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Fatty acids' profiles as indicators of stress induced by of a common herbicide on two marine bivalves species: *Cerastoderma edule* (Linnaeus, 1758) and *Scrobicularia plana* (da Costa, 1778)

A.M.M. Gonçalves [a,b,*], A.F. Mesquita [b], T. Verdelhos [a], J.A.P. Coutinho [c], J.C. Marques [a], F. Gonçalves [b]

[a] IMAR (Marine and Environmental Research Centre) & MARE (Marine and Environmental Sciences Centre), Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal
[b] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
[c] CICECO, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

In Europe, mainly the Mediterranean region, intensive use of fertilizers and pesticides has been recorded over the past 30 years, exceeding, in some cases, the limits of contamination authorized by the European Union. The intensive use of pollutants in fields near ecological coastal wetlands has led to implementation of pesticide monitoring programs to recover aquatic systems such as the Mondego estuary (Figueira da Foz, Portugal). According to information from the agricultural cooperatives of the Mondego valley, Primextra® Gold TZ is the most-used herbicide in corn crop fields. Biomarkers, such fatty acids (FAs), proved to be new and potentially powerful tools to detect, illustrate, and evaluate exposure to and the effects of contamination hazards. They play important roles in establishing neural levels in organisms' biochemical and physiological responses and are considered good bio-indicators of stress and potential indicators of ecosystem health. Bivalves are currently used in ecotoxicological bioassays because of their ecological importance, wide geographic distribution, ease of handling in the laboratory and in the field, and their ability to filter and ingest large volumes of water and sediment particles. Thus, the main goal of this work was to determine the toxic and biochemical (namely fatty acid profiles) responses of two size classes (small and big) of the two marine bivalve species *Cerastoderma edule* and *Scrobicularia plana* to the herbicide Primextra® Gold. Furthermore, we aimed to compare the fatty acid contents, and thus the nutritive values, of both species and size classes collected in the field with those under laboratory conditions. Results show *S. plana* is more sensitive to the herbicide than *C. edule*. In general, among the larger-sized specimens in the field, *S. plana* is more nutritive than *C. edule*, but among the smaller-sized specimens, the opposite tendency is seen, where *C. edule* presents a greater abundance of FAs.

1. Introduction

In Europe, mainly in the Mediterranean region, there is an overexploitation of farmland, and when combined with fertilizer and pesticide overuse, has adverse effects on surrounding aquatic systems. Because many estuaries are surrounded by farmland, residential, and industrial areas, they are subject to various anthropogenic pressures and behaviors that cause ecological stresses, affecting not only the water quality, but also the biological communities of these ecosystems (McCarthy et al., 2007; Cardoso et al., 2008; Gonçalves et al., 2010a, 2010b; Sameling et al., 2013; Verdelhos et al., 2005, 2014).

As in other estuaries, Mondego estuary, located near Figueira da Foz city, Portugal, is under strong anthropogenic pressures. The main stressors are related to port, beach, and industrial activities as well as the exploitation of marine resources. The eutrophication process is caused primarily by discharges of pollutants (e.g., fertilizers and inorganic compounds) from agricultural fields, particularly those used to grow rice and corn, where production is more intensive (Cardoso et al., 2008; Duarte et al., 2008). Pesticides used in agricultural practices have been found in surface

* Corresponding author at: IMAR (Marine and Environmental Research Centre) & MARE (Marine and Environmental Sciences Centre), Department of Life Sciences, University of Coimbra, 3004-517 Coimbra, Portugal/Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro, Portugal. Tel.: +351 239855760; fax: +351 239823603.
E-mail address: anamartagoncalves@ua.pt (A.M.M. Gonçalves).

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and ground waters; this contamination may have ecotoxicological effects on aquatic flora and fauna with consequences on human health (Carrasco et al., 2003; Macedo et al., 2005). According to information from the agricultural cooperatives of the Mondego valley, Primextra® Gold TZ is the most-used herbicide in corn fields. It is a selective and systemic herbicide that controls weeds, which are primarily grasses and *Cyperus esculentus*, with residual action and the major annual weeds found in corn crops. The herbicide is absorbed by the leaf and root, preventing growth; plants die before emerging or shortly after emergence (Syngenta®, 2014). Indeed, herbicides and fertilizers used in agricultural practices affect biologics at several organizational levels, from molecular to ecosystem and may be affective from early germination onward, leading to biochemical and physiological alterations and differences in enzymatic and non-enzymatic antioxidants, resulting in residues in plants, legumes, fruit, and non-target organisms (Parween et al., 2014). Primextra® Gold TZ, produced by Syngenta AG, is composed of two active ingredients (a.i.), terbuthylazine and S-metolachlor, which are also used by Syngenta AG in other commercial formulations used worldwide. Terbuthylazine is a selective systemic herbicide that acts as a photosynthesis inhibitor and is used as a large spectrum herbicide in maize, sorghum, vines, citrus, coffee, potatoes, vegetables, and forestry (Roberts et al., 1998). Nowadays, it is the second-most frequently used *s*-triazine (Velisek et al., 2014) and is adsorbed through roots and leaves and distributed throughout the plant, enabling it to be used in both pre- and post-emergent treatment. S-metolachlor, the major component of the herbicide, is potentially dangerous to environmental and aquatic systems. Metalochlor, and consequently Primextra® Gold TZ, was developed to control grass weeds following pre-emergent application (Karam et al., 2003). Its mode of action consists of inhibiting several biosynthesis processes, namely of lipids, fatty acids (FAs), leaf wax, terpenes, flavonoids, and protein synthesis, in addition to inhibition of cell division and interference with hormonal regulation (Weed, 1994; Liebl, 1995). Metolachlor is classified as an inhibitor of very long chain fatty acid formation. It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu and Xiong, 2009). The mode of action of this xenobiotic suggests that it affects the lipid (FA) profile of aquatic species.

Fatty acids are among the main constituents of the cell membrane, occurring in great concentrations in the neural system. They play key roles at the biological level, and are among the most important molecules transferred across the plant-animal interface in aquatic food webs. These compounds are involved in several biochemical pathways and are an important source of energy and constituent of cell membranes, acting on membrane permeability and influencing the traffic of cell compounds and the activity of membrane proteins and signals (Ibarguren et al., 2014; Liu et al., 2015). Therefore, as with other biomarkers, FAs are argued to be good bio-indicators of stress and potentially of ecosystem health (Martinez-Haro et al., 2015). Polyunsaturated fatty acids (PUFAs) are a family of lipids that contain subgroups and are identified by the position of the last double bond in their structures. They include many important compounds, such as essential fatty acids (EFAs). Although the terms *PUFA* and *EFA* are not synonymous, they are often used interchangeably because many biological functions of EFAs are exerted by EFA-derived PUFAs. Bret and Müller-Navarra (1997) pointed out that PUFAs are almost exclusively synthesized by plants, and that animals are able to convert PUFAs by elongation or desaturation. Only a few animals can synthesize this type of FA. The PUFAs play important roles in the organism, regulating cell membrane properties, serving as precursors to important hormones, and being essential to the organism (Neves et al., 2015). Highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are linked to growth, reproductive success, and neural development, playing key roles in the health and function of all animals, including plankton invertebrates, benthos, fish, and humans, at all levels. The HUFAs are essential metabolites that cannot be synthesized de novo in sufficient amounts to be taken up via food sources (Ladhar et al., 2014). Indeed, some groups of organisms fed high amounts of HUFAs present greater growth rates, showing the importance of FAs as ecophysiological indicators. Furthermore, lipid components are very sensitive to stressors and environmental changes. The HUFAs, as determined by EFAs such as EPA and DHA, are key nutritional constituents of the bivalves' diets and establish the nutritional value of algae consumed by bivalves (Hendriks et al., 2003). Bivalve species consume nutrients from many material particles, such as phytoplankton, resuspended benthic microalgae, and detritus from both bacterial and myco-heterotrophic sources; however, they are considered herbivorous, and phytoplankton is their primary food source (Pernet et al., 2012). In this study, we used two marine bivalve species, *Cerastoderma edule* and *Scrobicularia plana*, from the Mondego estuary. *C. edule* is a bivalve mollusc from the family *Cardiidae*, and is one of the most abundant shellfish in tidal flats, bays, and estuaries in Northern and Western Europe. It is widely distributed: it is found from North Africa to Northern Norway and Murmansk in the Arctic and on the east coast of the Atlantic, but not in the Mediterranean and Baltic Seas (Freitas et al., 2014). It is an infaunal suspension feeder living in intertidal shallow areas, burrowing just below the sediment surface and playing a key role as a link between primary producers and consumers (Verdelhos et al., 2015). Given its large filtration capacity and ability to accumulate a large amount of environmental pollutants, *C. edule* is widely used as an environmental bio-indicator (Paul-Pont et al., 2010a; Nilin et al., 2012; Cardoso et al., 2013; Freitas et al., 2014). *S. plana*, also a bivalve mollusc, is from the family *Semelidae* and is typically found in brackish waters. It is a dominant species in intertidal soft-substrate estuaries, lagoons, and bays along NE Atlantic seaboard communities from Norway to the Mediterranean and West African regions. It is a deposit filter feeder, inhabiting intertidal and subtidal areas and burrowing on mud to muddy sand sediments at depths up to 25 cm (Verdelhos et al., 2015). Like other bivalves, *S. plana* has a large capacity to filter pollutants that accumulate in the digestive gland (Paul-Pont et al., 2010a, 2010b; Freitas et al., 2014). In general, bivalve species play key roles in the trophic web because they act as links between primary producers and consumers and are the major prey of crustaceans, fish, and wading birds. They have a capacity to filter organic material, clean the freshwater column, and influence the available food and the energy flow in the entire community. Moreover, they can accumulate pollutants and parasite species (Verdelhos et al., 2014). Bivalves are considered standard species in ecotoxicological studies because of their sessile lifestyle, easy handling and collection, and sensitivity to pollutants. Physiological and biochemical responses are used as early indicators of potential ecosystem damage caused by pollutants such as metals and organic contaminants (Nilin et al., 2012). Furthermore, these species are of great economic value because they are significant as a food source (Paul-Pont et al., 2010a). Thus, ecotoxicological studies using bivalve species are extremely important and are crucial to determining their nutritive value as well as their responses to anthropogenic stressors. The main aims of this study were: (1) to determine the ecotoxicological effect of the herbicide Primextra® Gold TZ on two size classes (big [B] and small [S]) of both bivalve species (*C. edule* and *S. plana*), (2) to determine the biochemical response (namely FA profiles) of both size classes of both bivalve species when exposed to the commercial compound, and (3) to compare the nutritive value of both size classes of both bivalve species in the field and under exposure to the contaminant.

2. Materials and methods

2.1. Study area and sampling procedures

The Mondego estuary is located in a Mediterranean region on the Atlantic coast of Portugal (40°08′N, 8°50′W), near Figueira da Foz city, Portugal (Fig. 1). It is a small estuary extending about 8 km and covering an area of approximately 3.4 km^2. The estuary is divided in two arms, north and south, separated by the Morraceira island. The north arm is deeper (4–10 m during high tide; tidal range 1–3 m), highly hydrodynamic, and the main navigation channel, and thus is the location of the Figueira da Foz harbor. The south arm is shallower (2–4 m during high tide; tidal range 1–2 m) and is characterized by large areas of exposed intertidal flats during low tide. Until 1998, the south arm was almost silted up in the inner areas, and the river outflow occurred mainly via the north arm. Therefore, water circulation was mostly dependent on tides and freshwater input from the Pranto River, a small tributary whose flow was controlled by a sluice, regulated according to the water level of rice fields in the Mondego Valley. Sampling of *C. edule* and *S. plana* was conducted in the north and south arms, respectively (Fig. 1). The bivalves were captured using a dredge and put immediately in cold boxes with water from the estuary. The organisms were transported to the laboratory where they were divided in aquaria by species and sizes class.

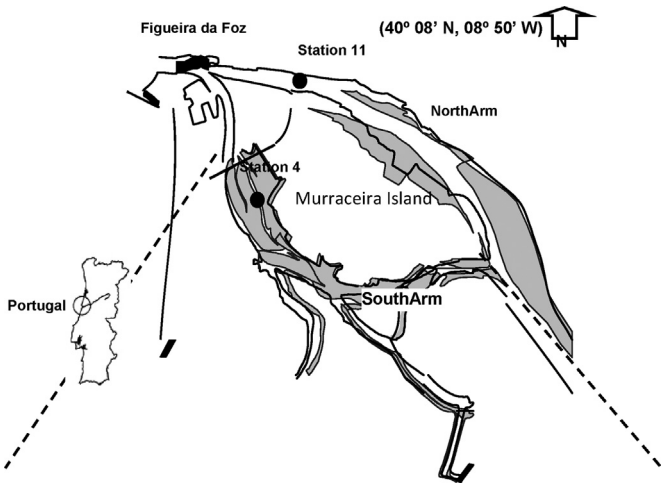

Fig. 1. Map of the Mondego estuary and sampling sites in both arms (north and south).

2.2. Laboratory and bioassays procedures

In the laboratory, condition indices (shell length, total weight, tissue weight, tissue weight without gonad, and tissue weight without gonad or digestive gland) of 10 larger individuals and 10 smaller individuals were evaluated. The muscle (foot) of each individual was then removed and stored at −80 °C until FA analysis. The remaining individuals were maintained in filtered sea water at 20 psu without food for depuration. Bioassays were performed on organisms undergoing one of 9 treatments, one negative control and eight concentrations of the herbicide ranging from 0 to 60 mg/L of the commercial formulation. Each bioassay was performed in ten replicates per treatment using 1000 mL test medium per vial. Bivalves were fed daily with a commercial mixture of rotifers and microalgae and transferred to new prepared test solutions every other day. They were checked every day at the same approximate hour for mortality and behavioral conditions (to evaluate the activity of the siphon, valve condition, and the organism's reactions during feeding). Tests were performed under a 12 h^L:12 h^D photoperiod at a temperature of 20 ± 2 °C in filtered sea water medium with 20 psu for 96 h. At the end of the bioassays, all surviving individuals were dissected, weighed, measured, and evaluated against condition indices.

2.3. Fatty acid analyses

Fatty acid analysis was performed on muscle tissue isolated from each organism. Extraction of total lipids and fatty acid methyl esters (FAMEs) was achieved by modifying one step of the method by Gonçalves et al. (2012). The boron trifluoride-methanol reagent was replaced by a 2.5% H_2SO_4-methanol solution since BF3-methanol can cause artifacts or loss of PUFA (Eder, 1995), in the extraction of fatty acid methyl esters by gas chromatography. The fatty acid Methylnonadecanoate C19:0 was added as an internal standard for later quantification (Fluka 74208). Samples were then centrifuged in a Thermo Scientific Heraeus Megafuge 16 R, stored, and frozen at −80 °C in new vials. Gas chromatography was used for separation and quantification of FAMEs. This was performed in a Trace 1300 Thermo Scientific gas chromatograph with an AI1310 auto sampler coupled with a flame ionization detector in a split injection system with a biodiesel FAME column (60 m × 0.250 mm × 0.20 μm). The column temperature was programmed to increase from 120 °C to 240 °C at a rate of 4 °C/min while the detector and injector were maintained at 250 °C. The carrier gas was helium at a flow rate of 0.6 mL/min.

2.4. Statistical analysis

Probit analysis (Finney, 1971) was applied to determine the LC_{10}, LC_{20}, and LC_{50} with corresponding 95% confidence intervals for each species and size class. The FA profiles were found by determining total (mg/ind) or relative %FA concentrations.

Multivariate statistical analyses were carried out using PRIMER-6 software (Clarke and Gorley, 2006) to examine the variation in FA composition through non-metric multidimensional scaling (n-MDS) plots. In addition, a dendogram obtained by a hierarchical clustering, using the data converted into similarity triangular matrices using Bray–Curtis resemblance measures (Clarke and Warwick, 2001) and a cluster mode based in group average distance linkage, and a variant by which a larger cluster is down weighted (Kindt and Coe, 2005), was used to assess the degree of similarity between FA samples. One-way analysis of similarity (ANOSIM) was used to test differences in FA profiles across species and size classes. The contribution of individual FAs to similarities and dissimilarities within and between sample groups was tested using the similarity percentage (SIMPER) analysis routine. The PCA analysis, without data transformation, samples center and standardize and species center by species, was used to highlight size or interspecific patterns in the bivalve species' diets using CANOCO version 4.5 (Ter Braak and Smilauer, 1998).

2.5. Fatty acid trophic markers

Fatty acid ratios were calculated and used as trophic markers based on Ezgeta-Balić et al. (2012) to determine whether animal, bacterial, or algae class ratios were maintained in the lipid extracts of both size classes of bivalves, thus reflecting dietary preferences. In general, carnivorous bivalves show higher contents of C18:1n9, C18:2n6, and DHA because these FAs are characteristic of zooplankton (Zhukova and Kharlamenko, 1999; Kharlamenko et al., 2001). The DHA/EPA ratio reflects the proportion of zooplankton and diatoms/dinoflagellates in the bivalves' diets (Budge and Parrish, 1998; Mansour et al., 1999; Dalsgaard et al., 2003). Because it is an important component of polar lipids, DHA is highly conserved in food webs. It is often dominant in zooplankton and

Table 1
Lethal concentration (LC) values of Primextra® Gold TZ for both size classes of *S. plana* and *C. edule*. The 95% confidence limits are given in brackets.

Chemical compound		Big size (mg/L)	Small size (mg/L)
Primextra® Gold TZ	***Scrobicularia plana***		
	LC10	6.338 (0.000; 11.573)	2.206 (0.000; 3.562)
	LC20	8.715 (0.737; 15.344)	3.351 (1.585; 4.729)
	LC50	13.263 (8.230; 27.168)	5.539 (4.199; 7.754)
	Cerastoderma edule		
	LC10	21.298 (11.008; 25.222)	22.873 (5.284; 26.133)
	LC20	23.868 (16.271; 27.422)	24.376 (11.008; 27.271)
	LC50	28.784 (24.731; 33.238)	27.252 (21.057; 30.349)

dinoflagellates (Budge and Parrish, 1998; Mansour et al., 1999; Zhukova and Kharlamenko, 1999; Kharlamenko et al., 2001; Dalsgaard et al., 2003), whereas EPA is found mainly in diatoms (Dunstan et al., 1994; Budge and Parrish, 1998; Dalsgaard et al., 2003). A high proportion of C15:0 and C17:0 denote the presence of bacteria in the bivalve diet (Mayzaud et al., 1989; Nadjek et al., 2002), a result of the large amounts of iso and ante-iso branched chains containing 15–17 carbons formed during bacteria biosynthesis (Gonçalves et al., 2012).

3. Results

3.1. Bioassays

The lethal concentration (LC) values of both size classes of the studied species show that *S. plana* is more sensitive than *C. edule* to the herbicide ($LC50_B$ = 13.26 mg/L (8.23–27.17), $LC50_S$ = 5.54 mg/L (4.20–7.75) and $LC50_B$ = 28.78 mg/L (24.73–33.24), $LC50_S$ = 27.25 mg/L (21.06–30.35), respectively). In an intraspecific analysis, this data indicates that bigger specimens are more tolerant in both *S. plana* and *C. edule*. This pattern is clearly observed in *S. plana* (Table 1).

3.2. Fatty acid profiles of bivalve species in the field and after exposure to the herbicide

In general, in the controls and those treated with Primextra® Gold TZ, the concentrations of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs decreased in all groups except the small sized *C. edule*, which showed increases in MUFAs and PUFAs. Comparing FA profiles of organisms from the field and after treatment, we found an increase in SFAs and a decrease in unsaturated fatty acids (UFAs) in all groups except the small sized *S. plana*, which showed the opposite trends in SFAs and UFAs. In the bioassay, we found that generally, *C. edule* showed higher FA content than *S. plana* in both size classes. However, organisms from the field showed the opposite trend; *S. plana* exhibited higher FA variety than *C. edule*. Thus, the FA profile of *S. plana* was much more affected by the herbicide than *C. edule*, demonstrating more response to the chemical stressor. Changes in FA profiles of *C. edule* were not as clearly observed as they were in *S. plana*, but it was clear that the FA composition of the small size class in both bivalve species showed greater changes than the big size class when exposed to the commercial formulation of the contaminant. The HUFAs (mainly DHA and EPA) occupy the largest proportion of the FA profile. It is noteworthy that at higher concentrations of the herbicide, there was a significant reduction in the overall amount of FAs, yet the EFAs remained dominant. In Tables 2 and 3, higher FA content is evident in the big size classes, compared to the small size classes, among those organisms from the field and those exposed to the herbicide.

3.3. Multivariate analyses for both size classes of the two bivalve species

After cluster analysis (Fig. 2), the samples were found to be distributed in 5 groups (I to V) according to their diversity and abundance of FAs. A clear separation between species is seen: *S. plana* occupies the first groups (I to III), and *C. edule*, the last two groups (IV and V). Furthermore, both size classes of *S. plana* present well-defined patterns where the sizes are completely separated in two groups (II and III), with an exception in the big size class of *S. plana* exposed to the lowest concentration of the herbicide (0.5 mg/L). These organisms occupy Group I, and the FA composition in this group is not related to any other organisms either of the same size class or exposed to higher concentrations. In fact, both sizes of *S. plana* present a clear and distinct fatty acid composition as observed by the cluster analysis. On the other hand, the analysis by size of *C. edule* does not show a pattern as obvious as that seen in *S. plana*. It is also observed that Groups IV and V include a mixture of both sizes of organisms from the field.

The n-MDS analysis (Fig. 3) shows a clear distribution by species and size class based on FA abundance and composition (stress = 0.06). In Group A, both size classes of *C. edule* are present, in Group B, the small size class of *S. plana* is present, and in Group C, the big size class of *S. plana* is present with the exception of those exposed to the lowest concentration of herbicide, which are in Group D.

The ANOSIM analysis indicated a clear separation of the groups defined ($R = 0.603$; $p = 0.001$). In comparing pairwise differences, almost all sizes classes were significantly different ($p < 0.05$) and presented high R values, showing good segregation (CeB/SpB: $R = 0.638$; p= 0.002; CeB/SpS: $R = 0.895$; p = 0.001; CeS/SpB: $R = 0.741$; $p = 0.001$; CeS/SpS: $R = 0.862$; $p = 0.001$; SpB/SpS: $R = 0.675$; $p = 0.002$). The classes CeB and CeS were also significantly different, but presented poor segregation ($R = 0.184$; $p = 0.048$). The SIMPER analysis (Table 4) shows that DHA and EPA are the two main FAs that contribute to the great similarity within each group (CeB/CeS; SpB; SpS). The dissimilarities between the groups (A/C; A/D; C/D; A/B; C/B; D/B) are also related to the differences between FA abundance and composition. The FAs mainly contributing to the dissimilarities between groups are C18:2n6cis, EPA, and DHA.

3.4. Dietary fatty acid trophic markers

During the bioassays, organisms from both species and size classes were fed a solution constituted of microalgae and rotifers (Fig. 4). Microalgae are mostly composed of PUFAs (86.42%) and small amounts of SFAs (7.71%), HUFAs (3.00%), and MUFAs (2.87%). Rotifers are mostly composed of SFAs (62.72%) and MUFAs (22.92%) and small quantities of PUFAs (9.00%) and HUFAs (5.36%). Tables 2 and 3 show an increase in SFAs and MUFAs in big class *S. plana* and in PUFAs in small class *S. plana*. They also show that the

Table 2
Abundance of fatty acids (Saturated Fatty Acids – SFAs, Monounsaturated Fatty Acids – MUFAs, Polyunsaturated Fatty Acids – PUFAs, and Highly Unsaturated Fatty Acids – HUFAs, in mg/ind) in the profiles of *C. edule* (small and big size classes) from the field and after exposure to the commercial formulation of Primextra® Gold TZ.

	Cerastoderma edule Big size															
FA	Field	±Std. Error	Ctl	±Std. Error	C1(0.5 mg/L)	±Std. Error	C2(2.5 mg/L)	±Std. Error	C3(5 mg/L)	±Std. Error	C4(10 mg/L)	±Std. Error	C5(20 mg/L)	±Std. Error	C6(30 mg/L)	±Std. Error
C15:0					0.008671	(0.002541)							0.003535	(0.003535)		
C16:0	0.027213	(0.008669)	0.029437	(0.016116)	0.052855	(0.010233)	0.017320	(0.004709)	0.048736	(0.002208)	0.041449	(0.009096)	0.050031	(0.000369)	0.030567	(0.002552)
C17:0	0.009083	(0.002295)	0.01483	(0.004067)	0.039383	(0.009304)	0.011641	(0.002438)	0.028385	(0.008726)	0.023724	(0.012756)	0.032169	(0.001498)	0.014424	(0.002104)
C18:0	0.014922	(0.004743)	0.021402	(0.007821)	0.044635	(0.010372)	0.011402	(0.001593)	0.028901	(0.001318)	0.025472	(0.009625)	0.033149	(0.000355)	0.017578	(0.00325)
C21:0			0.028418	(0.028418)			0.011277	(0.011277)								
C22:0	0.018032	(0.000269)	0.026112	(0.016231)	0.015266	(0.000111)	0.017421	(0.01012)	0.025235	(0.002845)	0.021315	(0.004317)	0.022386	(0.011063)	0.011894	(0.003898)
C23:0					0.019174	(0.002021)					0.00741	(0.00741)	0.008073	(0.008073)	0.008863	(0.001273)
C24:0	0.005115	(0.005115)	0.008389	(0.008389)	0.023908	(0.002843)	0.005305	(0.005305)	0.023569	(0.008074)	0.010909	(0.010909)	0.01557	(0.01557)	0.014658	(0.002309)
TOTAL SFAs	**0.074365**	**(0.021092)**	**0.128588**	**(0.081042)**	**0.203891**	**(0.037425)**	**0.074366**	**(0.035442)**	**0.154827**	**(0.023171)**	**0.130278**	**(0.054114)**	**0.164913**	**(0.040463)**	**0.097983**	**(0.015386)**
C15:1n5(cis10)					0.009312	(0.001905)										
C16:1					0.011177	(0.003369)					0.005284	(0.005284)				
C17:1n7(cis10)																
C18:1n9																
C22:1n9	0.018712	(0.003443)	0.040453	(0.022343)	0.036159	(0.002535)	0.023003	(0.002095)	0.039208	(0.003083)	0.030977	(0.007115)	0.038857	(0.001273)	0.02296	(0.000689)
C24:1n9																
Total MUFAs	**0.018712**	**(0.003443)**	**0.040453**	**(0.022343)**	**0.056649**	**(0.007809)**	**0.023003**	**(0.002095)**	**0.039208**	**(0.003083)**	**0.036261**	**(0.012398)**	**0.038857**	**(0.001273)**	**0.02296**	**(0.000689)**
C18:2n6cis																
C18:3n3	0.014301	(0.005984)	0.040279	(0.011307)	0.026594	(0.007915)	0.022147	(0.000785)	0.025295	(0.011211)	0.017743	(0.009551)	0.032849	(0.007638)	0.028326	(0.003187)
C20:2(cis11,14)	0.012296	(0.002473)	0.030502	(0.011671)	0.026714	(0.005367)	0.009294	(0.000329)	0.026601	(0.006512)	0.024015	(0.001668)	0.021697	(0.005206)	0.00556	(0.00556)
C22:2(cis13,16)	0.02477	(0.003292)	0.037473	(0.014903)	0.007517	(0.007517)	0.017464	(0.007362)	0.013866	(0.004486)	0.012605	(0.012605)	0.013947	(0.003238)	0.013855	(0.001143)
Total PUFAs	**0.051367**	**(0.011749)**	**0.108254**	**(0.037881)**	**0.060824**	**(0.020799)**	**0.048905**	**(0.008476)**	**0.065762**	**(0.022209)**	**0.054363**	**(0.023824)**	**0.068494**	**(0.016081)**	**0.047741**	**(0.00989)**
C20:5n3 (EPA)	0.046521	(0.009694)	0.084432	(0.022809)	0.09466	(0.002844)	0.04962	(0.008071)	0.069202	(0.007709)	0.074993	(0.016284)	0.069986	(0.003173)	0.061996	(0.007689)
C22:6n3 (DHA)	0.06678	(0.018097)	0.102206	(0.053557)	0.16353	(0.050044)	0.060649	(0.014979)	0.151619	(0.01512)	0.120699	(0.040878)	0.154137	(0.001925)	0.087938	(0.021378)
Total HUFAs	**0.113302**	**(0.027791)**	**0.186638**	**(0.076366)**	**0.25819**	**(0.052887)**	**0.11027**	**(0.023049)**	**0.220822**	**(0.022829)**	**0.195691**	**(0.057162)**	**0.224123**	**(0.005098)**	**0.149934**	**(0.029067)**
N	**11**		**12**		**15**		**12**		**11**		**13**		**13**		**12**	

Small size

FA	Field	±Std. Error	Ctl	±Std. Error	C1 (0.5 mg/:)	±Std. Error	C2 (2.5 mg/L)	±Std. Error	C3 (5 mg/L)	±Std. Error	C4 (10 mg/L)	±Std. Error	C5 (20 mg/L	±Std. Error	C6 (30 mg/L)	±Std. Error
C15:0																
C16:0	0.025649	(0.002925)	0.041286	(0.018301)	0.033914	(0.008357)	0.023714	(0.001064)	0.036714	(0.005001)	0.025512	(0.000629)	0.016258	(0.000677)	0.025363	(0.015103)
C17:0	0.011723	(0.00365)	0.020397	(0.008338)	0.012654	(0.002837)	0.003963	(0.003963)	0.016178	(0.00593)	0.013501	(0.000052)	0.004204	(0.004204)	0.009036	(0.009036)
C18:0	0.020499	(0.004737)	0.026104	(0.00633)	0.019069	(0.001528)	0.011665	(0.000946)	0.014863	(0.000429)	0.016156	(0.005462)	0.007775	(0.001641)	0.011851	(0.011851)
C21:0							0.009565	(0.009565)								
C22:0	0.021824	(0.010362)	0.0074	(0.005421)	0.032653	(0.0043)	0.029464	(0.009674)	0.032393	(0.013405)	0.024724	(0.000883)	0.032202	(0.00089)	0.039924	(0.004602)
C23:0			0.005615	(0.005615)												
C24:0							0.005943	(0.005943)	0.014485	(0.001754)					0.00108	(0.00108)
Total SFAs	**0.079695**	**(0.021674)**	**0.100802**	**(0.044005)**	**0.098291**	**(0.017022)**	**0.084314**	**(0.031154)**	**0.114633**	**(0.026519)**	**0.079894**	**(0.007025)**	**0.060439**	**(0.007412)**	**0.087253**	**(0.041671)**
C15:1n5(cis10)																
C16:1																
C17:1n7(cis10)																
C18:1n9																
C22:1n9	0.033144	(0.007765)	0.017232	(0.006043)	0.042078	(0.001925)	0.027566	(0.009795)	0.035718	(0.003834)	0.019388	(0.000637)	0.016704	(0.004284)	0.028935	(0.011064)
C24:1n9																
Total MUFAs	**0.033144**	**(0.007765)**	**0.017232**	**(0.006043)**	**0.042078**	**(0.001925)**	**0.027566**	**(0.009795)**	**0.035718**	**(0.003834)**	**0.019388**	**(0.000637)**	**0.016704**	**(0.004284)**	**0.028935**	**(0.011064)**
C18:2n6cis																
C18:3n3	0.025268	(0.017141)	0.008287	(0.003361)	0.023891	(0.006253)	0.019848	(0.008973)	0.016557	(0.003688)			0.029979	(0.005131)	0.039855	(0.009352)
C20:2(cis11,14)	0.026341	(0.011175)	0.012085	(0.007416)	0.020057	(0.002573)	0.01176	(0.002379)	0.017305	(0.004042)	0.006504	(0.006504)	0.014571	(0.002038)	0.010848	(0.010848)
C22:2(cis13,16)	0.022947	(0.005339)	0.016657	(0.008187)	0.037979	(0.007804)	0.033801	(0.023425)	0.028763	(0.001969)	0.019044	(0.019044)	0.010268	(0.000674)	0.038287	(0.016551)
Total PUFAs	**0.074555**	**(0.033655)**	**0.037029**	**(0.018964)**	**0.081926**	**(0.016631)**	**0.065409**	**(0.034776)**	**0.062625**	**(0.009699)**	**0.025548**	**(0.025548)**	**0.054817**	**(0.007843)**	**0.08899**	**(0.03675)**
C20:5n3 (EPA)	0.075548	(0.021367)	0.045181	(0.004682)	0.061342	(0.015267)	0.057902	(0.020827)	0.069382	(0.003989)	0.070987	(0.00499)	0.066114	(0.019564)	0.077097	(0.005812)
C22:6n3 (DHA)	0.066335	(0.00995)	0.109277	(0.047499)	0.084488	(0.0184689)	0.054056	(0.002612)	0.099557	(0.012371)	0.064978	(0.003552)	0.042912	(0.002025)	0.054241	(0.032418)
Total HUFAs	**0.141883**	**(0.031317)**	**0.154458**	**(0.052181)**	**0.14583**	**(0.033735)**	**0.111958**	**(0.023439)**	**0.168939**	**(0.01636)**	**0.135966**	**(0.008542)**	**0.109026**	**(0.021588)**	**0.131338**	**(0.03823)**
N	**10**		**11**		**10**		**11**		**11**		**9**		**10**		**11**	

Table 3
Abundance of fatty acids (Saturated Fatty Acids – SFAs, Monounsaturated Fatty Acids – MUFAs, Polyunsaturated Fatty Acids – PUFAs, and Highly Unsaturated Fatty Acids – HUFAs, in mg/ind) in the FA profiles of big and small size classes of *S. plana* from the field and after exposure to the commercial formulation of Primextra® Gold TZ.

	Scrobicularia plana Big size											
FA	Field	±Std. error	Ctl	±Std. error	0.5 mg/l	±Std. error	2.5 mg/l	±Std. error	5 mg/l	±Std. error	10 mg/l	±Std. error
C15:0												
C16:0	0,077385	(0.030997)	0.054408	(0.005677)	0.207946	(0.150382)	0.070647	(0.014376)	0.055207	(0.005116)	0.041252	(0.021497)
C17:0												
C18:0	0.06555	(0.023759)	0.043493	(0.003845)	0.130551	(0.087668)	0.053678	(0.016534)	0.040701	(0.003818)	0.029831	(0.013797)
C21:0	0.025861	(0.005847)	0.016827	(0.005396)								
C22:0	0.015595	(0.003512)	0.012975	(0.012975)	0.058912	(0.058912)	0.051839	(0.022801)			0.014296	(0.014296)
C23:0												
C24:0												
Total SFA	**0.18439**	**(0.064115)**	**0.127703**	**(0.027892)**	**0.39741**	**(0.296962)**	**0.176164**	**(0.053711)**	**0.095908**	**(0.008935)**	**0.085378**	**(0.049589)**
C15:1n5(cis10)												
C16:1	0.008572	(0.008572)									0.008545	(0.008545)
C17:1n7(cis10)					0.181803	(0.181803)	0.020053	(0.020053)				
C18:1n9			0.03605	(0.001204)	0.125876	(0.090945)	0.049175	(0.016573)	0.03906	(0.007748)	0.025142	(0.01146)
C22:1n9	0.055235	(0.020957)	0.042647	(0.001076)	0.169877	(0.133337)	0.067743	(0.005859)	0.038209	(0.009234)	0.029881	(0.012211)
C24:1n9											0.012293	(0.012293)
Total MUFA	**0.063808**	**(0.029529)**	**0.078698**	**(0.00228)**	**0.477556**	**(0.406085)**	**0.136971**	**(0.042485)**	**0.077269**	**(0.016982)**	**0.075861**	**(0.04451)**
C18:2n6cis	0.068503	(0.020702)	0.057538	(0.003245)	0.330211	(0.247572)	0.105483	(0.022177)	0.074257	(0.017972)	0.046747	(0.013102)
C18:3n3	0.082102	(0.022334)	0.069054	(0.000033)	0.195076	(0.141374)	0.073553	(0.024815)	0.064357	(0.008891)	0.037721	(0.005724)
C20:2(cis11,14)	0.008974	(0.008974)	0.007382	(0.007382)	0.203389	(0.178541)	0.051839	(0.022801)	0.020221	(0.006989)	0.017169	(0.017169)
C22:2(cis13,16)	0.018441	(0.001704)	0.023572	(0.000031)	0.517592	(0.50864)	0.08073	(0.007983)	0.038085	(0.011173)	0.015569	(0.015569)
Total PUFA	**0.178019**	**(0.053714)**	**0.157546**	**(0.01069)**	**1.246269**	**(1.076128)**	**0.311605**	**(0.077776)**	**0.196921**	**(0.045024)**	**0.117206**	**(0.051565)**
C20:5n3 (EPA)	0.105476	(0.031145)	0.075482	(0.006001)	0.628825	(0.562003)	0.126722	(0.006791)	0.079259	(0.012847)	0.053143	(0.012861)
C22:6n3 (DHA)	0.117675	(0.036426)	0.091177	(0.002789)	0.297381	(0.221103)	0.108052	(0.018182)	0.091614	(0.01029)	0.069011	(0.030191)
Total HUFA	**0.223151**	**(0.067571)**	**0.166659**	**(0.00879)**	**0.926206**	**(0.783106)**	**0.234773**	**(0.024973)**	**0.170873**	**(0.023136)**	**0.122153**	**(0.043052)**
N	**12**		**12**		**12**		**12**		**10**		**13**	

	Scrobicularia plana Small size									
FA	Field	±Std. error	Ctl	±Std. error	0.5 mg/l	±Std. error	2.5 mg/l	±Std. error	5 mg/l	±Std. error
C15:0										
C16:0	0.013322	(0.003386)	0.018357	(0.003306)	0.021622	(0.005252)	0.007367	(0.007367)	0.011165	(0.002312)
C17:0										
C18:0	0.009258	(0.002773)	0.015165	(0.003194)	0.014433	(0.001548)	0.022067	(0.011573)	0.002756	(0.002756)
C21:0	0.010695	(0.010695)	0.006244	(0.006244)						
C22:0	0.003968	(0.003968)	0.0075	(0.0075)					0.008918	(0.008918)
C23:0										
C24:0										
Total SFA	**0.037242**	**(0.020822)**	**0.047266**	**(0.020244)**	**0.036055**	**(0.0068)**	**0.029433**	**(0.018939)**	**0.02284**	**(0.013987)**
C15:1n5(cis10)										
C16:1										
C17:1n7(cis10)										
C18:1n9	0.0888	(0.04343)	0.008248	(0.00037)	0.012592	(0.002589)	0.03546	(0.003546)		
C22:1n9	0.011026	(0.003928)	0.010281	(0.00179)	0.022437	(0.007807)			0.006114	(0.006114)
C24:1n9										
Total MUFA	**0.019906**	**(0.008271)**	**0.018529**	**(0.00216)**	**0.035029**	**(0.010396)**	**0.003546**	**(0.003546)**	**0.009483**	**(0.009483)**
C18:2n6cis	0.012756	(0.001928)	0.019758	(0.000576)	0.029441	(0.021733)	0.006272	(0.006272)	0.009262	(0.009262)
C18:3n3	0.036864	(0.010585)	0.042461	(0.009586)	0.018494	(0.009424)	0.010233	(0.010233)	0.016803	(0.005241)
C20:2(cis11,14)	0.003761	(0.003761)	0.013978	(0.000987)	0.018765	(0.009624)	0.012215	(0.000742)	0.028182	(0.001868)
C22:2(cis13,16)	0.016731	(0.002148)	0.027954	(0.000143)	0.035097	(0.01741)	0.025346	(0.007758)	0.03161	(0.0157)
Total PUFA	**0.070112**	**(0.018422)**	**0.104153**	**(0.011292)**	**0.101798**	**(0.05819)**	**0.054067**	**(0.025005)**	**0.085857**	**(0.032071)**
C20:5n3 (EPA)	0.043697	(0.008739)	0.056743	(0.006069)	0.057553	(0.030515)	0.035566	(0.01063)	0.038604	(0.00842)
C22:6n3 (DHA)	0.020585	(0.007727)	0.025732	(0.004235)	0.031	(0.015174)	0.008299	(0.008299)	0.009323	(0.009323)
Total HUFA	**0.064282**	**(0.016466)**	**0.082475**	**(0.010304)**	**0.088553**	**(0.045689)**	**0.043864**	**(0.018928)**	**0.047926**	**(0.017742)**
N	**12**		**12**		**10**		**9**		**11**	

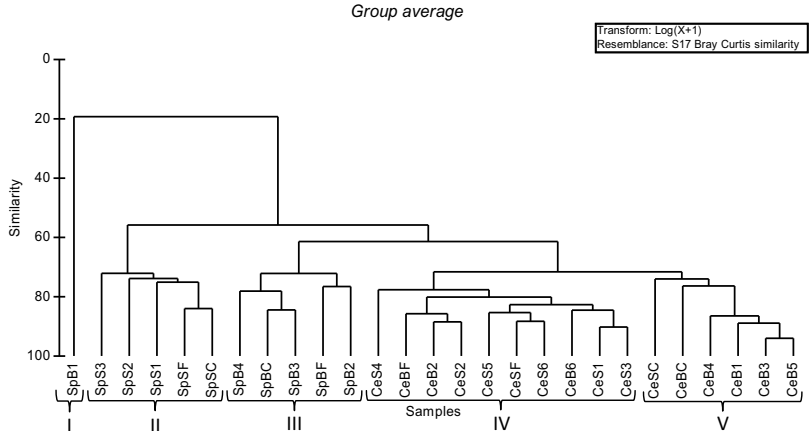

Fig. 2. Cladogram (cluster analysis) grouping the bivalve species *Scrobicularia plana* (Sp) and *Cerastoderma edule* (Ce) of two size classes, big (B) and small (S) based on their total fatty acid composition after exposure to the commercial formulation Primextra® Gold TZ. Numbers correspond to the ranges of concentrations performed and *F* to the field organisms.

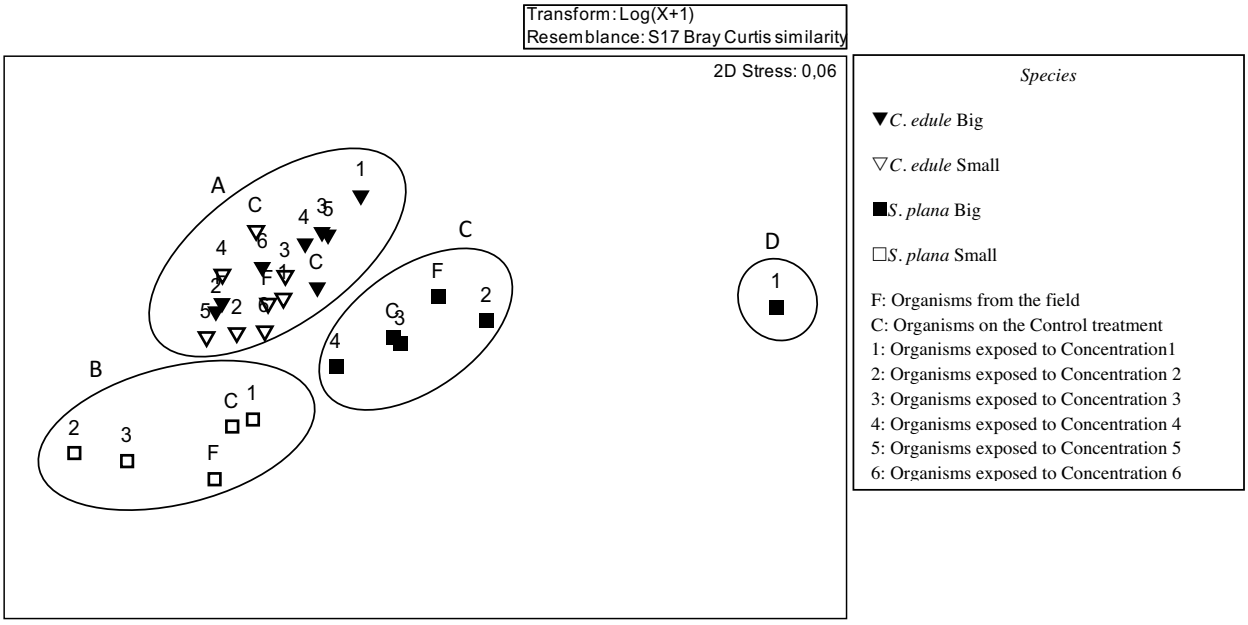

Fig. 3. Two-dimensional non-metric multidimensional scaling (n-MDS) ordination plot of fatty acid composition of both bivalve species and size classes. A, B, C, and D are the groups defined in the MDS.

big class *C. edule* shows an increase in SFAs and PUFAs while the small class *C. edule* shows an increase in SFAs.

Principal component analysis (PCA) of FA data matrix provided a global statistical distinction between the three groups, *C. edule*, *S. plana* and Food (microalgae and rotifers). The first and the second canonical axes explained 65.8% of the variance (PC1–39.3% and PC2–26.5%) (Fig. 5). PCA shows that *C. edule* presents higher amounts of SFAs (C17:0, C24:0, and C22:0) and HUFAs (mainly DHA), whereas *S. plana* presents higher amounts of MUFAs (C18:1n9 and C17:1n7) and PUFAs (C18:2n6cis, C18:3n3, and C18:2n6trans). Based on FA trophic markers (Table 5), the FA compositions of both species may suggest a diet based on zooplankton (carnivory) in the case of *C. edule*, whereas *S. plana* shows omnivorous behavior.

4. Discussion

This study confirmed the lethal effects of Primextra® Gold TZ on both bivalve species, reaching 100% mortality at higher concentrations. In both species, small class size individuals were more sensitive than the large class size ones. Furthermore, *S. plana* is more sensitive to the toxin than *C. edule*. To our knowledge, no studies have evaluated the effect of this herbicide in marine aquatic species. We found one report of toxicological and biochemical (namely FA) responses of the freshwater cladoceran species *Daphnia longispina* to this herbicide and its main active ingredient S-metholaclor (Neves et al., 2015). Comparing our results with this report, the cladoceran species is more tolerant to the herbicide (LC_{50} = 37.65 mg/L, 95% CI [32.89, 46.21] than the two bivalve species *C. edule* and *S. plana*. Ort et al. (1994) determined the effect of S-metolachlor in *Daphnia magna*, and it presented higher tolerance (LC50 = 23.5 mg/L) than *S. plana* ($LC50_B$ = 8.279 (5.137–16.958) mg/L; $LC50_S$ = 3.457 (2.621–4.840) mg/L) and *C. edule* ($LC50_B$ = 17.967 (15.437–20.747) mg/L; $LC50_S$ = 17.010 (13.144; 18.944) mg/L) to this active ingredient, as determined by our results. Comparing the tolerance of benthic and zooplankton species with phytoplankton species, marine bivalves (*C. edule* and *S. plana*) and the freshwater cladoceran species (*D. longispina*

Table 4
Results of SIMPER analyses showing average dissimilarity between sample groups according to non-metric multidimensional scaling (n-MDS) analysis.

MDS group	Similarity	Fatty acid	Av. Abund.	Av. Sim.	Sim./SD	Contrib.%	Cum.%
CeB + CeS (A)	76.64	C22:6n3 (DHA)	0.09	19.2	4.53	25.05	25.05
		C20:5n3 (EPA)	0.06	16.67	5.64	21.75	46.8
		C16	0.03	7.35	4.76	9.6	56.39
SpS (B)	71.13	C20:5n3 (EPA)	0.05	20.06	9.85	28.2	28.2
		C22:2 (cis13, 16)	0.03	11.71	3.75	16.46	44.65
		C18:3n3	0.02	8.14	2.53	11.44	56.09
SpB (C)	76.87	C22:6n3 (DHA)	0.09	14.07	8.66	18.31	18.31
		C20:5n3 (EPA)	0.08	11.73	6.77	15.26	33.57
		C18:2n6cis	0.07	9.67	8.04	12.57	46.15

MDS group	Dissimilarity	Fatty acid	Av. Abund.		Av. Diss.	Diss./SD	Contrib.%	Cum.%
CeB + CeS/SpB (A/C)	38.58	C18:2n6cis	0	0.07	7.28	6.14	18.87	18.87
		C18:3n3	0.02	0.06	4.36	2.32	11.29	30.16
		C22:6n3 (DHA)	0.09	0.09	3.36	1.54	8.71	38.87
CeB + CeS/SpB1 (A/D)	78.68	C20:5n3 (EPA)	0.06	0.49	14.23	17.91	18.08	18.08
		C22:2 (cis13, 16)	0.02	0.42	13.3	26.37	16.9	34.98
		C18:2n6cis	0	0.29	9.59	31.77	12.19	47.17
SpB/SpB1 (C/D)	65.21	C20:5n3 (EPA)	0.08	0.49	12.67	8.88	19.43	19.43
		C22:2 (cis13, 16)	0.03	0.42	12	9.23	18.41	37.83
		C18:2n6c	0.07	0.29	6.83	7.17	10.47	48.3
CeB + CeS/Sps (A/B)	44.67	C22:6n3 (DHA)	0.09	0.02	12.32	2.44	27.59	27.59
		C20:5n3 (EPA)	0.06	0.05	3.87	1.65	8.67	36.25
		C22	0.02	0	3.65	1.94	8.17	44.43
SpB/SpS (C/B)	51.72	C22:6n3 (DHA)	0.09	0.02	9.45	4.35	18.28	18.28
		C18:2n6cis	0.07	0.02	6.72	3.43	12.99	31.27
		C16	0.06	0.01	5.65	3.94	10.93	42.2
SpB1/SpS (D/B)	86.28	C20:5n3 (EPA)	0.49	0.05	15.68	24.23	18.18	18.18
		C22:2 (cis13, 16)	0.42	0.03	13.83	32.86	16.02	34.2
		C18:2n6cis	0.29	0.02	9.57	19.14	11.09	45.29

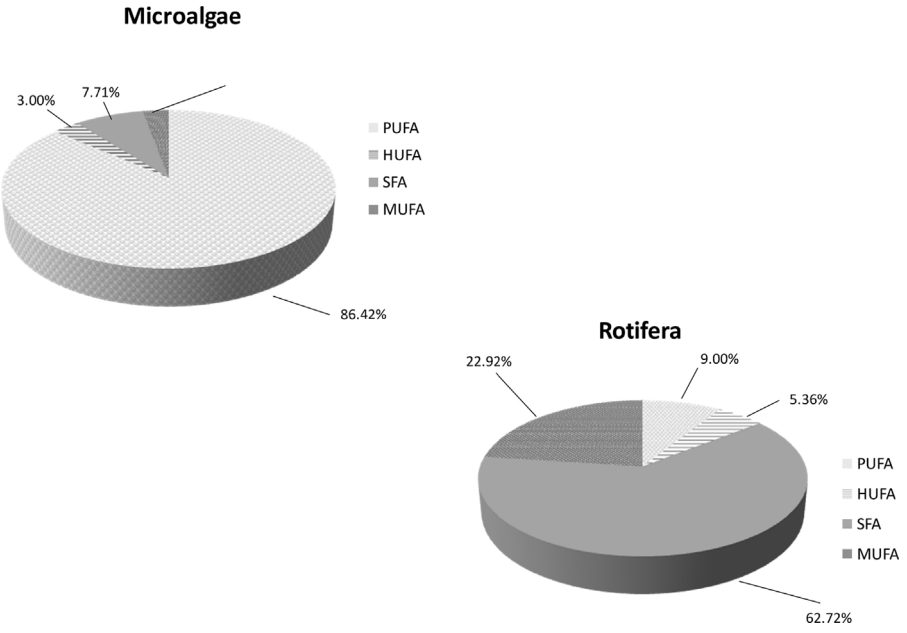

Fig. 4. Fatty acid composition of food sources (microalgae and rotifera) used to feed the organisms under laboratory conditions.

and *D. magna*) are more tolerant to the commercial formulation than the freshwater microalgae species *Scenedesmus vacuolatus* (LC50 = 2.3 mg/L; Liu and Xiong, 2009), which is in accordance with the guidelines (Syngenta®, 2014). On other hand, alterations were observed in the FA profiles of the organisms after exposure to the herbicide. Indeed, before exposure to the toxicant, *S. plana* showed more diversity and abundance in FAs than *C. edule*, whereas after exposure to Primextra® Gold TZ, *C. edule* presented higher nutritive values. In a general perspective, it was observed that larger organisms exhibited more FA diversity and abundance than smaller ones, and *C. edule* was more nutritive than *S. plana*. Indeed, the fatty acid composition of both sizes of *C. edule* is much more similar than in *S. plana*. Therefore, the FA profiles of the species and size classes prove that these molecules are good bio-indicators of stress in marine organisms. Indeed, Delaporte et al. (2005) reports that diet quality affects FA content in bivalves. Moreover, temperature and

Table 5
Fatty acid trophic markers (FATMS) of both size classes of *Cerastoderma edule* and *Scrobicularia plana* collected in the field and exposed to the commercial formulation of Primextra® Gold TZ.

	Marine bivalve species							
	Cerastoderma edule				*Scrobicularia plana*			
Origin	Field		Lab		Field		Lab	
Size	Big	Small	Big	Small	Big	Small	Big	Small
DHA/EPA	1.435	0.878	1.211	2.419	1.116	0.471	1.208	0.453
EPA	0.047	0.076	0.084	0.045	0.105	0.044	0.075	0.057
16:1					0.009			
16:0	0.027	0.026	0.029	0.041	0.077	0.013	0.054	0.018
DHA	0.068	0.066	0.102	0.109	0.118	0.021	0.091	0.026
18:1n9						0.009	0.036	0.008
18:2n6					0.069	0.013	0.058	0.020
∑iso and anteiso – C15 and C17	0.009	0.012		0.020				
	Sources							
	Dinoflagellates/Diatoms		Zooplankton		Detritus/Bacterial			

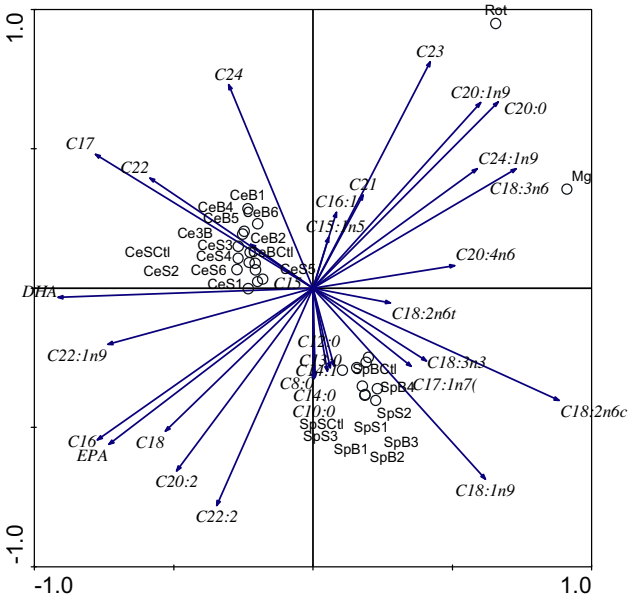

Fig. 5. Principal component analysis relating the FA profiles and fatty acid trophic markers in *Cerastoderma edule* and *Scrobicularia plana* (big and small size classes) collected in the field and exposed to a range of concentrations of the commercial formulation Primextra® Gold TZ.

food availability are two important factors in growth regulation of marine invertebrates including mollusc bivalves. Furthermore, differences in FA profiles may also result from filtration rates and seasonal changes in food sources. Some species exhibit selectivity in particles size, shape, nutritive value, or chemical composition, contributing to different diets between the species and size classes and resulting in different FA profiles (Ezgeta-Balić et al., 2012). Changes observed in the four main groups of FAs (SFAs, MUFAs, PUFAs, and HUFAs) after exposure to the contaminant are in accordance with food preferences that are detailed in the FA trophic markers. Alterations in the FA profiles may be related to the mode of action of the a.i. S-metolachlor, associated with inhibition of several biological processes, essentially biosynthesis, mainly affecting the lipids, FAs, and flavonoids and protein synthesis. It is classified as an inhibitor of very long fatty acid chain formations. However, according to the literature, metolachlor concentrations in Portuguese surface waters are around 0.056 mg/L, very low (Cerejeira et al., 2003) relative to the concentrations used in this study, which could correspond to catastrophic events of contamination or sporadic events such as herbicide application. The values reported by Cerejeira et al. (2003) surpassed the European threshold (0.0001 mg/L) for chloroacetamides in drinking water (Peña et al., 2013), justifying interest in this xenobiotic, mainly in the widespread use of S-metolachlor in several herbicide compositions intended for weed control in agriculture. Tested concentrations are high relative to reported records in natural waters, so we can conclude that the observed levels are safe in environmental terms.

However, studies evaluating the effect of the increasing anthropogenic pressures in estuarine ecosystems and in biological communities, mainly those with economic value (e.g., bivalve species), continue to be extremely important. Because these organisms play key roles in the aquatic ecosystem as a result of their capacity to filter and accumulate pollutants in their tissues (Nagarol et al., 2012), they contribute to the continual cleaning of the water. Moreover, they play crucial roles as links between primary producers and consumers. Furthermore, ecotoxicological studies associated with biomarker responses of living organisms to toxicants are of extreme importance for obtaining fast and low time-consuming results. The herbicide we tested, Primextra® Gold TZ, is applied to corn crops in large quantities. It is essential to study the toxicological and biochemical effects of it and of other pesticides widely used in agriculture near aquatic systems on non-target species.

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