Tetrabromobisphenol A inhibits carboxylesterase activity in marine organisms from different trophic levels

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20 Tetrabromobisphenol A (TBBPA), a brominated flame retardant used in synthetic polymers and
21 electronics, is present in the aquatic environment and recent evidence suggests it can be
22 potentially biomagnified in the marine ecosystem. However, the toxicity of TBBPA in the
23 marine biota has not been investigated in detail. In this study we aimed to understand the role of
24 carboxylesterases (CEs) in xenobiotic metabolism under the exposure of marine organisms to a
25 chemical of environmental concern, TBBPA. Specifically, we tested for *in vitro* inhibition of CE
26 activity in a range of marine organisms covering different ecological niches, from species from
27 low (mussels and copepods), medium (sardines and anchovies) and high trophic levels (tuna).
28 The results revealed that the highest inhibition of CE activity to 100 μ M TBBPA was recorded in
29 mussels (66.5% inhibition) and tunids (36.3-76.4%), whereas copepods and small pelagic fish
30 showed comparatively lower effects (respectively, 30% and 36.5-55.6%). Our results suggest
31 that CE-mediated detoxification and physiological processes could be compromised in TBBPA-
32 exposed organisms and could ultimately affect humans as many of them are market species.

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34 **Keywords:** carboxylesterases, tetrabromobisphenol A, *in vitro* inhibition, marine food web

Brominated flame retardants are routinely used as additives in many consumption goods (plastics, textiles and electronic equipment) to reduce flammability (Pittinger and Pecquet, 2018). Polybrominated diphenylether, hexabromocyclododecane and tetrabromobisphenol A (TBBPA) are the main polybrominated flame retardants used. Main emission sources of TBBPA into the environment are the manufacturing of brominated flame retardants, e-waste recycling and wastewater treatment plants (Malkoske et al., 2016). Because of their physicochemical characteristics, brominated flame retardants have been defined as recalcitrant pollutants, and TBBPA in particular has been detected in terrestrial and aquatic compartments (Morris et al., 2004; Abdallah, 2016; Malkoske et al., 2016;). However, the study of the toxicity of TBBPA has received little attention in marine ecosystems, despite its presence in marine water, sediment and biota. Recently, Choo et al. (2019) found species-specific differences in the accumulation of brominated flame retardants, including TBBPA, in marine organisms from South Korean waters. Interestingly, these authors detected higher concentrations of TBBPA in pelagic fish than in demersal fish and benthic invertebrates, suggesting a biomagnification of these chemicals through the food web. This assumption is reasonable since the estimate of lipophilia identified by the octanol-water partition coefficient ($\log K_{OW}$) for TBBPA is 5.90 (Abdallah, 2016).

The knowledge on the toxicity attributed to TBBPA originates mainly from studies on mammalian models that have shown it can function as endocrine disruptor (Lai et al., 2015), oxidative stress inducer (Jarosiewicz et al., 2019), alter adipocyte differentiation (Akiyama et al., 2015) and potential carcinogen (Lai et al., 2015). Although the toxic effects of TBBPA on non-model organisms (wildlife) are little documented (Pittinger and Pecquet, 2018), recent studies have shown TBBPA can cause growth impairment in marine bivalves (Jiang et al., 2019). Moreover, it is known that TBBPA degradation by-products resulting from abiotic and biotic transformation (e.g. debromination, deconjugation) in the environment in some instances could also be toxic to the organism (Liu et al., 2018). The interaction of this chemical (or any of its

byproducts) with phase-I metabolizing enzymes such as cytochrome P450-dependent monooxygenases and esterases has been scarcely investigated (Zhu, 2017). In this regard, carboxylesterases (CEs) are relevant members of the esterase family of enzymes (Lenfant et al., 2013), catalyzing the hydrolysis of a wide range of carboxylic esters, including different xenobiotics. In mammals, CEs are involved in important physiological processes such as lipid metabolism (Ross et al., 2010; Lian et al., 2018), pro-drug activation (Sato and Hosokawa, 2006), pesticide metabolism (Ross et al., 2006), and hydrolysis of phthalates (Ozaki et al., 2017). Likewise, CEs participate in the detoxification of organophosphorus pesticides via irreversible binding of the pesticide to the active site of the enzyme (Chambers et al., 2010), therefore reducing the circulation of toxic chemicals and thus, providing protection.

Given the potential impact of TBBPA in the marine ecosystem and the scarce knowledge on the responses of marine organisms to it, in this study we have assessed the *in vitro* inhibition species-specific differences in sensitivity of CEs to TBBPA in selected organisms from the northwestern Mediterranean Sea covering different ecological niches and trophic levels in the marine food-web. Our broad aim was to attain a deeper understanding of the interaction between TBBPA and CE activity, as a main phase I metabolic pathway, and test the use of CEs as a proxy for TBBPA-induced xenobiotic effects.

2. Material and methods

2.1. Samples

We used purified human recombinant CE1 (ref. E0162; lot #SBL4639) and CE2 (E0412; lot #SBLT8473), purchased from Sigma-Aldrich, as reference standards for CE activity. Invertebrate CE activity was first determined in the mussel *Mytilus galloprovincialis* (mean shell length 5.45 ± 0.27 cm; $n=8$), which were obtained from aquaculture farms in the Ebre Delta (St. Carles de la Ràpita, Spain). Mussels were transported to the laboratory in a cooler and dissected after arrival. Digestive glands were separated and immediately flash frozen in liquid nitrogen and

94 individually stored at -80°C until analysis. Samples were homogenized in (1:5, w/v) cold
95 phosphate buffer 0.1 M (pH 7.4) containing 150 mM KCl, 1 mM ethylenediaminetetraacetic acid
96 (EDTA), and 1 mM dithiothreitol (DTT). Homogenates were centrifuged at 10,000g for 30 min,
97 and the supernatant (post-mitochondrial fraction) used for CE determinations. Copepods used in
98 the study came from cultures of the copepods *Paracartia grani* and *Centropages typicus*, reared
99 in the ICM-CSIC facilities. Pools of 50 adult females (4 replicates per species) were
100 homogenized by sonication with 250 µl of 100 mM buffer phosphate containing 1 mM EDTA.
101 The livers of two small pelagic fish (*Sardina pilchardus* and *Engraulis encrasicolus*) and four
102 tuna species (*Euthynnus alletteratus*, *Auxis rochei*, *Sarda sarda* and *Thunnus alalunga*) were
103 sampled from the Catalan Sea (northwestern Mediterranean) by commercial fisheries. More
104 details are given in Table 1. Fish livers were individually homogenized in cold phosphate buffer
105 0.1 M (pH 7.4) containing 1 mM EDTA at the same w/v ratio as mussel digestive glands.

106 107 2.2. Enzyme activity

108 CE activity in mussels was measured using four different substrates: 4-nitrophenyl acetate (4-
109 NPA), 4-nitrophenyl butyrate (4-NPB), 1-naphthyl acetate (1-NA), and 1-naphthyl butyrate (1-
110 NB). Activity assay with the substrates 4-NPA and 4-NPB was performed as described in
111 Hosokawa and Satoh (2005), whereas assays with 1-NA and 1-NB were run according to the
112 method by Mastropaolo and Yourno (1981). More details on stock preparations and buffer
113 characteristics can be found in Sole and Sanchez-Hernandez (2018).

114 The in vitro inhibition assays consisted of the incubation of the samples (n=4 replicates) for
115 30 min at room temperature with either the buffer (control) or the presence of TBBPA (CAS
116 number 79-94-7) at a final concentration of 100 µM. Preliminary trials showed that the TBBPA
117 carrier (ethanol) did not interfere in the CE determinations at the highest concentration (0.1%).
118 After the 30-min incubation period, residual CE activity was recorded using the four substrates
119 as described above. Measurements were carried out in triplicates and at 25°C in a TECAN

Infinite 200 microplate reader. Linear reactions were registered using the kinetic assay mode (Magellan V6.0 data analysis software). Total protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as standard (0.05-1 mg/ml) and absorbance was read at 595 nm.

2.3. Statistical analysis

Basal activities were compared by t-Student and ANOVA tests. Significant inhibition of CE activity was detected by comparing the ranges between control and samples incubated with TBBPA using non-parametric Mann-Whitney tests. Statistical analyses were carried out using SPSS System Software v19 and significance level set at 0.05. CE activity is presented as mean $\pm$ SEM.

3. Result and Discussion

With the aim of identifying the most suitable reporter for esterase inhibition, we first tested the substrate-specific sensitivity of CE activity to TBBPA in the human recombinant purified proteins CE1 and CE2, as model enzymes, and also on the mussel CE obtained from the digestive gland. In agreement with previous comparable studies (Wheelock et al., 2008), including those with mussels (Solé and Sanchez-Hernandez, 2018), the inhibition of CE activity by TBBPA was substrate-specific for the human and mussel enzymes (Fig. 1A). The highest degree of inhibition of mussel CE and human CE1 activities was obtained with the 4 carbon length chain butyrate ester 4-NPB. Based on this higher sensitivity to TBBPA and together with the fact that this substrate also displayed higher hydrolysis rates in copepods, we chose 4-NPB as the substrate for further assays on TBBPA effects. Sensitivity of CE activity to TBBPA exposure significantly increased in high trophic level predators (tunids) compared to low trophic level organisms (copepods) (Fig. 1B). We also found that within the same trophic group there could be marked differences of CE inhibition. Thus, of the two copepods examined, *P. grani* displayed the

highest basal CE activity but it was also more sensitive to *in vitro* exposure to TBBPA (significant 30% inhibition). In the case of the two small pelagic fish, sardine and anchovy, their basal 4-NPB-CE activity did not differ significantly, but the *in vitro* sensitivity to TBBPA exposure did (56% and 36% inhibition, respectively). Among tunids, TBBPA strongly inhibited the esterase activity in *A. rochei* (76%) in comparison to *S. sarda* (36%). Our results revealed that *A. rochei* presented the lowest enzymatic basal activity together with the highest *in vitro* sensitivity, suggesting that this tuna species was the most sensitive to TBBPA. Among the studied tuna, the only species considered near-threatened by the IUCN, *T. alalunga*, showed intermediate basal CE activity and showed relative high sensitivity to *in vitro* TBBPA exposure (56% inhibition). Previous studies on pesticides have found a negative relationship between basal CE activity and IC50 in a range of marine fish species (Ribalta et al., 2015), suggesting that the potentially more protected fish (with higher basal CE activity) also presented the higher *in vitro* sensitivity to the pesticide (lower IC50 value). However, our results give evidence of large differences in TBBPA sensitivity within the same ecological group. In this study we have focused on representative key species from the Mediterranean pelagic ecosystem since recent reports from other latitudes have shown that TBBPA exposed pelagic species were more prone to experience biomagnification than demersal fish (Choo et al., 2019). In the Mediterranean Sea, the two small pelagic fish feed mostly on *C. typicus* (Costalago et al., 2014), and the tunid diet contains sardine and anchovy (Consoli et al., 2008; Navarro et al., 2017). In addition to uptake from water (Choo et al., 2019; Gu et al., 2017), food transfer of this chemical is likely to occur as all marine organisms have been reported to accumulate TBBPA to a certain extend. The occurrence of TBBPA in marine matrices including commercial species has been reported as largely variable (Abdelah et al., 2016 and references within). Although no clear suggestion of threat to humans through fish and shellfish consumption has been formerly outlined, the occurrence of metabolites of TBBPA and their higher associated toxicities (Liu et al., 2018) in marine fauna renders the biomagnification pathway worth exploring, especially for pelagic

species. Moreover, direct plastic ingestion by large predators is an increasing major concern as plastics contain additives (e.g. TBBPA) and other plasticizers chemically bound to the polymer, in addition to other environmental pollutants adhered to its surface (Hermabessiere et al., 2017). In fact, enhancement of the bioaccumulation of TBBPA has been reported in bivalves from aquaculture areas in relation to the use of plastic-based devices (Gu et al., 2017). Our study suggests that these negative effects would affect more strongly the top predators (e.g. tunids, cetaceans, sharks or seabirds) than the primary consumers (e.g. copepods) since the formers appear among the most TBBPA sensitive species in our essays. As many of the species tested are commercial and human CE inhibition with purified proteins was high, a concern for consumption of TBBPA-contaminated fish and shellfish should arise. Our findings also leaves open the question of whether TBBPA-exposed organisms would be more sensitive to other xenobiotics that require CE-mediated hydrolysis for their detoxification. In particular, the inhibitory action of TBBPA could compromise the metabolism of other plasticizers such as phthalates, contaminants seen to accumulate also in large predators such as tuna fish (Guerranti et al., 2016) and that are transformed to their mono-derivatives by the hydrolytic action of CEs (Ozaki et al., 2017). Thus, the interaction of TBBPA with CE activity may cause indirect toxicity (via xenobiotic metabolism) or even alter physiological processes mediated also by CEs (lipid metabolism) in aquatic wildlife, shellfish and humans as final consumers. Given the presence of TBBPA in a wide range of marine organisms, and the *in vitro* inhibition of CE activity by this exposure, the *in vivo* action of TBBPA and any of its degradation products deserves to be examined. The species covered in this study are either key primary consumers (copepods), responsible for the transfer of energy from primary producers to upper trophic levels in the pelagic ecosystem, or consumed by humans (bivalves, small pelagic fish and tunids), therefore the impact of TBBPA on CE-mediated reactions could have ecological, commercial and human health consequences.

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Figure legends

Figure 1. A) Carboxylesterase (CE) inhibition of mussel and human CE1 and CE1 incubated with 100 μ M TBBPA, and using nitrophenyl (4-nitrophenyl acetate [4-NPA] and 4-nitrophenyl butyrate [4-NPB]) and naphthyl (1-naphthyl acetate [1-NA] and 1-naphthyl butyrate [1-NB]) derivatives as substrates. B) Inhibition of CE activity to TBBPA in a range of marine organisms from the Mediterranean Sea. * denotes $p < 0.05$ from control.

Table 1. Main biological characteristics of the marine organisms used in this study and the basal carboxylesterase activity using 4-NPB as substrate. Activity as mean $\pm$ SEM (n=4). Different letters denote $p < 0.05$.

Ecological group	Species	Common name	Body Length (cm)	4-NPB-CE activity
Copepods	<i>Paracartia grani</i>	-	≈ 0.1	$133.2 \pm 9.63a$
(O. Calanoidea)	<i>Centropages typicus</i>	-	≈ 0.1	$66.6 \pm 2.24b$
Small pelagic	<i>Sardina pilchardus</i>	Sardine	16.6 ± 0.4	$193.1 \pm 60.3 a$
(O. Clupeiformes)	<i>Engraulis encrasicolus</i>	Anchovy	13.0 ± 0.2	$181.2 \pm 19.4 a$
Large pelagic	<i>Euthynnus alletteratus</i>	Little tunny	77.1 ± 11.1	$183.2 \pm 33.6 a$
(O. Perciformes)	<i>Sarda sarda</i>	Atlantic bonito	55.3 ± 2.2	$175.1 \pm 15.7 ab$
	<i>Auxis rochei</i>	Bullet tuna	34.7 ± 1.6	$92.8 \pm 8.07 b$
	<i>Thunnus alalunga</i>	Albacore tuna	68.8 ± 13.4	$137.5 \pm 14.8 ab$

Highlights

TBBPA inhibits carboxylesterases in a substrate dependent manner

Butyrate esters are more sensitive than naphthyl derived forms in mussels

Carboxylesterase inhibition by TBBPA in marine organisms is species dependent

High trophic level predators seem to be more sensitive to TBBPA than primary consumers

TBBPA

% CE inhibition