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Biochemical and toxicological effects of organic (herbicide Primextra® Gold TZ) and inorganic (copper) compounds on zooplankton and phytoplankton species

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ABSTRACT

In Europe, mainly in the Mediterranean region, an intensive usage of pesticides was recorded during the past 30 years. According to information from agricultural cooperatives of the Mondego valley (Figueira da Foz, Portugal), Primextra® Gold TZ is the most used herbicide in corn crop fields and one of the 20 best-selling herbicides in Portugal. Copper is mainly used in pesticide formulations. This study aims to determine the ecotoxicological and biochemical (namely fatty acid profiles) effects of the herbicide Primextra® Gold TZ and the metal copper on marine plankton. The organisms used in this study are three planktonic species: the marine diatom *Thalassiosira weissflogii*, the estuarine copepod *Acartia tonsa* and nauplii of the marine brine shrimp *Artemia franciscana*. Fatty acids (FAs) are one of the most important molecules transferred across the plant-animal interface in aquatic food webs and can be used as good indicators of stress. The conducted lab incubations show that *T. weissflogii* is the most sensitive species to the herbicide followed by *A. tonsa* (EC_{50} = 0.0078 mg/L and EC_{50} = 0.925 mg/L, respectively), whereas the copepod was the most sensitive species to the metal followed by *T. weissflogii* (EC_{50} = 0.234 mg/L and EC_{50} = 0.383 mg/L, respectively). *A. franciscana* was the most tolerant organism both to the herbicide and to the metal (EC_{50} = 20.35 mg/L and EC_{50} = 18.93 mg/L, respectively). Changes in the FA profiles of primary producer and primary consumers were observed, with the increase of saturated FA and decrease of unsaturated FA contents, especially of highly unsaturated FAs that can be obtained mainly from food and therefore are referred to as 'essential FA'. The study suggests that discharges of Primextra® Gold TZ or other pesticides mainly composed by copper may be a threat to plankton populations causing changes in the FA contents and thus in their nutritive value, with severe repercussions for higher trophic levels and thus the entire food web.

1. Introduction

Environmental pollution worldwide is an undesirable by-product of the increased demand for natural resources in modern civilization. However, since the advent of human societies, there have always been foci of environmental contamination, though nothing on the scale we see today. Practically all of the world's environments suffer from some degree of contamination in concentrations above those expected for the region (Bard, 1999). The pollutants that cause the most damage for the ecosystems are composed by pollutants from industries and mining that include toxic substances such as metals and organic pollutants. Little is known about how natural ecosystems respond to chronic and acute exposure to these contaminants, many of which, especially metals, are non-degradable and therefore accumulate in nature, where they continue to affect ecosystem functioning over the course of decades or even centuries. Thus, strategies to assess the effects of stressors on ecosystem functioning will need to take into consideration both old problems and new challenges. Anthropogenic pressures often decrease the health and stability of ecosystems, although the

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precise effects of these stressors on the biochemical components remain largely unknown. Despite the extensive literature under "anthropogenic pressures" issue, a more functional approach to trace changes in food webs due to the modified biochemical composition of interacting species is lacking so far (Silins and Högberg, 2011). These changes may have repercussions on the food quality and may play a key role to determine the pollution level of an aquatic system.

Under most conditions, stressors indirectly affect higher levels of the ecosystem hierarchy but directly affect processes at the biochemical and cellular levels.

In Europe, mainly in the Mediterranean region, there is an overexploitation of the farmlands that combined with an overuse of fertilizers and pesticides causes adverse effects on the surrounding aquatic systems. Since many estuaries are surrounded by farmland, residential and industrial areas, they are subject to various anthropogenic pressures and behaviors that cause ecological stresses, affecting not only the water quality, but also the biological communities of these ecosystems (Cardoso et al., 2008; Gonçalves et al., 2010a, 2016; Smalling et al., 2013). The intensive use of pollutants in agriculture areas near ecologically valuable coastal wetlands led to the implementation of the Pesticide-Monitoring programs to recover aquatic systems, such as in the Mondego estuary, Portugal, since 1998 (Galhano et al., 2011). Nowadays, and according to the information from agricultural cooperatives of Mondego valley, the herbicide Primextra® Gold TZ is the most used herbicide in corn crop fields and is one of the 20 best-selling herbicides in Portugal, whereas copper is mainly used in the constitution of pesticides (Gonçalves et al., 2016; Neves et al., 2015). Primextra® Gold TZ, produced by Syngenta AG, consists of two main active ingredients (a.i.), 17.75% (w/w) terbuthylazine (TBA) and 30.2% (w.w.) *S*-metolachlor, also used by Syngenta AG in other commercial formulations, used worldwide, plus coadjuvant substances supposedly inert (Neves et al., 2015), with a residual percentage in the composition of the herbicide. Metolachlor is classified as an inhibitor of very long chain fatty acid (VLCFA) formation (Liu and Xiong, 2009). It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu and Xiong, 2009). Due to the action mode of this xenobiotic, it is suggested that this a.i. affects the lipid (fatty acid-FA) profile of aquatic species.

TBA belongs to the group of triazines, inhibiting the photosynthesis at photosystem II, while metolachlor belongs to the family of chloroacetamides, inhibiting several biological processes, essentially biosynthesis of lipids, fatty acids, leaf wax, terpenes, flavonoids and proteins, in addition to inhibition of cell division and interfering with hormonal regulation (Liebl, 1995; Weed, 1994).

Copper belongs to a group of transitional essential metals, of vital importance for every organism at low concentration, however, becoming toxic at high amounts (Bae and Lim, 2012; Kennish, 2000). Kennish (2000) report copper on the third place as a toxic metal after cadmium and mercury, which present the highest toxicity. The presence of copper above the essential limits affects a great variety of metabolic and biochemical processes, such as respiration, cell division, photosynthesis, chlorophyll synthesis, carbohydrate synthesis, pigment synthesis and FA metabolism (Ritter et al., 2008; Sibi et al., 2014).

Nutrients, mainly lipids, are involved in many vital functions of aquatic individuals (Arts et al., 2001; Gotelli et al., 2012). Since some of them can only be obtained from food and therefore referred to as 'essential nutrients' they have proven to be useful trophic markers (Kelly and Scheibling, 2012). They are further essential for physiological functions, the overall metabolism of organisms and the prevention of diseases (Arts et al., 2001). Fatty acid (FA) analysis is a well-established tool for studying trophic interactions in aquatic habitats (Kelly and Scheibling, 2012). Biological specificity of FA and the fact that in most cases they are transferred from primary producers to higher trophic levels without change, make them suitable as bio-indicators (Gonçalves et al., 2012a, 2016). However, primary consumers such as benthic harpacticoid copepods have the ability to bioconvert FA (De Troch et al., 2012).

Besides, FA profiles can contribute to answer questions such as how structural changes in species composition are linked to functional changes in species or in response of species to environmental changes. Thus, the use of biomarkers to assess the effects of different stressors on biochemical processes that govern organismal health and fitness in complex ecosystems will provide much more relevant information than other indirect measurements alone (Fleeger et al., 2003; Neves et al., 2015). Environmental stressors interfere with sub-organismal constituents such as cells and tissues, therefore, biomolecular and biochemical levels are sensitive and quick-responding indicators to stressors (Adams and Greeley, 2000). Indeed, alteration in FA composition is a sensitive early warning bio-indicator of stress, as evidenced by numerous studies (Gonçalves et al., 2012b, 2016; Maazouzi et al., 2008; Ramírez et al., 2013; Sánchez-Muros et al., 2013).

Fatty acid (FA) profile consists of saturated FAs (SFA) that do not contain any double bonds and unsaturated FAs with one and more double bonds in the molecular structure. In accordance of the unsaturation level unsaturated FAs are divided into monounsaturated FAs (MUFA) with single double bond and polyunsaturated FAs (PUFA) with two and more double bonds. Among PUFAs, there are highly unsaturated FAs (HUFA), which are also termed as essential FAs (EFA) as well, since they cannot be synthesized *de novo* in animals.

Phytoplankton and zooplankton species are of high importance in ecotoxicological studies due to their key position in the trophic food web, making a link with higher trophic levels. In this work three planktonic species were used: the diatom *Thalassiosira weissflogii*, the copepod *Acartia tonsa* and the nauplii of the brine shrimp *Artemia franciscana*. The estuarine copepod *Acartia tonsa* is one of the most abundant copepod species in the Mondego estuary (Gonçalves et al., 2010b). The brine shrimp *Artemia franciscana* is a widespread invasive species in Portuguese marine waters (Pinto et al., 2013) and the marine diatom *Thalassiosira weissflogii* is a sensitive test-organism for seawater toxicity tests (Araújo and Souza-Santos, 2013) and is widely used as food source for zooplankton (Fields et al., 2011). Changes in their population or in their biochemical composition, including alterations in FA profiles, may lead to alterations along the food web. Therefore it is necessary and highly relevant to investigate the influence of the pollutants studied (a herbicide and a metal) on these target species.

The main aim of this study was to determine the ecotoxicological and biochemical effects of the metal copper and the herbicide Primextra® Gold TZ on three planktonic species. Therefore this study examined: (1) the ecotoxicological effect of the herbicide Primextra® Gold TZ and the metal copper (copper(II) sulphate pentahydrate) on diatom *T. weissflogii*, copepod *A. tonsa* and brine shrimp nauplii *A. franciscana*; (2) the biochemical response in terms of FA profiles of the studied species after exposure to the pollutants and (3) the nutritive value of the planktonic species after exposure to the organic and inorganic compounds.

2. Materials and methods

2.1. Culture maintenance

T. weissflogii were obtained from the Scottish Marine Institute, Dunbeg, PA37 1QA (UK) and were cultured for bioassay tests and zooplankton feeding. Guillard's f/2 medium [adapted after Rippingale and Payne, 2001] without EDTA, due to its ability to form a stable chelate complex with copper, was applied for diatom culti-

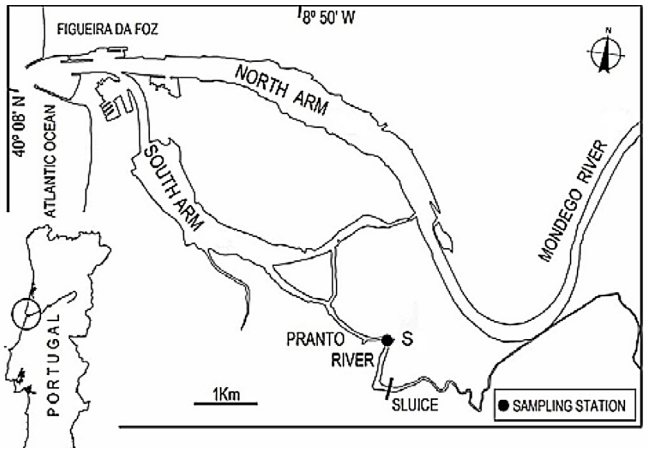

Fig. 1. Map of the Mondego estuary (40°08′N, 8°50′W), located along the west coast of Portugal.

vation and the incubation experiments. Once a week, algal culture was renewed with new medium.

A. tonsa was sampled from station S in the Mondego estuary (Fig. 1), where this species is one of the most abundant copepod species (Gonçalves et al., 2010b, 2012b,c).The Mondego estuary is a small mesotidal system covering an area of 8.6 km^2 along the West Atlantic coast of Portugal.

Horizontal subsurface tows with a bongo net (mesh size of 335 μm and mouth diameter of 0.5 m) were used for to collect copepods (Gonçalves et al., 2010b, 2012b,c). Samples were brought from the estuary to the laboratory in flasks of 2.5 L with estuarine water (Gonçalves et al., 2012a). *A. tonsa* specimens were separated from other species by means of Pasteur pipettes and moved to prepared aquaria with filtrated seawater and aeration for further maintenance and reproduction. Natural seawater was previously filtrated using VWR glass microfiber filters with 1.2 μm pores and diluted with distilled water to a salinity of 13–15. Aquaria were supplied with gentle aeration system and measurements of dissolved O_2 (%) were conducted every other day. Medium was renewed every 2–3 days, copepods were fed 3 times a week with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL. Applied maintenance and reproduction procedure of estuarine copepod *A.tonsa* was adapted from Marcus and Wilcox, 2007; Rippingale and Payne, 2001; Stottrup et al., 1986. Adult organisms, grown from the first cohort of nauplii, were used for the ecotoxicological bioassays.

A. franciscana specimens were hatched under laboratory conditions from dry cysts (Ocean Nutrition™) in a hatchery dish. ASPM reconstituted seawater of 35 g/L salinity (Guillard, 1983) was used as medium for hatching and bioassays, as synthetic medium significantly reduces bacterial infections during hatching (Lavens and Sargeloos, 1996). Temperature during the hatching process was maintained at 28 °C. Neonates (<24 h) were used for the bioassays.

Laboratory cultures were maintained at a temperature of 20 ± 2 °C, with photoperiod 16 h^L: 8 h^D, in filtrated seawater medium with a salinity of 13–15 for copepods and 35 for brine shrimps and 30 for diatom cultures.

2.2. Growth bioassays of microalgae

Prior to the beginning of the test, an inoculum of *T. weissflogii* was harvested from the bulk culture and incubated for three days under 20 ± 2 °C and a 16 h light and 8 h dark light regime. Briefly, the inoculum cell density was determined microscopically using a Neubauer haemocytometer and adjusted so that the initial test cell density was 10^4 cells/mL. The microalgae were then exposed to a geometric range of concentrations of each toxicant.

The herbicide and the metal solutions were obtained by successive dilutions of a stock solution of Primextra® Gold TZ or copper(II) sulphate pentahydrate in distilled water.

Based on literature data and preliminary trials, we used concentrations ranging from 0.005 to 0.040 mg/L for Primextra® Gold TZ and from 0.200 to 0.800 mg/L for copper(II) sulphate pentahydrate. The culture medium was used as the negative control treatment. Tests were carried out in glass (pesticide bioassays) or plastic (metal bioassays) flasks, three replicates per treatment, containing 40 mL of test solutions.

The tests were performed under the same photoperiod and temperature conditions as described for algal cultures during 96 h. Algal cell density was directly counted using a Neubauer chamber (APHA, 1995).

2.3. Acute zooplankton bioassays

Tests conditions from OECD protocol 202 (OECD 202, 2004) were adapted and applied for the acute immobilisation tests. Tests of adults of *A. tonsa* and neonates (<24 h) of *A. franciscana* were carried out under the same temperature and photoperiod regimes as described for rearing procedures with neonates from the same bulk cultures, born between first and second broods. The experiments were performed in glass (pesticide bioassays) and plastic (metal bioassays) vials containing 100 mL of the test solution. Geometric ranges of toxicants' concentrations were applied, and the culture medium was used as the negative control treatment. The experimental concentrations were obtained by successive dilutions of a stock solution of Primextra® Gold TZ and copper(II) sulphate pentahydrate in distilled water, with concentrations ranging from 0.100 to 3.700 mg/L and from 0.053 to 0.906 mg/L for *A. tonsa*, respectively, and ranging from 2.900 to 56.170 mg/L and from 2.000 to 21.400 mg/L for *A. franciscana*, correspondingly. The culture medium was used as the control treatment. A static design was employed, using twenty animals randomly assigned into four replicates with five animals *per* treatment. The organisms were exposed to the different toxicant concentrations for 48 h without food. Vessels were checked for immobilized individuals at 24 h and 48 h.

2.4. Population microcosm bioassays

Microcosm bioassays were conducted to determine changes in FA profiles after exposure to the herbicide Primextra® Gold TZ and the metal copper(II) sulphate pentahydrate, according to the results from toxicological bioassays.

Phytoplankton and zooplankton species were exposed in glass or plastic beakers with a final volume of corresponding test solution of each pollutant. Diatoms were exposed in four experimental treatments: (1) a negative control, consisting of uncontaminated culture medium; (2) a low level of each toxicant corresponding to the EC10 (0.1361 mg/L, for copper, and 0.0025 mg/L, for Primextra®) value; (3) an intermediate level which corresponds to the EC20 (0.1995 and 0.0038 mg/L) value and (4) a high level, which is close to the EC50 (0.3834 and 0.0078 mg/L) value (see Table 1 for details).

According to preliminary results with zooplankton species, exposed to the contaminants in single cases, the mortality greatly increased after 48 h, and, thus, the concentrations used were lower than the ECx (X = 10, 20, 50) values (for details, see Tables 2 and 3).

The larger amount of treatments in bioassays with copepod and copper was because of the fact that copepods showed the highest sensitivity to copper exposure (Table 1) and a wider range of concentrations was applied in order to be able to get information on the concentration at which the FA profile changes.

Table 1
EC_{10}, EC_{20} and EC_{50} values (mg/L) of copper and Primextra® Gold TZ for the three planktonic species with the respective time of exposure and 95% confidence limits (between brackets).

Species \ Toxicant	Copper(II) sulphate pentahydrate (mg/L)	Primextra® Gold TZ (mg/L)
T. weissflogii (96 h)	EC_{10}: 0.1361 (0.0292–0.2431) EC_{20}: 0.1995 (0.0820–0.3170) EC_{50}: 0.3834 (0.2669–0.4999)	EC_{10}: 0.0025 (0.0003–0.0047) EC_{20}: 0.0038 (0.0013–0.0063) EC_{50}: 0.0078 (0.0050–0.0106)
A. tonsa (48 h)	EC_{10}: 0.000 (0.000–0.011) EC_{20}: 0.005 (0.000–0.103) EC_{50}: 0.234 (0.149–0.338)	EC_{10}: 0.145 (0.006–0.583) EC_{20}: 0.289 (0.151–0.333) EC_{50}: 0.925 (0.589–1.449)
A. franciscana nauplii (48 h)	EC_{10}: 10.09 (7.07–12.03) EC_{20}: 13.13 (11.04–14.99) EC_{50}: 18.93 (16.84–22.43)	EC_{10}: 5.42 (0.00–10.21) EC_{20}: 10.54 (4.41–14.86) EC_{50}: 20.35 (16.04–26.43)

All treatments were replicated three times, with the glass or plastic beaker as the experimental unit. Microalgae and zooplankton incubations were conducted under the same laboratory conditions as described above for culture maintenance and bioassays.

According to the dynamics of microalgae growth by Lavens and Sorgeloos (1996), vessels of microalgae experiments were checked for growth inhibition after 7 days of exposure to the toxicants.

In each replicate 3.6×10^6 cells/mL were counted using a Neubauer chamber. The cell mass was then concentrated on a GF/F Whatman filter and frozen at −80 °C for further FA analysis.

The zooplankton experiments ran for 7 days for neonates of *A. franciscana* and 14 days for adults of *A. tonsa* according to preliminary data obtained after the series of preliminary microcosms bioassays for all species.

Organisms were fed daily with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL and moved to new test solutions every third day. Copepod experiments were conducted in vials with a final volume of 2000 mL and 200 individuals per replicate, whereas neonates of *A. franciscana* were kept at a final volume of 900 mL and 450 individuals per replicate. Each flask was connected to a gentle aeration system. At the end of each test alive organisms (60 individuals per replicate) were separated and concentrated on GF/F Whatman filters and stored frozen at −80 °C for further FA analysis.

2.5. FA analyses

The extraction of total lipids of planktonic species and methylation to fatty acid methyl esters (FAMEs) were done by a modified 1-step derivatisation method after De Troch et al. (2012) and Gonçalves et al. (2012a).

The fatty acid Methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard for the quantification of FA. Samples were centrifuged (eppendorf Centrifuge 5810R) three times for 15 min, at 10 °C, 1200 rpm and vacuum dried (Rapid Vap LABCONCO). The FAMEs thus obtained were analyzed using a Hewlett Packard 6890 N GC coupled to a mass spectrometer (HP 5973). Zooplankton samples were run in splitless mode, with a 1 μL injection per run, whereas phytoplankton samples were run in a split10 mode, with a 0.1 μL injection per run, both at an injector temperature of 250 °C, using a HP88 column (Agilent J & W; Agilent Co., USA) with a He flow of 1.5 mL min^{-1}.

The oven temperature was programmed at 50 °C for 2 min, followed by a ramp of 25 °C min^{-1} to 75 °C, then a second ramp at 2 °C min^{-1} to 230 °C with a final 14 min hold.

FAMEs were identified by comparison with the retention times and mass spectra of authentic standards and available ion spectra in Famedb23 and WILEY mass spectral libraries, and analyzed with the software Agilent MSD Productivity ChemStation.

Quantification of individual FAMEs was accomplished by the use of external standard (Supelco 37 Component FAME Mix, Supelco # 47885, Sigma-Aldrich, Inc., USA) and additional standards of 16:2ω6, 16:2ω4 and 16:3ω3 (Larodan Fine Chemicals). The quantification function of each FAME was obtained by linear regression applied to the chromatographic peak areas and corresponding known concentrations of the standards, ranging from 25 to 200 μg/mL for splitless mode and from 100 to 1000 μg/mL for split10 mode.

Shorthand FA notations of the form A:BωX are used, where A represents the number of carbon atoms, B gives the number of double bonds, and X gives the position of the double bond closest to the terminal methyl group.

2.6. Statistical analyses

The data obtained from the 96 h bioassays with microalgae were used to estimate concentrations promoting x% growth inhibition (EC_x values, with $x = 10, 20, 50$) and the corresponding 95% confidence intervals for each tested toxicant by non-linear regression, using the least-squares method to fit the data to the logistic equation.

Probit analysis (Finney, 1971) was used to estimate the concentration which caused 50%, 20% and 10% of effect (EC_{50}, EC_{20} and EC_{10}) in *A. tonsa* and *A. franciscana* after 48 h of exposure, together with the corresponding 95% confidence intervals. Multivariate statistical analyses were carried out using PRIMER-6 & PERMANOVA+ software (Clarke and Gorley, 2006) in order to examine the variation in FA composition through non-metric multidimensional scaling (*n*-MDS) plots. Data were converted into similarity triangular matrices using a Bray-Curtis resemblance measure (Clarke and Warwick, 2001).

One-way analysis of similarity (ANOSIM) was used to test differences in fatty acid profiles across the treatments to each species. The contribution of individual FAs to similarities and dissimilarities within and between sample groups were tested using similarity percentage analysis routine (SIMPER).

To determine significant differences between treatments, one-way analysis of variance (ANOVA) was performed, followed by Dunnett's multiple comparison test to discriminate significant differences between toxicant concentrations and the control treatment. The adopted level of significance was of 0.05.

3. Results

3.1. Experimental bioassays—algal growth bioassays and zooplankton acute bioassays

A clear inhibition of cells growth of *T. weissflogii* was observed after the exposure to both toxicants (Fig. 2).

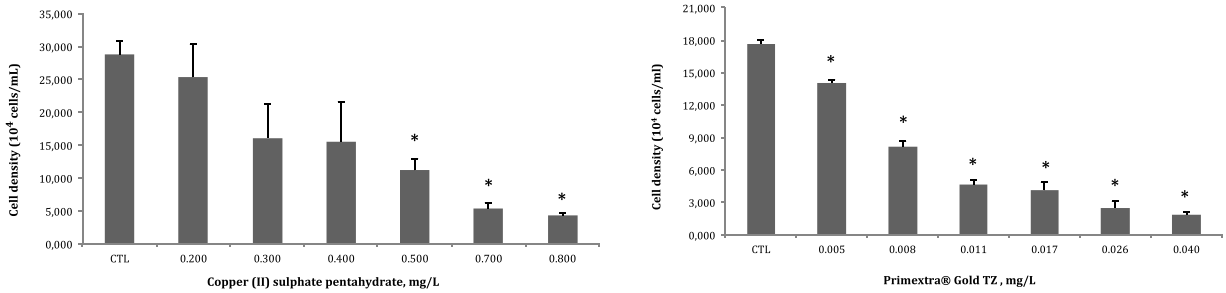

Fig. 2. Cell density of *T. weissflogii* after 96 h exposure to Primextra® Gold TZ (on the right) and copper(II) sulphate pentahydrate (on the left), where CTL refers to the negative control treatment. Symbol "*"—indicates a significant ($P < 0.05$) difference of the treatments compared to the CTL.

The one-way ANOVA revealed that treatments were significantly different in both tests (copper: $p = 0.002$; herbicide: $p = 0.000$). Dunnett test revealed that all treatments were significantly different from the control in case of exposure to Primextra® and last three treatments, with the highest concentration, were significantly different from the control in case of copper exposure.

The ECx (X = 10, 20 and 50) values determined to the three planktonic species after the exposure to both toxicants showed that the herbicide Primextra® Gold TZ is more toxic to the microalgae than to the zooplankton species (Table 1).

On the other hand, copper(II) sulphate pentahydrate revealed to be more toxic to *A. tonsa* than to *T. weissflogii*.

Indeed, both copper and Primextra® were highly toxic to the marine diatom *T. weissflogii* ($EC_{50} = 0.3834$ mg/L and $EC_{50} = 0.078$ mg/L, respectively) and to the calanoid copepod *A. tonsa* ($EC_{50} = 0.234$ mg/L and $EC_{50} = 0.925$ mg/L, respectively), but only slightly toxic to nauplii of brine shrimp *A. franciscana* ($EC_{50} = 18.93$ mg/L and $EC_{50} = 20.35$ mg/L, respectively).

3.2. Population microcosm bioassays and variation of FA profiles

The applied microcosm experiments showed that the herbicide and the metal interfere with the FA biosynthesis of the planktonic species. The FA content (values in%) of the three planktonic species exposed to the different treatments of the metal and the herbicide was compared with the control (see Tables 1 and 2).

The FA profile of diatoms exposed to copper and Primextra® Gold TZ was mainly represented by SFAs (16:0, 18:0, 14:0) and PUFA (16:2ω4, 16:3ω4), presenting also MUFA (16:1ω7) and HUFA (20:5ω3 (EPA)).

FA profiles of diatoms exposed to copper responded with the following changes in saturated and unsaturated levels: total SFAs slightly decreased from the control to C2 (0.1995 mg/L), however, increased 6% to C3 (0.3834 mg/L). Total MUFAs, PUFAs and HUFAs slightly increased from the control to C2 (0.1995 mg/L), decreasing once again to C3 (0.3834 mg/L), representing a difference of 0.21%, 3.46% and 1.90% with the control, respectively (Table 2, Fig. 4a1).

Although Primextra® led to a clear inhibition of the diatom growth, only slight changes are observed in total SFAs and MUFAs (increase of 2% and 1.43%, respectively), when the control is compared with the highest herbicide concentration.

HUFAs showed the highest sensitivity to the exposure of the herbicide, clearly decreasing from 16.48% in the control to 12.32% in the treatment concentration (Table 3, Fig. 4a2).

The FA profile of the copepods exposed to copper were represented mainly by SFAs – 18:0, 16:0 and 14:0, whereas MUFAs, PUFAs and HUFAs were only detected in trace amounts. Whilst after the exposure to the herbicide *A. tonsa* presents higher amounts of SFAs (16:0, 18:0 and 14:0), MUFA (16:1n-7) and HUFA (22:6n-3 (DHA) and 20:5n-3 (EPA)).

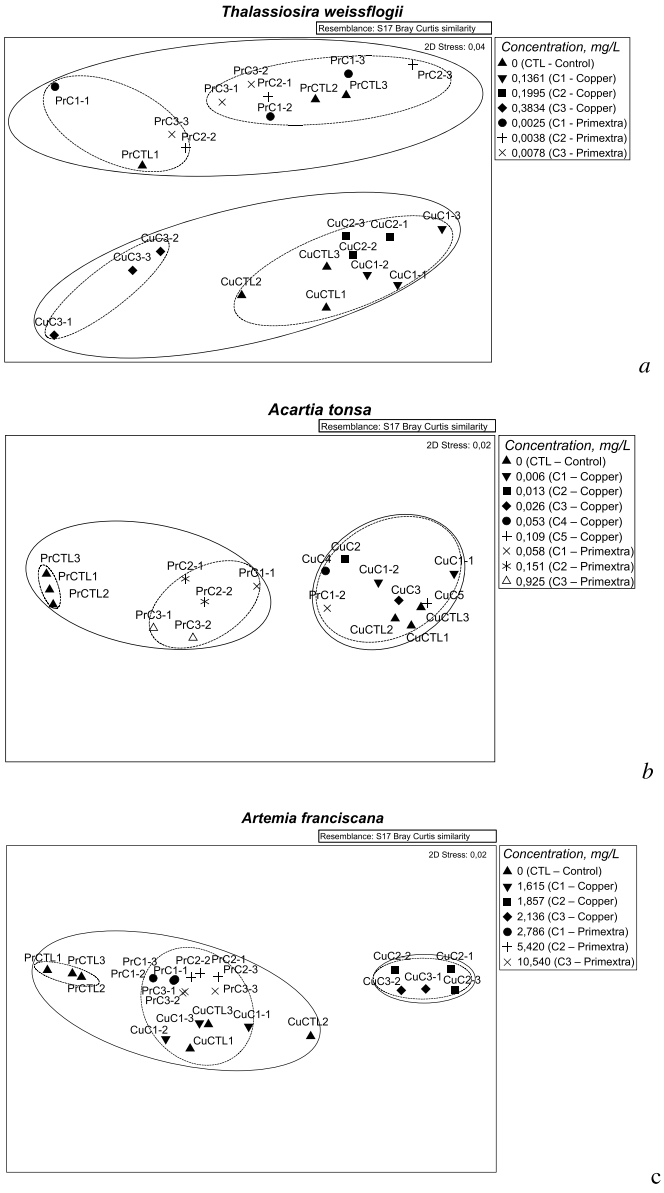

Fig. 3. Two-dimensional non-metric MDS ordination plots of FA profiles of the studied species exposed to copper(II) sulphate pentahydrate (Cu) and to the herbicide Primextra® Gold TZ (Pr): diatom *T. weissflogii* (a), copepod *A. tonsa* (b) and nauplii of brine shrimp *A. franciscana* (c). CTL, C1, C2, C3, C4, C5–treatments, referring to the concentration of the contaminants tested, where CTL < C1 < C2 < C3 < C4 < C5. Not inverted triangles represent negative control treatment.

Table 2
FA profiles of the three planktonic species (marine diatom *T. weissflogii*; estuarine copepod *A. tonsa* and marine shrimp *A. franciscana*) after exposure to copper(II) sulphate pentahydrate.

Species/FA profile		*Thalassiosira weissflogii*				*Acartia tonsa*						*Artemia franciscana* (nauplii)			
Copper (mg/L)		CTL	0.1361	0.1995	0.3834	CTL	0.006	0.013	0.026	0.053	0.109	CTL	1.615	1.857	2.136
SFA	C 14:0	8.71	8.38	8.03	6.74	8.13	4.95	5.88	6.86	4.94	6.34	2.23	2.25	3.13	3.36
	C 15:0	0.86	0.85	0.81	0.84	1.89	1.13	1.63	1.74	1.59	1.79	1.20	1.27	2.11	1.97
	C 16:0	23.15	21.18	20.73	21.79	36.57	33.22	29.70	34.75	31.40	36.67	17.45	21.92	36.33	33.84
	C 17:0	0.51	0.53	0.51	0.59	1.57	1.43	1.37	1.59	1.19	1.45	2.21	2.11	3.19	2.57
	C 18:0	15.10	10.71	12.66	23.69	46.78	52.17	42.72	47.59	41.27	50.80	26.17	32.11	48.98	48.25
	C 20:0	0.18	0.11	0.14	0.24	1.48	1.66	1.45	1.53	1.27	1.59	1.11	0.83	1.06	1.15
	C 22:0	0.00	0.00	0.00	0.00	0.41	0.41	0.39	0.44	0.39	0.43	0.81	0.77	1.40	1.20
	C 24:0	0.36	0.41	0.42	0.54	0.55	0.32	0.38	0.64	0.55	0.76	0.09	0.07	0.06	0.00
	Total% SFA	**48.85**	**42.16**	**43.30**	**54.44**	**97.39**	**95.27**	**83.50**	**95.15**	**82.61**	**99.83**	**51.27**	**61.33**	**96.25**	**92.35**
MUFA	C 16:1ω9	1.00	1.11	1.03	0.82	0.23	0.16	0.32	0.23	0.32	0.17	0.77	0.42	0.07	0.00
	C 16:1ω7	12.95	15.49	14.58	12.27	1.31	0.80	2.65	1.46	3.14	0.00	5.28	8.43	1.01	2.29
	C 16:1ω5	0.77	0.59	0.65	0.34	0.00	0.78	2.22	0.00	1.56	0.00	2.21	2.48	0.00	0.00
	C 17:1ω7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.29	0.00	0.00
	C 18:1ω9	0.39	0.42	0.43	0.99	0.04	0.20	0.50	0.15	0.53	0.00	5.24	4.18	0.58	1.23
	C 18:1ω7	0.05	0.09	0.11	0.53	0.13	0.18	0.64	0.18	0.69	0.00	9.79	8.41	1.08	2.21
	C 20:1ω9	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.07	0.00	0.19	0.24	0.00	0.00
	C 22:1ω9	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.19	0.00	0.00	0.00	0.28	0.00
	Total% MUFA	**15.17**	**17.70**	**16.81**	**14.96**	**1.71**	**2.12**	**6.54**	**2.02**	**6.49**	**0.17**	**23.75**	**24.44**	**3.03**	**5.74**
PUFA	C 16:2ω6	0.86	0.97	1.06	1.32	0.05	0.10	0.57	0.18	0.36	0.00	0.00	0.23	0.00	0.00
	C 16:2ω4	4.81	5.22	5.18	3.52	0.11	0.29	1.17	0.35	1.12	0.00	0.10	0.24	0.00	0.00
	C 16:3ω3	15.35	16.51	16.38	12.56	0.16	0.43	2.34	0.87	2.27	0.00	6.67	1.67	0.00	0.23
	C 18:2ω6 tr	0.46	0.50	0.51	0.61	0.04	0.15	0.47	0.13	0.49	0.00	1.53	1.54	0.15	0.44
	C 18:2ω6 cis	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
	C 18:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.15	0.00	0.00
	C 18:3ω3	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.10	0.00	1.40	1.96	0.16	0.30
	C 20:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	C 20:3ω3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00
	Total% PUFA	**21.48**	**23.21**	**23.14**	**18.02**	**0.36**	**0.97**	**4.67**	**1.53**	**4.34**	**0.00**	**9.98**	**5.79**	**0.31**	**0.97**
HUFA	C 20:4ω6 (ARA)	0.00	0.00	0.00	0.00	0.06	0.12	0.27	0.00	0.00	0.00	0.63	0.83	0.07	0.00
	C 20:5ω3 (EPA)	12.33	14.53	14.33	10.59	0.24	0.59	2.24	0.45	2.79	0.00	14.06	7.32	0.34	0.95
	C 22:63 (DHA)	2.16	2.40	2.42	2.00	0.25	0.92	2.78	0.85	3.77	0.00	0.31	0.28	0.00	0.00
	Total%HUFA	**14.49**	**16.93**	**16.75**	**12.59**	**0.55**	**1.63**	**5.29**	**1.30**	**6.56**	**0.00**	**14.99**	**8.43**	**0.42**	**0.95**
	N	18	18	18	18	19	20	23	18	22	9	25	24	17	14

The FA profile of the copepods exposed to copper responded with the following changes in saturated and unsaturated levels: the relative and absolute concentration of SFAs increased in a sinusoidal manner and at the highest concentration increased slightly with 2.44% compared with control treatment. The same variation was observed for MUFAs, PUFAs and HUFAs, however, their amount decreased at the level 0.109 mg/L Cu^{2+} with 1.94, 0.36 and 0.55% correspondingly compared with the control, resulting in a complete loss of PUFAs and HUFAs at the highest contaminant treatment (Table 2, Fig. 4b1).

Primextra® Gold TZ considerably changed the level of SFAs leading to an increase of 9.92% when compared to the control; the level of PUFAs also decreased significantly at the lowest concentration compared to the control, their abundance was decreased 3, and the same pattern was kept throughout the range of contaminant concentrations. The amount of MUFA and HUFA was reduced as well after the exposure to 0.925 mg/L of the herbicide with 3.06% and 0.47%, respectively, when compared to the control (Table 3, Fig. 4b2).

Considering the brine shrimp nauplii exposed to copper, SFAs (18:0 and 16:0) increased in all treatments while 16:1ω7, 18:1ω7 (MUFA) and 20:5ω3 (EPA) (HUFA) showed the highest values in CTL and 1.615 mg/L treatment. Likewise, 16:3ω4 (PUFA) showed the highest concentration in the CTL. A different pattern was observed for the FA profile of *A. franciscana* when exposed to the herbicide, with 18:0, 16:0, 20:5ω3 (EPA), 16:1ω7, 18:1ω7 being the most abundant FAs in all treatments.

Microcosm bioassays applied for the brine shrimp nauplii *A. franciscana* revealed that the herbicide and the metal altered its FA profiles much more than in the other studied species. Thus, in the case of exposure to copper, the level of SFAs increased almost twice from 51.27% in the control to 92.35% in the highest copper concentrations (C3 = 2.136 mg/L). Amounts of MUFAs, PUFAs and HUFAs decreased up to 4.5, 10 and 15 times, respectively, as well. The same significant changes were observed after exposure to C2 (1.857 mg/L). Although C1 concentration (1.615 mg/L) did not change the FA profile of *A. franciscana* significantly, an increase on SFAs and a reduction of the higher amounts of MUFAs, PUFAs and HUFAs were reported (Table 2, Fig. 4c1). Exposure of *A. franciscana* nauplii to Primextra® Gold TZ led to changes in FA profiles as well, although the changes were slighter than in the microcosm bioassay to copper. SFA level increased with 15.19%, whereas MUFAs, PUFAs and HUFAs decreased with 3.05, 6.78 and 5.36% respectively, between the control and the highest concentration (Table 3, Fig. 4c2).

The *n*-MDS plots revealed differences in the FA profiles among the treatments in each microcosm bioassay for each planktonic species (Fig. 3).

The *n*-MDS plot of the FA profile of *T. weissflogii* indicated a clear difference between the diatom exposed to copper and to the herbicide Primextra® Gold TZ. Changes in FA profiles between the control and the highest copper concentration (C3 = 0.3834 mg/L), referring to the EC_{50} value (Table 1), are easily observed. No significant differences were observed between the FA profiles of the control and

Table 3
FA profiles of three planktonic species (marine diatom *T. weissflogii*; estuarine copepod *A. tonsa* and marine shrimp *A. franciscana*) after exposure to Primextra® Gold TZ.

Species/FA profile		*Thalassiosira weissflogii*				*Acartia tonsa*				*Artemia franciscana*(nauplii)			
Primextra® Gold TZ, mg/L		CTL	0.0025	0.0038	0.0078	CTL	0.058	0.151	0.925	CTL	2.786	5.420	10.540
SFA	C 14:0	5.72	5.44	5.44	5.88	5.64	4.08	7.80	4.56	2.09	2.03	1.85	1.84
	C 15:0	0.95	0.88	1.15	1.21	2.17	1.16	1.62	2.32	1.00	0.97	1.01	1.15
	C 16:0	17.47	17.17	16.79	18.24	23.35	29.12	24.85	28.00	16.66	20.53	21.52	20.98
	C 17:0	0.71	0.70	0.70	0.64	0.81	1.12	0.97	1.22	1.58	1.61	1.61	1.80
	C 18:0	17.22	17.17	16.62	17.99	18.23	39.43	29.38	23.29	21.21	28.87	31.91	31.69
	C 20:0	0.19	0.13	0.19	0.19	0.58	1.07	0.74	0.71	0.69	0.77	0.87	0.87
	C 22:0	0.00	0.00	0.00	0.00	0.28	0.36	0.32	0.37	0.47	0.51	0.58	0.57
	C 24:0	0.25	0.24	0.29	0.37	1.11	0.82	0.86	1.63	0.07	0.06	0.07	0.07
	Total% SFA	**42.51**	**41.72**	**41.18**	**44.51**	**52.17**	**77.16**	**66.55**	**62.09**	**43.78**	**55.36**	**59.43**	**58.97**
MUFA	C 16:1ω9	1.22	1.32	1.59	1.72	1.06	0.36	0.45	1.11	0.49	0.49	0.46	0.37
	C 16:1ω7	11.96	11.87	12.63	12.62	6.93	3.01	3.14	3.47	10.82	7.00	6.12	6.53
	C 16:1ω5	0.65	0.40	0.55	0.33	0.40	0.21	0.19	0.00	0.42	0.28	0.24	0.22
	C 17:1ω7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.17	0.30	0.23
	C 18:1ω9	1.01	0.81	0.92	1.13	1.53	0.59	1.01	1.42	4.40	5.25	5.80	4.74
	C 18:1ω7	0.20	0.23	0.34	0.67	1.79	1.24	2.24	2.52	8.30	8.69	8.39	9.54
	C 20:1ω9	0.00	0.00	0.00	0.00	0.17	0.09	0.14	0.18	0.23	0.21	0.21	0.18
	C 22:1ω9	0.00	0.00	0.00	0.00	0.32	0.26	0.41	0.53	0.00	0.00	0.00	0.00
	Total% MUFA	**15.03**	**14.63**	**16.03**	**16.46**	**12.30**	**5.77**	**7.65**	**9.24**	**24.86**	**22.10**	**21.53**	**21.81**
PUFA	C 16:2ω6	1.25	1.45	1.55	1.50	0.69	0.26	0.07	0.00	0.83	0.33	0.00	0.29
	C 16:2ω4	5.17	5.12	5.36	4.91	1.86	0.55	0.45	0.56	1.34	0.63	0.13	0.34
	C 16:3ω3	18.82	19.11	20.01	19.70	5.53	1.25	0.86	1.28	7.75	2.55	1.04	1.18
	C 18:2ω trans	0.73	3.35	0.56	0.59	1.06	0.88	1.22	1.09	1.51	1.62	1.98	1.84
	C 18:2ω6 cis	0.00	0.00	0.00	0.00	0.18	0.08	0.07	0.13	0.12	0.00	0.00	0.10
	C 18:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.09	0.14	0.13
	C 18:3ω3	0.00	0.00	0.00	0.00	0.13	0.00	0.07	0.00	2.17	2.92	3.59	3.22
	C 20:3ω3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.23	0.28	0.26
	Total% PUFA	**25.98**	**29.03**	**27.48**	**26.70**	**9.45**	**3.01**	**2.74**	**3.05**	**14.13**	**8.36**	**7.16**	**7.35**
HUFA	C 20:4ω6 (ARA)	0.00	0.00	0.00	0.00	0.00	0.10	0.38	0.36	1.00	0.75	0.99	0.89
	C 20:5ω3 (EPA)	13.69	12.16	12.71	10.32	11.73	5.71	8.43	9.22	15.23	13.10	10.89	10.79
	C 22:6ω3 (DHA)	2.79	2.46	2.59	2.00	14.36	8.25	14.25	16.03	0.99	0.34	0.00	0.19
	Total% HUFA	**16.48**	**14.62**	**15.30**	**12.32**	**26.08**	**14.05**	**23.06**	**25.61**	**17.22**	**14.19**	**11.88**	**11.86**
	N	18	18	18	18	24	23	25	21	26	25	23	26

lower concentrations of copper (C1 = 0.1361 mg/L – EC_{10} value; C2 = 0.1995 mg/L – EC_{20} value).

In the case of *T. weissflogii*, exposed to the herbicide, no clear differences among the treatments were observed. Only organisms exposed to the highest herbicide concentration (C3 = 0.0078 mg/L) presented small FA profile changes when compared to the control and lower concentrations (Fig. 3a).

The *n*-MDS analysis revealed clear differences for FA profiles of copepods exposed to copper and to the herbicide. For copper, the highest percentages of MUFA, PUFA and HUFA were observed in C2 (0.013 mg/L) and C4 (0.053 mg/L) concentrations while SFA showed the highest percentage in C5 (0.109 mg/L) concentration. Interestingly, the percentage of the three first FA decreased abruptly from C4 to C5 and, inversely, the percentage of SFA increased to the highest value.

In the case of *Acartia tonsa* exposed to the herbicide, the percentage of MUFA, PUFA and HUFA in the treatments were below the values registered in the control while the opposite was observed for SFA (Fig. 3b).

ANOSIM confirmed a clear separation among treatments in both bioassays (Global R = 0.399). Pairwise differences indicated that treatments containing copper are highly different from treatments with the herbicide ($0.519 < R < 1$). In copper bioassay, C3 treatment in pair with control, C1 and C2, presents high R-values ($0.451 < R < 1$), therefore it is significantly different compared to lower copper concentrations and uncontaminated treatment. Still, the pair C1/C2 (R = 0.185) is slightly different. For Primextra®, pairwise differences indicated no differences between pair C1/C2 with 0.0025 and 0.0038 mg/L of herbicide (R = −0.259), but revealed a slight difference between FA profiles in control and each contaminated treatment ($0.074 < R < 0.142$), as well as between C1/C3 treatments with 0.0025 and 0.0078 mg/L of the herbicide (R = 0.148) and C2/C3 treatments with 0.0038 and 0.0078 mg/L of herbicide (R = 0.111).

An overall ANOSIM did not reveal significant differences among all treatments, considering the two contaminants, for *A. tonsa* (Global R = 0.015). However, pairwise differences indicated that copper treatments are highly different from herbicide treatments ($0.500 < R < 1$) with the exception of C1 herbicide treatment (0.058 mg/L) in pairs with following copper treatments: C2 (0.013 mg/L), C3 (0.026 mg/L) and C4 (0.053 mg/L), where R = 0. Pairwise analysis revealed slight segregation between control and C1 (0.006 mg/L) and C5 (0.109 mg/L) copper treatments (R = 0.104 and R = 0.222 correspondingly), but no differences between control and the rest of treatments contaminated by copper or the herbicide ($R < 0$). For all contaminated treatments with herbicide, pairwise differences showed high segregation ($0.750 < R < 1$).

The variation of FA profiles was confirmed by *n*-MDS and ANOSIM analyses. *n*-MDS ordination plot showed clear changes in FA profiles between the control and higher copper concentrations (C2 = 1.857 mg/L and C3 = 2.136 mg/L). FA abundance at the lowest concentration (C1 = 1.615 mg/L) is slightly different from the control (Fig. 3c). ANOSIM confirmed a clear separation between treatments in both bioassays (Global R = 0.464). Pairwise differences indicated that copper treatments are highly different from treatments exposed to herbicide ($0.741 < R < 1$). Pairwise analysis revealed high segregation between control and C2 (1.857 mg/L) and C3 (2.136 mg/L) copper treatments (R = 0.833 and R = 0.696 respec-

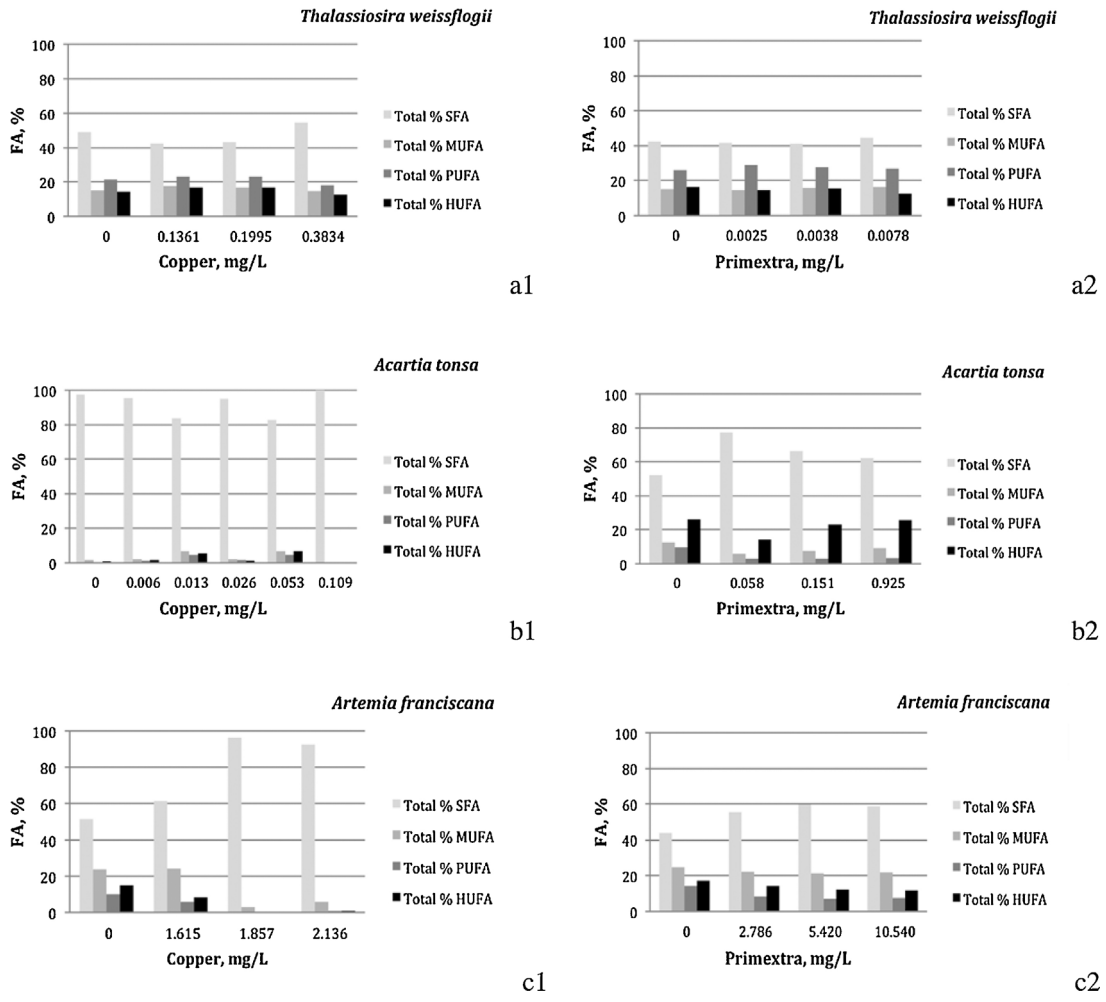

Fig. 4. Clustered column charts representing changes in SFA, MUFA, PUFA and HUFA (in%) of the studied species diatom *T. weissflogii* (a1, a2), copepod *A. tonsa* (b1, b2) and nauplii of brine shrimp *A. franciscana* (c1, c2) exposed to copper(II) sulphate pentahydrate (a1, b1, c1) and to the herbicide Primextra® Gold TZ (a2, b2, c2); CTL (=0) represents the negative control treatment.

tively), however revealed no differences between the control and the treatments contaminated by copper or the herbicide ($R \leq 0$).

Pairwise analysis among treatments contaminated by copper revealed that all these treatments are highly different between each other ($R = 1$), except for the pair C2/C3 (1.857/2.136 mg/L) with $R = -0.167$. In herbicide bioassay, pairwise differences revealed high segregation among all contaminated treatments ($0.370 < R < 0.926$).

4. Discussion

This study confirmed that organic and inorganic compounds are highly toxic to *T. weissflogii* and to *A. tonsa*, but only slightly toxic to nauplii of the brine shrimp *A. franciscana*.

Artemia sp. is known as one of the species with the highest resistance to changes in the environment, such as changes in temperature, salinity, dissolved oxygen. They also have a higher tolerance to contaminants compared with other species (Nunes et al., 2006). According to the literature, the nauplii larvae do not have a complete digestive tract and do not immediately feed. Only after 12–20 h when they have molted to metanauplius larvae they start filter-feeding (Lavens and Sorgeloos, 1996).

This morphological feature may explain the tolerance of this species to both contaminants, as they take up less chemicals during the first hours of the exposure than the other studied species.

A similar effect of Primextra® Gold TZ was observed in earlier studies with the freshwater zooplankton species *Daphnia longispina* (Neves et al., 2015) and marine bivalves *Cerastoderma edule* and *Scrobicularia plana* (Gonçalves et al., 2016), where *D. longispina* and *C. edule* showed to be slightly less sensitive to the herbicide with EC_{50} = 37.65 mg/L and LC_{50} = 28.784 mg/L, correspondingly, and *S. plana* was slightly more sensitive to Primextra® with EC_{50} = 13.263 mg/L.

The effects of copper on *T. weissflogii* and *A. tonsa* obtained in our study are in accordance with the results stated by other authors to marine diatoms and calanoid copepods, correspondingly (Manimaran et al., 2012; Pinho and Bianchini, 2010). Primextra® Gold TZ is recently used in agriculture fields, although similar results were obtained in other works that tested other toxicants, where marine copepod species were shown to be more tolerant to pesticides and more sensitive to metals (Diz et al., 2009; Hack et al., 2008; Stringer et al., 2012).

The higher sensitivity of the diatom *T. weissflogii* to the herbicide compared to the metal may be due to the fact that herbicides have been created to target plants and subsequently sensitivity of algae to many herbicides is very high (Prado et al., 2009). In addition, the terbuthylazine as one of the active ingredients of Primextra® Gold TZ, affecting the quality of the diatom cells, inhibits the process of photosynthesis at photosystem II with direct influence on the diatom growth rate (De Hoop et al., 2013).

Our study also revealed changes in the FA profile of the planktonic species after exposure to both toxicants. The FA profiles in the control are similar to those found in previous ecological studies for *Thalassiosira* sp. (Fisher and Schwarzenbach, 1978; Pratoomyot et al., 2005), for copepod *A. tonsa* (Gonçalves et al., 2012c; Veloza et al., 2006) and for *A. franciscana* (Figueiredo et al., 2009; Ruiz et al., 2008).

The metal copper and the herbicide Primextra® Gold TZ significantly interfere with the FA biosynthesis of the three planktonic species. Outcomes of population microcosm bioassays revealed a general pattern in FA alterations: increase of SFAs and decrease of MUFAs, PUFAs and HUFAs. The same pattern was observed by other authors when communities of marine bacteria were exposed to copper(II) sulphate and copper(II) chloride (De Carvalho, 2012; Popova et al., 2008) and the marine macroalgae *Gracilaria tenuistipitata* to copper sulphate(II) pentahydrate (Pinto et al., 2011).

Studies on the effects of Primextra® Gold TZ on FA profiles of marine and estuarine organisms are scarce in scientific literature. However, available data revealed that exposure of marine bivalves *Cerastoderma edule* and *Scrobicularia plana* to this herbicide significantly reduces the overall amount of their FAs, both saturated and unsaturated ones with strongest effects on the essential FAs (Gonçalves et al., 2016).

FA composition of the brine shrimp nauplii *A. franciscana* showed the highest sensitivity after exposure to both contaminants.

We assume that the highest sensitivity of FA in nauplii of *A. franciscana* to both pollutants may be due to the higher metabolic rate of the nauplius stage than the subsequent stages.

The other reason of sensitivities of nauplii may be due to the thinness of their exoskeleton leading to a higher absorption of contaminants within the cells and tissues (Hack et al., 2008) than in the other species, subsequently leading to the higher exposure to the toxin and influencing the FA biosynthesis.

The main effect was the increase of SFA, in particular palmic (16:0) and stearic (18:0) FAs. The increase in FA saturation could be a response of the organism's cells to promote the stability of the lipid membranes (Popova et al., 2008).

Alterations in FA profiles are adaptive responses with activation of defense and reparation mechanisms of the organism's cells to the impacts of toxicants (Rocchetta et al., 2006). Copper like some other metals induces oxidative stress by producing ROS *via* Fenton reaction, which compromises cell metabolism (Kumar et al., 2010).

PUFAs are not only key constituents in membranes of microalgae and zooplankton, but are also very sensitive to changes in the environment (Borges et al., 2011; Gonçalves et al., 2012c). Lipid peroxidation is one of the main effects of metal treatment. During lipid peroxidation, PUFAs are key molecules for the production of free radicals (Rocchetta et al., 2006), which may explain the notable decrease of PUFA and HUFA amounts after exposure to copper in the studied species.

In the case of the exposure to the herbicide Primextra®, the decrease in PUFAs and HUFAs may be due to the fact that metolachlor, which is the main active ingredient of Primextra® Gold TZ, constituting the majority of the studied herbicide, is known to inhibit several biosynthesis processes, namely lipid, fatty acid, leaf wax, terpene, flavonoid and protein synthesis, in addition to inhibition of cell division and interference with hormonal regulation (Liebl, 1995; Weed, 1994). It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu and Xiong, 2009). The synthesis of very long chain FAs (Robert et al., 2007) is inhibited by inactivation the enzyme involved in the condensation of acyl-CoA and malonyl-CoA to produce 3-ketoacyl-CoA and CO_2 (Thakkar et al., 2013) by metolachlor. Due to the mode of action of this xenobiotic, it is suggested that this contaminant affects the lipid (fatty acids-FA) profile of aquatic species. PUFAs and HUFAs decreased slightly after the exposure of diatom *T. weissflogii* to copper (1.2 times), were considerably reduced to the trace amounts in the brine shrimp nauplii (10 and 15 times respectively) and totally disappeared in the copepod *A. tonsa* exposed to the highest concentration of the contaminant.

This work revealed that the same contaminant, which led to slight changes in the amount of very long chain of FAs in primary producer species, led to considerable reduction of these FAs in primary consumer species, which are fed by healthy cultures of microalgae (noncontaminated cultures). This allows us to suggest that in the environment conditions the decrease of PUFAs and HUFAs would be even much more severe, since contaminant in nature influences all the biota, with the primary consumers suffering higher pressure from toxicants, which are obtained not only from water-borne but also from diet-borne sources.

Moreover, the cells of animals are not able to desaturate some positions of the fatty acyl chain and therefore, some PUFAs and HUFAs can only be synthesized from dietary fats. Required dietary FAs are known as 'essential fatty acids' (Vance and Vance, 2002). Among them are eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids, which play a key role in organism health and functioning. EPA and ARA serve as precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes, etc.), which are responsible for many immune and inflammatory responses, neural function, reproduction, and enhancing the organism's adaptation to the environment and to anthropogenic stressors (Fokina et al., 2013).

Consequently, changes in FA profiles, specifically, the decrease in the amount of PUFAs and HUFAs, influence the health status of the ecosystem on the base of food web, *i.e.* primary producer – primary consumer level, with profound and severe consequences along the entire trophic food web. In summary, our results confirmed that FA are good indicators of the presence of organic and inorganic chemical stressors in marine and estuarine organisms and can constitute important tools and endpoints for ecotoxicological studies.

5. Conclusions

Current research showed that the herbicide Primextra® Gold TZ and the metal copper imply a threat to the estuarine and marine systems, being toxic to the investigated planktonic species, decreasing the growth rate of phytoplankton and increasing the immobilisation of zooplankton species. Some biochemical effects of these pollutants on planktonic organisms were an increase of SFA and a decrease of unsaturated fatty acids, specifically a decrease of the essential fatty acids (EFA) that play important and crucial roles in the organisms' health. Thus, our study proves that changes in FA profiles of organisms may be used as an early-warning indicator of anthropogenic stressors for the assessment of the health status of aquatic species.

Authors' contributions

Conceived and designed the experiments: Ana M. M. Gonçalves, Fernando Gonçalves,

Performed the experiments: Valentina Filimonova, Ana M. M. Gonçalves,

Analyzed the data: Valentina Filimonova, Ana M. M. Gonçalves, Fernando Gonçalves,

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Provided some funding for reagents/materials/analysis tools: João C. Marques, Fernando Gonçalves,

Wrote the paper: Valentina Filimonova, Ana M. M. Gonçalves,

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References

APHA, 1995. Standard Methods for the Examination of Water and Wastewater, 19th ed. American Public Health Association, Washington, DC.

Adams, S.M., Greeley, M.S., 2000. Assess the health of aquatic ecosystems. Water Air Soil Pollut. 123, 103–115.

Araújo, C.F.C., Souza-Santos, L.P., 2013. Use of the microalgae *Thalassiosira weissflogii* to assess water toxicity in the Suape industrial-port complex of Pernambuco, Brazil. Ecotoxicol. Environ. Saf. 89, 212–221, http://dx.doi.org/10.1016/j.ecoenv.2012.11.032.

Arts, M.T., Ackman, R.G., Holub, B.J., 2001. Essential fatty acids in aquatic ecosystems: a crucial link between diet and human health and evolution. Can. J. Fish. Aquat. Sci. 58, 122–137, http://dx.doi.org/10.1139/f00-224.

Bae, J.H., Lim, S.Y., 2012. Heavy metals and biochemical composition of four sea bream species (Acanthopagrus schlegelii Bleeker, Pagurus major Temminck & Schlegel, Oplegnathus fasciatus Krøyer and Girella punctata Gray) Philipp. Agric. Scientist 95, 185–191.

Bard, S.M., 1999. Global transport of anthropogenic contaminants and the consequences for the Arctic marine ecosystem. Mar. Pollut. Bull. 38, 356–379, http://dx.doi.org/10.1016/S0025-326X(99)00041-7.

Borges, L., Morón-Villarreyes, J.A., D'Oca, M.G.M., Abreu, P.C., 2011. Effects of flocculants on lipid extraction and fatty acid composition of the microalgae Nannochloropsis oculata and *Thalassiosira weissflogii*. Biomass Bioenergy 35, 4449–4454, http://dx.doi.org/10.1016/j.biombioe.2011.09.003.

Cardoso, P.G., Raffaelli, D., Lillebø, A.I., Verdelhos, T., Pardal, M.A., 2008. The impact of extreme flooding events and anthropogenic stressors on the macrobenthic communities' dynamics. Estuar. Coast. Shelf Sci. 76, 553–565, http://dx.doi.org/10.1016/j.ecss.2007.07.026.

Clarke, K.R., Gorley, R.N., 2006. Primer V6: User Manual/Tutorial. Primer-E, Plymouth.

Clarke, K.R., Warwick, R.M., 2001. Change in marine communities. In: An Approach to Statistical Analyses and Interpretation. Primer-E, Plymouth.

De Carvalho, C.C.C.R., 2012. Adaptation of Rhodococcus erythropolis cells for growth and bioremediation under extreme conditions. Res. Microbiol. 163, 125–136, http://dx.doi.org/10.1016/j.resmic.2011.11.003.

De Hoop, L., De Troch, M., Hendriks, A.J., De Laender, F., 2013. Modeling toxic stress by atrazine in a marine consumer-resource system. Environ. Toxicol. Chem. 32, 1088–1095, http://dx.doi.org/10.1002/etc.2160.

De Troch, M., Boeckx, P., Cnudde, C., Van Gansbeke, D., Vanreusel, A., Vincx, M., Caramujo, M., 2012. Bioconversion of fatty acids at the basis of marine food webs: insights from a compound-specific stable isotope analysis. Mar. Ecol. Prog. Ser. 465, 53–67, http://dx.doi.org/10.3354/meps09920.

Diz, F.R., Araújo, C.V.M., Moreno-Garrido, I., Hampel, M., Blasco, J., 2009. Short-term toxicity tests on the harpacticoid copepod Tisbe battagliai: lethal and reproductive endpoints. Ecotoxicol. Environ. Saf. 72, 1881–1886, http://dx.doi.org/10.1016/j.ecoenv.2009.03.004.

Fields, D.M., Durif, C.M.F., Bjelland, R.M., Shema, S.D., Skiftesvik, A.B., Browman, H.I., 2011. Grazing rates of Calanus finmarchicus on *Thalassiosira weissflogii* cultured under different levels of ultraviolet radiation. PLoS One 6, 1–6, http://dx.doi.org/10.1371/journal.pone.0026333.

Figueiredo, J., van Woesik, R., Lin, J., Narciso, L., 2009. *Artemia franciscana* enrichment model – How to keep them small, rich and alive? Aquaculture 294, 212–220, http://dx.doi.org/10.1016/j.aquaculture.2009.05.007.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, WILEY-VCH Verlag, Cambridge, http://dx.doi.org/10.1002/bimj.19720140111.

Fisher, N.S., Schwarzenbach, R.P., 1978. Fatty-acid dynamics in thalassiosira-Pseudonana (*Bacillariophyceae*)—implications for physiological ecology. J. Phycol. 14, 143–150, http://dx.doi.org/10.1111/j.1529-8817.1978.tb02439.x.

Fleeger, J.W., Carman, K.R., Nisbet, R.M., 2003. Indirect effects of contaminants in aquatic ecosystems. Sci. Total Environ. 317, 207–233, http://dx.doi.org/10.1016/S0048-9697(03)00141-4.

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. Biol. Trace Elem. Res. 154, 217–225, http://dx.doi.org/10.1007/s12011-013-9727-3.

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. Aquat. Toxicol. 101, 367–376, http://dx.doi.org/10.1016/j.aquatox.2010.11.011.

Gonçalves, A.M.M., De Troch, M., Marques, S., Pardal, M., Azeiteiro, U., 2010a. Spatial and temporal distribution of harpacticoid copepods in Mondego estuary. J. Mar. Biol. Assoc. U. K. 90, 1279–1290, http://dx.doi.org/10.1017/S002531541000041X.

Gonçalves, A.M.M., Pardal, M.A., Marques, S.C., De Troch, M., Azeiteiro, U.M., 2010b. Distribution and composition of small-size zooplankton fraction in a temperate shallow estuary (Western Portugal). Fresenius Environ. Bull. 19, 3160–3176.

Gonçalves, A.M.M., Azeiteiro, U., Pardal, M., De Troch, M., 2012a. Fatty acid profiling reveals seasonal and spatial shifts in zooplankton diet in a temperate estuary. Estuar. Coast. Shelf Sci. 109, 70–80, http://dx.doi.org/10.1016/j.ecss.2012.05.020.

Gonçalves, A.M.M., Pardal, M., Marques, S., Mendes, S., Fernández-Gómez, M., Galindo-Villardón, M., Azeiteiro, U., 2012b. Diel vertical behavior of Copepoda community (naupliar, copepodites and adults) at the boundary of a temperate estuary and coastal waters. Estuar. Coast. Shelf Sci. 98, 16–30, http://dx.doi.org/10.1016/j.ecss.2011.11.018.

Gonçalves, A.M.M., Pardal, M., Marques, S., Mendes, S., Fernández-Gómez, M., Galindo-Villardón, M., Azeiteiro, U., 2012c. Responses of Copepoda life-history stages to climatic variability in a southern-European temperate estuary. Zool. Stud. 51, 321–335.

Gonçalves, A.M.M., Mesquita, A.F., Verdelhos, T., Coutinho, J.A.P., Marques, J.C., Gonçalves, F., 2016. Fatty acids' profiles as indicator of stress induced by of a common herbicide on two marine bivalves species: cerastoderma edule (Linnaeus, 1758) and Scrobicularia plana (da Costa, 1778). Ecol. Indic. 63, 209–218, http://dx.doi.org/10.1016/j.ecolind.2015.12.006.

Gotelli, N.J., Ellison, A.M., Ballif, B.A., 2012. Environmental proteomics, biodiversity statistics and food-web structure. Trends Ecol. Evol. 27, 436–442, http://dx.doi.org/10.1016/j.tree.2012.03.001.

Guillard, R.R.L., 1983. Culture of phytoplankton for feeding marine invertebrates. In: Berg, C.J. (Ed.), Culture of Marine Invertebrates. Hutchinson-Ross, Pensylvania, USA, pp. 108–132.

Hack, L.A., Tremblay, L.A., Wratten, S.D., Forrester, G., Keesing, V., 2008. Zinc sulfate and atrazine toxicity to the marine harpacticoid copepod Robertsonia propinqua. N. Zeal. J. Mar. Freshwater Res. 42, 93–98, http://dx.doi.org/10.1080/00288330809509939.

Kelly, J.R., Scheibling, R.E., 2012. Fatty acids as dietary tracers in benthic food webs. Mar. Ecol. Prog. Ser. 446, 1–22, http://dx.doi.org/10.3354/meps09559.

Kennish, M.J., 2000. Practical handbook of Marine Science, 3rd ed. CRC Press LLC, http://dx.doi.org/10.1017/CBO9781107415324.004.

Kumar, M., Kumari, P., Gupta, V., Anisha, P. a., Reddy, C.R.K., Jha, B., 2010. Differential responses to cadmium induced oxidative stress in marine macroalga Ulva lactuca (Ulvales, Chlorophyta). Biometals 23, 315–325, http://dx.doi.org/10.1007/s10534-010-9290-8.

Lavens, P., Sorgeloos, P., 1996. Manual on the production and use of live food for aquaculture. FAO Fish. Tech. Pap., p361.

Liebl, 1995. Cell growth inhibitors (Chloroacetamides, cabomothioates, napropamide, bensulide). Herbicide Action, vol. 1. Purdue University, West Lafayette, pp. 200–224.

Liu, H., Xiong, M., 2009. Comparative toxicity of racemic metolachlor and S-metolachlor to Chlorella pyrenoidosa. Aquat. Toxicol. 93, 100–106, http://dx.doi.org/10.1016/j.aquatox.2009.04.006.

Maazouzi, C., Masson, G., Izquierdo, M.S., Pihan, J.C., 2008. Chronic copper exposure and fatty acid composition of the amphipod Dikerogammarus villosus: results from a field study. Environ. Pollut. 156, 221–226, http://dx.doi.org/10.1016/j.envpol.2007.12.010.

Manimaran, K., Karthikeyan, P., Ashokkumar, S., Ashok Prabu, V., Sampathkumar, P., 2012. Effect of copper on growth and enzyme activities of marine diatom, Odontella mobiliensis. Bull. Environ. Contam. Toxicol. 88, 30–37, http://dx.doi.org/10.1007/s00128-011-0427-4.

Marcus, N.H., Wilcox, J.A., 2007. A Guide to the Meso-Scale Production of the Copepod *Acartia Tonsa*. Florida Sea Grant.

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

Nunes, B.S., Carvalho, F.D., Guilhermino, L.M., Van Stappen, G., 2006. Use of the genus Artemia in ecotoxicity testing. Environ. Pollut. 144, 453–462, http://dx.doi.org/10.1016/j.envpol.2005.12.037.

OECD 202, 2004. OECD Guidelines for testing of chemicals. OECD/OCDE 202. Daphnia sp. Acute Immobilisation Test.

Pinho, G.L.L., Bianchini, A., 2010. Acute copper toxicity in the euryhaline copepod *Acartia tonsa*: implications for the development of an estuarine and marine

biotic ligand model. Environ. Toxicol. Chem. 29, 1834–1840, http://dx.doi.org/10.1002/etc.212.
Pinto, E., Carvalho, A.P., Cardozo, K.H.M., Malcata, F.X., dos Anjos, F.M., Colepicolo, P., 2011. Effects of heavy metals and light levels on the biosynthesis of carotenoids and fatty acids in the macroalgae Gracilaria tenuistipitata (var. liui Zhang & Xia). Brazilian J. Pharmacogn. 21, 349–354, http://dx.doi.org/10.1590/S0102-695X2011005000060.
Pinto, P.M., Bio, A., Hontoria, F., Almeida, V., Vieira, N., 2013. Portuguese native Artemia parthenogenetica and *Artemia franciscana* survival under different abiotic conditions. J. Exp. Mar. Biol. Ecol. 440, 81–89, http://dx.doi.org/10.1016/j.jembe.2012.11.016.
Popova, O.B., Sanina, N.M., Likhatskaya, G.N., Bezverbnaya, I.P., 2008. Effects of copper and cadmium ions on the physicochemical properties of lipids of the marine bacterium Pseudomonas putida IB28 at different growth temperatures. Russ. J. Mar. Biol. 34, 179–185, http://dx.doi.org/10.1134/S1063074008030073.
Prado, R., García, R., Rioboo, C., Herrero, C., Abalde, J., Cid, A., 2009. Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide Paraquat. Environ. Int. 35, 240–247, http://dx.doi.org/10.1016/j.envint.2008.06.012.
Pratoomyot, J., Srivilas, P., Noiraksar, T., 2005. Fatty acids composition of 10 microalgal species. Songklanakarin J. Sci. Technol. 27, 1179–1187.
Ramírez, B., Montero, D., Izquierdo, M., Haroun, R., 2013. Aquafeed imprint on bogue (Boops boops) populations and the value of fatty acids as indicators of aquaculture-ecosystem interaction: are we using them properly? Aquaculture 414-415, 294–302, http://dx.doi.org/10.1016/j.aquaculture.2013.08.010.
Rippingale, R.J., Payne, M.F., 2001. Intensive cultivation of the calanoid copepod Gladioferens imparipes. Aquaculture 201, 329–342, http://dx.doi.org/10.1016/S0044-8486(01)00608-1.
Ritter, A., Goulitquer, S., Salaün, J.P., Tonon, T., Correa, J. a., Potin, P., 2008. Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp Laminaria digitata. New Phytol. 180, 809–821, http://dx.doi.org/10.1111/j.1469-8137.2008.02626.x.
Robert, S., Mansour, M.P., Blackburn, S.I., 2007. Metolachlor-mediated selection of a microalgal strain producing novel polyunsaturated fatty acids. Mar. Biotechnol. 9, 146–153, http://dx.doi.org/10.1007/s10126-006-6102-9.
Rocchetta, I., Mazzuca, M., Conforti, V., Ruiz, L., Balzaretti, V., De Molina, M.D.C.R., 2006. Effect of chromium on the fatty acid composition of two strains of Euglena gracilis. Environ. Pollut. 141, 353–358, http://dx.doi.org/10.1016/j.envpol.2005.08.035.
Ruiz, O., Amat, F., Navarro, J.C., 2008. A comparative study of the fatty acid profile of *Artemia franciscana* and *A. persimilis* cultured at mesocosm scale. J. Exp. Mar. Biol. Ecol. 354, 9–16, http://dx.doi.org/10.1016/j.jembe.2007.09.015.
Sánchez-Muros, M.J., Villacreces, S., Miranda-de la Lama, G., de Haro, C., García-Barroso, F., 2013. Effects of chemical and handling exposure on fatty acids, oxidative stress and morphological welfare indicators in gilt-head sea bream (Sparus aurata). Fish Physiol. Biochem. 39, 581–591, http://dx.doi.org/10.1007/s10695-012-9721-2.
Sibi, G., Anuraag, T.S., Bafila, G., 2014. Copper stress on cellular contents and fatty acid profiles in Chlorella species. OJBS 14, 209–217, http://dx.doi.org/10.3844/ojbssp.2014.209.217.
Silins, I., Högberg, J., 2011. Combined toxic exposures and human health: biomarkers of exposure and effect. Int. J. Environ. Res. Public Health 8, 629–647, http://dx.doi.org/10.3390/ijerph8030629.
Smalling, K.L., Kuivila, K.M., Orlando, J.L., Phillips, B.M., Anderson, B.S., Siegler, K., Hunt, J.W., Hamilton, M., 2013. Environmental fate of fungicides and other current-use pesticides in a central California estuary. Mar. Pollut. Bull. 73, 144–153, http://dx.doi.org/10.1016/j.marpolbul.2013.05.028.
Stottrup, J., Richardson, K., Kirgesgaard, E., Jorgen Pihl, N., 1986. The cultivation of *Acartia tonsa* Dana for use as a live food source for marine fish larvae. Aquaculture 52, 87–96, http://dx.doi.org/10.1017/S0022172400002345.
Stringer, T.J., Glover, C.N., Keesing, V., Northcott, G.L., Tremblay, L. a., 2012. Development of a harpacticoid copepod bioassay: selection of species and relative sensitivity to zinc, atrazine and phenanthrene. Ecotoxicol. Environ. Saf. 80, 363–371, http://dx.doi.org/10.1016/j.ecoenv.2012.04.008.
Thakkar, M., Randhawa, V., Wei, L., 2013. Comparative responses of two species of marine phytoplankton to metolachlor exposure. Aquat. Toxicol. 126, 198–206, http://dx.doi.org/10.1016/j.aquatox.2012.10.002.
Vance, D., Vance, J., 2002. Biochemistry of Lipids, Lipoproteins and Membranes, 4th ed. Elsevier.
Veloza, A.J., Chu, F.L.E., Tang, K.W., 2006. Trophic modification of essential fatty acids by heterotrophic protists and its effects on the fatty acid composition of the copepod *Acartia tonsa*. Mar. Biol. 148, 779–788, http://dx.doi.org/10.1007/s00227-005-0123-1.
Weed, 1994. Herbicide Handbook, 7th ed. Weed Science Society of America, Champaign.