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RESEARCH ARTICLE

Multibiomarker biomonitoring approach using three bivalve species in the Ebro Delta (Catalonia, Spain)

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Abstract
Bivalves have proved to be useful bioindicators for environmental pollution. In the present study, mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), and razor shells (*Solen marginatus*) were collected in the Ebro Delta, an extensive area devoted to rice farming and affected by pesticide pollution, from April to July, the heaviest rice field treatment period. Possible effects of pollution were assessed through biochemical markers (carboxylesterase (CE), antioxidant and neurotoxicity-related enzymes, and lipid peroxidation levels). Data on environmental variables, bivalve reproductive condition, and presence of organic pollutants, marine phycotoxins, pathogens, or histopathological conditions in bivalve's tissues were also evaluated. Although the bioaccumulated pesticides did not explain the patterns observed for biochemical responses, the obtained results point to an effect of environmental pesticide pollution on enzymatic markers, with a prominent contribution of CE to such changes. Mussels and razor shells provided a more sensitive biochemical response to pollution than cockles. Environmental variables, bivalve reproductive condition, and marine phycotoxins did not seem to have a relevant effect on the biomarkers assessed.

Keywords Mussel · Cockle · Razor shell · Biomarkers · Contaminants · Pesticides · Histology · Phycotoxins

Introduction

Estuaries are semi-enclosed coastal areas characterized by high biomass, biodiversity, and primary production, which favor the proliferation of aquatic organisms but are also highly exposed to anthropogenic impacts. Since these regions are often devoted to agriculture, pesticides derived from this activity are an important source of pollution that can threaten water quality (Mañosa et al. 2001; Ochoa et al. 2013; Rodrigues et al. 2018).

Since concentrations of toxic agents in environmental samples do not fully inform on their biological effects in organisms, environmental chemical analyses must be completed with the use of biomarkers defined as subindividual responses such as molecular, biochemical, and physiological responses that occur in exposed organisms and that allow identifying the effects of toxic agents in natural populations. In this sense, bivalves play a prominent role as bioindicators due to their filter-feeding behavior, widespread distribution, and easy sampling and have been widely used for ecotoxicological purposes (Farcy et al. 2013; Rodrigues et al. 2018).

Alterations in the activity levels of enzymes involved xenobiotic metabolism, neurotoxicity, and oxidative stress which are biochemical markers known to respond to environmental stress related to chemical exposure, and their combined use is strongly recommended (Capó et al. 2015; Mejdoub et al. 2017; Solé and Sanchez-Hernandez 2018). Carboxylesterases (CEs; EC 3.1.1.1) are hydrolases of wide specificity that hydrolyze esterified xenobiotics to the corresponding alcohol and carboxylic acid (Wheelock and Nakagawa 2010) and are inhibited by

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several different compounds, such as the oxon forms of organophosphate (OP) insecticides, carbamates, or sulfonamides (Wheelock et al. 2005). The enzyme acetylcholinesterase (AChE; EC 3.1.1.7) catalyzes the hydrolysis of the neurotransmitter acetylcholine at cholinergic nerve terminals and is also inhibited by OP and carbamate pesticides (Kristoff et al. 2010). Exposure to organic toxicants enhances the production of reactive oxygen species (ROS) that can react with important macromolecules, such as DNA, proteins, and lipids. Antioxidant enzymes, such as glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GR; EC 1.8.1.10), and glutathione S-transferases (GST; EC 4.4.1.20) carry out specific reactions preventing the adverse effects of ROS (Regoli and Giuliani 2014). These enzymes interact in a complex network and, alongside with lipid peroxidation (LPO), are frequently used as indicators of oxidative stress induced by chemical pollution in bivalves (Matozzo et al. 2018a). A wide range of other effect markers, such as histological alterations, microbiological measurements, or pathogenic conditions, have been commonly used to reveal signs of altered health status in bivalves in response to pollutants (Farcy et al. 2013; Izagirre et al. 2014; Matozzo et al. 2018b and references therein). Multidisciplinary studies that combine different sets of biomarkers that respond to both natural and anthropogenic stressors (e.g., contaminants) provide a broader perspective and a better understanding of the observed dynamics than more restricted approaches and are highly recommended in ecotoxicological studies (Cajaraville et al. 2000; Galloway et al. 2002; Matozzo et al. 2018b).

Many biomarkers are also known to vary according to environmental (e.g., temperature) and/or biological factors (e.g., nutritive status or reproductive conditions) in bivalves (Moore et al. 2007; Farcy et al. 2013; González-Fernández et al. 2015a, b). The physiologic alterations derived from these factors can cause misinterpretation of biomarker responses, and consequently, potentially confounding factors should be included in bivalve biomonitoring studies.

Mussels have been extensively used in ecotoxicological field studies as bioindicators (Farcy et al. 2013; Mundhe et al. 2016; Mejdoub et al. 2017; Matozzo et al. 2018b; Solé and Sanchez-Hernandez 2018). In contrast, knowledge on the potential use as bioindicator species of cockles and razor shells in the field is more limited, although a few studies have addressed the use of their enzymatic responses as biomarkers (Jebali et al. 2011; Nilin et al. 2012; Velez et al. 2016; Ferrante et al. 2014; Nunes and Resende 2017; Pearce and Mann 2006).

From this perspective, the present study aims to provide a multibiomarker approach with the use of the Mediterranean mussel, the common cockle, and the grooved razor shell in an estuarine area devoted to mariculture, under the impact of pesticides and other anthropogenic chemicals, in order to find the most suitable bioindicator. Changes in the levels of different biochemical markers (activity of CE, antioxidant and neurotoxicity-related enzymes, and LPO levels) were assessed under the hypothesis of a response mainly to pesticides derived from agricultural activity, but also to other organic contaminants potentially present in the study area. Additional factors that may influence biomarker responses, such as environmental variables, bivalve reproductive condition, and phycotoxins occurrence, or act as markers themselves, such as the presence of pathogens and/or histopathological conditions, were also taken into account.

Materials and methods

Sampling area and specimen collection

The Ebro Delta is an extensive estuarine area (320 km^2) located at the mouth of the Ebro River (Catalonia, NE Spain). It constitutes one of the most important aquatic environments in the western Mediterranean and is the most important bivalve and rice producer in the region (Guallar et al. 2016; Mañosa et al. 2001).

Mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), and razor shells (*Solen marginatus*) (approximate shell size range (cm): 4.5–7.0, 1.9–3.3, and 8.3–11.5, respectively) were collected in April, May, June, and July of 2017 from the Alfacs Bay of the Ebro Delta. Mussels were collected at 0.5 m depth from suspended cords located at the central part of the northern margin of the bay (40° 37.24′ N, 0° 39.22′ E), and cockles and razor shells were sampled by traditional techniques from the northern (40° 37.58′ N, 0° 39.66′ E) and southern (40° 36.17′ N, 0° 40.18′ E) margins of the bay at subtidal areas located at 0.5 and 1 m depth, respectively (Fig. 1). The number of specimens collected of each species and for each analysis is specified in the corresponding following sections.

Environmental variables

Records for temperature (in °C), salinity (in S_p), dissolved O_2 concentration (in mg/L), and chlorophyll-*a* concentration (in μg/L) were taken at the northern margin of the Alfacs Bay for mussels and cockles (40° 37.33′ N, 0° 39.49′ E) and at the southern margin for razor shells (40° 36.45′ N, 0° 39.37′ E) using an YSI multiparameter sounder (Fig. 1). Each parameter was measured in triplicate at a weekly frequency: on the sampling day and the two previous weeks. Data provided in Table 1 are the result of the mean of these three measurements.

Analysis of organic contaminants

A multiresidue analytical method (Álvarez-Muñoz et al., in preparation) was used for the extraction and quantification of a mixture of contaminants including pesticides, endocrine disruptors (EDCs), and pharmaceuticals (PhACs) in bivalves' soft tissues. A pool containing between 5 and 15 specimens was prepared for each species (i.e., *M. galloprovincialis*, *C. edule*,

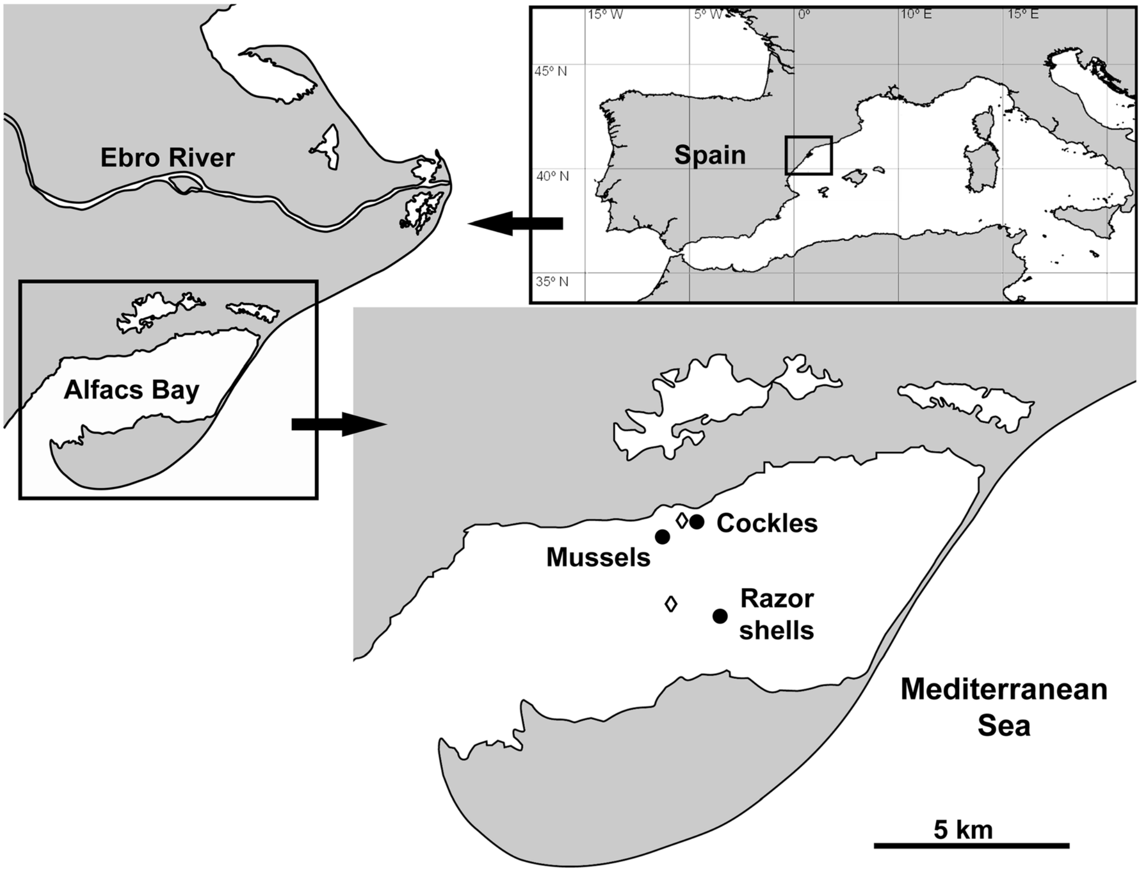


Fig. 1 Study area showing bivalve sampling sites (filled circles) and localities where environmental variables were measured (open diamonds) in the Alfacs Bay (Ebro Delta)

and *S. marginatus*) and sampling period (i.e., April, May, June, and July). Each pool was homogenized using a grinder and freeze-dried prior to the analysis. Approximately 1 g of sample was extracted per duplicate for each pool using QuEChERS (Bekolut® Citrat-Kit-01) with acetonitrile in aqueous condition followed by the addition of 4 g $MgSO_4$, 1 g NaCl, 1 g Na citrate, and 0.5 g disodium citrate sesquihydrate. Purification was carried out by means of dispersive solid-phase extraction (dSPE) using a sorbent mixture of 400 mg PSA and 400 mg C18 (Bekolut® PSA-Kit-04A). Samples were passed through a phospholipid removal plate (Ostro™ Pass-through Sample Preparation, from Waters) prior injection in ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) Orbitrap Q Exactive™ (Thermo Fischer Scientific, San Jose, CA, USA) for the identification and quantification of the target compounds.

Biochemical determinations

A total of 40 specimens of *M. galloprovincialis*, 80 of *C. edule*, and 40 of *S. marginatus* (mean shell length 5.54 ± 0.54, 2.72 ± 0.36, and 10.27 ± 0.9 cm, respectively) were used

Table 1 Physicochemical water parameters quantified in the two areas sampled throughout the length of the study

Area	Date	*T* (°C)	*S* (psu)	O_2 (mg/L)	*Chla* (μg/L)
Northern margin (mussels and cockles)	April	15.77	36.92	7.36	4.51
	May	17.80	37.57	6.41	4.70
	June	21.60	38.22	5.88	2.98
	July	23.63	38.62	6.41	2.34
Southern margin (razor shells)	April	16.57	36.57	7.21	2.48
	May	18.77	36.60	5.73	2.27
	June	22.90	35.47	6.00	2.29
	July	27.47	35.53	5.84	2.75

T, temperature; *S*, salinity; O_2, oxygen concentration; *Chla*, chlorophyll-*a* for biochemical determinations. Ten individual samples were considered for each sampled month in the case of mussels and razor shells. In the case of cockles, due to their small size, two specimens were included in each sample.

In the case of mussels, hemolymph was extracted from the adduct muscle immediately upon sampling, using a 1-mL syringe with a 0.21-gauge needle. Hemolymph was frozen at − 80 °C and centrifuged (5000*g* × 5 min at 4 °C) just before analyses to obtain a cell-free supernatant. Mussel gills and digestive glands of the three species were carefully dissected avoiding contamination by other tissues and immediately frozen in liquid nitrogen and stored at − 80 °C until further analyses.

As demonstrated by Solé and Sanchez-Hernandez (2018), the digestive gland is the most suitable tissue for measuring changes in CE activity in mussels. Therefore, and considering the special focus on CEs in the present study, this tissue was chosen as target for comparing biochemical results among the three species.

Tissue preparation

Tissues were homogenized (1:5, *w*/*v*) in ice-cold homogenization buffer using a Polytron® blender. In the case of gills, homogenization was carried out in a phosphate buffer (50 mM, pH 7.4) containing 1 mM ethylenediaminetetraacetic acid (EDTA), while for digestive glands, a phosphate buffer (100 mM, pH 7.4) containing 150 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT) was used. The resulting homogenates were centrifuged at 10,000*g* for 30 min at 4 °C, and the postmitochondrial supernatants were used for the enzymatic determinations (S10).

Enzymatic assays

Enzymatic activities quantified in the digestive glands of the three species and in mussel gills were CEs (using the commercial colorimetric substrates *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), 1-naphthyl acetate (1-NA), and 1-napthyl butyrate (1-NB)), GR, GPX, and GST. The use of an array of substrates for assessing CE activity has been recommended, since a variety of CE isozymes with dissimilar ability to hydrolyze different substrates can be found in the same tissue (Wheelock et al. 2005). CE activity was also assayed in mussel hemolymph using the substrate 1-NA, the only one for which CE shows high activity in this tissue (Solé and Sanchez-Hernandez 2018). AChE activity was also determined in mussel hemolymph and gills, where it shows higher activity than in the digestive gland. LPO levels, as indicator of oxidative stress damage, were also quantified in mussel digestive gland and gills.

CE and AChE activity determinations were carried out as described by Solé et al. (2018a). Sample volumes were 25 μL for CE (further S10 dilutions for substrates pNPA and pNPB were 1:2 for mussel gills, 1:10 for mussel and cockle digestive gland, and 1:20 for razor shell digestive gland; for substrates 1-NA and 1-NB, 1:5 for mussel gills, 1:20 for mussel and cockle digestive gland, 1:40 for razor shell digestive gland and undiluted hemolymph for mussel, and 25 μL of undiluted sample for AChE determinations). Regarding oxidative stress–related determinations, GR, GPX, and GST activities were determined as described in Koenig and Solé (2012). Sample volumes were 20 μL for GR (except for 10 μL in razor shell digestive gland), 10 μL for GPX (undiluted), and 25 μL for GST (1:10 in all cases). LPO assay in mussel gills and digestive gland was performed as described in Dallarés et al. (2016).

Assay conditions were kept similar and only the sample volume was adjusted in order to maintain linearity in the enzymatic measurements. All assays were carried out in triplicate at 25 °C in 96-well plates using a TECAN Infinite M200 microplate reader and blanks (sample free) accompanied the sample batches to correct for nonenzymatic reactivity of the substrates. Enzymatic activities are expressed as nmol/min/mg protein. LPO levels are expressed in nmol MDA (malondialdehyde)/g wet weight. Total protein content was determined by the Bradford method (Bradford 1976) adapted to microplate and using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin (BSA) as standard (0.05–1 mg/mL). Absorbance was read at 595 nm.

Histological assessment

A total of 40 specimens of *M. galloprovincialis*, 40 *C. edule*, and 40 *S. marginatus* (ten specimens for each sampled month, mean shell length 5.18 ± 0.62, 2.23 ± 0.21, and 9.51 ± 2.27 cm, respectively) were used for histological purposes. After dissection, a 5-mm section of each individual containing all main organs was fixed in Davidson's fixative (composition—10% glycerine, 20% formalin, 30% ethanol (95%), 30% seawater, and 10% glacial acetic acid) during 24–48 h for further histological processing. The rest of the body was conserved in 96% ethanol for further potential molecular assays. After fixation in Davidson's solution, tissues were embedded in paraffin, sectioned at 3 μm, mounted on slides, stained with hematoxylin and eosin, and examined under an Optech Biostar B5ICS light microscope.

The presence of pathogens, the condition of the different tissues, and gonadal development were also evaluated.

Microbiological and marine phycotoxins analysis

A minimum of ten specimens of *M. galloprovincialis*, *C. edule*, and *S. marginatus* were used for these analyses in order to obtain 100 g of homogenate tissue per species.

The presence of *Escherichia coli* was assessed in bivalves' tissues following the EU reference method BMS in ISO 16449-3. The procedure was based on the most probable number (MPN) method divided in two stages. The first stage consists of a five-tube three dilution with mineral modified glutamate broth (MMGB) inoculated with dilutions of shellfish homogenates (incubation 37 ± 1 °C for 24 ± 2 h). The presence of *E. coli* was confirmed by subculturing acid-producing and color change tubes in tryptone bile x-glucuronide medium (TBX) agar. The presence of blue-green colonies is positive for *E. coli*–positive β-glucuronidase (incubation 44 ± 1 °C for 21 ± 3 h).

Lipophilic marine toxins were analyzed by LC-MS/MS analysis according to the EU-Harmonised Standard Operating Procedure (SOP) procedure (ver. 5, 2015). Samples were analyzed under alkaline elution conditions (García-Altares et al. 2013). Briefly, an Agilent 1200 LC (Agilent Inc., Palo Alto, CA) coupled to a 3200 QTRAP mass spectrometer (AB Sciex, Concord, ON, Canada) was used. Analytical separation was performed on a X-Bridge C8 column (2.1 × 50 mm, 3.5 μm) protected with a precolumn (2.1 × 10 mm, 3.5 μm) from Waters (Milford, MA, USA). A binary gradient was programmed with water (mobile phase A) and acetonitrile/water (mobile phase B) both containing 6.7 mM of ammonium hydroxide.

Amnesic shellfish poisoning toxins (ASP) were analyzed by LC-UV analysis according to the EU-Harmonised SOP procedure for determination of domoic acid in shellfish by RP-HPLC using UV detection (ver. 1, 2008). For LC-UV analyses, an Alliance LC (Waters) was used. Analytical separation was performed on a X-Bridge C18 column (4.6 × 250 mm, 4.6 μm) protected with a precolumn (2.1 × 10 mm, 3.5 μm) from Waters (Milford, MA, USA). A mobile phase of acetonitrile/water (15:85) containing 0.1% formic acid was used. All runs were carried out at 40 °C using a flow rate of 1.2 mL/min. The injection volume was 20 μL and the autosampler was set at 4 °C. Detection was performed at 242 nm.

Paralytic shellfish poisoning toxins (PSP) were determined by the mouse bioassay (MBA) method according to the EURLMB SOP ver.1 (2004). Briefly, acetone extraction of the whole flesh or the hepatopancreas of molluscs was followed by evaporation and resuspension of the residue in a 1% solution of Tween 60 surfactant. One milliliter aliquots of the extract were ip injected into three male mice and observed for 24 h. The death of two of the three mice within 24 h was interpreted as a positive result. On the contrary, if none or only one of the mice died within this time, the test was considered to be negative.

Data analyses

General linear models (GLM) were applied to test the null hypothesis of no differences among the four sampling months for each enzymatic activity quantified, setting the variable month as factor, and post hoc S-N-K analyses. Prior to these analyses, data of some enzymes were logarithmically or square-root–transformed to meet both normality and homoscedasticity.

Permutation multivariate analyses (PERMANOVA) were also performed considering individual samples as replicates to test the null hypothesis of no differences in the enzymatic pool composition among the four sampling months for the three bivalve species addressed. The analyses were carried out using PERMANOVA+ for PRIMER v6 (Anderson et al. 2008) on a Bray–Curtis similarity matrix derived from untransformed data. Permutation p values were obtained under unrestricted permutation of raw data (9999 permutations). A similarity percentages analysis (SIMPER) was carried out using individual samples as replicates to identify the enzymatic activities that contributed most to the similarity/dissimilarity of individual samples within/among the samples of the four months sampled. Moreover, with the aim of visualizing patterns of dissimilarity in the enzymatic pool of the three bivalve species across the four months sampled, factorial correspondence analyses (FCA) were performed using STATISTICA v7 (StatSoft, Inc. 2004) on data matrices containing enzymatic data of each species. Hierarchical cluster analyses (Bray–Curtis similarity, average grouping method) were simultaneously performed based on the coordinates of the first two axes obtained in the corresponding FCA to identify month-related groups clearly. The previous multivariate analyses were not applied to enzymatic data of mussel hemolymph, due to the low number of biochemical markers assessed. In order to make the enzymatic pool activity patterns of the three bivalve species comparable, data of LPO levels in mussels were omitted. Finally, Spearman rank correlations were used to test the null hypothesis of no association among CE substrates and antioxidant enzymes within the tissues of the three bivalve species.

Results

Environmental variables

Most environmental variables measured presented temporal variation throughout the sampling period (Table 1). Water temperature markedly increased from April to July in the northern and southern margins, this trend being more marked in the latter locality. While salinity increased by nearly two points in the northern margin, it decreased over one point on the southern locality. Oxygen concentration did not show a clear trend in either locality. Finally, while chlorophyll-*a* concentration showed a marked decrease in the northern margin, it remained fairly stable in the southern one.

Concentration of contaminants

Mean concentrations, expressed in ng/g dry weight (dw) ± relative standard deviation ($n = 2$ replicates), of contaminants quantified in bivalves' soft tissues across the four sampled months are shown in Table 2. The following target chemicals were below the method detection limit in all cases and are thus not shown in the table: the organonitrogen pesticides metolachlor, simazine, and deethylatrazine; the organophosphorus pesticide malathion; the herbicides bentazone, MCPA, and propanil; the insecticides acetamiprid and imidacloprid; the endocrine disruptors bisphenol A, triclosan, and triclocarban; and the pharmaceuticals sulfamethozaxole, venlafaxine, and cabamazepine. Three of the quantified chemicals were pesticides, namely atrazine, thiabendazole, and diazinon, with levels ranging from below method quantification limit (MQL) to 14 ng/g dw of atrazine in razor shells from June (Supplementary material, Table 1). The other five contaminants detected were compounds considered endocrine disruptors such as caffeine, methylparaben, ethylparaben, propylparaben, and 1H-benzotriazole. The levels found ranged from below MQL up to 51 ng/g dw of caffeine measured in razor shells in July (Supplementary material, Table 1). Actually, caffeine was the contaminant presenting the highest concentrations in the three bivalves species analyzed. The mean levels of the majority of the contaminants measured were quite stable across months, and only atrazine measured in razor shell showed an increasing trend from April to July (Table 2).

Biochemical determinations

Activity levels of the different enzymes assayed in the three bivalve species, as well as LPO levels, are illustrated in Figs. 2, 3, 4, and 5.

In the case of the digestive gland of mussels, activity of CE progressively decreased with time, showing significantly lower activity values in July samples than in the other three months for the four substrates analyzed (GLM, $F_{(3,\ 36)} = 14.858$, $p < 0.001$ for 1-NA; $F_{(3,\ 36)} = 6.587$, $p = 0.001$ for 1-NB; $F_{(3,\ 36)} = 12.424$, $p < 0.001$ for pNPA; and $F_{(3,\ 36)} = 3.939$, $p = 0.016$ for pNPB) (Fig. 2a–d). The same trend was observed in cockles (GLM, $F_{(3,\ 36)} = 7.710$, $p < 0.001$ for 1-NA; $F_{(3,\ 36)} = 5.763$, $p = 0.003$ for pNPA; and $F_{(3,\ 36)} = 12.923$, $p < 0.001$ for pNPB), with the exception of 1-NB, which displayed the opposite pattern, although without showing significant differences among months (Fig. 2a–d). A similar decreasing trend was found in razor shells, although significantly lower activities were observed in April for the substrates 1-NA and pNPA (GLM, $F_{(3,\ 36)} = 4.190$, $p = 0.012$ and $F_{(3,\ 36)} = 4.709$, $p = 0.007$, respectively) (Fig. 2a–d).

A higher variability was observed among the responses of antioxidant enzymes: GR activity values significantly

Table 2 Mean concentration (ng/g dry weight) ± relative standard deviation (RSD) ($n = 2$ replicates) of the contaminants quantified in soft tissues of mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*), and grooved razor shells (*Solen marginatus*) in the four sampling performed in the Alfacs Bay, Ebro Delta

Family	Compounds	*Mytilus galloprovincialis*				*Cerastoderma edule*				*Solen marginatus*			
		April	May	June	July	April	May	June	July	April	May	June	July
Organonitrogen pesticides	Atrazine	1.56 ± 0.32	1.29 ± 0.10	<MQL	<MQL	0.82 ± 0.08	<MDL	<MDL	<MDL	3.04 ± 0.84	5.15 ± 1.02	13.63 ± 2.75	8.44 ± 0.23
Organophosphorus pesticides	Thiabendazole	<MDL	<MDL	<MDL	<MDL	<MDL	0.93 ± 0.18	0.43 ± 0.003	0.60 ± 0.00	<MDL	<MDL	<MDL	<MDL
	Diazinon	<MDL	<MDL	0.46 ± 0.00	0.51 ± 0.00	0.57 ± 0.00	1.53 ± 0.02	<MDL	<MDL	<MDL	<MDL	0.56 ± 0.01	0.55 ± 0.01
Endocrine disruptors (EDCs)	Caffeine	<MDL	<MDL	<MQL	11.82 ± 1.93	22.60 ± 5.48	33.62 ± 5.51	<MDL	<MDL	<MQL	<MQL	46.95 ± 6.29	50.96 ± 15.12
	Methylparaben	2.69 ± 0.02	1.01 ± 0.24	0.54 ± 0.08	0.83 ± 0.02	2.00 ± 0.06	3.54 ± 0.05	1.01 ± 0.11	1.13 ± 0.19	1.91 ± 0.59	1.71 ± 0.05	1.19 ± 0.08	1.03 ± 0.12
	Ethylparaben	1.85 ± 0.12	<MDL	<MDL	0.54 ± 0.06	0.55 ± 0.02	0.36 ± 0.03	0.73 ± 0.04	0.35 ± 0.02	<MDL	<MDL	<MQL	<MQL
	Propylparaben	<MDL	<MDL	<MDL	<MQL	1.38 ± 0.04	0.93 ± 0.06	0.94 ± 0.02	1.18 ± 0.06	<MDL	<MDL	<MQL	<MQL
	1H-benzotriazole	0.88 ± 0.76	1.38 ± 0.18	0.83 ± 0.51	0.66 ± 0.01	1.14 ± 0.53	2.24 ± 1.28	<MQL	1.44 ± 0.29	1.32 ± 0.16	1.26 ± 0.11	2.01 ± 0.24	1.73 ± 0.23

MDL, method detection limit; *MQL*, method quantification limit

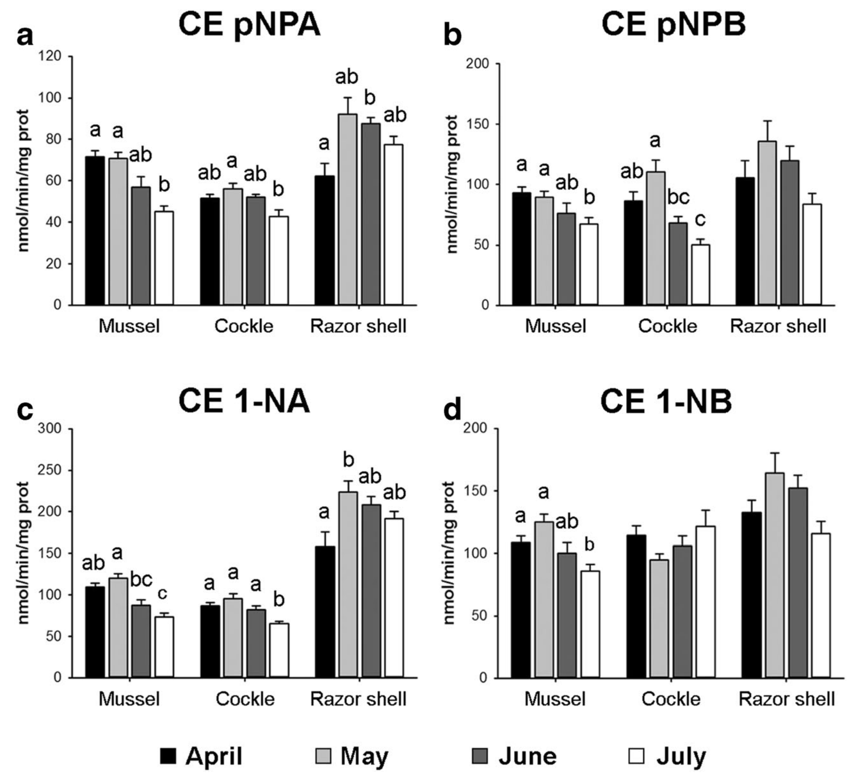


Fig. 2 **a**–**d** Levels of carboxylesterase activity using four different colorimetric substrates (*p*-nitrophenyl acetate, pNPA; *p*-nitrophenyl butyrate, pNPB; 1-naphthyl acetate, 1-NA; and 1-naphthyl butyrate, 1-NB) in the digestive gland of mussels, cockles, and grooved razor shells collected in the Alfacs Bay of the Ebro Delta in April, May, June, and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$)

decreased from April (mussels) and May (razor shells) to July samplings (GLM, $F_{(3,\ 36)} = 11.944$, $p < 0.001$ and $F_{(3,\ 35)} = 6.577$, $p = 0.001$, respectively), while a progressive increase in the activity of GPX and GST from April to July was observed in razor shells (GLM, $F_{(3,\ 36)} = 10.305$, $p < 0.001$ and $F_{(3,\ 35)} = 6.139$, $p = 0.002$, respectively) (Fig. 3a–c). No clear trends were detected for these enzymes in the case of cockles (GLM, $p > 0.05$).

Regarding enzymatic determinations in mussel gills, significant decreasing trends through time were found for CE activity with the substrates 1-NA, pNPA, and pNPB (GLM, $F_{(3,\ 36)} = 10.122$, $p < 0.001$; $F_{(3,\ 36)} = 11.128$, $p < 0.001$; and $F_{(3,\ 36)} = 4.359$, $p = 0.01$, respectively) (Fig. 4a–c) and also for GPX and GST activities (GLM, $F_{(3,\ 36)} = 3.005$, $p = 0.043$ and $F_{(3,\ 36)} = 11.663$, $p < 0.001$, respectively) (Fig. 5b, c). In the case of the enzymes tested in mussel hemolymph (i.e., CE with substrate 1-NA and AChE), no significant differences among months were detected in any case (GLM, $p > 0.05$).

The PERMANOVA applied to individual samples showed a significant effect of the factor month in the enzymatic pool of the digestive gland of the three bivalve species (pseudo-$F_{(3,}$

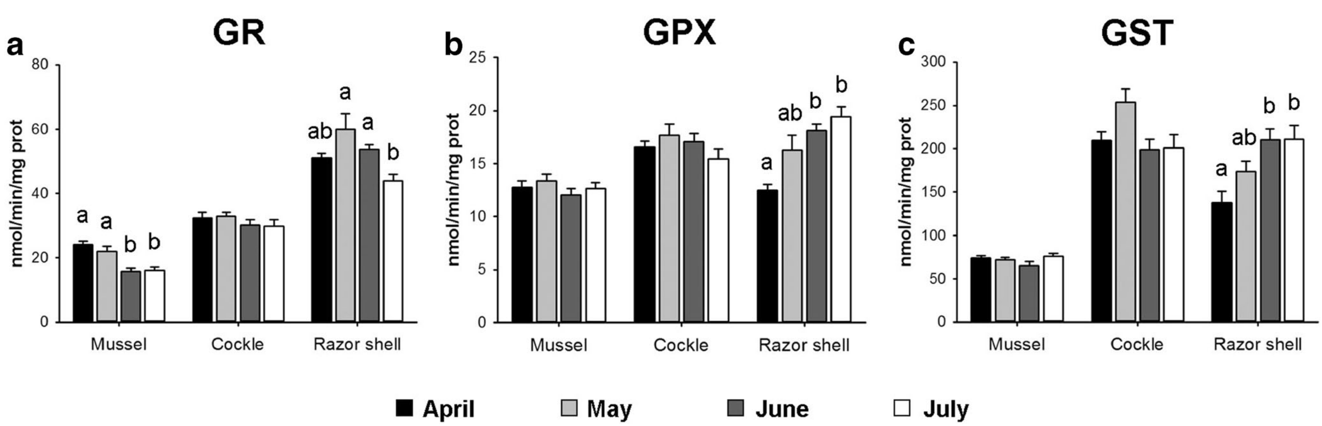


Fig. 3 **a**–**c** Levels of antioxidant enzymatic activities (glutathione reductase, GR; glutathione peroxidase, GPX; and glutathione S-transferases, GST) determined in the digestive gland of mussels, cockles, and grooved razor shells collected in the Alfacs Bay of the Ebro Delta in April, May, June, and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$)

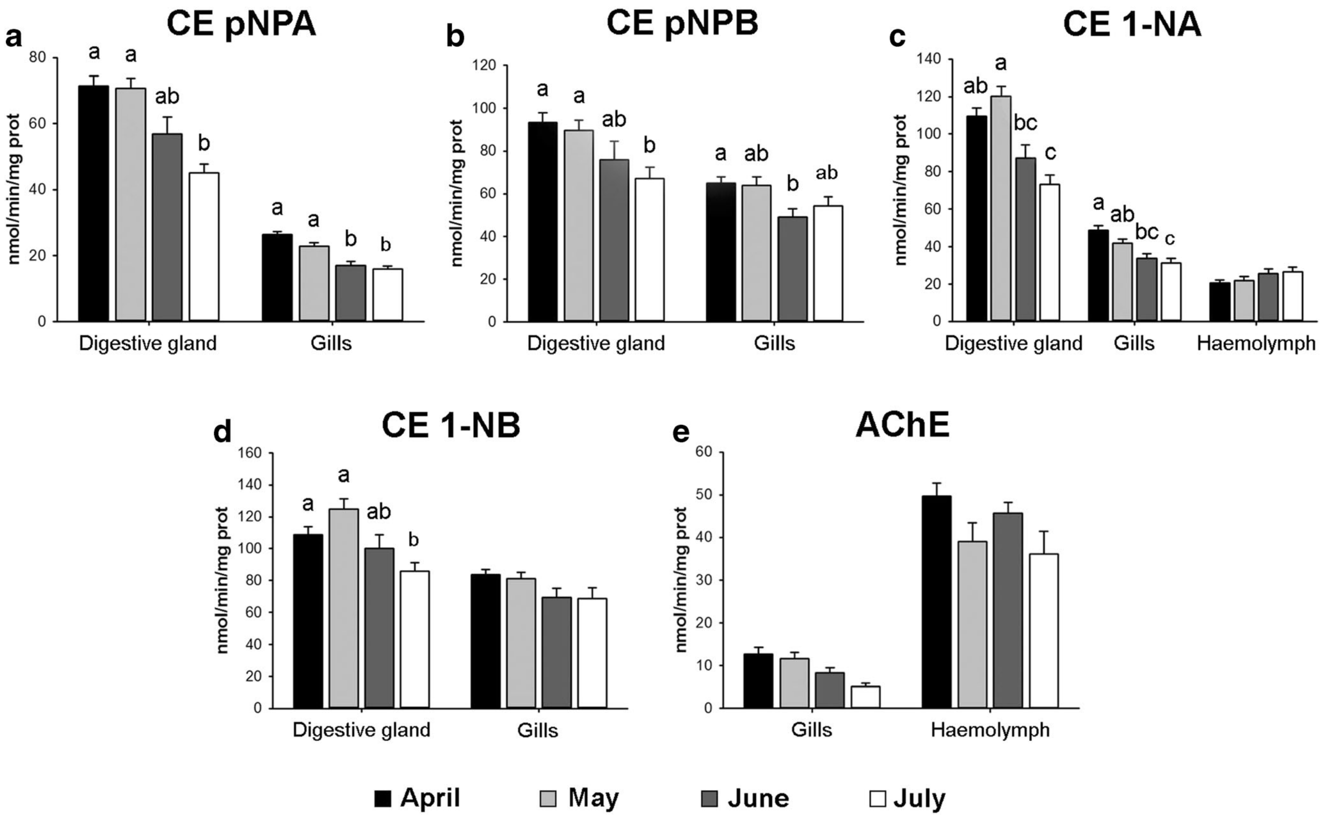
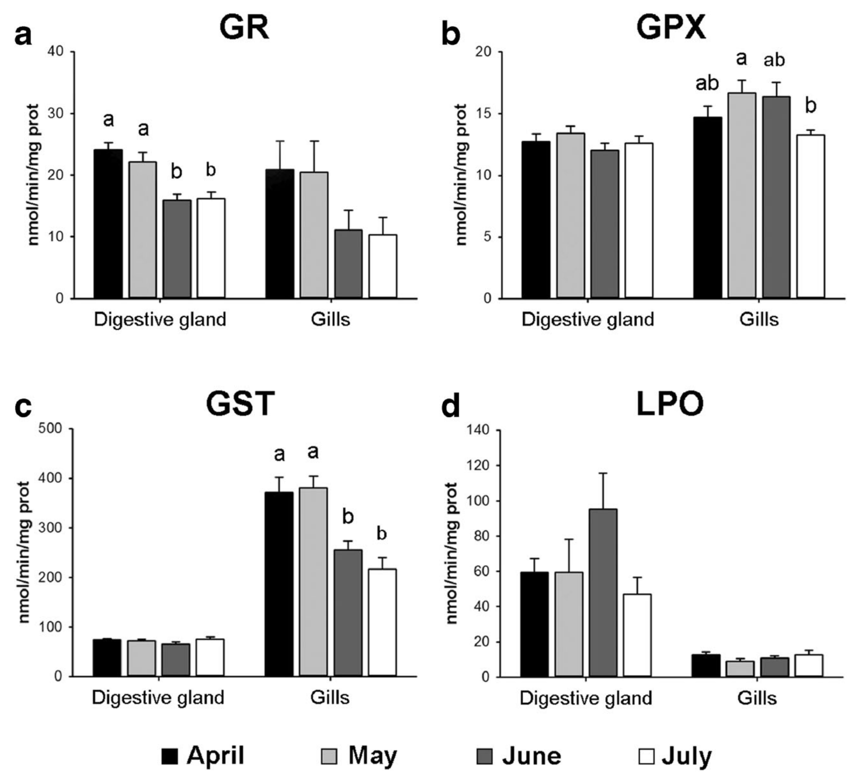


Fig. 4 **a**–**e** Levels of carboxylesterase activity using four different colorimetric substrates (*p*-nitrophenyl acetate, pNPA; *p*-nitrophenyl butyrate, pNPB; 1-naphthyl acetate, 1-NA; and 1-naphthyl butyrate, 1-NB) and acetylcholinesterase activity (AChE) in the digestive gland, gills, and hemolymph of mussels collected in the Alfacs Bay of the Ebro Delta in April, May, June, and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$)

Fig. 5 Levels of antioxidant enzymatic activities (glutathione reductase, GR; glutathione peroxidase, GPX; and glutathione S-transferases, GST) and lipid peroxidation (LPO) levels determined in the digestive gland and gills of mussels collected in the Alfacs Bay of the Ebro Delta in April, May, June, and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$)



$_{36)} = 6.3068$, $p_{(perm)} = 0.0003$ for mussels; pseudo-$F_{(3,\ 36)} = 5.0565$, $p_{(perm)} = 0.0003$ for cockles; and pseudo-$F_{(3,\ 35)} = 4.2551$, $p_{(perm)} = 0.0004$ for razor shells) and of the gills of mussels (pseudo-$F_{(3,\ 36)} = 9.2979$, $p_{(perm)} = 0.0001$). Figure 6 shows the resulting dendrograms of the cluster analyses simultaneously performed to the FCAs, which show the patterns of similarity among the enzymatic pools of the four sampled months in the three bivalve species. In the digestive gland of mussels and razor shells (Fig. 6a, d), April, May, and June clustered together, while July remained as a separate clade. For mussel gills (Fig. 6b), the two earliest samplings were separated from the two latter ones. In the case of cockles (Fig. 6c), enzymatic pools of April and June were most related, while May and July formed independent clades. The SIMPER allowed the identification, for each bivalve species, of the enzymatic activities that contributed most to the similarity/dissimilarity of the enzymatic pools within/between sampled months. These were CE with all substrates assayed (i.e., pNPA, pNPB, 1-NA, and 1-NB) and GST in the case of the digestive gland of mussels, cockles, and razor shells and CE with substrates 1-NB and pNPB and GST and GR for mussel gills (Supplementary material, Table 2).

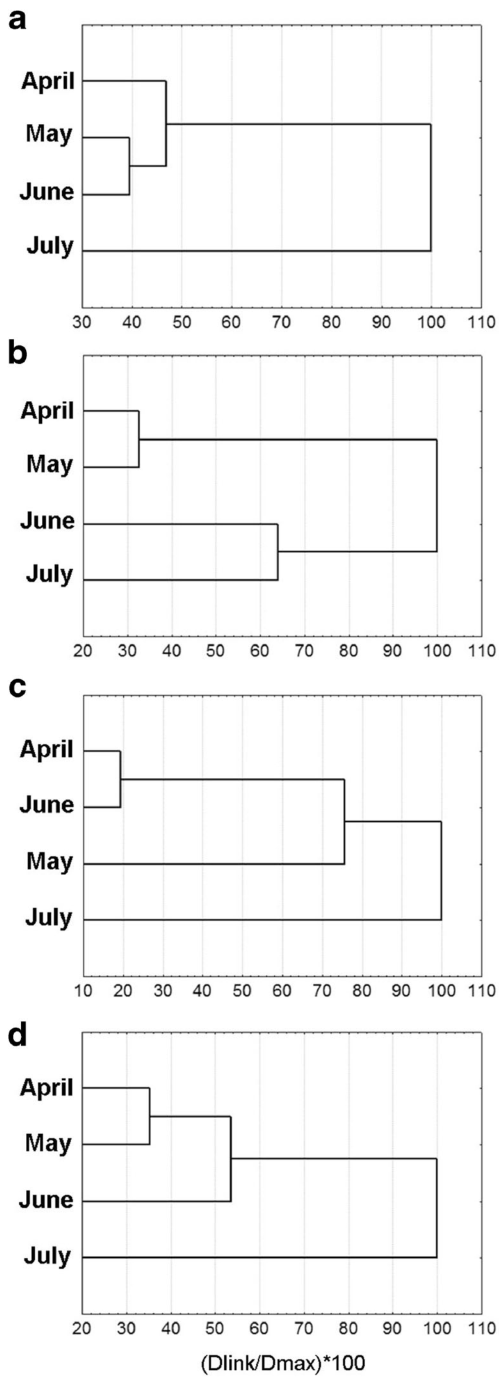


Fig. 6 Dendrograms resulting from the hierarchical cluster analyses based on enzymatic data in mussel digestive gland (**a**), mussel gills (**b**), cockles' digestive gland (**c**), and razor shells' digestive gland (**d**) collected in the Alfacs Bay of the Ebro Delta in April, May, June, and July of 2017

Significant positive correlations were found among all CE substrates in mussel digestive gland ($r_s = 0.700$–0.915, $p < 0.01$) and gills ($r_s = 0.663$–0.865, $p < 0.01$) and in the digestive gland of razor shells ($r_s = 0.460$–0.832, $p < 0.01$). In the case of the digestive gland of cockles, significant positive associations were detected among the CE substrates pNPA, pNPB, and 1-NA ($r_s = 0.755$–0.818, $p < 0.01$). In the case of antioxidant enzymes, significant positive correlations among GR, GPX, and GST were detected in the digestive gland of mussels ($r_s = 0.377$–0.503, $p < 0.05$), between GR and GPX and between GST and GPX in mussel gills ($r_s = 0.339$–0.439, $p < 0.05$), between GST and GR in the digestive gland of cockles ($r_s = 0.534$, $p < 0.05$), and between GST and GPX in digestive gland of razor shells ($r_s = 0.733$, $p < 0.05$).

Histology

The histological study revealed that the three bivalve species were in maturation and spawning reproductive phases during the sampling period. In the case of mussels, late spawning and gonadal reabsorption phases could be also observed in May and June, while nonobservable gonads were found in July.

The most relevant histopathological observations in mussels were unspecific lesions of hypertrophic nuclei with peripheral chromatin in the digestive gland in May, June, and July. Moreover, one sample was infected with moderate levels of the protozoan *Marteilia* sp. In the case of cockles, *Rickettsia*-like organisms (RLOs) were observed in gills in all samplings (Fig. 7), as well as ciliates of the genus *Trichodina* in the stomach and intestinal lumens in June. In regard to razor shells, RLOs were also detected in all samplings, in gills and the digestive system.

Microbiological and phycotoxin analysis

Low levels of *E. coli* were found in the tissues of mussels and razor shells (20–45 and 18–78 MPN/100 g, respectively) through the different samplings. In contrast, much higher

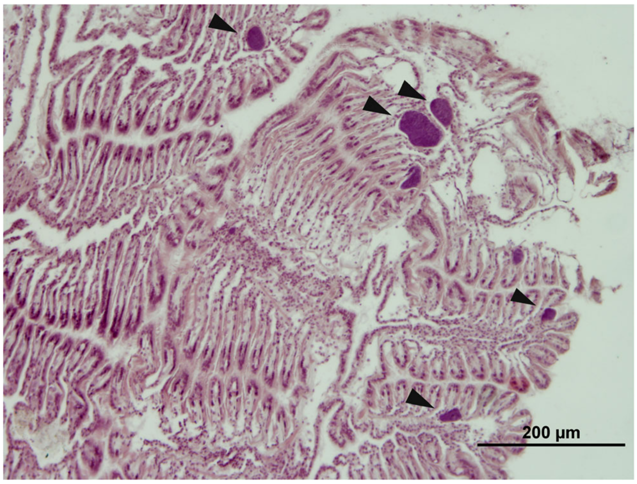


Fig. 7 *Rickettsia*-like organisms (RLOs) (arrowheads) in the gills of cockles collected in the Alfacs Bay of the Ebro Delta in June of 2017

numbers of these bacteria were detected in cockles, increasing in number from 790 MPN/100 g in April to 9200 MPN/100 g in July.

Levels of hydrophilic toxins (comprising PSP, paralytic shellfish poisoning and ASP, amnesic shellfish poisoning toxins) were low and did not reach the maximum permitted levels (MPLs) in edible shellfish tissues for human consumption according to the European Union (Regulation EC 853/2004: 20 mg domoic acid/kg (ASP) and 800 μg eq STX/kg (PSP)). Regarding lipophilic toxins, only two phycotoxins were detected: traces of yessotoxin in mussel samples in May and pinnatoxin-G at low concentrations in mussel samples of the four months (range 3.2–6 μg/kg) and in razor shell samples in late June (2.5 μg/kg). Pinnatoxin-G levels in mussel samples were higher in late June than in the other three sampling dates. Levels detected for lipophilic toxins did not reach the MPLs according to the EU (Regulation EC 853/2004 and Regulation EC 786/2013: 160 μg/kg okadaic acid (OA), equivalents for OA, dinophysistoxins (DTXs), and pectenotoxins (PTXs) together; 3.75 mg/kg for yessotoxins (YTXs) and 160 μg/kg for azaspiracids (AZAs)). Other lipophilic marine toxins are not yet regulated in the European Union, like cyclic imines mainly comprising spirolides (SPXs), gymnodimines (GYMs), and pinnatoxins (PnTXs).

Discussion

Biochemical responses and relationship to local contaminants

A general and coordinated change of enzymatic markers throughout the sampling period was revealed after multivariate analysis performed on enzymatic data by the PERMANOVA tests and cluster analyses and after GLMs for independent biochemical markers. The results of the SIMPER analyses highlighted the particular contribution of CE and, to a lesser extent, also GST to this response in the digestive gland of the three studied bivalves and also in mussel gills. However, the bioaccumulated pesticides apparently did not explain the patterns observed for the biochemical responses. Indeed, most of the herbicides and pesticides tested were not detected in any biological sample, and those that did, seemed to be below the threshold limit for altering the activity of the enzymes addressed in the present study, as deduced from the lack of correspondence between their concentrations and the biochemical responses observed (Table 2). Previous studies on the same bay indicated that after the period of pesticide application in the rice fields of the Ebro Delta, which takes place from April to June (Terrado et al. 2007; Köck et al. 2010; Suárez-Serrano et al. 2010), the maximum levels of pesticides in its drainage channels and bays are attained during the spring and summer periods (when this study takes place), soon after the field waters are discharged into the bays (Escartín and Porte 1997; Santos et al. 2000). It has already been reported that the measurement of contaminants' concentration in bivalves' tissues could lead to underestimation of the real pollution load in the surrounding waters (Lehotay et al. 1998; Köck et al. 2010). Indeed, these authors detected inexistent or very low levels of contaminants in oyster and mussel tissues despite that the concentrations of these same chemicals were high in water, which agrees with the low levels of atrazine, thiabendazole, and diazinon in the present research. The possible effect of environmental contaminants in the studied organisms, even though the former are nondetectable in their tissues, makes biomarkers highly recommended tools usually incorporated in ecotoxicological studies (Farcy et al. 2013; Solé and Sanchez-Hernandez 2018).

The presence of other anthropogenic chemicals accumulated in biota was low (few ng/g dw) except for caffeine, with levels much higher than previously reported in the study area and other locations (Dodder et al. 2014; Álvarez-Muñoz et al. 2015). No clear temporal trends were observed for the organic contaminants measured except for caffeine in mussel and razor shells, which increased in the summer months, and for atrazine in razor shells, which increased from April to June. Caffeine has been reported to produce alterations on metabolic activity and oxidative stress biomarkers in bivalves (Cruz et al. 2016). Therefore, this compound might explain part of the patterns observed for biochemical markers (see below), although as far as we are concerned, no reference concentrations have been recorded in bivalves' tissues; all studies addressing the effects of caffeine on invertebrates are based on water concentrations. In line with this influx of urban residues during the summer period, it stands out a higher presence of the fecal bacteria *E. coli* detected in cockle samples collected in July, which are in all likelihood explained by the raw sewage discharges of a nearby town during the touristic summer season.

Regarding CE activities, a former study conducted with mussels in the same area and over a longer period of time reported an inhibition of CE activity (1-NA as substrate) in the gills and digestive gland after the arrival of pollutants from rice fields to the Alfacs Bay in early summer (Escartín and Porte 1997), similar to the pattern observed in the present study. Moreover, Solé et al. (2018a) characterized baseline enzymatic activities for CEs in the digestive glands of the same three bivalve species addressed herein and collected from the same sites, and after in vitro exposure to the OP metabolite chlorpyrifos oxon, also concluded that CEs are potentially good indicators of pesticide pollution in bivalves. With regard to the tissues addressed, Solé and Sanchez-Hernandez (2018) and Escartín and Porte (1997) reported higher inhibition of CE in mussel gills than in digestive gland when exposed to pollutants in in vitro conditions, which waited to be confirmed under real field situations. However, a similar inhibitory trend on CE activity was observed in the two selected tissues in mussels from April–May to July for the substrates 1-NA and pNPA, and higher inhibition in digestive gland than in gills was detected for the longer-chain carbon esters pNPB and 1-NB (Fig. 4a–d), nonconfirming these previous expectations, and rather suggesting higher detoxification activity in the former tissue under field conditions. It could be hypothesized that the complexity of field conditions, where mixtures of chemicals occur, could yield a different pattern of CE activity in the selected tissues, with the digestive gland showing a greater participation in detoxification processes than when faced with in vitro conditions. Solé and Sanchez-Hernandez (2018) suggested that the substrates pNPB and 1-NB were potentially more suitable for detecting inhibition of CE activity in the field in mussels than the substrates pNPA and 1-NA based on their lower IC50 when exposed in vitro to the OP pesticide dichlorvos, but also in response to other pharmaceuticals and personal care products (PPCPs). This is not corroborated by present field results, since CE seemed more sensitive to inhibition from May to July when using the substrates 1-NA and pNPA both in gills and digestive gland. Although these and other authors (e.g., Otero and Kristoff 2016) have noted that the selection of appropriate substrates is species and tissue specific, the present results indicate that there might be additional variables influencing CE activity even within the same tissue and species. The inhibitory CE pattern observed in mussels was also apparent in *C. edule* and *S. marginatus*, although cockles showed an unexpected increase in activity with time when using the substrate 1-NB, which can explain the lack of correlation between this and the other three substrates. This result contrasts with the outcomes of the study by Solé et al. (2018a), in which all four substrates were significantly correlated in the three bivalves selected, including cockle. This result could point to a different contribution of CE isozymes in cockles compared to mussels and razor shells under conditions of pollution exposure. Razor shells demonstrated less sensitivity regarding the longer-chain carbon esters (i.e., pNPB and 1-NB), apparently due to a higher variability in the data (Fig. 2a–d). The observed substrate-specific variability in CE for the three bivalves addressed highlights the importance of using a battery of substrates for assessing the inhibition of this enzyme by pollutants.

The lack of effect on AChE activity in mussel gills and hemolymph supports former observations in bivalves that suggested that CE is a more adequate indicator of OP exposure (Galloway et al. 2002; Wheelock and Nakagawa 2010; Otero and Kristoff 2016; Solé et al. 2018b).

The responses of the antioxidant enzymes assessed were not consistent across the three species addressed. For GPX and GST, clear activation patterns were observed in razor shells (Fig. 3b, c), which point to chemical stress by pollutants and the involvement of these enzymes in the associated detoxification processes. Conversely, inhibitory patterns were observed for GR in the digestive glands of mussel and razor shells (Fig. 3a), as already reported by Mundhe et al. (2016) in the presence of the organophosphate pesticide monocrotophos. As regards tissue responsiveness in mussels, GR was inhibited in the summer months in the gills and digestive gland (Fig. 5a), although in the former organ, this inhibition was not significant ($p > 0.05$) due to high variability. By contrast, GPX and GST were more responsive in gills than in digestive gland, although an inhibition in the activity was revealed in July for GPX and in June and July for GST (Fig. 5b, c). The lack of a clear antioxidant response in mussels was concordant with no clear increase of oxidative stress damage measured as enhanced LPO levels, and both suggest that the chemical threat, rather than acting over ROS production, could be more selective toward CE inhibition in this case. Antioxidant responses in bivalves are complex and controversial because their activation can take place at low oxidative stress conditions, but depletion of antioxidant activities can occur in situations of severe oxidative stress, since antioxidant enzymes can be a target of ROS themselves (Regoli and Giuliani 2014).

Influence of temperature on biochemical markers

Temperature is considered one of the most important confounding factors in bivalve monitoring studies (Farcy et al. 2013), provided that it is known to increase metabolic rate and the production of ROS, to modify catalytic efficiency and influence phytoplankton abundance (Somero 1995; Pörtner 2002), among others. Very few studies have addressed CEs' response to temperature variations in animals and have yielded different conclusions: at higher temperatures, Owusu et al. (1994) reported an increase in CE activity in aphids, while Escartín and Porte (1997) found no significant change in mussels due to this factor. However, higher susceptibility of CE to pollutants under increasing temperatures has been validated in aquatic species (Laetz et al. 2014; Freitas et al. 2017) and would suggest that the generalized inhibition on CE activity with time due to pesticide pollution might have been enhanced in the warmer months. Activity levels of AChE are known to increase with higher temperatures (Pfeifer et al. 2005), and an increase of its activity across the four samplings would thus be expected in the absence of a pollution effect. The opposite trends observed in the present study, similar to those by Escartín and Porte (1997) in the same area, support the hypothesis of an effect of pesticide pollution on bivalves. Higher temperatures have also been reported to significantly increase the activity of antioxidant enzymes, yielding an oxidative stress-like response in mussels (Hu et al. 2015), which could be explained either by an increased production of ROS or by alterations of enzymatic catalytic efficiency (Somero 1995; Almeida and Mascio 2011). In this respect, a generalized increase in the activity of antioxidant enzymes was not observed, for we believe that the influence of temperature in this case might have either been weak or masked by other processes of greater influence.

Influence of reproductive condition on biochemical markers

Another important confounding factor in the assessment of pollution effects in bivalves is the reproductive condition, which interferes with biomarker responses and is closely related to temperature and nutritive status (Farcy et al. 2013; González-Fernández et al. 2015a, b). Although no specific measurements of nutritive condition were performed in the present study, the low variability in chlorophyll-*a* concentration across samplings suggests a fairly constant supply of food to the bivalves throughout the study. Reproduction represents a critical period with a major influence in gene expression, metabolism, and immune function, among others (Farcy et al. 2013; González-Fernández et al. 2017). All these energetically demanding processes can result in poorer physiological condition and make coping with stressful events difficult (Berthelin et al. 2000). This is why it is considered a major confounding factor for the interpretation of biomonitoring data, and assessing the effect of xenobiotics during the reproductive phase has been recommended (Farcy et al. 2013). Accordingly, the histological analysis revealed that bivalve populations were at the active reproductive stage during the sampling period. In the case of cockles and razor shells, the uniform reproductive condition across samplings suggests that observed changes in biomarkers should not be driven by reproduction events. In the case of mussels, in which final spawning and resting stages could be observed in June and July, alterations in biomarkers due to the reproductive condition could have occurred during these two months. However, decreasing trends in mussel CE and GR are concordant to those observed for cockles and/or razor shells, and no clear trends were observed for GPX and GST. We believe that reproduction effects on enzymatic activities in this case might have been weak and not clearly identified.

Histopathological analysis

Histological analysis of tissue damage was screened in all sampling groups (species and times), and no significant histological alterations could be associated to pesticide pollution. The frequent detection of RLOs in the gills of cockles and razor shells could potentially be associated to some type of contamination. For example, *Rickettsia* infections of gut/digestive tubules of oysters have been found to have a significant correlation with nickel contamination on the American coast (Kim et al. 1998; Morley 2010), and have also been found to be significantly higher in deep-sea mussels exposed to petroleum seeps (Powell et al. 1999), although further studies are required to demonstrate a direct effect to such exposure. The high prevalence and intensity reported for the ciliate *Trichodina* sp. in cockle gills in June is indicative of a great abundance of this ciliate in the environment, in some cases linked to environmental eutrophication (Palm and Dobberstein 1999). Bivalve gill ciliates have been found to be most common at heavily polluted sites on the northeast coast of America (Morley 2010). A unique sample of mussel was infected with *Marteilia* sp. (probably *M. refringens*), a pathogen of obligatory declaration according to the World Organisation for Animal Health (OIE). This parasite has been historically detected in mussels and flat oysters in the Ebro Delta production areas. However, in this case, prevalence seems to be low in an optimal period for the parasite (Carrasco et al. 2008).

Phycotoxin analysis

Recently, marine phycotoxins have been proposed as an additional confounding factor in studies assessing the effects of pollution (Farcy et al. 2013). However, they were found at very low concentrations in the present study, for it is unlikely that they have interfered with biomarkers' response. Harmful hydrophilic toxins ASP and PSP did not seem to pose a threat to bivalve consumption during the period of study as they were all below the legislation EU threshold.

Conclusions

The consistent responses of CE across species and tissues (in mussels) with respect to AChE and antioxidant enzymes suggest that CE activity can be a more sensitive and robust biomarker when evaluating pesticide pollution in bivalves. Among the three bivalve species, mussels provided the most sensitive response regarding CE activity. With respect to oxidative stress, it was better reflected by razor shells' enzymatic responses. In turn, cockles seemed to provide the less sensitive response, both considering CE and antioxidant enzymes. This interspecies dependence of the responses of different enzymatic functional groups points to the use of more than one bioindicator as the best approach to ecotoxicological studies using bivalves. Furthermore, biochemical markers seemed to provide a much more robust and sensitive response than histological ones. None of the confounding factors potentially influencing biomarker responses seemed to be relevant enough to modulate the assessed enzymatic activities in the study area.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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