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B-esterases activities in several tissues of the marine fish: sea bass and hake, as potential biomarkers of bisphenol A derivatives

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Abstract

In the marine environment, a new threat linked to plastic pollution relates to plastic additives. This threat encompasses multiple chemical compound groups with a high bioaccumulation potential for these chemical mixtures. Hence, informative biomarkers are needed to indicate the effects of environmentally realistic mixtures of these additives. This study proposes an *in vitro* approach using tissue homogenates of two marine fish, the European sea bass and hake, which are both of interest in aquaculture and fisheries. The selected biomarkers are B-esterase activities comprising acetylcholinesterase (AChE) and carboxylesterases (CEs). The physiological role of AChE in brain and muscle is mainly neural transmission, while CEs participate in liver detoxification processes. However, B-esterases are also widely distributed in other tissues/organs, where their role is yet to be determined, but their inhibition may have undesired biological consequences. Here, we compared the interaction of the plastic additives, bisphenol A and some of its derivatives, like tetrabromobisphenol A (TBBPA), with B-esterase activities. We particularly focused not only on the robust and broadly distributed CE enzymes in brain, gonad, liver, kidney, and plasma tissues of two marine fish, but also on the use of two commercial substrates, p-nitrophenyl butyrate and α-naphthyl butyrate, tentatively representing two CE isoforms. The results evidenced specific species and tissue responses that could be due to a diverse isoform composition. They identified sea bass as better protected against neurotoxic exposures, at least in terms of B-esterase composition. The flame retardant TBBPA was the

most reactive to B-esterases inhibition, although Bisphenol A bis (2,3-dihydroxy propyl) ether and Bisphenol F bis (3-chloro-2-hydroxypropyl) ether warrant further toxicology assessments.

Keywords: Acetylcholinesterase, carboxylesterase, marine fish *in vitro.* assessment, plastic additives, bisphenol A analogs

INTRODUCTION

Worldwide population growth boosts the demand for qualitative fish protein and, at the same time, the consequent enhanced anthropogenic discharges related to plastics in the marine environment may compromise fish quality and human health[1]. Among marine fish, European sea bass and hake are two very economically important species that face the widespread threat of worldwide ocean plastic pollution with high concentrations, particularly in the Mediterranean region[2,3]. Additionally, there may be some particular plastic inputs specifically associated with aquaculture practices[4,5]. Also, sea bass is easily cultured in captivity and is, therefore, adequate for aquaculture and laboratory experimentation[6], while hake is only available from extractive fisheries[7]. For pollution assessment purposes, fish samples obtained from laboratory or aquaculture facilities have some logistic advantages that facilitate sample preparation and storage as opposed to the fish coming from commercial fishing vessels with fewer logistics. Despite these limitations, some recent studies have targeted wild fish in terms of plastic pollution research, including hake and sea bass as sentinels[7-10]. Nonetheless, most research that has assessed plastic toxicity has been done on microplastic[11] or nanoplastic[12] exposures in sea bass under laboratory conditions. Less information is available about the threat posed by plastic additives, such as plasticizers like bisphenol A (BPA) which are purposely incorporated into plastic manufacturing and pose a risk to aquatic life and its consumers[13].

Biomarkers are measurable parameters at the suborganismal level capable of identifying the presence of environmental contaminants through changes in their responses or levels. Some biomarkers adopted in pollution monitoring are specific for particular chemical groups, such as metallothionein content for metals. Other non-specific ones inform about an oxidative stress condition, neurotoxicity and/or an imbalanced immunological status[14]. Chemicals associated with plastic pollution, such as those used as additives, can constitute a high percentage in the structural polymer and/or remain adhered to micro-/nanoplastic surfaces, from which they can easily leach to marine media[15]. Of the most frequently manufactured additives, TBBPA stands out for its use as a flame retardant. It results from the brominating of bisphenol A (BPA). BPA is a monomer used to form polycarbonate and epoxy resins. BPA itself and some of its analog derivatives exhibit endocrine disrupting properties[16-18], but also carcinogenicity and reproductive toxicity to humans and marine species[19,20]. Several studies, including those in fish, support the action of BPA as an endocrine disruptor, but also responsible for hepatotoxicity[21,22]. For this toxicity reason, chemically modified alternatives have been proposed, such as bisphenol A diglycidyl ether (BADGE), bisphenol F (BPF), and bisphenol S (BPS)[19]. Nevertheless, these BPA alternatives are not exempt from causing hepatotoxicity or acting as endocrine disruptors[23,24]. In NW Mediterranean Sea waters, plastic waste and associated additives such as BPA, BPS, and BPF have been reported to accumulate in relevant biota from fish to invertebrate groups[25]. Most of these BPA derivatives have also been detected in water, sediment, and several tissues of marine fish from the NW Mediterranean region[26]. Thus, there is a need to find biomarkers capable of integrating and reflecting exposures to environmentally relevant mixtures of chemicals of dissimilar nature, which are collectively considered plastic additives[27,28]. The main concern for the occurrence of BPA and its derivatives in marine seafood fish has been related to the migration from the cans in which they were preserved[29]. However, the concentrations naturally occurring in the muscle of wild fish are in the low ng/g dry weight (d.w) for TBBPA[30]. BPA and up to 6 analogs were found in three commercial Atlantic fish species at a maximum of 20 ng/g d.w[10], but a much higher concentration of BPA,

BPAF, and BADGE of about 20 μg/g d.w was detected in two wild fish species from the Persian Gulf[31]. BPA and two analogs (BPF and BPS) were present in the low μg/g d.w in *Sparus aurata* from the Spanish Mediterranean Sea[32]. BPA itself was detected in the low ng/g d.w in fish from the Tyrrhenian Sea of Italy[33]. Although these individual low concentrations may not represent an actual threat to wild populations of marine fish, higher concentrations occurring in liver, together with their combined action and the continuous release from plastics due to the weathering process at sea, alert the need to find biomarkers indicative of exposures.

B-esterases, such as acetylcholinesterase (AChE) and carboxylesterases (CEs), among others, are ester hydrolases that catalyze the hydrolysis of a carboxyl-ester substrate[34]. Both are susceptible to inhibition by organophosphorus (OP) pesticides, carbamates, and a larger number of environmental contaminants for which they unequivocally reflect neurotoxicity[35-38]. The physiological substrate for AChE is acetylcholine (ACh), a natural compound that participates in neural transmission, but is also a regulatory signaling molecule of immune responses in non-innerved organs, including in fish[39]. CEs constitute a family of enzymes of unknown physiological substrates because they have broad substrate specificity, including fatty acids, hormones, and many endogenous molecules with ester, amide, and thioester bonds[40-42]. They are often considered a first line of defense in the metabolism of drugs, xenobiotics, pesticides, insecticides, and plastics[43]. In fact, their promiscuous nature confers them the potential to reflect environmental exposures to a wide range of chemicals, including plastic additives[44]. To date, *in vitro* evidence exists based mostly on mammalian models, but also fish studies, which indicates that CEs respond to pharmaceutical drugs[45-47], per-polyfluorinated chemicals[48], flame retardants[37,49], and plastic additives, such as BPA and its derivatives[44]. In fish, there is also a large body of *in vivo* evidence for their inhibition by pesticides[50], with fewer data available on their modulation with plastic-related chemicals, such as the additive nonylphenol[51], triclosan[52,53], and BPA[54], microplastics[32,55], and nanoplastics[56,57].

Liver is the key metabolic organ where CE enzymes are more expressed and their physiological role is better understood. CEs also comprise several isoforms and the use of different commercial substrates is recommended for isoform estimation despite high overlapping specificities[38]. Of the available commercial substrates, p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB) are frequently used to relate *in vitro* CE activities[46,58] which, in conjunction with the inhibitor bis-(p-nitrophenyl) phosphate (BNPP)[59] can also help to identify CE activities in fish. From a methodological perspective, the inclusion of commercial purified human recombinant proteins (e.g., hCE1 and hCE2) in *in vitro* assays helps to identify the specificity of chemical-enzyme interactions and, at the same time, provides quality control assurance of the analytical protocols in less studied animal groups. Apart from liver, CEs are also expressed in many other tissues where their role is less obvious, including sea bass[60]. Despite this uncertainty, it is clear that any modulation of these enzymatic activities in a particular tissue/organ can disrupt its physiological role and, at the same time, act as a candidate biomarker of chemical exposure. Brain is particularly relevant because it regulates a vast range of physiological processes. Plastic additives, including BPA, have been detected in fish brains[61], which confirms that they can pass the blood-brain barrier and reach this organ with unpredicted toxicological consequences. The use of blood/plasma for B-esterase activity measurements is particularly valuable because it does not require sacrificing fish and can be easily withdrawn during laboratory experiments performed with anesthetics. However, fish obtained by commercial fishing arts do not allow for easy blood collection on board due to sampling constraints. Alternatively to the use of blood, and despite the scarification of the fish, the use of tissue homogenates is also a realistic approach because it considers the complete enzymatic/molecular load of the targeted tissue. The prospective application of B-esterase activities for monitoring plastic additive exposures in either lab or field scenarios is based on their robustness in thermal stability and storage terms[62]. Moreover, *in vitro* tools estimate potential *in vivo*

outcomes with the advantage of being able to screen a wide range of chemicals in a fast and high-throughput way.

The aim of this study was to characterize B-esterase activities in a species representative of aquaculture (sea bass) and one from traditional fisheries (hake) for bisphenols monitoring purposes. For this, we first determined: (i) baseline activities of B-esterases in selected tissues; (ii) their CE *in vitro* sensitivity to plastic additives of environmental concern nature; and (iii) hepatic cell distribution of CEs either in the soluble fraction of the cytosol or as a membrane bound in microsomes. The discussion is further focussed on the potential of B-esterases as candidate biomarkers of bisphenol pollution based on *in vitro* evidence in several tissues of two marine fish and commercially available purified proteins.

MATERIAL AND METHODS

Chemicals and quality control assurance

Acetylthiocholine iodide (ATC), DTNB (5,5′-dithio-bis- 2-nitrobenzoate), p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB), α-naphthyl acetate (αNA), α-naphthyl butyrate (αNB), purified proteins: recombinant human CE isoforms: CE1 (ref. E0162) and CE2 (ref. E0412) and AChE from electric eel (CAS 9000-81-4. ref. C2888) as well as the model inhibitors and emerging chemicals targeted and their purity were all provided by Sigma-Aldrich [Table 1]. The use of purified proteins and model inhibitors of well-known action over the targeted enzymes was adopted as quality control of the analytical protocols as detailed in our former *in vitro* studies[62-65].

Fish sampling

European sea bass (*Dicentrarchus labrax*; n = 32) was employed for characterization of basal B-esterase activities in several tissues corresponding to the controls used in another experimental trial[60,66]. They were available from the aquaria experimental facilities (ZAE) at our institute (ICM-CSIC) and were treated according to Spanish (RDL 53/2013) and European regulations on vertebrate uses for animal experimentation (2010/63/UE). Procedures were approved by the Ethical Committee of the Generalitat de Catalunya (FUE-2018-00813667).

Hake (*Merluccius merluccius*; n = 30) were obtained from fishing sites on the Catalan coast (NW Mediterranean). Since fish were obtained by traditional fisheries, they were not subjected to ethical regulations. Nonetheless, all steps were taken to speed up the process and use the minimum amount of individuals to limit animal suffering.

Sample preparation for B-esterase determinations in several fish tissues

For the determinations of B-esterase activities in fish tissues, brain, liver, gonad, kidney, and plasma were selected for sea bass, and also for hake, except plasma. The post-mitochondrial S10 extracts were obtained after the centrifugation step of the respective tissue homogenates with specific buffers at a particular weight:buffer ratio [Supplementary Table 1]. The centrifugation performed to obtain tissue S10 extracts was 10,000 g × 30 min. Plasma was obtained after blood centrifugation using heparinized syringes and a centrifugation of 3,000 g × 15 min. All these steps were conducted at 4 °C.

B-esterase activity measurements and protein content

For AChE activity, ATC was the employed substrate following Ellman's protocol adapted to microplate conditions[67]. Samples (25 µL of S10), formerly diluted to ensure the linearity of measurements, were pre-incubated with 150 µL of DTNB for 2 min to eliminate non-specific hydrolysis. Afterwards, the substrate was added (1 mM final concentration) and the reaction was monitored at 412 nm.

Table 1. List of the compounds used for the *in vitro* tests. All chemicals from Sigma-Aldrich were used at a single 50 µM concentration in the incubation mixture. Data from PubChem library

Acronym	Full name	CAS number	Purity	Log kow	Molecular mass	Formula
Diagnostic inhibidors						
BW284c51	1-5-Bis-(4-allydyimethyl)-ammoniumphenyl)pentan-3-one dibromide	402-40-4	≤ 100%	3.91	406.6	$C_{27}H_{38}Br_2N_2O$
BNPP	Bis(4-nitrophenyl) phosphate	645-15-8	99%	2.51	340.2	$C_{12}H_9N_2O_8P$
Eserine	Physostigmine	57-47-6	≤ 100%	1.33	275.4	$C_{15}H_{21}N_3O_2$
Iso-OMPA	Tetraisopropyl pyrophosphoramide	513-00-8	≤ 100%	1.22	342.4	$C_{12}H_{32}N_4O_3P_2$
Plastic additives						
TBBPA	3,3',5,5'-tetrabromobisphenol A	79-94-7	97%	4.50	543.9	$(CH_3)_2C[C_6H_2(Br)_2OH]_2$
BADGE	Bisphenol A diglycidyl ether	1675-54-3	≤ 100%	3.84	340.4	$C_{21}H_{24}O_4$
BPA	Bisphenol A	80-05-7	97%	3.32	228.3	$C_{15}H_{16}O_2$
BPA-E	Bisphenol A bis (2,3-dihydroxypropyl) ether	5581-32-8		2.10	376.4	$C_{21}H_{28}O_6$
BPA-P	Bisphenol A bis (3-cloro-2-hydroxypropyl) ether	4809-35-2	≤ 100%	4.60	413.3	$C_{21}H_{26}Cl_2O_4$
BPF	Bisphenol F bis (3-cloro-2-hydroxypropyl) ether	SIAL-15139	≤ 100%	3.98	385.3	$C_{19}H_{22}Cl_2O_4$

CE activities were measured using the hydrolysis rates of pNPA and pNPB as substrates as a proxy. They resulted from adding 25 µL of appropriately diluted tissue S10 to 200 µL of phosphate buffer, 100 mM, pH 7.4, containing a final concentration of 1 mM pNPA or pNPB. Nitrophenol formation was recorded at 405 nm[68]. CE activities were also measured in the UV mode with substrates αNA and αNB, each at 0.25 mM of the final concentration. Metabolite naphthol formation was recorded at 235 nm[69] .

The linearity of measurements was maintained during the 5-min kinetic readings in the TECAN Infinite 200 microplate spectrophotometer. Activities are expressed as nmol/min/mg prot.

The total protein content (mg/mL), to which to express enzyme activities, was determined by the Bradford method using the Bio-Rad Protein Assay reagent[70]. In parallel to samples, a standard of bovine serum albumin (0.05-0.5 mg/mL) was included for comparison and readings were at 495 nm.

Sample preparation for *in vitro* inhibitory tests

Three additional adult sea bass fish (mean weight of 2.02 ± 0.37 kg and size of 52.5 ± 3.97 cm) were used to assess the *in vitro* responses to plastic additives in brain, gonad, liver, and plasma. Similarly, three pools corresponding to six hake individuals (mean weight of 151.26 ± 69.25 g and size of 27.39 ± 4.07 cm) were made to obtain sufficient sample volumes to run tests under the same *in vitro* conditions as sea bass. Blood could not be obtained from hake due to sampling constraints. Instead, kidney was used for the *in vitro* comparisons.

In vitro inhibition tests of carboxylesterases in several fish tissues

The residual activity (RA) for the measurements of AChE (RA in %) and CE (using pNPB and αNB as substrates) was determined after 15-min incubations at room temperature with either the carrier (control) or the targeted chemicals from Table 1. For the *in vitro* tests, 5 µL of the carrier or test compound was incubated in a final reaction mixture of 100 µL with stocks at 20 mM (1 mM final incubation) or 1 mM (50 µM final incubation), depending on the targeted test. The amount of solvent (carrier) employed was previously confirmed to not interfere with B-esterase measurements, and it was also included in the controls. RA is expressed as a percentage of the hydrolysis rate of the respective carrier controls (100%) and it corresponds to three independent measurements for purified proteins, three distinct individuals (sea bass), or three pooled samples (hake) tissues (Section "Sample preparation for B-esterase determinations in several fish tissues"). The recombinant human CE isoforms hCE1 and hCE2, and the purified AChE from electric eel, were used to validate the *in vitro* protocol and for comparative purposes. It must be noted that the *in vitro* tests with the model compounds were conducted at two distinct concentrations: at the excess of 1 mM to confirm enzyme identity and at 50 µM to gain physiological relevance, as detailed later.

Cellular distribution of liver carboxylesterases (S10, microsomes and cytosol)

In both fish species, the S10 fraction obtained from the liver (n = 30-32) was further centrifuged at 100,000 g × 60 min and 4 °C, and used for analyzing CEs distribution either in the bulk S10 fraction or in the cytosol and microsomes fractions, as detailed elsewhere[66].

Statistical analysis

A non-parametric statistical analysis was done using the Kruskal-Wallis and Dunn *post hoc* test for the comparisons in the *in vitro* tests (n = 3). Modifications of activities greater than 20% were considered significant as adopted elsewhere in this type of study in the case of purified proteins[41]. The bivariate correlations between the CE measurements in the different liver fractions were done using Spearman test. The IBM SPSS system software, v27, was used as the statistical package.

RESULTS AND DISCUSSION

The fish species considered in this study are discussed as potential sentinels of bisphenols exposures during either laboratory experimentation (sea bass) or field monitoring (hake). The comparative *in vitro* approach herein taken refers mainly to the B-esterase enzymes encompassing AChE activities using the physiological substrate ATC and CEs by means of unnatural commercial substrates (pNPA, pNPB, αNA, and αNB).

Baseline B-esterase activities in several sea bass and hake tissues

In Table 2, AChE and CE activities per species and tissue are indicated according to the five independently assayed substrates due to the non-specificity and/or cross-reactivity of many of these substrates. For instance, it is known that butyrylcholinesterase (BuChE) and/or other carboxylesterases are also able to hydrolyze ATC[71,72]. The hydrolysis rates with ATC (mean ± SEM in nmol/min/mg protein) differed in the five compared sea bass tissues from 68.4 ± 4.0 (brain) to 3.8 ± 0.6 (kidney). The particular contribution of AChE activity in tissues in relation to the sum of the total B-esterases (based on the hydrolysis rates for all five substrates) in descending order in sea bass was brain (57%) > muscle (26%) > gonad (7%) ≈ liver (7%) > kidney (3%), which reflect their physiological role in neural transmission as formerly indicated in this species[60,73]. This trend has also been followed in other fish: *Haemulon plumieri*[74], *Sparus aurata*[75], *Anguilla anguilla*[76], and *Solea* spp.[77-79]. In contrast, the AChE hydrolysis rates in hake (in nmols/min/mg prot) were altered and ranged from 77.5 ± 5.6 (kidney) to 10 ± 0.9 (gonad) in this contribution order: kidney (33%) ≈ liver (26%) > muscle (20%) ≈ brain (17%) > gonad (4%). CE activities (also expressed as mean ± SEM in nmol/min/mg prot) were measured using four commercial substrates as being tentatively informative of diverse isoforms. In both fish species, the highest hydrolysis rates were reached in liver using αNB

Table 2. Enzymatic activities (in nmol/min/mg protein) based on the hydrolysis rates (mean ± SEM) using the listed commercial B-esterase substrates assayed in the S10 fraction of several tissues from sea bass and hake. Coefficient of variation indicated in brackets as a percentage (%). The ratio AChE/CE is considered a marker of susceptibility, as proposed elsewhere[73]

B-esterase substrate	Sea bass (*n* =32)	Ratio AChE/CE	Hake (*n* = 30)	Ratio AChE/CE
	Brain			
ATC	68.4 ± 4.0 (32.7)		41.0 ± 1.6 (21.8)	
pNPA	37.7 ± 1.9 (27.9)	1.8	12.7 ± 0.4 (17.1)	3.2
pNPB	41.9 ± 2.1 (28.0)	1.6	19.0 ± 1.2 (34.7)	2.2
αNA	51.5 ± 3.1 (32.7)	1.3	23.0 ± 0.9 (20.5)	1.8
αNB	48.0 ± 2.6 (29.8)	1.4	22.6 ± 1.6 (38.2)	1.8
	Gonad			
ATC	8.24 ± 1.4 (96.2)		10.0 ± 0.9 (48.0)	
pNPA	21.5 ± 3.0 (64.4)	0.4	5.8 ± 0.2 (17.3)	1.7
pNPB	19.4 ± 1.7 (39.5)	0.4	17.7 ± 0.9 (28.2)	0.6
αNA	15.0 ± 2.3 (71.2)	0.6	13.9 ± 0.6 (22.1)	0.7
αNB	11.3 ± 0.9 (35.8)	0.7	30.4 ± 1.6 (28.1)	0.3
	Liver			
ATC	8.7 ± 0.7 (42.5)		60.4 ± 60. (54.2)	
pNPA	122.2 ± 8.7 (41.0)	0.1	10.3 ± 0.29 (15.6)	5.9
pNPB	196.9 ± 6.7 (19.4)	< 0.1	81.6 ± 7.0 (47.1)	0.7
αNA	186.7 ± 8.2 (25.0)	0.1	70.6 ± 4.4 (34.0)	0.9
αNB	291.0 ± 8.7 (16.9)	< 0.1	72.4 ± 3.4 (25.6)	0.8
	Kidney			
ATC	3.8 ± 0.6 (85.2)		77.5 ± 5.6 (39.4)	
pNPA	77.3 ± 3.2 (23.6)	0.1	14.9 ± 0.6 (22.4)	5.2
pNPB	54.3 ± 1.5 (16.0)	0.1	41.2 ± 2.5 (33.1)	1.9
αNA	23.2 ± 1.3 (31.0)	0.2	42.4 ± 2.6 (33.7)	1.8
αNB	26.8 ± 1.2 (24.6)	0.1	49.6 ± 2.4 (26.6)	1.6
	Muscle			
ATC	31.2 ± 1.7 (29.9)		48.3 ± 2.0 (22.7)	
pNPA	7.4 ± 0.4 (29.2)	4.2	3.2 ± 0.1 (16.1)	15.1
pNPB	12.9 ± 0.5 (23.5)	2.4	4.8 ± 0.2 (19.3)	10.1
αNA	19.0 ± 1.1 (31.4)	1.6	24.6 ± 1.2 (25.8)	2.0
αNB	21.3 ± 1.0 (26.2)	1.5	17.5 ± 0.7 (23.4)	2.8

(291.0 ± 8.7) in sea bass and pNPB (81.6 ± 7.0) in hake. CE contribution according to tissue is depicted in Figure 1. Noticeable differences were seen between the liver pNPA-CE contributions in sea bass (46%) and hake (22%). The naphthyl-derived substrates were also more represented in the liver of both species, with a similar distribution in most organs, except in gonads.

As CEs show higher affinity than AChE for OP pesticides and other xenobiotics, they play a protective role in preventing AChE inhibition in several marine fish species[75,78]. In line with this, some authors have proposed the AChE/CEs ratio as a marker of susceptibility to chemical exposures[80]. Our results showed that liver, followed by kidney, was the organ that revealed the biggest species differences and indicated that sea bass was better protected because it displayed higher basal CE activities and the lowest AChE/CEs ratios. Likewise, hake muscle and, to a lesser extent, brain can be regarded as more susceptible tissues and species to neurotoxic substances, at least in B-esterases terms, since they display lower CE hydrolysis rates and a higher susceptibility ratio [Table 2].

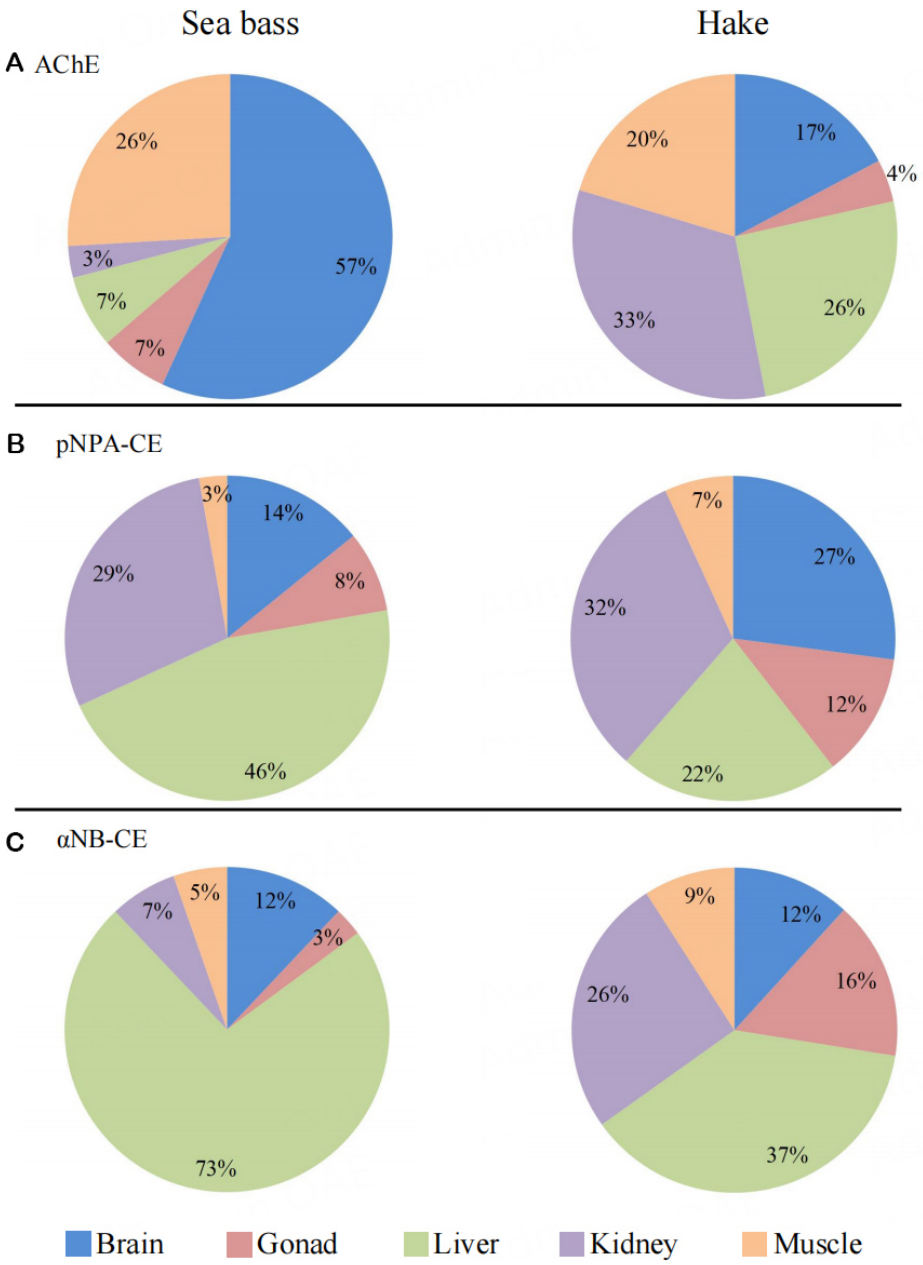

Figure 1. Percentage in contribution of the selected B-esterases: (A) Acetylcholinesterase (AChE), (B) pNPA-Carboxylesterase (pNPA-CE), and (C) αNB-Carboxylesterase (αNB-CE) in several tissues of the two fish species: sea bass and hake. The contribution in each tissue is based on the hydrolysis rates using the three commercial substrates independently.

Of the five B-esterases, two were responsible for species particularities: AChE and pNPA-CE measurements in gonad, liver, and kidney. Recently, a close association between AChE and pNPA-CE activities and immunological status in *S. aurata* has been proposed[81,82]. Therefore, while searching for their physiological role, it is necessary to characterize the true nature of their baseline activities in relevant tissues/organs. The confirmation of hake's singularities in the ATC and pNPA hydrolysis rates in gonad, liver, and kidney was possible with the help of specific inhibitors at an excess 1 mM concentration to ensure that the hydrolysis

rates truly corresponded to the targeted enzymes. The baseline activities of the individuals used for these additional *in vitro* tests are available as [Supplementary Table 2].

The hydrolysis rates in gonad for pNPA-CE and AChE activities were confirmed because poor inhibition was achieved after incubation with BNPP (5%) or BW284c51 (13%) as selective CE and AChE inhibitors, respectively. The extent of pNPA-CE inhibitions of 94.4% (hCE1) and 52.4% (hCE2) with BNPP assured the quality of the protocol. In contrast, when using substrate pNPB, the gonadal activity of the controls was markedly inhibited with BNPP (59.5%), but not with BW284c51 (7.5%). Likewise, the inclusion of hCE1 and hCE2 in the assay confirmed the adequacy of this substrate, with 95% and 100% inhibitions with BNPP. As expected, BW284c51 caused no inhibition of any CE measurements with either substrate (pNPA or pNPB). In contrast, AChE activity was inhibited by 86% after BW284c51 incubations, and not as much after BNPP (23.3%) and, thus, supports the fact that ATC can also be metabolized by enzymes other than AChE. The unexpected low hydrolysis rates attained for pNPA in hake liver were confirmed because BNPP caused no inhibition of basal pNPA-CE activity, whereas significant inhibitions were accomplished with the carbamate eserine (28.9%), the BuChE inhibitor iso-OMPA (15.5%), and BW284c51 (51.9%). Thus, the measurements taken with this broad-spectrum substrate (pNPA) likely corresponded to cholinesterases (AChE and BuChE) rather than to CEs. The third tissue in hake to present unexpected hydrolysis rates was kidney. In this case, BNPP led to lesser pNPA-CE inhibition (4.5%) than pNPB-CE (78.5%), while AChE activity was almost completely suppressed by BW284c51 (99%). Nonetheless, employing BNPP in the incubation mixture also yielded 19.4% inhibition of AChE, which suggests that a fraction of CEs can also hydrolyze ATC, as previously outlined[71,72]. Purified eel-AChE was included to confirm the suitability of the selected substrates and inhibitors. In this case, AChE activity was fully inhibited with BW284c51, while BNPP caused only 8% inhibition. These findings evidence poor cross-reactivity when using ATC as a substrate.

B-esterases inhibition of purified AChE and recombinant CEs by plastic additives

Additional *in vitro* tests were performed with environmentally relevant plastic additives (BPA and its derivatives) based on former pieces of evidence obtained in mammalian models at a lower 50 μM concentration to fit concentrations used in these types of *in vitro* approaches in other animal groups [Table 1]. To fulfill this goal, substrates ATC (for AChE) and pNPB (for the CE-related measurements) and purified proteins were used. Almost complete inhibitions occurred in AChE activity when using purified eel-AChE and BW284c51 or in the pNPB-CE activity of recombinant hCE1 or hCE2 and BNPP [Figure 2]. The flame retardant TBBPA caused greater AChE activity inhibition of purified eel-AChE at 65.6% and of pNPB-CE activity of hCE1 (95.6%) than hCE2 (51.5%). This high *in vitro* binding capacity of TBBPA has also been evidenced within a range of aquatic marine species, including zooplankton[49], several invertebrate and fish species[47,49,83,84], dolphins[64], and sea turtles[62]. In particular, TBBPA binds to CE, but also to plasmatic albumin[64] and could, therefore, be easily transported through the blood to all organs, and even cross the blood-brain barrier and interfere with many endocrine and physiological processes that are regulated in the brain. The BPA analogs BPA-E caused a significant and similar 20% inhibition of pNPB-CE activity for hCE1, hCE2, and eel-AChE activity, while BPF affected pNPB-CE activity with hCE2 (30%) and AChE activity on purified eel-AChE (40%). BADGE inhibited the AChE activity of purified eel-AChE and the pNPB-CE activity of hCE1, and both by 25%. The inhibitions of purified proteins hCE1, hCE2, and eel-AChE after the BPA and BPA-P incubations were not significant [Figure 2]. So, despite the reported endocrine disrupting properties for BPA and most of its analogs, no clear interaction with this purified eel-AChE and recombinant hCE1 and hCE2 was herein evidenced. However, the ability of BPA-E and BPF to interfere with this enzymatic system using purified proteins warrants further study. Other *in vitro* approaches using fluorometric probes have reported an interaction with CE2 activities (from human liver microsomes), which resulted in a conformational change and higher hydrolysis rates[39]. To the best of our knowledge, this is the only reported study to provide *in vitro* evidence for the interaction of BPA derivatives

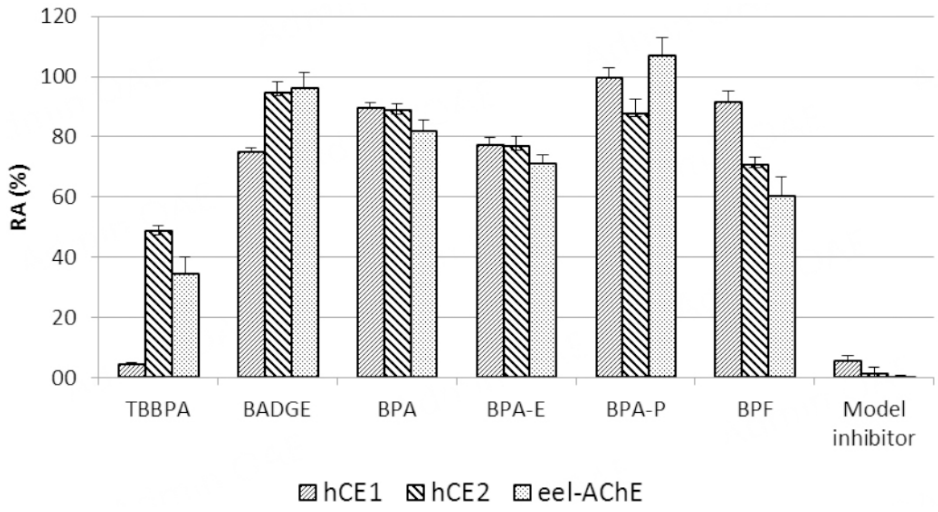

Figure 2. Percentage of remaining activity (RA) with respect to controls (representing 100% activity) after *in vitro* incubation with a single 50 μM concentration of several BPA derivatives and model inhibitors: BNPP for recombinant human carboxylesterases using pNPB as substrate and BW284c51 for AChE measurement with purified electric eel-AChE. Full names are shown in Table 1. The inhibition rate corresponds to 100-RA. All inhibitions > 20% are considered significant, as recommended elsewhere[41].

on CE enzymes using a unique screening concentration of 100 μM, which falls in line with the 50 μM concentration herein adopted. In NW Mediterranean waters, BPA derivatives have been detected at all levels of the marine trophic web, from zooplankton[85] to invertebrates and fish[25,26]. However, the toxicological consequences of environmental mixtures have only been recently explored in fish from this region[7,32,86-88]. Due to the complexity and high cost of comprehensive chemical analyses, using *in vitro* tools in early screening biomarker(s) tests is highly recommended.

CEs inhibition by plastic additives using several sea bass and hake tissues

In view of the stronger modulation of CEs by the BPA analogs, we extended the same *in vitro* protocol to tissue homogenates in sea bass and hake at the same 50 μM concentration. This concentration commonly used in pharmacology was applied as a first approach here as in mammalian models[41,85] and in fish exposed to pharmaceuticals and other environmental contaminants[42,68,72]. The selected tissues expressed pNPB and αNB activities to varying but measurable extents, and their interaction with the BPA derivatives could indicate the affinity of the enzyme for the chemical of concern, its buffering capacity, but also a potential disruptive action on the tissue.

Using the post-mitochondrial fraction (S10) of fish tissue homogenates, CE-related activities were significantly, but not completely, reduced in both sea bass and hake, and the extent of these inhibitions was tissue-dependent [Figure 3]. Note that plasma was available only for sea bass, and kidney was instead used in hake for comparative purposes. The inhibitory action of TBBPA on pNPB-CE activity was evident in all the sea bass organs in the liver > brain > gonad order, while only ≈20% inhibition was seen in plasma. In hake, the inhibitions after TBBPA incubation were around 80%, regardless of the tested tissue, and were greater in kidney. The use of the substrate αNB also revealed significant inhibition of its associated CE activity by TBBPA in sea bass and hake. However, the inhibitions in the latter were around 40% in all tissues, but lower than with pNPB (≈80%). The BPA analog, BPA-E, caused similar inhibition (40%-50%) of plasmatic pNPB- and αNB-CE basal activities in sea bass. pNPB-CE activity in the brain of sea bass was inhibited by 50%, but only by 25% in hake. pNPB-CE activity was also modified in sea bass liver (40% inhibition) and gonads (30%), but not in hake. Another BPA analog, BPF, only caused a significant

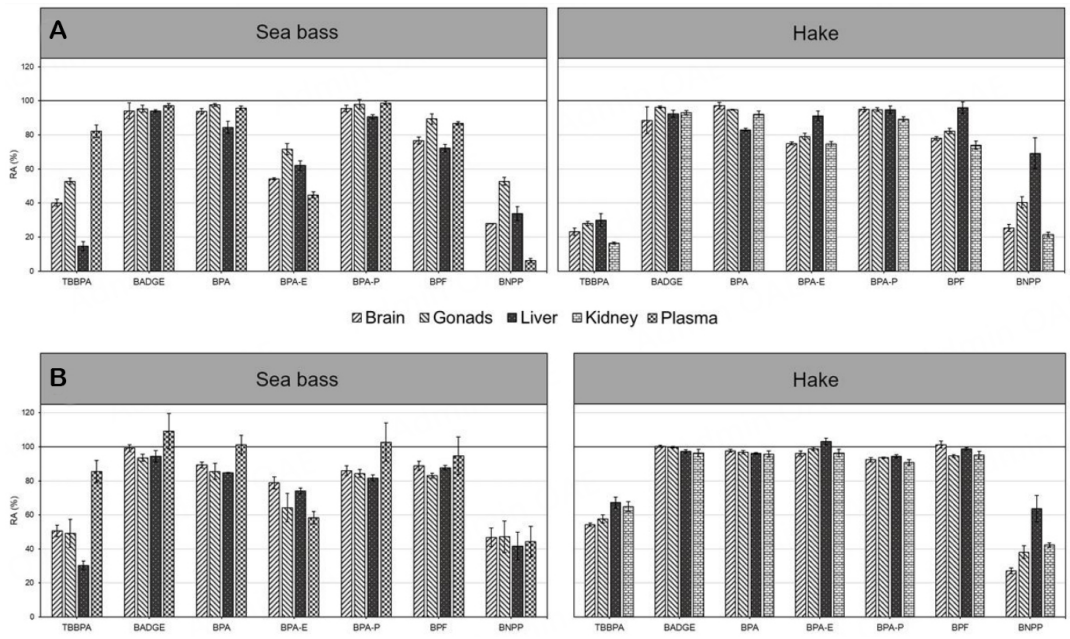

Figure 3. Percentage of remaining activity (RA) with respect to controls (representing 100% activity) after *in vitro* incubation with a 50 μM concentration of several BPA derivatives and the model inhibitor BNPP in several tissues of sea bass and hake using (A) pNPB and (B) αNB as substrates. Due to methodological constraints, plasma was only analyzed in sea bass and instead kidney in indicated hake. Full names are shown in Table 1. All inhibitions > 20% were seen as significant.

inhibition of pNPB-CE activity by 30% in sea bass liver. The inclusion of model CE inhibitor BNPP in the *in vitro* assay confirmed substrate-, species- and organ-/tissue-dependent differences. In sea bass, the BNPP modulation of pNPB-CE activity exhibited the inhibition gradient plasma > brain > liver > gonad, but it was of similar magnitude (about 50%) in all the tissues in the αNB-CE measurements. In hake, however, both substrates displayed a similar response, and liver was the least affected organ, while brain was the most affected one with a 73% inhibition.

In liver, CEs play a clear metabolic role, while a more protective task to prevent AChE inhibition has been appointed in plasma[75]. In other tissues, their role is still under study, but their modulation by BPA derivatives could have physiological consequences because they can bioaccumulate in several fish tissues[26]. In turn, a strong correlation of enzymatic measurements using diverse substrates is indicative of high overlapping specificity and, therefore, denotes the same isoform. Thus, a particular action of a chemical on a particular isoform is likely to alter this correlation and, consequently, be seen as a sign of disturbance. The present *in vitro* approach did not consider the analysis of isoforms, but used different substrates as a proxy of diverse isoforms[38]. From our results, it can be speculated that the two species showed a different isoform profile that, in turn, was tissue-dependent. This is not surprising because sea bass belongs to the Sparidae family (*O. gadiformes*) and hake to the Merluciidae family (*O. perciforms*), which are two very distant phylogenetic groups.

Out of the two assayed CE substrates, pNPB proved more sensitive to inhibition, as revealed by model pesticide BNPP, but also after TBBPA incubations, particularly in sea bass. The apparent lack of inhibition by TBBPA in plasma could be due to the presence of albumin, as seen in humans[89], and as supported herein when comparing sea bass (plasma) and hake (kidney). That is, in sea bass plasma, a high inhibition by BNPP (> 90%) contrasted with only a 20% one with TBBPA, while in hake kidney, the inhibition by

Table 3. Subcellular distribution of hepatic carboxylesterases (CE) based on the hydrolysis rates reached with each commercial substrate independently (in %). The sum of all hydrolysis rates in the 3 fractions for each measurement corresponds to 100 % and each specific CE activity represents its contribution to the total

	S10		Cytosol		Microsomes	
	Sea bass	Hake	Sea bass	Hake	Sea bass	Hake
pNPA-CE	17.3	29.6	5.9	30.7	76.8	39.6
pNPB-CE	24.4	16.3	26.1	13.5	49.5	70.2
αNA-CE	26.5	15.5	14.8	6.4	58.8	78.2
αNB-CE	24.7	10.5	26.5	8.8	48.8	80.2

BNPP and TBBPA was of a similar magnitude (about 80%). In sea bass, inhibition of pNPB-CE activity after the BPA-E incubations occurred in plasma but also in brain, liver, and gonad. Our results alert for CE modulation of BPA derivatives in the tissues of two commercial species with unknown *in vivo* consequences. This work also highlights the potential of CE as biomarkers to reflect exposure to bisphenols, particularly in sea bass.

CE subcellular distribution in liver of sea bass and hake

Pursuing the hypothesis that different substrates may inform about distinct isoforms, further separation of subcellular fractions was attempted in liver to discriminate between more soluble (cytosolic) and membrane-bound (microsomes) isoforms against the bulk S10 fraction[41]. Moreover, due to the aforementioned potential overlapping substrate specificity, the sum of the hydrolysis rates was calculated in the three subcellular fractions for all four substrates independently. In Table 3, the particular substrate contribution in the three factions is indicated relative to the total (sum of the 3 fractions; 100%). In sea bass, pNPA-CE activities predominated in microsomes (76.8%) but not cytosol (5.9%), while in hake contribution of this substrate in cytosol was 30.7% and microsomes were equally represented by the other three substrates (70.2%-80.2%). A balanced contribution was observed when considering the bulk S10 fraction (cytosol and microsomes) in sea bass (17.3%-26.5%) and hake (10.5%-29.6%). The preferential microsomal location in fish CEs coincides with the endoplasmic reticulum location in several mammalian tissues[41].

The Spearman correlation coefficients between the CE measurements in liver using different substrates were suggested as indicative of the degree of overlapping substrate specificities, and were also revealed to be species-dependent [Supplementary Table 3]. In sea bass liver, the Spearman correlation coefficients of the CE measurements in the S10 (ρ = 0.419-0.886), the cytosol (ρ = 0.787-0.953), and microsomes (ρ = 0.507-0.824) were all significant and positive with lower values on those involving S10 and the cytosolic measurements with pNPA. The number of positive and significant correlations was even smaller when considering all the substrates, except pNPA, in microsomal and cytosolic fractions. In hake, the correlations between the CE measurements in S10 (ρ = 0.370-0.609), the cytosol (ρ = 0.497-0868), and microsomes (ρ = 0.444-0.818) were lower and not all significant, particularly when the cytosol was included. As seen for sea bass, in liver of hake, the correlations were lower when including the cytosol.

Altogether, the subcellular hepatic distribution and the correlation coefficients observed suggest that in the two fish, CEs are mostly membrane-bound, but they may correspond to diverse isoforms. Thus, further studies into *in vitro* interaction with CE enzymes using the microsomal fraction may guarantee more sensitivity to bisphenols and be regarded as a first screening step in toxicity assessments.

CONCLUSION

B-esterase activities were characterized in the brain, gonad, liver, plasma, and kidney of two marine fish. Based on greater CE activities and lower AChE/CE ratios, sea bass seemed more protected from chemical inputs. However, other protective mechanisms that are not addressed in this study should be considered. The adopted *in vitro* protocol assessed the interaction of BPA and its derivatives, with B-esterase activities. It revealed that TBBPA and, BPA-E and BPF to a lesser extent, could interfere with CE enzymes in several relevant marine fish tissues, with hake being particularly sensitive to TBBPA and pNPB the most adequate substrate. The subcellular distribution of the associated CE activities and their correlations suggested that the liver of the two studied fish likely reflects a diverse isoform composition with preferential membrane-bound distribution in microsomes. Given the complexity of tissues and species, former baseline characterization and *in vitro* screening are recommended before validating CEs as biomarkers of bisphenols exposures. Any interactions of these chemicals of concern with CE enzymes could alter brain, reproductive organs, and xenobiotic metabolism functioning, and even prevent the transporting role of plasmatic proteins, such as albumin.

DECLARATIONS

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Authors' contributions

Contributed with the design, data analysis, interpretation, and writing: Solé M

Contributed to data adquisition, analysis and critically revising the manuscript: Omedes S, Brandts I, Esté vez J

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Procedures were approved by the Ethical Committee of the Generalitat de Catalunya (FUE-2018-00813667).

Consent for publication

Not applicable.

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