Irene Bermúdez Gutiérrez

IMPACTOS ECOTOXICOLÓGICOS E BIOQUÍMICOS DE S-METOLACLORO E TERBUTILAZINA EM *SCROBICULARIA PLANA*

Dissertação de mestrado em Biologia, orientada pela Doutora Ana Marta Mendes Gonçalves e Professor Doutor João Carlos Marques e apresentada no Departamento de Ciências da Vida da Universidade de Coimbra

Junho 2018

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Resumo

A sobre-exploração agrícola na região do Mediterrâneo tem contribuído para o uso excessivo de herbicidas, poluindo as áreas circundantes, como acontece no estuário do Mondego, localizado perto da cidade da Figueira da Foz (Portugal). Primextra® Gold TZ é o herbicida mais utilizado nos campos agrícolas que rodeiam o estuário do Mondego e é constituído por dois princípios ativos, S-metolacloro (SMOC) e terbutilazina (TBA), que têm exibido toxicidade em espécies aquáticas em estudos prévios. A espécie estudo deste trabalho é o bivalve *Scrobicularia plana,* a qual apresenta elevada capacidade de filtrar poluentes e uma importante posição na estrutura e no funcionamento das comunidades estuarinas. Organismos de duas classes de tamanho (grandes e pequenos) foram expostos individualmente a uma gama de concentrações de cada princípio ativo para avaliar as suas respostas e tolerâncias a ambos os compostos. *S. plana* mostrou uma sensibilidade claramente maior a SMOC (tamanho grande LC_{50} = 40,702 mg/L; tamanho pequeno LC_{50} = 41,517 mg/L) do que a TBA (tamanho grande LC_{50} = 118,590 mg/L; tamanho pequeno LC_{50} = 108,418 mg/L), e os organismos da classe de tamanho maior foram ligeiramente mais sensíveis a SMOC do que a classe pequena de organismos, mostrando uma tendência oposta quando expostos a TBA. A fim de avaliar áreas contaminadas e as suas potenciais consequências para a saúde humana e para o sistema aquático, é necessário desenvolver estratégias laboratoriais alternativas para estimar os potenciais impactos de poluentes de maneira rápida, simples e eficiente. Assim, selecionou-se um conjunto de biomarcadores que inclui as enzimas antioxidantes Glutationa S-transferase (GST), glutationa peroxidase total (tGPx) e glutationa redutase (GRed), e a atividade de peroxidação lipídica calculada pela quantificação das substâncias reativas do ácido tiobarbitúrico (TBARs). O presente trabalho revela que a enzima GRed e a enzima GST mostraram correlações positivas entre as atividades das enzimas e a toxicidade dos produtos químicos. Além disso, GRed mostra especificidade para detetar impactos de TBA, enquanto GST mostrou ser mais precisa para detetar a contaminação por SMOC. Por outro lado, os contaminantes orgânicos podem afetar os perfis de ácidos gordos de espécies aquáticas não-alvo, como por exemplo *S. plana*, além de que o valor nutricional de espécies edíveis pode ficar comprometido. Para compreender os efeitos bioquímicos destes compostos na espécie e classes de tamanhos estudadas, compararam-se os perfis de ácidos gordos e o teor de glicose total dos indivíduos expostos aos herbicidas em condições de laboratório e em indivíduos procedentes do campo. As análises bioquímicas foram realizadas em dois tecidos distintos: no músculo (ou pé) e na massa visceral restante, a fim de perceber se se complementam entre si ou se é possível identificar o tecido que poderá ser usado como indicador da presença dos tóxicos e ser usado em futuros trabalhos como endpoint em ecotoxicologia. Os resultados mostraram que o músculo é o tecido mais representativo de todo o organismo em termos de variações das quantidades de ácidos gordos e, portanto, o melhor tecido para determinar potenciais alterações bioquímicas. Os organismos de tamanho pequeno foram mais sensíveis a nível molecular do que os indivíduos maiores, coincidindo a sua sensibilidade em termos de variações no perfil de ácidos gordos e conteúdo total de glicose com a sua sensibilidade em termos de efeitos letais.

Palavras chave

Scrobicularia plana

S-metolacloro

Terbutilazina

Ensaios ecotoxicológicos

Biomarcadores

Abstract

The overexploitation of the farmlands in the Mediterranean region contributes to an overuse of herbicides and, thus, the pollution of the surrounding aquatic systems, like the Mondego estuary, located near the city of Figueira da Foz (Portugal). Primextra® Gold TZ is the most-used herbicide at agriculture fields that surround the Mondego estuary and it is constituted by two active ingredients (a.i.), S-metolachlor (SMOC) and terbuthylazine (TBA), that have exhibited toxicity to aquatic species in previous studies. The benthic bivalve species *Scrobicularia plana* was selected to carry out bioassays because of its capacity to filter pollutants and its important position in the structure and functioning of estuarine communities. Organisms of two size classes (big and small) were exposed individually to a range of concentrations of each a.i. to assess their responses and tolerance to both compounds. *S. plana* showed a clear higher sensitivity to SMOC (big size LC_{50} = 40.702 mg/L; small size LC_{50} = 41.517 mg/L) than to TBA (big size LC_{50} = 118.590 mg/L; small size LC_{50} = 108.418 mg/L), and big size class organisms were slightly more sensitive to SMOC than the small size class of organisms, showing an opposite trend when exposed to TBA. In order to evaluate contaminated areas and the potential adverse impacts on human health and the environment, it is necessary to develop alternative strategies to rapidly assess the potential impacts of pollutants, therefore, it is necessary to develop alternative laboratorial strategies to assess their potential impacts. Thus, a battery of biomarkers was selected that includes the antioxidant enzymes Glutathione S-transferase (GST), total glutathione peroxidase (tGPx) and glutathione reductase (GRed), and the activity of lipid peroxidation by quantification of the reactive substances of the thiobarbituric acid (TBARs). The present work reports that GRed and GST showed positive correlations between enzymes' activities and chemicals' toxicity. Moreover, GRed shows specificity to detect TBA impacts, whereas GST seems to be more accurate to detect SMOC contamination. Furthermore, organic contaminants may affect the fatty acid (FA) profiles of non-target aquatic species like *S. plana*, besides its nutritional value may be also compromised. To understand the biochemical effects of these chemicals at the studied species and size classes, it was compared the FA profiles and the total glucose content of the individuals exposed to the a.i. under laboratory conditions and at the field. Biochemical measurements were conducted at distinct tissues - the muscle (foot) and the remaining visceral mass, in order to compare if they complement each other or if is possible to identify the most appropriate and thus the best indicator tissue to be used as endpoint in ecotoxicology studies. The results showed that the muscle is the tissue most representative of the whole organism in terms of variations of FA content and thus the best tissue to determine potential biochemical changes. Besides, small size class is more sensitive at molecular level than big size class, coinciding its sensitiveness in terms of variations on FA profile and total glucose content with its sensitiveness in terms of lethal effects.

Key words

Scrobicularia plana

S-metolachlor

Terbuthylazine

Ecotoxicological bioassays

Biomarkers

Índex

Figures index

Table index

List of Abbreviations

Abbreviation	Designation
DHA	Docosahexaenoic acid
ECC	European Community Comission
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Organism for Food and Agriculture
FATM	Fatty acid trophic marker
GRed	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
HUFA	Highly unsaturated fatty acid
INE	*Instituto nacional de estatística*
LC	Lethal concentration
MUFA	Monounsaturated fatty acid
NMDPH	Nicotinamide adenine dinucleotide phosphate
PPP	Plant protection products
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SFA	Saturated fatty acid
SMOC	S-metolachlor
TBA	Terbuthylazine
TBAR	Thiobarbituric acid reactive substances
tGPx	Total glutathione peroxidase
UUA	Utilised Agricultural Area
VLCFA	Very long-chain fatty acid

1. Introduction

1.1. Importance and concerns associated to herbicides

World's population is constantly increasing and demanding agricultural food production, resulting in an excessive use of fertilizers and pesticides. Plant protection products (PPP) play an important role in improving agricultural production by protecting plants against harmful organisms and weeds. However, PPP can lead to detriments on plant production since its use may implicate taking risks of impairments on human health and on the environment. These hazards multiply in those cases in which PPP are placed on market without pertinent official tests for toxicity and authorisations, as well as in cases of improper use as excessive applications. Is inevitable that a percentage of pesticides end on non-target species, leading to undesirable side-effects that precede to repercussions in communities and on ecosystems as a whole (Van Der Werf, 1996). Given the global expansion of the use of pesticides during the past years, plus the fact that they are designed to harm biota, there is an ample risk for adverse impacts on environments. A proper risk assessment and knowledge of the chemicals that threat the communities is the key to the implementation of measures for reduction of the adverse consequences of pesticides application.

Agriculture is essential for the economy in the European Union (EU), farming employs over 20 million people among the 28 countries of the EU (EU-28), being many of them in peripheral and rural regions. The Utilised Agricultural Area (UUA) in the EU is 178.5 million of hectares (ha), corresponding to the Portuguese surface 3641600 ha, with 264400 holdings, a 59.8 % of the EU-28 is dedicated to arable land, mainly for cereal crops (Eurostat, 2016).

The use of PPP in agriculture has allowed to improve yields and to prevent crop losses. Regulation (EC) No 1185/2009 is the legal basis for the data on pesticide sales from national industries in EU Member States and it outlines the definitions and list of active substances. The sales registered to Portugal indicates that more than 3 kg of pesticides per hectare, being herbicides 2410.8 tonnes. In 2012 and 2013 was recorded in Portugal the herbicides with the greatest application of active ingredients after the well-known glyphosate were terbuthylazine and s-metolachlor (Figure 1) on surfaces of 61232 and 60786 ha, respectively, being the quantities applied to corn crops (in kg) 41367 and 47316, respectively (INE, 2013).

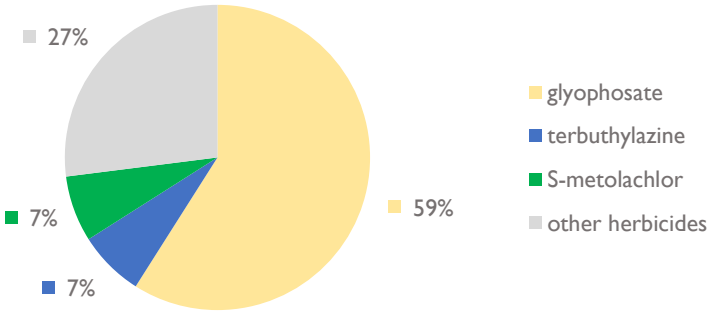

Figure 1. Representation of the percentages of herbicides applied on the Portuguese surface in 2012 and 2013, adapted from (INE, 2013).

Terbuthylazine (TBA) is a systemic herbicide, selective for corn crops, commonly used for the control of annual weeds with effectiveness on the control of graminid and dicotyledons and, similarly. This chemical belongs to the family of s-triazines and initially was use as a substitute of atrazine because of its high toxicity. TBA's mode of action is the inhibition of photosynthesis via blocking electron transport at the second stable electron acceptor, i.e., a protein-bound quinone, of the photosystem II (Steinback et al., 1981). It has low solubility in

water, allowing a persistence of 112-120 days (Nödler et al., 2013), moreover, it has showed toxicity to non-target aquatic animal species, as steatosis in egg of carp (Velisek et al., 2016), changes in structural cell organization of marbled crayfish (Velisek et al., 2017), cellular swelling and epithelial lifting in sea bass (Manera et al., 2016) and bioaccumulation and impairments in liver detoxification of rainbow trout (Tarja et al., 2003).

S-metolachlor (SMOC), is a chloroacetamide herbicide selective for corn crops generally applied post-emergently on weeds. Its utilization is authorized by most of the countries in the European Union (Regulation no. 540/2011). Metolachlor was developed to control grass weeds following pre-emergence application. Its mode of action involves the inhibition of several biological processes, mainly biosynthesis of proteins and lipids acting on meristematic zones of plants (Liu & Xiong, 2009). The herbicide acts by inhibiting elongase, which is responsible for the elongation of very long-chain fatty acids (VLCFAs) by inhibition of the expression of FAE1 gene (Trenkamp et al., 2004). Under aerobic conditions, SMOC has a half-life of 47 to 78 days in water (Nwani et al., 2015), besides, its action mode represents a danger to aquatic environments since studies have demonstrated that it cause damages to aquatic species, such anomalies in larval development in oysters (Gamain, et al., 2017), lethal effects and impairments in fecundity of cladocerans (Neves, et al., 2015), inhibition in cell algae density (Thakkar et al., 2013), malformations in zebrafish early life stages (Quintaneiro, Patrício, Novais, Soares, & Monteiro, 2017) and mortality in crustaceans (Maazouzi, et al., 2016).

Around the Mediterranean region, there is an overexploitation of the farmlands, associated to an overuse of herbicides with potential to pollute surrounding aquatic systems by different ways: aerial spraying, leaching until underground water, runoff or accidental spills (Vidal et al., 2012). An important portion of the agricultural lands are dedicated to irrigation techniques, with many of the irrigated areas placed in Mediterranean regions like Italy, Greece, Cyprus, Malta, Spain and the coast of Portugal. In southern European countries full irrigation is an essential element in many types of agricultural production. Portugal spends a volume of 7371 m^3 of water per hectare to irrigation, being the second country of the EU with the major expenses of water to this purpose, corresponding 75% of the irrigated area to maize crops (Eurostat, 2016). This information combined with the assumed risk of herbicide contamination suggest that the water used for irrigation eventually may reach dangerous concentrations of chemical pollutants, moreover, a vast volume of this water ends in surrounding aquatic systems spreading the risk of contamination to a larger area. Water contamination can cause adverse effects in aquatic species, including edible species, which indirectly may lead to risk on human health.

Food quality and safety concerns the consumers increasingly, therefore, to minimise the impacts produced by the use of chemicals on agriculture, international institutions have developed legislations and have implemented biomonitoring programs at locals under risk of pollution. Nationally, exists the law n° 26/2013 of April 11th, transposing the Directive 2009/128 / EC of the European Parliament and of the Council of October 21th that establishes a framework of action at the community level for a sustainable use of pesticides, being its aims: 1) to ascertain the implementation of necessary measures in order to promote phytosanitary protection, focusing in a low use of phytopharmaceutical products and giving priority to non-chemical solutions; 2) to monitor the good phytosanitary practices, giving preference to the phytopharmaceutical products with lower toxicity; and 3) to insure the respect to the instructions and the authorised conditions of utilization related to cultivations and agricultural products such as dosage, frequency of application, better period of application, correct use of the equipment of individual protection during applications and environmental precautions.

The indiscriminate use of pesticides is an increasing common exercise due to the intensive agricultural practices that occurs in many regions. Decontrolled applications of this chemical products lead to consequences on fauna, with special worry about the run-offs to aquatic environments. Despite the utilization of pesticides is currently diminishing in developed

countries, 40% of the production of herbicides occurs precisely in these regions. These products, used for plant protection and production optimization have effects at the different organizational levels, from molecular to ecosystem (Vidal et al., 2012), often resulting in residues in plants, vegetables, fruits, and non-target organisms (Parween et al., 2016).

One of the major environmental concerns is the bioaccumulation of pesticides on primary producers of aquatic systems and its propagation through the different levels of the trophic webs, becoming a threat to human health due to the consumption of commercial products (Filimonova et al., 2016a). Most of the xenobiotics introduced in aquatic environments does not disappear immediately so its effectiveness would not be reduced. Thus, they tend to persist and bioaccumulate, promoting the rise of the chemical concentrations in aquatic systems over time. Many factors determine the destiny of pesticides applied on agricultural soil, including techniques of application and type of soil, among others, influencing their persistence and the extension of the contamination (Torres et al., 2008). Moreover, it is difficult to monitor the presence of xenobiotics in aquatic environments since they can accumulate in one or many different locals or even in organisms, having different concentrations in each one (Figure 2). The impacts at the environment caused by herbicide pollution depend on how occur the dispersion, on the chemical concentration and on the toxicity of the substance.

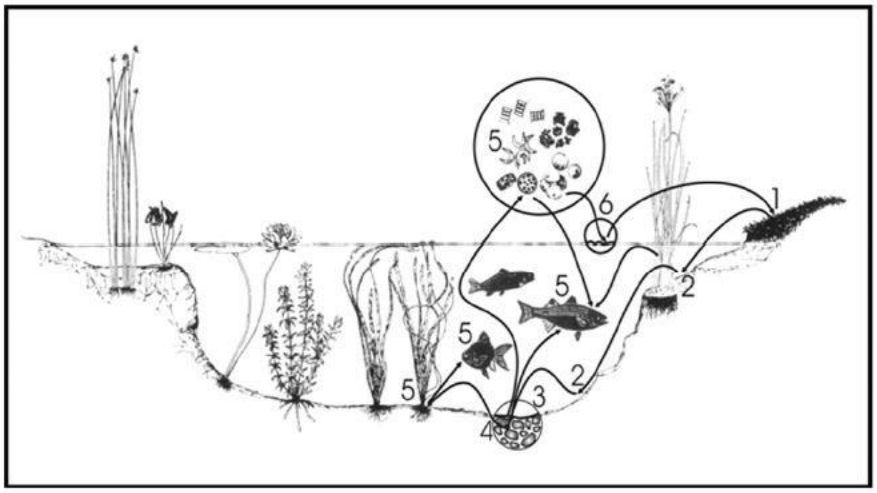

Figure 2. Scheme of the different parts of an aquatic ecosystem and its connection under the presence of xenobiotics. (1) Soil, (2) sediment, (3) water-sediment interphase, (4) interstitial water, (5) biota and (6) air-water interphase from (Torres et al., 2008).

1.2. Coastal ecosystems - Estuaries

Estuaries are complex systems highly productive with a huge quantity of inputs and outputs of water flows, species residents and migrators, nutrients, pollution, etc. They represent a major resource for the economic sector since they involve numerous activities as agriculture, fishing, aquaculture, port and industry. These ecosystems are commonly surrounded by farmland, residential and industrial areas which leads to pollution problems coming from rivers, run-offs, industrial residual discharges, intensive agriculture practices, marine traffic, among others. Thus, estuarine communities are under constant threat of organic and inorganic contaminants that affects the water quality and the aquatic species. Still, the way the xenobiotics are transferred between the different phases of the structure is poorly documented due to the difficulty associated to the non-conservative behaviour characteristic of these locals, to the assimilation of the substances and to the inclusion of the metabolites in the organic material cycle (Manuel Nicolaus et al., 2015).

The intensive use of pesticides in agricultural areas near ecological valuable coastal wetlands led to the implementation of pesticide-monitoring programs to recover aquatic systems, such as at the Mondego estuary, located in Figueira da Foz (Portugal) (Galhano et al., 2011), which was the local of sampling in the present study. The Mondego estuary is a mesotidal system, influenced by a temperate climate. It has an area of 8.6 km^2, approximately, and it comprises 2 channels (north and south), separated by the island of Morraceira, that join again near the mouth. The north channel is deeper, constituting the main channel of navigation and it is where the port of Figueira da Foz is located. The south channel is less deep and silted at the upper zones, being the entrance of fresh water into the estuary essentially through the north channel. Until 1998, the upstream areas of the south channel were practically silted up, with only a small connection with north channel; thus, water circulation in the south channel was mostly dependent on tides and freshwater input from the Pranto River, which was artificially controlled by a sluice, according to the rice fields irrigation needs in the lower Mondego Valley (Ansari & Gill, 2014). This local is a strongly threat area with anthropogenic pressures due to the crops, commercial port, leisure activities and industry, moreover, the estuary presents high levels of solid particles in suspension and diverse pollutants (Teixeira et al., 2008).. There are 15,000 ha of cultivated land, around the estuary producing mainly rice and corn, which represents a risk of organic contamination that can produce impacts from an ecological perspective by compromising the water quality and the aquatic communities, and even from an economic and health perspective since there are species being produced for human consume that might be affected. According to the agricultural cooperatives of the Mondego valley, the most-used herbicide on corn crops is Primextra Gold® TZ (Syngenta AG). The main active ingredients of this herbicide are terbuthylazine and S-metolachlor, which are among the most frequently used active principals in Portugal as mentioned (Introduction 1.1). S-metolachlor represents 30.2% (w.w.) and terbuthylazine, 17.75% (w/w) of the commercial formula but these active ingredients are also used by Syngenta AG in other commercial formulation used worldwide (Bicep II Magnum®, Gardo Gold®, Primagram Gold® and Primextra Gold®). Thus, in terms of risk assessment, it is important to study their effects and modes of action, individually, as well as joint at the commercial mixtures.

1.3. Bivalves as standard species in ecotoxicological studies

According to the Organism for Food and Agriculture (FAO) of the United Nations, in 2010 fishing and aquaculture produced more than 632000 tonnes of bivalves in Europe, and the production is still increasing worldwide in the past years (FAO, 2016). Bivalve species consume nutrients from organic material particles, such as plankton, resuspended benthic microalgae and detritus from both bacterial and myco-heterotrophic sources (Pernet et al., 2012). Benthic bivalves are defined as standard species for ecotoxicological studies of aquatic environments due to their trophic position, their ability to filtrate and ingest great volumes of water and sediments, including contaminants, and their easy handling and maintenance at the laboratory, because of their sessile lifestyle (Gonçalves et al., 2016).

Scrobicularia plana (da Costa, 1778), commonly known by peppery furrow shell, is a bivalve mollusc from the family *Semelidae*, typically found buried in brackish waters and muddy sand. 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. This species was selected to carry out bioassays because of its large capacity to filter organic material as a deposit filter feeder, including pollutants, and its important position in the structure and functioning of estuarine mudflats. In general, bivalves are important at all trophic levels, since they act as a link between producers and consumers, feeding on small organisms

and being a food source for crustaceans, fishes and wading birds. Moreover, *S. plana* is abundant at the Mondego estuary as an important food resource, owning a great economic value as edible species (Fossi Tankoua et al., 2013; Gonçalves et al., 2016, 2017, Verdelhos et al., 2014, 2015).

Figure 3. Scrobicularia plana (da Costa, 1778). Source: http://skaphandrus.com/media/cache/sk_widen_265/uploads/fotografias/8359.jpg

1.4. Oxidative stress against xenobiotics

Xenobiotics are substances that are alien on the environment and that produce toxicity on living beings by over-inducing natural physiological processes causing a phenomenon called oxidative stress. This event occurs when an organism, for example, response to the toxicity of a substance, translated as an overproduction of free radicals mainly reactive oxygen species (ROS), and develops adaptative strategies as the production of antioxidants to cope with the excess of radicals (Antunes et al., 2013). This overproduction depletes the antioxidant cell defences, eventually leading to impairments in cellular tissues if the antioxidant defences were not reset.

Pesticides contribute to the appearance of oxidative stress through diverse described mechanisms, such as (1) interfering on redox cycles (reversible oxidation) by accepting and giving electrons to determinate cellular compounds, increasing the level of ROS; (2) meddling on cellular metabolisms by requiring reduction substances (i.e. glutathione) as a consequence of the depletion of its reserves, factor that diminish the antioxidant capacity; (3) depending on the chemical nature of the pesticides, some can inactivate antioxidants enzymes leading to less recovering capacity; (4) troubling the production of energy, diminishing its availability for the metabolism and, subsequently, limiting detoxication processes; (5) contribute to the modification of vital biological processes in cellular nucleus, mainly transcription and translation of proteins (Lushchak, 2011). Recent studies show that the exposure of organisms to contaminants as pesticides and metals may potentiate the appearance of oxidative stress, decreasing its capability to eliminate them from its system and producing adverse effects (Cavalcante et al., 2010; Jordaan et al., 2013; Limón-Pacheco & Gonsebatt, 2009; Tejada et al., 2007). Ecotoxicological studies may potentiate the knowledge about biochemical parameters to detect the occurrence of oxidative responses, by applying biomarkers tools.

Biomarkers are biological parameters that are useful to measure the variations on behaviour, physiology, biochemical profile, cellular integrity and genomic expression in order to associate them to determinate situations of environmental perturbation. The biomarker approach detects disturbances that may accentuate over time if the factor that cause the stress persist or become stronger, allowing the implementation of recovery measures before the situation aggravates. Pollutants can be assessed by biomarkers that give information about their presence in certain environments and the effects that produce in parameters from the

molecular to the ecosystem level. Therefore, ecotoxicological studies that use biomarkers are well considered in the evaluation of the environmental health in aquatic systems (Cossu et al., 2000), since they allow the quantification of the impacts derived from the environmental stress monitoring the evolution of the systems guided to its recovery (Vasseur & Cossu-Leguille, 2003).

According to the literature, the variations at the levels of activity of antioxidant enzymes may result in suitable biomarkers in aquatic species, probably related to adaptative responses (Peixoto et al., 2006). Some contaminants may bind to the materials in water suspension, accumulated in the sediment or filtered by aquatic organisms, leading to consequent physiological and biochemical responses, such as effects on the antioxidant defence system (Jordaan et al., 2013). Under natural conditions, animals maintain normal generation and neutralization of reactive oxygen species (ROS) in the ecosystem (Kumar et al., 2013), nonetheless, when exposed to xenobiotics, the biotransformation process leads to higher generation of free radicals and consequent cellular oxidative stress. ROS are highly toxic and, in excess, produce reactions with biological macromolecules that, eventually, cause oxidative stress, lipoperoxidation, DNA damage and protein oxidation (Tejada et al., 2007), resulting in disturbances in cell physiological processes in aquatic organisms (Cavalcante et al., 2010). Thus, the sensitivity of an organism to the exposure to contaminants is associated to its capability to cope with the oxidative stress, which is shown as higher or lower activity of antioxidant enzymes, and the damage suffered is shown as lipid peroxidation. If the activity of antioxidant enzymes is induced by the exposure of the organisms to pollutants, it may suggest an adaptative response, although if it is inhibited it could mean a depletion of antioxidants that could lead to impairments and even mortality (Cossu et al., 2000).

The present work focus in three enzymes related to antioxidant responses and one indicator of lipid peroxidation to assess their responses to herbicide pollution. The battery of biomarkers selected comprise antioxidant enzymes (Glutathione-S-transferase, Glutathione Peroxidase and Glutathione Reductase) and the evaluation of the potential occurrence of lipid peroxidation by quantification of the thiobarbituric acid reactive substances (TBARs). These biomarkers are typically used to assess potential impacts of chemicals on aquatic ecosystems because of their high sensitiveness and they can be helpful to the understanding of adverse outcome pathways of toxicants.

The glutathione generally acts eliminating oxidant metabolites originated from the metabolization of a xenobiotic, preventing the aggravation of the oxidative damage. The tripeptide glutathione is present in many important processes including the detoxification of hydrogen peroxide (H_2O_2), metals, organic contaminants, at the maintenance of the normal conditions of proteins and at the synthesis of DNA precursors. Numerous reactions involve reduced glutathione (GSH) as redactor agent to be transform in its oxidized form (GSSG). On the other hand, GSH may not be produced in sufficient amount to eliminate the toxic reactive substances under oxidative stress, which will cause repercussions in the animal physiology.

The total glutathione peroxidase (tGPx) is involved in the inhibition of ROS, reducing H_2O_2 to H_2O to ensure optimum protection against oxidative stress (Gaté et al., 1999) with oxidation of GSH. Hence, under exposure to contaminants, an induction of tGPx provides a warning of higher H_2O_2 production in the organisms, suggesting that oxidative stress can be imposed by the presence of the herbicides and the ROS produced may subsequently react with biomolecules resulting in oxidative damages to cellular components (Nwani et al., 2015).

Glutathione reductase (GRed) reduces GSSG to GSH, which plays an important role in the GSH redox cycle, being essential a high GSH/GSSG ratio for protection against oxidative stress, therefore, GSH renewal in the cells is vital due to its condition as co-factor by both enzymes (Cheung et al., 2004; Maria & Bebianno, 2011).

Glutathione-S-transferase (GST) is involved in the degradation of organic contaminants (Fossi Tankoua et al., 2013). GST catalyse reactions of certain compounds, with GSH addition, forming thioethers (Habig et al., 1974), hence, this positive correlation denotes the importance of the coordinated regulation of those enzymes in maintaining the equilibrium between the cellular GSH and GSSG levels (Silva et al., 2012).

The evaluation of the levels of reactive substances of thiobarbituric acid (TBARs) reflects the status of the lipid peroxidation, that is, the degree of degradation of the polyunsaturated fatty acids that conform the phospholipid membrane of cells. Thus, by measuring TBARs, it is possible to reveal whether the defence of antioxidant enzymes has been overwhelmed (Bertrand et al., 2016). The detection of the lipid peroxidation is often used to understand the involvement of free radical reactions in toxicology. This degradation occurs as a consequence of stress produced by different factors, including xenobiotics. Peroxidised membranes turns rigid losing permeability and integrity (Valavanidis et al., 2006).

1.5. Fatty acid profiling as bioindicator of toxicity

Lipids are nutrients involved in many vital functions of aquatic species. Fatty acids (FA) comprise saturated FA (SFA), without any double bonds, and unsaturated FA (UFA), with one or more double bonds in the molecular structure. In accordance of the degree of unsaturation, UFA are divided in monounsaturated FA (MUFA), with a single double bond, polyunsaturated FA (PUFA), with two or more double bonds and a sub-group of PUFA, the highly unsaturated FA (HUFA) with three or more double bonds. Fatty acids molecules are important for several biological processes, being key constituents of phospholipid cell membranes to which confer special properties, affecting its permeability and the traffic of cell compounds (Ibarguren & López, 2014). Besides, FA are used as fuel in all metabolic systems at all trophic levels, having an important role on neural levels of biochemical and physiological response (Neves et al., 2015). Some of them only can be obtained from food and thus referred to as 'essential nutrients' or 'essential fatty acids' (EFA).

Polyunsaturated fatty acids (PUFA), where some EFA are included and some omega 6 and omega 3, are a family of lipids that contains some subgroups identified by the position of the last double bond in their structure. PUFA are mainly synthesized by plants, algae and bacteria, with only few animals being able to convert them through elongation or denaturation of other FA with a low ratio between production and consumption, thus, animals obtain this group of FA mainly by their diet (Brett & Muller-Navarra, 1997). PUFAs are precursors of important hormones and are present in neural system, being involved in cell signalling processes and playing a role on brains development (Liu et al., 2015). The group of the highly unsaturated fatty acids (HUFA) includes eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), essential metabolites that cannot be synthesized *de novo*, at least not in sufficient amounts, being taken up through food sources (Ladhar et al., 2014).

In aquatic food webs, FA are one of the crucial molecules transferred across the plant-animal interphase. and they are claimed to be a good bio-indicator of ecosystem health and bio-indicators of stress (Gonçalves et al., 2016), thus, FA serve in ecotoxicological studies as important tools to the determine the presence of toxicants (Filimonova et al., 2016b; Gonçalves et al., 2017). Studies about the distribution and abundance of lipids of aquatic species reveal important information about the ecosystem health, since they are sensitive and specific indicators of stress (Arts et al., 2009), moreover, they proved to be useful trophic markers (De Troch et al., 2012).Works about the occurrence of EFA on aquatic species among different trophic levels represent a suitable approach to understand the functioning of whole aquatic system. Organic and inorganic contaminants demonstrated to produce alterations on the FA profiles of several aquatic species, including cladocerans (Neves et al., 2015), diatoms and copepods (Filimonova et al., 2016a, 2018) and bivalves (Gonçalves et al., 2016; Mesquita et al., 2018). A decrease on EFA in primary consumer species may have

repercussions to higher trophic levels and, thus, to human-beings affecting their physiological condition and the resistance to some diseases (Brett et al., 2009). Since copepods get the majority of these EFA by feeding, they are largely depending on the FA content of phytoplankton, in addition to the efficiency to transfer those FA to the next trophic level which can be compromised under stress conditions. Therefore, the occurrence of EFAs in aquatic species from primary producer to consumers is crucial for a good quality of diets, rebounding in the healthy status and nutritional value of edible species populations and, consequently, is also important for the maintenance of a sufficient nutritional status of the human diet and health, being associated FA as omega-3 and omega-6 to the prevention of cardiovascular, inflammatory, ocular and neurological diseases, improvements on cognitive development and nervous system development, among others (Filimonova et al., 2016b).

Bivalves are rich on PUFAs and are strongly integrated on the Mediterranean diet, characteristic of the south of Europe. *S. plana* commonly has essential fatty acids on high concentrations and its occasional consume can lead to benefits to health, nonetheless it exposition to stressors may affect its fatty acid content and, consequently, compromises its nutritional value (Gonçalves et al., 2016; Mesquita et al., 2018; Verdelhos et al., 2015) Thus, the present study also includes the FA profiling of the species *Scrobicularia plana* to assess the impacts of the active ingredients of the herbicide Primextra Gold TZ.

1.6. Carbohydrates

Carbohydrates are the main and immediate energy sources to cope with stress, thus its metabolism may play an important role in the maintenance of cell homeostasis in bivalves during processes of immune defence. Theoretically, the presence of organic pollutants may impose high energetic costs for immune regulation resulting in an enhance of carbohydrate metabolism including glucose degradation, glycogenolysis, and gluconeogenesis (Lochmiller & Deerenberg, 2000). Hence, the quantification of the total carbohydrates content may offer precious information to determine the potential biochemical impacts of contaminants.

Glycogen is the main source of glucose reserves in soft tissues and constitutes approximately 90 % of the total carbohydrates in bivalves (Plana et al., 1996), thus was assumed that the variation on glucose/glycogen amounts caused by the herbicides contamination is calculated by the quantification of the total amounts of carbohydrates. Since glucose can be metabolized via glycolysis or oxidative phosphorylation pathway, then excessive glucose consumption can result in a rapid depletion of glucose reserves. Thus, in order to maintain glucose homeostasis, it can be obtained from the glycogen via glycogenolysis (Tiwari & Singh, 2005; Yoganandhan et al., 2003). Moreover, an enhanced exploitation of glycogen reserves for glucose availability has been reported in several aquatic animals under the presence of stressors (Flye-Sainte-Marie et al., 2007, 2009; Wang et al., 2015; Zhou et al., 2010).

2. Objetives

The present work aims to determine ecotoxicological and biochemical potential impacts of S-metolachlor (SMOC) and terbuthylazine (TBA) at the bivalve species *Scrobicularia plana* from the Mondego estuary. Therefore, the specific objectives are: to investigate (1) the mortality rate, (2) the oxidative stress (2) the FA profiles (3) the nutritious quality and (4) the total content of carbohydrates of two size classes of *S. plana* after exposure to S-metolachlor and terbuthylazine, individually. Moreover, this work also aims to identify the tissue to be used as the best indicator to assess the presence of these chemicals and thus to be used as endpoint at ecotoxicological studies.

Chapter I: Activity of antioxidant enzymes as biomarkers of the toxicity of S-metolachlor and terbuthylazine on *Scrobicularia plana*

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3. Chapter I: Activity of antioxidant enzymes as biomarkers of the toxicity of S-metolachlor and terbuthylazine on *Scrobicularia plana*

3.1. Introduction

World's population is constantly increasing and demanding agricultural food production, resulting in an excessive use of fertilizers and pesticides. Around the Mediterranean region, there is an overexploitation of the farmlands, associated to an overuse of herbicides with potential to pollute surrounding aquatic systems by different ways: aerial spraying, leaching until underground water, runoff or accidental spills (Vidal et al., 2012). The Mondego estuary, located near Figueira da Foz (Portugal), is influenced by the intensive agriculture practices in the river watershed that affect, not only the water quality, but the aquatic communities, including edible species, leading to potential consequences to our health (Gonçalves et al., 2016; Verdelhos et al., 2014). According to agricultural cooperatives of the Mondego valley, Primextra® Gold TZ is the most-used herbicide to control weeds found in corn crops (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015). It is composed by two main active ingredients, S-metolachlor and terbuthylazine, that appear in other commercial formulations of Syngenta AG (Bicep II Magnum®, Gardo Gold®, Primagram Gold® and Primextra Gold®). These products, used for plant protection and production optimization have effects at all of the organizational levels of life, from molecular to ecosystem (Pereira et al., 2009; Vidal et al., 2012), often resulting in residues in plants, vegetables, fruits, and non-target organisms (Parween et al., 2016). S-metolachlor (SMOC) is a chloroacetamide developed to control grass weeds following pre-emergent application (Karam et al., 1992), by inhibition of the biosynthesis of several molecules. SMOC's action mode represents a danger to aquatic environments, since some studies have demonstrated that it can cause damages to aquatic species, such anomalies in larval development in oysters (Gamain et al., 2017), lethal effects and impairments in fecundity of cladocerans (Neves et al., 2015), inhibition in cell algae density (Thakkar et al., 2013), malformations in zebrafish early life stages (Quintaneiro et al., 2017) and mortality in crustaceans (Maazouzi et al., 2016). Terbuthylazine (TBA), belongs to the group of s-triazines (Velisek et al., 2017) and acts selectively as a photosynthesis inhibitor in plants by both pre- and post- treatments, nonetheless it showed toxicity to non-target aquatic animal species, as steatosis in egg of carp (Velisek et al., 2016), changes in structural cell organization of marbled crayfish (Josef Velisek et al., 2017), cellular swelling and epithelial lifting in sea bass (Manera et al., 2016) and bioaccumulation and impairments in liver detoxification of rainbow trout (Tarja et al., 2003).

In aquatic environments, benthic bivalves are defined as standard species for ecotoxicological studies due to their trophic position, their ability to filtrate and accumulate pollutants, and their easy handling and maintenance at the laboratory (Gonçalves et al., 2016). The bivalve *Scrobicularia plana* (da Costa, 1778), typically found buried in intertidal mud and muddy sand, was selected to carry out bioassays because of its large capacity to filter organic material, as organic contaminants, and its important position in the structure and functioning of estuarine mudflats. In general, bivalves are important at all trophic levels, since they act as a link between producers and consumers, eating small organisms and being a food source for crustaceans, fishes and wading birds. Moreover, *S. plana* is commonly consumed by humans, being a species with a great economic value (Fossi Tankoua et al., 2013; Gonçalves et al., 2016, 2017; Verdelhos et al., 2014).

Organic pollutants in aquatic systems are difficult or extremely expensive to analyse, therefore it is necessary to develop strategies to evaluate the ecosystems health and to assess the impacts caused by stress factors (Bergayou et al., 2009). Currently, biomarkers are often used as tools for the assessment of pollutants' impacts. Few recent studies have used the biomarker approach in *S. plana* (Bergayou et al., 2009; Fossi Tankoua et al., 2013; Silva et al., 2012; Solé et al., 2009), although, there is a lack of information about suitable biomarkers for this species when exposed to herbicides.

Some contaminants may bind to the materials in water suspension, accumulate in the sediment or filtered by aquatic organisms, leading to consequent physiological and biochemical responses, such as effects on the antioxidant defence system (Jordaan et al., 2013). Thus, in our study, the battery of biomarkers selected comprise antioxidant enzymes (Glutathione Peroxidase and Glutathione Reductase), a biotransformation enzyme (Glutathione-S-transferase), and the evaluation of the potential occurrence of lipid peroxidation by quantification of the thiobarbituric acid reactive substances (TBARs). These biochemical biomarkers are typically used to assess potential impacts of chemicals on aquatic ecosystems because of their high sensitiveness and they can be helpful to the understanding of adverse outcome pathways of toxicants. Thus, the present work, besides it supports the lethal concentrations of the studied chemicals for *S. plana*, which may assurance more comprehensive and robust information for regulators and competent authorities to support their decisions in water protection; it also provides new relevant information about biomarkers useful to assay herbicide's impacts in bivalves.

3.2. Materials and methods

3.2.1. Study area and sampling procedures

The Mondego estuary is a small mesotidal system with an area of 8.6 km^2 on the western Atlantic coast of Portugal (40°08'N,8°50'W). It is divided in two main channels, north and south, that are separated by the island of Morraceira (Fig. 4). The bivalves were captured on the south channel, using a dredge and, immediately, were put in cold boxes with water from the estuary to be transported to the laboratory, where they were separate by size classes attending to their weight and length (medium body size: big = 4.20 cm, small =3.47 cm).

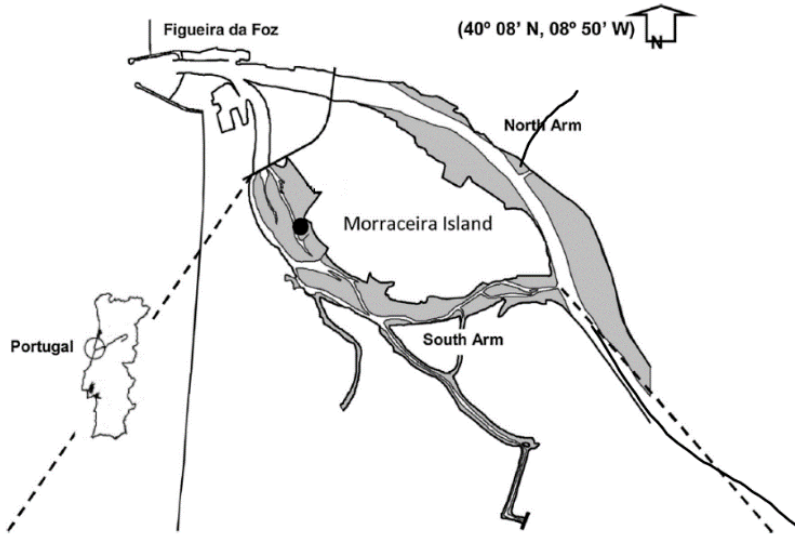

Figure1. Map of the Mondego estuary location and sampling site (black circle) at the south arm.

3.2.2. Laboratory and bioassays procedures

When arrived at the laboratory, from the total group of bivalves were picked ten individuals of big size class and ten individuals of small size class, whose muscles (foots) were then removed from the total biomass of the body, plus ten complete individuals. All muscles, visceral masses and complete individuals were weight and store at -80 °C, until biochemical analysis. The remaining individuals were maintained in filtered sea water diluted until 20 psu under controlled laboratorial conditions: temperature = 20 °C, photoperiod = $12h^{L}$:$12h^{D}$, and constant aeration. Before the bioassays, the organisms were submitted to a depuration period during which they were not fed. After this period, again were selected ten individuals of each size class to be dissected, weigh and stored at the same temperature, as previously described. The remaining organisms were submitted to an of 96 hours assay that consisted in various treatments, including ten individuals of each size class each, with growing concentrations of each chemical (SMOC: 2.048 mg/L, 5.24 mg/L, 13.42 mg/L, 25.46 mg/L, 30.5 mg/L, 34.36 mg/L, 39.40 mg/L, 42.5 mg/L, to 46.41 mg/L; TBA: 40 mg/L, 57.6 mg/L, 69.2 mg/L, 82.3 mg/L, 95 mg/L, 110,5 mg/L, 125 mg/L, 138 mg/L) and two negative controls, one per chemical. Organisms from big and small size classes were placed in aquariums filled with volumes of 1000 mL and 500 mL, respectively, of medium (stock solution of the active ingredient + filtered sea water at 20 psu), replaced after 48 hours. Bivalves were fed with a commercial frozen mixture of rotifers and microalgae and checked for mortality and behavioural conditions (evaluation of siphon activity, condition of the valves and reactions to the stimulus), daily at the same hour, approximately. After the exposures period, three of among the survived organisms were weighed, dissected, by separating the muscle (foot) (medium weight = 91.71 mg for big size class and 34.07 mg for small size class) of the remaining visceral mass (medium weight = 1007.46 mg for big size class and 264.29 mg to small size class) and stored at -80°C, as described, until biochemical analysis (Chapter II). Five remaining organisms were previously homogenized in phosphate buffer (50 mM, pH = 7.0 with TRITON X-100 0.1%) and centrifuged in a refrigerated centrifuge at 15000 g for 10 min at 4 °C and the supernatant was collected and stored at -80°C, afterwards, the quantification of enzymatic activities, lipid peroxidation and total contain of protein were proceeded in those organisms.

3.2.3. Molecular analyses

Antioxidant responses were evaluated through the enzymatic activities determinations of total glutathione peroxidase (tGPx), glutathione reductase (GRed) and glutathione–S–transferase (GST). The occurrence of lipid peroxidation was determined, through the measurement of thiobarbituric acid reactive substances (TBARs). The homogenization products were divided into five aliquots, one for each determination (tGPx, GRed, GST, TBARS and protein quantification). Enzymatic activities were determined in microplate reader and were expressed in nmol of substrate hydrolysed per min per mg of sample protein. Protein was quantified according to the method of (Bradford, 1976), based on spectrophotometry, adapted to microplate. Samples were read at 595 nm and the protein concentrations were calculated by comparison with a standard solution of bovine serum albumin. All biomarkers (tGPx, GRed, GST and TBARs) were expressed in function of the protein content of sample.

3.2.3.1. tGPx

tGPx (EC 1.11.1.9) activity was assessed, according to the method described by (Flohé & Günzler, 1984), following the oxidation of Nicotinamide adenine dinucleotide phosphate (NADPH) when GSSG is reduced back to GSH by GRed. Enzyme activity was determinate by monitoring the consumption of NADPH, by spectrophotometry at 37 °C and a wavelength of

340 nm (molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$), using cumene hydroperoxide (0.7 mM) as independent substrate.

3.2.3.2. GRed

GRed (EC 1.8.1.7) activity was determined, according the protocol based on spectrophotometry of(Carlberg & Mannervik, 1985). The quantification of the enzyme activity can be performed trough monitorization of the absorbance at 25 °C and 340 nm (molar extinction coefficient of 6.22 mM^{-1} cm^{-1}) when oxidation of NADPH mediated by GRed.

3.2.3.3. GST

GST (EC 2.5.1.18) activity was determined by the method described by (Habig et al., 1974), following the conjugation of the substrate 1-chloro-2, 4-dinitrobenzene (60 mM) with glutathione, catalysed by GST, forming a thioether (molar extinction coefficient of 9.6 mM^{-1} cm^{-1}) that can be assessed by spectrophotometry, following the increment of absorbance at 340 nm.

3.2.3.4. TBARs

The quantification of TBARs was achieved, according to the methodology described in (Correia et al., 2003), based on the reaction of lipid peroxidation by-products, such as malondialdehyde, with 2-thiobarbituric acid (1%). The amount of TBARS was determinate by spectrophotometry as a single determination at 535 nm (molar extinction coefficient of 1.56 9 105 M^{-1} cm^{-1}), and results were expressed in nmol per mg of sample protein.

3.2.4. Statistical analysis

In order to estimate mortality and correspondent LC_x values (with x = 10, 20, 50), with the corresponding 95% confidence intervals, a probit analysis (Finney, 1971) was applied, for both size classes of organisms of *S. plana*. One-way analysis of variance (ANOVA) was applied to the biochemical parameters, i.e. tGPx, GRed, GST and TBARs, to test significant differences among treatments. The Dunnett's multiple comparison test was further performed to determine the significant differences between each contaminated treatment and the control, for each substance. Moreover, two Tukey's tests were performed at the GRed and TBARs quantifications from terbuthylazine exposure. The used level of significance was of 0.05.

3.3. Results

3.3.1. Experimental bioassays

Table 1.
LC_{10}, LC_{20} and LC_{50} values (mg/L) of S-metolachlor and terbuthylazine to both size classes of *S. plana* with the respective 95% confidence limits (between brackets).

	S-metolachlor (mg/L)	terbuthylazine (mg/L)
	LC_{10}: 30.065 (20.011; 34.121)	LC_{10}: 46.284 (20.6; 70.039)
Big size	LC_{20}: 33.716 (26.91; 37.21)	LC_{20}: 71.105 (50.047; 91.289)
	LC_{50}: 40.702 (37.208; 46.021)	LC_{50}: 118.590 (97.638; 127.679)
	LC_{10}: 16.285 (5.434; 27.741)	LC_{10}: 35.988 (27.211; 63.241)
Small size	LC_{20}: 24.947 (11.237; 37.832)	LC_{20}: 60.852 (49.01; 82.938)
	LC_{50}: 41.517 (30.338; 52.941)	LC_{50}: 108.418 (86.763; 116.939)

The lethal concentration (LC) data of both size classes of *S. plana* reveals that this species is more sensitive to S-metolachlor (big - LC_{50}= 40.702 (37.208; 46.021) mg/L, small - LC_{50}= 41.517 (30.338; 52.941) mg/L) than to terbuthylazine (big - LC_{50}= 118.590 (97.638; 127.679) mg/L, small - LC_{50}=108.418 (86.763; 116.939) mg/L). In terms of size, generally, big size organisms are more tolerant to both chemicals except at LC_{50} of SMOC where small size organisms present a slightly higher value but practically the same (Table 1).

3.3.2. Enzymatic activity and lipid peroxidation

Table 2 presents the values of the enzymatic activities of total glutathione peroxidase (tGPx), glutathione reductase (GRed) and glutathione-S-transferase (GST), plus the quantifications of the TBARs, measured in both size classes of *S. plana*, comparing the situation at the field, after depuration and after the exposures to the concentrations of SMOC and TBA closer to the LC_{10}, that is, closer to the concentrations at which the mortality affected 10% of the organisms (big - 34.36 mg/L and small - 13.42 mg/L for SMOC, big – 40 mg/L and small – 57.6 mg/L for TBA).

After the period of depuration, all the enzymatic activities and the TBARs quantifications reduce when compared with values from field. tGPx's activities measured in organisms from bioassays of both substances also reduce, more evidently in small organisms, still at small size classes where registered slightly higher activities than at big size class; the differences between the chemical is minor, presenting SMOC a faintly higher activity. Oppositely, the levels of GRed activity increase after depuration under exposure to both chemicals, especially TBA, where the values increase by twice, reaching the levels from field in the case of big size class; under SMOC exposure, the highest value belongs to big size class as well. Big organisms from the field presented higher GST's activity than small organisms, while tGPx and GRed activities are lower. After the reduction produced by the depuration, GST's activity increases up to more than at field in big organisms under both exposures, whereas in small organisms the levels rise slightly, surpassing the values from field merely under SMOC presence. The quantifications of TBARs at the group from field were extremely upper than at the contaminated groups, including controls. Big organisms present the lowest amounts of TBARs, especially under TBA exposure, while small organisms show it low quantification under SMOC exposure. Quantifications at field, depuration and bioassays present higher values in small size class.

Table 2. Comparison of biomarker basal levels measured after field collection, after depuration and after exposure to the concentrations closer to the LC_{10} of S-metolachlor and terbuthylazine of both size classes of *S. plana* (mean ± SD). The values of tGPx, GRed and GST are expressed in nmol per min and mg of protein and the values of TBARs are expressed in nmol per mg of protein.

		Field	Depuration	S-metolachlor LC_{10}	terbuthylazine LC_{10}
Big size	tGPx	0.036 ± 0.009	0.028 ± 0.011	0.014 ± 0.004	0.012 ± 0.003
	GRed	0.243 ± 0.076	0.110 ± 0.013	0.192 ± 0.023	0.258 ± 0.018
	GST	2.639 ± 0.876	1.497 ± 0.25	3.214 ± 0.511	3.286 ± 0.279
	TBARs	0.165 ± 0.050	0.065 ± 0.011	0.032 ± 0.006	0.014 ± 0.003
Small size	tGPx	0.105 ± 0.011	0.059 ± 0.007	0.018 ± 0.008	0.016 ± 0.007
	GRed	0.336 ± 0.039	0.078 ± 0.022	0.137 ± 0.023	0.171 ± 0.017
	GST	2.175 ± 0.76	1.472 ± 0.388	2.734 ± 0.700	1.903 ± 0.375
	TBARs	0.853 ± 0.042	0.298 ± 0.023	0.055 ± 0.023	0.111 ± 0.034

The variations on the enzymatic activities of tGPx, GRed and GST and on the quantifications of TBARs through the range of concentrations of SMOC and of TBA are shown in Figure 2.

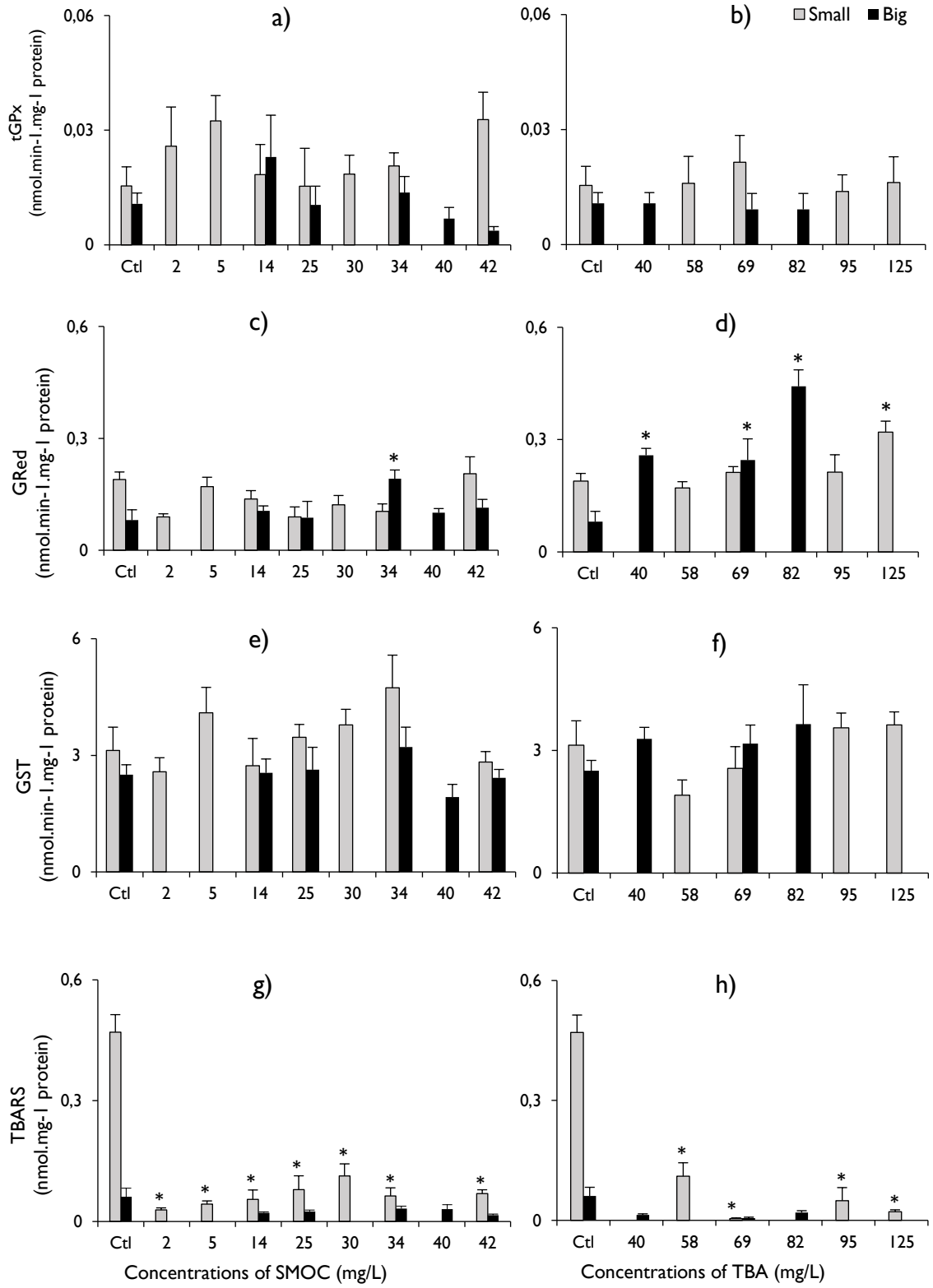

Figure 2. *Comparation of the values of the biochemical parameters at the controls (Ctl) and at the range of concentrations of SMOC (at left) and TBA (at right) in both size classes of S. plana, big (black bars) and small (grey bars). Mean and standard error of independent experiments are shown. Values with an asterisk on the top are statistically different from the control ($p<0.05$).*

The values of tGPx's activities determined through the rage of concentrations of SMOC are slightly higher than the control values for both size classes, especially for small organisms at first concentrations (Figure 2a). In the case of TBA, there are not remarkable differences in tGPx's activities, considering both size classes (Figure 2b). Considering GRed, the activities measured at SMOC's concentrations don't show significant changes in small size organisms, however, big size organisms rise significantly their activities at 34.36 mg/L comparatively with the control (Figure 2c). Exposure to TBA results in a gradual increase of the GRed's activity through growing concentrations in both size classes, more evidently in big size class (Figure 2d). The values from each concentration of TBA are all significantly higher than the control values, besides, the highest and the remaining concentrations are significantly superior between them (Figure 3a).

GST activity show increases in organisms under the exposure to SMOC, particularly in small size class despite without statistical significance (Figure 2e). The activities quantified at TBA's concentrations don't show any variations with statistical significance (Figure 2f), nonetheless, GST activities show gradual rises in a similar pattern as in GRed activities under the exposures to both chemicals, except small size class under SMOC exposure. Concerning TBARs quantifications, big size class don't show significant changes in its levels under both exposures, even it resembles a slightly decrease through concentrations when compared to the control. On the other hand, small organisms drastically decrease its TBARs under both exposures, being these reductions significantly low when compared to the control (Figure 2g and 2h); moreover, under TBA exposure, organisms from all concentrations present higher quantity of TBARs than the organisms from 69.3 mg/L, with statistical significance (Figure 3b).

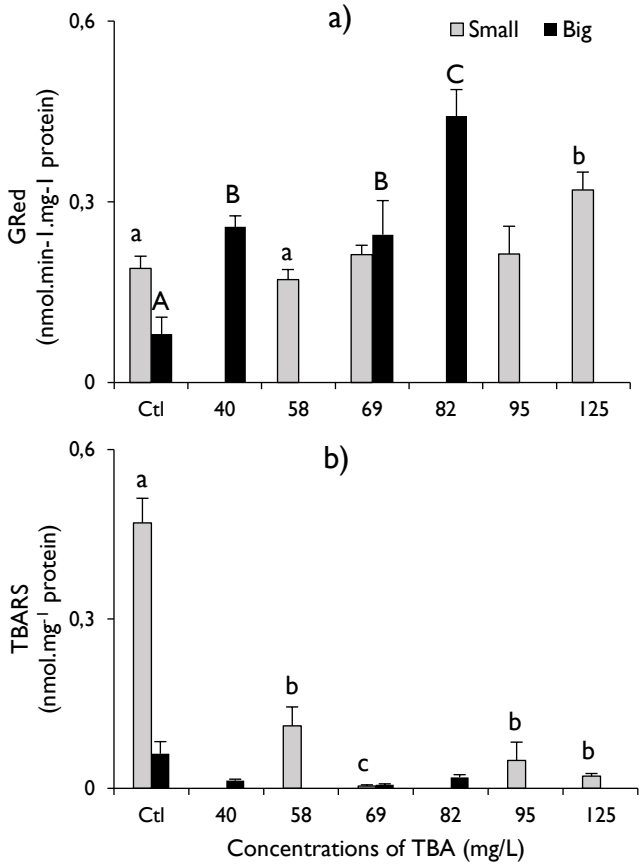

Figure 3. *Representation of the alterations of the biochemical parameters A) GRed and B) TBARs among the concentrations of the chemical TBA. Significant differences($p<0.05$) between all concentrations obtained by Tukey's tests are indicated by different letters on the top of the bars.*

3.4. Discussion

The main purpose of this work was to investigate the responses of *S. plana* when exposed to SMOC and TBA (main active ingredients of Primextra Gold® TZ), individually. Thus, the study comprises the determination of the lethal concentrations of each contaminant for the species and the analyses of biochemical biomarkers (enzymatic activities and lipid peroxidation), evaluating, at the same time, the suitability of these biomarkers to assess the effects of the selected chemicals in bivalve species in general and in *S. plana* in particular. Studying mortality as the biological endpoint, here is reported that *S. plana* is evidently more sensitive to SMOC than to TBA; furthermore, small size clams are, generally, more sensitive to TBA that big ones, agreeing with a previous experience in *S. plana* in which its tolerance to the herbicide was clearly higher in big size bivalves (Gonçalves et al., 2016), but at SMOC the trend is the opposite, coinciding with a different work that tests the effect of copper sulphate in *S. plana* and *Cerastoderma edule* (Mesquita et al., 2018). Therefore, as the size is related to the age of the organism, *S. plana* may be affected by pollutants exposures at different stages of life depending on the nature of the compound. The value of SMOC's lethal concentration here reported (big size LC_{50} = 40.702 mg/L; small size LC_{50} = 41.517 mg/L) is comparable with others found in literature for aquatic species, such as zebrafish (LC_{50} (96h) = 46.21 mg/L) (Quintaneiro et al., 2017), nonetheless, SMOC still shows higher toxicity to *S. plana*. There is a lack of information about bivalves' responses to SMOC, so far, a single study with Pacific oyster reports larval development impairments caused by the compound from a concentration of 10 ng/L (Gamain et al., 2017).

According to Gonçalves *et al.* (2016), the tolerance of *S. plana* to the herbicide Primextra Gold® TZ is (big size LC_{10} = 6.338 mg/L; small size LC_{10} = 2.206 mg/L), so, comparing with the present study, the tolerance of the species to the active ingredients, SMOC (big size LC_{10} = 30.065 mg/L; small size LC_{10} = 16.285 mg/L) and TBA (big size LC_{10} = 46.284 mg/L; small size LC_{10} = 35.988 mg/L), is higher than its tolerance to the commercial formulation. These results agree with Neves *et al.* (2015), whom concluded that Primextra has higher toxicity to *Daphnia longispina* than its active ingredient S-metolachlor. This event could be explained by a synergistic effect of both active ingredients here assessed in the herbicide formula, even because SMOC's toxicity to *S. plana*, which is much higher than TBA's toxicity, coincides with the fact that SMOC has a greater presence in the commercial formula, representing 30.2%(w.w.) of the total volume while TBA only represents 17.75%(w/w), so, almost half(Filimonova et al., 2016a; Neves et al., 2015). Nevertheless, further studies investigating the joint and separate effects of these substances and their underlying mechanisms of action are necessary to obtain confirmation. On the other hand, since most active ingredients are insoluble in water, there is a residual percentage of additionally solvents and potentiators in the composition of herbicides formulations such as Primextra Gold® TZ; these coadjuvant substances, supposedly inert, theoretically, may lead to changes on the magnitude of the effects of the active ingredients (Axelrad et al., 2002; Neves et al., 2015; Pereira et al., 2009).

Under natural conditions, animals maintain normal generation and neutralization of reactive oxygen species (ROS) in the ecosystem (Kumar et al., 2013), nonetheless, when exposed to xenobiotics, the biotransformation process leads to higher generation of free radicals and consequent cellular oxidative stress. ROS are highly toxic and, in excess, produce reactions with biological macromolecules that, eventually, cause oxidative stress, lipoperoxidation, DNA damage and protein oxidation (Tejada et al., 2007), resulting in disturbances in cell physiological processes in aquatic organisms (Cavalcante et al., 2010). Thus, enzymes that take part in the defence of cells against oxidative stress are argued to be good bio-indicators of stress due to their high sensitiveness. This work reports that tGPx activities

rise under SMOC exposure, indicating that a defence antioxidant mechanism is probably being activated. Moreover, under TBA exposure the activity remained unchanged and still values at the exposures are lower than at field. This enzyme is involved in the inhibition of ROS, reducing H_2O_2 to H_2O to ensure optimum protection against oxidative stress (Gaté et al., 1999) with oxidation of reduced glutathione (GSH). Hence, under exposure to contaminants, an induction of tGPx provides a warning of higher H_2O_2 production in the organisms, suggesting that oxidative stress can be imposed by the presence of the herbicides and the ROS produced may subsequently react with biomolecules resulting in oxidative damages to cellular components (Nwani et al., 2015).

GRed reduces oxide glutathione (GSSG) to GSH, which plays an important role in the GSH redox cycle, being essential a high GSH/GSSG ratio for protection against oxidative stress. Big size clams exposed to SMOC showed a significant rise in GRed activity at 34.36 mg/L, coincident with an increase in tGPX's activity, highlighting the importance of GSH renewal in the cells, due to its condition as co-factor by both enzymes (Cheung et al., 2004; Maria & Bebianno, 2011). GRed activity rise through growing concentrations of TBA concentrations and, evidencing its aptness as a biomarker to detect stress produced specifically by this chemical in *S. plana*, moreover, its values under chemical contamination surpass the values from field, thus, the detection of activities upper than the field values may be interpreted as a marker of lethal effects. This particular pattern suggests that the higher the concentration of the chemical was GSH is more required, perhaps due to the electrophilic metabolites of the compounds that under GST mediation, they conjugate with GSH for its detoxification. Further exposure assays are necessary to confirm this hypothesis.

GST is a biotransformation enzyme involved in the degradation of organic contaminants (Fossi Tankoua et al., 2013). This study reports that the enzyme is generally activated in a similar pattern as in GRed's activity. GST catalyse reactions of certain compounds, with GSH addition, forming thioethers (Habig et al., 1974), hence, the similitudes in their responses denote the importance of the coordinated regulation of those enzymes in maintaining the equilibrium between the cellular GSH and GSSG levels (Silva et al., 2012). This pattern is less clear in small size under SMOC exposure, still the activity of both size organisms is higher under SMOC exposure than in field. Besides, the activity of the enzyme in big size is activated trough growing concentrations, hence, GST rise of activity is related to SMOC toxicity, thus, GST resembles to be a good biomarker for impacts of SMOC in *S. plana*.

According to Solé et al., (2009), the use of biomarkers in bivalves could be influenced by physiological stage, sex, age and physical characteristics in the environment, so, size class could be included as a direct consequence of age, revealing that big clams are more suitable when using antioxidant enzymes. The presented results are in accordance with Pérez et al., (2004) that analysed GST after chemical exposures in *S. plana*, and with Fossi Tankoua et al., (2013) that reported GST higher activities in a contaminate estuary, linking that to impairments in behavioural and physiological biomarkers. Likely, during bioassays, the behavioural conditions concerned the enzymes' activities quantifications, as at the most elevated concentrations of SMOC, the clams responded by closing their valves most part of the time, reducing their filter activity and, eventually, their chemical responses. So, the biomarker levels at these concentrations should be interpreted with some reserve, giving more credibility to the results from the lasting concentrations.

By measuring thiobarbituric reactive acid substances it is possible to reveal whether the defence of antioxidant enzymes has been overwhelmed (Bertrand et al., 2016). The results showed a decrease or maintenance in TBARs quantifications under both exposures when compared with the control, so there were no evidences of lipid peroxidation. This situation could be due to the activities of the antioxidant enzymes that were enough to counteract the free redox cycling metabolites and to neutralize the oxidative stress produced by the presence

of the contaminants. The reported results agree with Almeida et al., (2017), whom exposed *S. plana*, sampled from a polluted site, to carbamazepine and they associated the decreases in lipid peroxidation levels with an adaptation of the clams to laboratorial conditions through time, suggesting that the habitat background of organisms could change their responses to contaminants, which make sense, especially if, as in this case, they were pre-exposed to the compounds at the local of sampling. Nowadays, this idea is poorly documented, and it represents an interesting topic for further investigation in the future. As well, as inhibition of lipid metabolism and synthesis is the main target of chloroacetamide herbicides such SMOC, it may produce lower lipid contents and, ultimately, lower levels of lipid peroxidation (Quintaneiro et al., 2017). The mechanism of action of TBA in animal cell has not yet fully elucidated (Velisek et al., 2017), although non-polar narcosis (non-specific toxicity) has been proposed (Bermúdez-Saldaña et al., 2005), consisting of an accumulation of hydrophobic chemicals within cell membranes disrupting interactions and impairing ions flux. Moreover, in carp early stages terbuthylazine-desethyl, a metabolite of TBA, diffused steatosis in the liver (J. Velisek et al., 2016), which plays a key role in lipid metabolism, increasing lipid inclusions in the hepatocytes; thus, to increase the synthesis or to reduce the release of lipoid substances may be a defence mechanism against chronic exposure. All this information indicates that the lipid content could be affected under exposure to SMOC and TBA, with a possible alteration at the fatty acid profiles. Therefore, in order to better understand the mechanism under toxic effect of these herbicides, fatty acid profiles are performed at the present work (Chapter II).

The biomarker levels measured in clams directly from the estuary are merely guidance for better understanding of the responses of the biochemical parameters, it must be taken into consideration that the biomarker approach is a simplification of a very complex exposure situation prevailing in study area. Measurement of biomarkers in field is still limited by temporal and spatial variability of biochemical responses, as well as by confounding factors, including temperature, salinity, season, sex, age, spawning period and reproductive cycle (Fossi Tankoua et al., 2013; Gamain et al., 2017; Silva et al., 2012). The differences observed between the organisms from the field and the organisms from the control treatment under laboratory conditions may be related to these confounding factors. Moreover, the site is not affected only by the presence of SMOC and TBA as single contaminants but by the mixture of both in the composition of Primextra® Gold TZ joint to several other chemicals present in the system. As well, organisms that inhabit areas of continuous stress conditions to compensate the presence of toxicants in non-optimal concentrations, frequently develop defence systems that includes enzymatic and non-enzymatic systems, whose function is to keep ROS at metabolically innocuous levels, thus preventing oxidative damage (Van der Oost et al., 2003). Enzymes, because of the non-specificity intrinsic to this kind of biomarkers does not allow identifying precisely the origin of their responses (Bergayou et al., 2009), thus, they are not appropriate to represent the actual situation at the environment. Besides, oxidative stress has been described as a highly seasonal phenomenon in bivalve mollusc (Bergayou et al., 2009), which is a factor not considered in this case. On the other hand, under controlled laboratorial conditions, antioxidant enzymes are considered as good biomarkers of the presence of chemical contaminants, so, this type of studies are very important to ascertain suitable biomarkers to use with certain contaminants in aquatic environments. The sediment quality of the natural environment may results impoverished by the low oxygen quantities associated to the presence of organic pollutants in water, leading to production of radicals and subsequent oxidative stress responses in field. *S. plana* as a deposit-feeder species, is exposed to the bioavailable fraction of sediment-bound contaminants, taking the risk of oxidative stress, however, this does not seem to be the case of the Mondego estuary according to Rodrigues et al., (2016), whom did not found pesticides in the sediments. However, they found 44 ng g^{-1} dw of TBA in *S. plana* organisms from the estuary, which may suggest that they could be

accumulating the pollutant under long-time exposures due to the elevated water persistence of the chemical. More information is required, even because there is not current regulation that establish the limit concentrations of SMOC and TBA in shellfish or water, whereas this situation may cause hazardous effects on bivalves and pose potential human health risk, since *S. plana* is edible and considered of economic interest, as many other species frequently captured and produced at the estuary for its consume.

According to the literature, the amounts of metolachlor and terbuthylazine detected in Portuguese surface waters are 0.056 mg/L and 0.0003 mg/L, respectively (Cerejeira et al., 2003; Palma et al., 2014; Silva et al., 2012). Although low, these values exceed the European threshold (0.0001 mg/L) of individual pesticides in water for human consumption by the Directive 98/83/EC (ECC, 1998). The ranges used to the bioassays are much higher, comparable to a catastrophic contamination event or sporadic herbicide massive application. Therefore, it must be concluded that previously mentioned levels at the environment, are safe. Nevertheless, in the Mediterranean region, waters are generally further contaminated by herbicides during dry months and soon after applications, taking the risk to rise until dangerous concentrations in these periods (Carabias-Martínez et al., 2003; Herrero-Hernández et al., 2013; Vryzas et al., 2009). In view of persistent applications of Primextra® Gold TZ and other products for plant protection or production optimization in farmlands, the concentration of its active ingredients may be high in most rivers, pools and other aquatic environments impaired by humans (Nwani et al., 2015); thus, this kind of ecotoxicological studies must continue elucidate the responses that organic pollutants can produce in non-target species and the responsible mechanisms behind.

3.5. Conclusion

In summary, this chapter reports that *S. plana* is a good bioindicator species to assess the impacts of SMOC and TBA in aquatic systems. The stated lethal concentrations of the active ingredients are accurate and can be used as support to reports about the toxicity of the chemicals. According to the results of enzymatic activity, GRed and GST revealed to be suitable biomarkers of lethal effects of the studied substances in *S. plana*. The specificity of GRed as biomarker for TBA impacts must be accentuate because of the statistical support at all the rises of activity when compared to the control, against the rises under SMOC exposure, which only obtained statistical significance at 34.36 mg/L. GST demonstrated to be more accurate biomarker for SMOC toxicity as it rises it activity under lower concentrations than of TBA. Now that we know that these biomarkers respond to toxic concentrations of the herbicides, further investigation should assess their potential as early warning indicators in order to detect dangerous concentrations before the appearance of lethal effects, allowing the implementation of solutions or mitigation measures in order to rehabilitate the environment before the damages turn irreversible.

Chapter II: Fatty acid profiling and carbohydrates total quantification to assess the impacts of S-metolachlor and terbuthylazine on *Scrobicularia plana*

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4.1. Introduction

Estuaries are complex systems that are commonly surrounded by farmland, residential and industrial areas, being under constant menace of anthropogenic pressures. Intensive agriculture practices are typical in this type of environments, leading to an overuse of fertilizers and pesticides, especially during the last 30 years. Eventually, the products employed for plant protection or production optimization outcomes in residues in non-target organisms, by aerial spraying, leaching, runoff, accidental spills or human faults during application (Vidal et al., 2012). Around the Mondego estuary, located near Figueira da Foz (Portugal), there are 15,000 ha of cultivated land, producing mainly rice and corn, which represents a risk of organic contamination. This can produce impacts from an ecological perspective by compromising the water quality and the aquatic communities, and even from an economic and health perspective since there are species being produced for human consumption that might be affected.

Bivalves are considered standard organisms for ecotoxicological studies because of their easy handling and maintenance at laboratory. The estuarine bivalve *Scrobicularia plana* (da Costa, 1778), commonly known by peppery furrow shell, is able to filtrate organic material, playing an important role on the estuaries' functioning and on the trophic chain since it consumes small organisms and serves to feed bigger species. Besides, it is amply produced at the Mondego estuary as an important food resource so it owns economic interest as edible species (Fossi Tankoua et al., 2013; Gonçalves et al., 2016, 2017, Verdelhos et al., 2014, 2015). For all these reasons, *S. plana* was chosen to carry out bioassay with the purpose of determinate the lethal concentrations of the herbicides S-metolachlor (SMOC) and terbuthylazine (TBA) and analyse their effects at the molecular level.

SMOC and TBA are the main active ingredients of Primextra® Gold TZ, the most-used herbicide in the estuary under study according to the agricultural cooperatives of the Mondego valley, making SMOC 30% (w/w) of the total volume of the commercial formulation and TBA 18% (w/w) (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015). These active ingredients also appear in other pesticides' commercial formulations used worldwide. In terms of risk assessment, it is important to study their effects and modes of action, separately, as well as joint at the commercial mixtures. S-metolachlor (SMOC) is the active principle of many herbicides authorized by most of the countries in the European Union to control grass. Metolachlor belongs to the cloroacetamides, known to interfere with fatty acid (FA) synthesis (Robert et al., 2007) by the inhibition of the elongase of elongation of very long-chain fatty acids (VLCFAs) synthesis, inhibiting the expression of FAE1 gene (Neves et al., 2015). Terbuthylazine (TBA) belongs to the triazine family and acts as a photosynthesis inhibitor in plants, but the mechanism of action in animal cells is yet undescribed (Josef Velisek et al., 2017), but some proposes point that it may cause alterations on lipid composition of cell membranes (Bermúdez-Saldaña et al., 2005). Besides, triazine herbicides has shown to produce superiority in numbers of lipid resorptive cells (De Hoop et al., 2013). Hence, these compounds are related to potential changes on lipid contents and, to understand its effects, it's conceivable to look for alterations of fatty acid profiles. Lipids are considered sensitive indicators of stress and disturbances, alterations on its distribution and concentrations can be an useful information to understand the processes by which the toxicants produce their effects (Arts et al., 2009). Thus, many recent studies used fatty acid profiles to assess the effects that different stressors cause in organisms (Filimonova et al., 2016a, 2016b, Gonçalves et al., 2012, 2016, 2017; Mesquita et al., 2018; Neves et al., 2015).

Fatty acids (FA) are important for several biological processes, being key constituents of phospholipid cell membranes to confer some specific properties, such as permeability and the traffic of cell compounds (Ibarguren & López, 2014). The group of the highly unsaturated fatty acids (HUFA), including eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), are essential metabolites that cannot be synthesized *de novo*, at least not in sufficient amounts, being taken up through food sources (Ladhar et al., 2014). Polyunsaturated fatty acids (PUFA), where are included some of the essential fatty acids (EFA) and some omega 6 and omega 3, are a family of lipids that contains some subgroups identified by the position of the last double bond in their structure. PUFAs are mainly synthesized by plants but not by humans, and only a few animals are able to convert them through elongation or desaturation of other FA with a low ratio between production and consumption, thus, animals obtain these groups of FA mainly from food sources, especially fish (Brett & Muller-Navarra, 1997). PUFAs are precursors of important hormones and are present in neural system, being involved in cell signalling processes and playing a role on brains development (J. J. Liu et al., 2015). Bivalves are rich in PUFAs and are strongly integrated on the Mediterranean diet, characteristic of the South of Europe. Besides, several epidemiological studies show that a diet enriched with omega-3 and omega-6 is highly recommended and it is related with prevention of cardiovascular, inflammatory, ocular and neurological diseases, improvements on cognitive and nervous system development, among other advantages (Filimonova et al., 2016b). *S. plana* commonly has essential fatty acids on high concentrations and its occasional consumption can lead to health benefits. Nonetheless, the exposure of this species to stressors can affect its fatty acid contents and, consequently, compromise its nutritional value (Gonçalves et al., 2016; Mesquita et al., 2018; Verdelhos et al., 2015).

Carbohydrates are the main and immediate energy sources to cope with stress, thus their metabolism may play an important role in the maintenance of cell homeostasis in bivalves during processes of immune defence. Theoretically, the presence of organic pollutants may impose high energetic costs for immune regulation resulting in an enhance of carbohydrate metabolism including glucose degradation, glycogenolysis, and gluconeogenesis (Lochmiller & Deerenberg, 2000). Hence, a quantification of the total carbohydrates content may offer relevant information about the biochemical effects of toxicants.

Therefore, the aims of the present study are: to investigate (1) the FA profiles and (2) the total carbohydrate content of two size classes of *S. plana* after exposition to S-metolachlor and terbuthylazine, separately, and (3) the nutritious quality of *S. plana* at the field and after the exposures under laboratory conditions.

4.2. Materials and methods

The procedures at the laboratory for preparation of the bioassays ran as described in Chapter I, 3.2. Materials and methods, 3.2.2. Laboratory and bioassays procedures.

4.2.1. Fatty acid profiling

Fatty acid analyses were performed on isolated muscle and visceral mass tissues from each survived organism. The extraction of total lipids and fatty acid methyl esters (FAMEs) was achieved by the method described in (Gonçalves et al., 2012). The internal standard, methyl nonadecanoate (C19:0, Fluka 74208) was added to all samples allowing the quantification of the methyl esters. Samples were centrifuged until obtention of the supernatants that were collected into vials to be stored at -80°C until quantification.

Separation and quantifications of FAMEs were performed through gas chromatography coupled with mass spectrometry (GC-MS), using an Agilent Technologies 6890N Network (Santa Clara, CA). This equipment was equipped with a 0.25 mm internal diameter, 0.1 µm film thickness and 30 m long DB-FFAP column. The injector port was lined with a splitless glass liner of 4.0 mm i.d. An Agilent 5973 Network Mass Selective Detector at 70 eV electron

impact mode and scanning the *m/z* range of 40-500 in 1 s cycle in full scan mode acquisition was used. The initial oven temperature was 80 °C, following a linear temperature increase of 25 °C min^{-1} to 160 °C, followed another temperature ramp of 2 °C min^{-1} to 190 °C and finally an increase of 40 °C min^{-1} until a final temperature of 230 °C which was maintained for 5 min. Helium was the carrier gas, at a flow rate of 4.4 mL min^{-1} and 2.66 psi of column head pressure. The detector starts operating 4 min after injection, corresponding to solvent delay. The injector and transfer line were maintained ate 220 °C and 280 °C, respectively. The output of the equipment's software is a chromatogram (Figure 1) where the integration of FAME peaks can be carried out using the software and the identification of each peak was done by retention time and mass spectrum of each FAME, in comparation with the Supelco ® 37 component FAME mix (Sigma-Aldrich, Steinheim, Germany) and mass spectra from the library (Willey). The calculation of the FAMEs content was performed as described in (Gonçalves et al., 2012).

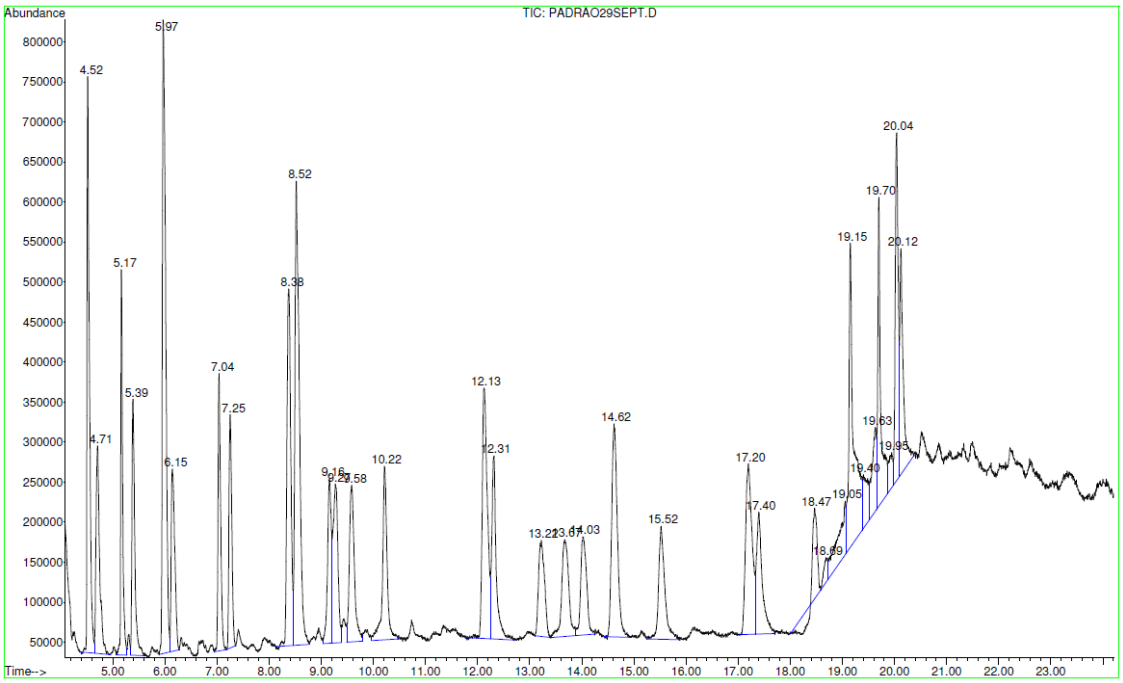

Figure 1. Chromatogram representing all the FAME peaks identifiable in a sample.

4.2.2. Fatty acid trophic markers

In order to ascertain the maintenance of bacterial, algae or animal ratios at lipids extracts, FA ratios were determined and applied (Ezgeta-Balić et al., 2012). Different food sources are rich in different fatty acids, with bivalves typically feeding on animals diets showing a raise at the quantities of oleic acid (C18:1n9) and linoleic acid (C18:2n6), since these fatty acids occur in zooplankton organisms (Zhukova & Kharlamenko, 1999). Another ratio to determine the food preferences of bivalve is DHA/EPA (Mansour et al., 1999). DHA is an omega 3 HUFA extremely important throughout life, with enormous benefits to the health of all organisms, with this ratio reflecting the proportion of zooplankton and diatoms/dinoflagellates in the bivalves' diet. DHA is often dominant in zooplankton and dinoflagellates (Kharlamenko et al., 2001; Mansour et al., 1999; Parrish, 1998; Zhukova & Kharlamenko, 1999), whereas EPA is found mainly in diatoms (Dunstan et al., 1993; Parrish, 1998). The proportion of all diatom markers (D = 16PUFA + 16:1n-7 + 20:5n-3) to all flagellate markers (F = 18PUFA + 18:2n-6+22:6n-3), D/F, was also used to distinguish between diatom and dinoflagellate-based diet (El-Sabaawi et al., 2009). A high proportion of C15:0 and C17:0 denotes the presence of bacterial species on bivalves diet (Mayzaud et al.,

1989), since bacteria biosynthesis large amounts of iso and ante-iso branched chains containing 15–17 carbons (Gonçalves et al., 2012).

4.2.3. Carbohydrates

The carbohydrates from tissue were obtained from the recollection of the remaining liquid after the removal of the supernatants from centrifugation in the above-mentioned protocol for fatty acid extraction. The quantification of total carbohydrates was performed by the phenol-sulfuric acid colorimetric method (Dubois et al., 1956), using 1 mL of 5% phenol in concentrated sulphuric acid for 80 mL of sample and heating for 10 min at 100 °C. The absorbances of the samples were determined at 490 nm using a microplate reader (Biotek). D-glucose was used to build a calibration curve. Results were expressed in mg of glucose equivalent per g of the biomass of *S. plana*.

The sugar analysis of the samples was performed to determine the composition in monosaccharides. Neutral sugars (NS) were converted to their alditol acetates, following the method of (Coimbra et al., 1994), and analysed by running through a Perkin-Elmer – Clarus 400 gas chromatography equipment, equipped with a flame ionization detector (GC-FID). A DB-225 (30m length, 0.25mm i.d., 0.15 µm film thickness) GC column was used and oven was programmed to an initial temperature of 200°C, following a linear temperature increase at 40°C min-1 to 220°C, this temperature was maintained for 7 min, after which followed another linear increase of 20°C min-1 to the final temperature of 230°C, maintaining this temperature for 1 min. The carrier gas was Hydrogen, at a flow rate of 1.7 mL min-1. Quantification of sugars was obtained by comparison of the sugar chromatographic peaks to the peaks obtained for the standard used (2-desoxiglucose).

4.2.4. Statistical analyses

Multivariate statistical analyses were carried out using PRIMER 5 software to study the variation at FA profiles via non-metric multidimensional (2D) scaling (n-MDS) plots, using the data converted into similarity triangular matrices through Bray Curtis resemblance measures. A one-way analysis of similarity (ANOSIM) was performed for each chemical to assess the grade of differences of FA contents between samples of both types of tissue and size classes. To analyse the contribution of individual FAs to similarities and dissimilarities within and between sample groups was performed a similarity percentage (SIMPER) analysis routine for each compound.

Sugar data are expressed as means with standard error of mean (S.E.M.). A one-way analysis of variance (ANOVA) was done to evaluate the results followed by Tukey's tests to compare results between groups. Differences were considered significant when the *p* value was lower than 0.05.

4.3. Results

4.3.1. Fatty acid profiles

Over the range of concentrations of both chemicals, equal profiles of FA are detected in both muscles and visceral masses meaning that same FAs are present in both types of tissue thought in different amounts, but the abundances are generally higher in muscles. The most abundant FA is DHA for all size classes and types of tissue, followed by C20:1, C16:0 and EPA (Annexe I).

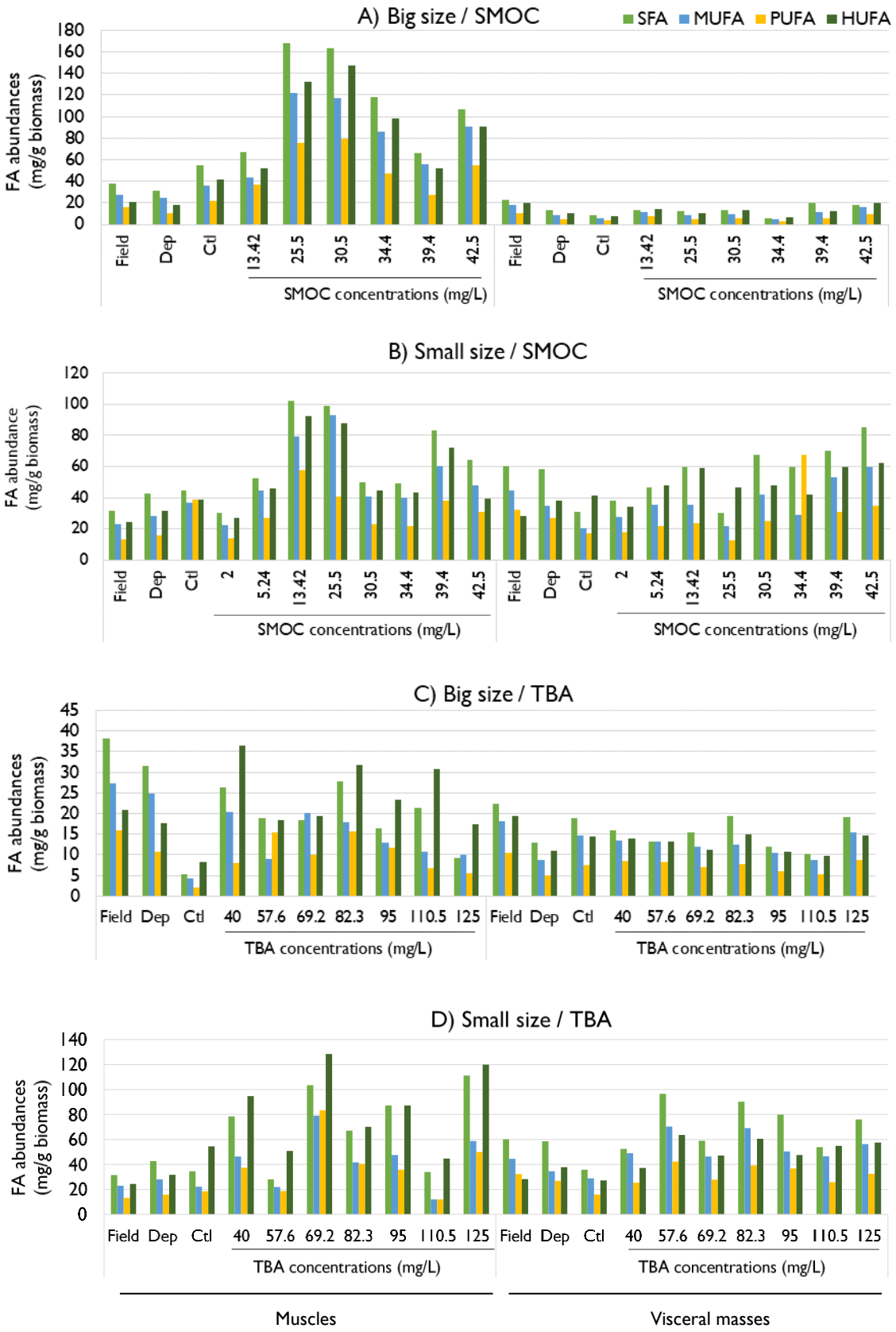

Figure2. Clustered column charts representing changes in total SFA, MUFA, PUFA and HUFA (in mg per g of biomass), for big (A) and small (B) size classes at SMOC exposures, and from big (C) and small (D) size classes at TBA exposures. Amounts of FA in organisms from the field (Field) and after depuration (Dep) and at the control treatments (Ctl) for big (A and C) and small (B and D) are also shown.

Big organisms from field have slightly higher FA contents at the muscle than at the rest of the visceral mass. After the period of depuration, it is observed a decrease of all the FA groups, appreciable at both types of tissue but acuter at visceral masses (42% SFA, 52% MUFA, 53% PUFA and 44% HUFA) than at muscles (17% SFA, 10% MUFA, 32% PUFA and 15% HUFA) (Fig. 1A and 1B). At the different concentrations of SMOC, most of the FA accumulate in the muscles, increasing up to more than twice at 42.5 mg/L when compared to the amounts at the field and at 30.5 mg/L. All groups experience a high peak, especially SFA and HUFA; while in the visceral masses the quantities are low and close to the values from the field (Figure 2A). On the other hand, at TBA bioassays, the content in SFA of muscles decrease by half, MUFA and PUFA by around 30% and HUFA by 6.64% at 69.2 mg/L when compared with field. However, HUFA shows high peaks at 40 mg/L, 82.3 and 110.5 mg/L where SFA presents its most elevated abundances as well. In visceral masses, the pattern is similar as at SMOC exposure, maintaining low amounts of FA close to the levels of field (Figure 2C). At highest concentrations, the abundance generally decreases, which is explained by the fact that organisms diminish their valvar activity and staying closed most of the time hindering from feeding.

FA content of small organisms from field are lower in muscles than in visceral masses, contrarily to the trend observed in big organisms. After the depuration, SFA, MUFA and PUFA amounts reduce in visceral masses by 3%, 23% and 17%, respectively, but at muscles they rise by 35%, 22% and 20%, respectively. On the other hand, HUFA increases in both, 30% at muscle and 34% at visceral mass (Figure 2B and 2D). For SMOC exposure, all FA generally increase at both types of tissue. In muscles, SFA, MUFA and PUFA increase up to twice and HUFA by 62% at the concentration of 42.5 mg/L, presenting peaks at 13%, 26% and 39.4 mg/L. In visceral masses, the quantifications values increase as well, SFA by 41%, MUFA by 34%, PUFA by 7% and HUFA by more than twice at 42.5 mg/L but maintain close to the levels at the field (Figure 2C). At TBA bioassays, in muscles, SFA and PUFA triplicate, MUFA rises by more than twice and HUFA increases 5 times at 125 mg/L, showing peaks at 40 and 69.2 mg/L. In visceral masses, SFA and MUFA increase by around 26% and HUFA up to twice (Figure 2D).

4.3.2. Multivariate analysis

In order to establish the separation among groups, a criterion based on R value was followed that considers groups as minimally separable ($R < 0.25$), overlapped but clearly different ($R > 0.5$) or completely separated ($R > 0.75$). Table 1 shown the results of the SIMPER analyse and the MDS groups are described by abbreviation of type of tissue (muscle (M) and visceral mass(V)) and size class (big (b) and small (s)).

For all cases, the type of tissue drives the formation of groups at concentrations, especially for TBA exposure, when compared with samples from field and from depuration, where the division by type of tissue is not so clear (Figure 3). Samples from SMOC exposure are divided into three groups ($R = 0.47$, $p = 0.001$). S1 and S2 include visceral masses and S3 includes muscles, being DHA the main contributor to the segregation (Table 1) which is explained by the fact that muscles samples have higher amount of DHA per gram than visceral masses samples (Annexe I, Tables 2 and 3). S1 and S2 ($R=0.745$, $p = 0.001$) split visceral masses by size classes (Figure 3) with DHA, C20:1, and C16:0 as the principal contributors, probably because they are more abundant in small size organisms (Annexe I, Table 2 and 3). In muscles (see group S3), only DHA follows the same pattern, and the low dissimilarity ($R=0.076$, $p = 0,143$) achieved does not allow to split S3 in two groups ruled by size classes.

On the other hand, samples from TBA exposure are divided in four groups ($R = 0.753$, $p = 0.001$) according to the type of tissue and the size class. T1 and T2 include muscle samples and T3 and T4 comprise visceral mass samples (Figure 3). The segregation into size classes is more evident in this case than at SMOC exposure, especially between T3 and T4 ($R = 0.983$, $p = 0.001$), representing DHA the major contribution (Table 2). Small size class present a higher DHA abundance than big size class (Annexe I, Table 4 and 5).

Table 1. SIMPER analyses average dissimilarity among groups of samples from S-metolachlor assays. For each FA, averages of abundances (Av. Abund), similarity (Av. Sim) and dissimilarity (Av. Diss) are shown with its standard deviations (Sim/SD and Diss/SD), plus the contributed percentage (Contrib%) and the cumulative contribution (Cum%).

MDS Groups	*Similarity*	*Fatty Acids*	*Av. Abund*		*Av. Sim*	*Sim/SD*	*Contrib%*	*Cum.%*
Mb	62.28	DHA	51.48		11.29	2.56	18.12	18.12
		C16:0	22.80		6.63	3.20	10.65	28.78
		C20:1	26.63		6.16	2.96	9.89	38.67
Ms	72.17	DHA	34.76		14.16	4.32	19.62	19.62
		C16:0	16.20		7.37	4.58	10.21	29.82
		C20:1	17.16		7.02	4.01	9.72	39.54
Vb	70.71	DHA	12.5		12.64	4.00	17.87	17.87
		EPA	8.07		8.40	4.58	11.88	29.76
		C16:0	7.37		8.30	3.77	11.74	41.50
Vs	69.75	DHA	53.75		12.72	3.06	18.23	18.23
		C20:1	32.12		8.16	2.99	11.71	29.94
		C16:0	28.47		7.79	3.19	11.17	41.11
	Dissimilarity		*Av. Abund*		*Av. Diss*	*Diss/SD*		
Mb/Ms	34.21	DHA	51.48	34.76	6.57	1.72	19.21	19.21
		C20:1	26.63	17.16	3.2	1.59	9.35	28.55
		C20:0	21.51	11.55	2.87	1.60	8.39	36.94
Mb/Vb	55.9	DHA	51.48	12.50	10.13	2.00	18.13	18.13
		C18:0	22.01	3.92	5.00	3.11	8.94	27.07
		C20:1	26.63	8.01	4.95	1.86	8.86	35.93
Mb/Vs	35.95	DHA	51.48	53.75	6.40	1.49	17.80	17.80
		C20:1	26.63	32.12	3.46	1.42	9.63	27.44
		EPA	21.06	26.76	3.00	1.41	8.35	35.79
Ms/Vs	37.91	DHA	34.76	53.75	6.18	1.41	16.30	16.30
		C20:1	17.16	32.12	3.91	1.60	10.32	26.62
		EPA	13.55	26.76	3.31	1.60	8.73	35.35
Vb/Vs	57.95	DHA	12.50	53.75	10.85	1.97	18.72	18.72
		C20:1	8.01	32.12	6.61	2.19	11.41	30.14
		C16:0	7.37	28.47	5.99	2.00	10.33	40.47

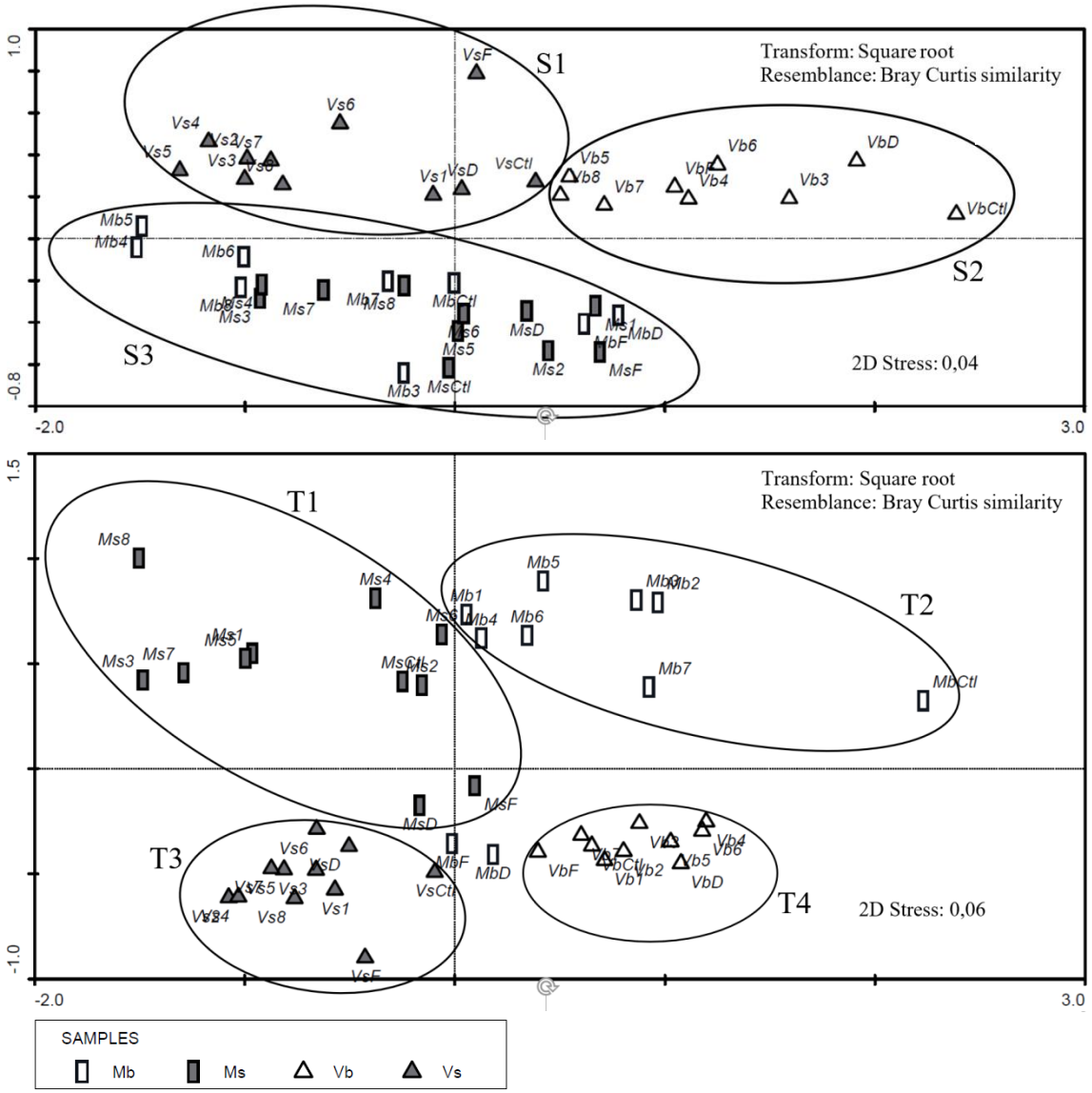

Figure3. *Two-dimensional non-metric MDS ordinations plots of FA profiles of both visceral masses and muscles from both size classes of S. plana, representing S-metolachlor at the top plot and terbuthylazine at the bottom plot. S1, S2 and S3 represent distinct groups at the plot for S-metolachlor and T1, T2, T3 and T4 represent distinct groups at the MDS plot for terbuthylazine. White symbols represent the big size class (b) of the bivalves and grey symbols represent the small size class (s). Samples of muscles (M); Samples of visceral masses (V); Mb – muscle big size class; Ms – muscle small size class; Vb – visceral mass big size class; Vs – visceral mass small size class; F – samples from field; D – samples from depuration; Ctl – samples exposed to control treatment; numbers – samples exposed to concentrations (SMOC: 1 - 2.048 mg/L, 2 - 5.24 mg/L, 3 - 13.42 mg/L, 4 - 25.46 mg/L, 5 - 30.5 mg/L, 6 - 34.36 mg/L, 7 - 39.40 mg/L, 8 - 42.5 mg/L; TBA: 1- 40 mg/L, 2 - 57.6 mg/L, 3 - 69.2 mg/L, 4 - 82.3 mg/L, 5 - 95 mg/L, 6 - 110,5 mg/L, 7 - 125 mg/L, 8 - 138 mg/L).*

Table 2. SIMPER analyses average dissimilarity among groups of samples from terbuthylazine assays. For each FA, averages of abundances (Av. Abund), similarity (Av. Sim) and dissimilarity (Av. Diss) are shown with its standard deviations (Sim/SD and Diss/SD), plus the contributed percentage (Contrib%) and the cumulative contribution (Cum%).

MDS Groups	*Similarity*	*Fatty Acids*	*Av. Abund*		*Av. Sim*	*Sim/SD*	*Contrib%*	*Cum.%*
Mb	57.16	DHA	17.8		18.87	2.84	33.01	33.01
		C23:0	5.62		5.57	2.23	9.75	42.75
		C22:2	5.44		4.91	1.45	8.59	51.34
Ms	62.14	DHA	68.55		20.36	2.77	32.76	32.76
		C22:2	23.10		6.18	2.71	9.94	42.70
		C23:0	24.60		5.86	2.13	9.44	52.14
Vb	82.53	DHA	7.97		13.92	9.12	16.86	16.86
		C20:1	5.93		10.17	4.54	12.32	29.19
		EPA	4.84		9.56	8.20	11.59	40.77
Vs	78.59	DHA	31.20		13.24	5.19	16.85	16.85
		C20:1	27.13		11.04	3.61	14.05	30.90
		C16:0	23.05		9.09	4.18	11.56	42.46
	Dissimilarity		*Av. Abund*		*Av. Diss*	*Diss/SD*		
Mb/Ms	54.67	DHA	17.8	68.55	16.39	1.95	29.99	29.99
		C23:0	5.62	24.60	5.72	1.40	10.46	40.44
		C24:0	4.46	19.70	5.60	1.72	10.24	50.69
Mb/Vb	48.85	DHA	17.90	7.97	9.15	1.47	18.73	18.73
		C22:2	5.44	0.55	4.24	1.41	8.68	27.41
		C16:0	3.96	5.24	4.07	2.02	8.34	35.75
Ms/Vb	61.38	C20:1	3.81	27.13	8.99	2.93	14.87	14.87
		C16:0	3.96	23.05	7.45	2.10	12.32	27.18
		DHA	17.8	31.20	6.11	1.58	10.10	37.29
Ms/Vs	49.65	DHA	68.55	31.20	8.74	1.36	17.60	17.60
		C20:1	9.44	27.13	4.72	1.80	9.50	27.09
		C22:2	23.10	2.75	4.45	1.66	8.96	36.06
Vb/Vs	59.57	DHA	7.97	31.20	9.48	3.15	15.91	15.91
		C20:1	5.93	27.13	8.55	3.05	14.35	30.26
		C16:0	5.24	23.05	7.20	2.36	12.09	42.35

4.3.3. Fatty acid trophic markers

Figure 4 shows the FA composition of rotifers and microalgae food mixture that was used to feed the organisms daily during the bioassays. Microalgae is mainly constituted by PUFA (≈87%) with small amount of SFA (≈8%), HUFA (≈3%) and MUFA (≈3%), whereas rotifers are mainly composed by SFA (≈63%) and MUFA (23%) plus a minor quantity of PUFA (9%) and HUFA (≈5%).

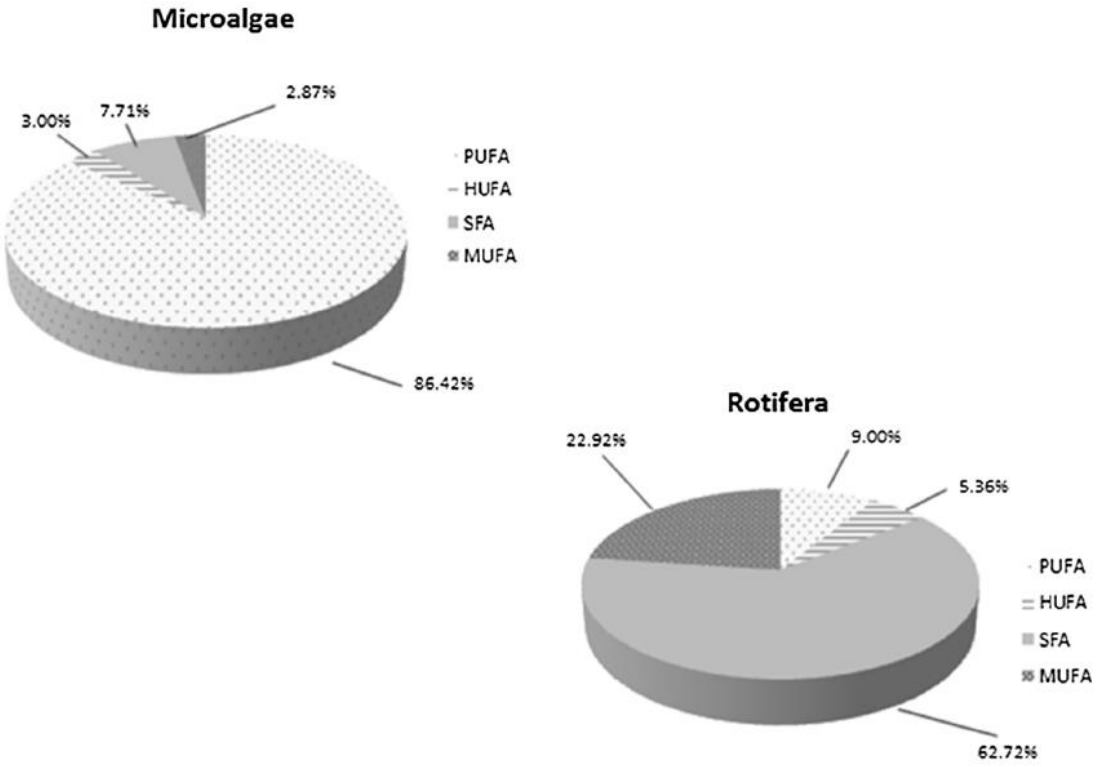

Figure4 *Fatty acid composition of food sources (microalgae and rotifera).*

Table 3 shows the concentrations of fatty acid trophic markers (FATMs) of organisms from field and from the exposures to the concentrations around LC_{10} values of SMOC (30.5 mg/L for big size and 13.42 mg/L for small size) and TBA (40 mg/L).

Table 3. Comparison of Fatty Acid Trophic Markers (FATMs) of muscles (M) and visceral masses (V) from small and big size *S. plana* sampling in the field and at the concentrations closer to the LC_{10} of SMOC and TBA.

Origin	Field				S-metalochlor LC_{10}				terbutylazine LC_{10}			
Size	Big		Small		Big		Small		Big		Small	
Tissue	V	M	V	M	V	M	V	M	V	M	V	M
DHA/EPA	2.28	1.93	2.04	3.66	1.45	2.31	2.11	3.05	1.79	14.91	1.97	8.61
EPA	5.95	7.12	9.30	5.22	5.44	44.61	34.27	22.90	4.98	2.30	12.54	9.85
16:1	4.65	2.20	6.25	1.10	1.60	6.94	7.79	3.21	1.80	1.07	6.44	2.60
16:0	8.40	14.03	35.22	10.61	4.19	40.61	34.74	26.54	5.33	2.44	13.31	8.43
18:1n9	5.07	10.76	27.51	6.19	2.83	35.81	23.10	18.71	3.42	3.39	13.60	9.96
18:2n6	1.06	0.73	7.98	0.73	0.59	4.58	4.21	3.09	0.65	0.35	2.72	1.29
Σ C15. C17	1.22	1.45	5.98	1.73	0.79	7.73	4.85	4.02	1.00	1.12	4.15	1.67

For organisms sampled in the field, the composition of fatty acids indicates high relative abundances of SFA and MUFA (≈35% and ≈26%, respectively, in both size classes and types of

tissue (Annexe I – Table I), besides, FATMs showed high values of EPA, 16:0 and 16:1n7, as well as, 15:0 + 17:0 (Table 3). The stress produced by the exposures originates a FA profile change, the relative abundance of HUFA increases above MUFA up to 20% in some cases, even above SFA at muscles exposed to TBA (Annexe I – Table 2-5). Concerning FATMs at the LC_{10} concentrations of SMOC, the values of EPA increase, as well as, 16:0 and 16:1; while at LC_{10} concentrations of TBA, the ratio DHA/EPA increases and 16:0 and 16:1 decrease (Table 3).

4.3.4. Total carbohydrate content

The composition analysis revealed that carbohydrates were constituted exclusively by glucose residues. Big organisms present higher carbohydrate content at field. However, small organisms increase its contents up to higher levels after the depuration and under chemical exposures, showing a higher sensitivity (Figure 5). In general, there is a tendency in carbohydrate content to raise through the concentrations of contaminants. At SMOC concentrations, the values of the quantifications surpassed the values at field, with statistical significance ($p<0.05$) at 39.4 mg/L in small size class. At TBA exposures, small organisms present significant increase in their carbohydrate content at 69.2 and 95 mg/L when compared to field and control samples ($p<0.05$). At the highest concentrations of both herbicides, carbohydrate concentrations tend to decrease relatively to the middle concentrations. The sugar analyses reveal that the percentage of polysaccharides of organisms from field, against the percentage of free monosaccharides, generally decrease at the bioassays until 26% at some concentrations.

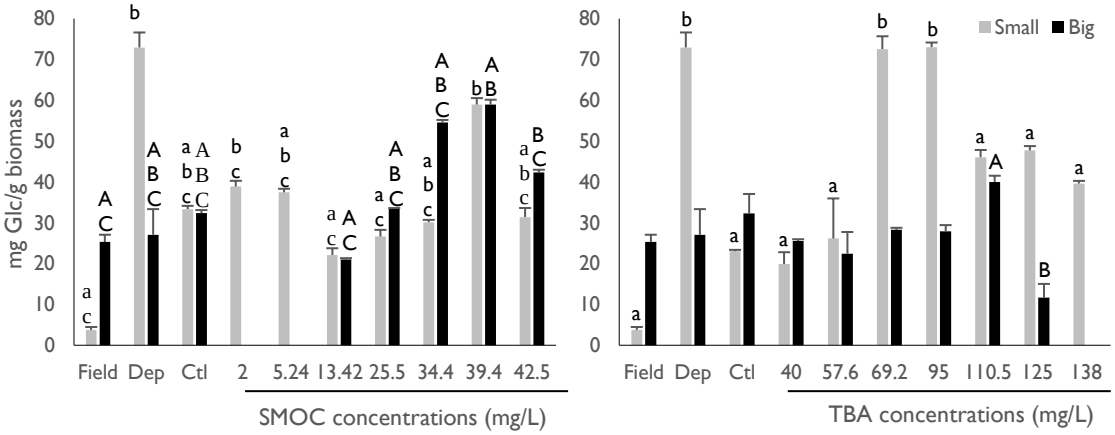

Figure5. Effects of the chemicals (SMOC and TBA) in glucose contents in mg per g of biomass to both size classes of S. plana, big (black bars) and small (grey bars) size classes. Organisms from the field (Field) and after depuration (Dep) and at the control treatments (Ctl) are shown. Letters on the top of the bars indicate significant differences statistically ($p<0.05$) between groups.

4.4. Discussion

The objective of this work is to explore the biochemical impacts of the herbicides, S-metolachlor and terbuthylazine, individually, on the estuarine species *S. plana*. Therefore, the study includes the analyses of the fatty acid profiles and the carbohydrate content of each treatment after the bioassays performed as described in Chapter I. Reviewing the results obtained in chapter I, *S. plana* showed to be clearly more tolerant to TBA than to SMOC in bioassays. Additionally, small size class revealed to be more sensitive to TBA, while big size was more sensitive to SMOC.

Regarding the effects of the chemicals at the molecular level, small size class exhibit generally higher FA content at the study area and under the exposures than big size class, agreeing with the study about the copper sulphate (Mesquita et al., 2018) but disagreeing with the exposure to Primextra commercial formulation (Gonçalves et al., 2016). Furthermore, alterations on FA profiles are more evident in small size class than in big size class. Toxic and biochemical effects are superior for small *S. plana*, which may be due to the role played by these molecules in the underlying mechanism of the herbicides to exert their effects.

Contrarily as spected, for both contaminants, is appreciable that the abundance of FA in muscles generally raise a vast rise at the middle herbicide concentrations, especially at those in which mortality start, that is, close to the LC_{10} of SMOC (13.42 mg/L and 30.5 mg/L for big and small size class, respectively) and TBA (40 mg/L). Therefore, the profiles from muscles resulted more positively affected than visceral masses that resemble unchanged. This division is well-defined at the big size class, since the abundances are generally higher at muscles. The diversity of the FA is equal at both types of tissue, except for the C18:3n3 (α LNA), a substrate at elongation and desaturation processes to synthesize DHA and EPA in mammals (Givens et al., 2008), that has been more easily found in visceral masses, despite it is also present in muscles. Thus, muscle is enough representative of the FA profile from the whole organism, being the better option to perform this kind of analyses. Besides, work with muscles represents other advantage since the biomass is significantly minor and, consequently, the needed amounts of the reagents to carry out the protocol of lipid extraction can be reduced by half, which means less expenses and more efficiency for this type of analysis.

SFA and MUFA occupy the major proportion of the FA profiles from organisms from field, but after the exposures to the chemicals, HUFA exhibit a massive increase, becoming dominant at muscles exposed to TBA and surpassing MUFA relative abundance at SMOC exposure. The increments of HUFA that are observable at both SMOC and TBA ranges of concentrations may be explained by a response to the stress caused by the toxicity. FAs, as EPA and C20:4n6, are precursors of eicosanoids and participate in several immune and inflammatory responses, neurologic and reproductive functions and improve the adaption of organisms to environmental and anthropogenic stressors (Fokina et al., 2013). PUFA is the less abundant group of the species, moreover, when compared with other species sampled at different coastal and estuarine systems of Portugal, as *Crassostrea angulata*, *Mytilus edulis*, *Cerastoderma edule* and *Venerupis pullastra,* the peppery furrow shell showed half of the PUFA contents (Ruano et al., 2012). There is a general reduction of C18:2n6 under the chemical exposures, being this FA involved, such as C20:4n6 and C18:3n6, in the biosynthesis of long chain PUFA (Givens, 2008). A decrease of the PUFA total abundance at TBA exposure, which is worrisome since PUFA are synthesized by conversion through elongation or desaturation, being these processes disturbed by pollutants. Animals are scarcely of high PUFA content, as is observable at SMOC bioassays, may be explained by the fact that PUFAs in membrane phospholipids protect the membranes from destruction by oxidation caused by chemical stressors exposure (Fokina et al., 2013) and their accumulation may become priority.

(Delaporte et al., 2005) reports that the diet quality affects FA content in bivalves. In order to investigate if bivalves show a selective behaviour to choose particles to filtrate, they were feed during bioassays with a mixture composed by microalgae and rotifers, each food having different FA contents that can reflect on the organisms FA profiles, to a later determination of FATMs as a tool to identify possible food sources. Based on the results of the FATMs, organisms from field presents component of zooplankton, phytoplankton plus a portion of bacteria, therefore, *S. plana* may be distinguished as an omnivorous specie. Under the exposures, organisms do not lost nutritious quality in terms of quantity of FA since it generally increased, however the FATMs shows that their FA profiles suffer changes. EPA content increased, specially at SMOC contamination, that may suggest a higher consume of phytoplankton because this FA is an indicator of a diet based on diatoms. On the other hand, the ratio DHA/EPA increased under TBA exposure, that is, a marker for a diet based on zooplankton, suggesting that the rotifers represent a relevant portion of the diet of the

orgasisms. Moreover, toxicants may produce effects indirectly by altering algal fitness food quality (Evens et al., 2012; Gorbi & Corradi, 1993). As TBA is a photosynthesis inhibitor it may affect the microalgae food source resulting in the low values for phytoplankton markers that are appreciable at TBA bioassays.

The exposure to organic contaminants affects the contents of fatty acids of bivalves. However, the real scope of the effects of these pollutants to non-target species is difficult to understand. Primary producers and consumers also present alterations on FA profiles when exposed to herbicides (Filimonova et al., 2016a). Thus, the occurrence of EFA at the whole contaminated aquatic environment may be compromised due to the interactions of the species through the trophic marine web. Therefore, savage organisms from edible species as bivalves, as organisms cultivated in pollute estuaries may suffer a loss of nutritional value plus other costs. This is a problem for the economy of aquacultures, such as lower rate of reproductive success due to C16:0, C16:1n7 and C18:1n9, that are related to embryonic development of bivalve eggs, or C18:3n3, C20:1n9 and C20:2n6, that have a key role in oocyte maturation of bivalve species (Baptista et al., 2014; Ojea et al., 2004).

Concerning the effects observed at the carbohydrate quantification, glucose content generally increase in a positive correlation with the concentrations of the stressors, agreeing with similar results observed at the onset of defence responses in fish, crustacean and bivalve species (Hall & Van Ham, 1998; Matozzo et al., 2011; Wang et al., 2015; Yoganandhan et al., 2003). Small size class of *S. plana* shows to be more sensitive relatively to the carbohydrate metabolism, coinciding with the results from the toxic effects and FA alterations. Glucose can be metabolized via glycolysis or oxidative phosphorylation pathway, then excessive glucose consumption can result in a rapid depletion of glucose reserves. Glycogen is the main source of glucose reserves in soft tissues and constitutes approximately 90 % of the total carbohydrates in bivalves (Plana et al., 1996). Thus, in order to maintain glucose homeostasis, it may be obtained from the glycogen via glycogenolysis (Tiwari & Singh, 2005; Yoganandhan et al., 2003). Moreover, the sugar analyses reveal a decrease at the quantification of polysaccharides, which can be interpreted as a decrease at the glycogen. An enhanced exploitation of glycogen reserves for glucose availability has been reported in several aquatic animals under the presence of stressors (Flye-Sainte-Marie et al., 2007, 2009; Wang et al., 2015; Zhou et al., 2010).

According to the Organism for Food and Agriculture (FAO, 2016), in 2010 aquaculture produce more than 632 000 tonnes of bivalves in Europe, increasing the production worldwide in recent years. Food quality and safety concerns by the consumers has been increasing. In order to minimise the impacts produced by the use of chemicals on agriculture, international institutions have developed legislations and have implemented biomonitoring programs at locals under risk of pollution as is the case of the Mondego estuary. The ranges of concentrations used to the bioassays exceed by far the amounts of metolachlor and terbuthylazine detected in Portuguese surface waters that are 0.056 mg/L and 0.0003 mg/L, respectively (Cerejeira et al., 2003; Palma et al., 2014; Silva et al., 2012). This is because the study is focus on study the biochemical impacts of the contaminants at concentrations with risk of mortality. Still, these concentrations are representative of a sporadic herbicide massive application. In the Mediterranean region, water contamination by pesticides is generally promoted during dry months and shortly after applications, rising the risk of reach hazardous concentrations in these periods. Furthermore, there is not current regulation establishing limit concentrations for SMOC and TBA neither in water or shellfish, while at certain concentrations, these chemicals may cause hazardous effects on aquatic species, rebounding in human health risk. Thus, ecotoxicological studies should continue clarifying the impacts of organic pollutants in aquatic species and the responsible mechanisms.

5. General conclusion

The present study highlights relevant information about the impacts to single exposure of *Scrobicularia plana* to S-metolachlor and terbuthylazine, reporting the occurrence of mortality and biochemical changes. *Scrobicularia plana* revealed to be a good bioindicator species for the assessment of the impacts and the presence of the selected toxicants in aquatic systems.

At Chapter I was stated accurate lethal concentrations of SMOC and TBA for the species *S. plana*, information that can support future reports of the toxicity of the chemicals, which is important since there is no current regulation about the limit concentrations at the environment to both active ingredients. Additionally, molecular analyses revealed that GRed and GST are suitable biomarkers of lethal effects of the studied substances in *S. plana*. Moreover, GRed showed to be a good biomarker to determine the presence of TBA in aquatic systems, whereas GST demonstrated to be the most accurate biomarker to SMOC. Thus, at future studies, the determination and quantification of the presence of such pollutants may be conducted applying the specific biomarker instead of a battery of biomarkers and, thus, the application of the most cost-effective approach, with less time consumption.

At Chapter II, FA profiling showed increments on the abundances of FA under chemical stress, revealing some kind of mechanism defence with a different trend than the usual. The elucidation of this mechanism at future works could be the key to understand the toxicity of pollutants in non-target species. Furthermore, from both types of tissues analysed (muscle and visceral mass), FA content in muscle was positively more affected by both toxicants than in visceral mass, with a raise in their content when compared to the FA profile in the field, at depuration and in visceral mass, respectively, with the amount in mg per gram of biomass higher in muscle. Thus, muscle is probably the best indicator tissue to carry out these biochemical analyses to pollutants since it shows a more accurate response to the toxicants. Moreover, the quantification of lipid extraction in muscle also revealed to be less expensive than at visceral mass due to the lower biomass used at the former tissue. In this chapter was also performed carbohydrate analyses that revealed glucose content generally increases with the raise of the chemical stressors' concentration. Generally, small size class of individuals show to be more sensitive at the molecular level, coinciding its sensitiveness to variations on FA profile, total glucose and the toxicity to the chemicals. Thus, this size class may be used at future toxic bioassays to determine the impact of TBA and / or SMOC at non-target species instead of big size class.

Although at the present study the biochemical analyses were carried out only at the end of the bioassays, this work suggests the need to determine biomarkers responses along the exposure to the chemicals, instead of a single analysis, to determine at which phase of the exposure period the effect is reversible and, thus to allow an earlier implementation of the recovery measurements. Furthermore, the investigation with analyses at distinct phases of the exposure will help to obtain more specific and detailed information about the suitability of the parameters measured, and their usage as early warning indicators to signalize the presence of the chemical stress before the appearance of lethal effects, allowing the implementation of mitigation measurements and, thus, to rehabilitate the environment before the damages turn irreversible.

Ultimately, to become aware of the impacts of these chemicals on the actual situation at the Mondego estuary and at other similar aquatic systems, and thus to the aquatic communities, further studies must be conducted to other species and also to mixture experiments. At future works a higher number of variables may be studied and combined with chemicals stressors, particularly those parameters related to the global changes such as salinity, temperature, pH and carbon dioxide sequestration forecasted by FAO (2016) . Therefore, the

main conclusions of this study highlights the tools to predict the impacts of these pollutants at the environment and to go deeper at the knowledge of toxicological impacts of toxicants in aquatic systems, their communities and at the biochemical level.

6. References

Almeida, Â., Calisto, V., Esteves, V. I., Schneider, R. J., Soares, A. M. V. M., Figueira, E., & Freitas, R. (2017). Toxicity associated to uptake and depuration of carbamazepine in the clam Scrobicularia plana under a chronic exposure. *Science of the Total Environment*, *580*, 1129–1145. https://doi.org/10.1016/j.scitotenv.2016.12.069

Ansari, A. A., & Gill, S. S. (2014). *Eutrophication: Causes, consequences and control: Volume 2*. *Eutrophication: Causes, Consequences and Control: Volume 2*. https://doi.org/10.1007/978-94-007-7814-6

Antunes, S. C., Freitas, R., Figueira, E., Gonçalves, F., & Nunes, B. (2013). Biochemical effects of acetaminophen in aquatic species: edible clams *Venerupis decussata* and *Venerupis philippinarum*. *Environmental Science and Pollution Research*, *20*(9), 6658–6666. https://doi.org/10.1007/s11356-013-1784-9

Arts, M. T., Brett, M. T., & Kainz, M. J. (2009). Lipids in aquatic ecosystems. *Lipids in Aquatic Ecosystems*, xv–xx. https://doi.org/10.1007/978-0-387-89366-2

Axelrad, J. C., Howard, C. V., & McLean, W. G. (2002). Interactions between pesticides and components of pesticide formulations in an in vitro neurotoxicity test. *Toxicology*, *173*(3), 259–268. https://doi.org/10.1016/S0300-483X(02)00036-7

Baptista, M., Repolho, T., Maulvault, A. L., Lopes, V. M., Narciso, L., Marques, A., … Rosa, R. (2014). Temporal dynamics of amino and fatty acid composition in the razor clam Ensis siliqua (Mollusca: Bivalvia). *Helgoland Marine Research*, *68*(4), 465–482. https://doi.org/10.1007/s10152-014-0402-7

Bergayou, H., Mouneyrac, C., Pellerin, J., & Moukrim, A. (2009). Oxidative stress responses in bivalves (Scrobicularia plana, Cerastoderma edule) from the Oued Souss estuary (Morocco). *Ecotoxicology and Environmental Safety*, *72*(3), 765–769. https://doi.org/10.1016/j.ecoenv.2008.09.012

Bermúdez-Saldaña, J. M., Escuder-Gilabert, L., Medina-Hernández, M. J., Villanueva-Camañas, R. M., & Sagrado, S. (2005). Chromatographic evaluation of the toxicity in fish of pesticides. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *814*(1), 115–125. https://doi.org/10.1016/j.jchromb.2004.10.007

Bertrand, C., Zalouk-Vergnoux, A., Giambérini, L., Poirier, L., Devin, S., Labille, J., … Mouneyrac, C. (2016). The influence of salinity on the fate and behavior of silver standardized nanomaterial and toxicity effects in the estuarine bivalve Scrobicularia plana. *Environmental Toxicology and Chemistry*, *35*(10), 2550–2561. https://doi.org/10.1002/etc.3428

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brett, M. T., & Muller-Navarra, D. C. (1997). The role of highly unsaturated fatty acids in aquatic food web processes. *FRESHWATER BIOLOGY*, *38*(3), 483–499. https://doi.org/10.1046/j.1365-2427.1997.00220.x

Brett, M. T., Muller-Navarra, D. C., & Persson, J. (2009). Crustacean zooplankton fatty acid composition. In *Lipids in Aquatic Ecosystems* (pp. 115–146).

Carabias-Martínez, R., Rodríguez-Gonzalo, E., Fernández-Laespada, M. E., Calvo-Seronero, L., & Sánchez-San Román, F. J. (2003). Evolution over time of the agricultural pollution of waters in an area of Salamanca and Zamora (Spain). *Water Research*, *37*(4), 928–938.

https://doi.org/10.1016/S0043-1354(02)00366-4

Carlberg, I., & Mannervik, B. (1985). [59] Glutathione reductase. *Methods in Enzymology*, *113*(C), 484–490. https://doi.org/10.1016/S0076-6879(85)13062-4

Cavalcante, D. G. S. M., Martinez, C. B. R., & Sofia, S. H. (2010). Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *695*(1–2), 41–46. https://doi.org/10.1016/j.mrgentox.2008.06.010

Cerejeira, M. J., Viana, P., Batista, S., Pereira, T., Silva, E., Valério, M. J., … Silva-Fernandes, A. M. (2003). Pesticides in Portuguese surface and ground waters. *Water Research*, *37*(5), 1055–1063. https://doi.org/10.1016/S0043-1354(01)00462-6

Cheung, C. C. C., Siu, W. H. L., Richardson, B. J., De Luca-Abbott, S. B., & Lam, P. K. S. (2004). Antioxidant responses to benzo[a]pyrene and Aroclor 1254 exposure in the green-lipped mussel, Perna viridis. *Environmental Pollution*, *128*(3), 393–403. https://doi.org/10.1016/j.envpol.2003.09.010

Coimbra, M. A., Waldron, K. W., & Selvendran, R. R. (1994). Isolation and characterisation of cell wall polymers from olive pulp (Olea europaea L.). *Carbohydrate Research*, *252*(C), 245–262. https://doi.org/10.1016/0008-6215(94)90019-1

Correia, A. D., Costa, M. H., Luis, O. J., & Livingstone, D. R. (2003). Age-related changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in whole body Gammarus locusta (Crustacea: Amphipoda). *J. Exp. Mar. Biol. Ecol*, *289*(1), 83–101. https://doi.org/10.1016/S0022-0981(03)00040-6

Cossu, C., Doyotte, A., Babut, M., Exinger, A., & Vasseur, P. (2000). Antioxidant biomarkers in freshwater bivalves, Unio tumidus, in response to different contamination profiles of aquatic sediments. *Ecotoxicology and Environmental Safety*, *45*(2), 106–121. https://doi.org/10.1006/eesa.1999.1842

De Hoop, L., De Troch, M., Hendriks, A. J., & De Laender, F. (2013). Modeling toxic stress by atrazine in a marine consumer-resource system. *Environmental Toxicology and Chemistry*, *32*(5), 1088–1095. https://doi.org/10.1002/etc.2160

De Troch, M., Boeckx, P., Cnudde, C., Van Gansbeke, D., Vanreusel, A., Vincx, M., & Caramujo, M. J. (2012). Bioconversion of fatty acids at the basis of marine food webs: Insights from a compound-specific stable isotope analysis. *Marine Ecology Progress Series*, *465*, 53–67. https://doi.org/10.3354/meps09920

Delaporte, M., Soudant, P., Moal, J., Kraffe, E., Marty, Y., & Samain, J. F. (2005). Incorporation and modification of dietary fatty acids in gill polar lipids by two bivalve species Crassostrea gigas and Ruditapes philippinarum. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *140*(4), 460–470. https://doi.org/10.1016/j.cbpb.2005.02.009

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

Dunstan, G. A., Volkman, J. K., Barrett, S. M., Leroi, J. M., & Jeffrey, S. W. (1993). Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). *Phytochemistry*, *35*(1), 155–161. https://doi.org/10.1016/S0031-9422(00)90525-9

El-Sabaawi, R., Dower, J. F., Kainz, M., & Mazumder, A. (2009). Characterizing dietary variability and trophic positions of coastal calanoid copepods: Insight from stable isotopes and fatty acids. *Marine Biology*, *156*(3), 225–237. https://doi.org/10.1007/s00227-008-1073-1

Eurostat. (2016). *Agriculture, forestry and fishery statistics*. *Statistical books*. https://doi.org/10.2785/917017

Evens, R., De Schamphelaere, K., De Laender, F., & Janssen, C. (2012). The effects of Zn-contaminated diets on Daphnia magna reproduction may be related to Zn-induced changes of the dietary P content rather than to the dietary Zn content itself. *Aquatic Toxicology*, *110–111*, 9–16. https://doi.org/10.1016/j.aquatox.2011.11.018

Ezgeta-Balić, D., Najdek, M., Peharda, M., & Blažina, M. (2012). Seasonal fatty acid profile analysis to trace origin of food sources of four commercially important bivalves. *Aquaculture*, *334–337*, 89–100. https://doi.org/10.1016/j.aquaculture.2011.12.041

FAO. (2016). *the State of World Fisheries and Aquaculture*. https://doi.org/10.5860/CHOICE.50-5350

Filimonova, V., Gonçalves, F., Marques, J. C., De Troch, M., & Gonçalves, A. M. M. (2016a). Biochemical and toxicological effects of organic (herbicide Primextra®Gold TZ) and inorganic (copper) compounds on zooplankton and phytoplankton species. *Aquatic Toxicology*, *177*, 33–43. https://doi.org/10.1016/j.aquatox.2016.05.008

Filimonova, V., Gonçalves, F., Marques, J. C., De Troch, M., & Gonçalves, A. M. M. (2016b). Fatty acid profiling as bioindicator of chemical stress in marine organisms: A review. *Ecological Indicators*. https://doi.org/10.1016/j.ecolind.2016.03.044

Filimonova, V., Nys, C., de Schamphelaere, K. A. C., Gonçalves, F., Marques, J. C., Gonçalves, A. M. M., & de Troch, M. (2018). Ecotoxicological and biochemical mixture effects of an herbicide and a metal at the marine primary producer diatom Thalassiosira weissflogii and the primary consumer copepod Acartia tonsa. *Environmental Science and Pollution Research*, pp. 1–16. https://doi.org/10.1007/s11356-018-2302-x

Finney, D. J. (1971). Probit Analysis. *Journal of Pharmaceutical Sciences*, *60*(9), 1432. https://doi.org/10.1002/jps.2600600940

Flohé, L., & Günzler, W. A. (1984). Assays of glutathione peroxidase. *Methods in Enzymology*, *105*(July), 114–120. https://doi.org/10.1016/S0076-6879(84)05015-1

Flye-Sainte-Marie, J., Jean, F., Paillard, C., & Kooijman, S. A. L. M. (2009). A quantitative estimation of the energetic cost of brown ring disease in the Manila clam using Dynamic Energy Budget theory. *Journal of Sea Research*, *62*(2–3), 114–123. https://doi.org/10.1016/j.seares.2009.01.007

Flye-Sainte-Marie, J., Pouvreau, S., Paillard, C., & Jean, F. (2007). Impact of Brown Ring Disease on the energy budget of the Manila clam Ruditapes philippinarum. *Journal of Experimental Marine Biology and Ecology*, *349*(2), 378–389. https://doi.org/10.1016/j.jembe.2007.05.029

Fokina, N. N., Ruokolainen, T. R., Nemova, N. N., & Bakhmet, I. N. (2013). Changes of blue mussels Mytilus edulis L. lipid composition under cadmium and copper toxic effect. *Biological Trace Element Research*, *154*(2), 217–225. https://doi.org/10.1007/s12011-013-9727-3

Fossi Tankoua, O., Buffet, P. E., Amiard, J. C., Berthet, B., Mouneyrac, C., & Amiard-Triquet, C. (2013). Integrated assessment of estuarine sediment quality based on a multi-biomarker approach in the bivalve Scrobicularia plana. *Ecotoxicology and Environmental Safety*, *88*, 117–125. https://doi.org/10.1016/j.ecoenv.2012.11.004

Galhano, V., Gomes-Laranjo, J., & Peixoto, F. (2011). Exposure of the cyanobacterium Nostoc muscorum from Portuguese rice fields to Molinate (Ordram®): Effects on the antioxidant system and fatty acid profile. *Aquatic Toxicology*, *101*(2), 367–376. https://doi.org/10.1016/j.aquatox.2010.11.011

Gamain, P., Gonzalez, P., Cachot, J., Clï¿½randeau, C., Mazzella, N., Gourves, P. Y., & Morin, B. (2017). Combined effects of temperature and copper and S-metolachlor on embryo-larval development of the Pacific oyster, Crassostrea gigas. *Marine Pollution Bulletin*, *115*(1–2), 201–210. https://doi.org/10.1016/j.marpolbul.2016.12.018

Gaté, L., Paul, J., Ba, G. N., Tew, K. D., & Tapiero, H. (1999). Oxidative stress induced in pathologies: The role of antioxidants. *Biomedicine & Pharmacotherapy*, *53*(4), 169–180. https://doi.org/http://dx.doi.org/10.1016/S0753-3322(99)80086-9

Givens, D. I., Baxter, S. J., Minihane, A. M., & Shaw, E. J. (2008). *Health benefits of organic food: Effects of the environment*. *Health Benefits of Organic Food: Effects of the Environment*.

Gonçalves, A. M. M., Azeiteiro, U. M., Pardal, M. A., & De Troch, M. (2012). Fatty acid profiling reveals seasonal and spatial shifts in zooplankton diet in a temperate estuary. *Estuarine, Coastal and Shelf Science*, *109*, 70–80. https://doi.org/10.1016/j.ecss.2012.05.020

Gonçalves, A. M. M., Barroso, D. V., Serafim, T. L., Verdelhos, T., Marques, J. C., & Gonçalves, F. (2017). The biochemical response of two commercial bivalve species to exposure to strong salinity changes illustrated by selected biomarkers. *Ecological Indicators*, *77*, 59–66. https://doi.org/10.1016/j.ecolind.2017.01.020

Gonçalves, A. M. M., Mesquita, A. F., Verdelhos, T., Coutinho, J. A. P., Marques, J. C., & Gonçalves, F. (2016). 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). *Ecological Indicators*, *63*, 209–218. https://doi.org/10.1016/j.ecolind.2015.12.006

Gorbi, G., & Corradi, M. G. (1993). Chromium Toxicity on Two Linked Trophic Levels I. Effects of Contaminated Algae on Daphnia magna. *Ecotoxicology and Environmental Safety*, *25*(1), 64–71. https://doi.org/10.1006/eesa.1993.1007

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases. The first step in mercapturic acid formation. *J. Biol. Chem.*, (22), 49–51. https://doi.org/10.14026/j.cnki.0253-9705.2010.23.013

Hall, M. R., & Van Ham, E. H. (1998). The effects of different types of stress on blood glucose in the giant tiger prawn Penaeus monodon. *Journal of the World Aquaculture Society*, *29*(3), 290–299. https://doi.org/10.1111/j.1749-7345.1998.tb00649.x

Herrero-Hernández, E., Andrades, M. S., Álvarez-Martín, A., Pose-Juan, E., Rodríguez-Cruz, M. S., & Sánchez-Martín, M. J. (2013). Occurrence of pesticides and some of their degradation products in waters in a Spanish wine region. *Journal of Hydrology*, *486*, 234–245. https://doi.org/10.1016/j.jhydrol.2013.01.025

Ibarguren, M., & López, D. J. (2014). The effect of natural and synthetic fatty acids on membrane structure, microdomain organization, cellular functions and human health. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1838*(6), 1518–1528. https://doi.org/10.1016/j.bbamem.2013.12.021

INE. (2013). *Estatísticas do Ambiente 2012*.

Jordaan, M. S., Reinecke, S. A., & Reinecke, A. J. (2013). Biomarker responses and morphological effects in juvenile tilapia Oreochromis mossambicus following sequential exposure to the organophosphate azinphos-methyl. *Aquatic Toxicology*, *144–145*, 133–140. https://doi.org/10.1016/j.aquatox.2013.10.007

Karam, D., Fara, F. R., Cruz, M. B., Filho, I. A. P., Pereira, F. T. F., Lara, F. R., & Biologia, B. S. (1992). Características do Herbicida S-Metolachlor nas Culturas de Milho e Sorgo. *Ministério Da Agricultura, Pecuária e Abastecimento*, (1983), 15–17.

Kharlamenko, V. I., Kiyashko, S. I., Imbs, A. B., & Vyshkvartzev, D. I. (2001). Identification of food sources of invertebrates from the seagrass Zostera marina community using carbon and sulfur stable isotope ratio and fatty acid analyses. *Marine Ecology Progress Series*, *220*, 103–117. https://doi.org/10.3354/meps220103

Kumar, P., Kumar, R., Nagpure, N. S., Nautiyal, P., Kushwaha, B., & Dabas, A. (2013). Genotoxicity and antioxidant enzyme activity induced by hexavalent chromium in Cyprinus carpio after in vivo exposure. *Drug and Chemical Toxicology*, *36*(4), 451–460. https://doi.org/10.3109/01480545.2013.776581

Ladhar, C., Ayadi, H., Denis, F., Tastard, E., & Sellami, I. (2014). The effect of environmental factors on the fatty acid composition of copepods and Artemia in the Sfax solar saltern (Tunisia). *Biochemical Systematics and Ecology*, *56*, 237–245. https://doi.org/10.1016/j.bse.2014.06.005

Limón-Pacheco, J., & Gonsebatt, M. E. (2009). The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*. https://doi.org/10.1016/j.mrgentox.2008.09.015

Liu, H., & Xiong, M. (2009). Comparative toxicity of racemic metolachlor and S-metolachlor to Chlorella pyrenoidosa. *Aquatic Toxicology*, *93*(2–3), 100–106. https://doi.org/10.1016/j.aquatox.2009.04.006

Liu, J. J., Green, P., John Mann, J., Rapoport, S. I., & Sublette, M. E. (2015). Pathways of polyunsaturated fatty acid utilization: Implications for brain function in neuropsychiatric health and disease. *Brain Research*. https://doi.org/10.1016/j.brainres.2014.11.059

Lochmiller, R. L., & Deerenberg, C. (2000). Trade-offs in evolutionary immunology: Just what is the cost of immunity? *Oikos*, *88*(1), 87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, *101*(1), 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Maazouzi, C., Coureau, C., Piscart, C., Saplairoles, M., Baran, N., & Marmonier, P. (2016). Individual and joint toxicity of the herbicide S-metolachlor and a metabolite, deethylatrazine on aquatic crustaceans: Difference between ecological groups. *Chemosphere*, *165*, 118–125. https://doi.org/10.1016/j.chemosphere.2016.09.030

Manera, M., Sayyaf Dezfuli, B., DePasquale, J. A., & Giari, L. (2016). Multivariate approach to gill pathology in European sea bass after experimental exposure to cadmium and terbuthylazine. *Ecotoxicology and Environmental Safety*, *129*, 282–290. https://doi.org/10.1016/j.ecoenv.2016.03.039

Mansour, M. P., Volkman, J. K., Jackson, A. E., & Blackburn, S. I. (1999). The fatty acid and sterol composition of two marine dinoflagellates. *Journal of Phycology*, *35*, 710–720. https://doi.org/10.1016/S0031-9422(00)82605-9

Manuel Nicolaus, E. E., Law, R. J., Wright, S. R., & Lyons, B. P. (2015). Spatial and temporal analysis of the risks posed by polycyclic aromatic hydrocarbon, polychlorinated biphenyl and metal contaminants in sediments in UK estuaries and coastal waters. *Marine Pollution Bulletin*, *95*(1). https://doi.org/10.1016/j.marpolbul.2015.03.012

Maria, V. L., & Bebianno, M. J. (2011). Antioxidant and lipid peroxidation responses in Mytilus galloprovincialis exposed to mixtures of benzo(a)pyrene and copper. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *154*(1), 56–63. https://doi.org/10.1016/j.cbpc.2011.02.004

Matozzo, V., Gallo, C., & Marin, M. G. (2011). Can starvation influence cellular and biochemical parameters in the crab Carcinus aestuarii? *Marine Environmental Research*, *71*(3), 207–212. https://doi.org/10.1016/j.marenvres.2011.01.004

Mayzaud, P., Chanut, J., & Ackman, R. (1989). Seasonal changes of the biochemical composition of marine particulate matter with special reference to fatty acids and sterols. *Marine Ecology Progress Series*. https://doi.org/10.3354/meps056189

Mesquita, A. F., Gonçalves, F., Verdelhos, T., Marques, J. C., & Gonçalves, A. M. M. (2018). Fatty acids profiles modifications in the bivalves Cerastoderma edule and Scrobicularia plana in response to copper sulphate. *Ecological Indicators*, *85*, 318–328. https://doi.org/10.1016/j.ecolind.2017.10.007

Neves, M., Castro, B. B., Vidal, T., Vieira, R., Marques, J. C., Coutinho, J. A. P., … Gonçalves, A. M. M. (2015). Biochemical and populational responses of an aquatic bioindicator species, Daphnia longispina, to a commercial formulation of a herbicide (Primextra® Gold TZ) and its active ingredient (S-metolachlor). *Ecological Indicators*, *53*, 220–230. https://doi.org/10.1016/j.ecolind.2015.01.031

Nödler, K., Licha, T., & Voutsa, D. (2013). Twenty years later - Atrazine concentrations in selected coastal waters of the Mediterranean and the Baltic Sea. *Marine Pollution Bulletin*, *70*(1–2), 112–118. https://doi.org/10.1016/j.marpolbul.2013.02.018

Nwani, C. D., Ifo, C. T., Nwamba, H. O., Ejere, V. C., Onyishi, G. C., Oluah, S. N., … Odo, G. E. (2015). Oxidative stress and biochemical responses in the tissues of African catfish Clarias gariepinus juvenile following exposure to primextra herbicide. *Drug and Chemical Toxicology*, *38*(3), 278–285. https://doi.org/10.3109/01480545.2014.947503

Ojea, J., Pazos, A. J., Martínez, D., Novoa, S., Sánchez, J. L., & Abad, M. (2004). Seasonal variation in weight and biochemical composition of the tissues of Ruditapes decussatus in relation to the gametogenic cycle. *Aquaculture*, *238*(1–4), 451–468. https://doi.org/10.1016/j.aquaculture.2004.05.022

Palma, P., Köck-Schulmeyer, M., Alvarenga, P., Ledo, L., Barbosa, I. R., López de Alda, M., & Barceló, D. (2014). Risk assessment of pesticides detected in surface water of the Alqueva reservoir (Guadiana basin, southern of Portugal). *Science of the Total Environment*, *488–489*(1), 208–219. https://doi.org/10.1016/j.scitotenv.2014.04.088

Parrish, C. C. (1998). Lipid biogeochemistry of plankton, settling matter and sediments in Trinity Bay, Newfoundland. I. Lipid classes. In *Organic Geochemistry* (Vol. 29, pp. 1531–1545). https://doi.org/10.1016/S0146-6380(98)00176-4

Parween, T., Jan, S., Mahmooduzzafar, S., Fatma, T., & Siddiqui, Z. H. (2016). Selective Effect of Pesticides on Plant—A Review. *Critical Reviews in Food Science and Nutrition*, *56*(1), 160–179. https://doi.org/10.1080/10408398.2013.787969

Peixoto, F., Alves-Fernandes, D., Santos, D., & Fontaínhas-Fernandes, A. (2006). Toxicological effects of oxyfluorfen on oxidative stress enzymes in tilapia Oreochromis niloticus. *Pesticide Biochemistry and Physiology*, *85*(2), 91–96. https://doi.org/10.1016/j.pestbp.2005.10.007

Pereira, J. L., Antunes, S. C., Castro, B. B., Marques, C. R., Gonçalves, A. M. M., Gonçalves, F., & Pereira, R. (2009). Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: Commercial formulation versus active ingredient. *Ecotoxicology*, *18*(4), 455–463. https://doi.org/10.1007/s10646-009-0300-y

Pérez, E., Blasco, J., & Solé, M. (2004). Biomarker responses to pollution in two invertebrate species: Scrobicularia plana and Nereis diversicolor from the Cádiz bay (SW Spain). In *Marine Environmental Research* (Vol. 58, pp. 275–279).

https://doi.org/10.1016/j.marenvres.2004.03.071

Pernet, F., Malet, N., Pastoureaud, A., Vaquer, A., Quéré, C., & Dubroca, L. (2012). Marine diatoms sustain growth of bivalves in a Mediterranean lagoon. *Journal of Sea Research*, *68*, 20–32. https://doi.org/10.1016/j.seares.2011.11.004

Plana, S., Sinquin, G., Maes, P., Paillard, C., & Le Pennec, M. (1996). Variations in biochemical composition of juvenile Ruditapes philippinarum infected by a Vibrio sp. *Diseases of Aquatic Organisms*, *24*(3), 205–213. https://doi.org/10.3354/dao024205

Quintaneiro, C., Patrício, D., Novais, S. C., Soares, A. M. V. M., & Monteiro, M. S. (2017). Endocrine and physiological effects of linuron and S-metolachlor in zebrafish developing embryos. *Science of the Total Environment*, *586*, 390–400. https://doi.org/10.1016/j.scitotenv.2016.11.153

Robert, S., Mansour, M. P., & Blackburn, S. I. (2007). Metolachlor-mediated selection of a microalgal strain producing novel polyunsaturated fatty acids. *Marine Biotechnology*, *9*(2), 146–153. https://doi.org/10.1007/s10126-006-6102-9

Rodrigues, E. T., Pardal, M. Â., Salgueiro-González, N., Muniategui-Lorenzo, S., & Alpendurada, M. F. (2016). A single-step pesticide extraction and clean-up multi-residue analytical method by selective pressurized liquid extraction followed by on-line solid phase extraction and ultra-high-performance liquid chromatography-tandem mass spectrometry for complex mat. *Journal of Chromatography A*, *1452*, 10–17. https://doi.org/10.1016/j.chroma.2016.05.036

Ruano, F., Ramos, P., Quaresma, M., Bandarra, N. M., & Pereira-da Fonseca, I. (2012). Evolution of fatty acid profile and Condition Index in mollusc bivalves submitted to different depuration periods. *Revista Portuguesa de Ciéncias Veterinárias*, *111*, 75–84.

Silva, C., Mattioli, M., Fabbri, E., Yáñez, E., DelValls, T. A., & Martín-Díaz, M. L. (2012). Benthic community structure and biomarker responses of the clam Scrobicularia plana in a shallow tidal creek affected by fish farm effluents (Rio San Pedro, SW Spain). *Environment International*, *47*, 86–98. https://doi.org/10.1016/j.envint.2012.06.008

Solé, M., Kopecka-Pilarczyk, J., & Blasco, J. (2009). Pollution biomarkers in two estuarine invertebrates, Nereis diversicolor and Scrobicularia plana, from a Marsh ecosystem in SW Spain. *Environment International*, *35*(3), 523–531. https://doi.org/10.1016/j.envint.2008.09.013

Steinback, K. E., Mcintoshtt, L., Bogoradt, L., & Arntzen, C. J. (1981). Identification of the triazine receptor protein as a chloroplast gene product. *Proc. Natl. Acad. Sci. USA*, *78*(12), 7463–7467. https://doi.org/10.1073/pnas.78.12.7463

Tarja, N., Kirsti, E., Marja, L., & Kari, E. (2003). Thermal and metabolic factors affecting bioaccumulation of triazine herbicides by rainbow trout (_Oncorhynchus mykiss_). *Environmental Toxicology*, *18*(4), 219–226.

Teixeira, H., Salas, F., Borja, Á., Neto, J. M., & Marques, J. C. (2008). A benthic perspective in assessing the ecological status of estuaries: The case of the Mondego estuary (Portugal). *Ecological Indicators*, *8*(4), 404–416. https://doi.org/10.1016/j.ecolind.2007.02.008

Tejada, S., Sureda, A., Roca, C., Gamundí, A., & Esteban, S. (2007). Antioxidant response and oxidative damage in brain cortex after high dose of pilocarpine. *Brain Research Bulletin*, *71*(4), 372–375. https://doi.org/10.1016/j.brainresbull.2006.10.005

Thakkar, M., Randhawa, V., & Wei, L. (2013). Comparative responses of two species of marine phytoplankton to metolachlor exposure. *Aquatic Toxicology*, *126*, 198–206. https://doi.org/10.1016/j.aquatox.2012.10.002

The Council of the European Union. (1998). Council Directive 98/93/EC of November 1998 on the equality of water intended for human consumption. *Official Journal of the European Communities*, *330*(5.12.98), 32–54. Retrieved from http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:31998L0083&from=EN

Tiwari, S., & Singh, A. (2005). Alterations in carbohydrates and the protein metabolism of the harmful freshwater vector snail Lymnaea acuminata induced by the Euphorbia tirucalli latex extract. *Environmental Research*, *99*(3), 378–386. https://doi.org/10.1016/j.envres.2004.12.008

Torres, M. A., Barros, M. P., Campos, S. C. G., Pinto, E., Rajamani, S., Sayre, R. T., & Colepicolo, P. (2008). Biochemical biomarkers in algae and marine pollution: A review. *Ecotoxicology and Environmental Safety*, *71*(1), 1–15. https://doi.org/10.1016/j.ecoenv.2008.05.009

Trenkamp, S., Martin, W., & Tietjen, K. (2004). Specific and differential inhibition of very-long-chain fatty acid elongases from Arabidopsis thaliana by different herbicides. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(32), 11903–11908. https://doi.org/10.1073/pnas.0404600101

Valavanidis, A., Vlahogianni, T., Dassenakis, M., & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, *64*, 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013

Van der Oost, R., Beyer, J., & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology*, *13*(2), 57–149. https://doi.org/10.1016/S1382-6689(02)00126-6

Van Der Werf, H. M. G. (1996). Assessing the impact of pesticides on the environment. *Agriculture, Ecosystems and Environment*, *60*(2–3), 81–96. https://doi.org/10.1016/S0167-8809(96)01096-1

Vasseur, P., & Cossu-Leguille, C. (2003). Biomarkers and community indices as complementary tools for environmental safety. *Environment International*, *28*(8), 711–717. https://doi.org/10.1016/S0160-4120(02)00116-2

Velisek, J., Stara, A., & Zuskova, E. (2016). Effect of single and combination of three triazine metabolites at environmental concentrations on early life stages of common carp (Cyprinus carpio L.). *Environmental Science and Pollution Research*, *23*(23). https://doi.org/10.1007/s11356-016-7689-7

Velisek, J., Stara, A., Zuskova, E., & Kouba, A. (2017). Effects of three triazine metabolites and their mixture at environmentally relevant concentrations on early life stages of marbled crayfish (Procambarus fallax f. virginalis). *Chemosphere*, *175*, 440–445. https://doi.org/10.1016/j.chemosphere.2017.02.080

Verdelhos, T., Cardoso, P. G., Dolbeth, M., & Pardal, M. A. (2014). Recovery trends of Scrobicularia plana populations after restoration measures, affected by extreme climate events. *Marine Environmental Research*, *98*, 39–48. https://doi.org/10.1016/j.marenvres.2014.03.004

Verdelhos, T., Marques, J. C., & Anastácio, P. (2015). The impact of estuarine salinity changes on the bivalves Scrobicularia plana and Cerastoderma edule, illustrated by behavioral and mortality responses on a laboratory assay. *Ecological Indicators*, *52*, 96–104. https://doi.org/10.1016/j.ecolind.2014.11.022

Vidal, T., Abrantes, N., Goncalves, A. M. M., & Goncalves, F. (2012). Acute and chronic toxicity of Betanal (R) Expert and its active ingredients on nontarget aquatic organisms from

different trophic levels. *Environmental Toxicology*, *27*, 537–548. https://doi.org/10.1002/tox.20671

Vryzas, Z., Vassiliou, G., Alexoudis, C., & Papadopoulou-Mourkidou, E. (2009). Spatial and temporal distribution of pesticide residues in surface waters in northeastern Greece. *Water Research*, *43*(1), 1–10. https://doi.org/10.1016/j.watres.2008.09.021

Wang, X., Wang, L., Zhang, H., Liu, R., & Song, L. (2015). The carbohydrate metabolism of scallop Chlamys farreri in the immune response against acute challenge of Vibrio anguillarum. *Aquaculture International*, *23*(5). https://doi.org/10.1007/s10499-014-9871-6

Yoganandhan, K., Thirupathi, S., & Sahul Hameed, A. S. (2003). Biochemical, physiological and hematological changes in white spot syndrome virus-infected shrimp,< i> Penaeus indicus</i>. *Aquaculture*, *221*(1), 1–11. Retrieved from http://www.google.mg/search?q=detection+WSSV+chez+les+crabes&hl=fr-MG&gbv=2&prmd=ivns&ei=SkvGVKuEEInvaLmwguAB&start=10&sa=N

Zhou, J., Zhu, X. shan, & Cai, Z. hua. (2010). Tributyltin toxicity in abalone (Haliotis diversicolor supertexta) assessed by antioxidant enzyme activity, metabolic response, and histopathology. *Journal of Hazardous Materials*, *183*(1–3), 428–433. https://doi.org/10.1016/j.jhazmat.2010.07.042

Zhukova, N. V, & Kharlamenko, V. I. (1999). Sources of essential fatty acids in the marine microbial loop. *Aquatic Microbial Ecology*, *17*(2), 153–157. https://doi.org/10.3354/ame017153

Annexes

Annexe I. Fatty acids profiles classified by saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) and highly unsaturated fatty acids (HUFA) from both size classes of *S. plana* measured after the collection from field and after depuration (Table 1). The profiles of big size class (Table 2) and small size class (Table 3) exposed to S-metolachlor concentrations and to the controls (Ctl) are shown, as well as the profiles of big size class (Table 4) and small size class (Table 5) exposed to terbuthylazine concentrations and to the controls (Ctl). All tables include the fatty acid contents from muscles (M) and visceral masses (V) of *S. plana.*

Table 1. FA profile (mg/g of biomass) of *S. plana* from field and after depuration.

Big size					Small size				
	Field		Depuration			Field		Depuration	
FA	M	V	M	V	FA	M	V	M	V
C14:0	0.49	1.02	0.54	0.93	C14:0	0.40	2.21	0.66	1.59
C15:0	0.64	0.87	0.77	0.67	C15:0	0.58	5.00	0.76	1.21
C16:0	14.03	8.40	12.08	5.68	C16:0	10.61	35.22	15.16	14.68
C17:0	0.81	0.35	1.20	0.38	C17:0	1.16	0.97	1.54	1.23
C18:0	10.75	4.66	8.65	2.50	C18:0	7.30	8.09	9.69	10.24
C20:0	7.72	4.41	4.81	1.70	C20:0	5.10	5.23	6.11	6.45
C23:0	3.71	1.96	3.58	0.94	C23:0	5.98	3.09	7.59	20.89
C24:0		0.65		0.18	C24:0	0.47	0.40	1.20	2.34
Total SFA	38.15	22.32	31.63	12.98	Total SFA	31.60	60.21	42.71	58.63
C16:1	2.20	4.65	1.72	2.66	C16:1	1.10	6.25	1.75	3.29
C18:1	10.76	5.07	10.06	2.82	C18:1	6.19	27.51	8.30	9.88
C20:1	10.08	7.49	7.54	3.17	C20:1	8.15	10.15	9.94	19.70
C22:1	1.06	0.50	2.83	0.08	C22:1	5.68	0.67	6.67	0.73
C24:1n9	3.24	0.54	2.59	0.04	C24:1n9	1.97		1.46	0.90
Total MUFA	27.34	18.25	24.74	8.77	Total MUFA	23.09	44.58	28.12	34.50
C18:2	0.73	1.06	1.06	0.83	C18:2	0.73	7.98	1.14	2.03
C18:3n6		0.73		0.63	C18:3n6		2.06		2.59
C18:3n3		0.50	0.53		C18:3n3		0.48	0.79	0.65
C20:2	1.84	2.33	2.72	1.31	C20:2	2.59	11.34	3.09	10.79
C20:4n6	5.51	4.93	3.84	1.83	C20:4n6	2.71	6.50	4.19	8.89
C22:2	7.77	0.86	2.61	0.33	C22:2	7.26	3.95	6.75	1.97
Total PUFA	15.85	10.41	10.76	4.93	Total PUFA	13.29	32.31	15.96	26.92
EPA	7.12	5.95	5.25	4.97	EPA	5.22	9.30	8.80	14.99
DHA	13.74	13.56	12.43	5.93	DHA	19.14	18.97	22.97	22.90
Total HUFA	20.86	19.51	17.68	10.90	Total HUFA	24.36	28.27	31.77	37.89
N	18	21	19	20	N	19	21	20	21

At the following tables, concentrations of S-metolachlor appear as: C1 - 2.048 mg/L, C2 - 5.24 mg/L, C3 - 13.42 mg/L, C4 - 25.46 mg/L, C5 - 30.5 mg/L, C6 - 34.36 mg/L, C7 - 39.40 mg/L, C8 - 42.5 mg/L; and concentrations of terbuthylazine, as: C1- 40 mg/L, C2 - 57.6 mg/L, C3 - 69.2 mg/L, C4 - 82.3 mg/L, C5 - 95 mg/L, C6 - 110,5 mg/L, C7 - 125 mg/L, C8 - 138 mg/L.

Table 2. FA profile (mg/g of biomass) of big size *S. plana* exposed to S- metolachlor

	Ctl		C3		C4		C5		C6		C7		C8	
FA	M	V	M	V	M	V	M	V	M	V	M	V	M	V
C14:0	0.64	0.30	0.45	0.50	6.11	0.54	1.85	0.51	1.08	0.44	0.71	0.47	1.09	0.70
C15:0	1.08	0.38	1.38	0.57	2.93	0.53	3.21	0.49	2.42	0.23	1.37	0.53	1.81	0.62
C16:0	14.71	2.64	14.92	4.42	36.66	4.34	40.61	4.19	29.21	2.06	18.94	4.39	24.02	5.92
C17:0	1.64	0.77	1.59	0.36	15.19	0.27	4.52	0.30	3.06	0.14	2.19	0.34	2.67	0.53
C18:0	13.62	1.33	12.77	2.53	37.04	1.95	40.85	2.26	31.47	1.01	18.10	2.47	24.80	4.01
C20:0	11.59	1.79	10.43	3.32	39.04	2.73	41.27	3.30	35.25	1.44	16.23	3.23	27.21	1.70
C23:0	7.19	0.97	12.76	1.43	28.54	1.33	28.51	1.63	14.60	0.47	8.02	8.51	20.77	2.48
C24:0	4.21	0.59	12.58	0.57	2.87	0.36	3.02	0.14	1.36	0.03	0.19	0.39	4.78	1.65
Total SFA	54.69	8.77	66.88	13.71	168.39	12.04	163.83	12.82	118.45	5.83	65.74	20.33	107.15	17.62
C16:1	1.90	0.90	1.14	1.52	6.87	1.52	6.94	1.60	4.65	1.12	2.72	1.41	3.73	1.50
C18:1	12.69	1.73	13.07	3.25	35.97	2.69	35.81	2.83	29.78	1.28	18.46	3.02	25.30	3.79
C20:1	14.98	2.98	14.59	5.99	45.97	3.86	52.18	4.64	36.85	1.87	25.02	5.62	32.44	10.05
C22:1	4.60	0.08	7.85	0.21	18.68	0.13	19.62	0.19	9.01	0.07	5.39	0.25	12.87	0.41
C24:1n9	2.04	0.30	6.90	0.42	14.39	0.11	2.16	0.31	5.99		3.95	1.14	16.78	0.78
Total MUFA	36.22	5.98	43.56	11.38	121.89	8.31	116.71	9.58	86.28	4.33	55.54	11.44	91.12	16.52
C18:2n6t	0.78	0.29	0.82	0.56	5.72	0.57	4.58	0.59	2.19	0.38	1.38	0.50	4.11	0.71
C18:3n6	0.27	0.33		1.04	0.81	0.59	0.85	0.78	0.06	0.30	0.07	0.40		1.02
C18:3n3	0.56		0.60	0.17	0.81	0.18	2.35	0.14	1.00	0.16		0.22	0.60	0.16
C20:2	4.91	0.47	3.76	1.41	12.99	0.70	13.64	0.91	6.51	0.34	7.15	1.39	9.64	2.05
C20:4n6	9.09	2.05	7.61	3.76	28.40	2.44	30.10	2.91	24.41	1.26	11.60	2.89	20.52	4.76
C22:2	6.08	0.27	23.97	0.51	26.51	0.43	27.55	0.46	12.71	0.22	7.28	0.53	19.90	0.98
Total PUFA	21.70	3.41	36.77	7.44	75.24	4.91	79.06	5.79	46.88	2.65	27.48	5.93	54.78	9.69
EPA	12.09	3.11	9.93	5.67	36.42	4.22	44.61	5.44	33.48	2.48	14.92	4.48	25.71	6.85
DHA	29.55	4.49	41.74	8.33	96.16	6.34	102.88	7.91	64.71	4.03	37.00	7.70	65.14	12.66
Total HUFA	41.64	7.59	51.67	14.00	132.58	10.56	147.48	13.35	98.19	6.52	51.92	12.18	90.85	19.52
N	21	20	20	21	21	21	21	21	21	20	20	21	20	21

Table 3. FA profile (mg/g of biomass) of small *S. plana* exposed to S-metolachlor.

	Ctl		C1		C2		C3		C4		C5		C6		C7		C8	
FA	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
C14:0	0.78	1.22	0.26	1.61	0.30	2.29	1.15	2.13	1.44	1.27	0.49	1.69	0.50	1.22	0.65	2.61	0.75	3.77
C15:0	0.95	0.96	0.29	0.71	0.54	1.44	0.95	1.51	1.41	1.13	0.76	1.46	0.76	1.29	1.18	1.95	0.60	2.87
C16:0	13.39	8.77	9.59	12.61	10.46	16.01	26.54	15.76	26.86	10.17	13.87	19.86	13.15	12.33	21.73	23.21	16.82	28.13
C17:0	1.51	0.75	1.16	1.15	1.31	1.56	3.07	1.52	2.66	0.71	1.74	1.83	1.58	0.89	2.68	1.92	2.08	2.40
C18:0	9.99	6.33	7.74	8.87	7.63	8.34	21.43	11.36	21.87	5.62	10.92	12.68	10.97	28.33	16.81	15.61	14.37	17.48
C20:0	2.87	8.26	6.98	8.73	6.21	10.52	21.59	10.31	22.01	7.06	11.31	11.89	12.52	9.43	16.59	14.81	15.74	16.03
C23:0	10.66	3.36	4.25	3.54	8.35	4.17	24.91	12.99	19.39	3.35	10.64	14.09	9.55	4.17	16.12	7.28	11.97	8.71
C24:0	4.39	1.12		1.02		2.42	2.31	4.01	3.50	0.96		4.29	0.14	2.20	7.66	2.87	2.20	5.58
T. SFA	44.54	30.77	30.27	38.24	34.80	46.75	101.95	59.58	99.14	30.28	49.73	67.80	49.17	59.87	83.42	70.27	64.53	84.96
C16:1	1.85	2.25	1.05	2.46	1.18	3.67	3.21	3.76	4.37	3.26	1.33	3.98	1.74	3.30	2.63	5.09	2.54	7.75
C18:1	10.78	6.32	6.80	8.57	6.77	10.16	18.71	10.35	25.40	6.98	9.78	12.75	10.19	8.44	17.19	16.98	14.52	17.13
C20:1	10.35	10.24	9.95	15.27	10.73	20.73	29.57	18.48	29.82	10.89	16.16	21.35	17.30	14.94	24.55	29.60	22.19	30.02
C22:1	7.68	0.51	2.32	0.79	5.14	0.92	11.23	0.82	13.11	0.16	8.03	1.04	6.60	0.30	7.75	0.65	6.49	1.09
C24:1n9	5.98	0.81	2.46	0.46	6.14	0.04	16.89	2.09	20.26	0.40	5.54	2.87	4.01	1.93	8.50	0.91	2.22	3.98
T. MUFA	36.64	20.12	22.58	27.54	29.96	35.52	79.61	35.50	92.96	21.68	40.84	42.00	39.84	28.91	60.62	53.22	47.96	59.96
C18:2	2.44	1.28	2.38	1.62	0.81	2.08	3.09	2.26	3.35	1.40	1.33	2.08	1.18	1.53	2.99	3.45	2.47	3.44
C18:3n6		3.64		2.35		2.64	0.69	2.87		1.49		2.80		1.99	1.02	4.03	0.09	4.27
C18:3n3	1.29		0.41	0.53	0.39	1.39	0.96		0.79	0.48	0.68	0.35	0.35	0.69		1.01		1.21
C20:2	10.90	2.00	2.55	3.43	3.41	3.65	8.85	3.41	8.99	2.20	4.62	4.48	5.18	54.73	7.05	5.90	6.69	6.79
C20:4n6	5.31	6.77	4.88	8.48	4.80	9.89	13.94	11.27	14.53	5.80	7.65	11.07	7.91	7.07	12.49	14.20	11.20	15.82
C22:2	18.63	3.59	3.73	1.57	8.53	2.01	30.46	3.71	12.92	1.12	8.61	3.93	7.23	1.41	14.33	2.35	10.27	3.04
T. PUFA	38.57	17.28	13.95	17.98	17.94	21.67	57.99	23.53	40.58	12.49	22.89	24.73	21.85	67.42	37.88	30.93	30.72	34.56
EPA	9.59	23.82	8.55	12.82	12.18	15.82	22.90	16.13	23.73	9.71	12.65	14.20	12.68	11.70	19.67	21.21	17.09	22.67
DHA	29.11	17.31	18.12	21.22	33.48	31.87	69.81	42.70	63.85	37.18	31.73	33.82	30.47	30.34	52.50	38.70	22.36	39.81
T. HUFA	38.70	41.13	26.67	34.04	45.66	47.69	92.71	58.83	87.58	46.89	44.38	48.03	43.14	42.04	72.17	59.91	39.45	62.48
N	20	20	19	21	19	21	21	20	20	21	19	21	20	21	20	21	20	21

Table 4. FA profile (mg/g of biomass) of big size *S. plana* exposed to terbuthylazine.

	Ctl		C1		C2		C3		C4		C5		C6		C7	
FA	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
C14:0	0.03	0.73	0.26	0.55	0.31	0.58	0.24	0.53	0.31	4.50	0.17	0.71	0.18	0.54	0.23	0.60
C15:0	0.18	0.81	0.51	0.56	0.18	0.40		0.53	0.29	0.50	0.15	0.33	0.19	0.39	0.28	0.79
C16:0	1.11	6.54	2.44	5.33	1.81	4.21	2.08	4.57	2.55	5.25	1.40	4.58	2.15	3.57	1.59	6.04
C17:0	0.24	0.45	0.61	0.44	0.31	0.40	0.46	0.40	0.21	0.41	0.31	0.30	0.19	0.26	0.10	0.47
C18:0	0.55	3.23	1.60	3.19	1.00	3.00	1.18	2.82	1.74	2.91	1.02	2.06	1.18	1.94	0.88	3.44
C20:0	0.25	4.18	1.63	4.66	1.47	3.01	1.51	2.53	1.97	2.38	0.61	2.60	1.17	2.14	1.37	4.22
C23:0	2.15	1.51	12.27	0.63	10.74	1.33	5.09	1.31	7.32	1.51	5.06	1.21	9.83	0.93	2.50	2.24
C24:0	0.80	1.46	6.89	0.46	3.16	0.26	7.79	2.66	13.49	1.90	7.72	0.20	6.41	0.36	2.29	1.37
T. SFA	5.30	18.91	26.21	15.82	18.98	13.19	18.34	15.35	27.88	19.36	16.44	11.99	21.30	10.13	9.24	19.17
C16:1	0.29	2.38	1.07	1.80	0.61	2.20	0.76	1.76	0.93	2.72	0.45	2.34	0.58	1.75	0.58	2.08
C18:1	1.39	4.38	3.39	3.42	2.28	3.69	2.99	3.10	3.18	3.46	1.73	2.94	2.58	2.42	2.20	4.08
C20:1	1.53	7.23	4.01	7.52	2.20	6.68	3.09	6.74	4.01	5.79	2.33	4.49	2.66	4.21	2.88	7.86
C22:1		0.19	1.26	0.33	0.51	0.28	3.66	0.23	6.18	0.30		0.11		0.15	0.63	0.22
C24:1n9	0.97	0.48	10.75	0.27	3.43	0.32	9.59	0.22	3.66	0.22	8.50	0.48	4.96	0.33	3.63	1.29
T. MUFA	4.18	14.66	20.48	13.34	9.03	13.17	20.08	12.05	17.96	12.50	13.01	10.36	10.78	8.86	9.92	15.53
C18:2		0.67	0.35	0.65	0.56	0.59		0.60	0.36	0.85	0.26	0.66	0.69	0.52	0.04	0.72
C18:3n6	0.15	0.92	0.24	0.89	0.43	1.07	0.15	0.75	0.55	0.79	0.82	0.84	1.05	0.69	0.46	0.96
C18:3n3		0.19		0.55	0.24	0.40		0.51		0.64	0.24	0.23		0.29		0.33
C20:2	0.12	1.59	0.58	1.73	0.62	1.90	0.46	1.60	0.76	1.46	0.46	1.23	1.01	1.26	1.66	2.08
C20:4n6	0.80	3.64	1.86	4.10	1.23	3.77	1.85	3.16	1.95	3.23	1.16	2.71	1.72	2.20	1.38	3.84
C22:2	1.07	0.59	4.92	0.63	12.45	0.52	7.62	0.50	12.01	0.82	8.75	0.43	2.25	0.41	1.95	0.71
T. PUFA	2.14	7.60	7.95	8.55	15.53	8.25	10.08	7.12	15.63	7.79	11.69	6.10	6.72	5.37	5.49	8.64
EPA	1.50	5.11	2.30	4.98	2.19	5.28	2.43	4.17	4.59	6.22	2.25	4.59	3.28	4.06	2.45	5.14
DHA	6.80	9.29	34.23	8.91	16.22	7.90	17.04	7.09	27.28	8.68	21.22	6.16	27.49	5.66	14.94	9.44
T. HUFA	8.29	14.40	36.53	13.89	18.41	13.18	19.47	11.26	31.87	14.90	23.47	10.75	30.77	9.72	17.39	14.58
N	18	21	20	21	21	21	18	21	20	21	21	21	19	21	20	21

Table 5. FA profile (mg/g of biomass) of small size *S. plana* exposed to terbuthylazine.

	Ctl		C1		C2		C3		C4		C5		C6		C7		C8	
FA	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
C14:0	0.34	1.02	1.01	2.36	0.50	2.52	1.84	1.86	0.36	3.03	0.68	3.21	0.38	1.61	1.13	2.75	0.73	2.63
C15:0	0.52	1.21	0.99	2.24	0.75	2.78	0.95	1.90	0.59	2.92	1.52	2.64	0.46	1.83	1.16	2.56	0.66	2.26
C16:0	5.04	11.74	8.43	13.31	5.77	37.59	13.96	18.44	5.13	30.08	7.58	27.73	3.21	16.54	12.87	25.45	6.10	22.72
C17:0	0.56	0.72	0.69	1.91	0.69	2.58	1.66	1.79	1.12	3.05	0.75	2.90	0.37	1.45	1.95	2.36	0.54	1.23
C18:0	2.91	7.76	5.07	14.38	2.64	20.23	6.85	13.75	3.01	22.52	4.40	18.87	1.97	10.83	7.09	17.58	3.51	15.94
C20:0	3.66	8.64	6.10	12.28	2.08	21.15	8.58	14.10	3.45	19.04	4.82	12.11	2.20	11.81	7.21	14.78	3.54	14.69
C23:0	14.23	3.37	24.59	5.39	7.63	8.72	38.64	5.06	18.12	7.43	43.77	6.28	8.13	5.90	44.85	6.95	63.14	5.56
C24:0	7.26	1.31	31.63	0.66	8.11	1.03	30.96	2.02	35.40	2.18	23.70	6.17	17.24	3.86	34.95	3.58	37.53	1.12
T. SFA	34.52	35.77	78.51	52.53	28.17	96.60	103.44	58.92	67.19	90.25	87.22	79.91	33.96	53.83	111.21	76.01	115.75	66.15
C16:1	1.68	4.17	2.60	6.44	1.68	7.04	3.72	5.38	1.42	8.02	3.16	8.70	0.85	5.18	4.28	6.38	1.99	5.98
C18:1	6.27	7.69	9.96	13.60	5.90	19.39	16.08	12.04	6.22	18.68	8.94	9.07	3.42	12.13	14.10	15.15	6.52	14.57
C20:1	7.97	15.49	11.63	27.17	6.52	41.33	19.07	27.30	6.97	40.52	11.78	28.59	4.32	25.05	11.51	32.15	8.33	30.93
C22:1		0.79	12.98	1.00	2.64	1.97	10.98	0.92	16.57	1.64	4.40	1.23		0.99	14.04	1.29	6.86	1.16
C24:1n9	6.23	0.79	9.25	0.72	5.33	0.58	29.18	0.73	10.45	0.28	19.35	2.84	3.44	3.32	14.83	1.33	47.61	0.12
T. MUFA	22.15	28.93	46.42	48.93	22.07	70.31	79.03	46.37	41.63	69.14	47.63	50.43	12.03	46.67	58.76	56.30	71.31	52.76
C18:2	0.50	1.55	1.29	2.72	0.20	3.86	1.62	2.19	0.50	3.81	0.58	2.86	0.82	2.17	2.05	2.82	1.37	2.60
C18:3n6	0.47	1.57	1.64	2.76	1.44	4.78	4.11	3.20	1.20	4.47	4.01	4.39	1.18	3.80	3.00	4.00	0.85	3.68
C18:3n3		1.21		1.49		2.67		1.60		2.78		2.46		1.67		2.18		1.64
C20:2	1.27	2.94	2.84	5.63	0.94	9.16	2.72	6.68	1.65	7.99	2.20	9.17	0.77	5.53	4.00	5.47	1.41	8.89
C20:4n6	3.63	7.13	6.02	10.82	2.89	17.75	9.56	11.72	4.00	16.85	6.41	14.41	1.75	10.18	9.22	15.26	4.61	12.63
C22:2	12.64	1.44	25.56	2.10	13.32	4.11	65.30	2.42	33.13	3.27	22.56	3.53	7.50	2.50	31.70	2.86	39.44	2.09
T. PUFA	18.51	15.84	37.35	25.52	18.79	42.33	83.31	27.81	40.48	39.17	35.76	36.82	12.02	25.85	49.97	32.59	47.68	31.53
EPA	6.21	9.43	9.85	12.54	7.14	21.15	14.67	12.80	6.41	20.91	12.79	16.74	3.63	13.09	13.76	17.56	29.36	16.64
DHA	48.30	17.85	84.81	24.65	43.68	42.53	113.71	34.45	63.66	39.68	74.41	30.88	41.02	41.75	106.07	40.00	157.55	29.52
T. HUFA	54.51	27.28	94.66	37.19	50.82	63.68	128.38	47.25	70.07	60.59	87.20	47.62	44.65	54.84	119.83	57.56	186.91	46.16
N	19	21	20	21	20	21	20	21	20	21	20	21	20	21	21	21	20	21

Annexe 2. Fatty acid trophic markers.

Table 1. FATMs (mg/g of biomass) of *S. plana* from field and from S-metolachlor exposure.

	Field				Ctl				C1		C2		C3				C4			
	Big		Small		Big		Small		Small		Small		Big		Small		Big		Small	
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
DHA/EPA	1.93	2.28	3.66	2.04	2.44	1.44	3.04	0.73	2.12	1.66	2.75	2.01	4.20	1.47	3.05	2.11	2.64	1.50	2.69	3.83
EPA	7.12	5.95	5.22	9.30	12.09	3.11	9.59	23.82	8.55	12.82	12.18	15.82	9.93	5.67	22.90	34.27	36.42	4.22	23.73	9.71
C16:1	2.20	4.65	1.10	6.25	1.90	0.90	1.85	2.25	1.05	2.46	1.76	3.67	1.14	1.52	3.21	7.79	6.87	1.52	4.37	3.26
C16:0	14.03	8.40	10.61	35.22	14.71	2.64	13.39	8.77	9.59	12.61	15.68	16.01	14.92	4.42	26.54	34.74	36.66	4.34	26.86	10.17
C18:1n9	10.76	5.07	6.19	27.51	12.69	1.73	10.78	6.32	6.80	8.57	10.16	10.16	13.07	3.25	18.71	23.10	35.97	2.69	25.40	6.98
C18:2n6	0.73	1.06	0.73	7.98	0.78	0.29	2.44	1.28	2.38	1.62	1.22	2.08	0.82	0.56	3.09	4.21	5.72	0.57	3.35	1.40
Σ(C15-C17)	1.45	1.22	1.73	5.98	1.64	1.15	2.46	1.71	1.45	1.86	2.78	3.00	2.97	0.93	4.02	4.85	18.12	0.80	4.07	1.84

Table 1 *(continued)*

	C5				C6				C7				C8			
	Big		Small		Big		Small		Big		Small		Big		Small	
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
DHA/EPA	2.31	1.45	2.51	2.38	1.93	1.62	2.40	2.59	2.48	1.72	2.67	1.82	2.53	1.85	1.31	1.76
EPA	44.61	5.44	12.65	14.20	33.48	2.48	12.68	11.70	14.92	4.48	19.67	21.21	25.71	6.85	17.09	22.67
C16:1	6.94	1.60	1.33	3.98	4.65	1.12	1.74	3.30	2.72	1.41	2.63	5.09	3.73	1.50	2.54	7.75
C16:0	40.61	4.19	13.87	19.86	29.21	2.06	13.15	12.33	18.94	4.39	21.73	23.21	24.02	5.92	16.82	28.13
C18:1n9	35.81	2.83	9.78	12.75	29.78	1.28	10.19	8.44	18.46	3.02	17.19	16.98	25.30	3.79	14.52	17.13
C18:2n6	4.58	0.59	1.33	2.08	2.19	0.38	1.18	1.53	1.38	0.50	2.99	3.45	4.11	0.71	2.47	3.44
Σ(C15-C17)	7.73	0.79	2.50	3.28	5.48	0.37	2.35	2.18	3.56	0.87	3.86	3.88	4.48	1.15	2.68	5.27

Table 2. FATMs (mg/g of biomass) of *S. plana* from terbuthylazine exposure.

	Ctl				C1				C2				C3				C4			
	Big		Small		Big		Small		Big		Small		Big		Small		Big		Small	
	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V	M	V
DHA/EPA	4.53	1.82	7.78	1.89	14.91	1.79	8.61	1.97	7.39	1.50	6.11	2.01	7.01	1.70	7.75	2.69	5.95	1.40	9.92	1.90
EPA	1.50	5.11	6.21	9.43	2.30	4.98	9.85	12.54	2.19	5.28	7.14	21.15	2.43	4.17	14.67	12.80	4.59	6.22	6.41	20.91
C16:1	0.29	2.38	1.68	4.17	1.07	1.80	2.60	6.44	0.61	2.20	1.68	7.04	0.76	1.76	3.72	5.38	0.93	2.72	1.42	8.02
C16:0	1.11	6.54	5.04	11.74	2.44	5.33	8.43	13.31	1.81	4.21	5.77	37.59	2.08	4.57	13.96	18.44	2.55	5.25	5.13	30.08
C18:1n9	1.39	4.38	6.27	7.69	3.39	3.42	9.96	13.60	2.28	3.69	5.90	19.39	2.99	3.10	16.08	12.04	3.18	3.46	6.22	18.68
C18:2n6		0.67	0.50	1.55	0.35	0.65	1.29	2.72	0.56	0.59	0.20	3.86		0.60	1.62	2.19	0.36	0.85	0.50	3.81
Σ(C15-C17)	0.42	1.26	1.08	1.93	1.12	1.00	1.67	4.15	0.49	0.80	1.44	5.36	0.46	0.93	2.61	3.69	0.50	0.91	1.70	5.97

Table 2. (*continued*)

	C5				C6				C7				C8	
	Big		Small		Big		Small		Big		Small		Big	
	M	V	M	V	M	V	M	V	M	V	M	V	M	V
DHA/EPA	9.43	1.34	5.82	1.84	8.38	1.39	11.29	3.19	6.11	1.84	2.28	7.71	5.37	1.77
EPA	2.25	4.59	12.79	16.74	3.28	4.06	3.63	13.09	2.45	5.14	17.56	13.76	29.36	16.64
C16:1	0.45	2.34	3.16	8.70	0.58	1.75	0.85	5.18	0.58	2.08	6.38	4.28	1.99	5.98
C16:0	1.40	4.58	7.58	27.73	2.15	3.57	3.21	16.54	1.59	6.04	25.45	12.87	6.10	22.72
C18:1n9	1.73	2.94	8.94	9.07	2.58	2.42	3.42	12.13	2.20	4.08	15.15	14.10	6.52	14.57
C18:2n6	0.26	0.66	0.58	2.86	0.69	0.52	0.82	2.17	0.04	0.72	2.82	2.05	1.37	2.60
Σ(C15-C17)	0.46	0.63	2.28	5.54	0.38	0.64	0.83	3.28	0.39	1.26	4.92	3.11	1.19	3.49