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1 Multibiomarker biomonitoring approach using three bivalve species in the Ebre Delta
2 (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 Ebre 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, bivalves 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

1. Introduction

Estuaries are semi-enclosed coastal areas characterized by high biomass, biodiversity and primary production, which favour 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 threat 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 sub-individual 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 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 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. 2004; 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 multi-biomarker 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.

2. Materials and methods

2.1. Sampling area and specimen collection

The Ebre Delta is an extensive estuarine area (320 km²) located at the mouth of the Ebre 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 Ebre 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.

2.2. Environmental variables

Records for temperature (in °C), salinity (in S_p), dissolved O₂ 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.

2.3. Analysis of organic contaminants

A multi-residue analytical method (Álvarez-Muñoz et al. en prep) 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* 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 MgSO₄, 1 g NaCl, 1 g NaCitrate 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.

2.4. 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 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, haemolymph was extracted from the adduct muscle immediately upon sampling, using a 1 mL syringe with a 0.21 gauge needle. Haemolymph was frozen at -80°C and centrifuged ($5,000\text{ g} \times 5\text{ min}$ 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.

2.4.1. Tissue preparation

Tissues were homogenised (1:5, w/v) in ice-cold homogenisation 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 1mM dithiothreitol (DTT) was used. The resulting homogenates were centrifuged at 10,000 g for 30 min at 4 °C, and the post-mitochondrial supernatants were used for the enzymatic determinations (S10).

2.4.2. Enzymatic assays

Enzymatic activities quantified in digestive glands of the three species and in mussel gills were: carboxylesterase (CE) (using the commercial colorimetric substrates p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), 1-naphthyl acetate (1-NA) and 1-naphthyl butyrate (1-NB)), glutathione reductase (GR), glutathione peroxidase (GPX) and glutathione S-transferase (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 hydrolyse different substrates can be found in the same tissue (Wheelock et al. 2005). CE activity was also assayed in mussel haemolymph using the substrate 1-NA, the only one for which CE shows high activity in this tissue (Solé and Sanchez-Hernandez 2018). Acetylcholinesterase (AChE) activity was also determined

187 in mussel haemolymph and gills, where it shows higher activity than in the digestive
 188 gland. Lipid peroxidation (LPO) levels, as indicator of oxidative stress damage, were
 189 also quantified in mussel digestive gland and gills.
 190 CE and AChE activity determinations were carried out as described by Solé et al.
 191 (2018a). Sample volumes were: 25 µl for CE (further S10 dilutions for substrates pNPA
 192 and pNPB were 1:2 for mussel gills, 1:10 for mussel and cockle digestive gland and
 193 1:20 for razor shell digestive gland; for substrates 1-NA and 1-NB, 1:5 for mussel gills,
 194 1:20 for mussel and cockle digestive gland, 1:40 for razor shell digestive gland and
 195 undiluted haemolymph for mussel and 25 µl of undiluted sample for AChE
 196 determinations. Regarding oxidative-stress-related determinations, GR, GPX and GST
 197 activities were determined as described in Koenig and Solé (2012). Sample volumes
 198 were: 20 µl for GR (except for 10 µl in razor shell digestive gland), 10 µl for GPX
 199 (undiluted) and 25 µl for GST (1:10 in all cases). LPO assay in mussel gills and
 200 digestive gland was performed as described in Dallarés et al. (2016).
 201 Assay conditions were kept similar and only the sample volume adjusted in order to
 202 maintain linearity in the enzymatic measurements. All assays were carried out in
 203 triplicate at 25°C in 96-wellplates using a TECAN Infinite M200 microplate reader and
 204 blanks (sample free) accompanied the sample batches to correct for non-enzymatic
 205 reactivity of the substrates. Enzymatic activities are expressed as nmol/min/mg protein.
 206 LPO levels are expressed in nmol MDA (malondialdehyde)/g wet weight. Total protein
 207 content was determined by the Bradford method (Bradford 1976) adapted to microplate
 208 and using the Bradford Bio-Rad Protein Assay reagent and bovine serum albumin
 209 (BSA) as standard (0.05–1 mg/mL). Absorbance was read at 595 nm.

211 *2.5. 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 24–48h 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 μ m, mounted on slides, stained with Haematoxylin 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.

2.6. Microbiological and marine phycotoxins analysis

A minimum of 10 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 is 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°C +/- 1°C for 24h +/- 2h). 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°C +/- 1°C for 21h +/- 3h).

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

245 Amnesic shellfish poisoning toxins (ASP) were analysed by LC-UV analysis according
246 to the EU-Harmonised SOP procedure for determination of domoic acid in shellfish by
247 RP-HPLC using UV detection (ver. 1, 2008). For LC-UV analyses, an Alliance LC
248 (Waters) was used. Analytical separation was performed on a X-Bridge C18 column
249 (4.6 × 250 mm, 4.6 µm) protected with a pre-column (2.1 × 10 mm, 3.5 µm) from
250 Waters (Milford, MA, USA). A mobile phase of acetonitrile/water (15:85) containing
251 0.1% formic acid was used. All runs were carried out at 40 °C using a flow rate of 1.2
252 mL/min. The injection volume was 20 µL and the autosampler was set at 4 °C.
253 Detection was performed at 242 nm.

254 Paralytic shellfish poisoning toxins (PSP) were determined by the Mouse Bioassay
255 (MBA) method according to the EURLMB SOP ver.1 (2004). Briefly, acetone
256 extraction of the whole flesh or the hepatopancreas of molluscs was followed by
257 evaporation and resuspension of the residue in a 1% solution of Tween 60 surfactant.
258 One milliliter aliquots of the extract were ip injected into three male mice and observed
259 for 24h. The death of two of the three mice within 24h was interpreted as a positive

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260 result. On the contrary, if none or only one of the mice died within this time, the test
261 was considered to be negative.

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263 *2.7. Data analyses*

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

269 Permutation multivariate analyses (PERMANOVA) were also performed considering
270 individual samples as replicates to test the null hypothesis of no differences in the
271 enzymatic pool composition among the four sampling months for the three bivalve
272 species addressed. The analyses were carried out using PERMANOVA+ for PRIMER
273 v6 (Anderson et al. 2008) on a Bray-Curtis similarity matrix derived from
274 untransformed data. Permutation p-values were obtained under unrestricted permutation
275 of raw data (9,999 permutations). A similarity percentages analysis (SIMPER) was
276 carried out using individual samples as replicates to identify the enzymatic activities
277 that contributed most to the similarity/dissimilarity of individual samples within/among
278 the samples of the four months sampled. Moreover, with the aim of visualizing patterns
279 of dissimilarity in the enzymatic pool of the three bivalve species across the four
280 months sampled, factorial correspondence analyses (FCA) were performed using
281 STATISTICA v7 (StatSoft, Inc. 2004) on data matrices containing enzymatic data of
282 each species. Hierarchical cluster analyses (Bray-Curtis similarity, average grouping
283 method) were simultaneously performed based on the coordinates of the first two axes
284 obtained in the corresponding FCA to identify month-related groups clearly. The

previous multivariate analyses were not applied to enzymatic data of mussel haemolymph, 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.

3. Results

3.1 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.

3.2. Contaminants concentration

Mean concentration, expressed in ng/g dry weight (dw) $\pm$ 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 organitrogen 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 sulfamethoxazole, 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 1-H-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, only atrazine measured in razor shell showed an increasing trend from April to July (Table 2).

3.3. Biochemical determinations

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

In the case of mussels digestive gland, 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 analysed (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) (Figs. 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 (Figs. 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) (Figs 2A–D).

A higher variability was observed among the responses of antioxidant enzymes: GR activity values significantly 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) (Figs. 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) (Figs. 4A–C), as 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) (Figs. 5B, C). In the case of the enzymes tested in mussel haemolymph (i.e. CE with substrate 1-NA and AChE), no significant differences among months were detected in any case (GLM, $p>0.05$).

The permutational multivariate analyses (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- $F_{(3, 36)}=6.3068$, $p_{(perm)}=0.0003$ 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 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 sampling 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 similarity percentages analysis (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 mussels, cockles and razor shells digestive gland and CE with substrates 1-NB and pNPB, GST and GR for mussel gills (Supplementary material, Table 2).

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 razor shells digestive gland ($r_s=0.460-0.832$, $p<0.01$). In the case of cockles digestive gland, 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 mussels digestive gland ($r_s=0.377-0.503$, $p<0.05$), 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 cockles digestive gland ($r_s=0.534$, $p<0.05$) and between GST and GPX in razor shells digestive gland ($r_s=0.733$, $p<0.05$).

3.4. 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 non observable 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.

3.5. Microbiological and phycotoxins analysis

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

4. Discussion

4.1. 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 Ebre 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 period (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

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434 been reported that the measurement of contaminants concentration in bivalves' tissues
435 could lead to underestimation of the real pollution load in the surrounding waters
436 (Lehotay et al. 1998; Köck et al. 2010). Indeed, these authors detected inexistent or very
437 low levels of contaminants in oyster and mussel tissues despite that the concentrations
438 of these same chemicals were high in water, which agrees with the low levels of
439 atrazine, thiabendazole and diazinon in the present research. The possible effect of
440 environmental contaminants in the studied organisms, even though the former are non-
441 detectable in their tissues, makes biomarkers highly recommended tools usually
442 incorporated in ecotoxicological studies (Farcy et al. 2013; Solé and Sanchez-
443 Hernandez 2018).

444 The presence of other anthropogenic chemicals accumulated in biota was low
445 (few ng/g dw) except for caffeine, with levels much higher than previously reported in
446 the study area and other locations (Dodder et al. 2014; Álvarez-Muñoz et al. 2015). No
447 clear temporal trends were observed for the organic contaminants measured except for
448 caffeine in mussel and razor shells, which increased in the summer months, and for
449 atrazine in razor shells, which increased from April to June. Caffeine has been reported
450 to produce alterations on metabolic activity and oxidative stress biomarkers in bivalves
451 (Cruz et al. 2016). Therefore, this compound might explain part of the patterns observed
452 for biochemical markers (see below), although as far as we are concerned, no reference
453 concentrations have been recorded in bivalves' tissues; all studies addressing effects of
454 caffeine on invertebrates are based on water concentrations. In line with this influx of
455 urban residues during the summer period, it stands out a higher presence of the fecal
456 bacteria *E. coli* detected in cockle samples collected in July, which are in all likelihood
457 explained by the raw sewage discharges of a nearby town during the touristic summer
458 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 gills and digestive gland after the arrival of pollutants from rice fields to the Alfacs Bay in early summer (Escartín and Porte 1997), similarly 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), non-confirming 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, 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; Sole 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 mussel and razor shells digestive glands (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 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 (Figs. 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 towards 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).

4.2. 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.

4.3. 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.

4.4. 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 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).

4.5. *Phycotoxins analysis*

Recently, marine phycotoxins have been proposed as an additional confounding factor in studies assessing 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 inter-species 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.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Almeida EA, Mascio P (2011) Hypometabolism and antioxidative defense systems in marine invertebrates. *Hypometab Strateg Surviv Vertebr Invertebr* 661:39–55

634

635 Álvarez-Muñoz D, Rodríguez-Mozaz S, Maulvault AL, Tediosi A, Fernández-Tejedor
636 M, Van den Heuvel F, Kotterman M, Marques A, Barceló D. (2015) Occurrence of
637 pharmaceuticals and endocrine disrupting compounds in microalgae, bivalves, and fish
638 from coastal areas in Europe. Environ Res 143:56–64

639

640 Álvarez-Muñoz D, Rambla-Alegre M, Carrasco N, de Alda M L, Barceló D. Fast
641 analysis of relevant contaminants mixture in commercial shellfish, in prep.

642

643 Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER: Guide to
644 Software and Statistical Methods. PRIMER-E, Plymouth, UK

645

646 Berthelin C, Kellner K, Mathieu M (2000) Storage metabolism in the Pacific oyster
647 (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West
648 Coast of France). Comp Biochem Physiol B: Biochem Mol Biol 125:359–369

649

650 Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A (2000) The
651 use of biomarkers to assess the impact of pollution in coastal environments of the
652 Iberian Peninsula: a practical approach. Sci Total Environ 247:295–311

653

654 Capó X, Tejada S, Box A, Deudero S, Sureda A (2015) Oxidative status assessment of
655 the endemic bivalve *Pinna nobilis* affected by the oil spill from the sinking of the Don
656 Pedro. Mar Environ Res 110:19–24

657

658 Carrasco N, Arzul I, Berthe FCJ, Fernández-Tejedor M, Durfort M, Furones MD (2008)
 659 Delta de l'Ebre is a natural bay model for *Marteilia* spp. (Paramyxean) dynamics and
 660 life-cycle studies. *Dis Aquat Org* 79:65–73
 661
 662 Cruz D, Almeida Â, Calisto V, Esteves VI, Schneider RJ, Wrona FJ, Soares AMVM,
 663 Figueira E, Freitas R (2016) Caffeine impacts in the clam *Ruditapes philippinarum*:
 664 Alterations on energy reserves, metabolic activity and oxidative stress biomarkers.
 665 *Chemosphere* 160:95–103
 666
 667 Dallarés S, Moyà-Alcover CM, Padrós F, Cartes JE, Solé M, Castañeda C, Carrassón M
 668 (2016) The parasite community of *Phycis blennoides* (Brünnich, 1768) from the
 669 Balearic Sea in relation to diet, biochemical markers, histopathology and
 670 environmental variables. *Deep-Sea Res I* 118:84–100
 671
 672 Dodder NG, Maruya KA, Lee Ferguson P, Grace R, Klosterhaus S, La Guardia MJ,
 673 Lauenstein GG, Ramirez J (2014) Occurrence of contaminants of emerging concern in
 674 mussels (*Mytilus* spp.) along the California coast and the influence of land use, storm
 675 water discharge, and treated waste water effluent. *Mar Pollut Bull* 81(2):340–346
 676
 677 Escartín E, Porte C (1997) The use of cholinesterase and carboxylesterase activities
 678 from *Mytilus galloprovincialis* in pollution monitoring. *Environ Toxicol Chem*
 679 16(10):2090–2095
 680
 681 EURLMB, Interlaboratory Validation Study of the EU-Harmonised SOP-ASP-LC-UV,
 682 2018 (version 1).

683

684 EURLMB, Interlaboratory Validation Study of the EU-Harmonised SOP-LIPO-LC-
685 MS/MS, 2011 (version 5).

686

687 Farcy E, Burgeot T, Haberkorn H, Auffret M, Lagadic L, Allenou J-P, Budzinski H,
688 Mazzella N, Pete R, Heydorff M, Menard D, Mondeguer F, Caquet T (2013) An
689 integrated environmental approach to investigate biomarker fluctuations in the blue
690 mussel *Mytilus edulis* L. in the Vilaine estuary, France. Environ Sci Pollut Res 20, 630–
691 650

692

693 Ferrante MC, Clausi MT, Naccari C, Fusco G, Raso GM, Santoro A, Meli R (2014)
694 Does the clam *Ensis siliqua* provide useful information about contamination y
695 polychlorinated biphenyls and organichlorine pesticides beyond that of mussel *Mytilus*
696 *galloprovincialis*? Bull Environ Contam Toxicol 92:636–641

697

698 Freitas JS, Felicio AA, Teresa FB, de Almeida EA (2017) Combined effects of
699 temperature and clomazone (Gamit (R)) on oxidative stress responses and B-esterase
700 activity of *Physalaemus nattereri* (Leiuperidae) and *Rhinella schneideri* (Bufonidae)
701 tadpoles. Chemosphere 185:548–562

702

703 Galloway TS, Millward N, Browne MA, Depledge MH (2002) Rapid assessment of
704 organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using
705 combined esterase activities as biomarkers. Aquat Toxicol 61:169-180.

706

707 García-Altare M, Diogène J, de la Iglesia, P (2013) The implementation of liquid
 708 chromatography tandem mass spectrometry for the official control of lipophilic toxins
 709 in seafood: Single-laboratory validation under four chromatographic conditions. J
 710 Chromatogr 1275:48–60
 711
 712 González-Fernández C, Albentosa M, Campillo JA, Viñas L, Fumega J, Franco A,
 713 Besada V, González-Quijano A, Bellas J (2015a) Influence of mussel biological
 714 variability on pollution biomarkers. Environ Res 137:14–31
 715
 716 González-Fernández C, Albentosa M, Campillo JA, Viñas L, Romero D, Franco A,
 717 Bellas J (2015b) Effect of nutritive status on *Mytilus galloprovincialis* pollution
 718 biomarkers: Implications for large-scale monitoring programs. Aquat Toxicol 167:90–
 719 105
 720
 721 González-Fernández C, Albentosa M, Sokolova I (2017) Interactive effects of nutrition,
 722 reproductive state and pollution on molecular stress response of mussels, *Mytilus*
 723 *galloprovincialis* Lamarck, 1819. Mar Env Res 131:103–115
 724
 725 Guallar C, Delgado M, Diogène J, Fernández-Tejedor M (2016) Artificial neural
 726 network approach to population dynamics of harmful algal blooms in Alfacs Bay (NW
 727 Mediterranean): Case studies of *Karlodinium* and *Pseudo-nitzschia*. Ecol Model
 728 338:37–50
 729

730 Hu M, Li L, Sui Y, Li J, Wang Y, Lu W, Dupont S (2015) Effect of pH and temperature
 731 on antioxidant responses of the thick shell mussel *Mytilus coruscus*. Fish Shellfish
 732 Immunol 46:573–583
 733
 734 Izagirre U, Garmendia L, Soto M, Etxebarria N, Marigómez I (2014) Health status
 735 assessment through an integrative biomarker approach in mussels of different ages with
 736 a different history of exposure to the Prestige oil spill. Sci Tot Environ 493:65–78
 737
 738 Jebali J, Ben-Khedher S, Kamel N, Ghedira J, Bouraoui Z, Boussetta H (2011)
 739 Characterization and evaluation of cholinesterase activity in the cockle *Cerastoderma*
 740 *glaucum*. Aquat Bioly 13:243-250
 741
 742 Kim Y, Powell EN, Wade TL, Presley BJ, Sericano J (1998) Parasites of sentinel
 743 bivalves in the NOAA status and trends program: distribution and relationship to
 744 contaminant body burden. Mar Pollut Bull 37(1-2):45–55
 745
 746 Köck M, Farré M, Martínez E, Gajda-Schrantz K, Ginebreda A, Navarro A, López de
 747 Alda M, Barceló D (2010) Integrated ecotoxicological and chemical approach for the
 748 assessment of pesticide pollution in the Ebro River delta (Spain). J Hydrol 383:73–82
 749
 750 Koenig S, Solé M (2012) Natural variability of hepatic biomarkers in Mediterranean
 751 deep-sea organisms. Mar Env Res 79:122–131
 752

753 Kristoff G, Guerrero NRV, Cochon AC (2010) Inhibition of cholinesterases and
 754 carboxylesterases of two invertebrate species, *Biomphalaria glabrata* and *Lumbriculus*
 755 *variegatus*, by the carbamate pesticide carbaryl. *Aquat Toxicol* 96(2):115–123
 756
 757 Laetz CA, Baldwin DH, Hebert VR, Stark JD, Scholz NL (2014) Elevated temperatures
 758 increase the toxicity of pesticide mixtures to juvenile coho salmon. *Aquat Toxicol*
 759 146:38–44
 760
 761 Lehotay SJ, Harman-Fetcho JA, McConnell LL (1998) Agricultural pesticide
 762 residues in oysters and water from two Chesapeake Bay tributaries. *Mar Pollut*
 763 *Bull* 37:32–44
 764
 765 Mañosa S, Mateo R, Guitart R (2001) A review of the effects of agricultural and
 766 industrial contamination on the Ebro Delta biota and wildlife. *Environ Monit Assess*
 767 71:187–205
 768
 769 Matozzo V, Febrello J, Masiero L, Ferraccioli F, Finos L, Pastore P, Di Gangi IM,
 770 Bogialli S (2018a) Ecotoxicological risk assessment for the herbicide glyphosate to non-
 771 target aquatic species: A case study with the mussel *Mytilus galloprovincialis*. *Environ*
 772 *Pollut* 233:623–632
 773
 774 Matozzo V, Ercolini C, Serracca L, Battistini R, Rossini I, Granato G, Quagliari E,
 775 Perolo A, Finos L, Arcangeli G, Bertotto D, Radaelli G, Chollet B, Arzul I, Quaglio F
 776 (2018b) Assessing the health status of farmed mussels (*Mytilus galloprovincialis*)
 777 through histological, microbiological and biomarker analyses. *J Inv Pathol* 153:165–179

778

779 Mejdoub Z, Fahde A, Loutfi M, Kabine M (2017) Oxidative stress responses of the
780 mussel *Mytilus galloprovincialis* exposed to emissary's pollution in coastal areas of
781 Casablanca. Ocean Coast Manag 136:95–103

782

783 Moore MN, Viarengo A, Donkin P, Hawkins AJ (2007) Autophagic and lysosomal
784 reactions to stress in the hepatopancreas of blue mussels. Aquat Toxicol 84(1):80–91
785

786 Morley NJ (2010) Interactive effects of infectious diseases and pollution in aquatic
787 molluscs. Toxicol 96:27–36
788

789 Mundhe AY, Bhilwade H, Pandit SV (2016) Genotoxicity and oxidative stress as
790 biomarkers in fresh water mussel, *Lamellidens marginalis* (Lam.) exposed to
791 monocrotophos. Indian J Exp Biol 54:822–828

792

793 Nilin J, Monteiro M, Domingues I, Loureiro S, Costa-Lotufo LV, Soares A (2012)
794 Bivalve Esterases as Biomarker: Identification and Characterization in European
795 Cockles (*Cerastoderma edule*). Bull Environ Contam Toxicol 88:707-711

796

797 Nunes B, Resende ST (2017) Cholinesterase characterization of two autochthonous
798 species of Ria de Aveiro (*Diopatra neapolitana* and *Solen marginatus*) and comparison
799 of sensitivities towards a series of common contaminants. Environ Sci Pollut Res 24:
800 12155-12167

801 Ochoa V, Riva C, Faria M, Barata C (2013) Responses of B-esterase enzymes in oysters
 802 (*Crassostrea gigas*) transplanted to pesticide contaminated bays from the Ebro Delta
 803 (NE, Spain). Mar Pollut Bull 66:135–142
 804
 805 Off J Eur Union L 226 (2004) 22. (Regulation (EC) No 853/2004)
 806
 807 Off J Eur Union L 220 (2013) 14. (Regulation (EC) No 786/2013)
 808
 809 Otero S, Kristoff G (2016) In vitro and in vivo studies of cholinesterases and
 810 carboxylesterases in *Planorbarius corneus* exposed to a phosphorodithioate insecticide:
 811 Finding the most sensitive combination of enzymes, substrates, tissues and recovery
 812 capacity. Aquat Toxicol 180:186–195
 813
 814 Owusu EO, Komi K, Horiike M, Hirano C (1994) Some properties of carboxylesterase
 815 from *Aphis gossypii* Glover (Homoptera: Aphididae). Appl Entomol Zool 29(1):7–53
 816
 817 Palm HW, Dobberstein RC (1999) Occurrence of trichodinid ciliates (Peritrichida:
 818 Urceolariidae) in the Kiel Fjord, Baltic Sea, and its possible use of as a biological
 819 indicator. Parasitol Res 85:726–732
 820
 821 Pearce NJG, Mann VL (2006) Trace metal variations in the shells of *Ensis siliqua*
 822 record pollution and environmental conditions in the sea to the west of mainland
 823 Britain. Mar Pollut Bull 52:739–755

824 Pfeifer S, Schiedek D, Dippner JW (2005) Effect of temperature and salinity on
 825 acetylcholinesterase activity, a common pollution biomarker, in *Mytilus* sp. from the
 826 south-western Baltic Sea. J Exp Mar Bio Ecol 320:93–103
 827
 828 Pörtner, HO (2002) Climate variations and the physiological basis of temperature
 829 dependent biogeography: systemic to molecular hierarchy of thermal tolerance in
 830 animals. Comp Biochem Physiol A 132:739–761
 831
 832 Powell EN, Barber RD, Kennicut II MC, Ford SE (1999) Influence of parasitism in
 833 controlling the health reproduction and PAH body burden of petroleum seep mussels.
 834 Deep-Sea Res Part I 46:2053–2078
 835
 836 Regoli F, Giuliani ME (2014) Oxidative pathways of chemical toxicity and oxidative
 837 stress biomarkers in marine organisms. Mar Environ Res 93:106–117
 838
 839 Rodrigues ET, Alpendurada MF, Ramos F, Pardal MÂ (2018) Environmental and
 840 human health risk indicators for agricultural pesticides in estuaries. Ecotoxicol Environ
 841 Saf 150:224–231
 842
 843 Santos TCR, Rocha JC, Barcelo D (2000) Determination of rice herbicides, their
 844 transformation products and clofibric acid using on-line solid-phase extraction followed
 845 by liquid chromatography with diode array and atmospheric pressure chemical
 846 ionization mass spectrometric detection. J Chromatogr A 879(1):3–12
 847

848 Solé M, Sanchez-Hernandez JC (2018) Elucidating the importance of mussel
 849 carboxylesterase activity as exposure biomarker of environmental contaminants of
 850 current concern: An in vitro study. *Ecol Indic* 85:432–439
 851
 852 Solé M, Rivera-Ingraham G, Freitas R (2018a) The use of carboxylesterases as
 853 biomarkers of pesticide exposure in bivalves: A methodological approach. *Comp*
 854 *Biochem Physiol Part C: Toxicol Pharmacol* 212:18–24
 855
 856 Solé M, Bonsignore M, Rivera-Ingraham G, Freitas R (2018b) Exploring alternative
 857 biomarkers of pesticide pollution in clams. *Mar Pollut Bull* 136:61-67.
 858
 859 Somero GN (1995) Proteins and temperature. *Annu Rev Physiol* 57:43–68
 860
 861 StatSoft, Inc (2004) STATISTICA (data analysis software system), version 7
 862 www.statsoft.com
 863
 864 Suárez-Serrano A, Ibáñez C, Lacorte S, Barata C (2010) Ecotoxicological effects of rice
 865 field waters on selected planktonic species: comparison between conventional and
 866 organic farming. *Ecotoxicol* 19:1523–1535
 867
 868 Terrado M, Kuster M, Raldúa D, Lopez de Alda M, Barceló D, Tauler R (2007) Use of
 869 chemometric and geostatistical methods to evaluate pesticide pollution in the irrigation
 870 and drainage channels of the Ebro river delta during the rice-growing season. *Anal*
 871 *Bioanal Chem* 387:1479–1488
 872

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58
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60
61
62
63
64
65

873 Velez C, Pires A, Sampaio L, Cardoso P, Moreira A, Leandro S, Figueira E, Soares
874 AMVM, Freitas R (2016) The use of *Cerastoderma glaucum* as a sentinel and
875 bioindicator species: Take-home message. Ecol Indic 62:228–241
876
877 Wheelock CE, Shan G, Ottea J (2005) Overview of carboxylesterases and their role in
878 the metabolism of insecticides. J Pestic Sci 30(2):75–83
879
880 Wheelock CE, Nakagawa Y (2010) Carboxylesterases – from function to the field: an
881 overview of carboxylesterase biochemistry, structure–activity relationship, and use in
882 environmental field monitoring. J Pestic Sci 35(3):215–217
883

Table 1. Physico-chemical water parameters quantified in the two areas sampled throughout the length of the study. T: temperature; S: salinity; O₂: oxygen concentration; Chla: Chlorophyll-a.

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

Table 2

Table 2. Mean concentration (ng/g dry weight) ± relative standard deviation (RSD) (n=2 replicates) of the concentration of mussels (*Mytilus galloprovincialis*), cockles (*Cerastoderma edule*) and grooved razor shells (*Solen marginatus*) performed in the Alfacs Bay, Ebre Delta. MDL: method detection limit; MQL: method quantification limit.

Family	Compounds	<i>Mytilus galloprovincialis</i>				<i>Cerastoderma edule</i>			
		April	May	June	July	April	May	June	July
Organitrogen pesticides	Atrazine	1.56 ± 0.32	1.29 ± 0.10	<MQL	<MQL	0.82 ± 0.08	<MDL	<MDL	<MDL
Organophosphorus pesticides	Thiabendazole	<MDL	<MDL	<MDL	<MDL	<MDL	0.93 ± 0.18	0.43 ± 0.003	0.60 ± 0.00
	Diazinon	<MDL	<MDL	0.46 ± 0.00	0.51 ± 0.00	0.57 ± 0.00	1.53 ± 0.02	<MDL	<MDL
Endocrine Disruptors (EDCs)	Caffeine	<MDL	<MDL	<MQL	11.82 ± 1.93	22.60 ± 5.48	33.62 ± 5.51	<MDL	<MDL
	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
	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
	Propylparaben	<MDL	<MDL	<MDL	<MQL	1.38 ± 0.04	0.93 ± 0.06	0.94 ± 0.02	1.18 ± 0.06
	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

Figure captions

Figure 1. Study area showing bivalves sampling sites (●) and localities where environmental variables were measured (◇) in the Alfacs Bay (Ebre Delta).

Figure 2. 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 Ebre Delta in April, May, June and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$).

Figure 3. 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 Ebre Delta in April, May, June and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$).

Figure 4. 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 haemolymph of mussels collected in the Alfacs Bay of the Ebre Delta in April, May, June and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$).

Figure 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 Alfac's Bay of the Ebre Delta in April, May, June and July of 2017. Different letters indicate statistical differences among months during the sampling period ($p < 0.05$).

Figure 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 Alfac's Bay of the Ebre Delta in April, May, June and July of 2017.

Figure 7. *Rickettsia*-like organisms (RLOs) (arrowheads) in gills of cockles collected in the Alfac's Bay of the Ebre Delta in June of 2017.

Figure 1

Figure 3

Figure 5

Figure 7