Ecological Indicators 53 (2015) 220–230

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

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)

M. Neves [a], B.B. Castro [a], T. Vidal [a], R. Vieira [a], J.C. Marques [b,c], J.A.P. Coutinho [d], F. Gonçalves [a], A.M.M. Gonçalves [a,b,c,*]

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

ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form 12 January 2015
Accepted 18 January 2015

Keywords:
Herbicide
S-metholaclor
Daphnia longispina
Life history parameters
Fatty acid analysis
Multi-generational effects

ABSTRACT

The growing demand of human populations for food supplies has led to an increase in the use of synthetic products, mainly pesticides, which induce adverse effects not only to target organisms, but also to non-target biota of agroecosystems. Aquatic ecosystems in the proximity of agricultural areas are particularly vulnerable to pesticides, which cause underperformance or extinction of non-target sensitive species. Once in the aquatic system, these chemicals can affect biological processes at multiple levels (molecular, individual, populational), causing ecosystem imbalance across multiple scales. In this study, the effect of a commercial formulation of a herbicide (Primextra® Gold TZ) and its main active ingredient (a.i., *S*-metolachlor) was studied on a freshwater cladoceran species (*Daphnia longispina*), at different levels of biological organization and temporal scales. *S*-metolachlor is used in many herbicide formulations applied in corn/maize cultures, which is a relevant culture worldwide. As a first step, the acute and chronic effects of both commercial formulation and a.i. were quantified, and both formulations negatively affected the cladoceran's survival and reproductive parameters (age at first reproduction, number of offspring and number of broods), as well as the population's rate of increase. Whilst acute effects were comparable, the commercial formulation was slightly more toxic (EC_{50} was two-times lower) than the a.i. in chronic exposures, being prejudicial to *D. longispina* populations above 4.0 mg/L of *S*-metolachlor. In a second experimental step, we focused on the potential multi-generational impacts of the exposure to the a.i. alone on biochemical (lipid biomarkers, namely fatty acids) and populational responses, because of the relevance of *S*-metolachlor as a biosynthesis inhibitor in many herbicidal formulations. The herbicide caused a significant decrease in *Daphnia* fecundity (in the size of the 1st clutch), but no concomitant alterations were found in fatty acid profiles of mothers or offspring. More important, this experiment showed that *S*-metolachlor did not cause effects in the subsequent generation, thus suggesting that biotic communities may recover after exposure to the xenobiotic.

1. Introduction

Pesticides are commonly used worldwide to face world's population need, increasing and improving agricultural food production by reducing crop losses both before and after harvest, and increase crop yields (Alavanja, 2009). Although the application of plant protection products occurs in the terrestrial environment, the ultimate receptors of many these xenobiotics are the aquatic ecosystems. The contamination in aquatic systems can occur via aerial spraying, leaching, runoff or accidental spills (Vidal et al., 2012). The ecotoxicological effects on plant protection products affect multiple levels of biological organization, from the molecular level to the ecosystem level (Pereira et al., 2009; Vidal et al., 2012). A proper comprehension of these xenobiotics' multiple scales action may help to understand how they affect non-target organisms, allowing

* Corresponding author at: University of Coimbra, Department of Life Sciences, IMAR-Marine and Environmental Research Centre (IMAR CMA) & MARE - Marine and Environmental Sciences Centre, 3004-517 Coimbra, Portugal.
Tel.: +351 239 855760; fax: +351 239823603.
E-mail addresses: ammendes@student.zoo.uc.pt, anamartagoncalves@ua.pt (A.M.M. Gonçalves).

http://dx.doi.org/10.1016/j.ecolind.2015.01.031
1470-160X/

an improved prediction on the potential changes caused in the ecosystem structure and functioning (Clements, 2000).

In the pursuit for the most efficient pesticide, there is a wide availability plant protection products that act against agricultural pests and that have the potential to affect non-target soil and aquatic biota. One of such xenobiotics is the pesticide Primextra® Gold TZ, manufactured by Syngenta AG, which is widely used for controlling weeds that emerge before corn plantules. Primextra® Gold TZ is composed of two active ingredients, *S*-metolachlor and terbuthylazine, which are used by Syngenta AG in other commercial formulations (Bicep II Magnum®, Gardo Gold®, Primagram Gold® and Primextra Gold®) used worldwide. Commercial formulations comprehend active ingredients plus coadjuvant substances (supposedly inert) that can potentiate the effects of the isolated active ingredients (Axelrad et al., 2002; Cedergreen and Streibig, 2005; Pereira et al., 2009; Dobšíková et al., 2011). It is therefore important, in terms of predictive risk assessment, to assess the effects of both active ingredients and commercial formulations.

S-metolachlor, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(1*S*)-2-methoxy-1-methylethyl]acetamide, is the active ingredient that makes up the majority of the herbicide being studied, and this compound is a potential danger to the environment and aquatic ecosystems (Liu et al., 2006). It is a component of many other herbicides and its use is still 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. According to USDA (1997) and Liu et al. (2004), metolachlor is the most widely used herbicide both in China and USA. It is part of the family of chloroacetamides (Kegley et al., 2011), whose mode of action consists in inhibiting several biological processes (essentially biosynthesis) acting on meristematic zones of plants (Karam et al., 2003). It 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). It also affects other important pathways that are responsible for the transformation of geranylgeranyl pyrophosphate in β-carotene and α-carotene (Cunningham et al., 1996), which leads to plants with higher predisposition to oxidative stress (Warner and Frankel, 1987). According to Rivard (2003) and Liu et al. (2006), *S*-metolachlor can also be moderately toxic to aquatic animals. Given the xenobiotic's mode of action, it is suggested that this active ingredient could affect the lipid (fatty acids—FA) profile of aquatic species.

Fatty acids are necessary for the production and permeability of cell membrane, and they are the main components of lipids (Pasquaud et al., 2007). They also act as gene transcription factors (Benatti et al., 2004; Davidson et al., 2009), activators of cellular pathways (e.g. protein kinase C; Stahl, 2004), and they are used as fuel in all metabolic systems at all trophic levels, having an important role on neural levels of biochemical and physiological response (Arts et al., 2009). Polyunsaturated fatty acids (PUFA) are a family of lipids that contain some subgroups identified by the position of the last double bond in their structure and includes many important compounds, such as essential fatty acids (EFA). Although the terms "PUFA" and "EFA" are not synonymous, they are often used interchangeably since many biological functions of EFAs are exerted by EFA–derived PUFAs. Brett and Müller-Navarra (1997) pointed out that PUFA are almost exclusively synthesized by plants, with animals being able of converting PUFA by elongation or desaturation, and only a few could synthesize this type of fatty acids. They also pointed that PUFA play an important role in the organism, regulating cell membranes properties, serving as precursors of important hormones and being essential to the organisms. Highly unsaturated fatty acids (HUFA) are nutritional key constituents of zooplankton diet and may determine the energetic efficiency across the plant–animal interface, in aquatic pelagic food webs (Perhar and Arhonditsis, 2012). Demott and Müller-Navarra (1997) demonstrated that zooplankton fed with high amounts of HUFA presented higher growth rate, thus strengthening the importance of fatty acids as ecophysiological indicators.

Zooplankton has long been used as a suitable group to assess the impact of environmental change, in part due to its key intermediate position in the trophic food web. Zooplanktonic organisms are frequently used as indicators of water quality due to the close relationship between environmental factors and species composition. Cladocerans, one of the most abundant primary consumer groups in lentic freshwater ecosystems, are good indicators of environmental changes (e.g. water quality and/or historical differences), especially regarding xenobiotic effects (Pereira et al., 2009; Vidal et al., 2012). *Daphnia longispina* (O. F. Müller, 1776) is a widespread and common species in Europe, often found in Portuguese freshwaters (mainly in reservoirs and lacustrine systems). This species plays a key role in the food web structure, as a link between primary producers (phytoplankton) and higher rank consumers (namely fish). Some authors (e.g. Colomer, 1996) have emphasized its potential as an indicator species in terms of water quality.

The present study investigated the toxicity of the herbicide Primextra® Gold TZ and its main active ingredient (a.i. *S*-metolachlor) in a model freshwater zooplankter, *D. longispina*, at different levels of biological organization and across temporal scales. The simultaneous testing of a.i. and commercial formulation is an approach that may guarantee more comprehensive and robust support for regulators and competent authorities. Thus, short and chronic exposures to the commercial formulation and a.i. were conducted incorporating relevant ecological receptors and standard ecotoxicological endpoints: (1) immobilisation and (2) reproductive and populational parameters of *D. longispina*. We additionally hypothesized that, because of *S*-metolachlor's interference with biosynthesis pathways, it could elicit effects at the molecular level, with potential carryover effects in offspring fitness. Few studies have addressed this so far. Our aim was to (1) identify qualitative and quantitative changes in fatty acid profiles of *D. longispina* after exposure to *S*-metolachlor and (2) to evaluate potential multi-generational post-exposure effects (relating lipid profile and population responses). To do this, we only focused on the main a.i. which is present in many herbicidal formulations. This work thus provides an integrated approach towards a more realistic assessment on the overall impacts of *S*-metolachlor on sensitive bioindicators.

2. Materials and methods

2.1. Daphnid cultures

Monoclonal cultures of *D. longispina* (clone EM7, *sensu* Antunes et al., 2003) were reared continuously in the lab as synchronized cohorts, under a $16\,h^{L}{:}8\,h^{D}$ photoperiod, at a temperature of $20 \pm 2\,^{\circ}C$, in synthetic ASTM hard water medium (ASTM 1980) supplied with an organic additive (described in Baird et al., 1989). Culture medium was renewed every other day and the organisms were fed with *Pseudokirchneriella subcapitata* at a ration of 1.5×10^5 cell/mL every two days. For further details on rearing procedures, see Antunes et al. (2004) and Loureiro et al. (2011, 2012).

2.2. Acute tests

Tests were performed according to standard protocols (ISO, 1996; OECD, 2000; EPA, 2002) under the same temperature and photoperiod regimes as described for rearing procedures. *D. longispina* experiments were initiated with neonates (<24 h old)

obtained from bulk cultures, born between the 3rd and 5th broods. The pesticide and active ingredient solutions were obtained by successive dilutions of a stock solution of Primextra® Gold TZ or *S*-metolachlor in distilled water. Based on literature data and preliminary trials, we used concentrations ranging from 8.00 to 28.13 mg/L *S*-metolachlor for Primextra® Gold TZ and from 11.5 to 26.5 mg/L for *S*-metolachlor. The culture medium was used as the negative control treatment. Tests were carried out in glass test tubes (four per treatment) containing 10 mL of test solutions. In both assays, a static design was employed, using 20 animals (randomly divided into four groups of five animals) per control and per toxicant concentration. Daphnids were exposed to the different toxicant concentrations during 48 h without food or organic extract. Vessels were checked for immobilized individuals, at 24 h and 48 h. Probit analysis (Finney, 1971) was used to estimate the concentration which caused 50%, 20% and 10% immobilization (EC_{50}, EC_{20} and EC_{10}) of daphnids in acute tests, and corresponding 95% confidence intervals.

2.3. Chronic tests

Chronic tests (Primextra® Gold TZ and *S*-metolachlor) were conducted for 21 days in accordance with standardized protocols (OECD, 1998, 2000; ISO, 2000; EPA, 2002). Each chronic assay was composed by 7 treatments (one negative control plus six nominal concentrations of respective toxicant) with a concentration range of 2.50 to 5.03 mg of *S*-metolachlor/L for the commercial formulation and 4.00 to 12.21 mg *S*-metolachlor/L. Each treatment consisted in ten glass beakers, with one daphnid per beaker, which were filled with 50 mL of test medium. Daphnids were fed daily with *P. subcapitata* at a ration of 1.5×10^5 cell/mL and transferred to freshly prepared test solutions every other day. For further details on test procedures with daphniids, including algal ration, see Gonçalves et al. (2007), Antunes et al. (2003, 2004) and Loureiro et al. (2011, 2012). Cladocerans were checked every day at the same approximated hour for mortality and reproductive state. When neonates were released, they were counted and discarded. Non-linear regression was used to estimate the concentration that caused 50%, 20% and 10% reduction in fecundity (EC_{50}, EC_{20} and EC_{10}) of daphnids in chronic tests, as well as corresponding 95% confidence intervals. A life history table was built with the data from the chronic assays, and the following parameters were determined: age at first reproduction, number of released broods, number of released neonates (fecundity), and per capita intrinsic rate of increase (r, day^{-1}). The latter was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where x stands for age class (d), l_x is the probability of surviving to age x, and m_x represents age-specific fecundity. This demographic parameter was calculated after pooling the life-history data of all individuals in each tested concentration; thus, for statistical purposes, replicate pseudo-values for r were generated with the jack-knifing technique described by Meyer et al. (1986).

2.4. Population (microcosm) tests

S-metolachlor (2-Chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(1*S*)-2-methoxy-1-methylethyl] acetamide—Table 1) was acquired from Sigma-Aldrich (Germany). Stock solutions of the herbicide were prepared in distilled water before each medium change. The appropriate amounts of the stock solution were introduced into culture medium to produce the final concentrations of the toxicity test.

The experimental procedure used for testing the effects of *S*-metolachlor in *Daphnia* populations had two different phases (Fig. 1). In phase 1, 40 *Daphnia* neonates (F_0 generation, <24 h old, born between the third and fifth broods of stock cultures) were exposed in glass beakers with 400 mL of corresponding test solution, in four experimental treatments: (1) a negative control, consisting of uncontaminated culture medium; (2) a low level of *S*-metolachlor (3.33 mg/L), corresponding to the LOEC value obtained in reproduction tests; (3) an intermediate level (5.00 mg/L), which corresponds to the reproductive EC_{20}; (4) a high level (7.50 mg/L), which is close to the reproductive EC_{50} (8.24 mg/L). All treatments were replicated six times, with the glass beaker as the experimental unit. Daphnids were fed daily with *P. subcapitata* at a ration of 0.75×10^5 cell/mL and transferred to freshly prepared test solutions every three days. Beakers were checked every day at the same approximated hour for mortality and reproductive state (presence of offspring). Neonates from the first clutch were counted and isolated for FA analysis (Fig. 1); individuals of two beakers were pooled in a single sample for fatty acid (FA) analysis, resulting in a total of 3 samples. Neonates from the second clutch were counted and transferred to new beakers, thus starting phase 2 (F_1 generation—Fig. 1). After all mothers had released the second clutch, they were also isolated for FA analysis (Fig. 1); again, individuals of two beakers were pooled in a single sample for fatty acid (FA) analysis, resulting in a total of 3 samples.

Phase 2 was similar to phase 1, except that organisms (F_1 generation) were allowed to grow and reproduce in control (uncontaminated) conditions, after maternal exposure to the four levels of *S*-metolachlor in the previous generation (phase 1). To do so, daphniids from each phase 1 glass beaker originated new experimental populations, initiated with 40 neonates from the second clutch of the F_0 mothers (Fig. 1). As in phase 1, all treatments were replicated six times, with each glass beaker constituting a replicate. Daphnids were fed at the same ration, and medium renewal occurred at the same periodicity. Similarly to phase 1, beakers were checked every day at the same approximated hour for mortality and reproductive state (presence of offspring), whilst FA analysis was performed in offspring from the first clutch and in mothers that had released their second clutch (Fig. 1). Again, individuals of two beakers were pooled in a single sample for fatty acid (FA) analysis, resulting in a total of 3 samples.

For both phases, the following population parameters were determined in each of the experimental units ($n = 6$): survival, number of *N1* offspring, total number of offspring, and per capita intrinsic rate of increase (r, day^{-1}). The latter was iterated from the Euler–Lotka equation:

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x,$$

where x stands for age class (d), l_x is the probability of surviving to age x, and m_x represents age-specific fecundity.

2.5. FA analysis

For FA analysis, individuals were isolated (neonates or adult females) in clean medium, and samples were concentrated in VWR glass microfibers filters (1.2 μm pores) and frozen at −80 °C. For each experimental treatment, 3 replicates were sampled, each containing 200 neonates or 68 adult females.

The extraction of total lipids of cladocerans and methylation to fatty acid methyl esters (FAMEs) was achieved by a modified one step derivatisation method, after Gonçalves et al. (2012). The fatty acid methylnonadecanoate C19:0 was added as an internal standard for later quantification (Fluka 74208). Samples were then centrifuged in a Thermo Scientific Heraeus Megafuge 16 R stored and frozen in new vials. Samples were subjected to gas chromatography (GC) for separation and quantification of FAMEs. This was

Table 1
Data on *S*-metolachlor.

CAS number	Empirical formula	Molecular weight	Water solubility	Adsorption Coefficient
87392-12-9	$C_{15}H_{22}ClNO_2$	283.79	480.00 mg/L	185.00 Koc

Source: http://www.sigmaaldrich.com/portugal.htm; Kegley et al. (2011).

performed in a Capillary GC (Varian CP-3800) coupled with a flame ionization detector (FID), in a split injection system with a biodiesel for FAME column (30 m × 0.32 mm × 0.25 μm). The column temperature was set at 120 °C and then programmed to increase up to 250 °C at a ratio of 4 °C/min. The detector and injector were set at 250 °C. The carrier gas was helium at a flow rate of 2 mL/min.

2.6. Statistical analysis

One-way analysis of variance (ANOVA), followed by Dunnett's test, was applied to each endpoint of the chronic assays to assign statistical differences between the concentrations tested and the control.

In the microcosm test, fecundity data and the rate of increase (r) were analyzed with a two-way ANOVA, using generation (=phase) and *S*-metolachlor concentration as factors. A significant interaction between both factors means that the potential effect of the herbicide differs between generations. When this was the case, the effect of *S*-metolachlor concentration was also analyzed for each generation separately. To do so, we used a one-way ANOVA – followed by a Dunnett test – to discriminate significant differences relatively to the control.

The FA profiles of *D. longispina* (neonates and adults females) were reported for each treatment by determining their total (mg/ind) or relative (%) concentrations, and analyzed with principal component analysis (PCA), using a correlation matrix. This ordination technique allows reducing a complex multivariate matrix to a few dimensions, without assuming an underlying data structure (ter Braak, 1995). Ultimately, PCA reduces the FA profiles to interpretable bidimensional plots that explained the highest proportion of variation in the data (following ter Braak, 1995). Three-way ANOVA were then performed on the PCA sample scores to assess significant differences among generation (F_0 vs. F_1 or phase 1 vs. phase 2), developmental stage or age (adult females vs. offspring), and *S*-metolachlor concentration, as well as their interactions. This provided an assessment of the sources of variation for the overall FA profile, including the effect of *S*-metolachlor on the FA profiles of *D. longispina*. Similar approaches have been described in the literature for other physiological data matrices (e.g. Loureiro et al., 2013; Correia et al., 2014). In order to explore potential differences in terms of specific FA, individual three-way ANOVAs (generation × age × concentration) were also employed using the proportional relative abundance of selected FAs.

All analyses were conducted with the software Minitab (v16) and the significance level used was 0.05. Prior to ANOVAs, data were analyzed for potential outliers, non-normality and heteroscedasticity with the aid of residual plots. Most variables satisfied the ANOVAs statistical assumptions; in the few cases of no compliance (e.g. AFR and number of broods), data transformation was unable to solve the problem. For coherence purposes, we simply assumed the ANOVA was robust enough to cope with the deviations in these few cases.

3. Results

3.1. Acute and chronic assays

The acute immobilization tests showed that *D. longispina* was slightly more tolerant to the commercial formulation than its active ingredient (a.i.). In fact, the acute EC_{50} value of the a.i. [18.71 (18.19–19.25) mg/L] was lower than the corresponding value for the commercial formulation [23.53 (20.60–28.90) mg/L], despite being very close. However, this was not observed for EC_{20} and EC_{10}, and the overall tendency was for a comparable acute toxicity between a.i. and formulation. These results are based in nominal concentrations. To allow proper comparison, we always refer to concentrations in mg/L of *S*-metolachlor, independently of presenting the results for the commercial formulation or a.i. (Table 2).

Commercial formulation and the a.i. caused a significant effect on the life history of *D. longispina*, affecting all parameters (Figs. 2 and 3). Indeed, a significant reduction in fecundity and a developmental delay (increase in age at first reproduction), as well as a significant decrease in the number of broods produced per female was observed. As a consequence of the lower fecundity and

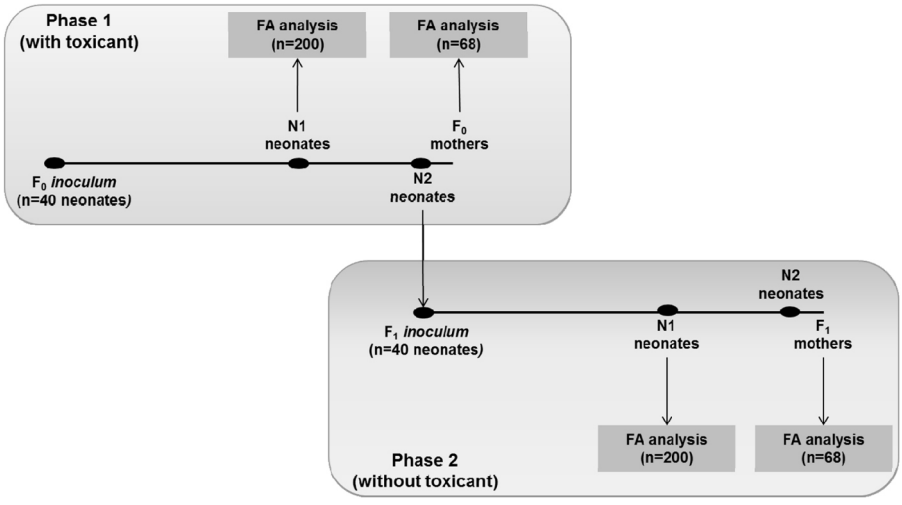

Fig. 1. Experimental design used for assessing potential multi-generational effects of *S*-metolachlor in population parameters and FA profiles.

Table 2
Acute EC_{10}, EC_{20} and EC_{50} values of Primextra® Gold TZ and *S*-metolachlor for *D. longispina*, and respective 95% confidence limits (in brackets). The acute EC_{10}, EC_{20} and EC_{50} values were derived from nominal concentrations.

Tested compound		Total active ingredients	*S*-metolachlor
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{10}	15.69 mg/L (5.56–20.91)	9.81 mg/L (3.48–13.06)
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{20}	23.23 mg/L (16.98–27.56)	14.52 mg/L (10.61–17.23)
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{50}	37.65 mg/L (32.89–46.21)	23.53 mg/L (20.60–28.90)
S-metolachlor	EC_{10}	12.73 mg/L (11.78–13.50)	
S-metolachlor	EC_{20}	14.78 mg/L (14.06–15.40)	
S-metolachlor	EC_{50}	18.71 mg/L (18.19–19.25)	

of the developmental delay, the intrinsic rate of increase (r) was also significantly reduced (Figs. 2 and 3). The number of offsprings was the most affected parameter, being drastically reduced with the increase of the herbicide and the a.i. mainly by the latter one.

Mortality was low (below or equal to 10%) with the commercial formulation, except at 7.00 mg/L (30%). In the case of the a.i. with increasing toxicant concentration, increasing mortality was observed. In the first three *S*-metolachlor treatments (4.00, 5.00 and 6.25 mg/L) there was 10% mortality, which increased to 30% at 7.81 mg/L, 40% at 9.77 mg/L, and 60% in the 12.21 mg/L treatment. In average, mortality started on the fourteenth day of the chronic assay.

The reproductive EC_{50} was two-times lower for the commercial formulation than the a.i. (Table 3). Therefore, *D. longispina* was more sensitive to the commercial formulation, contrary to the acute tests.

3.2. Microcosms experiments

The overall mortality of the microcosms experiments was very low (<10%). Consistent differences were found among generations (=experimental phases), with generation F_0 displaying a higher performance in terms of fecundity and rate of increase (Fig. 4). On the contrary, no significant differences were found for total fecundity or rate of increase between concentrations on both phases (Fig. 4). However, a significant interaction between generation and concentration was found in the fecundity of the first clutch. Indeed, there was a significant decrease of the number of neonates per female in the highest concentration of the assay (7.50 mg/L *S*-metolachlor) for phase 1 (F_0 generation), whereas no effects of *S*-metolachlor were observed in F_1 generation (Fig. 4).

The abundance (in %) of FA extracted from the sample is referred in Table 4. In general, the percentage of FA was higher in offsprings and mothers from F_0 than from F_1, mainly at the lower concentrations of *S*-metolachlor, being stronger on saturated fatty acids (SFA). We also observed higher percentage of FA with longer carbon chain and double bonds (e.g. MUFA, PUFA and HUFA) than FA with shorter carbon chain (e.g. SFA with shorter carbon chain). The total percentage of MUFA was higher in F_1 than in F_0, mainly at lower concentrations of *S*-metolachlor.

PCA revealed some differences in fatty acid abundances among samples, as seen by the data scatter in Fig. 5. However, these differences were inconsistent among experimental treatments (Fig. 6). Indeed, three-way ANOVAs revealed that no significant differences were found in PCA scores across generations, organism age, or *S*-metolachlor concentration (Table 5). Three-way ANOVAs applied to essential FA data (EPA and DHA) also showed inconclusive results about the effects of *S*-metolachor in this specific group of

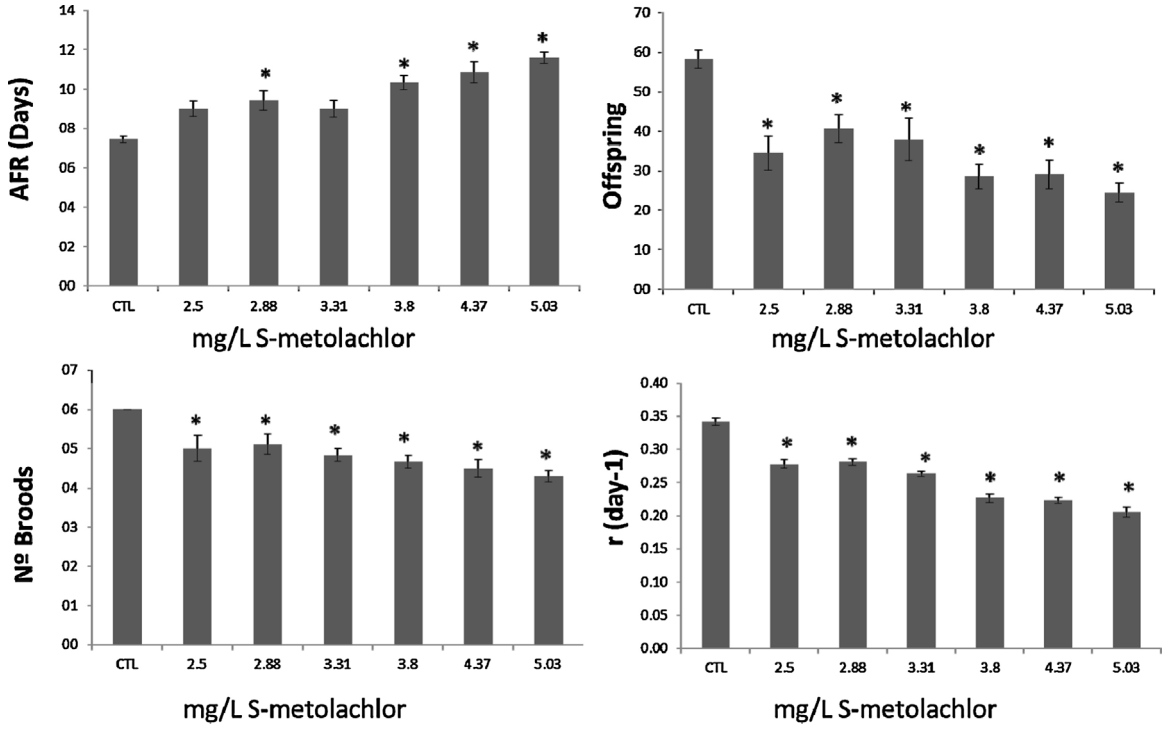

Fig. 2. Effects of the commercial formulation Primextra® Gold TZ on reproductive and populational parameters (age at first reproduction—AFR, total number of offspring, intrinsic rate of population increase (r), number of broods) monitored in the 21 d chronic assay with *Daphnia longispina*. In each case, the mean and respective standard error are presented, as well as significant differences from control (CTL). Concentrations of commercial formulation are presented in mg/L of *S*-metolachlor.

Table 3
Chronic EC_{10}, EC_{20} and EC_{50} values of Primextra® Gold TZ and *S*-metolachlor for *D. longispina*, and respective 95% confidence limits (in brackets). The chronic EC_{10}, EC_{20} and EC_{50} values were derived from nominal concentrations.

Tested compound		Total active ingredients	*S*-metolachlor
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{10}	4.94 mg/L (3.59–6.29)	3.09 mg/L (2.24–3.93)
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{20}	6.34 mg/L (5.34–7.35)	3.96 mg/L (3.34–4.59)
Primextra® GOLD TZ (*S*-metolachlor + terbuthylazine)	EC_{50}	6.58 mg/L (6.16–7.11)	4.11 mg/L (3.85-4.44)
S-metolachlor	EC_{10}	3.84 mg/L (2.89–4.79)	
S-metolachlor	EC_{20}	5.09 mg/L (4.19–5.98)	
S-metolachlor	EC_{50}	8.24 mg/L (7.38–9.10)	

FA (Table 6). Thus, we did not find any evidence that *S*-metolachlor could cause changes in the FA profile of *D. longispina*, nor that it could induce multi-generational effects, contrary to the alterations observed in the fecundity at the first clutch (found in the highest concentration).

4. Discussion

The purpose of the study was to determine the toxicity of the herbicide Primextra® Gold TZ and *S*-metolachlor (a.i.) for the species *D. longispina*, going beyond classical ecotoxicology by

Table 4
Fatty acid abundance mean values expressed in % for each *S*-metolachlor concentration (in mg/L), age (O stand for offspring and M stand for the mothers) and generation F_0 (light gray) and F_1 (dark gray).

Phase		Phase 1 (with toxicant—F_0)								Phase 2 (without toxicant—F_1)							
Concentration (in mg/L)		CTL		3.33		5.00		7.50		CTL		3.33		5.00		7.50	
Age		O	M	O	M	O	M	O	M	O	M	O	M	O	M	O	M
SFA	C6:0	0	0	1.75	0	0	0	0	0	0	0	0	0.1	0	0	0	0
	C8:0	0	0.0	3.13	0	0	1.1	0	0	0	0.3	0	0.1	1.2	0	0	0
	C10:0	0	0.0	0.53	0.3	0	0.7	0	0	0	0.1	0	0.1	0	0	0.3	0.07
	C12:0	1.7	0.0	0.49	0.2	0	0.2	0.03	0	0.02	0.2	0.6	0.1	0.1	0	0.1	1.1
	C13:0	0	0.0	0.05	0.1	0.1	0.1	0.06	0	0	0.2	0.4	0.1	0.1	0	0.2	0.1
	C14:0	2.6	0.0	1.93	0.1	0	0.02	0	0	0.07	0.3	0.2	0.1	0.1	0.02	0.3	0.1
	C15:0	0	0.1	0.16	0.1	0	0	0	0	0.03	0.2	0.2	0.1	0.1	0.13	0.2	0.4
	C16:0	0.1	2.4	7.24	0	0	0.02	3	0.2	1.5	13.4	11.8	2.7	17	0.03	6.2	0.4
	C17:0	2	0.5	0.87	0.6	0.2	0.1	0.4	0.1	0.1	0.3	1.1	2.2	0.4	0.08	0.5	0.6
	C18:0	1.5	3.1	2.13	6	0.7	1.5	0.3	2.5	0.8	3.3	2.4	0.4	0.4	0.3	1.8	0.6
	C20:0	2.2	9.8	0.93	22.4	11.1	15.3	9.4	23.4	12.9	4.7	2.4	6.3	8.9	15.9	3.1	11.5
	C21:0	0.8	14.0	0.29	0.8	1	1.9	0.8	4.8	1	1.4	2.5	0.4	5.4	1	2.5	2.6
	C22:0	0.7	1.5	0.85	0.3	0.5	1.1	0.4	0.5	0.7	0.2	0.4	1	3.9	0.3	0.3	1.2
	C23:0	1.7	2.2	2.82	2.8	0.8	0.5	3	0.6	2.2	0.6	2.8	4.2	0.4	1.4	1.2	1.9
	C24:0	2.6	0.7	4.07	0.3	0.3	1.6	0.9	1.6	0.2	2.2	2.9	2.3	1.3	3.5	10.6	1.8
	Total % of SFA	15.9	34.2	27.24	34	14.7	24.14	18.29	33.7	19.52	27.4	27.7	20.2	39.3	22.66	27.3	22.37
MUFA	C14:1	0	0.3	0.47	0.2	0.03	0.02	0.2	0	0.04	0.8	0.9	0.003	0.1	0.01	0.4	0.1
	C15:1w5(cis10)	0	0.1	0.13	0.2	0.1	0	0.08	0.06	0	0.1	0	0.05	0.1	0.02	0.04	0.3
	C16:1 (cis-9)	2.5	0.5	1.15	0.8	1.6	0.2	0.4	0.04	0.2	2	1.4	0.1	0.6	0.1	0.9	0.5
	C17:1w7(cis10)	3.1	0.3	1.12	0.7	0.1	1.6	0.5	0.9	0.5	1.4	0.2	0.9	0.6	0.2	0.2	1.1
	C18:1n9t	0.4	0.8	0.81	0.1	1.4	1.6	1.1	0.9	1.3	2.2	1.3	5.4	0.7	0.7	0.8	1.4
	C20:1n9(cis11)	0.5	4.0	0.02	2.9	7.2	17.6	6.4	12.4	17	6.1	13.6	8.7	8.6	17.7	3.3	12.6
	C22:1n9	5.3	0.5	1.09	1.1	0.2	1	1.5	0.2	0.2	1.3	0.9	0.6	1.1	0.3	0.7	0.9
	C24:1n9	7.3	4.4	2.90	3.4	1.7	0.6	2.4	1.2	1	6.2	15.3	1.5	1.4	3.4	2.4	2.9
	Total % MUFA	19.1	11.0	7.70	9.4	12.3	22.6	12.6	15.7	20.2	20.1	33.6	17.3	13.2	22.4	8.7	19.8
PUFA	C18:2n6c	0.1	1.8	1.29	0.1	0.6	3.7	0	1.7	0.3	1.3	0.5	3	0.9	1.4	0.02	1.6
	C18:2n6t	0	0.7	0.45	0.1	0.2	0.6	0.3	0.7	0.7	2.9	1.1	0	0	18.4	0	3.9
	C18:3n6	29.3	8.3	18.88	28.5	20.4	0.4	10.9	0.1	21	19.3	0	12.9	25.1	0.6	34.8	0.04
	C18:3n3	17.4	16.8	36.81	19.1	26.1	7.2	32.9	19.2	12	17.5	18.8	28.9	4.4	11.8	11.8	40.2
	C20:2w6	2.8	2.9	0.91	1.9	5.7	14	5.9	8.1	14.1	2.9	3.6	6.3	4.5	8.3	8	5.3
	C20:3n6	0.3	3.6	4.34	4.7	9.1	11.5	4	7.2	3.5	1.3	1.4	1.6	3.1	11	2.4	2
	C20:3n3	8.2	4.5	0.00	1	3	5.6	7.4	6.7	3.9	2.7	6.4	2.5	2.8	0.6	2.2	2.2
	Total% PUFA	58.1	38.6	62.68	55.4	65.1	43.0	61.4	43.7	55.5	47.9	31.8	55.2	40.8	52.1	59.2	55.2
HUFA	C20:4n6	0.7	4.9	0.04	0.4	3.7	4.2	3.1	1.8	2.4	3.4	3.4	4.6	2.3	1	2.2	0.5
	C20:5(EPA)	1	2.1	1.17	0.7	3.4	4	2.8	3.7	2.1	0.8	1.6	1	5.2	0.7	2.1	1.5
	C22:6(DHA)	5.3	6.1	2.82	0.7	0.8	2.1	1.8	1.3	0	0.6	2	2.3	0.5	1.2	0.9	1
	Total % HUFA	7	13.1	4.03	1.8	7.9	10.3	7.7	6.8	4.5	4.8	7	7.9	8	2.9	5.2	3
	Total %	100.1	96.9	101.65	100.6	100.0	100.1	99.97	99.9	99.8	100.2	100.1	100.6	101.3	100.09	100.5	100.4
	n	25	33	34	31	27	31	28	26	28	33	29	33	31	29	31	32

Table 5
Summary of three-way ANOVA on the fatty acid profiles (principal components 1 and 2—PC1 and PC2), showing the degrees of freedom (d.f.), variance (MS), *F* test and corresponding *P* value.

Data	Source of variation	Fatty acids scores for PC1				Fatty acids scores for PC2			
		d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Analysis of the FA profiles	Concentration	3	10.4	0.79	0.179	3	3.45	0.79	0.512
	Generation	1	0.150	0.62	0.875	1	2.72	0.62	0.438
	Age	1	6.71	0.05	0.298	1	0.24	0.05	0.817
	Concentration × generation	3	6.85	0.14	0.346	3	0.61	0.14	0.936
	Concentration × age	3	3.86	0.17	0.592	3	0.728	0.17	0.919
	Generation × age	1	5.55	1.26	0.433	1	5.55	1.26	0.270
	Concentration × generation × age	3	2.48	0.56	0.339	3	2.48	0.56	0.643
	Residual	29	5.967			29	4.397		

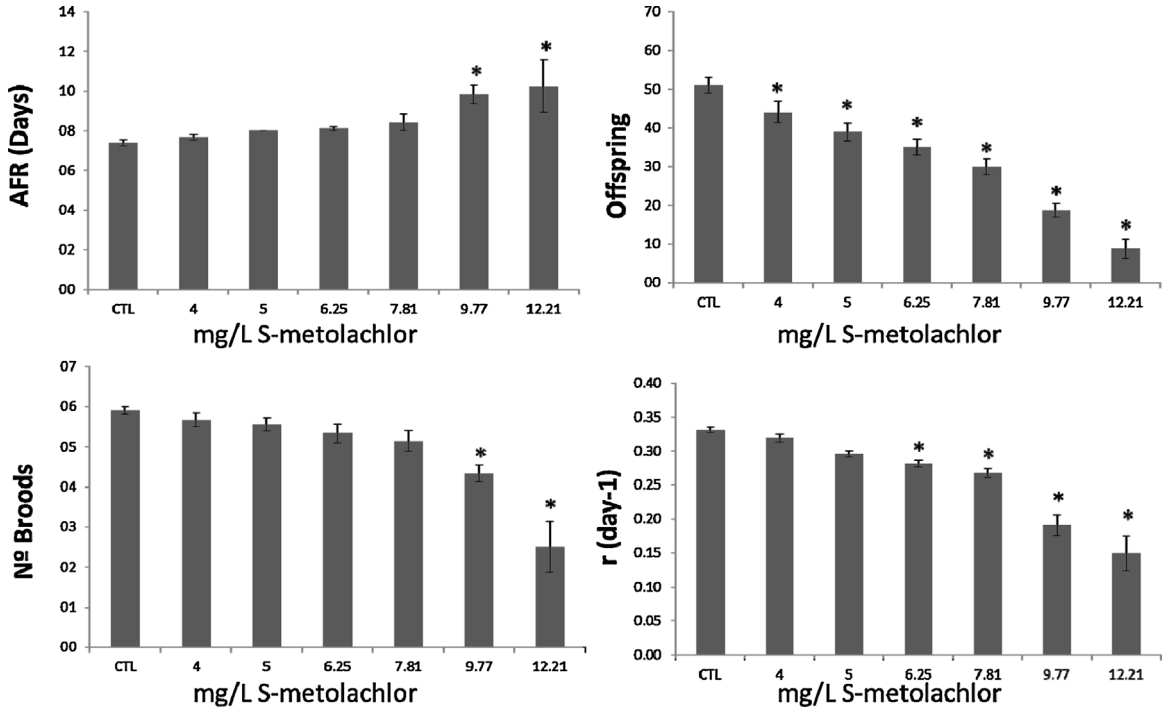

Fig. 3. Effects of the active ingredient *S*-metolachlor on reproductive and populational parameters (age at first reproduction—AFR, total of offspring, intrinsic rate of population increase (*r*), number of broods) monitored in the 21 d chronic assay with *Daphnia longispina*. In each case, the mean and respective standard error are presented, as well as significant differences from the control (CTL). Concentrations of active ingredient are presented in mg/L of *S*-metolachlor.

assessing the potential biochemical and multigenerational effects of the a.i. This species is ubiquitous in European aquatic ecosystems (lakes and reservoirs) and is a keystone species due to its role as grazer and transparency regulator (Castro and Gonçalves, 2007). Given the importance and area occupied by maize/corn crops, it is likely that this species, as well as other potentially sensitive ecoreceptors, are exposed to this xenobiotic. This study demonstrated deleterious effects of the herbicide in terms of fecundity reduction, but showed that neither FA profiles were affected by this xenobiotic nor it caused carry-over effects to the next generation, although a few nuances deserve further attention.

The acute assays showed overall similar toxicity between a.i. and commercial formulation, and both values were comparable to the *S*-metolachlor EC_{50} value found in the literature (25.1 mg/L for *D. magna*—Rivard, 2003). On the other hand, the chronic assay revealed that the formulation was more toxic than the isolated a.i. In both assays, the commercial formulation was expected to be more toxic, because of the presence of other toxic substances,

Table 6
Summary of three-way ANOVA on fatty acids (EPA and DHA), showing the degrees of freedom (d.f.), variance (MS), *F* test and corresponding *P* value.

Data	Source of variation	Fatty acids scores for EPA				Fatty acids scores for DHA			
		d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Analysis of EPA and DHA profiles	Concentration	3	0.00109	1.49	0.238	3	0.00145	0.71	0.556
	Generation	1	0.000200	0.28	0.604	1	0.00517	2.51	0.124
	Age	1	0.000416	0.57	0.455	1	0.000028	0.01	0.908
	Concentration × generation	3	0.000146	0.20	0.895	3	0.00203	0.98	0.414
	Concentration × age	3	0.000255	0.35	0.789	3	0.000881	0.43	0.734
	Generation × age	1	0.00136	1.87	0.182	1	0.000377	0.18	0.672
	Concentration × generation × age	3	0.000269	0.37	0.775	3	0.000753	0.37	0.778
	Residual	29	0.00073			29	0.002058		

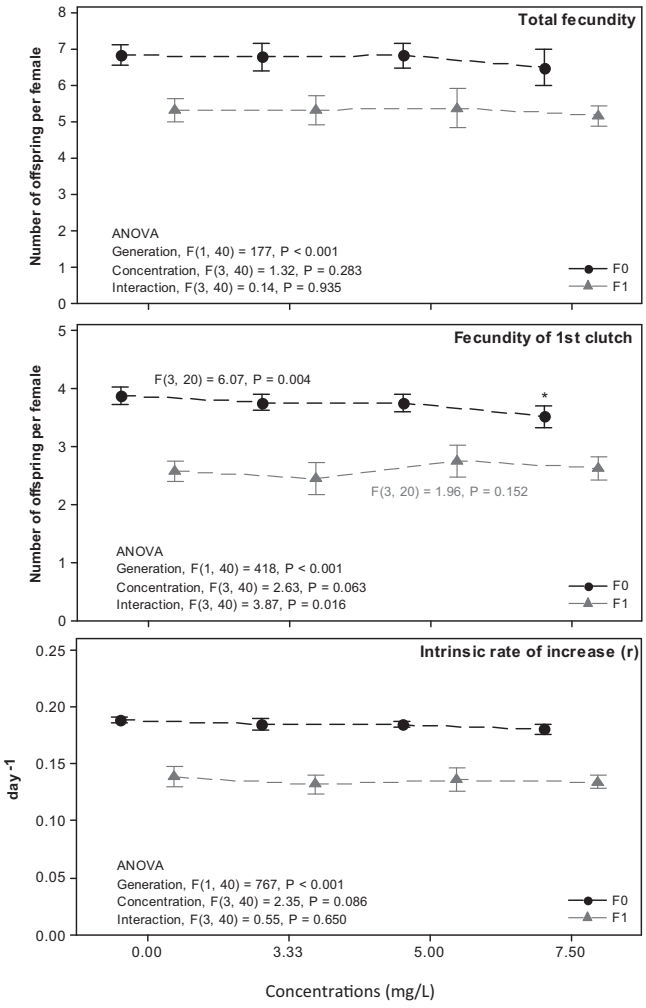

Fig. 4. Effects of the active ingredient *S*-metolachlor on reproductive and populational parameters of *D. longispina* (total fecundity, fecundity at 1st clutch and intrinsic rate of increase (*r*)) in two distinct experimental phases (see Fig. 3). Black circles represent data from *S*-metolachlor-exposed animals (see concentrations in *x* axis, in mg/L)—phase 1 or F_0 generation–and their offspring—phase 2 or F_1 generation (gray triangles). In each case, the mean and respective 95% confidence intervals are presented, as well as associated significance (ANOVA results and significant differences from the control, depicted with *).

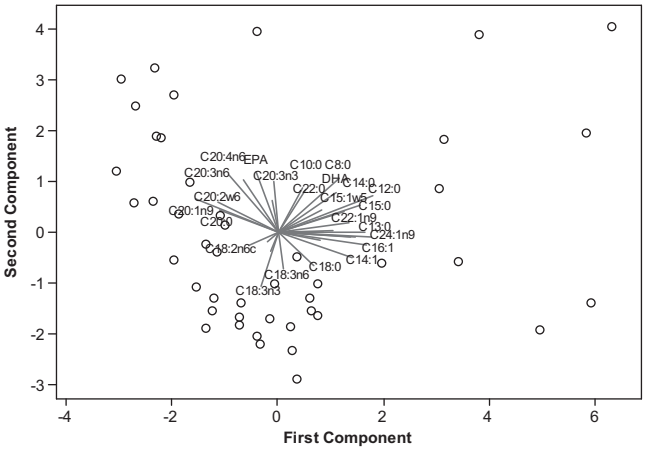

Fig. 5. PCA biplot of fatty acid profile of *D. longispina* illustrating their variation. Open circles depict samples (=experimental units) and arrows represent fatty acids.

such as terbuthylazine (herbicide) and coadjuvants (Pereira et al., 2009). Since most active ingredients are insoluble in water, the formulations of pesticides are also composed by a variety of solvents and potentiators where any component may interact with the other. Theoretically, these interactions may lead to potentiation effects (synergism) or weakening of the effects (antagonism) (Axelrad et al., 2002), or no effects. In our case, the contradictory results (acute vs. chronic) may be explained by concentration effects (Pérez et al., 2011a), since the ratio of concentrations is very important in the definition of their joint toxicity, as shown for combinations of *S*-metolachlor and terbuthylazine (Pérez et al., 2011a; Dobšíková et al., 2011). The acute and chronic EC_{50} values were derived from nominal, rather than effective (measured) concentrations. Because deviations between effective and nominal concentrations are common, these data are to be interpreted with some reserve. Nonetheless, this does not impair the present study, as its scope is mostly on the biological effects of the studied xenobiotic across multiple scales, rather than on the definition of quality criteria or legislator benchmarks.

Reproduction toxicity thresholds presented here were slightly lower than the value presented in literature (10 mg/L for *D. magna*—Liu et al., 2006) but much higher, for example, than the values obtained for the aquatic plant *Lemna gibba* (0.023 mg/L). The higher sensitivity of *L. gibba* is explained by the target mechanism of the a.i. a weed control agent that acts upon on meristematic zones of plants inhibiting biosynthesis of vital molecules (including lipids). Despite the generally lower sensitivity of animals to herbicides, it is important to look at potential side-effects caused by these xenobiotics in non-target aquatic fauna (e.g. Tatum et al., 2012). Aromatic anilides are a good example of herbicide-induced side effects (Pereira et al., 2007, 2009): although they are inhibitors of a specific pathway in autotrophs (involved in the electron transport chain of photosystem II), they cause reproductive impairment in daphniids. Indeed, 3,4-dichloroanilide (a herbicide) is recommended as a reference substance in toxicity tests with *Daphnia* (Loureiro et al., 2011). Other known examples include glyphosate, a fairly recent herbicide, which causes biochemical and behavioral alterations in invertebrates and vertebrates (Pérez et al., 2011b).

S-metolachlor can disrupt two mechanisms: protein synthesis and fatty acid (FA) metabolism (Rivard, 2003). The disruption of two separated and very important metabolic pathways can explain the low fecundity observed (Perrat et al., 2013) and the reduced fitness (measured as the intrinsic rate of population increase, *r*). On the other hand, toxicants may exert effects indirectly by altering algal fitness (ergo food quality), which then affects the performance of daphniids (Corbi and Corradi, 1993; Evens et al., 2012). This is especially relevant for a herbicide such as *S*-metolachlor, which is specifically designed to exert effects on autotrophs. However, studies support this phenomenon of culture algae in contaminated media (Corbi and Corradi, 1993; Evens et al., 2012), in our case, the residence time of algae in the herbicide-contaminated water column is too short to produce biochemical changes in the algae (daphnids only have a few hours to feed before the algae cells sink, and fresh food is provided daily). Assuming that this is not the case, we must therefore assume that the impacts of *S*-metolachlor in daphniids are direct effects, which may include subtle changes in FA or protein metabolism or feeding depression (Agatz et al., 2013). Such imbalances may be linked to populational effects, a hypothesis we tested with the multigenerational experimental set-up.

The populational results of phase 1 (F_0) showed a less toxic scenario than expected, taking into account the EC values obtained in the 21 d reproduction test. However, while reproduction tests were conducted in ten individualized replicate experimental units (with only one individual per 50 mL glass beaker), in this experiment larger beakers were used (400 mL) and organisms were exposed within a simulated population (40 individuals per beaker).

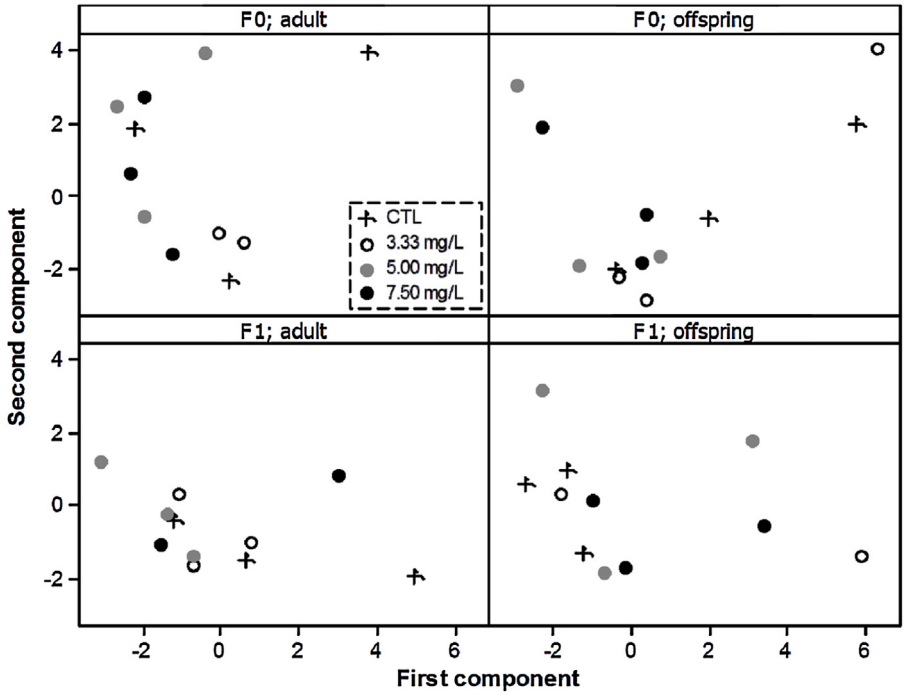

Fig. 6. PCA biplot of fatty acid profile of *D. longispina* illustrating their variation according to *S*-metolachlor concentrations (see legend), developmental stage (or age) of organisms (adult vs. offspring), and experimental phase or generation (phase 1 = F_0 generation; phase 2 = F_1 generation).

A putative density-dependent effect could be expected; still, several authors prefer assessing the effects of pesticides in mesocosm simulations, as they provide a more realistic assessment of noxious effects (e.g. Wendt-Rasch et al., 2003). Alternatively, our results could be due to a better performance of the batch of daphnids used in this experiment in comparison to those used in the chronic test. Unfortunately, this can sometimes happen as a consequence of food quality fluctuations. Despite this fact, there was a significant decrease in the fecundity in the first clutch in the highest concentration, thus confirming the toxicity of *S*-metolachlor. There was also an underperformance in the F_1 generation, for all treatments. This could be explained by phenomena of overpopulation in the glass beakers (Carvalho and Hughes, 2006). Because of the large number of animals necessary for FA analysis, we had to stock the beakers with a large number of mothers (in this case, 40 individuals for each 400 mL glass beaker). However, irrespective of these variations, the effect elicited by *S*-metolachlor was not perceptible in phase 2 of the experiment, i.e. there were no reproductive or populational effects of *S*-metolachlor in the subsequent generation. Several studies provide data about substances (mainly endocrine substances) with multi-generational effects (Brennan et al., 2006; Clubbs and Brooks, 2007; Dietrich et al., 2010; Marteinson et al., 2010). Brennan et al. (2006) showed that diethylstilbestrol and 4-nonylphenol (estrogens) can lead to multi-generational effects, such as lower number of offspring per clutch and altered moulting in posterior generations. The fact that the a.i. tested does not promote multi-generational effects is important from an ecological perspective, because it is indicative of full recovery of the cladoceran populations in the absence of the toxicant.

Results from the microcosm experiment could not link fecundity reduction to alterations in FA profiles. Although FA profiles of *D. longispina* were not altered by *S*-metalochlor, the percentage of FA was higher in organisms (neonates and mothers) exposed to *S*-metalochlor (F_0) than those not exposed to the toxicant (F_1). *S*-metolachor is a chloroacetamide used as pre-emergent herbicide that acts by inhibiting the biosynthesis of several molecules (most noticeable, proteins and lipids). Since cladocerans do not synthesize their own FA and take them up from food (algae), it is therefore unlikely that *S*-metolachlor could directly alter their FA profiles. Nonetheless, it could affect FA profiles through indirect ways (e.g. triglycerides) (Rico-Martínez et al., 2012). For example, some FA are bioconverted by daphnids and other consumers (Dalsgaard et al., 2003). FA are known to be of paramount importance to daphnids (Brett and Müller-Navarra, 1997), including embryo and neonate development (Ahlgren et al., 1990; Mourente and Odriozola, 1990; Forró et al., 2008; He et al., 2013). In particular, there are essential fatty acids (EFA) that must be acquired in the diet of heterotrophic organisms, such as unsaturated fatty acids of the omega 3 (EPA and DHA) and omega 6 (linoleic acid and arachidonic acid) groups (Watanabe et al., 1984; Bell et al., 1986; Dalsgaard et al., 2003). Despite the ability to produce EPA and DHA by conversion of linoleic acid (C18:2n6t), this capacity is insufficient to support high growth and reproduction rates in zooplankters (Dalsgaard et al., 2003). Therefore, when the nutritional uptake of long chain FA is already low, the effects of *S*-metolachlor could lead to lower performance of zooplankters.

According to Cerejeira et al. (2003), the amount of metolachlor found in Portuguese surface waters is very low (0.056 mg/L). Therefore, the concentrations used in our study could correspond to a catastrophic contamination event or sporadic event of herbicide application. The values reported by Cerejeira et al. (2003) surpassed the European threshold (0.0001 mg/L) for chloroacetamides in drinking water (Peña et al., 2013), which justifies further interest in this xenobiotic, particularly because of the widespread use of *S*-metolachlor in numerous herbicide formulations for weed-control in corn/maize cultures. Because tested concentrations were high, relatively to analytical records in natural waters, we must conclude that the observed levels are fairly safe in environmental terms. However, this type of studies must continue to elucidate the potential mechanisms of this type of biosynthesis-inhibitor agents in the biochemical profiles of non-target organisms, and associated potential ecological consequences. The experimental microcosm

design here-presented brings added-value and could be used in future studies for understanding multigenerational effects, in terms of both populational and biochemical/physiological endpoints.

5. Conclusion

Primextra® Gold TZ, which has *S*-metolachlor in its composition, has proven to be more toxic than *S*-metolachlor alone, since reproductive parameters were altered by lower equivalent concentrations of the commercial formulation. This is valid for lower, and more environmentally relevant concentrations of the xenobiotic (i.e. concentrations detected in the environment near areas of discharges of these compounds), as this (the alteration of reproductive parameters) was not observed for acute exposures. This allows us to say that Primextra® Gold TZ can endanger the aquatic ecosystems in the surroundings of agriculture fields by affecting important non-target species that live in freshwaters. *S*-metolachlor presents the ability of disturbing fatty acid and protein metabolism and further studies should be made to investigate whether *S*-metolachlor can lead to changes in the biochemical profiles of aquatic organisms.

The present study reveals important data about the toxic effects of *S*-metolachlor and one of its commercial formulations. It is important to notice that this herbicide is widely used in maize/corn crops (among others) in Portugal and other EU countries. Its application in agroecosystems with neighboring aquatic systems may represent a hazard for indigenous freshwater invertebrate species (such as *D. longispina*). This study also provides data about the effect of *S*-metolachlor on the FA profiles and their importance as ecophysiological indicators. Molecular biomarkers can provide ecophysiological data about metabolic changes, before the occurrence of populational effects. Several protein (Rocha and Souza, 2012; Badiou-Bénéteau et al., 2012), lipid (Lerch et al., 2011; Orhan et al., 2013), and fatty acids (Perrat et al., 2013) biomarkers have been used to assess the effects of xenobiotics on metabolic pathways. When combined with other ecophysiological indicators, such biomarkers could provide a better insight on the effects of xenobiotics in non-target organisms.

Acknowledgments

The authors are grateful for financial support from European Funds through COMPETE and by National Funds through the Portuguese Science Foundation (FCT) under the scope of the project PEst-C/MAR/LA0017/2013. The authors also thank FCT for financial support through the post-doctoral grants SFRH/BPD/79307/2011 and SFRH/BPD/97210/2013 of A.M.M. Gonçalves, SFRH/BPD/94562/2013 of T. Vidal and B.B. Castro's position (Ciência2008 contract), all of them co-funded by the Human Potential Operational Programme (National Strategic Reference Framework 2007-2013) and European Social Fund (EU).

References

Agatz, A., Cole, T.A., Preuss, T.G., Zimmer, E., Brown, C.D., 2013. Feeding inhibition explains effects of imidacloprid on the growth, maturation, reproduction, and survival of *Daphnia magna*. Environ. Sci. Technol. 47, 2909–2917.

Ahlgren, G., Lundstedt, L., Brett, M., Forsberg, C., 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. J. Plankton Res. 12 (4), 809–818.

Alavanja, M.C.R., 2009. Pesticides use and exposure extensive worldwide. Rev. Environ. Health 24 (4), 303–309.

Antunes, S.C., Castro, B.B., Gonçalves, F., 2003. Chronic responses of different clones of *Dapnhia longispina* to different food-levels. Acta Oecol. 823, S325–S332.

Antunes, S.C., Castro, B.B., Gonçalves, F., 2004. Effect of food level on the acute and chronic responses of daphnids to lindane. Environ. Pollut. 127, 367–375.

Arts, M.T., Brett, M.T., Kainz, M.J., 2009. Lipids in Aquatic Ecossystems. Springer, New York, NY.

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, 259–268.

Badiou-Bénéteau, A., Carvalho, S.M., Brunet, J.L., Carvalho, G.A., Buleté, A., Giroud, B., Belzunces, L.P., 2012. Development of biomarkers of exposure to xenobiotics in the honeybee *Apis mellifera:* application to the systemic insecticide thiamethoxam. Ecotoxicol. Environ. Saf. 82, 22–31.

Bell, M.V., Henderson, R.J., Sargent, J.R., 1986. The role of polyunsaturated fatty acids in fish. Comp. Biochem. Physiol. 83B, 711–719.

Benatti, P., Peluso, G., Nicolai, R., Calvani, M., 2004. Polyunsaturated fatty acids: biochemical, nutritional and epigenetics properties. J. Am. Coll. Nutr. 23, 281–302.

Brennan, S.J., Brougham, C.A., Roche, J.J., Forgaty, A.M., 2006. Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*. Chemosphere 64, 49–55.

Brett, M.T., Müller-Navarra, D.C., 1997. The role of highly unsaturated fatty acids in aquatic foodweb processes. Freshwater Biol. 38, 483–499.

Carvalho, G.R., Hughes, R.N., 2006. The effect of food availability, female culture-density and photoperiod on ephippia production in *Daphnia magna* Straus (Crustacea: Cladocera). Freshwater Biol. 13 (1), 37–46.

Castro, B.B., Gonçalves, F., 2007. Seasonal dynamics of the crustacean zooplankton of a shallow eutrophic lake from the Mediterranean region. Fundam. Appl. Limnol.—Archiv für Hydrobiologie 169, 189–202.

Cedergreen, N., Streibig, J., 2005. The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard. Pest Manage. Sci. 61, 1152–1160.

Cerejeira, M.J., Viana, P., Batista, S., Pereira, T., Silva, E., Valério, M.J., Silva, A., Ferreira, M., Silva-Fernandes, A.M., 2003. Pesticides in Portuguese surface and ground waters. Water Res. 37, 1055–1063.

Clements, W., 2000. Integrating effects of contaminants across levels of biological organization: an overview. J. Aquat. Ecosyst. Stress Recovery 7 (2), 113–116.

Clubbs, R.L., Brooks, B.W., 2007. *Daphnia magna* responses to a vertebrate estrogen receptor agonist and an antagonist: a multi-generational study. Ecotoxicol. Environ. Saf. 67 (3), 385–398.

Colomer, M.G.S., 1996. El uso del zooplancton como indicador biológico de la calidad del agua en 26 embalses españoles. Ingeniería Civil 105, 55–64.

Corbi, G., Corradi, M.G., 1993. Chromium toxicity on two linked trophic levels I. Effects of contaminated algae on *Daphnia magna*. Ecotoxicol. Environ. Saf. 25, 64–71.

Correia, B., Pintó-Marijuan, M., Neves, L., Brossa, R., Dias, M.C., Costa, A., Castro, B.B., Araújo, C., Santos, C., Chaves, M.M., Pinto, G., 2014. Water stress and recovery in the performance of two *Eucalyptus globulus* clones: physiological and biochemical profiles. Physiol. Plant 150, 580–592.

Cunningham Jr., F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D., Gantt, E., 1996. Functional analysis of the B and E lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8, 1613–1626.

Dalsgaard, J., St John, M., Kattner, G., Müller-Navarra, D., Hagen, W., 2003. Fatty acid trophic markers in the pelagic marine environment. Adv. Mar. Biol. 46, 225–340.

Davidson, L.A., Wang, N., Shah, M.S., Lupton, J.R., Ivanov, I., Chapkin, R.S., 2009. *n*-3 Polyunsatured fatty acids modulate carcinogen-directed non-coding microRNA signatures in rat colon. Carcinogenesis 12 (30), 2077–2084.

Demott, W.R., Müller-Navarra, D.C., 1997. The importance of highly unsaturated fatty acids in zooplankton nutrition: evidence from experiments with *Daphnia*, a cyanobacterium and lipid emulsions. Freshwater Biol. 38, 649–664.

Dietrich, S., Ploessl, F., Bracher, F., Laforsh, C., 2010. Single and combined toxicity of pharmaceuticals at environmentally relevant concentrations in *Daphnia magna*—a multi-generational study. Chemosphere 79 (1), 60–66.

Dobšíková, R., Blahová, J., Modrá, H., Škorič, M., Svobodová, Z., 2011. The effect of acute exposure to herbicide Gardoprim Plus Gold 500 SC on haematological and biochemical indicators and histopathological changes in common carp (*Cyprinus carpio* L.). Acta Vet. Brno 80, 359–363.

EPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. In: EPA-821-R-02-013, fourth ed. US Environment Protection Agency, Washington, DC, USA.

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. Aquat. Toxicol. 110–111, 9–16.

Finney, D.J., 1971. Probit Analysis. Cambrige University Press, Cambridge.

Forró, L., Korovchinsky, N.M., Kotov, A.A., Petrusek, A., 2008. Global diversity of *Cladocerans* (Cladocera; Crustacea) in freshwater. Hydrobiologia 595, 177–184.

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. Estuar Coast Shelf S 109, 70–80, http://dx.doi.org/10.1016/j.ecss.2012.05.020.

Gonçalves, A.M.M., Castro, B.B., Pardal, M.A., Gonçalves, F., 2007. Salinity effects on survival and life history of two freshwater cladocerans (*Daphnia magna* and *Daphnia longispina*). Ann. Limnol.—Int. J. Limnol. 43, 13–20.

He, H., Chen, G., Yu, J., He, J., Huang, X., Li, S., Guo, Q., Yu, T., Li, H., 2013. Individual and joint toxicity of three chloroacetanilide herbicides to freshwater cladoceran *Daphnia carinata*. Bull. Environ. Contam. Toxicol. 90, 344–350.

ISO, 1996. Water quality: determination of the inhibition of the mobility of *D.magna* Straus (Cladocera, Crustacea), Acute toxicity test. In: ISO International Standard 6341. International Organization for Standardization, Geneva, Switzerland.

ISO, 2000. Water quality: determination of long term toxicity of substances to Daphnia magna Straus (Cladocera, Crustacea). In: ISO International Standard 10706. International Organization for Standardization, Geneva, Switzerland.

Karam, D., Lara, F.R., Cruz, M.B., Filho, I.A.P., Pereira, F.T.F., 2003. Características do Herbicida S-Metolacloro nas Culturas de Milho e Sorgo in Circular Técnica. Ministério da Agricultura, Pecuária e Abastecimento, 36. Ministério da Agricultura, 65.

Kegley, S.E., Hill, B.R., Orme, S., Choi, A.H., 2011. PAN—Pesticide Database. Pesticide Action Network, North America, San Francisco, CA, ⟨http://www.pesticideinfo.org⟩.

Lerch, T.Z., Dignac, M.F., Barriuso, E., Mariotti, A., 2011. Effect of glucose on *Cupriavidus necator* JMP134 fatty acid composition during 2,4-D degradation: implications for lipid-based SIP methods. Appl. Environ. Microbiol. 77 (20), 1–39.

Liu, H.J., Liu, W.P., Chen, A.P., Yang, W.C., 2004. The reaction mechanism of metolachlor an *S*-isomer with urease. Spectrosc. Spectral Anal. 24, 166–168.

Liu, H., Ye, W., Zhan, X., Liu, W., 2006. A comparative study of rac- and *S*-metolachlor toxicity to *Daphnia magna*. Ecotoxicol. Environ. Saf. 63, 451–455.

Loureiro, C., Castro, B.B., Pereira, J.L., Gonçalves, F., 2011. Performance of standard media in toxicological assessments with *Daphnia magna*: chelators and ionic composition versus metal toxicity. Ecotoxicology 20 (1), 139–148.

Loureiro, C., Castro, B.B., Claro, M.T., Alves, A., Pedrosa, M.A., Gonçalves, F., 2012. Genetic variability in the tolerance of natural populations of *Simocephalus vetulus* (Müller, 1776) to lethal levels of sodium chloride. Ann. Limnol.–Int. J. Limnol. 48, 95–103.

Loureiro, C., Castro, B.B., Cuco, A.P., Pedrosa, M.A., Gonçalves, F., 2013. Life-history responses of salinity-tolerant and salinity-sensitive lineages of a stenohaline cladoceran do not confirm clonal differentiation. Hydrobiologia 702, 73–82.

Marteinson, S.C., Bird, D.M., Laird Schutt, J., Letcher, R.L., Ritchie, I.J., Fernie, K.J., 2010. Multi-generational effects of polybrominated diphenylethers exposure: embryonic exposure of male American kestrels (*Falco sparverius*) to DE-71 alters reproductive success and behaviors. Environ. Toxicol. Chem. 29 (8), 1740–1747.

Meyer, J.S., Ingersoll, C.G., McDonald, L.L., Boyce, M.S., 1986. Estimating uncertainty in population growth rates: Jackknife vs Bootstrap techniques. Ecology 67, 1156–1166.

Mourente, G., Odriozola, J.M., 1990. Effect of broodstock diets on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* L.). Fish Physiol. Biochem. 8 (2), 93–101.

OECD, 1998. *Daphnia magna* Reproduction Test. Test Guideline 211. Organization for the Economic Cooperation and Development, Paris, France.

OECD, 2000. *Daphnia* sp., Acute Immobilisation Test. Revised Proposal for Updating Guideline 202. Organization for the Economic Cooperation and Development, Paris, France.

Orhan, H., Sahin, A., Sahin, G., Aypar, U., Vermeulen, N.P.E., 2013. Urinary lipid and protein oxidation products upon halothane, isoflurane, or sevoflurane anesthesia in humans: potential biomarkers for a subclinical nephrotoxicity. Biomarkers 18 (1), 73–81.

Pasquaud, S., Lobry, J., Elie, P., 2007. Facing the necessity of describing estuarine ecosystems: a review of foodweb ecology study techniques. Hydrobiologia 588, 159–172.

Peña, D., Albarrán, Á., López-Piñeiro, A., Rato-Nunes, J.M., Sánchez-Llerena, J., Becerra, D., 2013. Impact of oiled and de-oiled olive mill waste amendments on the sorption, leaching, and persistence of *S*-metolachlor in a calcareous clay soil. J. Environ. Sci. Health 48B, 767–775.

Pereira, J.L., Mendes, C.D., Gonçalves, F., 2007. Short- and long-term responses of *Daphnia* spp. to propanil exposures in distinct food supply scenarios. Ecotoxicol. Environ. Saf. 68, 386–396.

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.

Pérez, J., Domingues, I., Soares, A.M.V.M., Loureiro, S., 2011a. Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva reservoir (Portugal): a bottom-up approach using binary mixtures. Ecotoxicology 20, 1167–1175.

Pérez, G.L., Vera, M.S., Miranda, L., 2011b. Effects of herbicide glyphosate and glyphosate-based formulations on aquatic ecosystems. In: Kortekamp, A. (Ed.), Herbicides and Environment. InTech, Rijeka, Croatia, pp. 343–368, Available from (http://www.intechopen.com/books/herbicides-and-environment/effects-of-herbicide-glyphosate-and-glyphosate-based-formulations-on-aquatic-ecosystems).

Perhar, G., Arhonditsis, G.B., 2012. Examination of the role of detritus food quality, phytoplankton intracellular storage capacity, and zooplankton stoichiometry on planktonic dynamics. Ecol. Inf. 11, 76–89.

Perrat, E., Couzinet-Mossion, A., Fossi Tankoua, O., Amiard-Triquet, C., Wielgosz-Collin, G., 2013. Variation of content of lipid classes, sterols and fatty acids in gonads and digestive glands of *Scrobicularia plana* in relation to environment pollution levels. Ecotoxicol. Environ. Saf. 90, 112–120.

Rico-Martínez, R., Arias-Almeida, J.C., Pérez-Legaspi, I.A., Alvardo-Flores, J., Retes-Pruneda, J.L., 2012. Adverse effects of herbicides on freshwater zooplankton. In: Hasaneen, M.N.A. (Ed.), Herbicides—Properties, Synthesis and Control of Weeds. , pp. 405–434, Available from ⟨http://www.intechopen.com/books/herbicides-properties-synthesis-and-control-ofweeds/adverse-effects-of-herbicides-on-freshwater-zooplankton⟩.

Rivard, L., 2003. Environmental Fate of Metolachlor. Department of Pesticide Regulation, 1001 I Street, Sacramento, CA 95812.

Rocha, C.T., Souza, M.M., 2012. The influence of lead on different proteins in gill cells from the freshwater bivalve, *Corbicula fluminea* from defense to repair biomarkers. Arch. Environ. Contam. Toxicol. 62, 56–67.

Stahl, A., 2004. A current review of fatty acid transport proteins (SLC27). Pflugers Ach.—Eur. J. Physiol. 447, 722–727.

Tatum, V.L., Borton, D.L., Streblow, W.R., Louch, J., Shepard, J.P., 2012. Acute toxicity of commonly used forestry herbicide mixtures to *Ceriodaphnia dubia* and *Pimephales promelas*. Environ. Toxicol. 27, 671–684.

Ter Braak, C.J., 1995. Ordination. In: Jongman, R.H., ter Braak, C.J., van Tongeren, O.F. (Eds.), Data Analysis in Community and Landscape Ecology. Cambridge University Press, Cambridge, pp. 91–173.

Trenkamp, S., Martin, W., Tietjen, K., 2004. Specific and differential inhibition of very-long-chain fatty acid elongases from *Arabidopsis thaliana* by different herbicides. Proc. Nat. Acad. Sci. U.S.A. 101 (32), 11903–11908.

USDA, 1997. The Acetanilide Herbicides: Alachlor, Metolachlor and Acetochlor. Office of Pesticide Program, Washington, DC.

Vidal, T., Abrantes, N., Gonçalves, A.M.M., Gonçalves, F., 2012. Acute and chronic toxicity of Betanal® Expert and its active ingredients in nontarget aquatic organisms from different trophic levels. Environ. Toxicol. 27 (9), 537–548.

Warner, K., Frankel, E.N., 1987. Effects of β-carotene on light stability of soybean oil. J. Am. Oil Chem. Soc. 64 (2), 213–218.

Watanabe, T., Arakawa, T., Kitajima, C., Fujita, S., 1984. Effect of nutritional quality of broodstock diet on reproduction of red sea bream. B. Jpn. Soc. Sci. Fish. 50 (3), 495–501.

Wendt-Rasch, L., Pirzadeh, P., Woin, P., 2003. Effects of metsulfuron methyl and cypermethrin exposure on freshwater model ecosystems. Aquat. Toxicol. 63, 243–256.