substrates pNPA and AChE were selected for CE and ChE measures, respectively. There was a clear inhibitory action by the model test compounds BNPP on CE, and BW284c51 on AChE measures. There were also confirmed specificities of particular CE isoforms by simvastatin, fenofibrate and loperamide which supports the suitability of this approach. Furthermore, this approach has also been demonstrated in recent literature (Solé et al., 2021a, 2021b). The expected and more evident action of CECs (and model chemicals) when using purified enzymes compared to when using incubated digestive gland extracts is because tissues contain complex mixtures of molecules. In tissues, the interaction between the selected substrate, the chemical of concern and the enzyme are not so straightforward. However, it is also possible that other enzymes are responsible for the hydrolysis of the test substrate as esterases are recognised as “promiscuous” enzymes with broad substrate specificity. The first hypothesis based on competition is also supported by the fact that octopus digestive gland incubations (1:5 diluted) were more responsive than undiluted haemolymph once exposed to the same chemicals, including those used as diagnostic models. Despite limitations on the use of homogenate extracts, TBBPA incubations revealed a high affinity for CEs in cephalopods’ digestive gland (and also with purified eel AChE) and in octopus’ haemolymph, with inhibitions ranging from 24% to 60% of the controls. High in vitro sensitivity of CEs in several marine groups towards TBBPA has been recently demonstrated (Nos et al., 2021). In fact, the toxicity associated to this brominated flame retardant has led to its progressive replacement by organophosphorus based flame retardants (OPFRs) with an expected lower toxicity. However, Tsugohi et al. (2020) recently demonstrated that the non-halogenated alkyl OPFRs selected in this study (EHDPP, TCP, DCP and TPHP) were also able to inhibit rat live microsomal CEs to a greater extent than the model pesticide BNPP. Our results support this inhibitory action using recombinant human CE1 following the inhibition capacity order DCP > TPhP > EHDPP > TCP, with a similar results (around 20%) for eel AChE. For digestive gland extracts, only TCP was responsible for AChE inhibition in cuttlefish. In the present in vitro approach, it has also been possible to verify that OPFR chemicals present a lower toxicity threat than TBBPA to cephalopods, at least in terms of B-esterase interactions.

In addition to flame retardants, other plastic additives, such as BPA and BPA-E interfered (caused an inhibition >20%) on CE1 while the analogue BPAF acted over the CE2 isofrom. Likewise, both cephalopod species revealed higher sensitivity to the BPA analogues, BPA-E and BPAF, which was also confirmed in octopus haemolymph. Former studies have revealed that these plastic additive monomers act on CEs although stronger evidence was achieved with other fluorometric substrates (Zhu, 2017). The antimicrobial agent TCS, also considered a plastic additive, caused a significant inhibitory action in recombinant human CE1 and eel AChE. While in vitro inhibitory action of TCS on CEs in other marine species has been formerly reported (Solé and Sanchez-Hernandez, 2015, 2018), the interaction of this antimicrobial agent in cephalopods had not yet been described. The reported action of TCS on B-esters, the fact that it can bioaccumulate in molluscs (Orvos et al., 2002), together with the CE sensitivity in cuttlefish warrants further in vivo consideration. Likewise, this applies to TCP which interacted in vitro on AChE activity of cuttlefish, and its bioaccumulation in other molluscs has already been proved (Mata et al., 2022).

Some authors have proposed the ratio between CEs/ChE (Kristoff et al., 2012) as a measure of the protective mechanisms conferred by particular CEs in front of neurotoxic chemicals (AChE inhibitors). In a comparative attempt using mean pNPB-CE and AChE activities in the digestive gland of both cephalopods as a proxy of CE and ChE measures, this ratio was close to 1 in octopus and about 10 times lower in cuttlefish (0.18). This means the latter is more vulnerable to marine neurotoxic compounds. Overall, the in vitro approach identified octopus as more sensitive to chemicals that interact with CEs while displaying higher basal activities; whereas cuttlefish were more responsive to neurotoxic compounds and express higher AChE activity. This negative association between basal activities and inhibition potential (seen as low IC50) was
formerly described for pesticides in a range of marine fish species (Ribalta et al., 2015).

5. Conclusions

The present study revealed that CE-related activities were significantly higher in all components of the octopus digestive tract; however, ChEs contribution was particular represented in the cuttlefish digestive gland. These singularities were confirmed by a stronger in vitro inhibitory action on CE in octopus by the diagnostic pesticide BNPP, and to a lower extent by BPAP. On the contrary, cuttlefish were more sensitive to neurotoxic effects since greater AChe inhibition was seen after incubations with the model inhibitor BW283c51 and TCP. TBPBA equally inhibited CE in both cephalopod species but it did not affect AChe. The suggested in vitro toxicities by CECs, such as plastic additives, was observed more strongly in purified enzymes than tissue homogenates and should be further confirmed by in vivo exposures, particularly in sensitive cephalopod populations. The use of octopus’ haemolymph showed similar trends to digestive gland responsiveness, but considering its non-destructive nature, its study should be further explored.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

To the institutional support of the ‘Severo Ohaco Centre of Excellence’ accreditation (CEX2019-000092-S). RV and OE were funded by the Spanish Ministry of Science, Innovation and Universities (OCTOSET project, RTI2018-097908-B-100, MCIU/AEI/FEDER, EU). To CESAM by FCT/MCTES (UIDP/50017/2020+UIDB/50017/2020+ LA/P/0094/ 2020) and the RED RIESCOS “ Evaluación de los Efectos de los Contaminantes Emergentes en Organismos Acuáticos y sobre la Salud Humana”, Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED) ref. 419RTU578.

Appendix A. Suplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.envres.2022.112961.

References

Alves, D.M., Cristo, M., Sendão, J., Borges, T.C., 2006. Diet of the cuttlefish Sepia officinalis (Cephalopoda: sepigidae) off the south coast of Portugal (eastern Algarve). J. Mar. Biol. Assoc. U. K. 86, 429–436. https://doi.org/10.1017/S0025315406013128.

Anderton, M.J., Golery, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods, 1st ed. PRIMER-E, Plymouth, UK.

Arechavala-Lopez, P., Capó, X., Oliver-Cordomi, M., Siller-Rios, J., Busquets-Cortés, C., Sánchez-Jerez, J., 2019. Fatty acids and elemental composition as indicators of aquatic ecosystem health. Integrated Environ. Assess. Manag. 15, 552–557. https://doi.org/10.1002/ieam.1530.

Gueria, A., 2006. Ecology of Sepia officinalis. Vie Milieu 56, 97–107.

Hofield, M.J., Potter, P.M., 2011. Carboxylesterase inhibitors. Expert Opin. Ther. Pat. 21 (8), 1159–1171. https://doi.org/10.1517/14714963.2011.586339.

Hook, S.E., Gallagher, E.P., Batley, G.E., 2014. The role of biomarkers in the assessment of aquatic ecosystem health. Integrated Environ. Assess. Manag. 10, 327–341. https://doi.org/10.1002/ieam.1530.

Hoskin, F.C.C., 1990. An organophosphorus detoxifying enzyme unique to squid. In: Adelman, W.J., Arnold, J.M., Gilbert, D.L. (Eds.), Squid as Experimental Animals. Springer, Boston, MA, pp. 469–480.

Hosokawa, M., 2008. Structure and catalytic properties of carboxylesterase isozymes involved in metabolic activation of prodrugs. Molecules 13, 412–421. https://doi.org/10.3390/molecules13020412.

Hosokawa, M., Satoh, T., 2001. Measurement of carboxylesterase (CES) activities. Curr. Protop. Toxicol. 10, 4. https://doi.org/10.1007/978-3-540-45043-3_4.

Ibarra-Garcia, I.E., Tovar-Ramírez, D., Rosas, C., Campa-Córdova, Á.L., Mazón-Suárez, I.M., 2018. Digestive enzymes of the californian two-spot octopus, Octopus bimaculoides (pickford and McClenagha, 1949). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 215, 10–18. https://doi.org/10.1016/j.cbpb.2017.10.001.

Jiang, H., Zhang, X.-J., 2008. Acetylcholinesterase and apotosis. FEBS J. 275, 612–617. https://doi.org/10.1111/j.1742-4658.2007.06236.x.

Kristoff, G., Chiny Barrionuevo, D., Cacciatorre, L.C., Verrengia Guerrero, N.R., Cochón, 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. 112–113, 19–26. https://doi.org/10.1016/j.aquatox.2012.01.016.

Kristoff, G., Guerrero, N.V., De D’Angelo, A.M.P., Cochón, A.C., 2006. Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: Biomphalaria glabrata and Lymnaea variegata. Toxicology 222 (3), 185–194. https://doi.org/10.1016/j.tox.2006.02.018.

Lischka, A., Lacoue-Labarthe, T., Bumantane, P., Piątkowski, U., Hoving, H.J.T., 2020. Trace element analysis reveals bioaccumulation in the squid Gunat (Lampea) from polar regions of the Atlantic Ocean. Environ. Pollut. 256, 113389. https://doi.org/10.1016/j.envpol.2019.113389.

Locatello, L., Fiorito, G., Finos, L., Rasotto, M.B., 2013. Behavioural and immunological responses to an immune challenge in Octopus vulgaris. Physiol. Behav. 122, 93–99. https://doi.org/10.1016/j.physbeh.2013.08.029.

Lockridge, O., 2015. Review of human butyrylcholinesterase structure, function, genetic variants, history of use in the clinic, and potential therapeutic uses. Pharmacol. Ther. 148, 24–46. https://doi.org/10.1016/j.pharmthera.2014.11.001.

Mancuso, M., Giordano, D., Genovese, L., Denaro, M.G., Caruso, G., 2014. Study of digestive enzymes in wild specimens of Sepia officinalis (Linnæus, 1758) and Octopus vulgaris (Cuvier, 1797). Cah. Biol. Mar. 55, 445–452. https://doi.org/10.21136/cbm.2014.034944.

Martínez-Morcillo, S., Rodríguez-Gil, J.L., Fernández-Rubio, J., Rodríguez-Mozaz, S., Míguez-Santiyán, M.P., Valdes, M.E., Barceló, D., Valcarcel, Y., 2020. Presence of
