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RESEARCH ARTICLE

Ecotoxicological and biochemical mixture effects of an herbicide and a metal at the marine primary producer diatom *Thalassiosira weissflogii* and the primary consumer copepod *Acartia tonsa*

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Abstract

Mixture effects of chemicals and their potential synergistic interactions are of great concern to the public and regulatory authorities worldwide. Intensive agricultural activities are leading to discharges of chemical mixtures to nearby estuarine and marine waters with possible adverse effects on the aquatic communities and for the trophic food web interlinking these communities. Further information about the impacts of these stressors on aquatic organisms is needed. This study addresses ecotoxicological and biochemical effects of single and mixtures of the metal copper and the herbicide Primextra® Gold TZ on the marine diatom *Thalassiosira weissflogii* and on the estuarine calanoid copepod *Acartia tonsa* by determining growth rate and survival, respectively, and changes on fatty acid(FA) profiles in both species. Mixture effects on diatom species revealed that copper and Primextra® acted most likely additively with respect to the concentration addition (CA) and independent action (IA) models with model deviation ratios (MDR), 0.752 and 1.063, respectively. For the copepod species, copper and Primextra® were most likely non-interactive with respect to the CA model (MDR = 1.521) but acted most likely synergistically with respect to the IA model (MDR = 2.026). A significant decline in the absolute FA concentration was observed for copepod species after mixture exposure including a considerable decrease of essential FAs that cannot be synthesized de novo by these grazers. We concluded that the mixture effects are more hazardous for primary consumer than for primary producer species in terms of both abundance and biomass quality, suggesting a potential for harmful effects for higher trophic levels and thus a decrease in energy flow through the ecosystem.

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Introduction

Herbicides and metal-containing pesticides play an important role in agricultural practices (Lepp 1981; Nguyen-Ngoc et al. 2009). However, their residues enter simultaneously to nearby estuarine and marine waters and cause ecological stress, getting in contact with organic matter (Hunting et al. 2016) and nutrients (Baker et al. 2016), directly affecting non-target biological communities (Gonçalves et al. 2016) and the related processes at the cellular and biochemical levels (Filimonova et al. 2016a).

The herbicide Primextra® Gold TZ (Syngenta AG) is one of the 20 best-selling herbicides in Portugal and the most used herbicide in corn crop fields covering 51% of the soil used for agricultural purposes in the lower part of the Mondego river following into the Mondego estuary (Figueira da Foz, Portugal), while metal copper is widely used in pesticides' constitution including fungicides (Ferreira et al. 2003; Cruzeiro et al. 2016; Filimonova et al. 2016a; Gonçalves et al. 2016).

Although both fungicide's and herbicide's consumption smoothly decreased for the last 10 years in Portugal and reached 5193 and 2122 t of active ingredients (a.i.), respectively, still both amounts are two times higher than they were in 1992 (http://www.fao.org/faostat).

The herbicide Primextra® Gold TZ consists of the two main a.i.: terbuthylazine (TBA, 17.75%, w.w.) and *S*-metolachlor (30.2%, w.w.) and adjuvants (surfactants) in a low amount (0–5%, w.w.). TBA is a triazine herbicide affecting diatom cells and inhibiting photosynthesis via blocking electron transport at the second stable electron acceptor, i.e., a protein-bound quinone, of the photosystem II (Steinback et al. 1981). Metolachlor is a chloroacetamide herbicide interfering with normal cell development and inhibiting essential biological processes, including biosynthesis of proteins, lipids, and fatty acids (FA) (Weed Science Society of America 1994; Liebl 1995; Liu and Xiong 2009). Although copper at low concentrations is an essential micronutrient for several physiological processes, this metal is known to be toxic at higher amounts affecting respiration, photosynthesis, synthesis of chlorophyll, carbohydrates and pigments, cell division, and metabolism of FA (Bae and Lim 2012; Chen et al. 2013).

Chemical risk assessments are performed for individual chemicals according to standardized frameworks such as the registration, evaluation, authorization, and restriction of chemical legislation (REACH) of the European Union (Lister et al. 2011). Traditional effect and risk assessment in such frameworks have routinely been focused on individual exposures to chemicals that may underestimate the risks related to toxic action of their mixtures. Currently, there are numerous studies that address the toxicity of mixtures of either organic contaminants (group of pesticides) or inorganic contaminants (group of metals) (Franklin et al. 2002; DeLorenzo and Serrano 2003; Geret et al. 2011; Mehler et al. 2011). However, a recent comprehensive review has reported that studies with mixture experiments of metals and pesticides still remain scarce (Cedergreen 2014). Thus, a better understanding of the interactive effects of organic-inorganic contaminant mixtures on non-target marine and estuarine species is necessary for a more comprehensive ecological risk assessment (Mehler et al. 2011; Chen et al. 2013).

Primary producer species, i.e., diatoms being at the base of the trophic food chain, are an important food source for various organisms and may be severely affected by pesticide and metal exposures. Zooplankton has long been used as a suitable group in ecotoxicological studies due to their key intermediate position in a trophic food web, as a link between primary producer and secondary consumer species (Debenest et al. 2010; Neves et al. 2015; Filimonova et al. 2016a). In the Mondego estuary, diatom species are one of the dominating phytoplankton groups (Flindt et al. 1997) and calanoid copepod *Acartia tonsa* is found in high abundance (Gonçalves et al. 2010). Therefore, in this work, the primary producer diatom *Thalassiosira weissflogii*—also widely used in seawater toxicity tests as a sensitive test organism (Araújo and Souza-Santos 2013) and the primary consumer *Acartia tonsa* were used.

In aquatic food webs, FA are one of the crucial molecules transferred across the plant-animal interface. They have the potential to be used in ecotoxicological studies as important tools and endpoints (Filimonova et al. 2016a, b). Our recent review about response of FA profiles of marine species to organic and inorganic chemical stressors revealed a knowledge gap about effects of metal-herbicide mixture exposure on this endpoint (Filimonova et al. 2016b).

In ecotoxicology, depending on the assumed mode of action of the mixture's components, two general reference models are generally used to predict the toxicity of mixtures: the concentration addition (CA) model (first introduced by Loewe and Muischnek 1926) and the independent action (IA) model (first introduced by Bliss 1939). Because modes of action of herbicide Primextra® and copper are various and not fully understood, and because we cannot exclude the possibility of partial similarity in their modes of action, we used both the reference model for similarly acting chemicals, i.e., the CA model and for dissimilarly acting chemicals, i.e., the IA model. Both models assume that there is no interaction among substances in the mixture (i.e., "noninteraction" or "additive effect"). However, if observed responses are stronger or weaker than predicted, then the mixture effect is described as being either synergistic or antagonistic, respectively (Sun et al. 2009; Hochmuth et al. 2014; Nys et al. 2015).

The objectives of the present study were (1) to determine whether or not there are interactive effects between the metal copper and the herbicide Primextra® Gold TZ on the relative growth rate (RGR) of diatom *Thalassiosira weissflogii* and the relative survival (RS) of the copepod *Acartia tonsa* and (2) to evaluate the effect of an applied organic-inorganic mixture on the FA composition of the investigated species.

The mechanisms involved in the joint toxicity of both contaminants and the ecological consequences of observed results are discussed.

Materials and methods

Test species: Culture conditions

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

Acartia tonsa (Copepoda, Calanoida) was sampled in the south arm of Mondego estuary (40° 08′ N, 8° 50′ W) near the Pranto River, where it was found in high abundance (Gonçalves et al. 2010, 2012b, c). The Mondego estuary is a tidal estuary located near Figueira da Foz City on the west coast of Portugal. Copepods were sampled with horizontal subsurface tows with a bongo net, placed to the 2.5-L flasks filled with the estuarine water and transported to the laboratory (Gonçalves et al. 2012a).

Separated from other species, *A. tonsa* was placed to aquaria with gentle aeration system and filtrated (1.2 μm pores) natural seawater diluted with distilled water to a salinity of 13–15 psu.

The medium renewal (30% from the total volume) and measurements of dissolved O_2 (%) were applied regularly. Feeding with the diatom *T. weissflogii* (2×10^4 cells/mL) was done three times a week.

The diatom species *Thalassiosira weissflogii* was acquired from the Scottish Marine Institute (Dunbeg, PA37 1QA, UK; strain number 1085/18). It was cultured with the Guillard's f/2 medium with a salinity of 30 psu, without EDTA due to its ability to form a stable chelate complex with copper (adapted after Rippingale and Payne 2001). A renew of algae culture was done weekly.

The Mondego estuary is still considered to be little contaminated by the studied chemicals (Vasconcelos et al. 2011; Cruzeiro et al. 2016). However, to eliminate potential adaption of the sampled copepod species to these contaminants, we used for the bioassays adult organisms of *A. tonsa* that were grown in the same laboratory conditions. Adult organisms were cultured during 14 days from the first cohort of nauplii of the copepods from the field (Filimonova et al. 2016a).

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

Individual and mixture acute zooplankton (immobilization) and microalgae growth bioassays

Acute zooplankton and microalgae growth bioassays were followed as described by Filimonova et al. (2016a).

An inoculum of *T. weissflogii* was harvested from the bulk culture (in exponential growth phase) and incubated under 20 ± 2 °C and a 16-h light:8-h dark photoperiod during 3 days before the beginning of bioassay. A Neubauer hemocytometer was used to determine the inoculum cell density. The initial test cell density was 10^4 cells/mL. A range of concentrations of each toxicant was applied to the diatom species presented in three replicates per treatment during 96 h with the same temperature and photoperiod conditions. At the end of the bioassay, a Neubauer chamber was used to count the algal cell density (APHA 1995).

The conditions of acute immobilization tests with the copepod *Acartia tonsa* were adapted for marine species based on the OECD protocol 202 (OECD 2004). Adult organisms of *A. tonsa* grown from neonates born between the first and second broods were used for the tests. Light and temperature regimes were the same as used for the culture maintenance. Static acute tests were applied with 20 animals allocated at random into four replicates with five individuals per replicate with three replicates per treatment. The organisms were exposed to a range of concentrations of each toxicant during 48 h incubation without food. After 24 and 48 h, immobilized individuals were counted.

The solutions of the metal and the herbicide were acquired by successive dilutions of a stock solution of copper(II) sulphate pentahydrate and Primextra® Gold TZ in distilled water and were added to the experimental flasks with culture medium in the calculated amounts.

We used nominal concentrations ranging from 0.0049 to 0.2247 mg/L and from 0.0024 to 0.1125 mg/L for copper and for Primextra® Gold TZ from 0.0003 to 0.0159 mg/L and from 0.0651 to 2.9979 mg/L exposed to the diatom and copepod species, respectively, in both individual and mixture bioassays. The corresponding culture medium was used as the uncontaminated (i.e., control) treatment. Tests were carried out in glass (pesticide and mixture bioassays) or plastic (metal bioassays) flasks, containing 40 and 100 mL of test solutions in the case of microalgae growth bioassays and the acute zooplankton tests, respectively. Bioassays with individual toxicants and their equitoxic mixture were run simultaneously. For the latter, we used the concentrations of each contaminant that give a similar toxic effect when applied individually.

At the end of each bioassay, Neubauer chamber was used to determine cellular density by counting the number of algae cells, whereas the survived copepod species were counted manually. Then the diatom's relative growth rate and the copepod's relative survival were determined by dividing the growth rate and the survival, respectively, at each

contaminated treatment by the respective control (uncontaminated) treatments.

Samples with the nominal concentrations of both contaminants from each bioassay were stored for the determination of measured values. Table S1 (Online resource) summarizes nominal and measured concentrations referring to the amount of copper (Cu) and the herbicide Primextra® (Pr) in the treatment combinations used.

The measurement of total copper concentrations was done with graphite furnace atomic absorption spectrophotometry (GFAAS Furnace Autosampler; ICE3500 from Thermo Scientific: limit of quantification for undiluted samples is 3 μg/L, seawater samples were diluted to eliminate the interference of the salt. Dilution factor was kept as low as possible). *S*-Metolachlor and TBA (two main a.i. of Primextra® Gold TZ) were quantified using gas-liquid chromatography-mass spectrometry after solid-phase extraction (GC/MS after SPE-extraction, Trace-GC / DSQ-MS from Thermo Scientific: limit of quantification for undiluted extracts and for 20 mL of sample is 0.4 μg/L) (ISO 10695 2000; Environmental & Agrochemical Applications Notebook 2002; CMA/3/H 2002).

Population microcosm individual and mixture bioassays

Population microcosm bioassays were applied to observe the FA profile alterations of *T. weissflogii* and *A. tonsa* after individual and mixture exposures of the herbicide and the metal, according to the results from microalgae growth bioassays and the acute zooplankton tests, respectively. In mixture exposure, the ratio of contaminants was 1:1 (concentrations that cause the same toxic effects on the study species, mg/L).

The diatom *T. weissflogii* and the copepod *A. tonsa* were exposed in four experimental treatments: a negative control (CTL), i.e., uncontaminated culture medium, and three contaminated treatments: a low level of each toxicant (C1), an intermediate level (C2), and a high level (C3), expressed as the sum of toxic units (TU) of copper and Primextra® combinations (Eq. 1):

$$\sum \mathrm{TU_{Cu\text{-}Prmix}} = \mathrm{TU_{Cu}} + \mathrm{TU_{Pr}} = \frac{x_{\mathrm{Cu}}}{\mathrm{EC50_{Cu}}} + \frac{x_{\mathrm{Pr}}}{\mathrm{EC50_{Pr}}} \qquad (1)$$

In Eq. (1), $\mathrm{TU_{Cu}}$ and TU_{Pr} are TU of copper and Primextra®, respectively, which are ratios of the relevant contaminant at the concentration x in the mixture and its EC50 value (Jonker et al. 2005).

Experiments with each species were conducted in glass (pesticide and mixture bioassays) or plastic (metal bioassays) beakers containing a final volume of corresponding test solution of each contaminant with three replicates per treatment. Light and temperature regimes were the same as used for the maintenance of cultures and toxicological bioassays.

The duration of microcosm bioassays after individual and mixture exposures for each species was 7 days. For the diatom species, it was in accordance with the dynamic of their growth as stated by Lavens and Sorgeloos (1996). In the case of the copepod species, the duration was limited to 7 days due to the high mortality (more than 75%) of *A. tonsa* at the high-contaminated treatment in the mixture bioassay.

Population microcosm bioassays with *A. tonsa* were performed using vials with established gentle aeration system and a final volume of 2500 mL and 250 individuals per replicate. The copepod species was 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 (Filimonova et al. 2016a). After 7 days of exposure to toxicants, alive organisms in the amount of 60 individuals per replicate were selected and collected on Whatman filters (GF/F) to be stored at − 80 °C for further FA analysis (Filimonova et al. 2016a).

At the end of each bioassay with the diatom species, flasks were checked for the growth inhibition. In each replicate, 3.6×10^6 cells were counted using a Neubauer chamber. Then cells were concentrated on Whatman filters (GF/F) to be stored at − 80 °C for further FA analysis (Filimonova et al. 2016a).

FA analyses

The used shorthand FA notations of the form *X*:*Y*ω*Z* denote the following: *X* is the number of carbon atoms, *Y* is the number of double bonds, and *Z* is the position of the double bond closest to the terminal methyl group (De Troch et al. 2012; Filimonova et al. 2016a).

Analyses of FA were followed as described by Filimonova et al. (2016a). The initial step was the extraction of total lipids of study species and their methylation to fatty acid methyl esters (FAMEs) that were performed with a modified one-step derivatization method after De Troch et al. (2012) and Gonçalves et al. (2012a). The internal standard of methylnonadecanoate C19:0 fatty acid (Fluka 74208) was added to each sample for the quantification of FA.

Fatty acid methyl esters were separated and quantified using a gas chromatograph (6890 N; Agilent Technologies, Diegem, Belgium) equipped with a quadrupole mass selective detector (5973 N; Agilent Technologies, Diegem, Belgium). The ion source and interface temperatures were maintained at 230 and 240 °C, respectively. The injector temperature was 250 °C, and the injection volume 1 μL with a split ratio of 4:1. A HP88 column (60 m × 0.25 mm (i.d.) × 0.25 μm thickness; Agilent J & W, Agilent Co., USA) with a He flow of 1.5 mL/min was used to separate fatty acid methyl esters. The oven temperature was programmed at 50 °C for 2 min, followed by a ramp of 25 °C/min to 75 °C, then a second ramp at 2 °C/min 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 (composed in the Marine Biology research group) and WILEY mass spectral libraries. The analyses of FAMEs were performed with the software Agilent MSD Productivity ChemStation. External (Supelco 37 Component FAME Mix, Supelco No. 47885, Sigma-Aldrich, Inc., USA), and additional standards of 16:2ω6, 16:2ω4, and 16:3ω3 (Larodan Fine Chemicals) were used to quantify the individual FAMEs. A linear regression was applied to the chromatographic peak areas and corresponding known concentrations of the standards (from 100 to 800 μg/mL) to define the quantification function of each FAME.

To test significant differences among treatments and to indicate significant changes in the species FA composition along the levels of contamination relatively to control, we performed a one-way analysis of variance (ANOVA) following the Dunnett's multiple comparison test. The used level of significance was 0.05. Prior to the analysis, the data were checked to meet the assumptions of normality (Shapiro-Wilk test) and homoscedasticity (Levene's test).

Statistical analysis

Analysis of interactive mixture effects on diatom growth rate and copepod survival

The data obtained after 96 h diatom growth bioassays with individual exposure to copper and Primextra® were analyzed by non-linear regression. Concentrations promoting 50% growth inhibition and the corresponding 95% confidence intervals for both toxicants were defined. The least-squares model was fitted to the data via the log-logistic equation (Eq. 2) in Statistica 7 (StatSoft):

$$y = \frac{100}{1 + \left(\frac{x}{\mathrm{EC50}}\right)^{\beta}} \tag{2}$$

In Eq. (2), y is the RGR (as a percentage, growth rate relative to a control); EC50 is the median effective concentration inducing a 50% effect on *T. weissflogii* growth rate; x is the contaminant concentration in the test medium; and β is the slope parameter (Nys et al. 2015; Filimonova et al. 2016a).

The concentration that caused 50% of effect in *A. tonsa* after 48 h bioassays with individual exposure to contaminants together with the corresponding 95% confidence intervals were estimated via Probit analysis (Finney 1971; Eq. 3) run in SPSS (Filimonova et al. 2016a):

$$\mathrm{probit}(p) = \beta_0 + \beta_x x \tag{3}$$

In Eq. (3), p is the probability; x is log-transformed contaminant concentration in the test medium; and β_0 and β_1 are the intercept and the slope parameter, respectively.

The interactive effects of the metal Cu and the Pr in mixture bioassays for both species were assessed through the mixture analysis framework developed by Jonker et al. (2005), further refined by Hochmuth et al. (2014) and described by Nys et al. (2015). This framework is based on both the CA and IA reference models and allows to analyze whether a mixture deviates from strict noninteraction.

The mean relative diatom growth rate and relative copepod survival for every Cu–Pr treatment were applied as input for the mixture analysis. The observed values of the RGR of diatom related to each replicate in every treatment were determined with Eq. (4). The observed values of the RS of copepod related to each replicate in every treatment were determined with Eq. (5) (Nys et al. 2015).

$$\mathrm{RGR}_{\mathrm{Cu}_x-\mathrm{Pr}_y} = \frac{\mathrm{GR}_{\mathrm{Cu}_x-\mathrm{Pr}_y}}{\mathrm{GR}_{\mathrm{CTL}}} \times 100\% \tag{4}$$

$$\mathrm{RS}_{\mathrm{Cu}_x-\mathrm{Pr}_y} = \frac{S_{\mathrm{Cu}_x-\mathrm{Pr}_y}}{S_{\mathrm{CTL}}} \times 100\% \tag{5}$$

In Eq. (4), $\mathrm{RGR}_{\mathrm{Cu}_x-\mathrm{Pr}_y}$ is the relative growth rate of the treatment with Cu at concentration x and Pr at concentration y; $\mathrm{GR}_{\mathrm{Cu}_x-\mathrm{Pr}_y}$ is the growth rate of the treatment with Cu at concentration x and Pr at concentration y; and $\mathrm{GR}_{\mathrm{CTL}}$ is the average growth rate of the control—uncontaminated treatment.

In Eq. (5), $\mathrm{RS}_{\mathrm{Cu}_x-\mathrm{Pr}_y}$ is the relative survival of the treatment with Cu at concentration x and Pr at concentration y; $S_{\mathrm{Cu}_x-\mathrm{Pr}_y}$ is the survival of the treatment with Cu at concentration x and Pr at concentration y; and S_{CTL} is the average survival at the control—uncontaminated treatment.

As described by Nys et al. (2015), the analysis of the interactive mixture effects was applied in three successive steps. In the first step, the predicted values (y) of the RGR in diatom or RS in copepod for the mixture combinations were predicted with the CA (Eq. 6) and IA (Eq. 7) reference models assuming no interaction and using the EC50 and the slope β after of Cu-only ($\mathrm{EC50}_{\mathrm{Cu}}$ and β_{Cu}) and Pr-only ($\mathrm{EC50}_{\mathrm{Pr}}$ and β_{Pr}) exposures calculated for diatom species after nonlinear regression with the least-squares method and for copepod species after probit analysis. The generalized reduced gradient iterative solver function (Excel 2011) was used to solve Eq. (6).

$$\frac{x_{\mathrm{Cu}}}{\mathrm{EC50}_{\mathrm{Cu}} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{\mathrm{Cu}}}}} + \frac{x_{\mathrm{Pr}}}{\mathrm{EC50}_{\mathrm{Pr}} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{\mathrm{Pr}}}}} = 1 \tag{6}$$

$$y = 100 \times \left(\frac{1}{1 + \left(\frac{x_{\mathrm{Cu}}}{\mathrm{EC50}_{\mathrm{Cu}}}\right)^{\beta_{\mathrm{Cu}}}}\right)\left(\frac{1}{1 + \left(\frac{x_{\mathrm{Pr}}}{\mathrm{EC50}_{\mathrm{Pr}}}\right)^{\beta_{\mathrm{Pr}}}}\right) \tag{7}$$

Then, in the second step, the IA and CA models were fitted to both single and mixture data. In the third step, in order to define antagonistic or synergistic deviations from the

reference models, the CA and IA models were extended with a deviation parameter a (Eqs. 8 and 9, respectively), which is a measure of the magnitude of the interactive effects. If $a < 0$, the mixture components interact synergistically, if $a > 0$ the mixture components interact antagonistically (Jonker et al. 2005; Nys et al. 2015).

$$\frac{x_{Cu}}{EC50_{Cu} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Cu}}}} + \frac{x_{Pr}}{EC50_{Pr} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Pr}}}} = \exp\left(\frac{a \times TU_{Cu} \times TU_{Pr}}{(TU_{Cu} + TU_{Pr})^2}\right) \quad (8)$$

$$y = 100 \times \Phi\left(\Phi^{-1}\left(\frac{1}{1+\left(\frac{x_{Cu}}{EC50_{Cu}}\right)^{\beta_{Cu}}}\right)\left(\frac{1}{1+\left(\frac{x_{Pr}}{EC50_{Pr}}\right)^{\beta_{Pr}}}\right) + \frac{a \times TU_{Cu} \times TU_{Pr}}{(TU_{Cu} + TU_{Pr})^2}\right) \quad (9)$$

where Φ is the standard cumulative normal distribution function.

The last two steps were performed using R (Ver 3.0.3 (2014-03-06)) and the software package RStudio (Ver 0.99.489).

In order to determine the best set of parameters predicted by the reference models, i.e., $EC50_{Cu}$, β_{Cu}, $EC50_{Pr}$, β_{Pr}, and parameter a for step 3, 20,000 sets were established to be sampled simultaneously and estimated in one sample run. The selection of the best set of the above-mentioned parameters from previously run 20,000 sets was based on the lowest sum of squared errors (Hochmuth et al. 2014).

Then an F test with a prior verification of the validity of assumptions was applied to the models corresponding to steps 2 and 3 in order to test whether the addition of the deviation parameter a to the model from step 3 significantly improved its predictions. Akaike Information Criterion (AIC) was used as a measure of the relative model fit of the reference models (Hochmuth et al. 2014; Nys et al. 2015). The visualization of interactive effects were performed by plotting the observed RGR in diatom or RS in copepod of the mixture treatments, together with the RGR or RS predicted by the CA and IA models in step 1 in the function of the sum of TU of the Cu–Pr combinations (Eq. 1) (Nys et al. 2015).

To express the deviation of observed toxicity from the toxicity predicted by the CA and IA models, i.e., to assess the model's accuracy, the model deviation ratio introduced by Belden et al. (2007) was calculated as

$$MDR = \frac{Predicted}{Observed} \quad (10)$$

where Predicted is the effective concentration to 50% of the population of the mixture predicted by the CA or IA models and Observed is the effective concentration to 50% of the population of the mixture obtained from the bioassays.

A model deviation ratio (MDR) greater than 1 denotes that the model underestimates the toxicity, whereas a MDR smaller than 1 indicates that the model overestimates the toxicity. MDR values smaller than 0.5, greater than 2, or within the range from 0.5 to 2 indicate that the mixture was most likely antagonistic, synergistic, or additive, respectively (Coors et al. 2013; Cedergreen 2014; Nweke et al. 2015).

FA response to interactive mixture effects

The analysis of interactive mixture effects was applied only for FA profiles of diatom species, since not all bioassays with copepod species were run simultaneously due to technical constraints.

The interactive mixture effects on FA profiles of diatom were determined with two-way ANOVA as described by De Coninck et al. (2013) that was used as a statistically significant deviation from the IA model of joint stressor effects.

FA data (Table S3 from Online resource) were log10 transformed prior to the statistical analysis to meet the assumptions of normality (Shapiro–Wilk test) and homoscedasticity (Levene's test).

Two-way ANOVA using copper and Primextra® treatments as factorial parameters was performed at each contamination level: C1, C2, and C3 for four groups of FA: saturated FA (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), and highly unsaturated FA (HUFA) and for the six major FAs with highest contribution to the FA profile of diatom *T. weissflogii* (Table 3). HUFA belongs to the group of PUFA and are also termed as essential FA (EFA) since they cannot be synthesized de novo in animal organisms.

As described by De Coninck et al. (2013), a significant interaction term at the 95% significance level ($p < 0.05$) found with this ANOVA carried out on log-transformed independent variables (here FA) implies a statistically significant deviation from the IA model.

When the two-way ANOVA revealed a statistically significant Cu–Pr interaction, synergistic or antagonistic effects were revealed through the comparison of the observed effect (Eq. 11) in the mixture treatment with the effect predicted with the IA model (Eq. 7).

$$E_{i\text{Observed}} = \frac{Y_{CTL} - Y_i}{Y_{CTL}} \quad (11)$$

where i is either copper, Primextra®, or mixture treatment of copper and Primextra®, $E_{i\text{Observed}}$ is the observed effect of treatment i on endpoint Y (FA group or top 6 FA) with Y_{CTL} referring to a FA amount from the uncontaminated treatment, and $E_{i\text{Observed}}$ can be both positive and negative, in case of a decrease or an increase of the endpoint compared with the control, respectively.

At the final stage, the interaction was classified as synergistic when the value of the observed effect in mixture treatment was "higher" than the value of the effect predicted with the IA model and as antagonistic when the value of the observed effect was "smaller" than the value of the predicted effect (see Eqs. 7 and 11 and De Coninck et al. (2013) for details).

Results

Interactive mixture effects on diatom growth rate and copepod survival

The EC50 values determined for both planktonic species revealed that the microalgae is more sensitive to the herbicide Primextra® Gold TZ than to the metal copper, whereas the copepod species is more sensitive to the metal copper than to the herbicide Primextra® (Table 1).

The individual concentration–response curves of each contaminant for both species are presented in Fig. S1 (Online resource).

The contaminant mixture revealed different tendencies in interactive effects on the diatom and copepod species. Mixture effects are presented in the plots of the observed and the predicted responses of the IA and CA models against the sum of the TU of copper and Primextra® (Fig. 1).

In the diatom *T. weissflogii*, at lower TU (< 1), the CA- and IA-predicted relative diatom growth rate values were mostly higher than the observed values, proposing synergisms, whereas at the intermediate TU ($1.4 < \Sigma TU < 2.8$), these were noticeably lower than the observed values, proposing antagonism. Only at the highest sum of TU (≈ 5–6), the predicted values of both models were clearly close to the observed relative diatom growth rate values suggesting noninteraction. On the other hand, these high TU values are in the realm of 100% effect, hence there is a possibility for synergistic or antagonistic effects that might be not visible.

Table 1 Summary table of the single-stressor concentration response parameters slope β and EC50 of copper (Cu) and herbicide Primextra® (Pr) for both planktonic species (± standard error)

Species/toxicant	Cu	Pr
T. weissflogii		
EC50 (96 h, mg/L)	0.0646 ± 0.0085	0.0365 ± 0.0021
β	4.70 ± 1.93	1.70 ± 0.14
A. tonsa		
EC50 (48 h, mg/L)	0.084 ± 0.013	3.947 ± 1.145
β	1.936 ± 0.384	2.732 ± 0.573

Calculations of parameters are based on measured concentration values

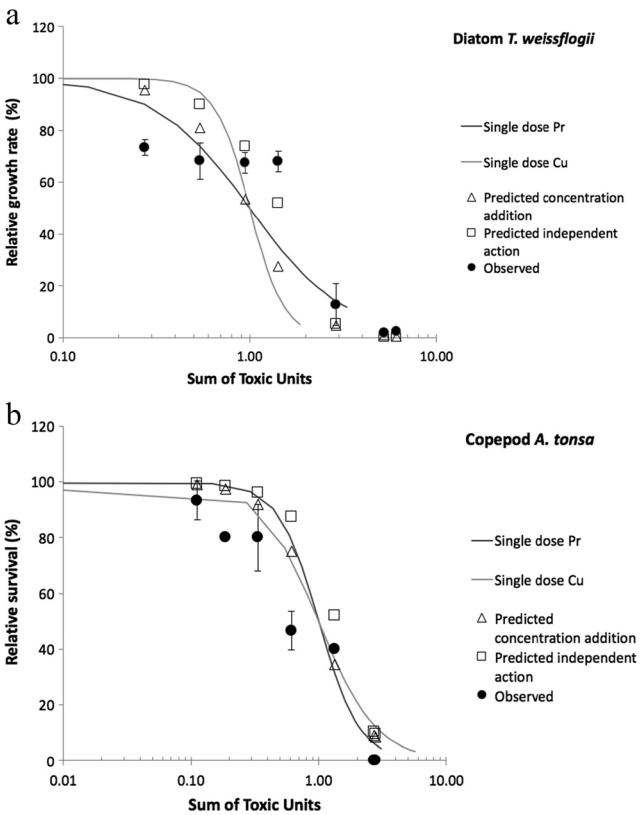

Fig. 1 Observed and predicted RGR (%) of the diatom species (**a**) and RS (%) of the copepod species (**b**) in the mixture combinations of the Cu–Pr mixture as a function of the sum of TU. Symbols are indicated as follows: observed effects (circles), predictions of CA (Eq. 6, triangles), predictions of IA (Eq. 7, squares). Predictions are based on the parameters (EC50 and β) of the single-stressor concentration response (Table 1). Cu, copper; Pr, Primextra® Gold TZ. Error bars represent standard errors. Standard error values for some observed data points are smaller than the symbol size

In the case of *A. tonsa* at lower and middle TU (< 1), the CA- and IA-predicted relative copepod survival values were higher than the observed values, proposing synergism, whereas at higher TU (> 1), predicted values of both models were relatively close to the observed relative copepod survival values, suggesting noninteraction.

The statistical analysis of mixture effects revealed that the Cu–Pr mixture acted significantly antagonistic on diatom growth, analyzed relative to the CA model ($p = 0.04$), while the mixture effects were noninteractive with respect to the IA model ($p = 0.55$) (Table S2; Fig. S2). There were no differences between the relative fit of the IA and CA reference models: values of AIC for each model were relatively similar to each other (Table S2). Calculated MDR values indicated that both models overestimated the toxicity of Cu–Pr mixture and that the applied mixture acted most likely additively on the diatom growth rate in relation to both the CA and IA models: MDR = 0.752 and MDR = 1.063, respectively.

Analysis of the global interactive mixture effects for copepod species showed the opposite trend: the Cu–Pr mixture acted significantly synergistic on the copepod survival, when

analyzed relative to the IA model ($p = 0.01$), while the mixture effects were non-interactive in respect to the CA model ($p = 0.18$) (Table S2; Fig. S2). The IA model with the deviation parameter *a* fitted the data slightly better than the IA model without *a*: lower AIC. However, the CA models showed no differences in the quality compared with the IA models: values of AIC were relatively similar to each other (Table S2). MDR values denoted that Cu–Pr mixture was most likely synergistically in relation to the IA model (MDR = 2.026) and additively on the copepod survival in relation to the CA model (MDR = 1.521) and that both models underestimated the toxicity of the applied mixture.

Variation of FA profiles and their response to interactive mixture effects

For population microcosm bioassays with the diatom species for further determination of FA profiles, a relatively high sum of TU (Fig. 1a; Table 2) was chosen in view of their relatively high growth rate after 96 h bioassay within this range of sum of TU combinations. Another reason was related to our research interest to determine FA response of the diatom species when a tendency in alteration of the mixture effects on the diatom growth rate was revealed within the same range: from synergistic to antagonistic and noninteractive (Fig. 1a).

On the contrary, a relatively low sum of TU (Fig. 1b; Table 2) was chosen for the copepod species in view of their relatively high survival after 48 h bioassay within this range of sum of TU combinations. Another reason was due to our research interest to determine copepod's FA response at the low sum of TU combinations when tendency to continuous synergism on the relative copepod survival was revealed (Fig. 1b).

In addition, these treatments were chosen in view of their effect on the RGR of *T. weissflogii* and the RS of *A. tonsa* after exposure to the equitoxic mixture of contaminants during 96 and 48 h bioassays, respectively. Related to C1, C2, and C3 contaminant concentrations caused 10, 20, and 50% effects, respectively, based on the nominal concentration values of the metal and the herbicide.

The conducted population microcosm bioassays revealed that Primextra® and copper individually and in equitoxic mixture interfered with the FA composition of both study species.

The FA content (absolute concentration in 10^{-9} μg FA/cell for the diatom and in 10^{-4} μg FA/individual for the copepod) of the diatom and copepod species exposed to the different treatments in each bioassay was compared with the uncontaminated treatment (Tables S3 and S4, Online resource). A *t* test revealed no significant differences between the FA profiles of uncontaminated (control) treatments for each species.

The metal copper and the herbicide Primextra® individually and in equitoxic mixture influenced the FA profile of copepod species more severe than the FA content of the diatom species (Fig. 2). The general trend in FA alteration of diatom species in each bioassay was a small increase of FA concentration with a peak at the low or the intermediate contaminated level. However, copper exposure led to the more significant changes: more than 50% increase of total MUFA, PUFA, and HUFA compared with the uncontaminated treatment (control) was observed with a peak of 126% of MUFA and 77% of HUFA at the lowest and intermediate levels of contamination, respectively (Fig. 2 (1a–c)).

The opposite trend was observed in FA response of the copepod species: the amount of total MUFA, PUFA, and HUFA decreased along the level of contamination from the control to the high-contaminated treatments slightly after the metal exposure, and more severely after the herbicide exposure and with the greatest significance after their equitoxic mixture exposure.

Thus, after single copper, single Primextra®, and the mixture exposures, total MUFA decreased by 40, 80, and 82%, respectively, total PUFA declined by 45, 78, and 81%, respectively, and total HUFA (including essential FA: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) has decreased by 50, 67, and 81%, respectively, at the high-contaminated level compared with the uncontaminated treatment. Only total SFA decreased slightly after the mixture exposure, by 42% after the herbicide treatments and after the metal exposure bottomed at the intermediate contaminated level with slight increase at the high-contaminated treatment (Fig. 2 (2a–c)).

Table 2 Summary of treatments applied for *T. weissflogii* and *A. tonsa* in population microcosm bioassays for further FA analysis

Treatments/species	Diatom *T. weissflogii*			Copepod *A. tonsa*		
	Cu (mg/L)	Pr (mg/L)	Sum of TU	Cu (mg/L)	Pr (mg/L)	Sum of TU
CTL	0	0	0	0	0	0
C1	0.0456	0.003	0.80	0.0046	0.120	0.09
C2	0.0549	0.004	0.96	0.0076	0.203	0.15
C3	0.0754	0.005	1.32	0.0209	0.555	0.39

CTL, C1, C2, and C3 are treatments referring to the uncontaminated treatment and the low, the intermediate, and the high levels of contaminants, expressed as sum of toxic units (TU) of copper and Primextra® combinations (Eq. 1), where CTL < C1 < C2 < C3

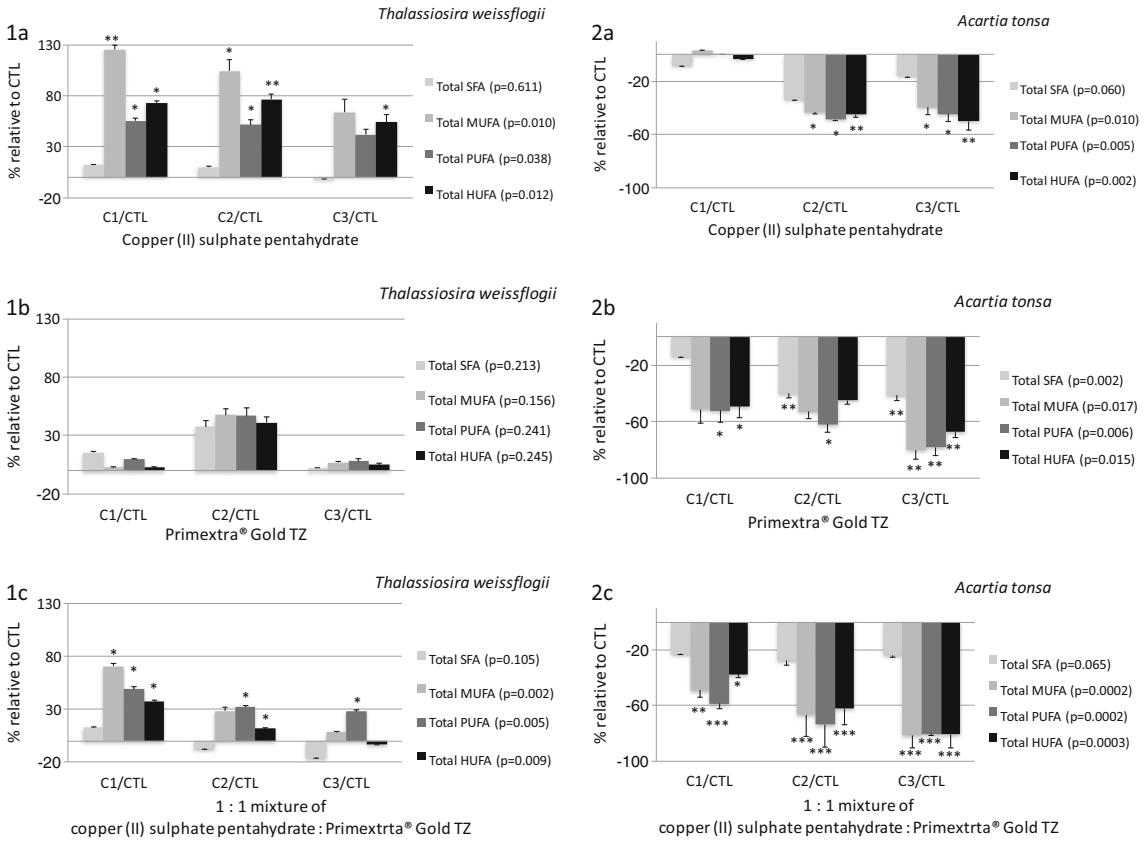

Fig. 2 Relative change of four FA groups: SFA, MUFA, PUFA, and HUFA in percent relative to uncontaminated (control (CTL)) treatment at the low (C1), the intermediate (C2), and the high (C3) levels of contamination for diatom *T. weissflogii* (1) and for copepod *A. tonsa* (2) after exposure to copper(II) sulphate pentahydrate (a), to the herbicide Primextra® Gold TZ (b), and their equitoxic mixture (c). A positive value refers to an increase of FA relative to the control. A negative value refers to a decrease of FA relative to the control. *p* values in parentheses represent the significant ($p < 0.05$) or insignificant ($p > 0.05$) differences among all treatments for each FA group. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, the significant difference of the treatment compared with the control for each FA group

The FA profiles of diatom and copepod were dominated by SFAs (14:0, 16:0, 18:0), MUFA (16:1ω7), and HUFA (20:5ω3 (EPA) and 22:6 ω3 (DHA)).

The interactive mixture analysis applied to FA profiles of the diatom species revealed that the effect was noninteractive for SFA (Table 3). However, a synergistic effect was observed for each group of unsaturated FA: MUFA, PUFA, and HUFA mostly at the intermediate levels of contamination. Thus, for the essential FA DHA and EPA, the interactive mixture effects were synergistic at the intermediate level of contamination. Only one case of antagonistic effect was revealed: for stearic acid 18:0 at the high level of contamination.

Discussion

Interactive mixture effects on diatom growth rate and copepod survival

Chemical analysis was conducted to compare nominal concentrations with the true concentrations in acute zooplankton and microalgae growth bioassays. Generally, measured copper concentrations are similar to the expected nominal values (Table S1). However, at the lowest doses, the measured concentrations are higher than expected. We assume it may be due to the presence of trace amounts of copper in the filtrated natural seawater sampled in the Mondego estuary (Vasconcelos et al. 2011), and we used it as a base of culture's mediums during maintenance and bioassays. Similarly, for the herbicide, both a.i. of Primextra® were registered in the Mondego estuary (Cruzeiro et al. 2016) that may be a reason of a difference between their nominal and measured values.

Single effects of copper and Primextra® on the RGR of *T. weissflogii* and the RS of *A. tonsa* are in accordance with other studies where primary consumer species showed higher sensitivity to metals compared with herbicides, whereas primary producer species responded with an opposite trend (Hack et al. 2008; Diz et al. 2009; Pinho and Bianchini 2010; Manimaran et al. 2012; Stringer et al. 2012).

Values of predicted no-effect concentrations (PNEC) for aquatic organisms of copper and the a.i. of Primextra® Gold TZ, metolachlor, and TBA are equal to 0.8, 0.76, and

Table 3 Summary of two-way ANOVA for FA profiles (log10) of diatom *T. weissflogii* with three levels of contamination (C1 < C2 < C3)

FA	Contamination level	*p* value	Obverved effect ($E_{CuPr\ observed}$)	$SD_{Observed}$	Predicted effect ($E_{CuPr\ predicted}$)	Interaction type, relatively to the IA model	Deviation size
SFA	C1	0.092	0.104	0.007	0.149	Non-interactive	1.423
	C2	0.952	0.089	0.013	0.113	Non-interactive	1.274
	C3	0.147	0.082	0.007	0.158	Non-interactive	1.932
MUFA	C1	*0.050*	0.011	0.005	− 0.009	Synergism	0.803
	C2	*0.000*	− 0.012	0.015	− 0.068	Synergism	5.606
	C3	0.173	− 0.025	0.007	− 0.065	Non-interactive	2.634
PUFA	C1	0.971	0.017	0.005	0.014	Non-interactive	0.823
	C2	*0.015*	0.008	0.007	− 0.027	Synergism	3.349
	C3	0.996	0.006	0.007	− 0.019	Non-interactive	3.187
HUFA	C1	0.520	0.010	0.007	0.006	Non-interactive	0.565
	C2	*0.001*	− 0.007	0.011	− 0.052	Synergism	6.980
	C3	0.220	− 0.019	0.010	− 0.040	Non-interactive	2.080
14:0	C1	0.251	0.009	0.005	− 0.003	Non-interactive	0.276
	C2	*0.002*	− 0.008	0.011	− 0.051	Synergism	6.649
	C3	0.312	− 0.020	0.008	− 0.040	Non-interactive	2.010
16:0	C1	0.194	0.020	0.007	− 0.012	Non-interactive	0.588
	C2	0.370	0.001	0.014	− 0.052	Non-interactive	0.052
	C3	0.366	− 0.010	0.007	− 0.014	Non-interactive	1.324
18:0	C1	*0.009*	0.048	0.009	0.010	Synergism	0.203
	C2	*0.042*	0.037	0.013	− 0.014	Synergism	0.366
	C3	*0.012*	0.036	0.008	0.065	Antagonism	1.790
16:1ω7	C1	*0.024*	0.012	0.005	− 0.014	Synergism	1.110
	C2	*0.000*	− 0.014	0.016	− 0.081	Synergism	5.885
	C3	0.116	− 0.027	0.007	− 0.079	Non-interactive	2.892
20:5ω3 (EPA)	C1	0.493	0.011	0.007	0.005	Non-interactive	0.480
	C2	*0.001*	− 0.006	0.011	− 0.052	Synergism	8.683
	C3	0.214	− 0.017	0.009	− 0.041	Non-interactive	2.384
22:6ω3 (DHA)	C1	0.635	0.003	0.008	0.006	Non-interactive	1.844
	C2	*0.000*	− 0.012	0.008	− 0.040	Synergism	3.196
	C3	0.241	− 0.024	0.009	− 0.025	Non-interactive	1.072

Significant *p* values at the 95% significance level are shown in *italics*. The observed effect in the mixture treatment (Eq. 11) and its standard deviation, the predicted effect based on the independent action model (Eq. 7), the interaction type, and the size of deviation (absolute values) calculated as the ratio between the predicted and observed effects are provided

SD, standard deviation of the observed effect

0.0032 μg/L, respectively (Lopez-Roldan et al. 2013). Recently, metolachlor and TBA were registered in the Mondego River estuary with the following maximum values of 0.266 and 0.088 μg/L correspondingly (Cruzeiro et al. 2016), exceeding PNEC of TBA for aquatic organisms and the limit value of 0.100 μg/L for a single pesticide in drinking water established by the EC Drinking Water Directive (98/83/EC DWD) regarding metolachlor (Spoljaric et al. 2011; Lopez-Roldan et al. 2013) which justifies further interest in these xenobiotics, particularly because of the widespread use of metolachlor and TBA in numerous pesticide formulations for weed control in corn/maize cultures.

In view of the continuous intensive agriculture practices at the surrounding fields of the Mondego estuary, there is a tendency for a raise in the concentration of both a.i. of Primextra® in this ecosystem (Cruzeiro et al. 2016; Gonçalves et al. 2016).

In other aquatic basins, in the EU and worldwide, high concentration levels (compared with PNEC values and values established by EC DWD) of the investigated chemical stressors, resulting from the continuous anthropogenic impacts to those aquatic ecosystems have been registered for copper from 10.20 to 50 μg/L (Gabrielides 1995; Brix et al. 2006; Ruas et al. 2008), for metolachlor from 2.84 to 36 μg/L (Cook and Moore 2008; Dores et al. 2009; Nwani et al. 2014),

and for TBA from 0.53 to 8.50 μg/L (Köck et al. 2010; Fiori et al. 2013; Nödler et al. 2013; Palma et al. 2014; Pereira et al. 2017). Moreover, some values in situ exceed the EC50 values obtained in this study for the diatom *T. weissflogii* and the copepod *A. tonsa*: for copper up to 80.4 μg/L (Lekkas et al. 2004), for metolachlor from 40 to 460 μg/L (Cerejeira et al. 2003; Cook and Moore 2008; EPA 2008; Mai et al. 2012; Thakkar et al. 2013), and for TBA from 47 to 100 μg/L (Otto et al. 1999; Wenneker et al. 2010).

Therefore, the concentrations used in our study correspond to realistic contamination events of these contaminants' application which allows to determine and predict ecological consequences and elucidating about the potential mechanisms of this type of biosynthesis-inhibitor agents in biochemical profiles of nontarget species.

Although there is information on the individual effects of metals and herbicides on nontarget species, studies that examine the interactive mixture effects of these compounds are scarce in the literature. Our study revealed that the mixture acted most likely additively on the RGR of the diatom species in respect to both the CA and IA reference models: MDR = 0.752 and MDR = 1.063, respectively, and on the copepod's survival relative to the CA model (MDR = 1.521). However, mixture effects were most likely synergistically relative to the IA reference model (MDR = 2.026) on the RS of *A. tonsa*.

Besides active ingredients, a fully formulated pesticide product usually contains various adjuvants, such as surfactants, potentiators and other solvents to increase the uptake and half-life of an a.i. Theoretically they may interact all with each other and this subsequently may lead to potentiating or weakening of the interactive effects or even be a reason of additive effects (Neves et al. 2015). The herbicide Primextra® Gold TZ contains 0–5% (w.w.) of adjuvants. However, they might be a potential cause for the interactions observed in this study (Castro et al. 2013).

The calculated EC50 values for algae and crustacean species as "the base dataset" and the applied mixture toxicity concepts, i.e., the CA and IA models can serve for further assessment of environmental hazard and risk of the studied chemical mixture (Backhaus and Faust 2012).

A simple additivity is occurring frequently as a result of inorganic and organic compounds in mixture exposure to both aquatic plants and animals as reported by Pantani et al. (1990) and Lister et al. (2011).

Studies addressing the mixture effect of pesticides and metals are still remaining scarce (Cedergreen 2014). However, more than 50% of the available studies revealed the synergistic interactions between these contaminants (Uwizeyimana et al. 2017).

Additivity may occur when chemicals individually and in mixture combination act on similar action sites or have similar modes of action on the exposed species or when they have similar accumulation rates (Mahar and Watzin 2005; Lister et al. 2011). The observed noninteractive effects in this study may happen due to one or more of these causes.

Synergistic interaction may appear when metabolites from the metabolization process that occurs with the components of mixture after absorption by the organism are more toxic than the mixture's components before the absorption (Uwizeyimana et al. 2017). The other cause of synergism may be due to the alteration in a metal ion speciation when components of mixture interact outside the exposed organism (Binderup et al. 2003). Thus, the toxicity of copper to amphipods greatly increased in the presence of lipid-soluble ligands as well due to the formation of the complexes with copper diffusing through the cell membrane and participating in injurious reaction (Ahsanullah and Florence 1984). Indeed, copper is known for its tendency for complexation (Undabeytia et al. 1996). Recently it was discovered that triazine herbicide atrazine and the metal copper ion are able to form metal-organic complexes in aqueous media (Kumar et al. 2015). One of the a.i. of the herbicide Primextra® Gold TZ is TBA—lipophilic compound: K_{ow} = 1096 (WHO 2003), which also belongs to the group of triazine herbicides. Therefore, we may assume the formation of TBA-Cu complexes that were difficult to interact with the algal cell wall and easy for diffusion through the copepod cell membrane. These could be a possible reason of synergistic effect of observed mixture on *A. tonsa* survival relatively to the IA model (MDR = 2.026).

A few available studies revealed both synergistic and additive effects of copper–pesticide mixtures on invertebrate animals: for the marine copepod *Tigriopus brevicornis*, a mixture of the metal copper and insecticide malathion had strong synergistic effect relatively to the IA model (Forget et al. 1999), for the terrestrial red warm *Eisenia fetida*, a co-existence of a pesticide and a heavy metal led to the synergism already at low effect levels relatively to the combination index-isobologram equation method (Chen et al. 2015), for the freshwater daphnid *Ceriodaphnia dubia*, a combination of copper and insecticide diazinon acted additively on its survival in relation to the CA model (Mahar and Watzin 2005), and both additive and antagonistic effects of copper–organic mixtures on aquatic plant organisms: a mixture of the herbicide diuron with copper for duckweed *Lemna minor* acted additively in relation to the applied multifactorial model but antagonistically according to Abott's formula (Teisseire et al. 1999). For the unicellular algae *Chlorella ellipsoidea* and *Scenedesmus obliquus*, a combination of copper with pentachlorophenol and copper with herbicide imazethapyr, respectively, resulted in antagonism (Aoyama et al. 1987; Chen et al. 2013).

Variation of FA profiles and their response to interactive mixture effects

Our study revealed that copper and Primextra® Gold TZ individually and in equitoxic mixture differently interfered with

the FA composition of study species. Notwithstanding, the individual effects of metals and herbicides on the FA profiles of nontarget organisms are well studied, a few studies about the mixture effects of a group of metals and a group of pesticides are available, whereas the studies examining the effect of metal-pesticide mixture compounds on the FA profiles of marine species have not been performed (Filimonova et al. 2016a).

Our findings demonstrated that the metal copper, the herbicide Primextra® single, and in their combination interfered with the FA composition of the diatom *T. weissflogii* to a greater extent than to the copepod *A. tonsa* (Fig. 2).

In the case of the diatom species, the greatest significant change in FA amount was observed for highly unsaturated FA, i.e., essential FA, after exposure to copper. At each level of copper contamination, the amount of HUFA increased 1.5–2 times compared with the control treatment. This is in accordance with another study (Sibi et al. 2014) with microalgae species when under copper stress the increase of percent composition of FA was observed. An increase in HUFA (i.e., 20:5ω3, EPA) may show the photosynthetic dysfunction, since these FA presumably replace linolenic acid in the diatom species (Sicko-Goad et al. 1989a, b, c, d). The other reason may relate with galactolipids and phospholipids that are substrates in the process of desaturation, which plays the role in the synthesis of HUFA in marine microalgae (Henderson et al. 1990; Filimonova et al. 2016b).

For the copepod *A. tonsa*, and in general among all bioassays with both species, it is notable that the equitoxic mixture of contaminants led to the greatest alteration in copepod's FA profile and significantly decreased MUFA, PUFA, and HUFA amounts ($p < 0.001$, Fig. 2 (2c)). These results are in agreement with the interactive mixture effect on the RS of *A. tonsa* for which a synergistic effect relative to the IA model (MDR = 2.026) was observed after exposure to the equitoxic mixture of copper and Primextra®. Therefore, stronger effects of the applied mixture on the FA profile of the copepod species were expected. As was mentioned earlier, this could be due to the easy diffusion of contaminants, including the possible TBA-Cu complexes into the cell membrane of *A. tonsa*. In addition, *S*-metolachlor—being the main a.i. of Primextra® is known to inhibit the synthesis of long chain FA (Neves et al. 2015; Filimonova et al. 2016a; Gonçalves et al. 2016). Therefore, mixture and single Primextra® treatments had larger interference with copepod FA composition compared with the effect after single copper exposure.

A synergistic effect of equitoxic mixture on unsaturated FA of the diatom species, including EFA (i.e., EPA and DHA) at the intermediate level of contamination and noninteractive effects on its SFA at all three levels of contamination are in accordance with the known fact that PUFA are target molecules for reactive oxygen species (Gabryelak et al. 2000), whereas SFA are less vulnerable for lipid peroxidation (Rael et al. 2004) which may be induced by copper and herbicide exposures (Letelier et al. 2005; Martins and Costa 2014; Filimonova et al. 2016a).

Generally, individual and mixture exposure to copper and Primextra® had a larger effect on the FA composition of the primary consumer *A. tonsa* than on the primary producer *T. weissflogii* with the most harmful effect on the essential FA of copepod species after exposure to the metal-herbicide mixture. We hypothesize the lower alteration of FA composition of the diatom *T. weissflogii* in view of the presence of the cell wall in the algae cell structure that serves as a defensive barrier against environmental stressors to the cell membrane containing fatty acids (Keegstra 2010) due to the known fact that algae and plants are able to synthesize PUFA and HUFA de novo, whereas most animals do not have this ability. In case they do have it, the produced amount is not sufficient and they have to obtain these FA from their food sources (Brett and Müller-Navarra 1997). Hence, these FA are termed EFA. However, De Troch et al. (2012) proved that harpacticoid copepods are able to convert short-chain FA (i.e., C 18:0) to long-chain PUFA (i.e., EPA and DHA) via the Δ-5, Δ-6 desaturase, and elongase enzymes. However, the production of PUFA by planktonic calanoid copepods, including our test species *Acartia tonsa*, is not significant since these species are limited with the amount of the enzymes that take part in this process (De Troch et al. 2012). As copepods get the majority of these EFA from their food, they are largely depending on the FA concentration of the diatoms. However, here, the diatom produced more FA under stress as a kind of defense mechanism but yet it was not very efficiently transferred to the next trophic level under stress. The physiological condition of the copepod should be studied more in detail to understand the mechanism for this.

A significant decrease of essential FA in the primary consumer species after exposure to the study contaminants individually and in mixture are of high concern in relation to the next trophic level, i.e., secondary consumers. Thus, EFA are crucial for the overall well being of juvenile fish including their growth and the resistance to the diseases (Brett et al. 2009). Therefore, the presence of EFAs in fish diets is crucial for the healthy status of fish populations and is consequently also important for the maintenance of a sufficient nutritional status of the human diet and health.

Conclusions

There was a stronger effect of equitoxic mixture of the herbicide Primextra® Gold TZ and the metal copper on the RS and essential FA of copepod *A. tonsa*. The observed synergism (relatively to the IA model, MDR = 2.026) at the low levels of toxicants for this species suggested a potential ecotoxicological risk related to a higher possibility of the co-occurrence

of these contaminants at environmentally relevant concentrations. A lower sensitivity of the primary producer diatom *T. weissflogii* and a greater response of the primary consumer copepod *A. tonsa* to the applied mixture of copper and Primextra® in terms of abundance and FA composition, suggest a potential worse effect on higher trophic levels than on primary producers that may lead to a decrease in available biomass and energy flow through the ecosystem. In addition, these results may contribute to future ecological risk assessments of potentially hazardous metal-herbicide mixtures on nontarget species.

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