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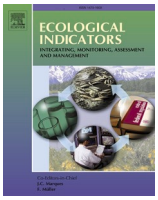

Carboxylesterase activities as potential biomarkers of pollution in marine pelagic predators

David Nos [a,*], Joan Navarro [a], David Macías [b], Montserrat Solé [a]

[a] Institut de Ciències del Mar - CSIC, Passeig Marítim de la Barceloneta, 37-49, 08003 Barcelona, Spain
[b] Centro Oceanográfico de Málaga (IEO), Puerto Pesquero, s/n, 29640 Fuengirola, Spain

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ABSTRACT

Assessment of chemical exposures in the marine environment is frequently undertaken in sedentary organisms inhabiting coastal environments. However, predatory pelagic fish should be considered sentinel species, as they play an important role in the sustainability of the ecosystems due to their high position in trophic webs. In this study, carboxylesterase (CE) activities were analysed in four predatory tuna species of commercial interest along the western Mediterranean Sea: little tunny (*Euthynnus alletteratus*), Atlantic bonito (*Sarda sarda*), bullet tuna (*Auxis rochei*) and albacore tuna (*Thunnus alalunga*). CEs are potential biomarkers of chemical exposure, as they are an important family of enzymes involved in the metabolism of xenobiotic and endogenous compounds. CE measures were taken from the liver of these tuna species using five commercial substrates: 4-nitrophenyl acetate (4NPA), 4-nitrophenyl butyrate (4NPB), 1-naphthyl acetate (1NA), 1-naphthyl butyrate (1NB), and 2-naphthyl acetate (2NA). Butyrate substrates (1NB and 4NPB) yielded the highest hydrolysis rates, and were thus the best substrates for these measures. CE activities differed between species. The larger differences were attained with 1NB-CE, with higher activities seen in bullet tuna, followed by little tunny, Atlantic bonito and albacore tuna. Individual size was identified as one of the main factors modulating CE activities, while there was no evidence for a role for trophic level (measured as δ^{15}N). Using little tunny as sentinel, no geographical differences but inter-annual variation in CE activity was observed. The kinetic parameters and *in vitro* exposure to the pesticide dichlorvos provided complementary information on the sensitivity of tuna CEs to this model pesticide. Our results propose that the little tunny could be considered a potential bioindicator species in the pelagic realm.

1. Introduction

The variety and quantity of chemicals released into the environment by human activities has become a problem at a global scale, with most of these chemicals having damaging effects on ecosystems (Aktar et al., 2009). The oceans receive inputs of pollutants from urban wastewater treatment plants, residual waters from industry, runoff waters from agriculture, among other sources (Boehm et al., 2017; Ramirez-Llodra et al., 2011). Pollutant concentrations are expected to be higher close to coastal areas, but there is also evidence of ubiquitous chemical contamination in open-ocean ecosystems (Brumovský et al., 2017). The study area, which encompasses the Catalan Sea and Alboran Sea (western Mediterranean Sea), receives from the north the inputs of the Rhône and Ebro rivers and from the South the influence of the Atlantic Ocean waters. Pollution monitoring of its coastal waters has been periodically analyzed in the frame of the Marine Strategy Framework Directive (EU, 2008). The last evaluation report (Campillo et al., 2019a, 2019b), confirmed the presence of EU targeted chemicals, highlighting "the need to enlarge the number of compounds and biomarkers considered in the monitoring, as well to include new indicator species in areas further away from the coast".

Biomarkers are specific parameters that provide information about the biological condition of an organism after exposure to those environmental stressors, indicating possible damage or toxicity (Van der Oost et al., 2003). Marine monitoring programs are mainly based on *in situ* analysis of xenobiotic chemicals and the use of different types of biomarkers in mollusks and fish inhabiting coastal and benthic environments (Campillo et al., 2019a, 2019b; Okay et al., 2014; Siscar et al., 2015; Solé et al., 2010; Solé and Sanchez-Hernandez, 2018). However, the predominant use of sedentary organisms does not take into account the effects of pollutants in organisms from open-sea pelagic environments. Moreover, marine species belonging to high trophic levels are not

* Corresponding author.
E-mail address: david.nos@icm.csic.es (D. Nos).

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usually included in biomonitoring programs although they also experience biomagnification processes, as tuna species (Chouvelon et al., 2017; Maisano et al., 2016; Ramos-Miras et al., 2019). Currently, there is little information on how marine predators perform as bioindicators, underestimating the great potential they may offer in assessing the integrative effects of marine pollution in higher trophic levels. Today, studies involving enzymatic biomarkers in high trophic level or pelagic species are limited to some shark species (Alves et al., 2015, 2016; Nos et al., 2017), large teleost fish (Fossi et al., 2004, 2002), seabirds (Narvaez et al., 2015), or marine mammals (Bengtson Nash et al., 2014). In this study, we selected four tuna species representative of high trophic levels within the pelagic marine environment: Atlantic little tunny (*Euthynnus alletteratus*), Atlantic bonito (*Sarda sarda*), bullet tuna (*Auxis rochei*) and albacore tuna (*Thunnus alalunga*).

Among the different biomarkers employed in the monitoring of harmful chemicals, carboxylesterases (CEs, EC 3.1.1.1) are considered suitable enzymes for pollution monitoring (Wheelock et al., 2008). CEs are enzymes composed of a variable number of isozymes belonging to the family α,ß-serine hydrolases that catalyze the hydrolysis of substrates containing ester, amide and thioester bonds (Casey Laizure et al., 2013). CEs play a double role in the organism, taking part in the transformation of endogenous molecules, i.e., cholesterol esters (Ross and Edelmann, 2012) but also in the metabolism of xenobiotic chemicals, i.e. activation of pharmaceutical drugs and metabolism personal care products and pesticides (Hatfield et al., 2016; Nos et al., 2020; Satoh and Hosokawa, 1998; Wheelock and Nakagawa, 2010). CEs are considered useful biomarkers of susceptibility and exposure to pesticides and other chemical esters with an also recognized protective role of acetylcholinesterase due to their high affinity for organophosphorus pesticides (Estévez et al., 2019). Moreover, from a monitoring perspective, CEs are interesting robust enzymes that can be measured after longer storage periods and freezing conditions, better than other more short-lived and thermolabile metabolic enzymes (Sood et al., 2018). Several commercial substrates can be used for CEs activity measures and their combined use is highly recommended in pollution monitoring as they can be indicative of different enzyme isoforms (Wheelock et al., 2008). Furthermore, in order to validate the use of these biomarkers, it is essential to understand the main environmental and biological factors that can modulate these enzymatic activities. Spatial or seasonal variability due to environmental and biological factors has been assessed in other fish species (Alpuche Gual and Gold Bouchot, 2008; Koenig and Solé, 2012; Solé and Sanchez-Hernandez, 2018; Ribalta et al, 2015). Similarly, species-specific differences in CE activities or the potential influence of the trophic level, using δ^{15}N as a proxy (Boecklen et al., 2011), are also important factors to be considered when biomarkers are used in pollution monitoring (Martínez-Morcillo et al., 2019; OSPAR Commission, 2013; Solé et al., 2010; Wheelock et al., 2008).

The main aims of this study were 1) to characterize CEs activity in four Mediterranean tuna species using different commercial substrates, 2) to test *in vitro* sensitivity of tuna CEs to a model pesticide and 3) to search for the suitability of tuna CEs for future biomonitoring studies in high trophic level species from the pelagic environment. For this purpose, the spatial and temporal variation and the influence of different biological parameters affecting the selected biomarker (size, liver weight and trophic level) were considered. This work sets the ground for the development of CEs as biomarkers of marine pollution in large pelagic fish.

2. Material and methods

2.1. Sampling

A total of 110 individuals of little tunny, Atlantic bonito, bullet tuna and albacore tuna were captured by commercial fisheries in the western Mediterranean Sea between the years 2015 and 2017 (Fig. 1).

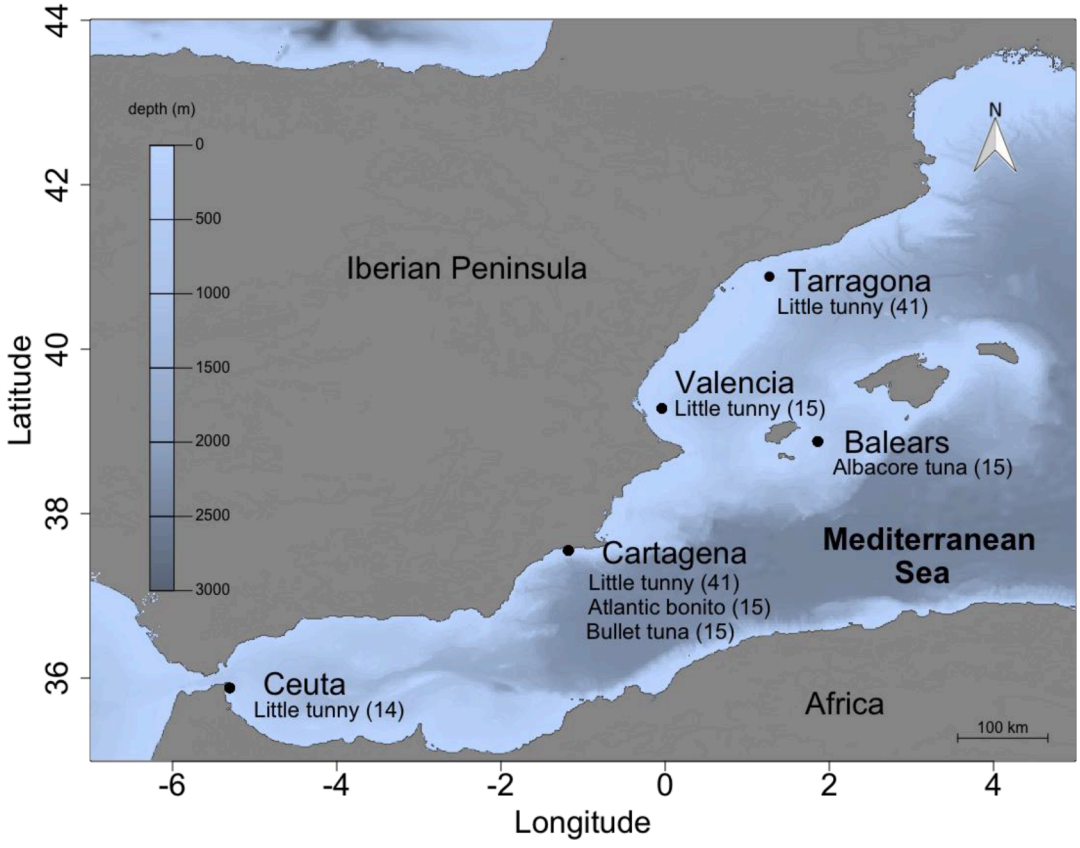

Fig. 1. Sampling zones indicating in brackets the number of individuals captured for each species. Latitude (N) and longitude (E) in degrees.

Individuals from Cartagena and Ceuta were captured with "almadraba" fishing, while in Tarragona, Valencia and Balears were captured with longlines. Species differences in CE activities were tested using, at least, 15 individuals of each species. Geographical and temporal variations were assessed for the most abundant species, the little tunny, employing between 9 and 17 individuals by site and year. For each individual, furcal length (FL, in cm) and liver weight (LW, in g) were taken and the mean for each species, site and sampling time is reported (Table 1). In the laboratory, the entire fish viscera were dissected and immediately frozen at −20 °C. The same samples of each liver were used for enzymatic assays (frozen at −80 °C) and for stable isotope determinations (dried and lyophilized).

2.2. Enzymatic activity determinations

About 0.3 g of each individual liver were homogenized in ice-cold 100 mM K-phosphate buffer (pH = 7.4) containing 150 mM KCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) in a 1:5 (w:v) ratio with a Polytron® blender. The homogenate was centrifuged at 10,000 × g for 20 min at 4 °C and the supernatant (S10) obtained was stored at −80 °C until analysis. Carboxylesterase (CE) activity of each liver was individually measured using the commercial colorimetric substrates 4-nitrophenyl acetate (4NPA), 4-nitrophenyl butyrate (4NPB), 1-naphthyl acetate (1NA), 1-naphthyl butyrate (1NB) and 2-naphthyl acetate (2NA). The hydrolysis rate of 4NPA and 4NPB was determined according to Hosokawa and Satoh (2002), adapted to the microplate format. The kinetic assay was performed in a 50 mM phosphate buffer (pH = 7.4) medium mixed with the respective substrates (each at 1 mM final concentration) and the S10 sample. The formation of 4-nitrophenolate was measured at 405 nm. A millimolar extinction coefficient of $1.8{\cdot}10^4$ $mM^{-1}{\cdot}cm^{-1}$ was used to express the hydrolysis of these nitrophenyl esters as $nmol{\cdot}min^{-1}{\cdot}mg\ protein^{-1}$ (Hosokawa and Satoh, 2002). The hydrolysis of 1NA, 1NB and 2NA was measured using the ultraviolet spectrophotometric method by Mastropaolo and Yourno (1981), adapted to microplate format. The reaction mixture consisted of the same 50 mM buffer phosphate (pH = 7.4) together with the selected substrates (each at 0.25 mM final concentration) and the sample. The formation of 1-naphtol was measured at 235 nm. An extinction coefficient of $2.34{\cdot}10^4$ $mM^{-1}{\cdot}cm^{-1}$ was used for enzymatic activity calculations (Mastropaolo and Yourno, 1981). Total protein content was determined by the Bradford method (1976), using the Bradford Bio-Rad Protein Assay reagent. A standard curve made with bovine serum albumin (0.05–0.5 $mg{\cdot}mL^{-1}$) was used for total protein quantification. All determinations were carried out in triplicate for each sample at 25 °C using a TECAN infinite 200 microplate reader.

2.3. Kinetic parameters

The maximal hydrolysis rates (V_{max}, in $nmol{\cdot}min^{-1}{\cdot}mg\ protein^{-1}$) and Michaelis-Menten constant values (K_m, in mM) were determined in livers of the four species using five concentrations of the substrates (for 4NPA and 4NPB: 0.0625–2 mM, and for 1NA and 1NB: 15.6–500 μM). V_{max} and K_m were calculated implementing the Michaelis-Menten equation ($V = V_{max}\,[S]/K_m + [S]$) with the different concentrations and using the linearity transformation of Lineweaver-Burk plot.

2.4. In vitro inhibition by a model pesticide

The commonly used model organophosphorus (OP) pesticide dichlorvos was selected to comparatively evaluate the *in vitro* sensitivity of the targeted tuna and other species. For this approach, the post-mitochondrial fraction (PMF) of the liver was incubated with a 7-point range concentration of the pesticide (0.02–320 μM). The IC50, concentration of dichlorvos at which inhibits activity by 50% after a 30 min incubation at room temperature (25 °C), was calculated by a logistic regression to a sigmoidal function (Sigmaplot 8.0)."

2.5. Stable isotopic analysis

Liver samples collected for isotopic determination were freeze-dried and powdered, and 0.28–0.33 mg of each sample packed into tin capsules. Isotopic analyses were carried out at the Doñana Biological Station (EBD) Stable Isotope Laboratory (LIE) (www.ebd.csic.es/lie/index.html). Samples were combusted at 1020 °C using a continuous flow isotope-ratio mass spectrometry system by means of a Flash HT Plus elemental analyser coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The isotopic composition was reported in the conventional delta (δ) per mil notation (‰), relative to atmospheric N_2. Replicate assays of standards routinely inserted within the sampling sequence indicated analytical measurement errors of ± 0.2. The standards used were: EBD-23 (cow horn, internal standard), LIE-BB (whale baleen, internal standard) and LIE-PA (razorbill feathers, internal standard). These laboratory standards were previously validated with the use of international standards from the International Atomic Energy Agency (IAEA, Vienna).

2.6. Data analysis

Generalized Additive Models (GAMs) were adopted to evaluate interspecific differences in CE activity. Spatial differences in CE activity

Table 1
Mean and standard error of the mean (SEM) of the furcal length (FL) and $\delta^{15}N$ values (proxy of trophic level) of little tunny, Atlantic bonito, bullet tuna and albacore tuna by sampling area (Tarragona, Valencia, Mallorca, Cartagena, Ceuta) and year (2015, 2016, 2017). Lowercase letters indicate significant differences between years, tested only for the localities Tarragona and Cartagena. Capital letters show differences between the four zones in 2015 for the little tunny. Letters α, β and γ indicate differences between species.

Species	Zone	Year	N	FL (cm)	$\delta^{15}N$ (‰)
Little tunny (*E. alletteratus*)	Tarragona	2015	11	85.1 ± 2.8 A	8.76 ± 0.68 A
		2016	15	94.1 ± 0.9	9.12 ± 1.01
		2017	15	80.3 ± 6.3	9.05 ± 0.86
	Valencia	2015	15	25.7 ± 0.4B	7.56 ± 1.00 A
	Cartagena	2015	9	60.1 ± 1.0 a, C	10.1 ± 0.6 a, b, B
		2016	15	74.0 ± 3.4b, **α**	10.3 ± 0.4b, **α**
		2017	17	55.2 ± 2.0 a	9.39 ± 0.98 a
	Ceuta	2015	14	37.8 ± 0.5 D	8.88 ± 0.48 A
Atlantic bonito (*S. sarda*)	Cartagena	2016	15	34.6 ± 0.4 **β**	8.05 ± 0.31 **β**
Bullet tuna (*A. rochei*)	Cartagena	2016	15	53.7 ± 0.6 **γ**	11.2 ± 0.5 **γ**
Albacore tuna (*T. alalunga*)	Mallorca	2015	15	72.1 ± 2.1 **α**	7.56 ± 0.92 **γ**

of little tunny was assessed between four geographical zones (Tarragona, Valencia, Cartagena, and Ceuta) and inter-annual differences in two localities (Tarragona and Cartagena) for three consecutive years (2015–2017). As fish body length and δ15N values differed between years, the furcal length (FL) and $\delta^{15}N$ were included as covariables in the GAMs. Since the influence of the trophic level ($\delta^{15}N$) was not revealed as a significant factor modulating CE activity, it was not further considered in the equation. Correlations between CE activities using the different substrates were evaluated using the non-parametric Spearman's rank test (R_s). The significance level was set at $\alpha = 0.05$. All statistical analyses were carried out with R software.

3. Results

3.1. Interspecific differences in CE activity

Differences on CE activities between species were confirmed regardless of the substrate assayed (Fig. 2). All CE-related activities using the five commercial substrates were positive and significantly correlated ($R_s = 0.75–0.95$, $p < 0.05$, $n = 60$). Little tunny CE activities were inversely and significantly correlated to fish size and liver weight (FL: R_s = -0.21 to −0.51, $p < 0.05$; LW: −0.22 to −0.53, $p < 0.05$; $n = 110$). The other species did not show significant correlations with TL or LW, in those species the sample number was lower ($n = 15$). The highest CE activities were recorded using the longer-chain butyrate substrates (4NPB and 1NB) while the lowest were recorded when using acetate-derived substrates (4NPA, 1NA, and 2NA). This trend agrees with the estimated maximal hydrolysis rates (V_{max}) recorded. The Michaelis-Menten constant (K_m) values indicated that 1NB (0.06–1.09 mM) and 1NA (0.15–0.34 mM) had higher enzyme affinity in the different species, and the catalytic efficiency values (V_{max}/K_m) also supported these results (1180-6879 with 1NB and 837-1732 with 1NA). The observed K_m values around 1 mM confirmed that 4-nitrophenyl substrates were not rate-limiting. However, in Atlantic bonito, the use of 1-naphthyl substrates at 250 μM could be rate-limiting (Table 2). While the 2NA substrate resulted in slightly higher hydrolysis rates than 4NPA, as both activities showed strong positive correlation and, since 4NPA is one of the most frequently used substrates in CE determination, it was further selected as more adequate for comparative purposes.

The *in vitro* inhibition of CEs by the model pesticide dichlorvos was tested in the four tuna species using IC50 as a proxy of the sensitivity of tuna CEs to pesticides (Table 2). Dichlorvos had a variable inhibitory effect on liver CE activity in all species. In general terms, the little tunny CEs were the most sensitive to dichlorvos (IC50 = 0.40–0.45 μM), followed by bullet tuna (IC50 = 0.30–0.88), Atlantic Bonito (IC50 = 0.35–0.65), and albacore tuna, (IC50 = 0.44–1.36), which had the less sensitive CEs. Dichlorvos barely caused a 50% inhibition of baseline CE activity when using 4NPA as a substrate and consequently, IC50 calculation was not possible (Fig. S1 supplementary material).

3.2. Spatial and temporal trends of CE activity in little tunny

Geographical variation on CE activities in little tunny was followed up in specimens from four geographical areas collected in 2015. CE activities varied among sampling zones. However, when considering the individuals' size, site differences were not supported (Area: $F_{4,60} = 0.024$, $p = 0.88$, FL: $F_{1,60} = 12.650p = 0.001$) Table S1.

The temporal trends only suggested interannual variation of CE activities in specimens from Cartagena with consistently lower values in those sampled in 2016 for all substrates assayed, even after including FL in the model Year: $F_{3,45} = 3.243$, $p = 0.003$, FL: $F_{1,45} = -2.07$, $p = 0.045$). There were no temporal trends found in Tarragona specimens (Year: $F_{1,45} = 0.01p = 0.922$, FL: $F_{3,45} = 3.28p = 0.078$) (Fig. 3).

4. Discussion

A large number of studies have addressed marine pollution, however, little information is available on how pollution affects predators inhabiting pelagic and oceanic areas. In this study, the basal activity of liver CEs in four species of tuna is described, taking in consideration the potential influence of specific biological traits like fish size and trophic level. Some methodological aspects of CE measures such as the use of different substrates, kinetic parameters V_{max} (maximal hydrolysis rate) and K_m (substrate affinity), and *in vitro* sensitivity to the model

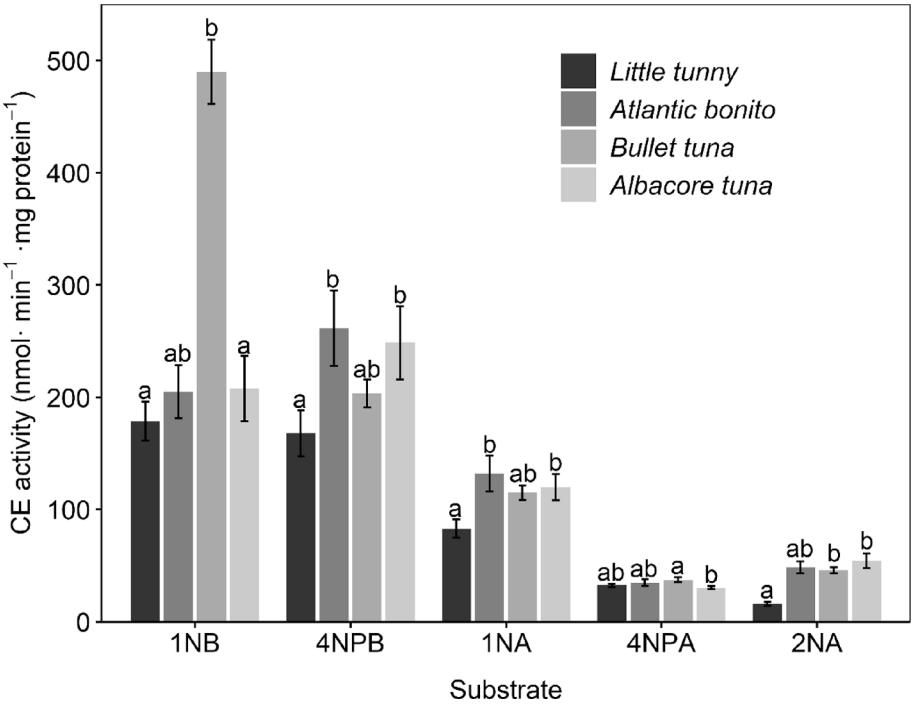

Fig. 2. Carboxylesterase (CE) activity in mean ± SEM (n = 15) of little tunny, Atlantic bonito, bullet tuna and albacore tuna from the western Mediterranean Sea measured with five substrates. Letters indicate significant differences in CE activity between species for each substrate.

Table 2
Kinetic parameters V_{max} (in nmol·min^{-1}·mg protein^{-1}), k_m (mM), catalytic efficiency (V_{max}/K_m) and dichlorvos IC_{50} (concentration causing 50% inhibition, in μM) for little tunny, Atlantic bonito, bullet tuna and albacore tuna with the substrates 4NPA, 4NPB, 1NA and 1NB (n = 4). *NC*: not calculated.

			4NPA-CEs	4NPB-CEs	1NA-CEs	1NB-CEs	2NA-CEs
Little tunny	(*Euthynnus alletteratus*)	V_{max}	49.2 ± 7.2	321.9 ± 41.0	284.6 ± 66.3	562.6 ± 39.8	68.6 ± 12.5
		K_m	0.87 ± 0.16	1.16 ± 0.14	0.34 ± 0.11	0.16 ± 0.02	0.21 ± 0.06
		V_{max}/K_m	56.6	277.5	837.1	3516.25	326.7
		IC_{50}	*NC*	0.42 ± 0.02	0.40 ± 0.01	0.45 ± 0.09	*NC*
Atlantic bonito	(*Sarda sarda*)	V_{max}	25.7 ± 11.1	477.4 ± 160.6	244.0 ± 71.9	1826.5 ± 627.5	108.3 ± 40.5
		K_m	0.70 ± 0.32	1.28 ± 0.14	0.28 ± 0.02	1.09 ± 0.18	0.30 ± 0.08
		V_{max}/K_m	36.71	373.0	871.4	1180.3	361.0
		IC_{50}	*NC*	0.35 ± 0.01	0.47 ± 0.03	0.65 ± 0.09	*NC*
Bullet tuna	(*Auxis rochei*)	V_{max}	59.87 ± 2.1	259.4 ± 26.4	166.4 ± 31.9	481.5 ± 121.6	72.7 ± 18.8
		K_m	0.64 ± 0.06	0.81 ± 0.05	0.15 ± 0.05	0.07 ± 0.02	0.19 ± 0.06
		V_{max}/K_m	93.5	320.2	1109.3	6878.6	382.6
		IC_{50}	*NC*	0.30 ± 0.03	0.54 ± 0.03	0.88 ± 0.07	*NC*
Albacore tuna	(*Thunnus alalunga*)	V_{max}	52.8 ± 4.0	397.6 ± 117.6	363.7 ± 112.3	344.3 ± 129.3	77.5 ± 18.6
		K_m	0.91 ± 0.06	0.97 ± 0.11	0.21 ± 0.04	0.06 ± 0.03	0.10 ± 0.01
		V_{max}/K_m	58.0	409.9	1731.9	5738.3	775.0
		IC_{50}	*NC*	0.63 ± 0.05	0.44 ± 0.06	1.36 ± 0.71	*NC*

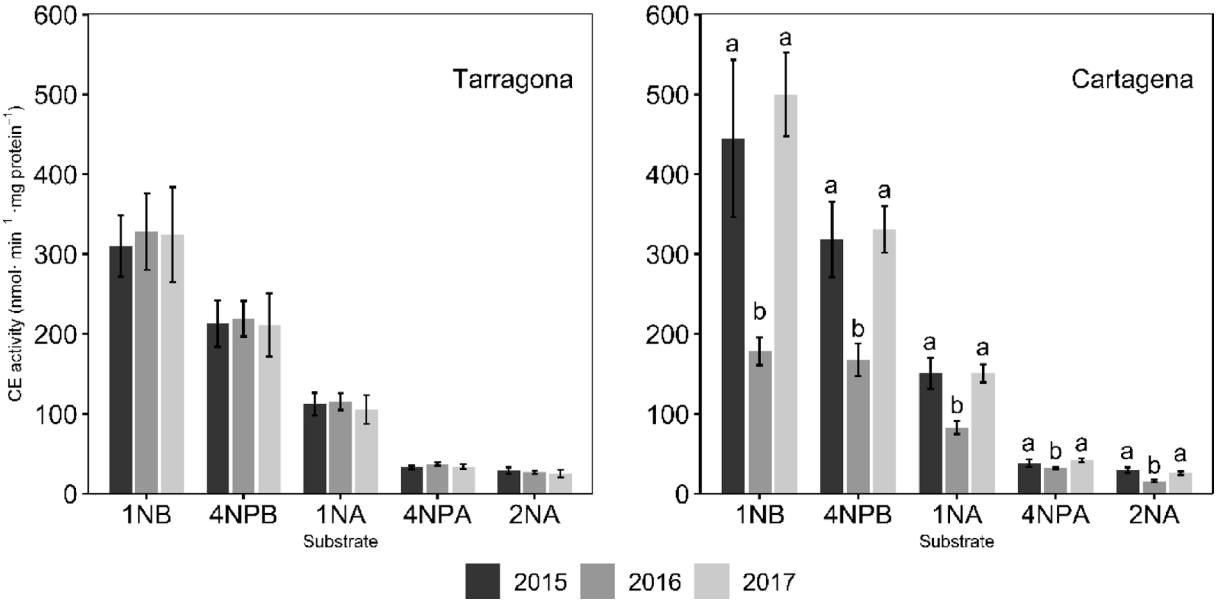

Fig. 3. Mean and standard error of the mean (SEM) of carboxylesterase (CE) activity of little tunny in Tarragona and Cartagena between 2015 and 2017 (n between 9 and 17). Letters indicate significant differences of CE activity between years for each substrate.

organophosphate pesticide dichlorvos were also contrasted in the four tuna species.

The influence of trophic level as a possible source of variability in tuna CE activity determinations was assessed. However, because of the small $\delta^{15}N$ fluctuations (7.56–11.2‰), a proxy of the trophic level (Navarro et al., 2011), confirmed this variable was not seen as relevant and all species were equally representative of medium and high stages of the trophic marine chain. Some studies have shown the influence of food availability on several enzyme activities, including CEs (Barron et al., 1999; Koenig and Solé, 2012; Leinweber, 1987; Whyte et al., 2000). Since CEs also interact with lipid metabolism, food ingestion and diet are parameters likely to affect them. The trophic level values of common tuna species had already been described in Navarro et al. (2017), confirming similarities in $\delta^{15}N$ values when tuna are of similar size and habitat. A previous study on seabirds testing the influence of the diet and nitrogen content on CE activity, showed no significant role for these parameters. However, diet did affect other enzymes such as lipases and cholinesterases (Narvaez et al., 2015). The association between body size and hepatic CE activities have been formerly assessed in fish, with correlations being either positive or negative depending on the species (Alpuche Gual and Gold Bouchot, 2008; Solé et al., 2009). In the present study with tuna, most relationships between species' size and CE activities were negatively related to the FL.

The use of different substrates can be indicative of different CE isozymes which, in turn, may reveal different sensitivities to foreign chemicals. While there is little information on CE isoforms in fish, CE variability between species, tissues and responsiveness to chemicals suggests the existence of different isoforms (Solé et al., 2012, 2010). In the present tuna study, CE activities using acetate-derived substrates (4NPA, 1NA, and 2NA) were one order of magnitude lower than those using butyrate-derived esters (4NPB and 1NB). Moreover, CE activities measured using 1NA were higher than with 4NPA, similar to those observed in demersal fish (Martínez-Morcillo et al., 2019; Ribalta et al., 2015). The variable but significant correlation between CE activities using several substrates found in the present study (0.51–0.95, $p < 0.05$) confirms a high degree of overlapping activity between substrates as formerly suggested in gilthead seabream (*Sparus aurata*) (Soto-Mancera et al., 2020). Even though 1NB and 4NPB were seen as more adequate substrates for CE determinations in tuna species, we adopted the substrate 4NPA for comparative purposes as it is the most widely used in the fish studies available for comparison.

Tuna mean CE hydrolysis rates using the common 4NPA as substrate,

ranged between 32.4 and 42.1 nmol·min^{-1}·mg protein^{-1} while a broader range was reported in the study by Martínez-Morcillo et al. (2019) considering several demersal fish species (12.2–52.8 nmol·min^{-1}·mg protein^{-1}). Kinetic values of V_{max} and K_m confirmed the related trends in species activities and substrates preferences that have been formerly described. To our knowledge, no information on these kinetic parameters for CEs in tuna species is available. The closest possible comparison can be made with the benthic mesopredator small-spotted catshark (*Scyliorhinus canicula*) which showed, for the same substrates (4NPA and 1NA), higher V_{max} and K_m (Nos et al., 2017). Conversely, the Senegalese sole (*Solea senegalensis*) showed lower V_{max} and K_m values for the substrate 1NA using comparable protocols (Solé et al., 2012).

Another parameter that suggests differences in the sensitiveness to the pesticide among the species was the estimated IC50 values after *in vitro* exposure to a model pesticide. Species displaying low IC50 values (high dichlorvos affinity) and high CE activity in liver, are regarded as better protected from AChE inhibition by pesticides (Küster, 2005; Wheelock et al., 2008). On the contrary, those species exhibiting high IC50 values and low CE activities would be more vulnerable to neurotoxic chemicals. In fact, a negative relationship between these two parameters has been reported in some marine fish species (Ribalta et al., 2015). In the case of tuna fish, the relationship between CEs and IC50 was not inversely related and the balance between both variables could render similar *in vitro* sensitivities. These former observations on the hydrolysis rates, kinetic parameters and IC50 sensitivity to dichlorvos revealed notable interspecific differences in CE activities in a substrate-dependent manner.

Of all four species, the little tunny accomplished to express high enzymatic activity with most substrates and a high *in vitro* responsiveness to dichlorvos (low IC50). In addition, it is an abundant and broadly distributed species representative of high trophic levels, being exposed to pollution biomagnification processes, thus making of it a good candidate as sentinel in future monitoring studies of high trophic level species inhabiting the pelagic environment.

In this sense, a first step for the validation of this species as sentinel, a temporal and geographical scale comparison was approached. An interannual variation in CE activity (2015, 2016 and 2017) was confirmed in little tunny individuals sampled only off Cartagena, with significantly lower activities in 2016 regardless of the assayed substrates. Despite it could not be confirmed as no *in situ* chemical analysis were done, a possible explanation for the decrease in this activity could be the presence of CE inhibitors (e.g. pesticides) in the water. Cartagena is a location chronically affected by pollution derived from intensive agricultural activities along its coast, which involves the massive use of pesticides. Also, this is an area the input of anthropogenic pollutants from nearby coastal activities into the Cartagena marine waters has been repeatedly demonstrated (Manteca et al., 2017; Moreno-González et al., 2013; Moreno-González and León, 2017; Quesada et al., 2014). A confirmation of this connectivity further comes from presence in this zone, a metabolite of the pharmaceutical salbutamol in surface waters (Moreno-González et al., 2014) and its confirmed presence in the bile of the same tuna individuals caught in Cartagena in the same 2016 sampling (Peña-Herrera et al., 2018). Temporal variations of CE activity in fish sampled off Tarragona were not observed as it corresponds to individuals sampled in a broader area.

Geographical variation in hepatic CE activities of little tunny at a comparable sampling time (2015) was not observed. CEs site differences were not confirmed once fish size as a modulating factor was considered. This is in agreement with the high mobility reported for this pelagic species and supports its potential use as a sentinel of the pelagic realm, which are also susceptible to the bioaccumulation of pollutants. The occasional reported differences in activity could correspond to the fish maintained for some time confined in a coastal area of Cartagena in 2016. The data here obtained on CE activities for each of the assayed substrates could be regarded as baseline activities for this tuna species.

5. Conclusions

This study provides basal CE activities in liver of four tuna species representative from the pelagic Mediterranean waters. High CEs activity and high *in vitro* sensitivity to the model pesticide dichlorvos (low IC50) in little tuna species indicate that this species could be considered good sentinel of anthropogenic chemicals in the pelagic environment and high trophic levels. Among the tested variables that could affect CEs activity in fish, only length had a significant influence while trophic level was similar in all of them. In the case of little tunny, as sentinel candidate, temporal and spatial differences were investigated. Although no geographical variations in CE activities were indicated as corresponds to fish with high mobility, a significant decrease in 2016 CEs activities in Cartagena was observed that could be justified. Tuna fish have a great potential as bioindicator as they integrate pollution trough the trophic nets and they are also integrating pollution of broader pelagic areas, therefore they should be considered in marine pollution monitoring.

CRediT authorship contribution statement

David Nos: Formal analysis, Writing - original draft, Investigation, Visualization. **Joan Navarro:** Supervision, Funding acquisition, Resources, Writing - review & editing. **David Macías:** Resources. **Montserrat Solé:** Supervision, Funding acquisition, Resources, Writing - review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.107217.

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