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# Effects of a herbicide and copper mixture on the quality of marine plankton

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## Abstract:

Pesticides and metals are often used in agriculture and are therefore often simultaneously discharged to nearby estuarine and marine areas. The effects of this organic-inorganic chemical mixture on food quality of aquatic organisms are currently unknown. In this study we test if a mixture of copper (inorganic) and the herbicide Primextra<sup>®</sup> Gold TZ (organic) affects the quality of the diatom *Thalassiosira weissflogii* and the copepod *Acartia tonsa* – two key species that fuel the local food-web. We quantified quality (i.e. energy content as food for the next trophic level) in terms of fatty acids, proteins and thiobarbituric acid reacting substances. We found non-additive effects (positive and negative) of the metal-herbicide mixture on the diatom and copepod species. In general, nutritionally important biochemical parameters of *Acartia tonsa* were most sensitive to the chemical stressors.

**Keywords:** food quality; generalized linear model; plankton; species sensitivity; biomarkers; fatty acids

37        *Abbreviations:* FA, fatty acids; EFA, essential fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic  
38 acid; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reacting substances; GLM, generalized linear  
39 model; FAMES, fatty acid methyl esters; ROS, reactive oxygen species.

## 40        **1. Introduction**

41        The amount of available biomass has important effects on ecosystem functions, as it drives  
42 the amount of food available in food webs. In plankton communities, low food quality of  
43 phytoplankton biomass leads to poor energy and nutrient transfer through the food web (Perhar  
44 and Arhonditsis, 2009).

45        Important indicators of quality (i.e. energy content as food for the next trophic level) are the  
46 fatty acids (FA), especially the essential ones (EFA), and proteins. The dietary protein content  
47 influences the biochemical composition of invertebrates and their growth rate and production.  
48 Fatty acids are the main components of lipids and fuel all metabolic systems. EFA belong to the  
49 group of polyunsaturated fatty acids (PUFA) and play a key role in the health and function of all  
50 animals at all trophic levels.

51        Chemical stressors can catalyze the production of reactive oxygen species, which may lead to  
52 the lipid peroxidation in organisms. Consequently, lipid peroxidation may severely change the  
53 nutritional quality by breaking down EFA. The measurement of thiobarbituric acid reacting  
54 substances (TBARS) content is the most common test to assess the lipid peroxidation and thus  
55 stress response but it is also used as one of the standardized parameters of food quality (Huss,  
56 1995).

57        Most ecotoxicological studies expose species to single doses of chemical stressors (Chen et  
58 al., 2015). Few studies are focused on mixtures of stressors from one chemical group (e.g.  
59 organic-organic or inorganic-inorganic) (Hanazato, 2001). However, even fewer studies have  
60 reported on mixtures of contaminants from different chemical groups (e.g. organic and  
61 inorganic). Such studies are urgently needed to better predict the changes that can be expected  
62 from the exposure of aquatic communities to environmental stress (Filimonova et al., 2016a).  
63 The simultaneous presence of organic and inorganic contaminants is especially relevant for  
64 estuarine and marine areas with intensive agricultural activities, because the discharge of  
65 pesticides and metals can be substantial in these areas (Gonçalves et al., 2016).

66        At present, according to the information obtained from agricultural cooperatives of Mondego  
67 valley located in West Atlantic coast of Portugal, the herbicide Primextra<sup>®</sup> Gold TZ is one of the  
68 20 best-selling herbicides in Portugal, being widely used in corn fields, whereas copper is in  
69 general one of the main constituents of fungicides, herbicides, molluscides, and pesticides  
70 (Filimonova et al., 2016b; Gonçalves et al., 2016; Neves et al., 2015).

71        Copper (II) sulphates pentahydrate belongs to the group of fungicides. The Portuguese market  
72 is largely dominated by fungicides. In the last 10 years their consumption has been decreasing  
73 smoothly and by 2015 reached 5193 tonnes of active ingredients (a.i.). being still 1.5 times  
74 higher than in 1992 and 2.5 times higher than the herbicide consumption in 2015  
75 (<http://www.fao.org/faostat>).

76

77 Primextra<sup>®</sup> Gold TZ (Syngenta AG) consists of two main a.i., 30.2% (w/w) S-metolachlor  
78 and 17.75% (w/w) terbuthylazine (TBA). It contains as well a residual percentage of coadjuvant  
79 substances that are supposed to be inert (Filimonova et al., 2016b; Neves et al., 2015). According  
80 to groundwater ubiquity score (GUS) that estimates the pesticide's potential to move towards  
81 groundwater and ranks their leaching potential from extremely low (below 0), via low (0 – 1.8)  
82 and moderate (1.8 – 2.8) to high (above 2.8) ones, metolachlor and TBA have a high leaching  
83 potential: GUS index of 3.5 and 3.1, respectively. Therefore, they are expected to contaminate  
84 aquatic ecosystems. In addition, these compounds are relatively hydrophobic and have a high  
85 potential for bioaccumulation ( $\log K_{ow} = 3.40$ ), which indicates their possible accumulation in  
86 aquatic organisms and therefore finally of their bioamplification in the food chains (Cruzeiro et  
87 al., 2016; Gustafson, 1989). A recent study revealed the presence of both active ingredients of  
88 Primextra<sup>®</sup> Gold TZ in the Mondego River estuary (Cruzeiro et al., 2016). Although the amount  
89 of TBA was lower than the established legislation value of 0.100 µg/L – 0.088 µg/L (in winter) –  
90 metolachlor exceeded this value – 0.266 µg/L (in spring) was obtained (Cruzeiro et al., 2016).

91 Copper sulphate is known to be toxic to invertebrates, i.e. rotifers, cladocerans and copepods  
92 and above a specific concentration – to fish including economically valuable species such as  
93 salmonids, cyprinids and catfish (Abdel-Tawwab et al., 2007). At higher than essential amounts  
94 copper negatively influences on numerous important processes, including metabolism of fatty  
95 acid and protein synthesis. Metolachlor as well inhibits the biosynthesis of several crucial  
96 molecules including proteins and very long chain fatty acid that are essential for all living  
97 organisms, whereas TBA inhibits the photosynthesis at photosystem II (Filimonova et al.,  
98 2016b). Only a few studies analysed the toxic and biochemical effects of Primextra<sup>®</sup> Gold TZ on  
99 aquatic species: e.g. marine bivalves *Cerastoderma edule* and *Scrobicularia plana* (Gonçalves et  
100 al., 2016), freshwater zooplanktonic species *Daphnia longispina* (Neves et al., 2015) and  
101 freshwater fish species *Clarias gariepinus* and *Clarias albopunctatus* (Asomba and Ugokwe,  
102 2015; Nwani et al., 2014). Therefore, it is essential to study the toxicological and biochemical  
103 effects of this herbicide on other non-target species (Gonçalves et al., 2016) especially in  
104 combination with inorganic substances, i.e. metals, such as copper.

105 In order to clarify whether this combination will lead to additive or non-additive effects, in the  
106 present study, we analysed the effects of two different chemical stressors on the FA, EFA,  
107 protein and TBARS contents, using planktonic species at two trophic levels. We considered the  
108 herbicide Primextra<sup>®</sup> Gold TZ and the metal copper, i.e. copper (II) sulphates pentahydrate, both  
109 individually and in an equitoxic mixture. We selected two plankton species (one primary  
110 producer, one consumer) that are well-known test species in marine ecotoxicology: the marine  
111 diatom *Thalassiosira weissflogii* and the estuarine copepod *Acartia tonsa* (one of the most  
112 abundant copepod species in the Mondego estuary (Gonçalves et al., 2010).

113 In the Mondego estuary diatoms are one of the dominating phytoplankton groups (Flindt et  
114 al., 1997) therefore in this study the diatom *T. weissflogii* was chosen as one of the main primary  
115 producer species.

116 The main aims of this study were to determine: (1) whether there is an additive (without  
117 interaction of chemicals) or non-additive (with interaction of chemicals) effect of a relevant  
118 chemical mixture on the FA, EFA, protein and TBARS contents

119 (as proxy for energy content or food quality for the next trophic level) of selected phytoplankton  
120 and zooplankton species, and (2) how this mixture differentially affects different trophic levels.

## 121 **2. Materials and methods**

### 122 *2.1. Cultures maintenance*

123 Culture conditions and its maintenance were followed as described by Filimonova et al.  
124 (2016b).

125 *Acartia tonsa* (Copepoda, Calanoida) was sampled in the south arm of Mondego estuary  
126 (40°08`N, 8°50`W) near the Pranto river, where it was found in high abundance (Gonçalves et  
127 al., 2010). The Mondego estuary is a tidal estuary located near Figueira da Foz city on the west  
128 coast of Portugal.

129 Copepods were sampled with horizontal subsurface tows with a bongo net, placed to the 2.5 L  
130 flasks filled with the estuarine water and transported to the laboratory (Gonçalves et al., 2012).  
131 Adults of *A. tonsa* were separated from other species and moved to prepared 10 L – aquaria at a  
132 concentration of 1 individual per 10 ml of medium for further maintenance and reproduction  
133 (Rippingale and Payne, 2001). Aquaria were supplied with gentle aeration system. Filtrated (1.2  
134 µm pores: to exclude the possibility of nanoplankton penetration) natural seawater diluted with  
135 distilled water to a salinity of 13-15 psu was used as medium. These values reflected the salinity  
136 found in the sampling site and allowed to maintain the successful reproduction and growth of *A.*  
137 *tonsa*. The medium renewal (30 % from the total volume) and measurements of dissolved O<sub>2</sub> (%)  
138 were applied regularly. Feeding with the diatom *T. weissflogii* (2×10<sup>4</sup> cells/mL) was done 3 times  
139 a week. A Neubauer chamber was used to count the algae cells. Adult organisms, grown during  
140 14 days from the first cohort of nauplii were used for the bioassays.

141 The diatom species *Thalassiosira weissflogii* was acquired from the Scottish Marine Institute  
142 (Dunbeg, PA37 1QA, UK; strain number 1085/18). It was cultured with the Guillard's f/2  
143 medium with a salinity of 30 psu, without EDTA [adapted from Rippingale and Payne, 2001] that  
144 was used as well for algae dilution. A renew of algae culture was done weekly.

145 Zooplankton and phytoplankton culture maintenance was conducted with a 16h light and 8h  
146 dark light photoperiod and at a temperature of 20±2°C.

### 147 *2.2. Population microcosm bioassays for the determination of the effect on the quality of the* 148 *diatom and copepod species*

149 Microcosm bioassays for the determination of the effect on the quality of studied species were  
150 conducted to determine changes in their FA profiles, EFA, protein and TBARS contents after  
151 exposure to the herbicide Primextra® Gold TZ and the metal copper (II) sulphates pentahydrate  
152 individually and in equitoxic mixture. These bioassays were performed according to Filimonova  
153 et al. (2016b) under the same laboratory conditions described above for culture maintenance.

154 The diatom *T. weissflogii* and the copepod *A. tonsa* were exposed in four experimental  
155 treatments: (1) a negative control – CTL, consisting of uncontaminated culture medium; (2) a  
156 low level of each toxicant – C1; (3) an intermediate level – C2 and (4) a high level – C3 (Table  
157 S1, Supplemental Data).

158 These treatments were chosen in view of their negative effect (C1, C2, C3 caused 10%, 20%  
159 and 50% effect, respectively) on the relative growth rate of *T. weissflogii* (i.e. growth inhibition)  
160 and the relative survival of *A. tonsa* (i.e. immobilized individuals) after exposure to the equitoxic  
161 mixture of contaminants during 96h and 48h bioassays respectively that were performed  
162 according to Filimonova et al. (2016b).

163 For both species, all treatments were replicated three times in bioassays to conduct further FA  
164 analysis and five times in bioassays to conduct further TBARS and protein contents  
165 determination. The duration of bioassays for further TBARS and protein content determination  
166 was 96h, whereas for further FA analyses – 7 days. Exposures of both species were performed in  
167 glass (pesticide and mixture bioassays) and plastic (metal bioassays) beakers: copper is able to  
168 bind with silica constituting the chemical structure of the glass, therefore plastic flasks were used  
169 in bioassays with copper; the glass dishes are typically used when the test material is  
170 unknown (Rand, 1995). That was the case for bioassays with the herbicide formulation and its  
171 mixture with the metal.

172 Bioassays with *T. weissflogii* were carried out in flasks with the final volume of the medium  
173 40 mL, i.e. the Guillard's f/2 medium with a salinity of 30 psu. Initial cell density was  $10^4$  cells /  
174 mL. At the end of each bioassay  $3.6 \times 10^6$  cells / mL of diatom were counted in each replicate that  
175 was then concentrated and stored frozen at  $-80^\circ\text{C}$  for further biochemical analyses. Neubauer  
176 haemocytometer was used to calculate the cell density. For further FA analyses cells were  
177 concentrated on GF/F Whatman filters, for the TBARS and protein contents measurement the  
178 diatom cells were separated from the culture medium by centrifugation ( $4^\circ\text{C}$ , 4000 rpm, 10 min).

179 Bioassays with *A. tonsa* were done in vials with a final volume of 2500 mL with 250  
180 individuals per replicate for further FA analyses and of 5000 mL with 500 individuals per  
181 replicate for further TBARS and protein contents determination. Each flask was connected to a  
182 gentle aeration system. *A. tonsa* were fed daily with *T. weissflogii* cells in the exponential growth  
183 phase at a concentration of  $2 \times 10^4$  cells/mL and moved to new test solutions every third day.  
184 When feeding the copepod species with the diatom culture the salinity was adjusted back to 13-  
185 15 psu adding distilled water to the experimental flasks. This was a very small volume and had  
186 no substantial influence on the final volume. At the end of each bioassay for further FA analyses  
187 60 individuals per replicate were concentrated on GF/F Whatman filters and stored frozen at -  
188  $80^\circ\text{C}$ . At the end of each bioassay for further TBARS and protein contents determination 250  
189 individuals per replicate were separated manually without medium and stored frozen at  $-80^\circ\text{C}$ .  
190 The further TBARS and protein analyses were performed with individuals of the same flask in  
191 each bioassay.

192

### 193 2.3. Population microcosm bioassays for a comparison of the effects between trophic levels

194 In order to compare the effects between trophic levels, both the primary consumer *A. tonsa*  
195 and the primary producer *T. weissflogii* were exposed to the same test conditions and to the same  
196 levels of contaminants.

197 Biochemical analyses of the species required the collection of live organisms at the end of  
198 each bioassay. Therefore, the contaminant's concentrations applied to *A. tonsa* (Table S1) were  
199 used for both species (Table S2, Supplemental Data)".

200 Due to the low cell density of diatoms at the end of each bioassay the separation of diatom  
201 from the culture medium was possible only with the GF/F Whatman filter. Therefore, the  
202 samples were stored only for further FA analysis.

#### 203 2.4. Biochemical analyses

204 Analyses of fatty acids including essentials fatty acids were followed as described by  
205 Filimonova et al. (2016b).

206 The initial step was the extraction of total lipids of study species and their methylation to fatty  
207 acid methyl esters (FAMES) that were performed with a modified 1-step derivatisation method  
208 from De Troch et al. (2012) and Gonçalves et al., (2012). The internal standard of  
209 methylnonadecanoate C19:0 fatty acid (Fluka 74208) was added to each sample for the  
210 quantification of FA. The FAMES thus obtained were analyzed using a gas chromatograph (HP  
211 6890N GC) coupled to a mass spectrometer (HP 5973).

212 All samples were run in split4 mode with a 0.25  $\mu\text{L}$  injection per run at an injector  
213 temperature of 250  $^{\circ}\text{C}$ , using a HP88 column (Agilent J & W; Agilent Co., USA) with a He flow  
214 of 1.5  $\text{mL min}^{-1}$ . The oven temperature was programmed at 50  $^{\circ}\text{C}$  for 2 min, followed by a ramp  
215 of 25  $^{\circ}\text{C min}^{-1}$  to 75  $^{\circ}\text{C}$ , then a second ramp at 2  $^{\circ}\text{C min}^{-1}$  to 230  $^{\circ}\text{C}$  with a final 14 min hold.

216 FAMES were identified by comparison with the retention times and mass spectra of authentic  
217 standards and available ion spectra in Famedb23 (composed in the Marine Biology research  
218 group) and WILEY mass spectral libraries. The analyses of FAMES were performed with the  
219 software Agilent MSD Productivity ChemStation. External (Supelco 37 Component FAME Mix,  
220 Supelco # 47885, Sigma-Aldrich, Inc., USA) and additional standards of 16:2 $\omega$ 6, 16:2 $\omega$ 4 and  
221 16:3 $\omega$ 3 (Larodan Fine Chemicals) were used to quantify the individual FAMES.

222 A linear regression was applied to the chromatographic peak areas and corresponding known  
223 concentrations of the standards (from 100 to 800  $\mu\text{g/mL}$ ) to define the quantification function of  
224 each FAME.

225 The used shorthand fatty acids notations of the form X:Y $\omega$ Z denote the following: X is the  
226 number of carbon atoms, Y is the number of double bonds, and Z is the position of the double  
227 bond closest to the terminal methyl group (De Troch et al., 2012; Filimonova et al., 2016b).

228 Samples of copepod and diatom for further TBARS and protein contents determination were  
229 homogenized and sonicated respectively at 4  $^{\circ}\text{C}$  in 50 mM  $\text{NaH}_2\text{PO}_2/\text{Na}_2\text{HPO}_4$  buffer, pH 7.0,  
230 containing 0.1% Triton X-100 and then centrifuged at 15000 G for 10 min. at 4  $^{\circ}\text{C}$ . The  
231 supernatant 1 of each sample was divided in two aliquots, one for protein content determination  
232 and the other for determining the TBARS' content. For TBARS the supernatant 1 of each sample  
233 was treated with 10% trichloroacetic acid and then centrifuged at 10000 G for 1 min. at room  
234 temperature. Supernatant 2 was treated with 1% thiobarbituric acid and then boiled for 10 min.  
235 After cooling it was centrifuged a second time at 10000 G for 1 min. at room temperature.

236 Supernatant 3 was taken and its absorbance measured at 535 nm using a microplate reader  
237 LabSystems Original Multiskan EX and a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$   
238 was used to calculate TBARS concentration. The values were expressed as nanomoles of  
239 malondialdehyde (MDA, one of the main co-products of lipid peroxidation with 2-thiobarbituric  
240 acid) per milligram of protein (Buege and Aust, 1978).

241 Protein concentration was measured in supernatant 1 by the Bradford method with Coomassie  
242 Brilliant Blue G-250 (Bradford, 1976) and using  $\gamma$ -globulin bovine as a standard. The protein  
243 assay was performed using a microplate reader LabSystems Original Multiskan EX at 595 nm  
244 and expressed as milligrams per milliliter.

245 One-way analysis of variance (ANOVA) was applied to the biochemical parameters, i.e.  
246 protein and TBARS contents, that were significantly predicted by the contaminant/s to test  
247 significant differences among treatments. The Dunnett's multiple comparison test was further  
248 performed to determine the significant differences between contaminated treatments and  
249 uncontaminated treatment, i.e. the control. The used level of significance was of 0.05. Prior to  
250 the analysis, the data were checked to meet the assumptions of normality (Shapiro-Wilk test) and  
251 homoscedasticity (Levene's test).

## 252 2.5. Modelling of the data

253 Generalized Linear Models can be used to test the presence or absence of a non-additive  
254 effect. To achieve the main aims of the study we fitted generalized linear models with interaction  
255 (GLM<sub>i</sub>) and without interaction (GLM<sub>n/i</sub>) terms to experimentally observed responses of  
256 biochemical composition to the single substances and the mixture.

257 The further comparison of their Akaike Information Criteria (AICs) was used to evaluate  
258 the predictive capacity of each model and allowed us to test if effects were additive or not. A  
259 lower AIC was interpreted as a better trade-off between predictive capacity and model  
260 complexity.

261 Thus, a GLM with gamma distribution and inverse link function (model 1) was used to  
262 estimate the effect of the chemical mixture on the quality of the planktonic species with  
263 biochemical parameters: FA, EFA, TBARS and protein contents as response variables and the  
264 treatments of copper (II)sulphates pentahydrate and the herbicide Primextra<sup>®</sup> as predictors.

265 The presence of non-additive effects was tested via applying the GLM with interaction  
266 (GLM<sub>i</sub>) and without interaction of contaminants (GLM<sub>n/i</sub>).

$$267 \quad BP_i = \beta_0 + \beta_1 \times T_{CuSP,i} + \beta_2 \times T_{Pr,i} \text{ (model 1, GLM}_{n/i}\text{)}$$

$$268 \quad BP_i = \beta_0 + \beta_1 \times T_{CuSP,i} + \beta_2 \times T_{Pr,i} + \beta_3 \times T_{CuSP,i} \times T_{Pr,i} \text{ (model 1, GLM}_i\text{)}$$

269  $BP_i$  represents the biochemical parameter (FA, EFA, TBARS or protein contents) at the  
270 concentration  $i$  of the contaminant;  $T_{CuSP,i}$  and  $T_{Pr,i}$  are treatments of copper (II) sulphate  
271 pentahydrate and Primextra<sup>®</sup> at the concentration  $i$  (Table S1);  $\beta_0$  and  $\beta_1/\beta_2/\beta_3$  are the intercept  
272 and the related slopes, respectively.

273 Homogeneity of model residuals was inspected by plotting the standardized residuals versus  
274 the predicted values. The goodness of the model fit was estimated by plotting the observed  
275 values versus the predicted values (Zuur et al., 2009).

276 To compare the effects of the chemical mixture between the two different trophic levels, we  
277 made two models:

278 (1) the GLM model 1, where for each species equal levels of contaminants (Table S2) were  
279 predictors and species FA profiles were dependent variables;

280

281 (2) the GLM model 2, where the saturated FA (SFA) and polyunsaturated FA (PUFA) of the  
282 copepod species were response variables and the SFA and PUFA of the diatom species were  
283 predictors.

$$284 \quad FA_{At,i} = \beta_0 + \beta_1 \times SFA_{Tw,i} + \beta_2 \times PUFA_{Tw,i} \text{ (model 2)}$$

285  $FA_{At}$  represents the response of either saturated FA or polyunsaturated FA of the copepod *A.*  
286 *tonsa* at the concentration  $i$  of the contaminant,  $SFA_{Tw,i} / PUFA_{Tw,i}$  are saturated /  
287 polyunsaturated FA of the diatom *T. weissflogii* at the concentration  $i$  of the contaminant,  $\beta_0$  and  
288  $\beta_1 / \beta_2$  are the intercept and the related slopes.

289 FA data of the copepod species were log10 - transformed to meet the assumptions of  
290 generalized linear model regarding homoscedasticity, i.e. homogeneity of model residuals was  
291 tested by plotting the standardized residuals versus the predicted values (Zuur et al., 2009). The  
292 goodness of the model fit were tested by plotting the observed values versus the predicted values  
293 (Zuur et al., 2009).

294 All calculations were performed in R ver. 3.2.2, using RStudio ver. 0.99.489 and the packages  
295 lattice, mgcv, nlme.

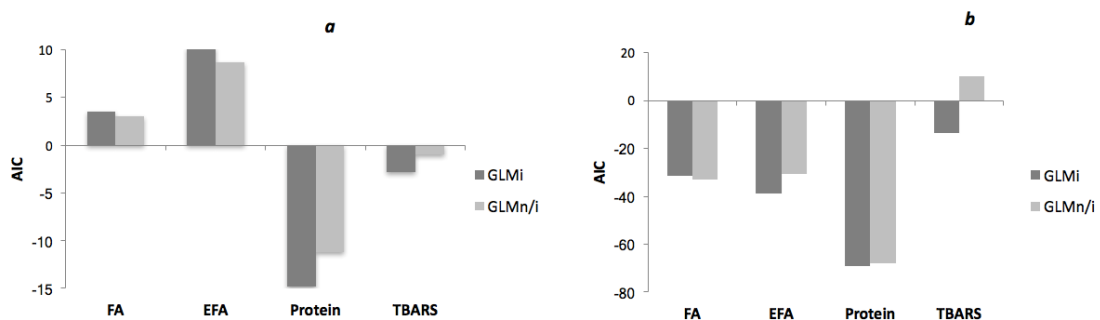
296

297

### 3. Results

#### 298 3.1. The effect of chemical mixture on the quality of the diatom and copepod species

299 We found non-additive effects (both positive and negative) of the chemical mixture on most  
300 biochemical parameters. The GLM<sub>i</sub> model predicted better the essential FA of the copepod  
301 *Acartia tonsa* and the TBARS and protein contents of both species than the model without  
302 interaction (GLM<sub>n/i</sub>). Only for the total FA profile of both species and for the essential FA data  
303 of the diatom *T. weissflogii* a lower AIC value for the GLM<sub>n/i</sub>, suggesting that interactions were  
304 not improving the predictive capacity (Fig. 1), was reported. Plots indicating the goodness of the  
305 model fit are presented at Fig. S1 (Supplemental Data).



306

307 **Fig. 1.** The Akaike Information Criterion (AIC) values determined for generalized linear models with interaction (GLM<sub>i</sub>) and without interaction  
308 (GLM<sub>n/i</sub>) term for the diatom *T. weissflogii* (a) and for the copepod *A. tonsa* (b). The lower value of AIC indicates better model fit to the data. FA  
309 – total fatty acid profile, EFA – essential FA, protein – protein content, TBARS - thiobarbituric acid reacting substances content.

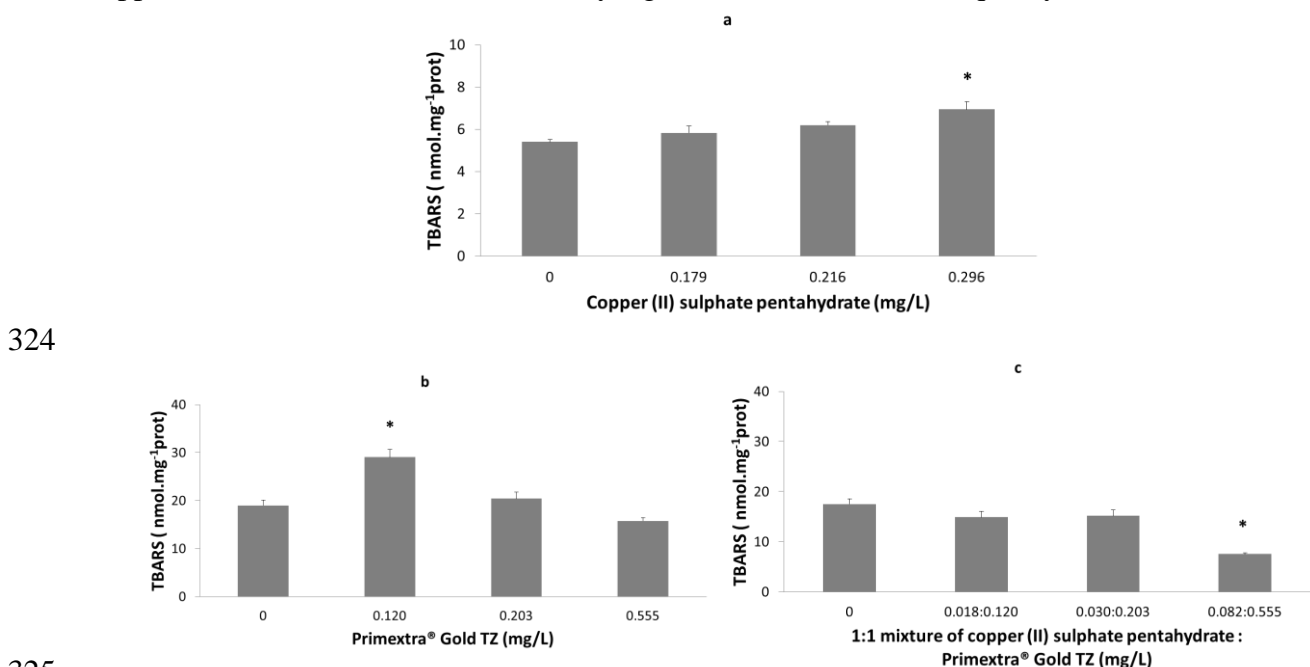
310 Modelling results revealed that the treatments affected most of the biochemical parameters  
311 only for the copepod species. For the diatom species, only a few parameters had significant  
312 effects (Table 1).

313 **Table 1.** Results of generalized linear models with lower AIC value predicting the effect of contaminants on the quality of *T. weissflogii* and *A. tonsa*,  
 314 where copper (II) sulphates pentahydrate (CuSO<sub>4</sub>·5H<sub>2</sub>O) is represented as "CuSP" and Primextra® Gold TZ is indicated as "Pr"; 1:1 mixture – equitoxic  
 315 mixture of contaminants; SE – the standard error on the estimated coefficients; statistically significant values are in bold.

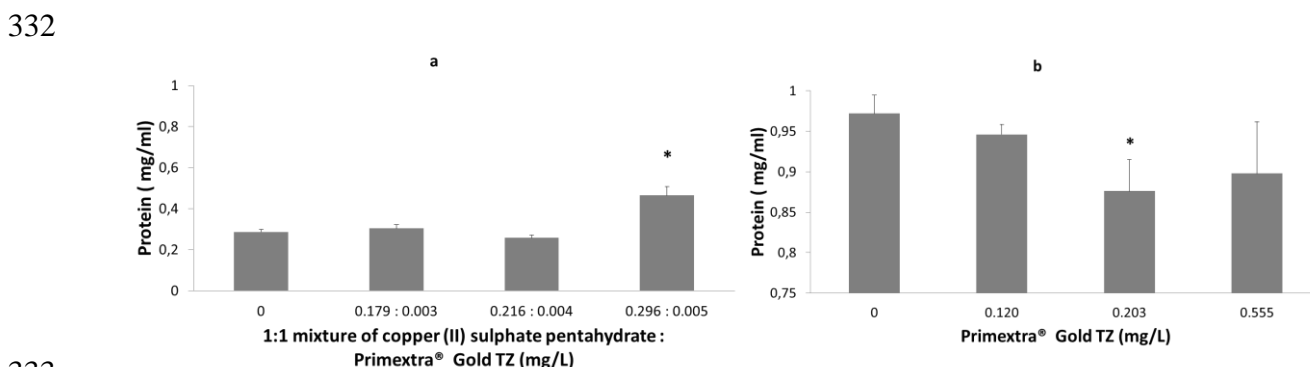
Biochemical parameter	Predictor	Coefficients	SE	t	p	Median deviance of residuals	Null deviance / Degrees of freedom	Residual deviance / Degrees of freedom	AIC	Effect
<i>T. weissflogii</i>										
FA	CuSP	-0.290	0.317	-0.915	0.369					
	Pr	24.200	16.299	1.485	0.151	-0.018	1.185 / 26	0.989 / 24	3.005	additive
EFA	CuSP	-0.062	0.300	-0.208	0.837					
	Pr	46.671	15.241	3.062	0.055	-0.024	1.498 / 26	0.994 / 24	8.985	additive
Protein	CuSP	0.652	0.743	0.878	0.385					
	Pr	32.371	40.692	0.796	0.431	0.029	2.888 / 44	1.471 / 41	-14.792	non-additive
	1:1 mixture	-412.121	170.654	-2.415	<b>0.020</b>					
TBARS	CuSP	-1.730	0.703	-2.460	<b>0.018</b>					
	Pr	-69.870	39.558	-1.766	0.085	-0.017	2.050 / 44	1.727 / 41	-2.816	non-additive
	1:1 mixture	312.731	162.984	1.919	0.062					
<i>A. tonsa</i>										
FA	CuSP	4.794	2.092	2.292	<b>0.031</b>					
	Pr	1.847	0.344	5.369	<b>&lt;0.0001</b>	0.009	2.089 / 26	0.846 / 24	-36.534	additive
EFA	CuSP	13.274	5.210	2.548	<b>0.018</b>					
	Pr	3.863	0.953	4.053	<b>&lt;0.001</b>	-0.005	3.791 / 25	1.524 / 22	-38.438	non-additive
	1:1 mixture	-10.542	21.830	-0.483	0.634					
Protein	CuSP	0.825	0.833	0.992	0.327					
	Pr	0.376	0.133	2.835	<b>0.007</b>	-0.018	0.597 / 44	0.487 / 41	-69.167	non-additive
	1:1 mixture	-3.987	2.398	-1.663	0.104					
TBARS	CuSP	-0.247	1.232	-0.200	0.842					
	Pr	0.736	0.237	3.111	<b>0.003</b>	-0.021	6.736 / 44	1.739 / 41	-13.705	non-additive
	1:1 mixture	33.795	6.337	5.333	<b>&lt;0.00001</b>					

316 Due to the used link function, a negative value of coefficient refers to the increase of content  
 317 of the biochemical parameter and a positive value to its decrease in the presence of the related  
 318 contaminant.

319 Thus, the quality of the diatom species was significantly predicted by the metal copper and  
 320 the equitoxic mixture of contaminants in terms of TBARS and protein contents  
 321 respectively ( $p < 0.05$ , Table 1 and Figs. 2a, 3a). The latter parameter indicated the presence of the  
 322 lipid peroxidation in *T. weissflogii*. However, the single treatments of the herbicide Primextra®  
 323 applied to the diatom did not reveal any significant influence on its quality.



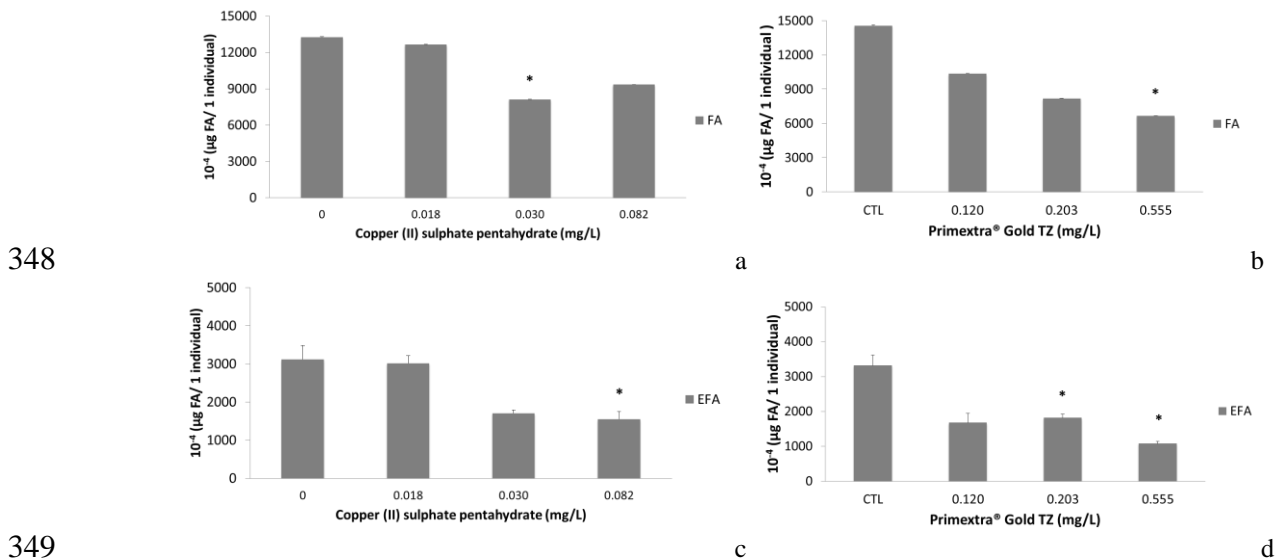
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 327 Fig. 2. Absolute concentrations ( $\pm$  standard error) of TBARS,  $\text{nmol.mg}^{-1}\text{prot}$ . for diatom *T. weissflogii* (a) and for  
 328 copepod *A. tonsa* (b, c) significantly predicted by one of the contaminant/s, i.e. copper (II) sulphate pentahydrate (a),  
 329 herbicide Primextra® Gold TZ (b) and the equitoxic mixture of contaminants (c). Values are means,  $n = 5$ . Symbol  
 330 “\*” indicates the significant difference of the treatments compared to the CTL (Fig. a –  $p = 0.007$ ; Fig. b –  $p = 0.000$ ;  
 331 Fig. c –  $p = 0.000$ ).



333  
 334 Fig. 3. Absolute concentrations ( $\pm$  standard error) of protein,  $\text{mg/ml}$ , for diatom *T. weissflogii* (a) and for copepod  
 335 *A. tonsa* (b) significantly predicted by the equitoxic mixture of contaminants and the herbicide Primextra® Gold TZ  
 336 respectively. Values are means,  $n = 5$ . Symbol “\*” indicates the significant difference of the treatments compared to  
 337 the CTL (Fig. a –  $p = 0.000$ ; Fig. b –  $p = 0.482$ )

338

339 An opposite trend was revealed for *A. tonsa*: the herbicide Primextra® Gold TZ affected  
 340 significantly all nutritionally important biochemical parameters ( $p < 0.05$ , Table 1) by reducing  
 341 the amount of FA, EFA and protein (Figs. 4b, d; 3b) and increasing TBARS content (Fig. 2b),  
 342 whereas the single treatments of the metal copper significantly predicted only the total FA and  
 343 EFA contents in the copepod species ( $p < 0.05$ , Table 1) in terms of a decrease of its amount (Figs.  
 344 4a, c). The single treatments of the herbicide (Fig. 2b) and the equitoxic mixture of copper and  
 345 herbicide (Fig. 2c) significantly impacted the TBARS content proving the interference in the  
 346 process of lipid peroxidation in the copepod *A. tonsa*.  
 347

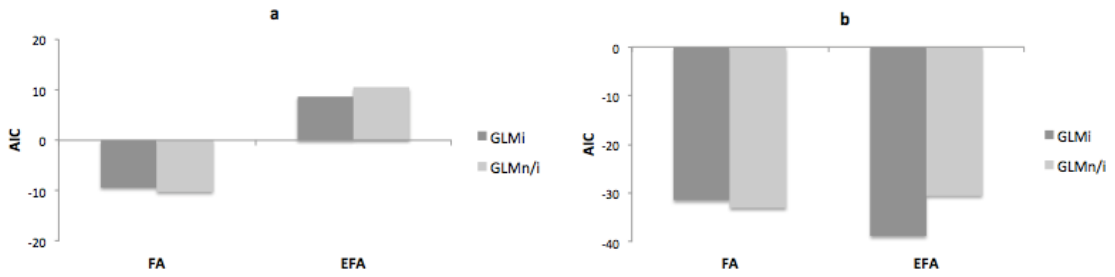


350 Fig. 4. Absolute concentrations ( $\pm$  standard error) of total FA (a, b) and EFA (c, d) in  $10^{-4}$   $\mu\text{g}/1$  individual for  
 351 copepod *A. tonsa* significantly predicted by Primextra® Gold TZ (b, d) and copper (II) sulphates pentahydrate (a, c).

352 Values are means,  $n = 3$ . Symbol “\*\*” indicates the significant difference of the treatments compared to the CTL  
 353 (Fig. a –  $p = 0.020$ ; Fig. b –  $p = 0.003$ ; Fig. c –  $p = 0.002$ ; Fig. d –  $p = 0.006$ )  
 354

### 355 3.2. Comparison of the effects of chemical mixture between trophic levels

356 An additive effect of the chemical mixture was revealed for the total FA profiles of both  
 357 species (Fig.5). However, a non-additive effect was revealed for the essential FA of both primary  
 358 producer and primary consumer. For both species, the AIC suggested the model without  
 359 interactions ( $\text{GLM}_{n/i}$ ) for the total FA profile's data, and the model with interactions ( $\text{GLM}_i$ ) for  
 360 the essential FA data (Fig. 5). Plots indicating the goodness of the models fit for the models 1  
 361 and 2 are presented in the supplemental material section as well (Figs. S2 and S3, respectively).  
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**Fig. 5.** The Akaike Information Criterion (AIC) values determined for generalized linear models with interaction (GLM<sub>i</sub>) and without interaction (GLM<sub>n/i</sub>) term for FA profiles (FA) and the essential FA (EFA) of the diatom *T. weissflogii* (a) and the copepod *A. tonsa* (b) after exposure to the same levels of contamination. A lower value of AIC indicates a better trade-off between predictive capacity and model complexity.

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The GLM with inverse link function fitted to the biochemical data of each species exposed to equal levels of contaminants revealed no significant impact on the total FA profile and essential FA of the primary producer diatom *T. weissflogii*, whereas in the case of the primary consumer copepod *A. tonsa*, significant effects of the herbicide Primextra® and metal copper on these parameters were reflected in a decrease of their amount (Table 2, Fig. 4).

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**Table 2.** Results of generalized linear models with lower AIC value predicting the effects of contaminants between trophic levels, where copper (II) sulphates pentahydrate (CuSO<sub>4</sub>.5H<sub>2</sub>O) is represented as "CuSP" and Primextra® Gold TZ is indicated as "Pr"; 1:1 mixture – equitoxic mixture of contaminants; SE – the standard error on the estimated slopes; statistically significant values are in bold, n/a – not applicable.

Biochemical parameter	Predictor	Coefficients	SE	t	p	Median deviance of residuals	Null deviance / Degrees of freedom	Residual deviance / Degrees of freedom	AIC	Effect
<i>T. weissflogii</i>										
FA	CuSP	-0.370	1.096	-0.337	0.739	-0.020	0.783	0.754	-10.161	additive
	Pr	0.143	0.165	0.866	0.395					
EFA	CuSP	-2.417	3.379	-0.717	0.481	-0.021	3.038	2.431	8.648	non-additive
	Pr	-0.161	0.528	-0.305	0.763					
	1:1 mixture	20.486	11.296	1.814	0.083					
<i>A. tonsa</i>										
FA	CuSP	4.794	2.092	2.292	<b>0.031</b>	0.009	2.089 / 26	0.846 / 24	-36.534	additive
	Pr	1.847	0.344	5.369	<b>&lt;0.0001</b>					
EFA	CuSP	13.274	5.210	2.548	<b>0.018</b>	-0.005	3.791 / 25	1.524 / 22	-38.438	non-additive
	Pr	3.863	0.953	4.053	<b>&lt;0.001</b>					
	1:1 mixture	-10.542	21.830	-0.483	0.634					
<i>Correlation between trophic levels</i>										
SFA ( <i>A. tonsa</i> )	SFA ( <i>T. weissflogii</i> )	-4.845 × 10 <sup>4</sup>	8.498 × 10 <sup>3</sup>	-5,702	<b>&lt;0.00001</b>	0.022	0.578	0.285	-63.985	n/a
	PUFA ( <i>T. weissflogii</i> )	3.088 × 10 <sup>4</sup>	6.328 × 10 <sup>3</sup>	4.881	<b>&lt;0.0001</b>					
PUFA ( <i>A. tonsa</i> )	SFA ( <i>T. weissflogii</i> )	-1.691 × 10 <sup>4</sup>	2.058 × 10 <sup>4</sup>	-0.821	0.417	-0.039	1.713	1.673	-0.311	n/a
	PUFA ( <i>T. weissflogii</i> )	5.601 × 10 <sup>3</sup>	1.532 × 10 <sup>4</sup>	0.366	0.717					

376 An output of the model 2 indicated that both saturated and polyunsaturated fatty acids of the  
377 primary producer *T. weissflogii* significantly correlate with saturated fatty acids of the primary  
378 consumer *A. tonsa*. However, no relationship was observed between FA profile of the diatom  
379 species and PUFAs of the copepod *A. tonsa* (Table 2).

## 380 4. Discussion

### 381 4.1. *The effect of the chemical mixture on the quality of the diatom and copepod species*

382 Studies examining the influence of the organic and inorganic contaminants on the nutritional  
383 quality of planktonic species are very scarce. Baker et al. (2016) revealed that simultaneous  
384 exposure to glyphosate herbicide and nutrients (ammonium nitrate and phosphoric acid) led to  
385 the decline in edible carbon content and thus in dietary quality of phytoplankton and zooplankton  
386 communities. This effect was not found after exposure to only the herbicide.

387 To the best of our knowledge, the current study is the first that determines the interaction  
388 effect of the organic and inorganic toxicant`s mixture on the species quality. The overall results  
389 of our study demonstrated that non-additive effects allow a better prediction of the mixture effect  
390 of the herbicide Primextra® and the metal copper on the nutritional quality, compared to a model  
391 that assumes purely additive effects.

392 It is noteworthy that studies about the interaction effect of organic and inorganic contaminants  
393 on non-target species are scarce in the literature and are aimed mostly at measuring species  
394 growth, survival or reproduction (Chen et al., 2015; Filimonova et al., 2016a; Klerks and  
395 Moreau, 2001).

396 **Herbicides and metals both adversely affect the same biological component: EFA**, via  
397 interactions of these contaminants to various biochemical processes, i.e. glutathione peroxidase`s  
398 inhibition that leads to peroxidation of EFA`s membranes, inhibition of FA elongation and  $\omega 3$ ,  
399  $\omega 6$  – desaturation processes; and creation of new adverse biochemical reactions, i.e. production  
400 of reactive oxygen species (ROS) that enter into a reaction with EFA molecules (Cohen et al.,  
401 1993; Robert et al., 2007). PUFA, including EFA are almost exclusively synthesized by algae  
402 and plants. **Diatoms contain much EFA, which may allow the effects of both chemicals to be  
403 added up.** The other reason may be the presence of the cell wall in the algae cell structure that  
404 plays an important role in the defence responses against potential stressors (Keestra, 2010).  
405 Animals can convert one form of PUFA to another through elongation and desaturation, but very  
406 few can synthesize PUFA *de novo* (Brett and Müller-Navarra, 1997). **Planktonic calanoid  
407 copepods (i.e. our study species *A. tonsa*) have limited ability of FA bioconversion as they lack  
408 the necessary enzymes to produce significant amounts of PUFA (De Troch et al.,  
409 2012). Therefore, they have a limited amount of EFA, and thus the effects were non-additive.**

410 Most of nutritionally important biochemical parameters of the investigated planktonic species  
411 were significantly affected by at least one of the chemical stressors. This is in accordance with  
412 other studies where biochemical composition of aquatic organisms was affected by herbicides or  
413 metals.

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416 The effect on protein content was revealed after exposure of aquatic species from different  
417 trophic levels to metal copper (Pytharopoulou et al., 2013), to Metolachlor (Martins et al., 2011),  
418 to triazine herbicides (El-Sheekh et al., 1994) and to the combination of the metal copper and  
419 chloroacetanilide herbicide applied to phytoplankton species (Lu et al., 2015). The latter is in  
420 agreement with our result that the metal-herbicide mixture significantly predicted protein content  
421 of the diatom species.

422 TBARS content as well revealed to be affected by copper (Bazihizina et al., 2015) and  
423 triazine herbicides (Velisek et al., 2014). In our case TBARS content was significantly predicted  
424 by the metal copper in the case of the diatom *T. weissflogii* and by the herbicide Primextra® in  
425 the case of the copepod *A. tonsa*. The metal copper and two main active ingredients of the  
426 herbicide Primextra®: S-metolachlor and terbuthylazine, which belong to the groups of  
427 chloroacetanilide and triazine herbicides respectively induce reactive oxygen species (ROS).  
428 (Filimonova et al., 2016a, 2016b). ROS attack the polyunsaturated fatty acids (including the  
429 essential FA) producing secondary products such as hydroperoxides or their aldehyde  
430 derivatives, which inhibit the protein synthesis (Repetto et al., 2012). Among them are MDA  
431 molecules – biomarkers of lipid ( $\omega 3$ ,  $\omega 6$ ) peroxidation that is estimated by the amount of  
432 TBARS. We hypothesize that the herbicide Primextra® generates ROS more intensively than the  
433 metal copper due to the presence of two active ingredients that subsequently lead to a greater  
434 production of MDA molecules and thus to a greater influence on TBARS content. Therefore,  
435 TBARS content was significantly predicted by the herbicide in *A. tonsa*. Indeed, in another study  
436 Velisek and co-authors (2014) showed that the activity of the antioxidant enzyme superoxide  
437 dismutase, was negatively affected in animals exposed to terbuthylazine, which can present  
438 profound impacts on production of MDA. On the other hand, another study (Mohammed, 2014)  
439 reported that a stimulatory effect of the activity of antioxidant enzymes occurred in a copepod  
440 species after being exposed to a metal (Ni). This led to a reduction of MDA in those organisms.  
441 For the diatom *T. weissflogii* we assume that metal copper has a significant impact on its TBARS  
442 content due to the presence of silica in the cell wall of this species which is known to serve as a  
443 barrier to the herbicide transport (Ferreira et al., 2007). In addition, there are reports of copper  
444 excess being able to disrupt photosynthesis resulting in an increase of ROS and consequently of  
445 MDA (Bazihizina et al., 2015).

446 A limited number of studies on the herbicide Primextra® have shown its effect on fatty acid  
447 profiles, including the essential FA (Filimonova et al., 2016b; Gonçalves et al., 2016; Neves et  
448 al., 2015). Effect on FA content of different marine and freshwater species was observed as well  
449 after exposure to S-metolachlor (Neves et al., 2015; Robert et al., 2007), triazine herbicides (De  
450 Hoop et al., 2013), and copper (numerous studies reviewed by Filimonova et al. 2016a).

451 Our modelling results revealed that nutritionally important biochemical parameters of the  
452 copepod *A. tonsa* were generally more sensitive to the chemical stressors than those of the  
453 diatom *T. weissflogii*.

454 This is in agreement with a few available studies that documented that the terrestrial  
455 invertebrate *Eisenia fetida* (Chen et al., 2015) was more sensitive to a pesticide-metal mixture  
456 (eight – component mixture of five insecticides: chlorpyrifos, avermectin, imidacloprid,  $\lambda$ -  
457 cyhalothrin, and phoxim; two herbicides: atrazine and butachlor; and the metal cadmium), i.e.  
458 synergism on the survival rate was observed, whereas the aquatic plant *Lemna minor* (Teisseire

459 et al., 1999) and the aquatic algae *Chlorella ellipsoidea* (Aoyama et al., 1987) were more  
460 tolerant to organic-copper mixtures: herbicide diuron-copper and pentachlorophenol-copper  
461 respectively, i.e. antagonism on the growth rate was evident. A greater tolerance of *T. weissflogii*  
462 to the applied chemicals may be also due to the ability of diatom species generates  
463 morphological changes and activates chemical defensive mechanisms in the presence of different  
464 contaminants (Debenest et al., 2010) that finally may imply difficulties in penetration of  
465 molecules of toxicants into diatom cell membrane.

466 In general, we conclude that the quality referring to the species at the higher trophic level  
467 (here first-level consumers) was found to be more sensitive to the chemical stressors than the one  
468 at the lower trophic level, and that the contaminants mixture mostly acted non-additively on  
469 studied biochemical parameters.

470 The sensitivity of the producer and the primary consumer species and the observed non-  
471 additive effect of the applied metal-pesticide mixture, on the most nutritionally important  
472 biochemical parameters, can serve to the future risk assessment of organic and inorganic  
473 chemical mixtures.

#### 474 4.2. Comparison of the effects of chemical mixture between trophic levels

475 When species were exposed to the same levels of contamination, effects of the copper-  
476 Primextra<sup>®</sup> mixture on the essential FA content of both species were non-additive. These results  
477 have important consequences since essential FA ( $\omega 3$ ) determine the nutritional quality of algae  
478 and calanoid copepods need to take up EFA from their food source (Brett and Müller-Navarra,  
479 1997; De Troch et al., 2012).

480 A healthy food web requires adequate food quality in sufficient quantities. In an aquatic  
481 ecosystem, “good” quality phytoplankton lead to better quality of zooplankton and therefore to  
482 larger and more diverse fish populations (Kelble, 2012) with high nutritional values. Aquatic  
483 organisms have been and continue to be our primary source of readily available EFA, which  
484 have proven their effects in preventing/mitigating cardiovascular diseases, ontogenesis  
485 (particularly neural development), atherosclerosis, neural disorders, and, potentially, some  
486 cancers, as well as autoimmune diseases (Arts et al., 2001).

487 No significant correlation was observed for PUFA of the copepod species with SFA and  
488 PUFA of the diatom species, whereas an opposite trend was revealed for saturated FA of *A.*  
489 *tonsa*.

490 Under non-stress conditions, PUFA of the copepod species are expected to be significantly  
491 correlated with PUFA of the diatom species due to the fact mentioned above, namely that PUFA  
492 of the primary consumer species are usually taken up from food and that some PUFA (i.e.  
493 essential FA: 20:5 $\omega 3$ ) are dietary tracers between diatom and copepod species (Arts et al., 2001).  
494 Similarly, in the absence of stressors, non-significant correlation is expected between SFA of the  
495 copepod and diatom species, since SFA (i.e. 16:0, 18:0) can be synthesized by both algae and  
496 animal cells *de novo* from acyl-CoA and don't depend on the food source availability.

497 Therefore, we conclude that the herbicide Primexrta<sup>®</sup> and the metal copper as stress factors  
498 intervened into processes of SFA's synthesis and of PUFA's transfer, including essential FA  
499 along the trophic level, i.e. primary producer – primary consumer in the case of the studied

500 planktonic species. Indeed, the main active ingredient of Primextra® – S-metolachlor is known  
501 to inhibit FA elongase that aims to catalyze the first step of FA elongation process: the reaction  
502 of condensation of acyl-CoA and malonyl-CoA (Filimonova et al., 2016a; Thakkar et al.,  
503 2013). On the other hand, metal copper may bind to the thiol-group of coenzyme that interferes  
504 with the production of acyl-CoA and malonyl-CoA and therefore with SFA synthesis  
505 (Filimonova et al., 2016a).

506 In general, nutritionally important biochemical parameters of the primary consumer *A. tonsa*  
507 was more sensitive to the chemical stressors than of the primary producer *T. weissflogii*, when  
508 species were exposed to the equal levels of contamination. Therefore, we assume that the quality  
509 decreases when moving up the food web, which would have important implications for the  
510 human diet.

## 511 5. Conclusions

512 The major findings of this study are: (1) effects of the metal-herbicide mixture on the quality  
513 of phytoplankton and zooplankton species, were non-additive and (2) nutritionally important  
514 biochemical parameters of the species from the higher trophic level were most sensitive to the  
515 chemical stressors. This information is valuable for future risk-assessment procedures of  
516 organic-inorganic contaminant mixtures, can assist in the determination of the effects for higher  
517 trophic levels (i.e. secondary consumers) and can help in the assessment of estuarine and marine  
518 ecosystem health.

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530

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