Universidade de Aveiro Departamento de Biologia
2016

VALENTINA FILIMONOVA

EFEITOS DO STRESS ANTRÓPICO NA QUALIDADE ALIMENTAR DE SISTEMAS ESTUARINOS

THE EFFECTS OF ANTHROPOGENIC STRESSORS ON THE FOOD QUALITY IN ESTUARINE SYSTEMS

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Tese apresentada à Universidade de Aveiro e à Universidade de Ghent para cumprimento dos requisitos necessários à obtenção do dual grau de Doutor em Ciências do Mar e Doutor em Ciência: Ciências do Mar, respectivamente, realizada sob a orientação científica da Doutora Ana Marta Gonçalves, Professor Doutor Fernando Gonçalves do Departamento de Biologia & CESAM da Universidade de Aveiro e Professora Doutora Marleen De Troch da Faculdade de Ciências, Departamento de Biologia, Biologia Marinha da Universidade de Ghent

Thesis submitted to University of Aveiro and University of Ghent for compliance with the requirements for the dual degree of Doctor in Marine Sciences and Doctor of Science: Marine Sciences, respectively, held under the supervision of Dr. Ana Marta Gonçalves, Prof. Dr. Fernando Gonçalves from Department Biology & CESAM, University of Aveiro and Prof. Dr. Marleen De Troch from Faculty of Science, Department of Biology, Marine Biology, University of Ghent

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«Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time»

Thomas A. Edison

o júri

presidente

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palavras-chave

sistemas estuarinos; herbicida; metal; plâncton; ecotoxicidade; misturas; biomarcadores; modelo linear; qualidade alimentar

resumo

De um modo geral, os poluentes constituem uma ameaça para os ecossistemas aquáticos, originando grande preocupação nas entidades responsáveis pela gestão destas áreas. Por exemplo, o uso intensivo e continuado de poluentes em áreas agrícolas, perto de zonas húmidas costeiras, como o estuário do Mondego (Portugal), levou à execução de programas de monitorização, ao longo dos últimos 20 anos, para proteger e recuperar este sistema aquático. De acordo com informações recentes, obtidas junto de cooperativas agrícolas do vale do Mondego, Primextra® Gold TZ é o herbicida mais utilizado em campos de cultura de milho, sendo um dos 20 herbicidas mais vendidos em Portugal. Por outro lado, o cobre é largamente utilizado na formulação de pesticidas.

A avaliação ecológica e de risco têm sido rotineiramente focadas na exposição individual de substâncias químicas, o que pode subestimar os riscos associados à ação tóxica de misturas. Assim, os potenciais efeitos sinergísticos, que podem levar a consequências mais graves e imprevisíveis para os ecossistemas estuarinos e marinhos, são subestimados. Apesar do recente aumento do número de estudos relacionados com a toxicidade de misturas de contaminantes orgânicos (grupo de pesticidas) e de contaminantes inorgânicos (grupo de metais), há ainda falta de informação científica sobre os efeitos destas misturas.

A avaliação de risco ambiental normalmente tem como objetivo estudar os efeitos de contaminantes em *endpoints*, como a sobrevivência, o crescimento ou a reprodução, uma vez que a quantidade de biomassa disponível tem efeitos importantes sobre os níveis tróficos subsequentes e o funcionamento global do ecossistema. No entanto, um aspeto muitas vezes esquecido é a qualidade do alimento disponível, que tem implicações importantes na transferência de energia e de nutrientes ao longo da cadeia trófica. Portanto, para melhor compreender os efeitos das perturbações antrópicas, são necessários mais estudos sobre o efeito de mistura de substâncias orgânica-inorgânica na qualidade do alimento de espécies aquáticas não-alvo.

Assim, este trabalho visa determinar os efeitos do herbicida Primextra® e do cobre, usados individualmente e em mistura, sobre a qualidade alimentar em sistemas estuarinos, considerando as respostas populacionais e bioquímicas nutricionais de organismos chave não-alvo: a diatomácea marinha (*Thalassiosira weissflogii*) e o copépode estuarino calanoide (*Acartia tonsa*). As diatomáceas como os copépodes são grupos dominantes na comunidade planctónica no estuário do Mondego, constituindo uma cadeia trófica simples: produtor primário –consumidor primário. Foram também usados náuplios de uma terceira espécie (*Artemia franciscana*) para determinar os efeitos tóxicos e bioquímicos individuais de ambos os poluentes e para comparar as suas respostas com as das outras duas espécies planctónicas

resumo (cont.)

Para isso foram desenvolvidos ensaios de toxicidade, envolvendo exposições a substâncias químicas individuais e a misturas equitóxicas, para obtenção e modelação de dados bioquímicos indicadores da qualidade alimentar: teor de ácidos gordos (FA), incluindo ácidos gordos essenciais, teor de proteína e teor de substâncias reativas de ácido tiobarbitúrico (TBARS). Esta escolha baseou-se no facto dos ácidos gordos serem uma das mais importantes moléculas transferidas através da cadeia trófica, em teias alimentares aquáticas, podendo ser usados como um bom indicador de *stress*.

Os resultados mostraram que *T. weissflogii* foi a espécie mais sensível ao herbicida seguida por *A. tonsa* (CE_{50} = 0,008 mg / L e CE_{50} = 0,925 mg / L, respetivamente), enquanto o copépode foi a espécie mais sensível ao metal, seguido por *T. weissflogii* (CE_{50} = 0,234 mg / L e CE_{50} = 0,383 mg / L, respetivamente).

A análise estatística dos efeitos da mistura metal-herbicida sustentou um efeito sinergístico significativo sobre a sobrevivência do copépode (relativamente ao modelo de ação independente), e um efeito significativamente antagonístico sobre o crescimento da diatomácea (relativamente ao modelo de adição de concentração).

A composição em ácidos gordos das espécies zooplanctónicas respondeu com elevada sensibilidade aos dois tipos de exposição (individual e de mistura),tendo-se observado o efeito mais nocivo na concentração de ácidos gordos essenciais de *A. tonsa* após a exposição à mistura de metal-herbicida: o seu valor diminuiu significativamente (5 vezes) em comparação com o controlo.

Os resultados dos modelos lineares generalizados, baseados na variação da composição bioquímica (total de FA, FA essenciais, teor de proteína e TBARS), para as substâncias individuais e para a mistura, revelaram que os efeitos desta foram não-aditivos, para o conteúdo de FA essenciais de ambas as espécies planctónicas. Já a qualidade alimentar de *A. tonsa* (consumidor primário) foi mais sensível aos *stressors* químicos comparativamente a *T. weissflogii* (produtor), quando as duas espécies foram expostas a níveis iguais de contaminação. O presente estudo sugere que a exposição simultânea a um metal e a um herbicida pode afetar negativamente a qualidade alimentar de espécies planctónicas, em diferentes níveis tróficos. Esse efeito pode, potencialmente, ser transferido para níveis tróficos superiores e causar alterações importantes no fluxo de energia através do sistema estuarino e, posteriormente, para a dieta humana.

keywords

estuarine systems; herbicide; metal; plankton; ecotoxicity; mixture; biomarkers; generalized linear model; food quality

abstract

Contaminants constitute a threat to aquatic communities and, thus to aquatic ecosystems. Pesticides are widely used to control pests and diseases in crop production. Among these agrochemicals, herbicides are commonly applied on crops to control adventive infestations. Thus, the intensive usage of pollutants in agriculture areas near ecological coastal wetlands led to the implementation of the Pesticide-Monitoring programs to recover aquatic systems, such as in the Mondego estuary, Portugal, since 1998. Nowadays, and according to the information from agricultural cooperatives of Mondego valley, the herbicide Primextra® Gold TZ is the herbicide most used in corn crops fields and is one of the 20 best-selling herbicides in Portugal, whereas copper is mainly used in pesticide`s formulation.

Traditional effect and risk assessment have been routinely focused on exposures to single chemicals, which may underestimate the risks associated with toxic action of mixtures. Thus, the potential synergistic effects that may lead to more severe and unpredictable consequences for estuarine and marine ecosystems are ignored. Recently, there are an increasing number of studies dealing with toxicity of mixtures of either organic contaminants (group of pesticides) or inorganic contaminants (group of metals). However, studies with mixture experiments of metals and pesticides still remain scarce. Furthermore, environmental risk assessment typically aims to study the effects of contaminants on endpoints such as survival, growth or reproduction, since the available quantity of the biomass has important effects on the subsequent trophic levels and the overall ecosystem functioning. However, an often overlooked aspect of food availability is food quality, which has important implications on the energy and nutrient transfer through the food web. Further information about the effect of organic-inorganic mixture on the food quality of aquatic organisms, which are typical non-target species for these contaminants, is needed.

This research aims to determine the effects of anthropogenic stressors on the food quality in estuarine systems by means of both, individual and mixture exposures, to the herbicide Primextra® and the metal copper, on the toxicity and nutritionally important biochemical parameters of key non-target organisms: a marine diatom (e.g. *Thalassiosira weissflogii*) and a estuarine calanoid copepod (e.g. *Acartia tonsa*) – both are dominant plankton groups in the Mondego estuary, constituting simple trophic food chain: primary producer – primary consumer. A third species, the brine shrimp *Artemia franciscana* (nauplii), was also added to the study to determine toxic and biochemical individual effects of both pollutants and to compare its response with the two other planktonic species.

abstract (cont.)

In this study a joint approach was applied, i.e. controlled laboratory experiments (toxicity tests and microcosm bioassays), involving single and equitoxic mixture exposures to chemical stressors combined with the further modelling of the obtained biochemical data: fatty acids (FA) content, including the essential FA, protein content and content of thiobarbituric acid reacting substances (TBARS) as important indicators of the food quality. Moreover, fatty acids are one of the most important molecules transferred across the plant-animal interface in aquatic food webs and can be used as good indicators of stress.

The conducted lab incubations showed that *T. weissflogii* was the most sensitive species to the herbicide followed by *A. tonsa* (EC_{50} = 0.008 mg/L and EC_{50} = 0.925 mg/L, respectively), whereas the copepod was the most sensitive species to the metal in comparison to *T. weissflogii* (EC_{50} = 0.234 mg/L and EC_{50} = 0.383 mg/L, respectively). The statistical analysis of mixture effects revealed that the metal-herbicide mixture acted significantly synergistic on the copepod survival (relatively to the independent action model), while acted significantly antagonistic on the diatom growth (relatively to the concentration addition model).

FA composition of zooplanktonic species responded with higher sensitivity to both, the individual and mixture exposures with the most harmful effect on the essential FA of *A. tonsa* after exposure to the metal-herbicide mixture: their amount significantly decreased (5 times) compared to the uncontaminated treatment.

The results of the generalized linear models fitted to the experimentally observed responses of biochemical composition (total FA, essential FA, protein and TBARS contents) to the single substances and the mixture revealed that effects of the copper-Primextra® mixture were non-additive for the essential FA content of both planktonic species. They further showed that the food quality of the species from higher trophic level, i.e. primary consumer *A. tonsa* was more sensitive to the chemical stressors than for the primary producer *T. weissflogii*, when species were exposed to the equal levels of contamination. The study concludes that simultaneous exposure to metal and pesticide contaminants adversely affect the food quality of planktonic species at different trophic levels and this can potentially be transferred to higher trophic levels and cause important implications for the energy flow through the estuarine system and subsequently the human diet.

trefwoorden

estuariene systemen; herbicide; metalen; plankton; ecotoxiciteit; mengsel; biomerkers; gegeneraliseerd lineair model; voedselkwaliteit

abstract

Verontreinigingen vormen een bedreiging voor in het water levende gemeenschappen en daarom ook voor aquatische ecosystemen. Pesticiden worden op grote schaal gebruikt om ziekten en plagen in de plantaardige productie te controleren. Onder deze landbouwchemicaliën, worden herbiciden vaak toegepast op gewassen om adventive besmettingen te controleren. Zo heeft het intensief gebruik van verontreinigende stoffen in landbouwgebieden in de buurt van ecologische wetlands geleid tot de uitvoering van Pesticide-Monitoring-programma's sinds 1998 om aquatische systemen, zoals in het Mondego estuarium (Portugal) te herstellen. Op basis van de huidige informatie uit agrarische coöperaties van de Mondego-vallei blijkt dat de herbicide Primextra® Gold TZ de meest gebruikte herbicide is in maïsvelden en dat het één van de 20 best verkopende herbiciden in Portugal is waarbij koper het voornaamste element van de samenstelling vormt.

Traditionele effect- en risicobeoordeling zijn routinematig gericht op blootstelling aan enkele chemische stoffen, die de risico's van de toxische werking van mengsels kunnen onderschatten. Zo worden de potentiële synergetische effecten die kunnen leiden tot meer ernstige en onvoorspelbare gevolgen voor estuariene en mariene ecosystemen genegeerd. Recent zijn er een toenemend aantal studies gebeurd die toxiciteit van mengsels van ofwel organische verontreinigingen (groep pesticiden) of anorganische verontreinigingen (groep metalen) onderzoeken. Studies met mengselexperimenten van metalen en pesticiden zijn nog steeds schaars. Bovendien heeft milieurisicobeoordeling typisch tot doel om de effecten van contaminanten op eindpunten zoals overleving, groei of reproductie te bestuderen, omdat de beschikbare hoeveelheid biomassa belangrijke gevolgen heeft voor de hogere trofische niveaus en het algemeen functioneren van het ecosysteem. Echter, een vaak vergeten aspect van de voedselbeschikbaarheid is de kwaliteit van het voedsel, wat belangrijke gevolgen kan hebben voor de energie- en voedingsstoffenoverdracht in de voedselketen. Verdere informatie over het effect van de organische-anorganische mengsel op de voedselkwaliteit van het aquatische organismen, die vaak geen doelsoorten voor deze verontreinigingen zijn, is daarom noodzakelijk.

Dit onderzoek heeft tot doel de effecten van antropogene stressoren op de voedselkwaliteit in estuariene systemen te bepalen door middel van blootstelling aan zowel individuele concentraties als mengsels van herbicide Primextra® en het metaal koper, op de toxiciteit en de nutritioneel relevante biochemische parameters van belangrijke non-target organismen: een mariene diatomee (bijv *Thalassiosira weissflogii*) en een estuariene calanoid copepod (bijv *Acartia tonsa*) - beide zijn één van de belangrijkste planktongroepen in de Mondego estuarium, die een eenvoudig trofische voedselketen voorstellen tussen primaire producent en primaire consument.

abstract (cont.)

Een derde soort, (de nauplii van) *Artemia franciscana*, werd ook toegevoegd aan de studie om de toxische en biochemische individuele effecten van beide polluenten te bepalen en om zijn respons te vergelijken met de twee andere planktonsoorten.

In deze studie werd een gezamenlijke aanpak toegepast waanbij laboratoriumexperimenten (toxiciteitstesten en microcosmos bioassays), met blootstelling aan één of aan een equitoxische mengsel van chemische stressoren, werden gecombineerd met het verder modellering van de verkregen biochemische data: vetzuren, met bijzondere aandacht voor essentiële vetzuren, eiwitgehalte en de inhoud van thiobarbituurzuur reagerende stoffen (TBARS) als belangrijke indicatoren voor voedselkwaliteit. Daarenboven zijn vetzuren één van de belangrijkste moleculen overgedragen aan de plant-dier interface in aquatische voedselwebben en kunnen ze gebruikt worden als goede indicatoren van stress.

De uitgevoerde labincubaties toonden dat *T. weissflogii* de meest gevoelige soort was voor de herbicide gevolgd door *A. tonsa* (EC_{50} = 0.008 mg / L en EC_{50} = 0.925 mg / L, respectievelijk), terwijl de copepoden het meest gevoelig waren voor het metaal tenopzichte van *T. weissflogii* (EC_{50} = 0.234 mg / L en EC_{50} = 0.383 mg / L respectievelijk). De statistische analyse van mengseleffecten bewees dat het metaal-herbicide mengsel significant synergistisch werkte op de overleving van de copepoden (ten opzichte van de onafhankelijke actiemodel), terwijl significante antagonistische effecten op de diatomeeëngroei optraden (ten opzichte van de concentratieadditiemodel).

FA samenstelling van de zooplanktonische soorten reageerde met een hogere gevoeligheid op beide, individuele en mengsel, blootstellingen met de meest schadelijke gevolgen voor de essentiële vetzuren van *A. tonsa* na blootstelling aan het metaal-herbicide mengsel: de vetzuurconcentratie daalde aanzienlijk (tot 5 maal) ten opzichte van de onbesmette behandeling.

De resultaten van de gegeneraliseerde lineaire modellen toegepast op de experimenteel waargenomen responsen van biochemische samenstelling (totale vetzuren, essentiële vetzuren, eiwit- en TBARS concentraties) tenopzichte van de afzonderlijke stoffen en van het mengsel toonden aan dat effecten van het koper-Primextra® mengsel niet additief waren voor de concentratie van de essentiële vetzuren van beide planktonische soorten. Verder bleek de voedselkwaliteit van de soort op het hoger trofisch niveau, namelijk de primaire consument *A. tonsa*, gevoeliger te zijn voor de chemische stressoren dan de primaire producent *T. weissflogii*, wanneer soorten werden blootgesteld aan gelijke verontreinigingsniveaus. De studie concludeert dat gelijktijdige blootstelling aan metalen en pesticiden verontreinigingen een negatieve invloed heeft op de voedselkwaliteit van planktonische soorten op verschillende trofische niveaus en dit kan mogelijk worden overgedragen naar hogere trofische niveaus met belangrijke gevolgen voor de energiestroom door het estuariene systeem en vervolgens ook voor het menselijke dieet.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

a.i.	Active ingredient
AIC	Akaike information criterion
AP	Alkylphenols
ARA	Arachidonic acid
CA	Concentration addition
Cu	Copper
CuSP	Copper (II) sulphate pentahydrate
DHA	Docosahexaenoic acid
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAME	Fatty acid methyl esters
FFA	Free fatty acids
GLM	Generalized linear model
HUFA	Highly unsaturated fatty acids
IA	Independent action
LP	Lipid peroxidation
MTs	Metallothioneins
MUFA	Monounsaturated fatty acids
OC	Organochlorine
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzofurans
POPs	Persistent organic pollutants
Pr	Primextra® Gold TZ
PUFA	Polyunsaturated fatty acids
RGR	Relative growth rate
ROS	Reactive oxygen species
RS	Relative survival
SFA	Saturated fatty acids
SSE	Sum of squared errors
TBA	Terbuthylazine
TBARS	Thiobarbituric acid reacting substances
TU	Toxic units
VLCFA	Very long chain fatty acid

CHAPTER 1. Introduction

1.1. Estuarine systems and anthropogenic stressors

Environmental stress refers to physical, chemical, and biological constraints on the productivity of species and on the development of ecosystems. Stressors can be natural environmental factors, or they may result from the activities of humans, i.e. anthropogenic stressors (Iyyer, 2009). All ecosystems are potentially vulnerable to their negative impacts. Anthropogenic stressors often exceed the range of natural environmental stressors, thus reducing ecosystem resistance, resilience, and biodiversity (Darling and Côté, 2008; Folt et al., 1999; Pinto et al., 2010).

Estuaries have been claimed to be amongst the most productive natural habitats in the world, providing most important services, such as food production, water quality, resources for industry and agriculture, carbon sequestration, pleasant areas for recreational activities (Bulling et al., 2006; McLusky and Elliott, 2004; Ribeiro et al., 2016).

Estuary was defined by Fairbridge (1980) as “an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually being divisible into three sectors: a) a marine or lower estuary, in free connections with the open sea; b) a middle estuary subject to strong salt and freshwater mixing; and c) an upper or fluvial estuary, characterized by freshwater but subject to strong tidal action. The limits between these sectors are variable and subject to constant changes in the river discharges” (McLusky and Elliott, 2004).

Due to their natural characteristics, estuaries have been poles of attraction for human habitation and therefore urban and industrial settlements and intensive agriculture, constituting sources of pressures to these valuable ecosystems (Chapman et al., 2013; Ferreira et al., 2004). The human population living at or near the coast has more than doubled from 1.2 billion in 1990 to 2.5 billion in 2002 and is expected to increase even further over the coming years (O’Gorman et al., 2012). Therefore it will considerably rise the pressure to local coastal and estuarine ecosystems caused by huge variety of anthropogenic stressors.

Noteworthy, within the sea, almost all pollution is concentrated into estuaries and near shore coastal zones and so the problem of pollution in the sea is very often a problem of pollution in the estuarine systems (McLusky and Elliott, 2004).

In heavy urbanized and industrialized areas, human-derived chemical contamination (i.e. chemical stressors) is considered one of the most important threats estuarine biodiversity and ecosystem functioning.

Such chemicals may enter into the estuaries directly or indirectly as a result of lixiviation of nearby agriculture soils, contaminated tributaries, discharges from industrial and urban sources, estuarine traffic and near coast chemical spills (Martins and Costa, 2014; Ribeiro et al., 2016). Therefore the estuarine systems are under constant influence of multiple chemical stressors: phytosanitary products, products of pharmaceutical synthesis, hydrocarbons, dioxins, pesticides and metals. Consequently, exposure to organic and inorganic pollutants can lead to a contamination of the biomass, rendering it unfit for human consumption (Loizeau and Tusseau-Vuillemin, 2014).

Agriculture activities are the most dominant European land use, accounting for almost half of the total EU-27 land area (Stoate et al., 2009) and an overuse of pesticides (e.g. herbicides, fungicides, insecticides and others) and metals contained in their compositions causes adverse effects on the surrounding aquatic ecosystems.

One of the major environmental concerns about herbicide contamination is its bioaccumulation in the ecosystem's primary producers and its subsequent propagation through the trophic chain (Galhano et al., 2011). Metals are persistent in estuarine environments. They tend as well to accumulate in marine aquatic organisms through the different kind of processes and effects, such as bioaccumulation, bioconcentration, and the food chain process and ultimately endanger the health of humans by seafood consumption (Al-Malki and Moselhy, 2011; Kennish, 2016).

In Portugal, many studies reported the presence of pesticide contamination in both surface and ground waters, in areas occupied by intensive horticulture, maize and rice fields with some exceeding the 0.100 µg/L EU limit (Stoate et al., 2009, 2001). Thus, levels of triazine herbicides (i.e. atrazine) in groundwater have been found to exceed maximum allowable concentrations near the areas with the intensively managed crops such as maize (Stoate et al., 2001).

As in other estuaries, the Mondego estuary, located near Figueira da Foz city, Portugal (Fig. 1.1) is under strong anthropogenic pressures. The main stressors are related to port, beach, surrounding agriculture fields and industrial activities as well as the exploitation of marine resources (Gonçalves et al., 2016).

The Mondego river following into the Mondego estuary, during its course passes through agriculture fields (12286 ha), mainly of rice and corn: these cultures cover 45% and 51%, respectively, of the soil used for agricultural purposes in the lower part of the Mondego river (from Coimbra until Figueira da Foz) (Cruzeiro et al., 2016; Ferreira et al., 2003).

The Pranto river (Fig. 1.1) is considered the main anthropogenic source of nutrients (agricultural and domestic) to the South channel. Agricultural practices upstream in the Pranto are based as well on rice and maize cultures (Ferreira et al., 2003).

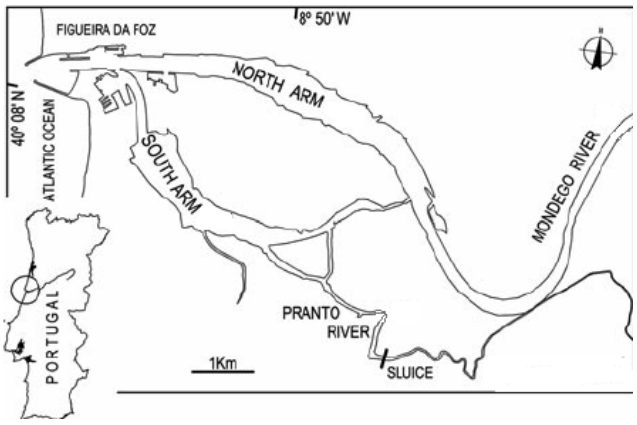

Fig. 1.1. Map of the Mondego estuary (40°08`N, 8°50`W), located along the west coast of Portugal

The intensive use of pollutants in agriculture areas near ecologically valuable coastal wetlands led to the implementation of the Pesticide-Monitoring programs to recover aquatic systems, such as in the Mondego estuary, since 1998 (Galhano et al., 2011).

Fifty three pesticides from three different categories were quantified in the Mondego river estuary from 2010 to 2011. Still, several pesticides were above the maximum amounts established by the European Directives (98/83/EC and 2013/39/EU), some of them able to cause mortality (LC_{50}) in some animals (fish, invertebrates and crustaceans). Seasonally, the average total concentrations were always above the legal limits (Cruzeiro et al., 2016).

Nowadays, and according to the information from agricultural cooperatives of Mondego valley, the herbicide Primextra® Gold TZ is the most used herbicide in corn crop fields and is one of the 20 best-selling herbicides in Portugal, whereas copper is mainly used in the constitution of pesticides (Gonçalves et al., 2016; Neves et al., 2015).

Primextra® Gold TZ, produced by Syngenta AG, consists of two main active ingredients (a.i.): 17.75% (w/w) terbuthylazine (6-chloro-N^2-ethyl-N^4-*tert*-butyl-1,3,5-triazine-2,4-diamine, TBA) and 30.2% (w/w) S-metolachlor (2-chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)- 2-methoxy-1-methylethyl] acetamide), also used by Syngenta AG in other commercial formulations, used worldwide, plus coadjuvant substances supposedly inert (Neves et al., 2015), with a residual percentage in the composition of the herbicide.

Metolachlor is classified as an inhibitor of very long chain fatty acid (VLCFA) formation (Liu and Xiong, 2009). It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu and Xiong, 2009). Due to the action mode of this xenobiotic, it is suggested that this a.i. affects the lipid (fatty acid-FA) profile of aquatic species. TBA belongs to the group of triazines, inhibiting the photosynthesis at photosystem II, while metolachlor belongs to the family of chloroacetanilides, inhibiting several biological processes, essentially biosynthesis of lipids, fatty acids, leaf wax, terpenes, flavonoids and proteins, in addition to inhibition of cell division and interfering with hormonal regulation (Liebl, 1995; Weed, 1994).

Metolachlor and TBA have a high leaching potential: GUS index of 3.5 and 3.1, respectively, which makes them prone to contaminate surface and ground waters (Cruzeiro et al., 2016; Palma et al., 2015).

In addition, these compounds are relatively hydrophobic and have a high potential for bioaccumulation (log K_{ow} = 3.40), which points out a risk associated to their possible accumulation in animals and plants, as well as eventual bio-amplification in the food chains (Cruzeiro et al., 2016; Palma et al., 2015).

Copper belongs to a group of transitional essential metals, of vital importance for every organism at low concentration, however, becoming toxic at high amounts (Bae and Lim, 2012; Kennish, 2000a). Kennish (2000) reported copper on the third place as a toxic metal after cadmium and mercury, which present the highest toxicity. The presence of copper above the essential limits affects a great variety of metabolic and biochemical processes, such as respiration, cell division, photosynthesis, chlorophyll synthesis, carbohydrate synthesis, pigment synthesis and FA metabolism (Ritter et al., 2008; Sibi et al., 2014). Copper sulphate is seldom directly toxic to fish, but may kill large numbers of invertebrate food organisms such as rotifers, cladocerans and copepods. However, above a specific concentration, copper is toxic to fish including economically valuable species as salmonids, cyprinids and catfish (Abdel-Tawwab et al., 2007).

In addition, both, copper compounds and two main active ingredients of the herbicide Primextra® Gold TZ: terbuthylazine and S-metolachlor are the most extensively used plant protection products within the European Union (Eurostat, 2007). The concentrations of these herbicides in European surface waters often exceed the limit value of 0.100 µg/L for a single pesticide and 0.500 µg/L for total pesticides in drinking water set by the EC Drinking Water Directive (98/83/EC) (Spoljaric et al., 2011).

Thus, a national survey from 2000 to 2005 in Hungary revealed that more than half of the samples (59%) contained pesticide residues: mainly chloroacetanilide herbicide acetochlor and triazine herbicide atrazine. The main pollution source was agriculture (Stoate et al., 2009).

A recent study revealed the presence of both active ingredients of Primextra® Gold TZ in the Mondego River estuary (Cruzeiro et al., 2016).

Although the amount of TBA was lower then the established legislation value of 0.100 µg/L – 0.088 µg/L (in winter) – metolachlor exceeded this value – the average annual value of 0.146 µg/L (with maximum value of 0.266 µg/L revealed in spring) was obtained (Cruzeiro et al., 2016).

Due to the important role of the estuarine ecosystems (i.e. the Mondego estuary), understanding the effects and consequences of human activities (i.e. chemical stressors) on these systems is of primordial importance to implement action plans to minimize or reduce the impacts or likelihood of the risk of it occurring.

1.2. Ecotoxicology and mixture toxicity

Due to the anthropogenic pollution of aquatic environments, all the associated ecosystems are subject to the toxic stress (Loizeau and Tusseau-Vuillemin, 2014).

Ecotoxicology, which studies the effects of the above stressors, is a relatively new discipline. The term “ecotoxicology” was initially coined by Truhaut in 1969. It is defined as the science that predicts effects of potentially toxic agents or other stressors to natural ecosystems and to target species (Blasco et al., 2016). Ecotoxicological studies are part of the “tool kit” for risk assessment. Effective environmental management as well requires having relevant ecotoxicological data specific to the environment of concern (Reichelt-Brushett, 2012).

Marine ecotoxicology is a very recent avenue of research (Loizeau and Tusseau-Vuillemin, 2014). Marine and estuarine pollution with associated impacts to marine and estuarine organisms and food webs are important issues to be assessed. Ecotoxicological test methodology puts emphasis on metrics such as survival, growth and reproduction since these changes in individual fitness are considered to be “ecologically relevant” and influence directly the status of the population (Segner, 2007).

The toxicological data usually have been obtained from short-term toxicity tests performed with single pollutants. Chemical risk assessments are conducted for individual chemicals according to standardized frameworks such as the registration, evaluation, authorization and restriction of chemicals legislation (REACH) of the European Union (Lister et al., 2011).

Traditional effect and risk assessment in such frameworks have been routinely focused on exposures to single chemicals (Chen et al., 2015; Everaert et al., 2016). However, it is the fact that organisms, like the marine and estuarine ones, are often simultaneously exposed to multiple stressors in nature. Exposure to one stressor may change their response and sensitivity to a second stressor. Thus, impact by one contaminant may provoke only subtle changes but it may be enhanced by interaction with other chemical stressor (Segner, 2007).

Recently, there are an increasing number of studies dealing with toxicity of mixtures of either organic contaminants (group of pesticides) and/or inorganic contaminants (group of metals) (Mehler et al., 2011). Thus, in the period 2008 – 2013 around 207 studies with pesticide mixtures were published, of which 194 were binary and 13 consisted of more than two pesticides and around 28 studies with metal mixtures, where 21 mixtures were binary and remaining 7 consisted of more than two metals (Cedergreen, 2014).

Although a few decades ago Aoyama et al. (1987) have stated that there are a limited number of papers on the interaction effects between heavy metals and organic chemicals, 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 needed for more accurate assessment of ecological risk (Chen et al., 2013; Mehler et al., 2011).

Responses to multiple stressors can be classified relative to the effects of individual stressors in three simple ways: additive effects, where the multi-stressor effect equals the sum of the individual stressor effects; synergistic effects, where the impact of multiple stressors is greater than the sum of individual stressor effects; and antagonistic effects, where the impact of multiple stressors is less than predicted by the sum of the individual stressor effects.

In ecotoxicology, depending on the presumed mode of action of the mixture's components two general reference models are generally used to predict the effects of mixtures: the concentration addition (CA) model for similarly acting chemicals (or Loewe Additivity, first introduced by Loewe and Muischnek, 1926) and the independent action (IA) model for dissimilarly acting chemicals (first introduced by Bliss, 1939).

Both models assume that there is no interaction among the substances in the mixture (i.e., "noninteraction" or "additive effect"). However, if the observed responses are stronger or weaker than expected, then the combined effect is described as being either synergistic or antagonistic, respectively (Hochmuth et al., 2014; Nys et al., 2015; Sun et al., 2009). A priori both the reference models are used, since it is still under debate how 'similar' the substances actually must behave in order to justify the choice of CA (Coors and De Meester, 2008).

Microcosms experiments are important tools for assessing the effects stressors on aquatic communities because results are considered applicable to natural systems (Loizeau and Tusseau-Vuillemin, 2014).

Primextra® Gold TZ is recently used in agriculture fields. Only a few studies are available about toxic and biochemical effects of this herbicide on aquatic species: e.g. marine bivalves *Cerastoderma edule* and *Scrobicularia plana* (Gonçalves et al., 2016), freshwater zooplanktonic species *Daphnia longispina* (Neves et al., 2015) and freshwater fish species *Clarias gariepinus* and *Clarias albopunctatus* (Asomba and Ugokwe, 2015; Nwani et al., 2014). Therefore, it is essential to study the toxicological and biochemical effects of the herbicide Primextra® on other non-target species (Gonçalves et al., 2016) especially in a combination with inorganic substances, i.e. metals, such as copper.

1.3. Bioindicators of chemical stress and food quality

Bioindicators can be defined as “anthropogenically-induced variations in biochemical or physiological components or functions (i.e., biomarkers) that have been either statistically correlated or causally-linked, in at least a semiquantitative manner, to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization” (Adams and Greeley, 2000).

As described by Mussali-Galante et al. (2013), in relation to toxicological responses of biomarkers may be indicators of exposure, effect or susceptibility and may overlap sometimes:

(1) biomarkers of exposure: “An exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that it is measured in a compartment within an organism”. These types of biomarkers are also known as “biological dosimeters” or biomarkers of internal dose, and when they measure the product of the interaction with target molecules they are regarded as “biomarkers of biological effective dose”;

(2) biomarkers of effect: “A measurable biochemical, genetic, physiological, behavioral or other alteration within an organism that, depending on the magnitude, can be recognized as associated with an established or early health impairment or disease” and (3) biomarkers of susceptibility: “An indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance” (Mussali-Galante et al., 2013).

Over the past decades, the bioindicator and biomarker approach has attracted considerable attention of scientists, mainly toxicologists, and the international regulatory agencies as a new and potentially powerful tool for detecting, documenting and evaluating exposure to and the effects of contamination hazards for wildlife (Adams and Rowland, 2003; Huggett et al., 1992; Mussali-Galante et al., 2013).

In 1980s the amount of research applying biomarkers and their use in environmental and toxicological studies significantly increased. Outcomes showed implemented biologically and ecologically important methods that allow measurement of direct impairment of key endpoints in the test organisms or non-target species caused by contaminants.

There were defined key parameters estimating biomarkers applications on molecular, cellular and whole animal levels, including biochemical biomarkers as well. The latter were used for different purposes, in most cases for environmental monitoring with estimation of the pollution levels *in situ*, and to a lesser extent for predictions – conducting experiments in laboratory conditions (Jemec et al., 2009; Nunes, 2011). Nowadays, laboratory experiments (e.g. bioassays) and biomarkers have been found to be the most valuable in assessing environmental contamination (Segner, 2007).

Frequent response of biomarkers provides a high degree of sensitivity to environmental impacts. Thereby they act as “early warning” signals of the presence of potentially toxic xenobiotics (Adams and Rowland, 2003; Picado et al., 2007).

Environmental stressors first affect sub-organismal components such as cells and tissues, and, if a stressor continues to be of sufficient duration and magnitude, effects will reach higher levels of biological organization.

Thus, biochemical biomarkers are quick-responding and sensitive indicators to environmental (including the anthropogenic) stressors and used to be considered the most promising tools for ecotoxicological applications (Fig. 1.2.) (Adams and Greeley, 2000a; Gonçalves et al., 2016; Jemec et al., 2009; Neves et al., 2015).

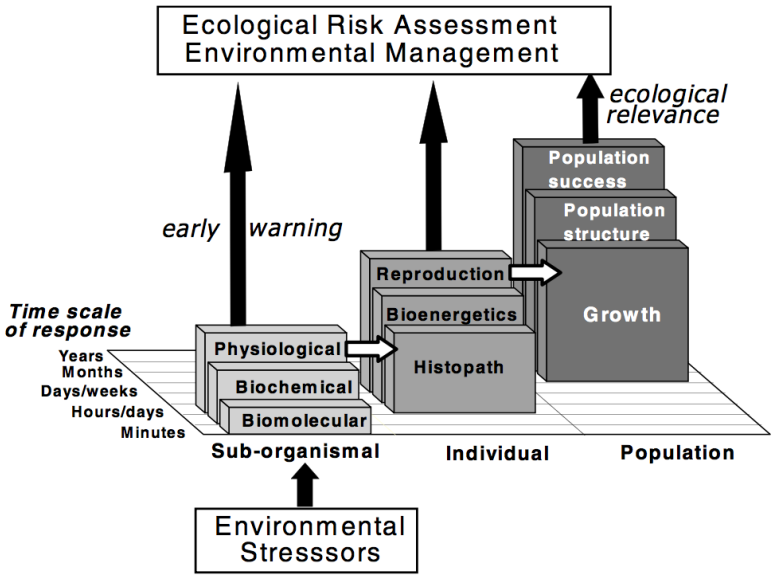

Fig 1.2. Hierarchical response of organisms to environmental stressors (from Adams and Greeley, 2000).

Nutrients, mainly lipids and proteins, are involved in many vital functions of aquatic individuals. They are carbon-rich compounds with a very high energy value that make them important fuels in marine ecosystems (Parrish et al., 2000).

Since some of them can only be obtained from food and therefore referred to as ‘essential nutrients’ (e.g., essential fatty acids or EFA), they proved to be useful trophic markers and are essential for physiological functions, the overall metabolism of organisms and their prevention of diseases (De Troch et al., 2012; Gladyshev et al., 2009; Kelly and Scheibling, 2012).

Analysis of fatty acids composition are used as biochemical markers in order to indicate bacterial symbiosis, demonstrating as a valuable screening tool for detecting symbionts in species (e.g., Zhukova and Eliseikina, 2012). They have been advocated as qualitative markers for tracing or confirming predator-prey relationships in the marine environment (Budge et al., 2006; Grahl-Nielsen et al., 2003; Iverson et al., 2004).

On the other hand, as FA are one of the most important molecules transferred across the plant-animal interface in aquatic food webs, they were claimed to be a good bio-indicator of ecosystem health (Maazouzi et al., 2008; Ramírez et al., 2013) and bio-indicators of stress (Gonçalves et al., 2016; Sánchez-Muros et al., 2013).

Fatty acids, especially, the long chain ω3 polyunsaturated fatty acids (PUFA), i.e. essential FA (20:4ω6 – ARA, 20:5ω3 – EPA, 22:6ω3 – DHA), play a key role in the health and function of all animals at all trophic levels, including planktonic invertebrates, fish and humans and are one of the key nutrients that influence food quality (Arts et al., 2001; Breteler et al., 2004; Gonçalves et al., 2012a; Koski et al., 1998; Veloza et al., 2006).

Food quality is at least as important as food quantity to sustain viable consumer populations and to maintain the sufficient conversion of plant material to herbivore biomass (Perhar and Arhonditsis, 2009).

Thus, the lipids contained in plants and other organisms at the base of the food chain determine the quality of food for the higher trophic levels. In aquatic environments ω3 polyunsaturated fatty acids, including EFA, are almost exclusively synthesized by plants.

Animals can convert from one form of PUFA to another through elongation and desaturation, but very few can synthesize PUFA *de novo* (Brett and Müller-Navarra, 1997) and require their obtain from plant food (Breteler et al., 2004). In the pelagic system, planktonic algae are the primary producers of DHA and EPA and higher consumers such as zooplankton, shellfish and finfish must acquire them via trophic accumulation up the food chain (Tang and Taal, 2005).

Some harpacticoid copepods may modify ω3 precursors to EFA, but such capability has not been shown in calanoid copepods, the dominant zooplankton in the marine pelagic environment (De Troch et al., 2012; Tang and Taal, 2005). Available studies proved the correlation of the dietary intake of these fatty acids with growth and development in copepods (Koski et al., 1998; Lacoste et al., 2001; Tang et al., 2001), larval fish and invertebrates (Levine and Sulkin, 1984; Rainuzzo et al., 1997).

Besides FA and EFA, proteins are as well important indicators of food quality. They are the most abundant organic molecules within invertebrate cells, often comprising 50% or more of the dry weight, and are often used to assess the nutritional quality of algae (Rausch, 1981).

The dietary protein content influences the biochemical composition of invertebrates and their growth rate. In general, organismal growth and production are usually positively correlated with the protein content of the diet (Carpenter and Capone, 1983; Houde and Roman, 1987). In microalgae protein is always the major organic constituent, followed usually by lipid and then by carbohydrate molecules and is one of the major factors in crustacean nutrition (Lavens and Sorgeloos, 1996).

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

Ecotoxicological studies associated with biomarker responses of living organisms to toxicants are of extreme importance for obtaining fast and low time-consuming results (Gonçalves et al., 2016). Furthermore, integrating multiple specific biomarkers to assess biochemical responses of estuarine and marine species in response to anthropogenic stressors provides a powerful tool to determine their effects on the species food quality and thus on the related aquatic ecosystems.

1.4. Study species

Phytoplankton and zooplankton species are of high importance in ecotoxicological studies due to their key position in the trophic food web, making a link with higher trophic levels.

Marine microalgae are typically the most abundant species in aquatic environments occupying the base of the food chain and therefore may be seriously affected by various organic and inorganic contaminants.

Being a food source, microalgae may contribute their uptake into higher organisms with increasing the possibility of toxicity (Debenest et al., 2010; Pratoomyot et al., 2005; Torres et al., 2008). Laboratory microalgal tests are the most commonly used methods for estimation of effects of toxicants on marine primary producers (Walsh, 1997). As described by Araújo and Souza-Santos (2013) the microalgae to be applied in toxicological studies should meet the following criteria: “sensitivity to the reference substances; well-known nutrient requirements; good taxonomic characterization of strains; low genetic and phenotypic variability; available to obtain and cultivate; easy to handle in a laboratory and population growth rates that allow a density estimate within approximately three days of the moment of inoculation”. After taking into account all of above factors the marine diatom *Thalassiosira weissflogii* was stated as an adequate species for toxicity tests (Araújo and Souza-Santos, 2013).

In addition, *T. weissflogii* is a common coastal diatom species and widely used as food source for zooplankton (i.e. copepods species) in laboratory experiments (Fields et al., 2011). Moreover, in the Mondego estuary diatoms are one of the dominating phytoplankton groups (Flindt et al., 1997).

Zooplankton has long been used as a valuable group to assess the ecosystem disturbance (Cardoso et al., 2008; Neves et al., 2015).

In estuarine coastal waters copepods are usually the dominant group of zooplankton and play a pivotal ecological role in terms of biomass and energy transfer between primary producers and higher trophic levels, indicating the importance of this group in ecological studies (Cardoso et al., 2008; Gonçalves et al., 2012c, 2010a). This transfer is essential for the optimal functioning of an ecosystem but at the same time it also implies a vulnerable point in a changing environment (Gonçalves et al., 2010a). Planktonic copepods are important components of marine pelagic foods webs, especially in the rich productive estuaries of coastal regions. Their nauplius stage is a key component of the diet of many fish larvae that develop in inshore nursery habitats (Marcus, 2004).

In the Mondego Estuary copepods are the dominant zooplankton group and the estuarine calanoid copepod *Acartia tonsa* is one of the most abundant copepod species, especially in the Southern arm (Cardoso et al., 2008; Gonçalves et al., 2012b, 2012c, 2010b).

Another zooplankton species the brine shrimp *Artemia franciscana* is a widespread invasive species in the Mediterranean region and has been recorded in numerous marine waters in Portugal.

A. franciscana compared to the native *Artemia sp.* has greater sexual competence, either in terms of its reproductive period or in terms of the number and quality of its offspring (Pinto et al., 2013). *Artemia* has been proved to be a valuable organism in toxicity tests. This species has high prolificacy and short life cycles, easy for laboratory culture and maintenance. Bioassays with *Artemia* are rapid and cost-effective. Therefore, it continues to be used extensively in research and applied toxicology laboratories worldwide (Persoone and Wells, 1987; Zhou et al., 2010).

Changes induced by anthropogenic stressors in planktonic population and in their biochemical composition, including alterations in FA profiles and protein content may interfere with their food quality status and lead to alterations along the food web.

Therefore it is necessary and highly relevant to investigate the influence of the above-referred contaminants (i.e. herbicide Primextra® and metal copper) on these key non-target species.

1.5. Objectives and outline of the thesis

This thesis intends to address the influence of human-induced environmental changes on the food quality in estuarine systems. Thus, we will use related species that constitute a simple trophic food chain (primary producer – primary consumer) to determine the influence of anthropogenic activities on their growth rate and survival, nutritionally important biochemical parameters and thus on the food quality.

The specific aims of the research are:

(1) To assess individual effects of chemical stressor (e.g. metal copper and herbicide Primextra® Gold TZ) on the growth rate, survival and biochemical composition of species from different trophic levels.

(2) To assess multiple-stressor scenarios (e.g. effect of equitoxic chemical mixture) on the growth rate, survival and biochemical composition of species from different trophic levels.

(3) To develop an appropriate statistical model to analyze the effect of environmental contaminants on species food quality and related food chain (primary producer – primary consumer), using experimentally obtained biochemical data (FA content, including essential FA, proteins and TBARS contents).

To achieve established goals three main tasks were defined:

Task 1. Maintenance of stock cultures;

Task 2. Laboratory bioassays and biochemical analysis of planktonic species exposed to toxicant stressors individually and in equitoxic mixture;

Task 3. Modelling of the obtained biochemical data for the determination of the effects of anthropogenic stressors on species food quality.

The following main hypothesis of the study was proclaimed: planktonic species show different biochemical responses to stressors, being more severe for the primary consumer species at the highest concentrations of mixture exposure and therefore more adverse to their food quality. As expected results, we stated that: (1) at the highest concentrations of the metal copper and the herbicide, concentrations of fatty acids would be lower; (2) a mixture of contaminants is much more detrimental to key species than their individual exposure.

The thesis is structured in six chapters. In this first chapter, the basic principles and concepts supporting this work are drawn. In the chapters two to five the work is detailed in the form of four Manuscripts that were published (Chapters 2 and 3) or submitted (Chapters 4 and 5) to SCI journals. Finally, the major achievements of this study are drawn in the "General conclusions" chapter (Chapter 6).

It follows a brief explanation of the steps taken and the rationale.

(i) in **Chapter 2** entitled "*Fatty acid profiling as bioindicator of chemical stress in marine organisms: A review*" we address a thorough literature review concerning the response of fatty acid profiles of marine organisms after exposure to various organic and inorganic pollutants (mainly pesticides and metals) and the impact at the molecular level. Because of the growing usage worldwide of contaminants, especially in agriculture practices, it became crucial to evaluate and predict the presence of toxicants in aquatic (marine and estuarine) systems in a cost-effective and rapid way by the application of biomarkers tools in order to implement and manage monitoring programmes to recover polluted aquatic systems or maintain the water quality of these systems.

More than determine the toxicity levels it is pivotal to relate the response of marine organisms exposed to toxicants to changes in molecular processes. Fatty acids are involved in many vital functions of aquatic individuals and are one of the most important molecules transferred across the plant-animal interface in aquatic food webs. Indeed, FA were claimed to be a good bio-indicator of ecosystem health and bio-indicators of stress.

The present chapter underlines the consistent directional trends in changes of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, and summarizes the mechanisms of action leading to their alteration and possible consequences of these changes to marine species from different trophic levels.

(ii) in **Chapter 3** entitled "*Ecotoxicological and biochemical effects caused by individual exposure to a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species*" we address the results of our research considering the biochemical (namely fatty acid profiles) and ecotoxicological responses of two marine (diatom *Thalassiosira weissflogii* and brine shrimp nauplii *Artemia franciscana*) and an estuarine (copepod *Acartia tonsa*) planktonic species to a single exposure of organic and inorganic compounds: the herbicide Primextra® Gold TZ and the metal copper, correspondingly. The determination of the toxicity levels is pivotal to relate the response of aquatic organisms exposed to toxicants to changes in the molecular processes.

Fatty acids proved to be good bioindicators of the presence of chemical stressors and thus to be applied as an early-warning signal to assess levels of contamination, resulting in an increased application of FA profiling in ecotoxicological studies in the last two decades.

Due to the revealed high tolerance of *Artemia sp.* to both contaminants the further research regarding the chemical mixture exposure with its effect on the species food quality and related general conclusions (presented in Chapters 4, 5 and 6) were focused only on the sensitive planktonic organisms: the primary producer diatom *T. weissflogii* and the primary consumer copepod *A. tonsa*.

(iii) in **Chapter 4** entitled "*Ecotoxicological and biochemical mixture effects of a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species*" we address the results considering ecotoxicological and biochemical effects of single and equitoxic mixtures of the herbicide Primextra® Gold TZ and the metal copper on the marine diatom *Thalassiosira weissflogii* and the estuarine calanoid copepod *Acartia tonsa* by determining growth rate and survival, respectively, and changes on fatty acids (FA) profiles in both species, latter being a good biomarker of stress.

There still exist an important gap in literature on the mixture toxicity of metals and pesticides and their simultaneous effect on the FA composition. Therefore potential synergistic effects that may lead to more severe and unpredictable consequences for estuarine and marine ecosystems are ignored. Thus, a better understanding of the interactive effects of organic-inorganic contaminant mixtures on non-target marine and estuarine species is needed.

(iv) in **Chapter 5** entitled "*Effects of a herbicide-metal mixture on the food quality of marine and estuarine primary producers and primary consumers"* we address the results of our research where generalized linear models were applied to determine the effect of organic-inorganic chemical mixture (herbicide Primextra® Gold TZ – metal copper) on the food quality of phytoplankton (the diatom *Thalassiosira weissflogii*) and zooplankton (the copepod *Acartia tonsa*) species in terms of their fatty acid content, especially essential fatty acids, proteins and thiobarbituric acid reacting substances content as important indicators of the food quality;

and how this mixture differentially affects different trophic levels, i.e. primary producer and primary consumer. Environmental risk assessment has been routinely focused on study the effects of contaminants on endpoints such as survival, growth or reproduction giving the important information how the amount of biomass changes following the exposure of the ecosystem to stress. However, food quality has important implications on the energy and nutrient transfer through the food chain and is often an overlooked aspect of food availability.

It is important to determine the effects of simultaneous exposure to metal and pesticide contaminants, since they may adversely affect the food quality of planktonic species being more severe for the organisms from higher trophic level and thus potentially cause crucial consequences for the human diet.

(v) in **Chapter 6** entitled "*General conclusions*", the results and findings of the preceding chapters are discussed.

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CHAPTER 2.

Fatty acid profiling as bioindicator of chemical stress in marine organisms: A review

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2.1. Abstract

The development of industrial, anthropogenic, and agricultural activities is the main factor leading to contaminants' increasing in marine ecosystems. Contaminants include the great variety of pesticides and heavy metal pollutants. One of the major environmental concerns about herbicides and heavy metals contamination is their bioaccumulation in the ecosystem's primary producers and its subsequent propagation through the trophic chain. Over the last decades, the use of biochemical markers considerably contributed to the evaluation of contamination hazards. The fatty acid composition proved to be a good bioindicator to assess contamination levels. This paper provides a review of current knowledge on the fatty acids response in marine species after exposure to the chemical stressors including organic and inorganic pollutants, mainly pesticides and heavy metals. This review underlines the consistent directional trends in changes of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, and summarizes the mechanisms of action leading to their alteration and possible consequences of these changes to marine species from different trophic levels.

2.2. Introduction

In the last decades, contaminants' discharges are one of the themes that concerned the scientific community and politicians and received special attention from media because of the threat and adverse effects that they may cause in aquatic ecosystems (McKnight et al., 2012). The development of industrial, anthropogenic, and agricultural activities are the main factors leading to their increase in aquatic ecosystems (Peixoto et al., 2006).

Marine ecosystems play the role of the ultimate storage for a great variety of organic and inorganic pollutants, which are constantly discharging due to the active anthropogenic activities (Kennish, 1996).

There are five main pollutant sources of marine ecosystems: (1) landscape level causes; (2) industrial and domestic discharges; (3) inflows from rivers; (4) shipping and (5) precipitation from atmosphere. The great input to the sea pollution gives land-based sources – more than 80% (Kennish, 2000).

Among them are intensive agriculture activities discharging the great variety of chemical stressors with organic and inorganic nature, contaminating the marine aquatic environment.

Contaminants include the different kind of pesticides and heavy metal pollutants. Pesticides represent a large group of toxic chemicals.

Their use is reducing in most developed countries, although the world level of herbicides production is still up to 40% (Peixoto et al., 2006). One of the major environmental concerns about herbicide contamination is its bioaccumulation in the marine ecosystem's primary producers and its subsequent propagation through the trophic chain (Galhano et al., 2011).

Metal contaminants may not directly damage the organisms when entering the marine aquatic ecosystem; however, as pesticides, they can be accumulated into marine aquatic organisms through the different kind of processes and effects, such as bioaccumulation, bioconcentration, and the food chain process and ultimately endanger the health of humans by seafood consumption (Al-Malki and Moselhy, 2011). The various anthropogenic pressures and behaviors that cause ecological stress at these systems, affect not only the water quality, but also the biological communities of these ecosystems (Gonçalves et al., 2012, 2016; Neves et al., 2015). Thus, marine contamination directly affects marine aquatic biota. The review and meta-analysis of numerous studies about this issue proved that anthropogenic contamination of marine habitats is clearly reduces marine species richness and evenness (Johnston and Roberts, 2009).

Over the past decades, the bioindicator and biomarker approach has attracted considerable attention of scientists, mainly toxicologists, and the international regulatory agencies as a new and potentially powerful tool for detecting, documenting and evaluating exposure to and the effects of contamination hazards for wildlife (Huggett et al., 1992).

In 1980s the amount of research applying biomarkers and their use in environmental studies significantly increased. Outcomes showed regularities between biomarkers responses and chemical exposure or biological response in both population and community levels.

There were defined key parameters estimating biomarkers applications on molecular, cellular and whole animal levels, including biochemical biomarkers as well. The latter were used for different purposes, in most cases for environmental monitoring with estimation of the pollution levels *in situ*, and to a lesser extent for predictions – conducting experiments in laboratory conditions (Jemec et al., 2009).

Nutrients, mainly lipids and proteins, are involved in many vital functions of aquatic individuals. Since some of them can only be obtained from food and therefore referred to as 'essential nutrients' (e.g., essential fatty acids or EFA), they proved to be useful trophic markers (De Troch et al., 2012; Kelly and Scheibling, 2012).

On the other hand, analysis of fatty acids composition are used as biochemical markers in order to indicate bacterial symbiosis, demonstrating as a valuable screening tool for detecting symbionts in species (e.g., Zhukova and Eliseikina, 2012) and have been advocated as qualitative markers for tracing or confirming predator-prey relationships in the marine environment (Grahl-Nielsen et al., 2003; Iverson et al., 2004; Budge et al., 2006).

Fatty acids (FA) are necessary for the production and permeability of cell membrane, they are the main components of lipids and used as fuel in all metabolic systems at all trophic levels, having an important role on neural levels of biochemical and physiological response (Neves et al., 2015).

As FA are one of the most important molecules transferred across the plant-animal interface in aquatic food webs, they were claimed to be a good bio-indicator of ecosystem health (Maazouzi et al., 2008; Ramírez et al., 2013) and bioindicators of stress (Sanchez-Muros et al., 2013; Gonçalves et al., 2016).

Polyunsaturated fatty acids (PUFA) include many important compounds, such as EFA. PUFA and EFA are often used interchangeably since many biological functions of EFAs are exerted by EFA – derived PUFAs.

PUFA are almost exclusively synthesized by plants, with animals being able of converting PUFA by elongation or desaturation, and only a few could synthesize this type of fatty acids (Neves et al., 2015).

Highly unsaturated fatty acids (HUFA) (e.g. ARA, EPA and DHA) play a key role in the health and function of all animals at all trophic levels, including plankton invertebrates, fish and humans and cannot be synthesized *de novo*, or at least not in sufficient amounts (Saito and Aono, 2014; Gonçalves et al., 2012). Indeed eicosapentaenoic acid (20:5ω3, EPA) is an excellent energy source and precursor of eicosanoids, docosahexaenoic acid (22:6ω3, DHA) is involved in the maintenance of membrane structures and functions, while ω6 arachidonic acid (20:4ω6, ARA) is identified as affecting the growth and survival of scallops larval and post-larval stages (Costa et al., 2015).

The long-chain ω3 PUFA are originated from phytoplankton and accumulate in marine animals in higher trophic levels through the food web (Saito and Aono, 2014). Some groups of organisms feed with high amounts of HUFA present higher growth rate, which strength the importance of fatty acids as ecophysiological indicators (Neves et al., 2015). In addition, ARA and DHA are considered most important for infants (Saito and Aono, 2014). Furthermore, lipid components are very sensitive to stressors and environmental changes (Gonçalves et al., 2012, 2016).

2.3. Toxic effects of chemical stressors and their action to metabolism

2.3.1. Metals effects

Heavy metals are one of the most significant groups of pollutants generated by industrial activities. It is known to seriously impact coastal zones and their inhabitants, influencing both organisms and ecosystem processes.

This class of pollutants directly affects aquatic organisms, altering metabolic pathways, consequently compromising the structural and physicochemical properties of the membrane, and damaging cells, tissues and organs.

This effect, when occurring during long term, can lead to higher mortality among a population, and change community structure and diversity (Bae and Lim, 2012; Gabryelak et al., 2000).

For living organisms all metals can be divided into essential elements, for instance copper (Cu), zinc (Zn) and toxic elements, such as mercury (Hg), cadmium (Cd), chromium (Cr). Therefore, some heavy metals become toxic only at very high amounts, because they are biologically essential and are natural components of the aquatic ecosystem. However, the rest of heavy metals are toxic to living organisms even at remarkably low concentrations (Bae and Lim, 2012). For example, in fish muscle, contents of essential elements (e.g., Zn, Cu, Ni) are higher than those of toxic elements (e.g., Hg, Cd, Pb) (Bae and Lim, 2012).

As mentioned above, copper is an essential element, necessary to maintain healthy cellular functioning (Mayor et al., 2013), acting as enzyme cofactor and key participant in several metabolic pathways (Ritter et al., 2008).

However, this heavy metal quickly becomes toxic when its supply exceeds the demand. The toxicity of dissolved copper depends on the pH and temperature of water, thus it will increase in the coming years as seawater pH decreases and temperature increases (Mayor et al., 2013). An excess of this metal may lead to detrimental effects on photosynthesis, chlorophyll synthesis, fatty acid metabolism, carbohydrate synthesis (Ritter et al., 2008), as well as on cellular respiration, ATP production, pigment synthesis and inhibition in cell division (Sibi et al., 2014).

There are three main processes explaining the mode of action of cupric and cuprous (Cu (II), Cu (I)) ions, which at high amounts significantly affect organismal cells: (1) the affinity of Cu (II) with thiol-, imidazole-, and carboxyl-groups of amino acid leads to their interactions and therefore to protein inactivation;

(2) Cu (I) can be obtained from interactions between Cu (II) and deoxidants, take part in Fenton's reaction as a catalyzing agent for formation of hydroxyl radicals, belonging to the group of reactive oxygen species (ROS); (3) both ions of copper displace essential cations from specific binding sites.

By affecting organisms, at the cellular level, copper ions interfere in the metabolism of FAs and proteins, and may inhibit respiration and nitrogen fixation processes in photosynthetic organisms (Maazouzi et al., 2008; Ritter et al., 2014).

Another essential element is zinc that plays a pivotal role in processes such as cell division, growth, metabolic mechanisms, and physiological dynamics concerning immunity maintenance. It can act alone or as an enzyme cofactor in the prevention of damage caused by highly ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. The mechanism of action still remains unclear, but it is believed that proteins and PUFAs are the main target for these radicals. Furthermore, this metal may interact with thiol-groups or some amino acids, whereby altering protein functions (Gabryelak et al., 2000).

Hexavalent chromium belongs to the group of high-toxic heavy metals. It damages considerably many aquatic ecosystems. Chromium is highly permeable for cell membranes due to the fact that chromate anion $CrO4^{2-}$ is a strong oxidant and structurally similar to other inorganic anions, thus is able to substitute other substrates in the sulphate transport system (Rocchetta et al., 2006).

Even at trace concentration, cadmium induces oxidative stress by producing ROS that compromise cell metabolism. Cadmium has high affinity for sulfhydryl and oxygen containing groups therefore it is able to block the essential functional groups of biomolecules and it consequently inhibits the functioning of nutrient transport system (Kumar et al., 2010b).

Mercury as well disturbs cell metabolic processes: respiration, photosynthesis and lipid biosynthesis, and inhibits enzymatic activity, including activity of catalase, urease, alcohol dehydrogenase, fatty acid synthetase and oleyl-CoA desaturase (Jones et al., 1987).

Cadmium also affects algal photosynthesis and inhibits a wide range of enzymes. Both metals interfere with the wide range of cell processes, such as permeability of highly important ions: Na^+ and K^+, ATPase activity and molecular transport (Jones et al., 1987).

2.3.2. Pesticides effects

Pesticides are one of the hazardous groups of chemicals, which are used worldwide. They are known to interfere with many vital functions of organisms, including the inhibition of important biochemical processes. Pesticides can be grouped according to their biochemical mode of action (Table 2.1) (Corbett, 1974).

Table 2.1. Pesticides classification according to their biochemical mode of action

Inhibited biochemical process	Chemical group	Pesticide class
Respiration:		
Oxidative phosphorylation	Dinitrophenol derivatives	Acarcides, powdery mildevicides, fungicides, nematicides, herbicides, insecticides
	Hydroxibenzonitriles	Herbicides, anthelmenitic, molluscicides, fungicides
	Salicylanilides	Anthelmenitic, molluscicides, fungicides
Photosynthesis:		
Photosynthetic electronic transport	Ureas, thioureas, triazines, alcylanilides, pyridazinones, uracils, hydroxybenzonitriles, dinitrophenols, pyrimidinones, triazinones, n-phenylcarbamates	Herbicides
Nervous system:		
Acetylcholine esterase activity	Organophosphates, carbamates	Insecticides
Axonal transmission	Chlorinated hydrocarbons, pyrethroids	Insecticides
Biosynthetic reactions:		
Nucleic acid and protein synthesis	Antibiotics: cycloheximide, streptomycin, blasticidin S, kasugamycin, validamycin A	Fungicides, bactericides
	Benzimidazoles	Fungicides
	Glyphosate-based pesticides [http://osufacts.okstate.edu, Armstrong]	Herbicides
Carotenoid synthesis		
	Triazoles, carbamates, hydroxipiridines, pyridazinones	Herbicides
Lipid synthesis (fatty acids, neutral fats and steroids)	Triazoles [http://edis.ifas.ufl.edu/pi117, Fishel], pyridazinones [Cohen et al., 1993, Kachroo et al., 2006], chloracetamides [Robert et al., 2007], thiolcarbamates	Herbicides
	Triarimol	Fungicide

Pyridazinone herbicides not only influence on the synthesis of carotenoids, but also change processes of FA biosynthesis, in particular – inhibit FA desaturation, primarily delta-6-desaturation (Cohen et al., 1993; Kachroo et al., 2006).

Chloroacetamide herbicide metolachlor as well interfere with FA synthesis, inhibiting the biosynthesis of very long-chain FAs in higher plants and microalgae (Robert et al., 2007).

One of the triazine herbicides atrazine affects the quality of the diatom cells, as indicated by a change in the maximum quantum yield of photosynthetic activity and total FA concentrations in the cells (De Hoop et al., 2013). The glyphosate based herbicide Roundup® inhibits EPSP (5-enolpyruvylshikimate-3-phosphate) synthase preventing protein synthesis (Bayir et al., 2013).

Urea herbicide Diuron affects the photosynthesis in algae (Kumar et al., 2010a), social behavior, survival and inhibition of the nervous system and anemia in fishes (Sanchez-Muros et al., 2013). Some pesticides interfere with the activity of nuclear receptors (NR), which are important in the regulation of physiological functions of organisms. Thus, impact of these pesticides, such as triazole pesticides, can either damage reproduction functions or intensify the development of other serious diseases (Sun et al., 2013).

2.3.3. Effects of other organic pollutants

It is necessary to mention the mechanisms of action of other organic pollutants as they also have a large influence on the FA profiles of marine species. For instance, persistent organic pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment.

This group of priority pollutants consists of pesticides (such as DDT), industrial chemicals (such as polychlorinated biphenyls (PCBs)) and unintentional by-products of industrial processes (polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs)). POPs are transported across international boundaries far from their sources, even to regions where they have never been used or produced. Consequently, persistent organic pollutants pose a threat to the environment and to human health all over the world (European Commission – http://ec.europa.eu/environment/ chemicals/international conventions/index en.htm, http://ec. europa.eu/environment/pops/; EUGRIS – http://www.eugris.info).

In the past decades, many POPs were used as pesticides, however, some of them are continuing to be applied (Belcham, 2014).

The extensive use of lipophilic POPs, such as organochlorine (OC) pesticides, PCBs in the past and domestic chemicals (e.g. brominated flame retardants) nowadays, led to their accumulation in marine ecosystems.

This has proven to be hazardous for marine life, as POPs have a high resistance to degradation and are lipophilic, which means they can build up in the fat tissues of animals, e.g. the blubber of cetaceans and threaten their health and reproductive activity (Ellisor et al., 2013).

Exposure of marine mammals by OCs is leading to endocrine disruption, depression of the immune system, reproductive impairment, hypothyroidism, carcinogenicity, neurotoxicity and alterations in skeletal growth and ontogenetic development (Waugh et al., 2014).

PAHs belong to the group of POPs, accidentally formed or eventually released as a byproduct from various activities, such as industrial or combustion processes (http://www.eugris.info). PAHs have mutagenic and carcinogenic properties and therefore are in priority pollutant lists of several federal agencies such as the Environmental Protection Agency (EPA), the Agency of Toxic Substances and Disease Register (ATSDR), the International Agency for Research on Cancer (IARC), and the European Community (EC) (Yebra-Pimentel et al., 2014).

PAHs have acutely toxic effects and able to biomagnify through the food web, impair growth inhibition, cellular toxicity, alter cell permeability, damage to cellular lipids, membranes, and/or cellular integrity (Nilsen et al., 2015), interfere with the fatty acid metabolism and silica shell formation in diatoms (Bopp and Lettieri, 2007), lipid and fatty acid metabolism in bivalve molluscs (Signa et al., 2015).

The other group of organic contaminants is alkylphenols (AP). Industrial development led to the appearance of these compounds in the aquatic ecosystems. For instance nonylphenol and octylphenol, which are used as surfactants and emulsifying agents, were observed in concentrations up to 369 µg/l in highly polluted areas of freshwater and coastal marine zones.

Even at low concentrations, APs affect steroid levels and damage gonadal development in fish of both genders. In addition, APs interfere with enzymatic activity, for instance with enzymes which catalyze first two phases of detoxification reaction and contaminants excretion (Meier et al., 2007).

2.4. Response of FA profiles in marine species from different trophic levels

The FA profiles alterations in marine species after exposure to organic and inorganic contaminants has been the goal of numerous investigations. The earliest studies are reported in 1980s.

Several researches at that time examined changes in FA profiles of microalgae, exposed to polychlorinated biphenyl (Fisher and Schwarzenbach, 1978), copper and zinc (Gillan et al., 1983), chlorinated benzenes (Sicko-Goad et al., 1989 a, b, c, d), pyridazinone herbicide Sandoz 9785/SAN 9785 (Cohen et al., 1993; Henderson et al., 1990); on sea periwinkles exposed to diesel oils (Grahl-Nielsen and Barnung, 1985), bivalves exposed to cadmium (Chelomin and Belcheva, 1991) and on amphipods exposed to aromatic hydrocarbons and plasticizers (Morris et al., 1982).

Potential use of FA composition as bioindicator still remained to be established in the 90s. Thus, the first attempt to determine the effects of organochlorine contaminants on the FA composition in cetaceans dates from 1995 by R. Guitart (Guitart et al., 1996).

Nowadays a wide range of marine species, representing different trophic levels, has been studied. A lot of research attention was spent to study the response of FA profiles in micro- and macro algae and fishes to pollutants impact. Only a few works on marine mammals and marine zooplankton species are available (Table 2.2).

Table 2.2. Summary of alterations in FA profiles of marine species after exposure to contaminants. SFA – Saturated Fatty Acids; MUFA – Monounsaturated Fatty Acids; PUFA – Polyunsaturated Fatty Acids; FFA – Free Fatty Acids. N/A – not available; N/I – not influenced or not significant changes. "↗" – increase of FA relatively to increase of contaminant concentration, "↘" – decrease of FA relatively to increase of contaminant concentration.

Contaminants	Conditions of exposure (time/ concentration)	Contaminants mode of action	Type of organism	Species name	Analyzed part of organism	SFA	MUFA	PUFA	FFA	Reference
				Metals						
Copper (II) sulphate pentahydrate	30.2 – 603.4 mg Cu [kg wet sediment]$^{-1}$ 10 days	Affect the biomass and metabolic activities of sediment-associated bacteria, change community structure, affects the activity and survival of marine metazoan fauna, impact their capacity for bioturbation	Marine sediment microbial community	N/A	Whole community	30mg: ↘ 14:0, 15:0, 16:0, 17:0, 18:0 >91 mg: ↗ 15:0, 17:0, 19:0	30mg: ↘ 16:1ω5 >91 mg: ↗ 16:1ω7 18:1ω7	30 mg: ↘ 20:4ω5, 8,11,14 20:5ω3 N/A	N/A	[Mayor et al., 2013]
Copper (II) sulphate	0.01-1.00 % (w/v)	Effect on the bacterial cytoplasmic membrane, decrease in the percentage of adapted cells with polarized membranes	Bacterium	*Rhodococcus erythropolis*	Whole organism	↗	↘	↘	N/A	[De Carvalho et al., 2012]
Copper (II) chloride dihydrate	4.0 mg L^{-1} 15 days	Interfere with biomass content and lipid productivity	Microalga	*Chlorella sp.*	Whole organism	↗ 15:0, 16:0, 18:0	↗ 16:1, 17:1, 18:1	↗ 18:2	N/A	[Sibi et al., 2014]

Table 2.2. Continued

Copper (II) chloride	300 µg L^{-1} 24h	Detrimental effects on photosynthesis, chlorophyll synthesis, fatty acid metabolism, carbohydrate synthesis; detrimental action on enzyme activity and reactive oxygen species (ROS) accumulation, which react with proteins, nucleic acids and lipids, causing deleterious effects on cell structure.	Brown algal kelp	*Laminaria digitata*	Algae tissue	N/A	N/A	N/A	↗	[Ritter et al., 2008]
Copper – Cu (II)	250 µg L^{-1} 8h		Brown algae	*Ectocarpus siliculosus*	Algae tissue	N/A	N/A	N/A	↗	[Ritter et al., 2014]
Copper (*in situ*: sites polluted by run-off from old copper mines)	N/A	Alteration in the rate of lipid synthesis, inhibition of growth or death, influence enzyme reactions	Brown algae	*Fucus serratus and Fucus vesiculosus*	Algae tissue	↘ 16:0	↗ 18:1	↘ 18:3ω3 18:4, 22:5	N/A	[Smith et al., 1985]
Copper	4-40 µg Cu L^{-1} Chronic exposure, field study	Generates excessive free radicals leading to the accumulation of ROS, which react with cellular components resulting in peroxidation of lipids, oxidation of proteins, and changes in the cellular redox status leading ultimately to cell death	Amphipod	*Dikerogammarus villosus*	N/A	↗ major 16:0 14:0 18:0	↘ major 18:1ω7 18:1ω9 16:1	↗ major 18:2ω6 20:2ω6 20:4ω6 20:5ω3 18:3ω3 22:6ω3	N/A	[Maazouzi et al., 2008]
Copper (II) chloride Cadmium (II) chloride	300 mg L^{-1} 80 mg L^{-1}	Alter the permeability of the cytoplasmic membrane in metal-resistance bacteria, impair the bacterial growth	Bacterium	*Pseudomonas putida*	Whole organism	Cu^{2+}: ↗ Cd^{2+}: ↗	Cu^{2+}:↘ Cd^{2+}: ↘	Cu^{2+}: ↘ Cd^{2+}: ↘	N/A	[Popova et al., 2008

Table 2.2. Continued

Cadmium (II) chloride Copper (II) sulphate pentahydrate	200 ppb 200 ppb	Accumulated by some species of macroalgae, interfere with photosynthesis, induce oxidative stress	Macroalgae	*Gracilaria tenuistipitata*	N/A	↗ 14:0 16:0 18:0	↗ 18:1ω7 18:1ω9	↘ 18:2ω6 18:3ω6 18:5ω4 20:4ω6 20:5ω3 22:6ω3	N/A	[Pinto et al., 2011]
Copper (II) chloride Cadmium (II) chloride	5, 50, 250 µg L^{-1} 10, 100, 500 µg L^{-1}, 24 and 72 h	Enhance formation of free radicals and other reactive species; disturb lipid metabolism, inhibit enzymes activity, damage the cellular membrane structure and functions. Cadmium suppresses the activity of hepatic stearoyl-CoA desaturase microsomal delta9-desaturase.	Blue mussels	*Mytilus edulis L.*	Gills and digestive glands	N/A	N/A	↗	N/A	[Fokina et al., 2013]
Cadmium (II) chloride	0.4 mM 4 days	Disturbs the cellular metabolic process by producing excessive ROS, which react with proteins, nucleic acids and lipids; generates oxygenated polyunsaturated fatty acids (Ox-PUFAs) defending the oxidative stress.	Macroalgae	*Ulva lactuca*	Whole organism	↘ 14:0 16:0 20:0 ↗ 18:0	↘ 16:1ω7 18:1ω9	↗ 18:2ω6 20:4ω6 20:5ω3 18:3ω6 22:6ω3	N/A	[Kumar et al., 2010]

Table 2.2. Continued

Cadmium	0.25 ppm, 21 days.	Change RNA and protein synthesis, decline in ATP deviate in lipid peroxidation and glutathione (GSH) cell balance, inhibits respiration process, Ca^{2+} transport and enzymatic activity; influence on microsomal lipid composition and metabolism in gill cells of marine bivalves	Bivalve molluscs	*Mizuhopecren yessoensis,*	Gill	↘ 16:0 14:0 18:0	↘ 16:1, 18:1 ↗ 20:1	↘ 20:5ω3 ↗ 20:2ω9 20:4ω6 18:2ω6 22:5ω3 22:6ω3	N/A	[Chelomin et al., 1991]
Chromium - Cr (VI)	1.3 - 96.4 µM	Reduces pigment content, lead to chloroplast disorganization, mitochondrial damage, cytoskeleton alterations, changes in the membrane lipid content	Microalga	*Euglena gracilis*	Whole organism	↗ 14:0, 16:0	N/A	↘ 18:2ω6 18:3ω6	N/A	[Rocchetta et al., 2006]
				Pesticides						
Urea herbicide: Diuron	60 days: exposure 24h per week. 0.20 mg L^{-1}	Moderately toxic to fish, affect fish social behaviour, the survival of juvenile fish, inhibition of the nervous system and anaemia; causes an increase in liver and spleen weight; induce a decrease in the percentage of muscle and carcase without decrease in total weight, muscular fat, perivisceral fat or fatty acid profile change.	Gilt-head sea bream	*Sparus aurata*	Muscle Periviceral fat	↗	↗	↘ ω3 ω6	N/A	[Sanchez-Muros et al., 2013]

Table 2.2. Continued

Triazine herbicide: atradex	245 mg L^{-1} 8 days	Alter microbial respiration, photosynthesis, biosynthetic reactions, organic matter mineralisation, cell growth, division and molecular composition	Microbial communities	N/A	Whole community	↘ 12:0,14:0	↗ trans-18:1 cis-18:1 ↘ 16:1	↗ 18:3ω3 18:2	N/A	[Littlefield-Wyer et al., 2008]
Triazine herbicide: atrazyne	100 and 150 mg L^{-1}	Interferes with the photosynthesis function of plants, including some algae, affects the quality of the diatom cells: photosynthetic activity	Diatom	*Seminavis robusta*	Whole organism	N/I	N/I	N/I	N/A	[De Hoop, et al., 2013]
Triazine herbicides Irgarol 1051 (2-(tert -butylamino)-4-cyclopropylamino) -6-(methylthio)-1,3,5-triazine)	10%, 30% and 50% of the 96 h LC50= 0.535 mg/ml; 21 days	Induce significant increase of larval malformation and sperm fertilization rate, interact with calcium homoeostasis, inhibit ATP synthesis, decrease normal chemoreceptive response	Asian sea-bass	*Lates calcarifer*	Liver	↘	↘	↘	N/A	[Ali et al., 2015]
1,2,4-trichlorobenzene (used in the production of organochlorine pesticides)	0.245 ppm 5 days	Produces the greatest number of alterations in morphological and fatty acid composition after longer exposure periods	Diatom	*Cyclotella meneghiniana*	Whole organism	↘ 16:0 ↗ 15:0, 18:0	↗ 16:1	↗ 20:5	N/A	[Sicko-Goad et al., 1989a]

Table 2.2. Continued

1,3,5-trichlorobenzene (used in the production of organochlorine pesticides)	0.245 ppm 5 days	Produces the greatest number of alterations in morphological and fatty acid composition after 24 hours, alters photosynthetic capacity, decreases in cellular lipid reserves	Diatom	*Cyclotella meneghiniana*	Whole organism	↘ 16:0, 18:0 ↗ 14:0	↗ 18:1	↘ 20:5	N/A	[Sicko-Goad et al., 1989b]
1,2,3-trichlorobenzene (used in the production of organochlorine pesticides)	0.245 ppm 5 days	The consistent increase in lipid volume, accompanied by a decrease in the structure we refer to as "fibrous" vacuole and fewer significant changes in FAs composition	Diatom	*Cyclotella meneghiniana*	Whole organism	↘ 18:0	↗ 16:1, 18:1	↘ 20:5	N/A	[Sicko-Goad et al., 1989c]
Pentachloroben-zene (used in the production of organochlorine pesticides)	0.245 ppm 5 days	Results in changes in diatom FA composition and cell structure increase the lipid volume in diatom	Diatom	*Cyclotella meneghiniana*	Whole organism	↘ 15:0, 18:0	↗↘↗ 18:1	↗↘ 20:5	N/A	[Sicko-Goad et al., 1989d]
Glyphosate-based herbicide: Roundup®	10 and 20 mgL^{-1} 30 days	Highly toxic for aquatic organisms: bacteria, alga, protozoa, zooplankton. Effects on hematological and immunological parameters for fish. Inhibits activity of enzyme preventing protein synthesis, affects gene expression in FA metabolism.	Brown trout	*Salmo trutta*	Muscle 10 mg L^{-1} 20 mg L^{-1} Liver 10 mg L^{-1} 20 mg L^{-1}	 ↘ ↗ ↘ N/I	 N/I N/I ↗ ↗	 ↘ω3 ↘ω3 ↘ ω3 ↘ ω3	N/A	[Bayir et al., 2013]

Table 2.2. Continued

Triazol pesticide: Paclobutrazol	1000 ng/L 50days	Results in elevated expression of $PPAR_{\Upsilon}$ in the liver, responsible for the accumulation of lipid and catabolism of fatty acids	Rockfish	*Sebastiscus marmoratus*	Liver	N/A	N/A	N/A	↗	[Sun et al., 2013]
Chloroacetamide herbicide: metolachlor	20 µM added once	Inhibitor of long-chain fatty acid biosynthesis	Diatom	*Melosira cf. moniliformis*	Whole organism	↘ 14:0 16:0 18:0	↗ 16:1ω7 ↘ 18:1ω7	↗ 16:2, 16:3, 16:4 18:4 (5,8,11,14) 18:5 (5,8,11,14, 17) ↘ 18:4(6,9,12,15) 20:5ω3	N/A	[Robert et al., 2007]
Chloroacetamide and triazine herbicide Primextra® Gold TZ: a.i. S-metolachlor a.i. terbuthylazine	0.5, 2.5, 5.0, 10.0, 20.0 and 30.0 mg/L exposed to *C. edule;* 0.5, 2.5, 5.0, 10.0 mg/L exposed to *S. plana* 96h	Terbuthylazine: photosynthesis inhibitor; Metolachlor: Inhibition of several biosynthesis processes: lipids, fatty acids (FAs), leaf wax, terpenes, flavonoids, and protein synthesis, inhibition of cell division and interference with hormonal regulation, inhibitor of very long chain fatty acid formation, interferes with normal cell development and inhibits both cell division and cell enlargement	Bivalve	*Cerastoderma edule; Scrobicularia plana*	Muscle tissue	↘	↘	↘	N/A	[Gonçalves et al., 2016]

Table 2.2. Continued

Pyridazinone herbicide: norflurazon (SAN 9785)	0.08 – 0.40 mM	The most effective known inhibitor of n-3, n-6 desaturation; Inhibits photosynthesis, carotenoid biosynthesis, chlorophyll accumulation and growth	Red microalga	*Porphyridium cruentum*	Whole organism	↗ 16:0	↗ 18:1	↘ 20:5ω3, 20:4ω6 18:2ω6, 18:3ω6	N/A	[Cohen et al., 1993]
Pyridazinone herbicide: norflurazon (SAN 9785)	0.2 mM		Cianobacterium	*Spirulina platensis*	Whole organism	↘16:0 ↗18:0	↘ 18:1	↘ 18:2, 18:3	N/A	[Kachroo et al., 2006]
			Unicellular alga	*Chlorella minutissima*		↘16:0 N/I 18:0	↘ 18:1	↘ 18:2, 18:3		
Substituted pyridazinone herbicide: 4-chloro-5-(dimethylamino)-2-phenyl-3(2H)-pyridazinone (Sandoz 9785)	N/A	Inhibitor of long chain FA desaturation	Microalga	*Chroomonas salina*	Whole organism	N/A	In digalactosyl-diacylglycerol: ↘ 20:1	In phospholipid fraction: ↘ 20:5ω3 22:6ω3 in digalactosyl-diacylglycerol: ↘ 18:4 ↗ 20:5ω3		[Henderson et al., 1990]
Other organic pollutants (POPs: PAHs, PCB, PCMB, DDT and others)										
Polycyclic aromatic hydrocarbons, PAHs (*in situ*)	N/A	Influence the structure of microbial communities	Microbial communities	N/A	Whole community	↗ 19:0	↘ 18:1ω9t 16:1ω9 ↗ 18:1ω9c	N/A	N/A	[Wang et al., 2012]
Petroleum hydrocarbons: blend arabian light petroleum (BAL 250)	2 g L^{-1} 21 days	Assimilated by bacteria, interfere with its FA profiles	Marine hydrocarbon-degrading bacteria	N/A	Bacteria consortium	↗ 15:0, 17:0, 18:0	↗ 17:1, 19:1 ↘ 16:1, 18:1	N/A	N/A	[Aries et al., 2001]

Table 2.2. Continued

Lipophilic materials: aromatic hydrocarbons and plasticizers (phthalates)	Pollutant levels in the animal lipids (expressed as % total lipid): Hydrocarbons: 0.6 – 14.4 % Plasticizers: 0.2 - 2.1 %	Concentrate in the lipids of fish and amphipod gills, change saturation/unsaturation ratio of FAs profiles	Amphipod	*Gammarus duebeni*	Gill	↘ 14:0, 16:0 ↗ 18:0	↗ 16:1, 18:1, 22:1	↗ 20:5, 22:6 ↘ 20:4, 18:2	N/A N/A	[Morris et al., 1982]
					Full body	↘ 16:0, 18:0	↗ 16:1 ↘ 20:1	↗ 22:6		
Dioxin Polycyclic aromatic hydrocarbon	136-512 109-570 contaminant levels in ng/g TOC -total organic carbon (*in situ*)	Interfere with total lipid concentration, percentages of glycolipids	Bivalve	*Scrobicularia plana*	Gonads Digestive glands	N/I	N/I	↘ 20:5ω3	N/A	[Perrat 2013]
Trace elements (As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, V, Zn) and polycyclic aromatic hydrocarbon	N/A *In situ* . Reference and contaminated sites	Accumulate in mussels, influence on lipid metabolism and FA biosynthesis	Bivalve molluscs	*Mytilus galloprovincialis*	Soft tissues	Differences in FA composition, especially polyunsaturated (PUFA), essential (EFA) and non-methylene interrupted dienoic (NMID) FAs between reference and impacted sites				[Signa et al., 2015
Oil: PAH	N/A *In situ* Sites around oil spill	Histopathological alterations in the cells of the digestive gland. Reduction in triacylglycerol synthesis and a decrease in mobilization of fatty acids, result in deficiency of essential fatty acids, changes in lipid classes and suppression of the transport of triglycerides	Bivalve molluscs	*Mytilus galloprovincialis*	Soft tissues	N/I	↗ 16:1ω7	↘ 22:6ω3	N/I	[Labarta et al., 2005]

Table 2.2. Continued

Organomercurial p-chloromercuri-benzoate (PCMB) and cadmium	PCMB: 0.2, 0.6 mM; Cd: 2.0, 6.0 mM	Mercury interferes with respiration, photosynthesis, lipid biosynthesis, inhibits the activity enzymes: catalase, urease, alcohol dehydrogenase, fatty acid synthetase, oleyl- CoA desaturase. Cadmium affects algal photosynthesis and inhibits a wide range of enzymes. Both metals impair many membrane-related functions including Na+ and K+ ion permeability, ATPase activity and molecular transport, change composition of cellular fatty acids, sterols	Diatom	*Asterioneilu glacialis*	Whole organism	PCMB: ↗ Cd: 2.0 mM ↘ 6.0 mM ↗	PCMB: ↗ Cd: 2.0 mM ↘ 6.0 mM ↗	PCMB: ↘ Cd: ↘	N/A	[Jones et al., 1987]
Polychlorinated biphenyl (PCB)	N/A	Growth inhibition and changes in FA profile	Diatom	*Thalassiosira pseudonana*	Whole organism	↗ 16:0	↗ 16:1	N/A	N/A	[Fisher et al., 1978]
Organochlorine pesticides (OP). Polychlorinated biphenyls (PCBs)	N/A *in situ* (Mediterranean epizootic)	Accumulate in tissues and interfere with FA profiles	Striped dolphin	*Stenella coeruleoalba*	Cerebrum Cerebellu m Lung Liver Kidneys Melon Skeletal muscle	↗	↗	↗ ↘ 20:4ω6	N/A	[Guitart et al., 1996]
n-eicosane	1 g L^{-1}	Promotes the highest FA variation	marine denitrifying bacterium	*Pseudomonas nautica*	Whole organism	↗ 16:0, 20:0	↗ 20:1ω9	N/A	N/A	[Doumenq et al., 1999]

Table 2.2. Continued.

Diesel oil	25 and 100 ppb	Interfere with FA profile	Periwinkle	*Littorina littorea*	Tissue from the tip of the antennae	The resulting demonstrates a distinct difference of fatty acids between the periwinkles exposed by 25 and 100 ppb			[Grahl-Nielsen et al., 1985]
Heavy metals and POPs: DDTs, PCBs, HCHs, Cd, Cu, Hg, and Pb	N/A *in situ*	Accumulate in tissues, pose risks of teratogenic and carcinogenic effects, interfere with FA profile	Large yellow croaker Silvery pomfret	*Pseudosciaena crocea* *Pampus argenteus*	Tissues	N/A	N/A	↗ 20:5ω3 22:6ω3	N/A	[Geng et al., 2015]
POPs: dioxins [poly-chlorinated dibenzo-p-dioxins (PCDDs); polychlorinated dibenzofurans (PCDFs)]; dioxin-like polychlorinated biphenyls (DL-PCBs) polybrominated diphenyl ethers (PBDEs)	N/A *in situ*	High affinity for lipid; accumulate through the food chain	Atlantic bluefin tuna	*Thunnus thynnus, L.*	Tissues	↗ 22:0 (DL- PCBs and PCDD/Fs) ↘16:0 (PCDD/Fs)	N/A	↗ 18:4ω3, 16:3 (DL-PCBs and PCDD/Fs) ↗ 18:3ω6 (PCDD/Fs) ↘ 20:2ω6, 20:4ω6 (PCDD/Fs)	N/A	[Sprague et al., 2012]
PCBs	N/A *in situ*	Biomagnify to high levels in top predators	Labrador ringed seals	*Pusa hispida*	Blubber	↗ 14:0	↗ 20:1ω9 22:1ω9 20:1ω11	↘ 16:2ω4 18:3ω1, 20:4ω6, 22:4ω6, 22:6ω3	N/A	[Brown et al., 2015]

Table 2.2. Continued.

4-tert-butylphenol, 4-n-pentylphenol, 4-n-hexylphenol, 4-n-heptylphenol (AP) 17 β-estradiol (E2) (surfactants and emulsifying agent)	0.02 to 80 mg AP/kg, 5 weeks 5 mg E2/kg, 5 weeks	Have hormone-disrupting effects: interfere with hormonal processes in cod, affect central enzymatic systems; affect the estrogen receptors and thereby affect reproduction of fish; interfere with FA profiles	Atlantic Cod	*Gadus morhua*	Liver (polar lipids) Brain (total lipids) Brain (neutral lipids)	E2: ↗ AP: ↗ E2: ↗ AP: ↗ E2: N/I AP: ↗	E2: ↘ AP: N/I E2: N/I AP: N/I E2: ↘ AP: ↘	E2: ↘ AP: ↘ E2: N/I AP: N/I E2: ↗ AP: ↘	N/A	[Meier et al., 2007]
Wasted food from sea cages of fish farms	N/A (*in situ*)	Changes in body condition and FA profiles	Bogue	*Boops boops*	Muscle Liver	N/I	↗ 18:1ω9	↗ 18:2ω6	N/A	[Ramírez et al., 2013]
PCB, DDT, As, Cd, Cu, Pb residues in muscle	N/A	Accumulates in adipose tissues, interfere with enzymatic activity, change the level of essential FAs,	European seabass	*Dicentrarchus labrax*	Muscle	N/I	↘	↗ 22:6ω3 20:5ω3 ↘ ω6	N/A	[Ferreira et al., 2010]

2.4.1. Microbial communities

Microorganisms are one of the most sensitive aquatic organisms to the deviation of the water quality. Thus, their structural and functional changes can cause huge impact to aquatic system from an integral point of view (Littlefield-Wyer et al., 2008).

Since the native microbial communities can accumulate or metabolize the pesticides, their introduction may affect their biological processes in many ways, such as altering cellular respiration, photosynthesis, biosynthetic reactions, organic matter mineralization, cell growth, division and molecular composition (Littlefield-Wyer et al., 2008). Metals also affect microbial communities. For example, binding with particulate matter, copper ions could settle onto the seafloor and damage the metabolic functioning of soft sediment microbial communities and their composition (Mayor et al., 2013). PAHs can be toxic to bacteria and consequently microbial communities have shown rapid and profound changes in response to PAH contamination (Nilsen et al., 2015).

Membrane lipids, especially FAs are one of the most useful biomarkers for microbial communities, since they have great structural diversity and high biological specificity, essential for every living cell. For instance, the total FA records and the PAHs were significantly negatively correlated in the study, which determined the effects of these hazardous contaminants on estuarine microbial biomass (Nilsen et al., 2015). Wang and Tam (2012) reported that the response of microbial communities to PAH was reflected in an increase in SFA: 19:0, MUFA: 18:1ω9c, but a decrease in other MUFAs: 16:1ω9 and 18:1ω9t.

Research with herbicides revealed the decrease of FA saturation level and increase of the FA unsaturation level in a microbial community after 8 days of exposure by 245 mg L^{-1} Atradex (2-chloro-4-6-isopropylamine-S-triazine) consisting of 90% (w/v) atrazine and 10% (w/v) related triazine herbicides (Littlefield-Wyer et al., 2008).

Study with metal exposure showed that after 10 days of exposure by 30 mg/kg wet sediment of copper (II) sulphate pentahydrate FA profile responded with decrease of both saturated and unsaturated levels, but after exposure by concentration of >91mg/kg wet sediment an reverse effect was observed: the amount of SFAs and MUFAs increased (Mayor et al., 2013) (see Table 2.2).

2.4.2. Bacteria

As in other aquatic organisms (Gabryelak et al., 2000), in marine bacteria heavy metals interfere with the cytoplasmic membrane (Popova et al., 2008), with Aries et al. (2001) showing that bacteria membranes are able to adapt to a contaminated environment. Therefore, the analysis of bacterial FA membrane as bioindicator of chemical stress was of interest for numerous studies. Several studies have been dedicated to this subject, including influences of inorganic and organic compounds (e.g. heavy metals, aromatic and aliphatic alcohols, organic solvents) on the FA composition of bacteria, emphasizing the consistent directional trends in changes of FA after exposure to a certain pollutants. Thus, an increase of SFAs and MUFAs and a decrease of PUFAs were observed in *Rhodococcus erythropolis* and *Pseudomonas putida* after exposure to 0.01–1.00% (w/v) of copper (II) sulphate (De Carvalho, 2012), and 300 mg L^{-1} of copper (II) chloride and 80 mg L^{-1} of cadmium (II) chloride (Popova et al., 2008), respectively (Table 2.2).

Furthermore, the raise of saturation and monounsaturation levels in marine hydrocarbon-degrading bacteria and the marine denitrifying bacterium *Pseudomonas nautical* was observed after 21 days of exposure to 2 g L^{-1} of petroleum hydrocarbon BAL 250 (Aries et al., 2001) and to 1 g L^{-1} of n-eicosane (Doumenq et al., 1999), simultaneously (Table 2.2).

The adaptation of bacteria to metal ions was produced by the activation of saturation mechanism from unsaturated FA, the increase in FA saturation probably is a response of bacteria to promote the stability of the lipid membranes (Popova et al., 2008). The appearance of SFA in bacteria is mainly due to the direct incorporation of monoterminal oxidation products into cellular lipids (Doumenq et al., 1999; Aries et al., 2001).

2.4.3. Microalgae

Algal lipids are important source of energy since they take the main part in the primary consumer diet (Arts et al., 2009). Algal FAs play the role of fundamental components in the biosynthesis of polar and non-polar lipids, which are important components of algal membranes (Kumar et al., 2010b). In algae, PUFA largely involve in lipid fraction responsible for structural function, whereas SFA and MUFA mostly constitute the storage lipid fraction (Olofsson et al., 2012).

Therefore, FA profile in algae may be good bioindicator of key energy reserves and membrane adaptation to the changing environmental conditions (Fisher and Schwarzenbach, 1978).

Summarized results from Table 2.2 revealed that in the majority of studies with microalgae, in the presence of pesticides, their FA profiles responded with decrease in the level of PUFAs, especially EPA (20:5ω3) and PUFAs with 18-C, such as linoleic acid (18:2), linolenic acid (18:3); but with increase in the level of MUFAs, especially palmitoleic acid (16:1) and oleic acid (18:1) (Table 2.2).

In the presence of several herbicides the process of FA desaturation is inhibited, as a result of a direct inhibition of the desaturase enzyme. For instance, inhibition of ω3 desaturation and subsequent reduce of 18:3ω3 concentrations in the glycolipids of higher plants and algae is observed upon exposure to substituted pyridazinone herbicide SAN 9785 (BASF 13-338, 4-chloro-5(dimethylamino)-2-phenyl-3(2H) pyridazinone), inhibition of the ω6-desaturation system is a response on the presence of the herbicide norflurazon SAN 9789 (Sandoz) (Cohen et al., 1993).

The fatty acids 18:2ω6 and 18:3ω3 are possible precursors of 20:5ω3 (Cohen et al., 1993). Therefore inhibition of ω3- and ω6-desaturation leads to decrease of linoleic and linolenic acids and as consequently to a decrease of EPA in microalgae. Another reason of EPA decreasing is related to FA elongase (FAE). For instance, the diatom *Melosira cf. moniliformis* contains a FAE-type elongase that converts 16:0-ACP into 18:0-ACP (ACP – acyl carrier protein is an important component in FA biosynthesis (Berg et al., 2002)), and 18:1 acid is then produced by a ω9 desaturation.

E.g., the chloroacetamide herbicide metolachlor primarily interfere the synthesis of very long chain fatty acids by inactivating the enzyme involved in the condensation of acyl-CoA and malonyl-CoA to produce 3-ketoacyl-CoA and CO_2 (Thakkar et al., 2013) and affects FAE elongation in *Melosira cf. moniliformis*, as evidenced by the reduction in proportion of the FA 18:0, 18:1ω9, 18:4 (6,9,12,15) and 20:5 (5,8,11,14,17) – being FA in the "conventional" pathway to EPA. A consequence of this inhibition is an accumulation of the precursor of the FAE elongation, 16:0, as well as its desaturation product 16:1ω9 (Robert et al., 2007).

However, a few studies (Henderson et al., 1990; Sicko-Goad et al., 1989a) showed that the level of PUFAs can be increased after exposure to some pesticides. Thus, the amount of PUFA in *Chroomonas salina* increased after exposure to substituted pyridazinone herbicide SAN 9785 (Sandoz) and the level of EPA in the diatom *Cyclotella meneghiniana* rised after exposure to 0.245 ppm 1,2,4-Trichlorbenzene in 5 days. An increase in EPA may give evidence of photosynthetic disfunction, since this FA probably substitutes linolenic acid in diatoms (Sicko-Goad et al., 1989 a, b, c, d).

The other possible reason of PUFA increase could be associated with galactolipids and phospholipids participating as substrates in desaturations, which take part in the synthesis of HUFA in marine microalgae (Henderson et al., 1990).

It is the fact, that heavy metals exposure to algal cells lead to the significant changes in their morphology and biochemistry (Rocchetta et al., 2006). Thus, after exposure of phytoplankton to metals FA profile responded with the following directional trend anchored by the increase of SFAs and MUFAs, and the decrease of PUFAs (Table 2.2). PUFAs are not only key constituents in membranes of microalgae, but also very sensitive to changes in the aquatic environment (Borges et al., 2011). The reason of changes in SFAs and PUFAs levels caused by metal exposure can be explained by activation of defense or reparation mechanisms in order to neutralize cellular damage (Rocchetta et al., 2006).

Lipid peroxidation is one of the main mechanisms caused by metal exposure. During lipid peroxidation, PUFAs are key objects for free radicals (Rocchetta et al., 2006).

For example, decrease of PUFAs and increase of SFAs content were indicated in the microalgae *Euglena gracilis* after exposure to hexavalent chromium through the lipid peroxidation mechanism (Rocchetta et al., 2006). Mercury and cadmium complexes can interact with thiol-containing enzymes involved in lipid biosynthesis and metabolism, which are physiologically important. Two ways are suggested in which mercury and cadmium could affect FA synthesis in diatoms. One of them concerns the metal binding to the reactive thiol-group of coenzyme that interferes with the production of acetyl- and malonyl-coenzyme A and finally causes the inhibition of FA synthesis. The other, involves the binding to glutathione peroxidase, which results to the inhibition of this enzyme, leads to the peroxidation of membrane polyunsaturated fatty acids, and therefore, decreases the amount of PUFAs (Jones et al., 1987).

2.4.4. Macroalgae

Macroalgal FAs synthesized in chloroplast vary their quality and quantity according to the different environmental conditions: such as light, temperature, nutrient level and salinity (Dethier et al., 2013).

Heavy metals can be accumulated by some species of macroalgae and interfere with the photosynthesis and the oxidative stress (Pinto et al., 2011).

Mechanisms of metals' action to FA profiles of macroalgae are similar to the ones for microalgae species. It involves mostly oxidative stress and the production of reactive oxygen/nitrogen species that lead to oxidation of lipids. PUFAs are most affected and their levels are reduced in the presence of heavy metals.

E.g. the concentration of PUFAs in the macroalga *Gracilaria tenuistipitata* significantly decreased after exposure to 200 ppb cadmium (II) chloride and 200 ppb copper (II) sulphate pentahydrate, which induced oxidative stress with further adverse effect on FA biosynthesis (Pinto et al., 2011). On the other hand, an increase in the amount of PUFAs in macroalgae is observed after metal exposure (Kumar et al., 2010b).

For instance, exposure of the macroalgae *Ulva lactuca* to cadmium (II) chloride led to significant accumulation of both di and tri unsaturated (18:2ω6, linoleic and 18:3ω6, linolenic) FAs at the expense of dominant saturated (16:0) and monounsaturated FAs (16:1, 18:1) that indicates the induction of desaturation process of FAs during cadmium stress.

As was stated by (Kumar et al., 2010b), there are several reasons to explain the high level of lipids unsaturation: (1) to sustain fluidity level needed for the diffusion of lipophilic compounds; (2) to give an appropriate geometry to the lipid molecules and (3) to maintain the activities of membrane-bound enzymes.

2.4.5. Zooplankton species

Compared to phytoplankton species, research on the response of FA profiles in marine zooplankton species to the impact of contaminants is scarce.

However, the limited number of studies that exist show responses in the FA profiles of these species with an increased PUFA level after exposure to marine pollutants. For example, the amphipod *Dikerogammarus villosus*, collected from copper concentration of 40 mg L^{-1}, showed the highest PUFAs content, mainly the long-chained ones (Maazouzi et al., 2008). The cause may be the effective processes of regulation/detoxification in order to prevent PUFAs peroxidation.

For the amphipod *Gammarus locusta* it was conclude that metallothioneins (MTs) defend from copper prooxidant effects, by the binding of free Cu or by damaging ROS. Despite that, and like a warning signal, higher lipid peroxidation (LP) was induced before MTs, further MTs production were increased with LP returning to the control values (Maazouzi et al., 2008).

Morris et al. (1982) stated that the amphipod *Gammarus duebeni* responds to the increase of concentrations of lipophilic materials (aromatic hydrocarbons and plasticizers (phthalates)) also with the increase of PUFAs level. These authors explained such changes by different structures of SFAs and UFAs. Thus, saturated lipids keep straight chain configuration whereas UFAs have breakings and higher stiffening at the area of their double bonds. These kinks trouble connection between nearby molecules and therefore, conduce difficulties with the establishment of strong Van der Waals interactions.

Subsequently, membranes with higher lipid unsaturation incline to be more fluid, compared with membranes containing low unsaturated and saturated lipids. Probably the decrease of fluidity is one of the first responses of membranes to lipophilic pollutants. This can be explained by two causes: (1) unsaturated double bonds of membrane FAs may construct loose linkages with the alien compounds and (2) as a result of inclusion of relatively saturated compounds saturation and unsaturation levels are changing. Therefore, probably the organism react using the same mechanism as it use for adaption to lowering of environmental temperature, videlicet by increasing the level of PUFAs in the affected membrane (Morris et al., 1982).

2.4.6. Bivalve Molluscs

Several researches investigated the influence of heavy metals on marine bivalve molluscs (e.g., Chelomin and Belcheva, 1991; Perrat et al., 2013; Fokina et al., 2013). Results showed that these contaminants influence on feeding, growth, reproduction, cardiac activity, and also maturity of molluscs (Fokina et al., 2013), interfere with the wide range of biochemical processes in bivalves' tissues, e.g. decline in ATP, deviation in lipid peroxidation and glutathione cell balance, inhibition of respiration process, Ca^{2+} transport, enzymatic activity, protein synthesis and changes in RNA (Chelomin and Belcheva, 1991).

Bivalve molluscs are valuable sources of PUFAs (Brett and Müller-Navarra, 1997; Anacleto et al., 2014). Reviewed studies showed that the level of EPA decreased after exposure to metals and organic pollutants (Table 2.2).

For instance, the bivalve *Mizuhopecren yessoensis*, after 21 days of exposure to 0.25 ppm $CdCl_2$ and the bivalve *Scrobicularia plana* inhabiting a site contaminated by dioxin and polycyclic aromatic hydrocarbon, showed lower value of EPA, compared with control and the reference site, correspondingly (Chelomin and Belcheva, 1991; Perrat et al., 2013).

Recent research with herbicide Primextra® Gold TZ exposed to marine bivalves *Cerastoderma edule* and *S. plana* showed significant reduction of the overall amount of their FAs, both saturated and unsaturated ones with strongest effects on the essential FAs (Gonçalves, 2016).

By contrasting reference and impacted sample sites, *Mytilus galloprovincialis* was found to respond to PAH in terms of polyunsaturated (PUFA), essential (EFA) and non-methylene interrupted dienoic (NMID) FAs (Signa et al., 2015).

Explanation regarding the loss of ω3 PUFAs in bivalves can be due to the activation of the lipid peroxidation mechanism: since PUFAs are primary targets of ROS, when the process of lipid radical formation begins, higher lipid saturation and high oxygen concentrations cause the increase in the velocity of lipid radical chain reactions (Anacleto et al., 2014).

Nonetheless, the amount of other PUFAs, from ω6 series, was increased in the bivalve *M. yessoensis* and *Mytilus edulis L.,* after exposure by cadmium (Chelomin and Belcheva, 1991) and cadmium with copper (Fokina et al., 2013) correspondingly. High PUFA content maybe explained due to the fact that PUFAs in membrane phospholipids protects the membranes from oxidation destruction caused by chemical stressors exposure (Fokina et al., 2013). Lipid biosynthesis in membranes is affected by any changes in the lipid metabolism. Such phases of FA biosynthesis as elongation and desaturation may become primary targets of metals effects on lipid metabolism. Therefore, it may be considered that alterations in these phases influence on the label distribution in saturated, mono- and diene-FAs under conditions of Cd accumulation in *M. yessoensis* gills. Consequently, it can be the reason for some increase in the amount of the ω6 PUFAs of microsome lipids of these molluscs (Chelomin and Belcheva, 1991).

2.4.7. Other invertebrates

A few studies discuss the influence of heavy metals on lipid and on fatty acid contents of cephalopods. One of the largest proportions of FAs is contained in their digestive glands, taken from their preys (Ahmad et al., 2015).

Cephalopods have high amount of ω3 FAs and are great sources of proteins and large number of essential elements (Rjeibi et al., 2015). Being active predators with high feeding rates, they became easily accessible for penetration for many elements, including heavy metals: copper and zinc, through their diets (Rjeibi et al., 2015).

This study stated that these marine organisms are “potentially” hazardous for consumers, due to the ability to accumulate cadmium in great amount even from the environment with low metal concentration (Rjeibi et al., 2015).

However, in terms of mercury exposure, despite of the high FAs content in the digestive gland of cephalopods, amount of this heavy metal, which has low fat – solubility would not be so high in this organ as after exposure by organic pollutants DDT and PCBs due to their high octanol–water distribution coefficients (K_{ow}) (Ahmad et al., 2015).

2.4.8. Fish

Fish presents high nutritional benefits mainly due to the high content of two kinds of PUFAs: eicosapentaenoic acid (EPA, 20:5 ω3) and docosahexaenoic acid (DHA, 22:6ω3) – both are omega-3 highly polyunsaturated fatty acids (HUFAs), which provide many health effects (Bayir et al., 2013; Ferreira et al., 2010; Sprague et al., 2012; Geng et al., 2015).

These FAs are important for somatic growth of marine fish (Sprague et al., 2012) and is considered as essential, since these species do not have the ability to bio-convert shorter FAs into these FAs, due to the very low activity of delta-6 and delta-5 desaturase in fish (Ramírez et al., 2013). Since fisheries products are the major source of these HUFA in the human diet (Ferreira et al., 2010) and the modifications of FA profiles are sensitive indicators of stress (Sanchez-Muros et al., 2013), there was a great concern on the influence of pesticides and metals on fish FA profile.

In the majority of the reviewed studies with marine fish, the decrease in the level of PUFAs from ω3 and ω6 series and increase in the level of SFAs were revealed after exposure to organic and inorganic pollutants (Table 2.2).

The main reason of changes in PUFAs is that they could be oxidized under oxidative stress conditions induced by metals or other compounds (Gabryelak et al., 2000). For instance, the amount of EPA and DHA in brown trout *Salmo trutta* decreased because of higher oxidation of these omega-3 HUFAs after exposure to glyphosate-based herbicide Roundup® with concentrations of 10 and 20 mg L^{-1} during 30 days (Bayir et al., 2013).

Similar picture was observed after exposure of gilt-head sea bream *Sparus aurata* by the pesticide Diuron: the amount of ω3 FAs decreased after exposure to 0.20 mg L^{-1} of Diuron during 24 h per week during 60 days (Sanchez-Muros et al., 2013).

FA profile of Asian sea-bass *Lates calcarifer* exposed to 10%, 30% and 50% of the 96 h LC50 = 0.535 mg/ml of triazine herbicides Irgarol 1051 during 21 days responded with the decrease in both, saturated (SFAs) and unsaturated FAs (MUFAs and PUFAs) even at the low level of exposure (10% and 30% LC_{50} values) (Ali et al., 2015).

In the study with the exposure by POPs of the *Atlantic bluefin* tuna, it was revealed that the amount of the SFA 22:0, and PUFA, 18:4ω3 and 16:3 positively correlates with the amount of dioxin-like polychlorinated biphenyls (DL-PCB).

The same picture was observed for polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/DFs), the following FAs: 22:0, 18:4ω3, 16:3 and 18:3ω6 were examined in higher amounts, but 16:0, 20:2ω6 and 20:4ω6 with the lower quantity, where the last one is an essential highly unsaturated arachidonic FA (Sprague et al., 2012). Similar results were obtained in the study with two marine fishes *Pseudosciaena crocea* and *Pampus argenteus*, where the summary amount of two essential FAs: EPA and DHA positively correlated with the amount of investigated POPs (Geng et al., 2015).

In another study, the increase in SFAs and MUFAs is related to FA synthase activity. Thus, the levels of palmitic acid (16:0) and oleic acid (18:1ω9) in rockfish *Sebastiscus marmoratus* were elevated by FAS which was up-regulated by triazol pesticide Paclobutrazol with concentration of 1000 ng/L after 50 days of exposure (Sun et al., 2013).

2.4.9. Marine Mammals

Changes in FA of marine mammals determined in parallel with persistent organic pollutants residues (polychlorinated biphenyls, organochloride contaminants, DDTs, polybrominated diphenyl ethers) in different kind of organs and tissues: cerebellum, lung, liver, kidneys, melon, skeletal muscle, blubber tissue.

Researches are made on bottlenose dolphin *Tursiops truncatus* (Ellisor et al., 2013), striped dolphins *Stenella coeruleoalba* (Guitart et al., 1996), humpback whale *Megaptera novaeangliae* (Waugh et al., 2014) and labrador ringed seal *Pusa hispida* (Brown et al., 2015). In the presence of high concentrations of POPs FA profile of marine mammals responded with decreasing in the amount of PUFAs, e.g. the eicosanoid production (20:4ω6) in striped dolphin *S. coeruleoalba* was reduced due to extremely high concentrations of PCBs and DDTs (Guitart et al., 1996).

A similar pattern was observed for ringed seal *P. hispida*, where FA profiles from specimens collected near the PCBs oil spill did not contain PUFAs, whereas specimens sampled further away from the spill had FA profiles including PUFAs: 16:2ω4, 18:3ω1, 20:4ω6, 22:4ω6, 22:6ω3 (Brown et al., 2015).

Review data (Table 2.2) indicates that the FA profiles of marine species at different trophic levels significantly change after exposure to pesticides and metals, especially the content of PUFAs, among which are essential FAs (e.g. EPA and DHA) which maintain the health status of organisms and can be obtained only by food sources. FA desaturation process has been shown inhibited in the presence of herbicides. Metals induce production of reactive oxygen species that is leading to lipid peroxidation, which directly influence on PUFAs content. The decrease of PUFAs in the presence of heavy metals is a consequence of reaction between double bond in unsaturated FA and binding to it heavy metal or metal interaction with organic-groups of enzymes participating in FA biosynthesis.

2.5. Conclusions

FAs are important components of membranes, essential for every living cell, have great structural diversity and high biological specificity, react very sensitively to stress conditions. FAs are one of the most useful bioindicators for the determination of the effects of chemical stressors (e.g., metals and pesticides).

FA biosynthesis is inhibited when exposed to these pollutants, along with alterations in the processes of FA desaturation and elongations as stated in this review. Despite the information in the literature concerning the response of FA profiles of organisms along the whole trophic marine food web from primary producers to the top carnivores, little attention has been given to the alterations in FA composition of primary consumers in the presence of metals and/or pesticides.

Zooplankton plays a pivotal ecological role in terms of biomass and energy transfer between primary producers and higher trophic levels, indicating the importance of this group in ecological studies. Determining changes in FA profiles of primary consumers in future studies can contribute to obtain more comprehensive results to estimate the impacts of pollutants in the marine environment.

Furthermore, the nutritional content of marine organisms (from algae to fish through zooplankton) is of the highest importance in the human diet, especially thanks to omega-3 highly polyunsaturated fatty acids which have important roles in human health, including promoting cardiovascular health and protecting against neurological and inflammatory diseases.

Thus, it becomes a potential process to predict the influence of contaminants on the human being through the trophic marine food web.

In the most studies, FA profile's response was investigated when marine species were exposed to a single pollutant (pesticide or metal). Only few studies present the FAs alterations when organisms exposed to two or more contaminants, but from one chemical group (two metals or group of pesticides). It is the fact that marine organisms are often simultaneously exposed to multiple stressors in nature, therefore investigations on the species biochemical response (e.g. FA profiles) to combined exposure of different pollutants (e.g. pesticide and metal) is a potential research contributing to foresee future changes in the health of aquatic communities and the implementation of monitoring programs in order to avoid severe repercussions to ecosystem level and thus to ecosystem services. Indeed, it could give further information concerning anthropogenic activities, and thus global changes, predicting changes on aquatic ecosystems and thus on trophic food webs and food quality.

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CHAPTER 3.

Ecotoxicological and biochemical effects caused by individual exposure to a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species

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

3.1. Abstract

In Europe, mainly in the Mediterranean region, an intensive usage of pesticides was recorded during the past 30 years. According to information from agricultural cooperatives of the Mondego valley (Figueira da Foz, Portugal), Primextra® Gold TZ is the most used herbicide in corn crops fields and one of the 20 best-selling herbicides in Portugal. Copper is mainly used in pesticides' formulation. This study aims to determine the ecotoxicological and biochemical (namely fatty acids profiles) effects of the herbicide Primextra® Gold TZ and the metal copper on marine plankton. The organisms used in this study are three planktonic species: the marine diatom *Thalassiosira weissflogii,* the estuarine copepod *Acartia tonsa* and nauplii of the marine brine shrimp *Artemia franciscana*. Fatty acids (FAs) are one of the most important molecules transferred across the plant-animal interface in aquatic food webs and can be used as good indicators of stress. The conducted lab incubations show that *T. weissflogii* is the most sensitive species to the herbicide followed by *A. tonsa* (EC_{50}=0.0078 mg/L and EC_{50}=0.925 mg/L, respectively), whereas the copepod was the most sensitive species to the metal followed by *T. weissflogii* (EC_{50}=0.234 mg/L and EC_{50}=0.3834 mg/L respectively). *A. franciscana* was the most tolerant organism to the herbicide and the metal (EC_{50}=20.35 mg/L and EC_{50}=18.93 mg/L, respectively). Changes in the FA profiles of primary producer and primary consumers were observed, with the increase of saturated FA and decrease of unsaturated FA contents, especially of highly unsaturated FAs that can be obtained mainly from food and therefore are referred to as 'essential FA'. The study suggests that discharges of Primextra® Gold TZ or other pesticides mainly composed by copper may be a threat to plankton populations causing changes in the FA contents and thus in their nutritive value, with severe repercussions for higher trophic levels and thus the entire food web.

3.2. Introduction

Environmental pollution worldwide is an undesirable by-product of the increased demand for natural resources in modern civilization.

However, since the advent of human societies, there have always been foci of environmental contamination, though nothing on the scale we see today. Practically all of the world's environments suffer from some degree of contamination in concentrations above those expected for the region (Bard, 1999). The pollutants that cause the most damage for the ecosystems are composed by pollutants from industries and mining that include toxic substances such as metals and organic pollutants. Little is known about how natural ecosystems respond to chronic and acute exposure to these contaminants, many of which, especially metals, are non-degradable and therefore accumulate in nature, where they continue to affect ecosystem functioning over the course of decades or even centuries. Thus, strategies to assess the effects of stressors on ecosystem functioning will need to take into consideration both old problems and new challenges. Anthropogenic pressures often decrease the health and stability of ecosystems, although the precise effects of these stressors on the biochemical components remain largely unknown. Despite the extensive literature under "anthropogenic pressures" issue, a more functional approach to trace changes in food webs due to the modified biochemical composition of interacting species is lacking so far (Silins and Högberg, 2011). These changes may have repercussions on the food quality and may play a key role to determine the pollution level of an aquatic system.

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

In Europe, mainly in the Mediterranean region, there is an overexploitation of the farmlands that combined with an overuse of fertilizers and pesticides cause adverse effects on the surrounding aquatic systems. Since many estuaries are surrounded by farmland, residential and industrial areas, they are subject to various anthropogenic pressures and behaviors that cause ecological stresses, affecting not only the water quality, but also the biological communities of these ecosystems (Cardoso et al., 2008; Gonçalves et al., 2010a; Gonçalves et al., 2016; Smalling et al., 2013).

The intensive usage of pollutants in agriculture areas near ecological coastal wetlands led to the implementation of the Pesticide-Monitoring programs to recover aquatic systems, such as in the Mondego estuary, Portugal, since 1998 (Galhano et al., 2011). Nowadays, and according to the information from agricultural cooperatives of Mondego valley, the herbicide Primextra® Gold TZ is the most used herbicide in corn crops fields and is one of the 20 best-selling herbicides in Portugal, whereas copper is mainly used in pesticides' constitution (Gonçalves et al., 2016; Neves et al., 2015). Primextra® Gold TZ, produced by Syngenta AG, consists of two main active ingredients (a.i.), 17.75 % (w/w) terbuthylazine (TBA) and 30.2% (w.w.) S-metolachlor, also used by Syngenta AG in other commercial formulations, used worldwide, plus coadjuvant substances supposedly inert (Neves et al., 2015), with a residual percentage at the composition of the herbicide. Metolachlor is classified as an inhibitor of very long chain fatty acid (VLCFA) formation (Liu and Xiong, 2009). It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu and Xiong, 2009). Due to the action mode of this xenobiotic, it is suggested that this a.i. affects the lipid (fatty acids-FA) profile of aquatic species.

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

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

Nutrients, mainly lipids, are involved in many vital functions of aquatic individuals (Arts et al., 2001; Gotelli et al., 2012). Since some of them can only be obtained from food and therefore referred to as 'essential nutrients' they proved to be useful trophic markers (Kelly and Scheibling, 2012). They are further essential for physiological functions, the overall metabolism of organisms and the prevention of diseases (Arts et al., 2001). Fatty acid (FA) analysis is a well-established tool for studying trophic interactions in aquatic habitats (Kelly and Scheibling, 2012).

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

Fatty acid (FA) profile consists of saturated FA (SFA) that does not contain any double bonds and unsaturated FA with one and more double bonds in the molecular structure. In accordance of the unsaturation level unsaturated FA are divided into monounsaturated FA (MUFA) with single double bond and polyunsaturated FA (PUFA) with two and more double bonds.

Among PUFA are highly unsaturated FA (HUFA), which are also termed as essential FA (EFA) as well since they cannot be synthesized *de novo* in animal organisms.

Phytoplankton and zooplankton species are of high importance in ecotoxicology studies due to their key position in the trophic food web, making a link with higher trophic levels.

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

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

3.3. Materials and methods

3.3.1. Cultures maintenance

T. weissflogii was obtained from the Scottish Marine Institute (strain number 1085/18), Dunbeg, PA37 1QA (UK) and was cultured for bioassay tests and zooplankton feeding.

Guillard's f/2 medium [adapted after Rippingale and Payne, 2001] without EDTA, due to its ability to form a stable chelate complex with copper, was applied for diatom cultivation and the incubation experiments. Once a week, algae culture was renewed with new medium.

A. tonsa was sampled from station S in the Mondego estuary (Fig. 3.1), where this species is one of the most abundant copepod species (Gonçalves et al., 2010b; Gonçalves et al., 2012b, 2012c).The Mondego estuary is a small mesotidal system covering an area of 8.6 km^2 along the West Atlantic coast of Portugal.

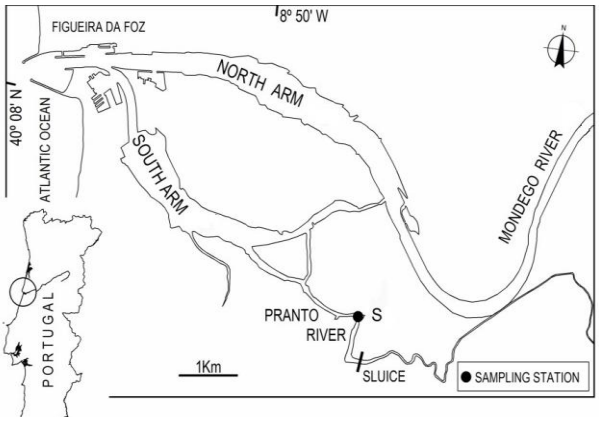

Fig.3.1. Map of the Mondego estuary (40°08`N, 8°50`W), located along the west coast of Portugal and the sampling station (S).

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

Adult organisms, grown from the first cohort of nauplii, were used for the ecotoxicological bioassays.

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

Laboratorial cultures were maintained at a temperature of 20±2ºC, with photoperiod $16h^{L}$: $8h^{D}$, in filtrated seawater medium with a salinity of 13-15 psu for copepods and 35 psu for brine shrimps and 30 psu for diatom cultures.

3.3.2. Growth bioassays of microalgae

Prior to the beginning of the test, an inoculum of *T. weissflogii* was harvested from the bulk culture and incubated for three days under 20 ± 2°C and a 16h light and 8h dark light regime. Briefly, the inoculum cell density was determined microscopically using a Neubauer haemocytometer and adjusted so that the initial test cell density was 10^4 cells mL^{-1}. The microalgae were then exposed to a geometric range of concentrations of each toxicant.

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

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

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

3.3.3. Acute zooplankton bioassays

Tests conditions from OECD protocol 202 (OECD 202, 2004) were adapted and applied for the acute immobilisation tests.

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

3.3.4. Population microcosm bioassays

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

Phytoplankton and zooplankton species were exposed in glass or plastic beakers with a final volume of corresponding test solution of each pollutant. Diatom was exposed in four experimental treatments: (1) a negative control, consisting of uncontaminated culture medium; (2) a low level of each toxicant corresponding to the EC_{10} (0.1361 mg/L, for copper, and 0.0025 mg/L, for Primextra®) value; (3) an intermediate level which corresponds to the EC_{20} (0.1995 and 0.0038 mg/L) value and (4) a high level, which is close to the EC_{50} (0.3834 and 0.0078 mg/L) value (see Table 3.1 for details).

According to preliminary results with zooplankton species, exposed to the contaminants in single cases, the mortality greatly increased after 48h, and, thus, the concentrations used were lower than the EC_x (X=10, 20, 50) values (for details, see tables 3.2 and 3.3).

The larger amount of treatments in bioassay with copepod and copper was because of the fact that copepod showed the highest sensitivity to copper exposure (Table 3.1) and wider range of concentrations was applied in order to able to get information on the concentration at which the FA profile will change.

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

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

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

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

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

3.3.5. FA analyses

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

The fatty acid Methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard for the quantification of FA.

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

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

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

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

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

3.3.6. Statistical analyses

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

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

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

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

3.4. Results

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

A clear inhibition of cells growth of *T. weissflogii* was observed after the exposure to both toxicants (Fig. 3.2).

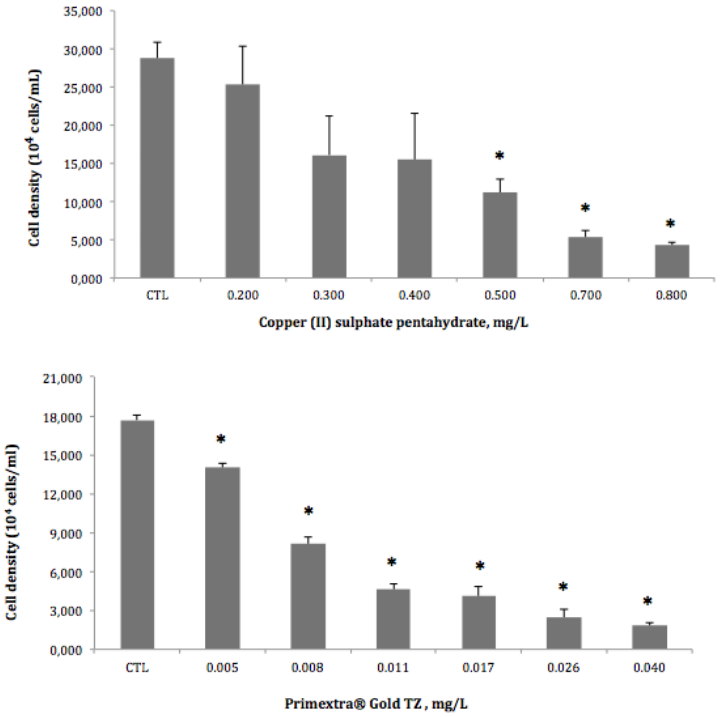

Fig. 3.2. Cell density of *T. weissflogii* after 96h exposure to Primextra® Gold TZ (down) and copper (II) sulphate pentahydrate (up), where CTL refers to the negative control treatment. Symbol "*" – indicates the significant difference of the treatments compared to the CTL.

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

The ECx (X=10, 20 and 50) values determined to the three planktonic species after the exposure to both toxicants showed that the herbicide Primextra® Gold TZ is more toxic to the microalgae than to the zooplankton species (Table 3.1).

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

Table 3.1. EC_{10}, EC_{20} and EC_{50} values (mg/L) of copper and Primextra® Gold TZ for the three planktonic species with the respective time of exposure and 95% confidence limits (between brackets)

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

3.4.2. Population microcosm bioassays and variation of FA profiles

The applied microcosm experiments showed that the herbicide and metal interfere with the FA biosynthesis of the planktonic species. The FA content (values in %) of the three planktonic species exposed to the different treatments of the metal and the herbicide was compared with the control (see Tables 3.1 and 3.2).

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

FA profiles of diatom exposed to copper responded with the following changes in saturated and unsaturated levels: total SFAs slightly decreased from the control to C2 (0.1995 mg/L), however, increased 6% to C3 (0.3834 mg/L). Total MUFAs, PUFAs and HUFAs slightly increased from the control to C2 (0.1995 mg/L), decreasing once again to C3 (0.3834 mg/L), representing a difference of 0.21%, 3.46% and 1.90% with the control, respectively (Table 3.2, Fig. 3.4 a1).

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

HUFAs showed the highest sensitivity to the exposure of the herbicide, clearly decreasing from 16.48% in the control to 12.32% in the treatment concentration (Table 3.3, Fig. 3.4 a2).

The FA profile of the copepod exposed to copper were represented mainly by SFAs - 18:0, 16:0 and 14:0, whereas MUFAs, PUFAs and HUFAs were only detected in trace amounts. Whilst after the exposure to the herbicide, *A. tonsa*, presents higher amounts of SFAs (16:0, 18:0 and 14:0), MUFA (16:1ω7) and HUFA (22:6ω3 (DHA) and 20:5ω3 (EPA)).

The FA profile of the copepod exposed to copper responded with the following changes in saturated and unsaturated levels: amount of SFAs increased in a sinusoidal manner and at the highest concentration level increased slightly with 2.44% compared with control treatment. The same variation was observed for MUFAs, PUFAs and HUFAs, however, their amount decreased at the level 0.109 mg/L Cu^{2+} with 1.94, 0.36 and 0.55% correspondingly compared with the control, resulting to a complete loss of PUFAs and HUFAs at the highest contaminated treatment (Table 3.2, Fig. 3.4 b1).

Primextra® Gold TZ considerably changed the level of SFAs leading to an increase of 9.92% when compared to the control; the level of PUFAs also decreased significantly at the lowest concentration compared to the control, reducing 3 times its abundance, and keeping the same pattern along the range of concentrations. The amounts of MUFA and HUFA were reduced as well after the exposure to 0.925 mg/L of the herbicide with 3.06% and 0.47%, respectively when compared to the control (Table 3.3, Fig. 3.4 b2).

Considering the brine shrimp nauplii, and for copper, SFAs (18:0 and 16:0) presented higher quantities in all treatments while 16:1ω7, 18:1ω7 (MUFA) and 20:5ω3 (EPA) (HUFA) showed the highest values in CTL and 1.615 mg/L treatment. Likewise, 16:3ω4 (PUFA) showed the highest concentration in the CTL.

A different pattern was observed for the FA profile of *A. franciscana* when exposed to the herbicide, with 18:0, 16:0, 20:5ω3 (EPA), 16:1ω7, 18:1ω7 being the most abundant FAs in all treatments.

Microcosm bioassays applied for the brine shrimp nauplii *A. franciscana* revealed that the herbicide and the metal altered its FA profiles much stronger compared with the other studied species. Thus, in the case of exposure to copper, the level of SFAs increased almost twice from 51.27% in the control to 92.35% in the highest concentration level (C3 = 2.136 mg/L). Amounts of MUFAs, PUFAs and HUFAs were as well considerably declined up to 4.5, 10 and 15 times, respectively. The same significant changes were observed after exposure to C2 (1.857 mg/L). Although C1 concentration (1.615 mg/L) did not change significantly the FA profile of *A. franciscana*, an increase on SFAs and a reduction of the higher amounts of MUFAs, PUFAs and HUFAs were reported (Table 3.2, Fig. 3.4 c1). Exposure of *A*. *franciscana* nauplii to Primextra® Gold TZ led as well to changes in FA profiles, still more slightly than in the microcosm bioassay to copper. SFA level increased with 15.19%, whereas MUFAs, PUFAs and HUFAs decreased with 3.05, 6.78 and 5.36% respectively, between the control and the highest concentration (Table 3.3, Fig. 3.4 c2).

Table 3.2. FA profiles of the three planktonic species (marine diatom *T. weissflogii*; estuarine copepod *A. tonsa* and marine shrimp *A. franciscana*) after exposure to copper (II) sulphate pentahydrate.

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

Table 3.2. Continued

HUFA	C 20:4ω6 (ARA)	0.00	0.00	0.00	0.00	0.06	0.12	0.27	0.00	0.00	0.00	0.63	0.83	0.07	0.00
	C 20:5ω3 (EPA)	12.33	14.53	14.33	10.59	0.24	0.59	2.24	0.45	2.79	0.00	14.06	7.32	0.34	0.95
	C 22:63 (DHA)	2.16	2.40	2.42	2.00	0.25	0.92	2.78	0.85	3.77	0.00	0.31	0.28	0.00	0.00
	Total % HUFA	**14.49**	**16.93**	**16.75**	**12.59**	**0.55**	**1.63**	**5.29**	**1.30**	**6.56**	**0.00**	**14.99**	**8.43**	**0.42**	**0.95**
	N	18	18	18	18	19	20	23	18	22	9	25	24	17	14

Table 3.3. FA profiles of three planktonic species (marine diatom *T. weissflogii*; estuarine copepod *A. tonsa* and marine shrimp *A. franciscana*) after exposure to Primextra® Gold TZ.

Species/ FA profile		***Thalassiosira weissflogii***				***Acartia tonsa***				***Artemia franciscana* (nauplii)**			
Primextra® Gold TZ, mg/L		CTL	0.0025	0.0038	0.0078	CTL	0.058	0.151	0.925	CTL	2.786	5.420	10.540
SFA	C 14:0	5.72	5.44	5.44	5.88	5.64	4.08	7.80	4.56	2.09	2.03	1.85	1.84
	C 15:0	0.95	0.88	1.15	1.21	2.17	1.16	1.62	2.32	1.00	0.97	1.01	1.15
	C 16:0	17.47	17.17	16.79	18.24	23.35	29.12	24.85	28.00	16.66	20.53	21.52	20.98
	C 17:0	0.71	0.70	0.70	0.64	0.81	1.12	0.97	1.22	1.58	1.61	1.61	1.80
	C 18:0	17.22	17.17	16.62	17.99	18.23	39.43	29.38	23.29	21.21	28.87	31.91	31.69
	C 20:0	0.19	0.13	0.19	0.19	0.58	1.07	0.74	0.71	0.69	0.77	0.87	0.87
	C 22:0	0.00	0.00	0.00	0.00	0.28	0.36	0.32	0.37	0.47	0.51	0.58	0.57
	C 24:0	0.25	0.24	0.29	0.37	1.11	0.82	0.86	1.63	0.07	0.06	0.07	0.07
	Total % SFA	**42.51**	**41.72**	**41.18**	**44.51**	**52.17**	**77.16**	**66.55**	**62.09**	**43.78**	**55.36**	**59.43**	**58.97**
MUFA	C 16:1ω9	1.22	1.32	1.59	1.72	1.06	0.36	0.45	1.11	0.49	0.49	0.46	0.37
	C 16:1ω7	11.96	11.87	12.63	12.62	6.93	3.01	3.14	3.47	10.82	7.00	6.12	6.53
	C 16:1ω5	0.65	0.40	0.55	0.33	0.40	0.21	0.19	0.00	0.42	0.28	0.24	0.22
	C 17:1ω7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.17	0.30	0.23
	C 18:1ω9	1.01	0.81	0.92	1.13	1.53	0.59	1.01	1.42	4.40	5.25	5.80	4.74
	C 18:1ω7	0.20	0.23	0.34	0.67	1.79	1.24	2.24	2.52	8.30	8.69	8.39	9.54
	C 20:1ω9	0.00	0.00	0.00	0.00	0.17	0.09	0.14	0.18	0.23	0.21	0.21	0.18
	C 22:1ω9	0.00	0.00	0.00	0.00	0.32	0.26	0.41	0.53	0.00	0.00	0.00	0.00
	Total % MUFA	**15.03**	**14.63**	**16.03**	**16.46**	**12.30**	**5.77**	**7.65**	**9.24**	**24.86**	**22.10**	**21.53**	**21.81**

Table 3.3. Continued

PUFA	C 16:2ω6	1.25	1.45	1.55	1.50	0.69	0.26	0.07	0.00	0.83	0.33	0.00	0.29
	C 16:2ω4	5.17	5.12	5.36	4.91	1.86	0.55	0.45	0.56	1.34	0.63	0.13	0.34
	C 16:3ω3	18.82	19.11	20.01	19.70	5.53	1.25	0.86	1.28	7.75	2.55	1.04	1.18
	C 18:2ω trans	0.73	3.35	0.56	0.59	1.06	0.88	1.22	1.09	1.51	1.62	1.98	1.84
	C 18:2ω6 cis	0.00	0.00	0.00	0.00	0.18	0.08	0.07	0.13	0.12	0.00	0.00	0.10
	C 18:3ω6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.09	0.14	0.13
	C 18:3ω3	0.00	0.00	0.00	0.00	0.13	0.00	0.07	0.00	2.17	2.92	3.59	3.22
	C 20:3ω3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.23	0.28	0.26
	Total % PUFA	**25.98**	**29.03**	**27.48**	**26.70**	**9.45**	**3.01**	**2.74**	**3.05**	**14.13**	**8.36**	**7.16**	**7.35**
HUFA	C 20:4ω6 (ARA)	0.00	0.00	0.00	0.00	0.00	0.10	0.38	0.36	1.00	0.75	0.99	0.89
	C 20:5ω3 (EPA)	13.69	12.16	12.71	10.32	11.73	5.71	8.43	9.22	15.23	13.10	10.89	10.79
	C 22:6ω3 (DHA)	2.79	2.46	2.59	2.00	14.36	8.25	14.25	16.03	0.99	0.34	0.00	0.19
	Total % HUFA	**16.48**	**14.62**	**15.30**	**12.32**	**26.08**	**14.05**	**23.06**	**25.61**	**17.22**	**14.19**	**11.88**	**11.86**
	N	18	18	18	18	24	23	25	21	26	25	23	26

The n-MDS plots revealed differences in the FA profiles among the treatments in each microcosm bioassay for each planktonic species (Fig. 3.3).

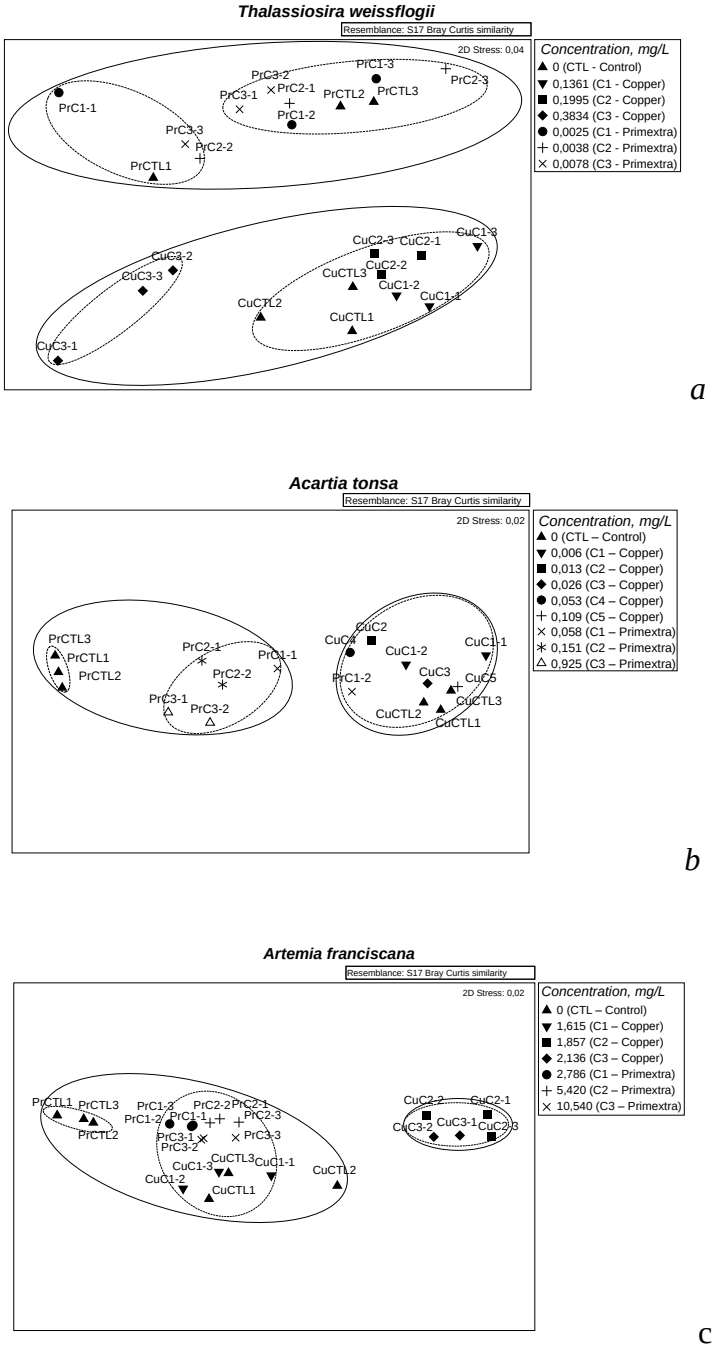

Fig. 3.3 – Two-dimensional non-metric MDS ordination plots of FA profiles of the studied species exposed to copper (II) sulphate pentahydrate (Cu) and to the herbicide Primextra® Gold TZ (Pr): diatom *T. weissflogii* (a), copepod *A. tonsa* (b) and nauplii of brine shrimp *A. franciscana* (c). CTL, C1, C2, C3, C4, C5 – treatments, referring to the concentration of the contaminants tested, where CTL<C1<C2<C3<C4<C5. Not inverted triangles represent negative control treatment

The n-MDS plot of the FA profile of *T. weissflogii* indicated a clear difference between the diatom exposed to copper and to the herbicide Primextra® Gold TZ. Changes in FA profiles between the control and the highest copper concentration (C3 = 0.3834 mg/L), referring to the EC_{50} value (Table 3.1), are well observed. No significant differences were observed among the FA profiles at the control and at lower concentrations of copper (C1 = 0.1361 mg/L – EC_{10} value; C2 = 0.1995 mg/L – EC_{20} value).

In the case of *T. weissflogii*, exposed to the herbicide, no clear differences among the treatments were observed. Only organisms exposed to the highest herbicide concentration (C3 = 0.0078 mg/L) presented small FA profiles changes when compared to the control and lower concentrations (Fig. 3.3 a).

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

In the case of *Acartia tonsa* exposed to the herbicide, the percentage of MUFA, PUFA and HUFA in the treatments were below the values registered in the control while the opposite was observed for SFA (Fig. 3.3 b).

ANOSIM confirmed a clear separation among treatments in both bioassays (Global R = 0.399). Pairwise differences indicated that treatments containing copper are highly different from treatments with the herbicide ($0.519<R<1$). In copper bioassay, C3 treatment in pair with control, C1 and C2, presents high R-values ($0.451<R<1$), therefore it is significantly different compared to lower copper concentrations and uncontaminated treatment. Still, the pair C1/C2 (R = 0.185) is slightly different.

For Primextra®, pairwise differences indicated no differences between pair C1/C2 with 0.0025 and 0.0038 mg/L of herbicide (R = -0.259), however, revealed slight segregation between FA profiles in control and each contaminated treatments (0.074<R<0.142), as well as between C1/C3 treatments with 0.0025 and 0.0078 mg/L of the herbicide (R = 0.148) and C2/C3 treatments with 0.0038 and 0.0078 mg/L of herbicide (R = 0.111).

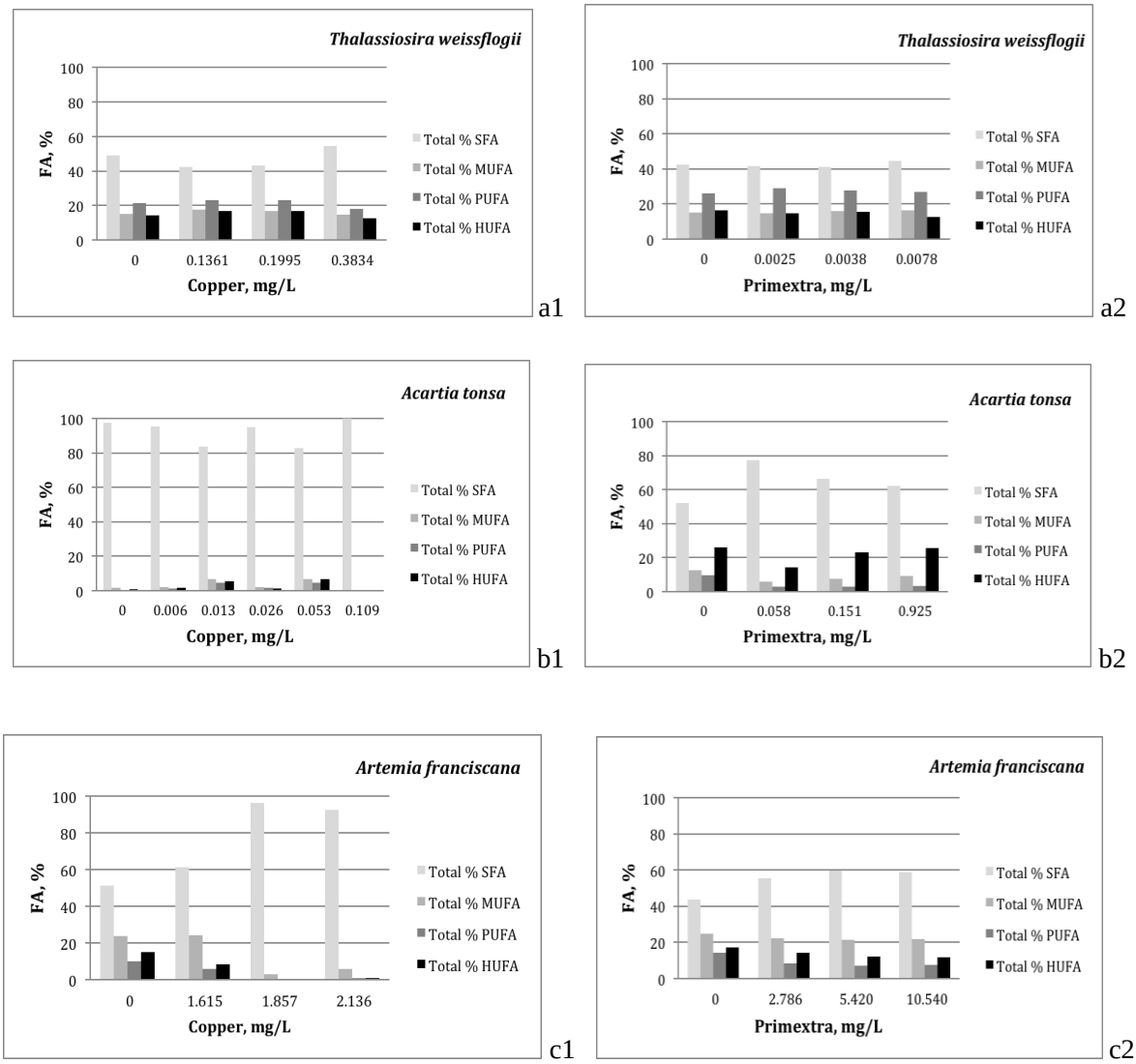

Fig. 3.4. Clustered column charts representing changes in SFA, MUFA, PUFA and HUFA (in %) of the studied species diatom *T. weissflogii* (a1, a2), copepod *A. tonsa* (b1, b2) and nauplii of brine shrimp *A. franciscana* (c1, c2) exposed to copper (II) sulphate pentahydrate (a1, b1, c1) and to the herbicide Primextra® Gold TZ (a2, b2, c2); CTL (=0) represents the negative control treatment

An overall ANOSIM did not reveal significant differences among all treatments, considering two contaminants, for *A. tonsa* (Global R = 0.015).

However, pairwise differences indicated that treatments contaminated with copper are highly different from treatments exposed to the herbicide (0.500<R<1) with exception of C1 herbicide treatment (0.058 mg/L), in pairs with following copper treatments: C2 (0.013 mg/L), C3 (0.026 mg/L) and C4 (0.053 mg/L), where R=0. Pairwise analysis revealed slight segregation between control and C1 (0.006 mg/L) and C5 (0.109 mg/L) copper treatments (R=0.104 and R=0.222 correspondingly), however revealed no differences between control and the rest of treatments contaminated by copper or the herbicide (R<0). For all contaminated treatments with herbicide, pairwise differences showed high segregation (0.750<R<1).

The variation of FA profiles was confirmed by n-MDS and ANOSIM analyses. n-MDS ordination plot showed clear changes in FA profiles between the control and higher copper concentrations (C2 = 1.857 mg/L and C3 = 2.136 mg/L). FA abundance at the lowest concentration (C1 = 1.615 mg/L) is slightly different from the control (Fig. 3.3 c). ANOSIM confirmed a clear separation between treatments in both bioassays (Global R=0.464). Pairwise differences indicated that copper treatments are highly different from treatments exposed to herbicide (0.741<R<1). Pairwise analysis revealed high segregation between control and C2 (1.857 mg/L) and C3 (2.136 mg/L) copper treatments (R=0.833 and R=0.696 respectively), however revealed no differences between the control and the treatments contaminated by copper or the herbicide (R≤0).

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

3.5. Discussion

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

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

This morphological feature may explain the tolerance of this species to both contaminants due to the less uptake of chemicals during the first hours of the exposure.

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

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

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

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

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

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

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

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

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

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

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

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

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

This work revealed that the same contaminant led to slight changes in the amount of very long chain of FAs in primary producer species, however, led to considerable reduction of these FAs in primary consumer species, which are fed by healthy cultures of microalgae (no contaminated cultures).

This allows us to suggest that in the environment conditions the decrease of PUFAs and HUFAs, would be even much more severe, since contaminant in nature influences all the biota, with the primary consumers suffering higher pressure not only through water-borne but also through the diet-borne sources.

Moreover, animals' cells are not able to desaturate at some positions along the fatty acil chain and therefore, some PUFAs and HUFAs can only be synthesized from dietary fats. Required dietary FAs are known as 'essential fatty acids' (Vance and Vance, 2002).

Among them are eicosapentaenoic (EPA), docosahexaenoic (DHA) and arachidonic (ARA) acids, which play a keyrole in organism health and functioning. Thus, EPA and ARA serve as precursors of eicosanoids (prostaglandins, thromboxanes, leukotrienes, etc.), which are responsible for the immune and inflammatory responses, neural function, reproduction, and enhancing the organism’s adaptation to the environment and to anthropogenic stressors (Fokina et al., 2013).

Consequently, changes in FA profiles, specifically, the decrease in the amount of PUFAs and HUFAs, influence the health status of the ecosystem and thus on the basis of food web: primary producer – primary consumer, with profound and severe consequences along the entire trophic food web. In summary, our results confirmed that FA are good bio-indicators of the presence of organic and inorganic chemical stressors in marine and estuarine systems being important tools and ecotoxicological endpoints in toxic studies.

3.6. Conclusions

Current research showed that the herbicide Primextra® Gold TZ and the metal copper implies a threat to the estuarine and marine systems, being toxic to the investigated planktonic species, decreasing the growth rate of phytoplankton and increasing the immobilisation of zooplankton species. Biochemical effects of these pollutants on planktonic organisms revealed an increase of SFA and a decrease of unsaturated fatty acids, specifically a decrease of the essential fatty acids (EFA) that develop important and crucial roles and functions in the organisms' health.

Thus our study proves that changes in FA profiles may be used as an early-warning bio-indicator of anthropogenic stressors for the assessment of the health status of aquatic species.

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CHAPTER 4.

Ecotoxicological and biochemical mixture effects of a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species

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4.1. Abstract

Mixture effects of chemicals and their potential synergistic interactions are of great concern to both the public and regulatory authorities worldwide. Intensive agricultural activities are leading to discharges of chemical mixtures (pesticides and metals) 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 ecotoxic and biochemical effects of single and equitoxic mixtures of the herbicide Primextra® Gold TZ, which consists of two main active ingredients: 17.75% (w/w) terbuthylazine and 30.2% (w/w) S-metolachlor, and the metal copper 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 acids (FA) profiles in both species. Mixture effects on diatom species revealed that copper and Primextra® acted antagonistically relative to the concentration addition (CA) model, but non-interactively relative to the independent action (IA) model. For the copepod species, copper and Primextra® were non-interactive relative to the CA model, but acted synergistically relative to the IA model. The interactive mixture analysis applied to FA profiles of diatom species revealed that the effect was non-interactive for saturated FA and synergistic for unsaturated FA: mono-, poly- and highly unsaturated FA mostly at the intermediate levels of contamination. In general, FA profiles of diatom responded to a greater extent to the single copper exposure by increasing MUFA and HUFA contents. A significant decline in the absolute copepod FA concentration was observed 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.

4.2. 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, affecting non-target biological communities of these ecosystems (Gonçalves et al., 2016) and the related processes at the biochemical and cellular 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 crops fields located near the Mondego estuary area (Figueira da Foz, Portugal), whereas metal copper is widely used in pesticides' constitution (Filimonova et al., 2016a; Gonçalves et al., 2016). Moreover, copper compounds and two main active ingredients of the herbicide Primextra® Gold TZ: terbuthylazine (TBA, 17.75 % w.w.) and S-metolachlor (30.2 % w.w.), are the most extensively used plant protection products within the European Union (Eurostat, 2007).

TBA is a triazine herbicide affecting the diatom cells and inhibiting the photosynthesis at photosystem II, while metolachlor belongs to the family of chloroacetamides interfering with normal cell development and inhibiting several biological processes, essentially biosynthesis of lipids, fatty acids (FA), leaf wax, terpenes, flavonoids and proteins. Although copper at low concentrations is an essential micronutrient for numerous physiological processes, this metal becomes toxic when its supply exceeds the demand, affecting respiration, cell division, photosynthesis, chlorophyll synthesis, carbohydrate synthesis, pigment synthesis and FA metabolism (Chen et al., 2013; Filimonova et al., 2016a, 2016b; Gonçalves et al., 2016; Neves et al., 2015).

Chemical risk assessments are conducted for individual chemicals according to standardized frameworks such as the registration, evaluation, authorization and restriction of chemicals legislation (REACH) of the European Union (Lister et al., 2011). Traditional effect and risk assessment in such frameworks have been routinely focused on exposures to single chemicals, which may underestimate the risks associated with toxic action of mixtures (Chen et al., 2015; Everaert et al., 2016).

Recently, there are an increasing number of studies dealing with toxicity of mixtures of either organic contaminants (group of pesticides) or inorganic contaminants (group of metals) (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 needed for more accurate assessment of ecological risk (Chen et al., 2013; Mehler et al., 2011).

At the base of the trophic food chain, primary producers such as diatoms, which represent a source of food for numerous other organisms, may be seriously affected by pesticide and metal exposure. Zooplankton has long been used as a suitable group in ecotoxicological studies due to their key intermediate position in the trophic food web, as a link between primary producer and secondary consumer species (Debenest et al., 2010; Filimonova et al., 2016a; Neves et al., 2015). In this work the primary producer diatom *Thalassiosira weissflogii* – a sensitive test-organism for seawater toxicity tests (Araújo and Souza-Santos, 2013) and the primary consumer calanoid copepod *Acartia tonsa* – one of the most abundant copepod species in the Mondego estuary (Gonçalves et al., 2010b) were used.

Fatty acids are one of the most important molecules transferred across the plant-animal interface in aquatic food webs. They have the potential to be important tools and endpoints for ecotoxicological studies (Filimonova et al., 2016a, 2016b).

The intensive recent review about the response of fatty acid profiles of marine species to organic and inorganic chemical stressors revealed a knowledge gap about the effects of metal-herbicide mixtures exposure on this endpoint (Filimonova et al., 2016b).

In ecotoxicology, depending on the presumed mode of action of the mixture's components two general reference models are generally used to predict the effects of mixtures: the concentration addition (CA) model (or Loewe Additivity, first introduced by Loewe and Muischnek, 1926) and the independent action (IA) model (first introduced by Bliss, 1939).

Because the modes of action of copper and herbicide Primextra® 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 the substances in the mixture (i.e., "noninteraction" or "additive effect"). However, if the observed responses are stronger or weaker than expected, then the combined effect is described as being either synergistic or antagonistic, respectively (Hochmuth et al., 2014; Nys et al., 2015; Sun et al., 2009)

The objectives of the present study were: (1) to determine whether or not there are interactive effects between the herbicide Primextra® Gold TZ and the metal copper on the relative growth rate of diatom *Thalassiosira weissflogii* and the relative survival of the copepod *Acartia tonsa*; and (2) to evaluate the effect of an applied organic-inorganic mixture on the FA profile of the investigated species. The mechanisms involved in the joint toxicity of the two contaminants and possible incorporation of determined mixture effects into risk-assessment procedures are discussed.

4.3. Materials and methods

4 3.1. Test species: culture conditions

The diatom species *Thalassiosira weissflogii* (strain number 1085/18) was obtained from the Scottish Marine Institute, Dunbeg, PA37 1QA (UK) and was cultured for bioassay tests and zooplankton feeding. Guillard's f/2 medium [adapted after Rippingale and Payne, 2001] without EDTA, due to its ability to form a stable chelate complex with copper, was applied for diatom cultivation and the experimental treatments. Once a week, algae culture was renewed with new medium.

Acartia tonsa (Copepoda, Calanoida) was sampled in the south arm of Mondego estuary (40°08`N, 8°50`W) near the Pranto river, where this species is one of the most abundant copepod species (Gonçalves et al., 2010b; 2012b, 2012c). The Mondego estuary is a small mesotidal system covering an area of 8.6 km^2 along the West Atlantic coast of Portugal.

Horizontal subsurface tows with a bongo net (mesh size of 335 µm and mouth diameter of 0.5 m) were used to collect copepods (Gonçalves et al., 2012a). Samples were brought from the estuary to the laboratory in flasks of 2.5 L with estuarine water (Gonçalves et al., 2012a). *A. tonsa* was separated from other species by means of glass Pasteur pipettes and moved to prepared aquaria with filtrated seawater and aeration for further maintenance and reproduction.

Natural seawater was previously filtrated using VWR glass microfiber filters (1.2 µm pores) and diluted with distilled water to a salinity of 13-15 psu. Aquaria were supplied with gentle aeration system and regular measurements of dissolved O_2 (%) were conducted every other day. Medium (30 % from the total volume) was renewed every 2-3 days, copepods were fed 3 times a week with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL.

The applied maintenance and reproduction procedures of estuarine copepod *A. tonsa* were adapted from Marcus and Wilcox (2007); Rippingale and Payne (2001) and Stottrup et al. (1986). Adult organisms, grown during 14 days from the first cohort of nauplii (Drillet et al., 2006) were used for the bioassays.

Laboratory cultures were maintained at a temperature of 20±2ºC, with photoperiod $16h^L$: $8h^D$, in filtrated (1.2 µm pore size) seawater medium with a salinity of 13-15 psu for copepods and 30 psu for diatom cultures.

4.3.2. Individual and mixture acute zooplankton (immobilisation) and microalgae growth bioassays

Prior to the start of the test, an inoculum of *T. weissflogii* was harvested from the bulk culture (in exponential growth phase) and incubated for three days under 20 ± 2°C and a 16h light and 8h dark light regime. Briefly, the inoculum cell density was determined microscopically using a Neubauer haemocytometer and adjusted so that the initial test cell density was 10^4 cells mL^{-1}. The microalgae were then exposed to a geometric range of concentrations of each toxicant.

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

Based on previous results (Filimonova et al., 2016), we used nominal concentrations ranging from 0.0192 to 0.8828 mg/L for copper (II) sulphate pentahydrate and from 0.0003 to 0.0159 mg/L for Primextra® Gold TZ individually and in equitoxic mixture in the bioassays with diatom. The culture medium was used as the negative control treatment. Tests were carried out in glass (pesticide and mixture bioassays) or plastic (metal bioassays) flasks, three replicates per treatment, containing 40 mL of test solutions.

The tests were performed during 96 h under the same photoperiod and temperature conditions as described for the growth of the algal stock cultures. In all cases the effects of the individual toxicants and their equitoxic mixture were analyzed simultaneously. At the end of the incubation algal cell density was counted using a Neubauer chamber (APHA, 1995).

Tests conditions from OECD protocol 202 (OECD 202, 2004) were adapted for marine species and applied for the acute immobilisation tests with copepod *Acartia tonsa*. Tests of adults of *A. tonsa* were carried out under the same temperature and photoperiod regimes as described for rearing procedures with neonates from the same bulk cultures, born between the first and second broods. The experiments were performed in glass (pesticide and mixture bioassays) and plastic (metal bioassays) vials containing 100 mL of the test solution. Geometric series of toxicants' concentrations were applied, and the culture medium was used as negative control treatment.

The experimental concentrations were obtained by successive dilutions of a stock solution of copper (II) sulphate pentahydrate and Primextra® Gold TZ in distilled water, with nominal concentrations ranging from 0.0096 to 0.4421 mg/L and from 0.0651 to 2.9979 mg/L respectively, individually and in equitoxic mixture. The culture medium was used as the control treatment. A static design was employed, using twenty animals randomly assigned into four replicates with five animals per treatment.

The organisms were exposed to the different toxicants' concentrations during 48 h incubation without food. After 24 h and 48 h, vessels were checked for immobilized individuals. In all cases the effects of the individual toxicants and their equitoxic mixture were tested simultaneously.

The samples with the nominal concentrations of both contaminants from each bioassay were stored for the determination of the measured values. Table A1 (Annexes) summarizes nominal and measured concentrations referring to the amount of copper (Cu) and the herbicide Primextra® (Pr) in the treatment combinations used. Total copper concentrations were measured using 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).

Concentrations of S-metolachlor and terbuthylazine (two main active ingredients of Primextra® Gold TZ) were performed 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 20ml of sample is 0.4 µg/L).

4.3.3. Population microcosm individual and mixture bioassays

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

Phytoplankton and zooplankton species were exposed in glass (pesticide and mixture bioassays) or plastic (metal bioassays*)* beakers with a final volume of corresponding test solution of each pollutant. Diatom *T. weissflogii* and copepod *A. tonsa* were exposed in four experimental treatments: a negative control (CTL), consisting of uncontaminated culture medium, and three contaminated treatments: a low level of each toxicant (C1): 0.80 for diatom and 0.09 for copepod; an intermediate level (C2): 0.96 for diatom and 0.15 for copepod; and a high level (C3): 1.32 for diatom and 0.39 for copepod, expressed as the sum of toxic units of copper and Primextra® combinations (see Equation 1 and Table 4.1 for details):

$$\sum TU_{Cu-Pr\,mix} = TU_{Cu} + TU_{Pr} = \frac{x_{Cu}}{EC50_{Cu}} + \frac{x_{Pr}}{EC50_{Pr}} \quad (1)$$

In Equation 1, TU_{Cu} and TU_{Pr} are toxic units (TU) of copper and Primextra® respectively, that are ratios of the relevant contaminant at the concentration x in the mixture and its EC_{50} value (Table 4.1).

A relatively high sum of toxic units was chosen for diatom in view of their relatively high growth rate after 96h bioassay within this range of sum of toxic units 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 non-interactive (Fig. 4.1 a).

On the contrary, a relatively low sum of toxic units was chosen for the copepod species in view of their relatively high survival after 48h bioassay within this range of sum of toxic units combinations. Another reason was due to our research interest to determine copepod`s FA response at the low sum of toxic units combinations when tendency to continuous synergism on the relative copepod survival was revealed (Fig. 4.1 b).

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

All treatments were replicated three times. Microalgae and zooplankton incubations were conducted under the same laboratorial conditions described above for culture maintenance and bioassays.

According to the dynamics of microalgae growth as reported by Lavens and Sorgeloos (1996), vessels of microalgae experiments were checked for growth inhibition after 7 days of exposure to the toxicants. In each replicate 3.6×10^6 cells / mL were counted using a Neubauer chamber that was then concentrated on a GF/F Whatman filter and frozen at -80 °C for further FA analysis.

The duration of zooplankton experiments after individual and mixture exposure were limited to 7 days due to the high mortality (more than 75 %) of copepod species at the high-contaminated treatment in the mixture bioassay. Organisms were fed daily with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL and moved to new test solutions every third day.

Copepod experiments were conducted in vials with a final volume of 2500 mL and 250 individuals per replicate. Each flask was connected to a gentle aeration system. At the end of each test alive organisms (60 individuals per replicate) were separated and concentrated on GF/F Whatman filters and stored frozen at -80°C for further FA analysis.

4.3.4. FA analyses

The extraction of total lipids of planktonic species and methylation to fatty acid methyl esters (FAMEs) were done by a modified 1-step derivatisation method after De Troch et al. (2012); Gonçalves et al. (2012a). The fatty acid Methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard for the quantification of FA. The FAMEs thus obtained were analyzed using a gas chromatograph (HP 6890N GC) coupled to a mass spectrometer (HP 5973).

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

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

Quantification of individual FAMEs was accomplished by the use of external standards (Supelco 37 Component FAME Mix, Supelco # 47885, Sigma-Aldrich, Inc., USA) and additional standards of 16:2ω6, 16:2ω4 and 16:3ω3 (Larodan Fine Chemicals). The quantification function of each FAME was obtained by linear regression applied to the chromatographic peak areas and corresponding known concentrations of the standards, ranging from 100 to 800 µg/mL.

The fatty acid (FA) profile consists of saturated FAs (SFA) that do not contain any double bonds and unsaturated FAs with one or more double bonds in the molecular structure.

In accordance with the unsaturation level unsaturated FAs are divided into monounsaturated FAs (MUFA) with single double bond and polyunsaturated FAs (PUFA) with two and more double bonds. Among PUFAs, there are highly unsaturated FAs (HUFA), which are also termed as essential FAs (EFA) as well, since they cannot be synthesized *de novo* in animals. Shorthand FA notations of the form A:BωX are used, where A represents the number of carbon atoms, B gives the number of double bonds, and X gives the position of the double bond closest to the terminal methyl group.

4.3.5. Statistical analysis

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

The data obtained from the microalgae 96h bioassays with individual exposure to copper and Primextra® were used to estimate concentrations promoting 50 % growth inhibition and the corresponding 95% confidence intervals for each tested toxicant by non-linear regression, using the least-squares method to fit the data to the logistic equation (equation 2) in Statistica 7 (StatSoft):

$$y = \frac{c}{1+(\frac{x}{EC50})^{\beta}} \quad (2)$$

In Equation 2, y is the relative growth rate (as a percentage, growth rate relative to a control); c is fixed at the maximum growth rate referring to the control (i.e.100%); EC_{50} 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.

Probit analysis (Finney, 1971) run in SPSS was used to estimate the concentration which caused 50% of effect in *A. tonsa* after 48h bioassays with individual exposure to contaminants together with the corresponding 95% confidence intervals.

The interactive effects of the metal copper (Cu) and the herbicide Primextra® Gold TZ (Pr) in mixture bioassays for both species were assessed through the mixture analysis framework developed by Jonker et al. (2005) and further refined by Hochmuth et al. (2014). This framework is based on both the concentration addition (CA) and the independent action (IA) reference models and allows to analyse whether a mixture deviates from strict noninteraction. The mean relative diatom growth rate and relative copepod survival for every Cu–Pr treatment were used as input for the mixture analysis.

The observed values of the relative growth rate (RGR) of diatom of every replicate for all treatments were calculated with Equation 3. The observed values of the relative survival (RS) of copepod of every replicate for all treatments were calculated with Equation 4.

$$RGR_{Cu_x-Pr_y} = \frac{GR_{Cu_x-Pr_y}}{GR_{CTL}} \times 100\% \quad (3)$$

$$RS_{Cu_x-Pr_y} = \frac{S_{Cu_x-Pr_y}}{S_{CTL}} \times 100\% \quad (4)$$

In Equation 3, $RGR_{Cu_x-Pr_y}$ is the relative growth rate of the treatment with copper (Cu) at concentration x and Primextra® (Pr) at concentration y; $GR_{Cu_x-Pr_y}$ is the growth rate of the treatment with Cu at concentration x and Pr at concentration y; GR_{CTL} is the average growth rate of the control - uncontaminated treatment. In Equation 4, $RS_{Cu_x-Pr_y}$ is the relative survival of the treatment with Cu at concentration x and Pr at concentration y; $S_{Cu_x-Pr_y}$ is the survival of the treatment with Cu at concentration x and Pr at concentration y; S_{CTL} is the average survival at the control - uncontaminated treatment.

As described by Nys et al. (2015), the analysis of the interactive effects was performed in 3 steps. In the first step, the predicted values (y) of the relative growth rate in diatom or relative survival in copepod for the mixture combinations were predicted with the concentration addition (Equation 5) and independent action (Equation 6) reference models assuming no interaction and using the EC50 and the slope β after of Cu-only ($EC50_{Cu}$ and β_{Cu}) and Pr-only ($EC50_{Pr}$ and β_{Pr}) exposures calculated for diatom species after non-linear regression, using the least-squares method and for copepod species after probit analysis (Table 4.1).

The generalized reduced gradient iterative solver function (Excel 2011) was used to solve equation 5.

$$\frac{x_{Cu}}{EC50_{Cu} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Cu}}}} + \frac{x_{Pr}}{EC50_{Pr} \times \left(\frac{100-y}{y}\right)^{\frac{1}{\beta_{Pr}}}} = 1 \qquad (5)$$

$$y = 100 \times \left(\frac{1}{1+\left(\frac{x_{Cu}}{EC50_{Cu}}\right)^{\beta_{Cu}}}\right)\left(\frac{1}{1+\left(\frac{x_{Pr}}{EC50_{Pr}}\right)^{\beta_{Pr}}}\right) \qquad (6)$$

Then, in the second step, the reference models were fitted to both single and mixture data. In the third step, in order to account for synergistic or antagonistic deviations from the reference models, the above reference models were extended with a deviation parameter a, 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 (Hochmuth et al., 2014; Jonker et al., 2005; Nys et al., 2015).

The last two steps were performed in the software package RStudio (Ver 0.99.489). In order to fit the reference models 20000 sets of parameter values (i.e. $EC50_{Cu}$, β_{Cu}, $EC50_{Pr}$, β_{Pr} and parameter a for step 3) were sampled simultaneously and estimated in one sample run. Each parameter value was taken from a normal distribution, with the mean and standard deviation from the parameter values originating from the single stressor concentration response. The best set of the above-mentioned parameters was selected based on the lowest sum of squared errors (SSE). We tested whether the addition of the deviation parameter a significantly improved the predictions of the nested models from steps 2 and 3 using an F test with a prior verification of the validity of assumptions. The 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 interactive effects were visualized by plotting the observed relative growth rate in diatom or relative survival in copepod of the mixture treatments, together with the relative growth rate or relative survival predicted by the concentration addition and independent action models in step 1 in function of the sum of toxic units (TU) of the Cu–Pr combinations (Equation 1).

4.3.5.2 Analysis of FA profiles and their response to interactive mixture effects

Multivariate statistical analyses were carried out using PRIMER-6 & PERMANOVA+ software (Clarke and Gorley, 2006) in order to examine the variation in FA composition of both species through non-metric multidimensional scaling (n-MDS) plots. Data were converted into similarity triangular matrices using a Bray-Curtis similarity index (Clarke and Warwick, 2001).

One-way analysis of similarity (ANOSIM) was used to test differences in fatty acid profiles across the treatments to each species.

The analysis of the 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 independent action model of joint stressor effects. FA data (Table A2 from Annexes) 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 4 groups of FA: saturated FA, monounsaturated FA, polyunsaturated FA and highlyunsaturated FA and for top 6 FAs with highest contribution to FA profiles of diatom *T. weissflogii* (Table A5). 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 dependent variables (here FA) implies a statistically significant deviation from the IA model.

When the 2-way ANOVA revealed a statistically significant Cu-Pr interaction, synergistic or antagonistic effects were revealed through the comparison of the observed effect in the mixture treatment with the effect predicted with the IA model. This model was originally formulated by Bliss (1939) and can be as well expressed as follows (De Coninck et al., 2013):

$$E_{CuPr\ Predicted} = E_{Cu} + E_{Pr} - E_{Cu} \times E_{Pr} \quad (7)$$

where

$$E_{i\ Observed} = \frac{Y_{CTL} - Y_i}{Y_{CTL}} \quad (8)$$

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

At the final stage, the interaction was classified as synergistic when the observed effect in mixture treatment was 'higher' than the effect predicted with the IA model and as antagonistic when the observed effect was 'smaller' than the predicted effect (see Equations 7 and 8 and De Coninck et al. (2013) for details).

4.4. Results

4.4.1. Interactive mixture effects on diatom growth rate and copepod survival

The EC_{50} 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 zooplankton species is more sensitive to the metal copper than to the herbicide Primextra® (Table 4.1). The individual concentration–response curves of each contaminant for both species are presented in Fig. A1 (Annexes).

Table 4.1. Summary table of the single stressor concentration response parameters slope β and EC_{50} of copper (Cu) and herbicide Primextra® (Pr) for both planktonic species (±standard error). Calculations of parameters are based on measured concentration values.

Species \ Toxicant	Cu	Pr
T. weissflogii		
EC_{50} (96h, mg/L)	0.0646±0.0845	0.0365±0.0021
β	4.70±1.93	1.70±0.14
A. tonsa		
EC_{50} (48h, mg/L)	0.084±0.013	3.947±1.145
β	1.936±0.384	2.732±0.573

The contaminant mixture revealed different tendencies in the interactive effects on the diatom and copepod species. Mixture effects are presented in the plots of the observed responses and the predicted responses of the reference models against the sum of the toxic units of copper and Primextra® (Fig. 4.1).

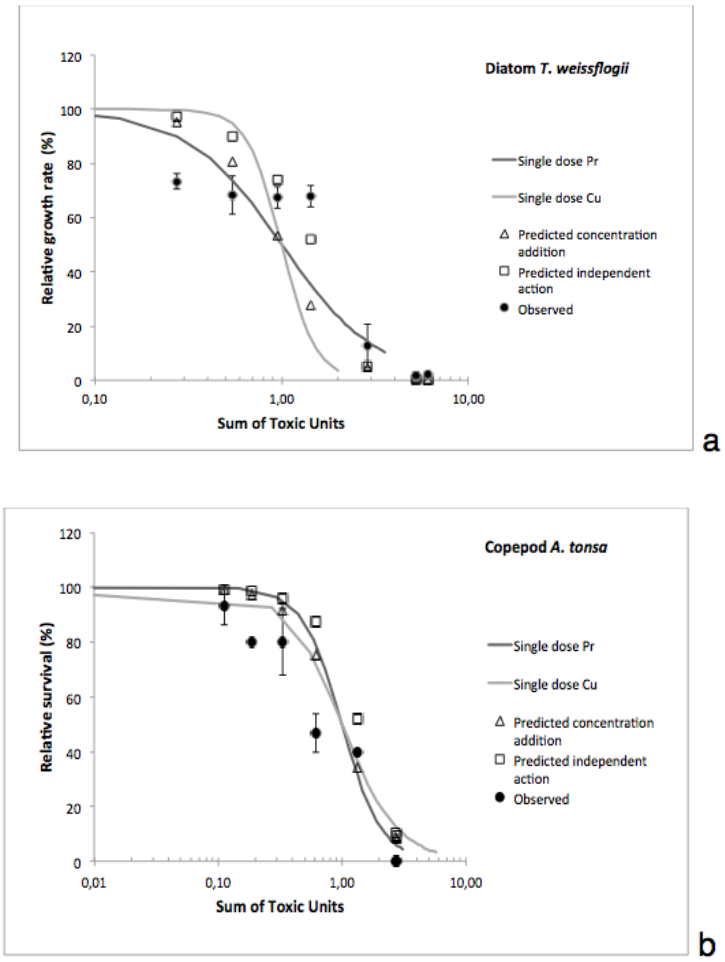

Fig. 4.1. Observed and predicted relative growth rate (%) of the diatom species (a) and relative survival (%) of the copepod species (b) in the mixture combinations of the Cu-Pr mixture as a function of the sum of toxic units. Symbols are denoted as follows: observed effects (circles), predictions of concentration addition (Equation 5, triangles), predictions of independent action (Equation 6, squares). Predictions are based on the parameters (EC_{50} and β) of the single stressor concentration response (Table 4.1). Cu=copper; Pr= Primextra® Gold TZ; CA=concentration addition; IA=independent action. Error bars represent standard errors. Standard error values for some observed data points are smaller than the symbol size.

In the diatom *T. weissflogii*, at lower toxic units (<1) the concentration addition– and independent action–predicted relative diatom growth rate values were mostly higher than the observed values, suggesting synergisms, whereas at the intermediate toxic units (1.4<ΣTU<2.8) these were noticeably lower than the observed values, suggesting antagonism. Only at the highest sum of toxic units (≈5-6) the predicted values of both models were clearly close to the observed relative diatom growth rate values indicating noninteraction.

In the case of *A. tonsa* at lower and middle toxic units (<1) the concentration addition– and independent action–predicted relative copepod survival values were higher than the observed values, suggesting synergism, whereas at higher toxic units (>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, analysed relative to the CA model (p=0.04,), while the mixture effects were non-interactive relative to the IA model (p=0.55) (Table A2, Fig A2). There were no differences in the IA and CA reference models quality: values of Akaike information criterion for each model were relatively similar to each other (Table A2).

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 analysed relative to the IA model (p=0.01), while the mixture effects were non-interactive relative to the CA model (p=0.18) (Table A2, Fig A2). The IA model with the deviation parameter a fitted the data slightly better than the IA model without a: lower Akaike information criterion. However, the CA models showed no differences in the quality compared to the IA models: values of Akaike information criterion were relatively similar to each other (Table A2).

4.4.2. Variation of FA profiles and their response to interactive mixture effects

The applied microcosm experiments showed that the herbicide and metal individually and in equitoxic mixture interfered with the FA composition of the planktonic 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 uncontaminated treatment (Tables A3 and A4, Annexes).

The n-MDS plots highlighted the concentration effect on FA profiles in each microcosm bioassay for both planktonic species (Fig. 4.2).

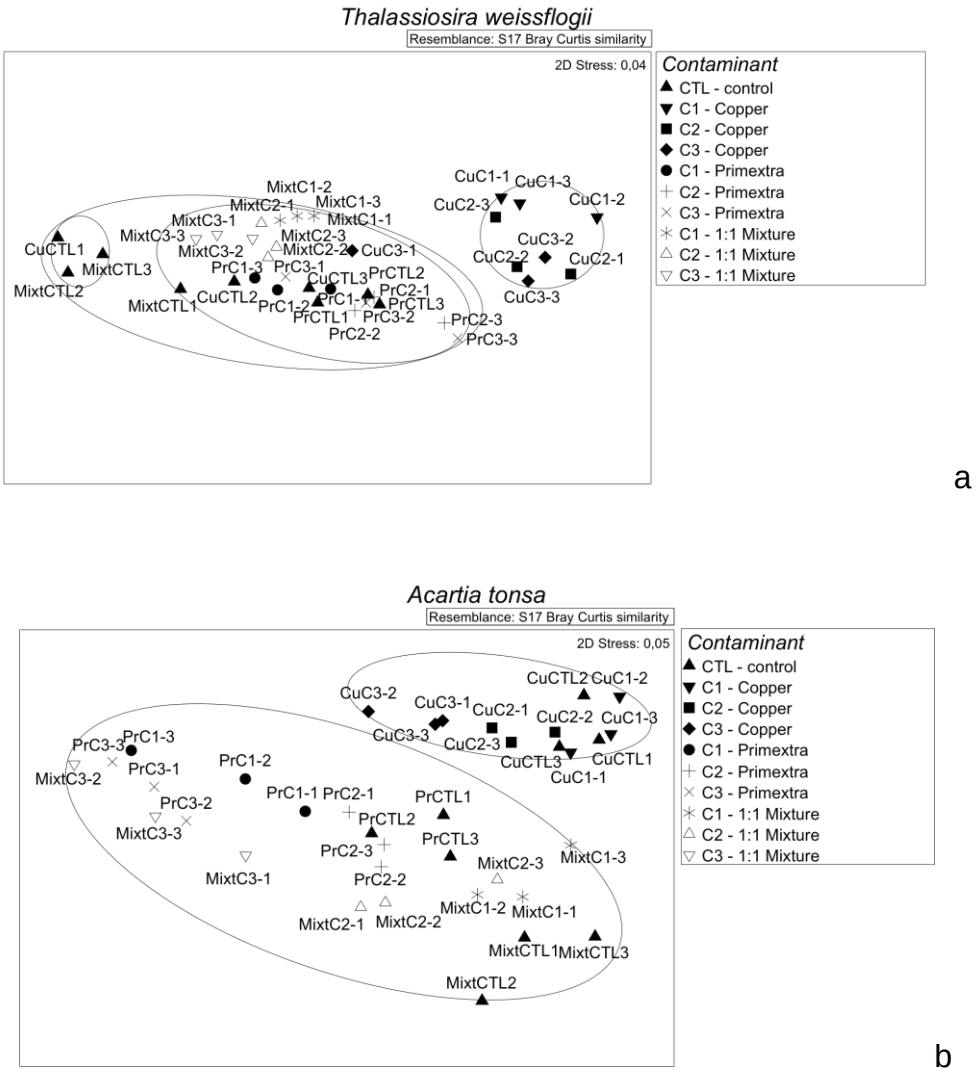

Fig. 4.2 – Two-dimensional non-metric MDS ordination plots of FA profiles of the studied species exposed to copper (II) sulphate pentahydrate (Cu), to the herbicide Primextra® Gold TZ (Pr) and their equitoxic mixture (Mixt): diatom *T. weissflogii* (a), copepod *A. tonsa* (b). CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment (not inverted triangles) and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3.

The n-MDS plot of the FA profile of *T. weissflogii* indicated a clear difference between the exposure to copper and the one to the herbicide Primextra® Gold TZ and the equitoxic mixture of contaminants. Changes in FA profiles between the control and all copper contaminated treatments were clearly observed. ANOSIM confirmed a clear separation among all treatments (Global R=0.361) and between all contaminated treatments (0.556<R<1) in all bioassays.

Significant differences between the FA profiles of the control and of all levels of contamination were observed only in case of copper exposure (Fig. 4.2 a; 0.472<R<0.728). In case of the herbicide exposure significant differences were revealed only between FA profiles at the lowest and the intermediate levels of contamination (R=0.630), after exposure to the equitoxic mixture of contaminants - only among contaminated treatments (0.481<R<1).

As was mentioned earlier, due to technical constraints not all bioassays with copepod species were run simultaneously. The nMDS analysis showed temporal variations among uncontaminated treatments from three bioassays (Fig. 4.2 b). Therefore, the nMDS and ANOSIM analyses of FA profiles of *A. tonsa* were applied for each bioassay separately (Fig. A3, Annexes).

The n-MDS analysis (stress 0.02) applied to FA profiles of the copepod species exposed to the metal copper revealed clear differences for FA profiles of the high and the intermediate contaminated treatments versus the low contaminated and uncontaminated treatments (Fig. A3 a). ANOSIM confirmed a clear separation between all treatments (Global R=0.620), among all contaminated treatments (0.667<R<1) and between control and contaminated treatments only at the high and the intermediate contamination levels (0.444<R<1).

A similar trend was observed for FA profiles of *A. tonsa* exposed to the herbicide Primextra®. The nMDS analysis (stress 0.01) indicated clear differences for FA profiles of the high and the low contaminated treatments versus the intermediate contaminated and the control treatments (Fig. A3 b). ANOSIM confirmed significant differences among all treatments (Global R=0.645), between the low and both the intermediate and the high-contaminated treatments (0.556<R<1) and between uncontaminated and contaminated treatments only at the high and the low contamination levels (0.741<R<1).

The equitoxic mixture of contaminants led to the highest interference with FA composition in the copepod species. The nMDS analysis (stress 0.02) revealed clear differences between FA profiles at the control treatments and all contaminated treatments being more severe at the high contamination level (Fig. A3 c). ANOSIM proved significant differences among all treatments (Global R=0.836), among all contaminated treatments (0.444<R<1) and between control and all contaminated treatments (R=1).

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. 4.3).

The general trend in FA alteration of diatom species in each FA group 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 increase of 2 and 1.5 times in total MUFA and HUFA respectively compared to the uncontaminated treatment (Fig. 4.3: 1 a, b, c).

The opposite trend was observed in FA response of the copepod species: amount of total SFA, MUFA, PUFA and HUFA decreased along the level of contamination from the control to the high-contaminated treatments slightly after the metal exposure, 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 1.5, 4 and 5 times, respectively, total PUFA declined 1.5, 2 and 5 times, respectively and total HUFA (including essential EPA and DHA) has decreased 2, 1.5 and 5 times, respectively at the high-contaminated level compared to the uncontaminated treatment. Only total SFA decreased slightly after the mixture exposure, 1.5 times after the herbicide treatments and after the metal exposure bottomed at the intermediate contaminated level with slight increase at the high-contaminated treatment (Fig. 4.3: 2 a, b, c).

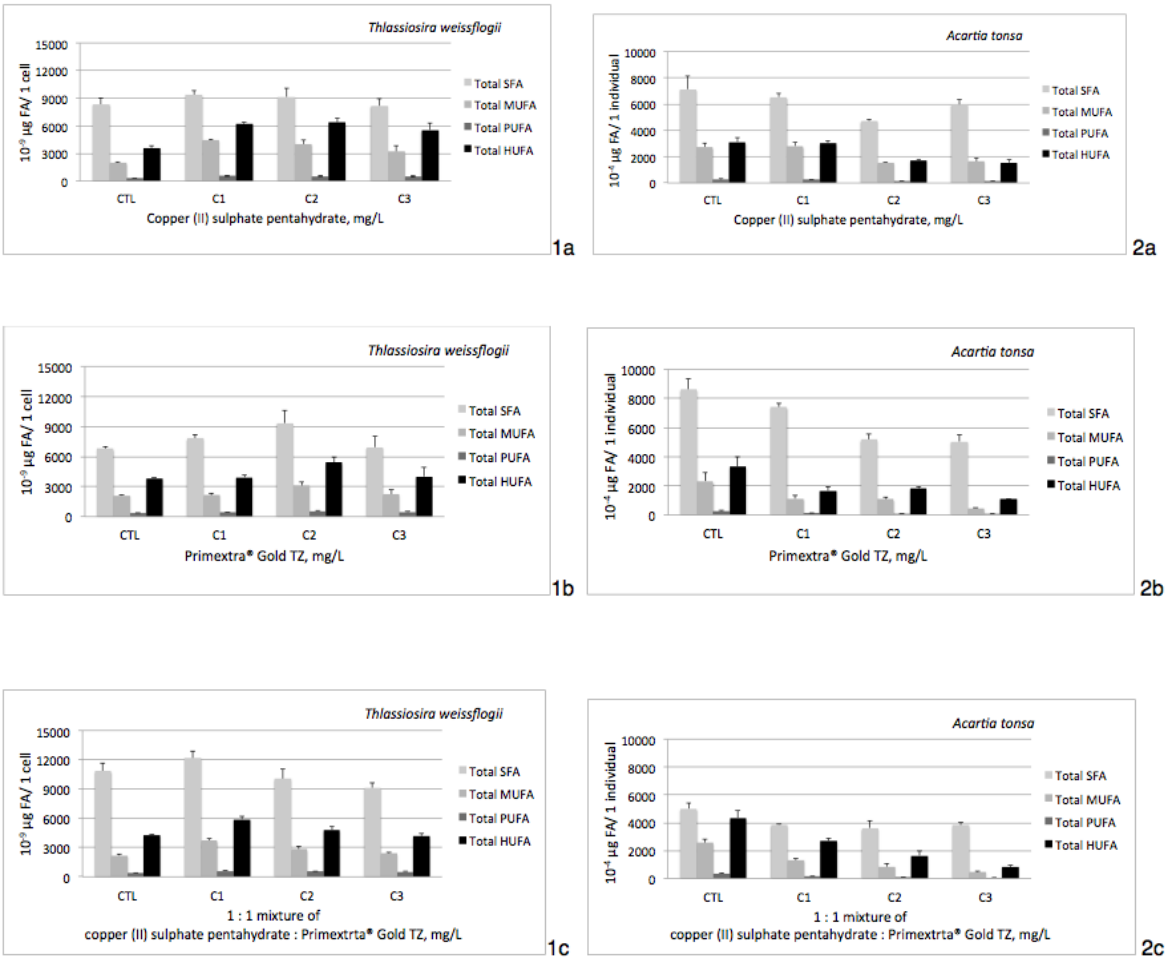

Fig. 4.3. Absolute concentrations (± standard error) of SFA, MUFA, PUFA and HUFA in 10^{-9} µg/1 cell for diatom *T. weissflogii* (1) and in 10^{-4} µg/1 individual for copepod *A. tonsa* (2) exposed to copper (II) sulphate pentahydrate (a), to the herbicide Primextra® Gold TZ (b) and their equitoxic mixture (c). CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3.

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 non-interactive for saturated fatty acids (SFA). 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 (Table A5, Annexes).

4.5. Discussion

4.5.1. Interactive mixture effects on diatom growth rate and copepod survival

Single effects of copper and Primextra® on the relative growth rate of *T. weissflogii* and the relative survival of *A. tonsa* are in accordance with the results from our previous study (Filimonova et al., 2016a) and the results stated by other authors to marine diatoms and calanoid copepods, where primary consumer species showed higher sensitivity to metals and greater tolerance to the herbicides, whereas primary producer species responded with an opposite trend (Diz et al., 2009; Hack et al., 2008; Manimaran et al., 2012; Pinho and Bianchini, 2010; Stringer et al., 2012).

Although there is information on the individual effects of metals and herbicides on non-target species, studies that examine the interactive mixture effects of organic and inorganic compounds are scarce in the literature.

Our study revealed different mixture effects depending on the test species relative to both reference models. The mixture acted antagonistically on the relative growth rate of the diatom species relative to the concentration addition reference model, while mixture effects were non-interactive relative to the independent action model. For copepod survival, mixture effects were non-interactive relative to the concentration addition model, but synergistically relative to the independent action reference model.

A different mixture effect of copper and Primextra®: i.e. antagonism on *T. weissflogii* relative to the CA model and synergism on *A. tonsa* relative to the IA model, may be explained by the absence of the cell wall in the animal cell structure and by the ability of the diatom species to activate specific defensive mechanisms against the contaminant mixtures in the cell wall. Indeed, diatom species induce morphological and chemical defences as a result of exposure to various xenobiotics (Debenest et al., 2010). It has been reported that heavy metals (i.e copper) and herbicides increase the cell wall surface and its roughness (Rijstenbil et al., 1994) and induce the formation of abnormal frustules and their disrupted orientation in the diatom species (Debenest et al., 2008).

It possibly led to the increased adsorption capacity of the contaminants by the diatom cell walls (Rijstenbil et al., 1994) and therefore more difficult penetration of the herbicide and metal molecules into diatom cell membranes that. Thus, it promoted the additional resistance of the diatom species to this contaminant mixture. Furthermore, some herbicides when combined with copper may prevent metal-induced decreases of chlorophyll content in aquatic plants and hence prevent their growth inhibition (Teisseire et al., 1999).

In addition, interactions between heavy metals and organic contaminants could result in speciation changes of heavy metals. A decrease of copper toxicity to algal cells was observed after simultaneous treatment with the herbicide imazethapyr. The latter was explained by the formation of an octahedral complex between imazethapyr and copper. Once copper combines with imazethapyr, it was difficult for it to interact with the algal cell wall (Chen et al., 2013).

In another study, 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). In addition, recently it was discovered that triazine based compounds and the metal copper are able to form metal-organic complexes (Lyapchenko et al., 2004; Wang et al., 2015).

One of the active ingredients of Primextra® Gold TZ is terbuthylazine (TBA) – lipophilic compound: K_{ow}=1096 (WHO, 2003), which belongs to the group of triazine herbicides. Therefore, we may assume the formation of the TBA-Cu complexes that were difficult to interact with the diatom cell wall and easy for a direct diffusion through the copepod cell membrane. These could be possible reasons of differences in observed mixture effects on *T. weissflogii* growth and *A. tonsa* survival.

A few available studies revealed similar effects: for the terrestrial invertebrate *Eisenia fetida* a co-existence of a pesticide and a heavy metal led to the synergism already at low effect levels (the combination index-isobologram equation method, Chen et al., 2015), while for the aquatic plant *Lemna minor* and the aquatic algae *Chlorella ellipsoidea* a combination of the herbicide diuron with copper (Abott's formula, Teisseire et al., 1999) and the organic-compound pentachlorophenol with copper (Stratton's modified procedure and analytical method by isobologram for the combination of two chemicals, Aoyama et al., 1987), respectively, resulted in antagonism .

4.5.2. Variation of FA profiles and their response to interactive mixture effects

Our study revealed that the metal copper and the herbicide Primextra® Gold TZ individually and in equitoxic mixture differently interfered with the FA profile of planktonic species.

Notwithstanding, the individual effects of metals and herbicides on the FA profiles of non-target organisms are well studied, few studies about the mixture effects of group of metals and 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 interfered with the FA composition of the diatom *T. weissflogii* to a greater extent than the herbicide Primextra® and their equitoxic mixture. We hypothesise the greater alteration of FA composition of the diatom *T. weissflogii* after copper exposure in view of the presence of the cell wall containing of silica which is known in giving greater resistance to the herbicide transport (Ferreira et al., 2007).

Although we expected a decrease of HUFA at the highest level of copper contamination compared with the control treatment (Filimonova et al., 2016a), an increase of 1.5 times was observed. However, similar results were obtained in another study where microalgae under copper stress increased percent composition of fatty acids (Sibi et al., 2014).

An increase in HUFA (i.e 20:5ω3, EPA) may give evidence of photosynthetic disfunction, since these FA probably substitutes linolenic acid in diatoms (Sicko-Goad et al., 1989a, 1989b, 1989c, 1989d). The other possible reason could be associated with galactolipids and phospholipids participating as substrates in desaturations, which take part in the synthesis of HUFA in marine microalgae (Henderson et al., 1990).

However, an opposite trend was revealed in the response of the FA profiles of the copepod *A. tonsa*: the equitoxic mixture of contaminants led to the greatest alteration in copepod`s FA profiles and significantly decreased MUFA, PUFA and HUFA. The metal copper showed the weakest interference with FA composition of *A. tonsa*. These results are in agreement with the interactive mixture effect on the relative survival of *A. tonsa* for which a synergistic effect relative to the IA model 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 absence of the cell wall in the animal cell structure and the easy direct diffusion of contaminants, including the possible TBA-Cu complexes into the cell membrane of *A. tonsa*. In addition, S-metolachlor – being the main active ingredient of Primextra® is known to inhibit the synthesis of long chain FA (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015). Therefore mixture and single Primextra® treatments had larger interference with copepod FA composition compared to 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 non-interactive effects on its saturated FA 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 (Filimonova et al., 2016a; Letelier et al., 2005; Martins and Costa, 2014).

Generally, individual and mixture exposure to copper and Primextra® had greater 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 metal-herbicide mixture. These results are of high concern since these molecules are critical for the growth, disease resistance, and general well being of juvenile fish (Brett et al., 2009). Omega-3 FA have important roles in human health, including promoting cardiovascular health and protecting against neurological and inflammatory diseases (Gladyshev 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.

4.6. Conclusions

There was a stronger effect of equitoxic mixture of the metal copper and herbicide Primextra® Gold TZ on the relative survival and essential FA of copepod *A. tonsa*. The observed synergism at the low effect levels for this species indicated a potential ecotoxicological risk associated with a higher possibility of the co-occurrence of these chemicals at environmentally relevant concentrations. A higher tolerance of the primary producer diatom *T. weissflogii* and a greater sensitivity of the primary consumer copepod *A. tonsa* to the applied mixture of copper and Primextra® in terms of abundance and FA composition, suggest 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 non-target species.

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CHAPTER 5.

Effects of a herbicide-metal mixture on the food quality of marine and estuarine primary producers and primary consumers

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5.1. Abstract

Environmental risk assessment has been routinely focused on study the effects of contaminants on endpoints such as survival, growth or reproduction, since the available quantity of the biomass has important effects on the subsequent trophic levels and the overall ecosystem functioning. However, an often overlooked aspect of food availability is food quality, which has important implications on the energy and nutrient transfer through the food web. Pesticides and metals are the key compounds used in agriculture practices and their simultaneous discharges to the nearby estuarine and marine areas are obvious. Further information about the effect of organic-inorganic mixture on the food quality of aquatic organisms, which are typical non-target species for these contaminants, is needed. This study determines whether if there is an effect of the chemical mixture– the inorganic metal copper and the organic herbicide Primextra® Gold TZ – on food quality of the diatom *Thalassiosira weissflogii* and the copepod *Acartia tonsa* in terms of their fatty acid content, especially essential fatty acids, proteins and thiobarbituric acid reacting substances content as important indicators of the food quality. This work also aims to assess how this mixture differentially affects different trophic levels, i.e. primary producer and primary consumer. Generalized linear models were fitted to experimentally observe responses of biochemical composition to the single substances and the mixture. Our study revealed non-additive effect of the metal-herbicide mixture on the food quality of the test phytoplankton and zooplankton species and that nutritionally important biochemical parameters of our test species from the higher trophic level was the most sensitive to the chemical stressors. The study suggests that simultaneous exposure to metal and pesticide contaminants adversely affect the food quality of planktonic species at different trophic levels and this can potentially be transferred to higher trophic levels and cause important implications for the human diet.

5.2. Introduction

Environmental risk assessment typically aims to study the effects on endpoints such as survival, growth and reproduction.

These are considered as ecologically relevant parameters, because they directly affect population size (Hanazato, 2001; Segner, 2007). Thus, such data gives important information about how the amount of biomass changes following exposure of the ecosystem to stress.

The amount of available biomass has important effects on ecosystem functions, as it drives the amount of food available in food webs. However, an often overlooked aspect of food availability is food quality. Food quality is at least as important as food quantity to sustain viable consumer populations and to maintain the sufficient conversion of plant material to herbivore biomass (Perhar and Arhonditsis, 2009). In plankton communities, low food quality of phytoplankton biomass leads to poor energy and nutrient transfer through the food web (Danielsdottir et al., 2007; Perhar and Arhonditsis, 2009), whereas high phytoplankton quality promotes zooplankton community size (Perhar and Arhonditsis, 2009) and therefore energy transfer along the food web. Effects on food quality may become greater when moving up the food web. For instance if the food quality of primary producers and primary consumers are both affected directly and to the same degree, then there is a potential to have an underestimated effect, which may be worse to higher trophic levels in terms of quality of biomass built along the food web.

Important indicators of food quality are the fatty acids (FA) content, especially the essential ones (EFA), and proteins. Proteins are the most abundant organic molecules within invertebrate cells, often comprising 50% or more of the dry weight, and are often used to assess the nutritional quality of algal (Rausch, 1981). The dietary protein content influences the biochemical composition of invertebrates and their growth rate. In general, organismal growth and production are usually positively correlated with the protein content of the diet (Carpenter and Capone, 1983; Houde and Roman, 1987).

Fatty acids are the main components of lipids and fuel all metabolic systems, being the most important molecules transferred across the plant-animal interface in aquatic food webs.

EFA (20:4ω6 – ARA, 20:5ω3 – EPA, 22:6ω3 – DHA) belong to the group of polyunsaturated fatty acids (PUFA) and play a key role in the health and function of all animals at all trophic levels, including planktonic invertebrates, fish and humans.

These cannot be synthesized *de novo*, or at least not in sufficient amounts, and therefore have to be obtained from food (Filimonova et al., 2016b). Chemical stressors can catalyze the production of reactive oxygen species, which may lead to the lipid peroxidation in organisms. Consequently, lipid peroxidation may severely change the nutritional quality by breaking down EFA. The measurement of thiobarbituric acid reacting substances (TBARS) content is the most common test to assess the lipid peroxidation and thus stress response but it is also used as one of the standardized parameters of food quality (Huss, 1995).

Phytoplankton and zooplankton are two main trophic groups that include important test species in ecotoxicology and occupy a key position in food webs since they serve as food to higher trophic levels (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015). The aim of most ecotoxicological studies is to expose species to single doses of chemical stressors (Walsh, 1978). Few studies are focused on mixtures of stressors from one chemical group (e.g. organic-organic or inorganic-inorganic) (Hanazato, 2001). However, very few studies account for mixtures of contaminants from different chemical groups (e.g. organic and inorganic). Such studies are urgently needed to better predict the changes that can be expected from the exposure of aquatic communities to environmental stress (Filimonova et al., 2016b; Mehler et al., 2011). The simultaneous presence of organic and inorganic contaminants is especially relevant for estuarine and marine areas with intensive agricultural activities, because the discharge of pesticides and metals can be substantial in these areas (Gonçalves et al., 2016; Martins and Costa, 2014).

At present, according to the information obtained from agricultural cooperatives of Mondego valley located in West Atlantic coast of Portugal, the herbicide Primextra® Gold TZ is one of the 20 best-selling herbicides in Portugal, being widely used in corn fields, whereas copper is mainly used in the constitution of pesticides (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015).

In the present study, we observed the effects of two different chemical stressors on the food quality of planktonic species at two trophic levels. We considered the organic compound herbicide Primextra® Gold TZ and the metal copper, both individually and in an equitoxic mixture. We selected two plankton species (one primary producer, one consumer) that are well-known test species in marine ecotoxicology: the marine diatom *Thalassiosira weissflogii* (Araújo and Souza-Santos, 2013), which is widely used as food source for zooplankton (Fields et al., 2011), and the estuarine copepod *Acartia tonsa* (one of the most abundant copepod species in the Mondego estuary, (Gonçalves et al., 2010b)). We measured food quality in terms of FA profiles including EFA, protein and TBARS contents.

The main aims of this study were to determine: (1) if there is an effect of a relevant chemical mixture on the food quality of the selected phytoplankton and zooplankton species, and (2) how this mixture differentially affects different trophic levels. To achieve both goals we fitted generalized linear models (GLM) to experimentally observed responses of biochemical composition to the single substances and the mixture. This allowed us to test if effects were additive or not.

5.3. Materials and methods

5 3.1. Cultures maintenance

The diatom species *Thalassiosira weissflogii* was obtained from the Scottish Marine Institute (strain number 1085/18), Dunbeg, PA37 1QA (UK) and was cultured for bioassay tests and zooplankton feeding.

Guillard's f/2 medium [adapted after Rippingale and Payne, 2001] without EDTA, due to its ability to form a stable chelate complex with copper, was applied for diatom cultivation and the experimental treatments. Once a week, the algae culture was renewed with new medium.

Acartia tonsa (Copepoda, Calanoida) was sampled from the southern arm of Mondego estuary (40°08`N, 8°50`W) near the Pranto river, where this species is one of the most abundant copepod species (Gonçalves et al., 2010b; 2012b, 2012c). The Mondego estuary is a small mesotidal system covering an area of 8.6 km^2 along the West Atlantic coast of Portugal.

Horizontal subsurface tows with a bongo net (mesh size of 335 µm and mouth diameter of 0.5 m) were used for copepod collection (Gonçalves et al., 2012a). Samples were brought from the estuary to the laboratory in flasks of 2.5 L with estuarine water (Gonçalves et al., 2012a). *A. tonsa* was separated from other species by means of glass Pasteur pipettes and moved to prepared aquaria with filtrated seawater and aeration for further maintenance and reproduction. Natural seawater was previously filtrated using VWR glass microfiber filters (1.2 µm pores) and diluted with distilled water to a salinity of 13-15 psu. Aquaria were supplied with gentle aeration system and regular measurements of dissolved O_2 (%) were conducted every other day.

Medium (30 % from the total volume) was renewed every 2-3 days, copepods were fed 3 times a week with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL. Applied maintenance and reproduction procedure of estuarine copepod *A. tonsa* were adapted from Marcus and Wilcox (2007); Rippingale and Payne (2001) and Stottrup et al. (1986). Adult organisms, grown during 14 days from the first cohort of nauplii (Drillet et al., 2006) were used for the bioassays.

Laboratorial cultures were maintained at a temperature of 20±2ºC, with photoperiod $16h^L$: $8h^D$, in filtrated seawater medium with a salinity of 13-15 psu for copepods and 30 psu for diatom cultures.

5.3.2. Population microcosm bioassays for the determination of the effect on the planktonic food quality

Microcosm bioassays for the determination of the effect on the planktonic food quality were conducted to determine changes in species FA profiles, protein and TBARS contents after exposure to the herbicide Primextra® Gold TZ and the metal copper (II) sulphate pentahydrate individually and in equitoxic mixture.

Phytoplankton and zooplankton species were exposed in glass (pesticide and mixture bioassays) and plastic (metal bioassays) beakers with a final volume of corresponding test solution of each pollutant. The diatom *T. weissflogii* and the copepod *A. tonsa* were exposed in four experimental treatments: (1) a negative control – CTL, consisting of uncontaminated culture medium; (2) a low level of each toxicant – C1; (3) an intermediate level – C2 and (4) a high level – C3 (Table 5.1).

These treatments were chosen in view of their effect (C1, C2, C3 caused 10%, 20% and 50% effect, respectively) on the relative growth rate of *T. weissflogii* and the relative survival of *A. tonsa* after exposure to the equitoxic mixture of contaminants during 96h and 48h bioassays respectively (Filimonova et al., 2017).

Table 5.1. Summary of the treatments applied to both investigated species in microcosm bioassays for the determination of the effect on the planktonic food quality with copper and Primextra® individually and in an equitoxic mixture, with copper (II) sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) represented as "CuSP" and Primextra® Gold TZ indicated as "Pr". CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3.

Treatments / Species	Diatom *T. weissflogii*		Copepod *A. tonsa*	
	CuSP, mg/L	Pr, mg/L	CuSP, mg/L	Pr, mg/L
Metal copper single dose treatments				
CTL	0.000	0.000	0.000	0.000
C1	0.179	0.000	0.018	0.000
C2	0.216	0.000	0.030	0.000
C3	0.296	0.000	0.082	0.000

Table 5.1. Continued

Herbicide Primextra® single dose treatments				
	CTL	0.000	0.000	0.000	0.000
	C1	0.000	0.003	0.000	0.120
	C2	0.000	0.004	0.000	0.203
	C3	0.000	0.005	0.000	0.555
Equitoxic mixture treatments					
	CTL	0.000	0.000	0.000	0.000
	C1	0.179	0.003	0.018	0.120
	C2	0.216	0.004	0.030	0.203
	C3	0.296	0.005	0.082	0.555

All treatments were replicated three times in bioassays to conduct further FA analysis and five times in bioassays to conduct further TBARS and protein contents determination, with the glass or plastic beaker as the experimental unit. Microalgae and zooplankton incubations were conducted under the same lab conditions described above for culture maintenance.

According to the dynamics of microalgal growth as reported by Lavens and Sorgeloos (1996), vessels with microalgae from bioassays to conduct further FA analysis were checked for growth inhibition after 7 days of exposure to the toxicants. The duration of zooplankton bioassays to conduct further FA analysis after individual and mixture exposure were limited to 7 days due to the high mortality (more than 75 %) of copepod species at the high-contaminated treatment in the mixture bioassay. The duration of the bioassays to conduct further TBARS and protein contents determination for both species was limited to 96h due to known earlier responses of these endpoints to the chemical stressors.

In each replicate 3.6×10^6 cells / mL of diatom were counted using a Neubauer chamber that was then concentrated and stored frozen at -80 °C for further biochemical analyses.

The copepod *A. tonsa* were fed daily with the diatom *T. weissflogii* at a concentration of 2×10^4 cells/mL and moved to new test solutions every third day.

Bioassays with copepod species to conduct further FA analysis and bioassays to conduct further TBARS, protein contents determination were done in vials with a final volume of 2500 mL with 250 individuals per replicate and 5000 mL with 500 individuals per replicate respectively.

Each flask was connected to a gentle aeration system.

At the end of each test alive organisms, i.e. 60 individuals and 250 individuals per replicate were separated and stored frozen at -80°C for further FA analysis and TBARS, protein contents determination respectively.

Organisms (cells and individuals) for further FA analyses were concentrated on GF/F Whatman filters. For the TBARS and protein content measurement, adult copepods were separated manually without medium and the diatom cells were separated from the culture medium by centrifugation (4°C, 4000 rpm, 10 min).

5.3.3. Population microcosm bioassays for a comparison of the effects between trophic levels

In order to compare the effects between trophic levels, the primary producer *T. weissflogii* was exposed to the same test conditions and to the same levels of contaminants as the primary consumer *A. tonsa* (Table 5.2).

Table 5.2. Summary of the treatments applied for both investigated species in microcosm bioassays for a comparison of the effects between trophic levels with copper and Primextra® individually and in an equitoxic mixture, with copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) represented as "CuSP" and Primextra® Gold TZ indicated as "Pr". CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3.

Treatments / Species		Diatom *T. weissflogii*		Copepod *A. tonsa*	
		CuSP, mg/L	Pr, mg/L	CuSP, mg/L	Pr, mg/L
Metal copper single dose treatments					
	CTL	0.000	0.000	0.000	0.000
	C1	0.018	0.000	0.018	0.000
	C2	0.030	0.000	0.030	0.000
	C3	0.082	0.000	0.082	0.000

Table 5.2. Continued

Herbicide Primextra® single dose treatments				
CTL	0.000	0.000	0.000	0.000
C1	0.000	0.120	0.000	0.120
C2	0.000	0.203	0.000	0.203
C3	0.000	0.555	0.000	0.555
Equitoxic mixture treatments				
CTL	0.000	0.000	0.000	0.000
C1	0.018	0.120	0.018	0.120
C2	0.030	0.203	0.030	0.203
C3	0.082	0.555	0.082	0.555

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

5.3.4 Biochemical analyses

The extraction of total lipids of planktonic species and the methylation to fatty acid methyl esters (FAMEs) were done by a modified 1-step derivatisation method after De Troch et al. (2012) and Gonçalves et al. (2012a). The fatty acid Methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard for the quantification of FA. The FAMEs thus obtained were analyzed using a gas chromatograph (HP 6890N GC) coupled to a mass spectrometer (HP 5973).

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

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

Quantification of individual FAMEs was accomplished by the use of external standards (Supelco 37 Component FAME Mix, Supelco # 47885, Sigma-Aldrich, Inc., USA) and additional standards of 16:2ω6, 16:2ω4 and 16:3ω3 (Larodan Fine Chemicals).

The quantification function of each FAME was obtained by linear regression applied to the chromatographic peak areas and corresponding known concentrations of the standards, ranging from 100 to 800 µg/mL. Shorthand FA notations of the form A:BωX are used, where A represents the number of carbon atoms, B gives the number of double bonds, and X gives the position of the double bond closest to the terminal methyl group.

Samples of copepod and diatom for further TBARS and protein contents determination were homogenized and sonicated respectively at 4 °C in 50 mM NaH_2PO_2/Na_2HPO_4 buffer, pH 7.0, containing 0.1% Triton X-100 and centrifuged at 15000 G for 10 min. at 4 °C. The supernatant 1 of each sample was divided in two aliquots, one for protein content determination and the other for determining the TBARS' content.

For TBARS the supernatant 1 of each sample was treated with 10% trichloroacetic acid and then centrifuged at 10000 G for 1 min. at room temperature. Supernatant 2 was treated with 1% thiobarbituric acid and then boiled for 10 min. After cooling it was centrifuged a second time at 10000 G for 1 min. at room temperature. Supernatant 3 was taken and its absorbance measured at 535 nm and a molar extinction coefficient of 1.56×10^5 $M^{-1}.cm^{-1}$ was used to calculate TBARS concentration. The values were expressed as nanomoles of TBARS per milligram of protein (Buege and Aust, 1978).

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

5.3.5. Modelling of the data

A generalized linear model (GLM) with gamma distribution and inverse link function (model 1) was used to estimate the effect of the chemical mixture on the food quality of the planktonic species with biochemical parameters: FA, EFA, TBARS and protein contents as response variables and the treatments of copper (II) sulphate pentahydrate and the herbicide Primextra® as predictors.

The presence of non-additive effects was tested via applying the GLM with interaction (GLM_i) and without interaction of contaminants ($GLM_{n/i}$).

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

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

BP_i represents the biochemical parameter (FA, EFA, TBARS or protein contents) at the concentration i of the contaminant; $T_{CuSP,i}$ and $T_{Pr,i}$ are treatments of copper (II) sulphate pentahydrate and Primextra® at the concentration i (Table 5.1); β_0 and β_1 / β_2 / β_3 are the intercept and the related slopes.

The Akaike Information Criterion (AIC) was used as a measure to evaluate the predictive capacity of the GLM_i and $GLM_{n/i}$ models. A lower AIC was interpreted as a better trade-off between predictive capacity and model complexity. Homogeneity of model residuals was inspected by plotting the standardized residuals versus the predicted values. The goodness of the model fit was estimated by plotting the observed values versus the predicted values (Zuur et al., 2009).

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

(1) the GLM model 1, where for each species equal levels of contaminants (Table 5.2) were predictors and species FA profiles were dependent variables;

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

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

FA_{At} represents the response of either saturated FA or polyunsaturated FA of the copepod *A. tonsa* at the concentration i of the contaminant, $SFA_{Tw,i}$ / $PUFA_{Tw,i}$

are saturated / polyunsaturated FA of the diatom *T. weissflogii* at the concentration i of the contaminant, β_0 and β_1 / β_2 are the intercept and the related slopes.

FA data of the copepod species were log10 - transformed to meet the assumptions of homoscedasticity.

Homogeneity of model residuals and the goodness of the model fit were tested as for the model 1, i.e. by plotting the standardized residuals versus the predicted values and the observed values versus the predicted values respectively (Zuur et al., 2009).

All calculations were performed in R ver. 3.2.2, using RStudio ver. 0.99.489 and the packages lattice, mgcv, nlme (Pinheiro et al., 2016; Sarkar, 2015; Wood, 2016).

5.4. Results

5.4.1. The effect of chemical mixture on the planktonic food quality

The data used for the modelling of the effect of contaminants on the planktonic food quality are presented in the annexes section: fatty acid (FA) data, including the essential FA for the diatom species (Table A6), for the copepod species (Table A7), and the protein and TBARS data of both species (Table A8). Plots indicating the homogeneity of model residuals and the goodness of the model fit are presented at Figs. A4 and A5 respectively (Annexes).

We found non-additive effects of the chemical mixture on most biochemical parameters. The GLM_i model predicted better the total FA profile of the diatom *T. weissflogii*, the essential FA of the copepod *Acartia tonsa* and the TBARS and protein contents of both species. Only for the total FA profile of the copepod *A. tonsa* and for the essential FA data of the diatom *T. weissflogii* the models without interactions ($GLM_{n/i}$) had a lower AIC, suggesting that interactions were not improving the predictive capacity (Fig.5.1).

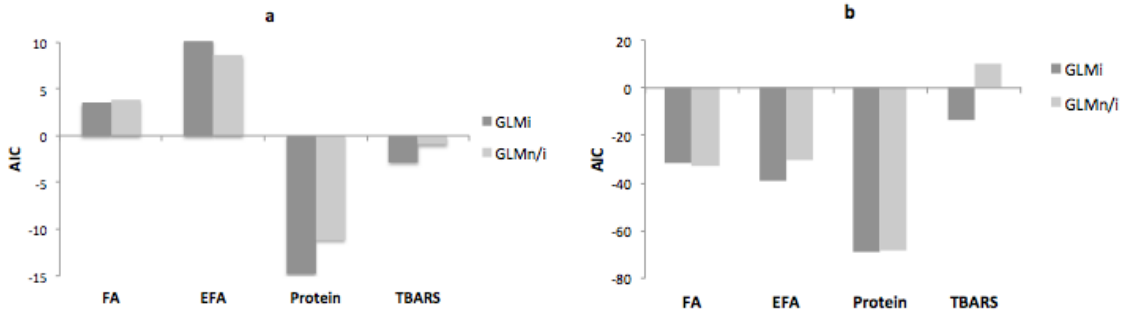

Fig. 5.1. The Akaike Information Criterion (AIC) values determined for generalized linear models with interaction (GLM_i) and without interaction ($GLM_{n/i}$) term for the diatom *T. weissflogii* (a) and for the copepod *A. tonsa* (b). The lower value of AIC indicates better model fit to the data. FA – total fatty acid profile, EFA – essential FA, protein – protein content, TBARS - thiobarbituric acid reacting substances content.

Modelling results revealed that the single treatments of the metal copper, the herbicide Primextra® and their equitoxic mixture significantly affected most of the biochemical parameters (Table 5.3).

Table 5.3. Results of generalized linear models predicting the effect of contaminants on the planktonic food quality, where copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) 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.

Biochemical parameter	Predictor	Slope	SE	t	p
T. weissflogii					
FA	CuSP	0.789	0.841	0.938	0.358
	Pr	86.316	47.550	1.815	0.083
	1:1 mixture	-281.472	203.609	-1.382	0.180
EFA	CuSP	0.326	0.740	0.440	0.664
	Pr	68.601	42.372	1.619	0.119
	1:1 mixture	-110.512	182.036	-0.607	0.550
Protein	CuSP	0.652	0.743	0.878	0.385
	Pr	32.371	40.692	0.796	0.431
	1:1 mixture	-412.121	170.654	-2.415	**0.020**
TBARS	CuSP	-1.730	0.703	-2.460	**0.018**
	Pr	-69.870	39.558	-1.766	0.085
	1:1 mixture	312.731	162.984	1.919	0.062

Table 5.3. To be continued

A. tonsa

FA	CuSP	3.908	2.740	1.427	0.168
	Pr	1.668	0.492	3.389	**0.003**
	1:1 mixture	6.615	11.043	0.599	0.555
EFA	CuSP	13.274	5.210	2.548	**0.018**
	Pr	3.863	0.953	4.053	**<0.001**
	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	**0.007**
	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	**0.003**
	1:1 mixture	33.795	6.337	5.333	**<0.00001**

The food quality of the diatom species was significantly predicted by the metal copper and the equitoxic mixture of contaminants in terms of TBARS and protein contents respectively ($p<0.05$, Table 5.3). However, the single treatments of the herbicide Primextra® did not reveal any significant influence on the food quality of the diatom *T. weissflogii*.

An opposite trend was revealed for *A. tonsa*: the herbicide Primextra® Gold TZ affected significantly on all nutritionally important biochemical parameters ($p<0.05$, Table 5.3), whereas the single treatments of the metal copper significantly predicted only the EFAs content in the copepod species ($p<0.05$, Table 5.3). The equitoxic mixture of copper and Primextra® significantly impacted the TBARS content, proving the presence of the significant lipid peroxidation in copepod *Acartia tonsa,* when exposed to the single treatments of the herbicide and to the mixture of pollutants.

5.4.2. Comparison of the effects of chemical mixture between trophic levels

The data used for the comparison of the effects of contaminants between different trophic levels are presented in the annexes section (Tables A7, A9, A10). Plots indicating the homogeneity of model residuals and the goodness of the models fit for the models 1 and 2 are presented in the annexes section as well (Figs. A6, A7 and A8).

An additive effect of the chemical mixture was revealed for the total FA profiles of both species. However, a non-additive effect was revealed for the essential FA of both primary producer and primary consumer. For both species the AIC suggested the model without interactions ($GLM_{n/i}$) for the total FA profile`s data, and the model with interactions (GLM_i) for the essential FA data (Fig. 5.2).

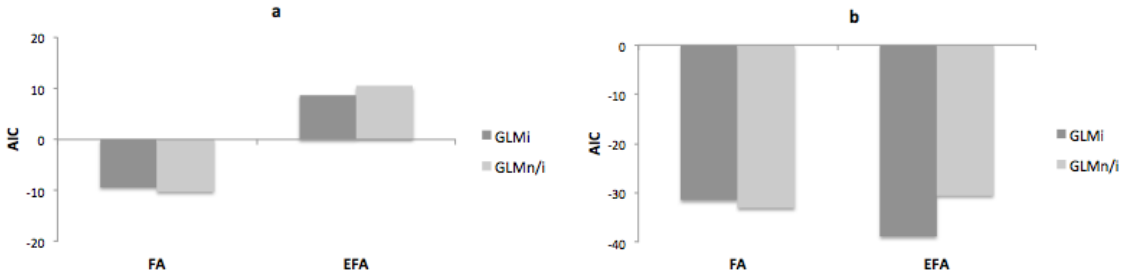

Fig. 5.2. The Akaike Information Criterion (AIC) values determined for generalized linear models with interaction (GLM_i) and without interaction ($GLM_{n/i}$) term for FA profiles (FA) and the essential FA (EFA) of the diatom *T. weissflogii* (a) and the copepod *A. tonsa* (b) after exposure to the same levels of contamination. A lower value of AIC indicates a better trade-off between predictive capacity and model complexity.

The equal levels of contaminants applied to the diatom *T. weissflogii* and to the copepod *A. tonsa* revealed no significant impact on the total FA profile and essential FA of the primary producer species, whereas in the case of the primary consumer species, significant effects of the herbicide Primextra® and metal copper on these parameters were observed (Table 5.4).

Table 5.4. Results of generalized linear models predicting the effects of contaminants between trophic levels, where copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) 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.

Biochemical parameter	Predictor	Slope	SE	t	p
Primary producer *T. weissflogii*					
FA	CuSP	-1.464	1.548	-0.945	0.354

Table 5.4. Continued.

	Pr	-0.036	0.244	-0.148	0.884
	1:1 mixture	4.401	4.517	0.974	0.340
EFA	CuSP	-2.417	3.379	-0.717	0.481
	Pr	-0.161	0.528	-0.305	0.763
	1:1 mixture	20.486	11.296	1.814	0.083
Primary consumer *A. tonsa*					
FA	CuSP	3.908	2.740	1.427	0.168
	Pr	1.668	0.492	3.389	**0.003**
	1:1 mixture	6.615	11.043	0.599	0.555
EFA	CuSP	13.274	5.210	2.548	**0.018**
	Pr	3.863	0.953	4.053	**<0.001**
	1:1 mixture	-10.542	21.830	-0.483	0.634
Correlation between trophic levels					
SFA (*A. tonsa*)	SFA (*T. weissflogii*)	-4.845×10^4	8.498×10^3	-5,702	**<0.00001**
	PUFA (*T. weissflogii*)	3.088×10^4	6.328×10^3	4.881	**<0.0001**
PUFA (*A. tonsa*)	SFA (*T. weissflogii*)	-1.691×10^4	2.058×10^4	-0.821	0.417
	PUFA (*T. weissflogii*)	5.601×10^3	1.532×10^4	0.366	0.717

An output of the model 2 indicated that both saturated and polyunsaturated fatty acids of the primary producer *T. weissflogii* significantly correlate with saturated fatty acids of the primary consumer *A. tonsa*.

However, no relationship was observed between FA profile of the diatom species and PUFAs of the copepod *A. tonsa* (Table 5.4).

5.5. Discussion

5.5.1. The effect of chemical mixture on the planktonic food quality

To the best of our knowledge, this is the first study determining the interaction effect of the organic and inorganic contaminants on the food quality of planktonic species. The overall results of this study demonstrated that non-additive effects allow a better prediction of the mixture effect of the herbicide Primextra® and the metal copper on planktonic food quality, compared to a model that assumes purely additive effects.

It is noteworthy that studies about the interaction effect of organic and inorganic contaminants on non-target species are scarce in the literature and are aimed mostly at measuring species growth, survival or reproduction (Ahsanullah and Florence, 1984; Chen et al., 2015, 2013; Filimonova et al., 2016b; Klerks and Moreau, 2001; Pringault et al., 2016).

Our previous study with the equitoxic mixture of the herbicide Primextra® and the metal copper revealed its antagonistic effect on the relative growth rate of *T. weissflogii,* synergistic effect on the relative survival of *A. tonsa*, synergistic and additive effects on the diatom FA content (Filimonova et al. 2017).

Our models predicted similar results: non-additive effects were necessary for the prediction of the contaminant's mixture effect on the total FA profile in the diatom species, whereas additive effects – on their essential FA.

One could assume that effects of the chemical mixture on the essential FA profile of the diatom species and on the essential FA profile of the copepod species are non-additive, in view of the well know fact that PUFA, including EFA are almost exclusively synthesized by plants. Animals can convert from one form of PUFA to another through elongation and desaturation, but very few can synthesize PUFA *de novo* (Brett and Müller-Navarra, 1997).

Thus, recent study (De Troch et al., 2012) proved that harpacticoid copepods are able to convert short chain FA (i.e. C18) to the long chain PUFA (i.e. EPA and DHA) via the Δ-5, Δ-6 desaturase and elongase enzymes. However, planktonic calanoid copepods (i.e. our study species *A. tonsa*) have limited ability of FA bioconversion since they have lack of the necessary enzymes to produce significant amounts of PUFA (De Troch et al., 2012).

The other reason may be due to the presence of the cell wall in the plant cell structure that plays an important role in the defence responses against potential stressors (Keegstra, 2010).

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

Thus, the effect on protein content was revealed after exposure of aquatic species from different trophic levels to metal copper (Balczon and Pratt, 1994; Olkhovych et al., 2016; Pytharopoulou et al., 2013), to metolachlor (Hartgers et al., 1998; Martins et al., 2011), to triazine herbicides (El-Sheekh et al., 1994; Hartgers et al., 1998) and to the combination of the metal copper and chloroacetanilide herbicide applied to phytoplankton species (Lu et al., 2015). The latter is in agreement with our result that the metal-herbicide mixture significantly predicted protein content of the diatom species.

TBARS content as well revealed to be affected by copper (Gioda et al., 2007; Ransberry et al., 2016) and triazine herbicides (Blahova et al., 2013; Stara et al., 2013, 2012). In our case TBARS content was significantly predicted by the metal copper in the case of the diatom *T. weissflogii* and by the herbicide Primextra® in the case of the copepod *A. tonsa*.

The metal copper (Filimonova et al., 2016a; Letelier et al., 2005) and two main active ingredients of the herbicide Primextra®: S-metolachlor and terbuthylazine, which belong to the groups of chloroacetanilide and triazine herbicides respectively (Filimonova et al., 2016a; Martins et al., 2011; Spoljaric et al., 2011) induce reactive oxygen species (ROS).

ROS attack the polyunsaturated fatty acids (including the essential FA) educing secondary products such as hydroperoxides or their aldehyde derivatives, which inhibit the protein synthesis (Repetto et al., 2012). Among them are malondialdehydes – biomarkers of lipid (ω3, ω6) peroxidation that is estimated by the amount of TBARS (Ayala et al., 2014).

A limited number of studies on the herbicide Primextra® have shown its effect on fatty acid profiles, including the essential FA (Filimonova et al., 2016a; Gonçalves et al., 2016; Neves et al., 2015). Effect on FA content of different marine and freshwater species was observed as well after exposure to S-metolachlor (Neves et al., 2015; Robert et al., 2007), triazine herbicides (Ali et al., 2015; De Hoop et al., 2013; Littlefield-Wyer et al., 2008), and copper (numerous studies reviewed by Filimonova et al. 2016a).

In our study only the total FA profile and the essential FA of the diatom species were not predicted significantly by any of the contaminant treatments. This could be due to the following features of the plant cells mentioned above: plant cells are able to synthesize PUFA, including EFA *de novo* and the cell wall in plant cells is important defence barrier against the potential stressors (Brett and Müller-Navarra, 1997; Keegstra, 2010). In addition, this result is in line with our previous results: absolute values (µg FA/cell) of the diatom's fatty acids did not change significantly in the majority of treatments related to the individual and mixture exposures of the herbicide and the metal (Filimonova et al, 2017).

Our modelling results revealed that nutritionally important biochemical parameters of the copepod *A. tonsa* were generally more sensitive to the chemical stressors than those of the diatom *T. weissflogii*. This is in agreement with a few available studies that documented that the terrestrial invertebrate *Eisenia fetida* (Chen et al., 2015) was more sensitive to the pesticide-metal mixture, i.e. synergism on the survival rate was observed, whereas the aquatic plant *Lemna minor* (Teisseire et al., 1999) and the aquatic algae *Chlorella ellipsoidea* (Aoyama et al., 1987) were more tolerant to the organic-copper mixtures, i.e. antagonism on the growth rate was evident.

In addition, the chemical mixture affected the essential FA of the copepod in a non-additive way but affected the essential FA of the diatom in an additive way.

In general, we conclude that the food quality of the species at the highest trophic level (here first-level consumers) was found to be more sensitive to the chemical stressors than the one at the lower trophic level, and that the contaminants mixture mostly acted non-additively on nutritionally important biochemical parameters. This result can have important consequences for the risk assessment of organic and inorganic chemical mixtures.

5.5.2. Comparison of the effects of chemical mixture between trophic levels

When species were exposed to the same levels of contamination, effects of the copper-Primextra® mixture on the essential FA content of both species were non-additive.

These results have important consequences since essential FA (ω3) determine the nutritional quality of algae (Ahlgren et al., 1990), and polyunsaturated FA – including the essential FA – are almost exclusively synthesized by plants. As referred above animals are able to convert EFA from other PUFA through elongation and desaturation but mostly are not able to synthesize them *de novo* (Brett and Müller-Navarra, 1997; De Troch et al., 2012) and therefore need to take up EFA from food.

A healthy food web requires adequate food quality in sufficient quantities. In an aquatic ecosystem, “good” quality phytoplankton lead to better quality of zooplankton and therefore to larger and more diverse fish populations (Kelble, 2012) with high nutritional values. Aquatic organisms have been and continue to be our primary source of readily available EFA (Arts et al., 2001), which have proven their effects in preventing/mitigating cardiovascular diseases, ontogenesis (particularly neural development), atherosclerosis, neural disorders, and, potentially, some cancers, as well as autoimmune diseases (Gladyshev et al., 2009).

No significant correlation was observed for PUFA of the copepod species with SFA and PUFA of the diatom species, whereas an opposite trend was revealed for saturated FA of *A. tonsa*. However, it was expected that the PUFA of the copepod species were significantly correlated with PUFA of the diatom species due to the fact mentioned above, namely that PUFA of the primary consumer species are usually taken up from food and that some PUFA (i.e. essential FA: 20:5ω3) are dietary tracers between diatom and copepod species (Arts et al., 2001; Kelly and Scheibling, 2012).

Similarly, non-significant correlation was expected between SFA of the copepod and diatom species, since SFA (i.e. 16:0) can be synthesized by both algae and animal cells *de novo* from acetyl-CoA and don’t depend on the food source availability.

In general, the food quality of the primary consumer *A. tonsa* was more sensitive to the chemical stressors than of the primary producer *T. weissflogii*, when species were exposed to the equal levels of contamination.

Therefore, there is the possibility that food quality decreases when moving up the food web, which would have important implications for the human diet.

5.6. Conclusions

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

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CHAPTER 6. General conclusions

6.1. General conclusions

In the present chapter we summarize the main conclusions of each chapter of this dissertation. Additionally, the main research findings are discussed and perspectives for future research are identified.

Chapter 2 – Fatty acid profiling as bioindicator of chemical stress in marine organisms: A review

The literature review proved fatty acids are good bioindicators to be applied at the assessment of contamination levels, resulting in an increased application of FA profiling in environmental studies in the last two decades. Furthermore, FA are essential to normal function of all marine organisms from all trophic levels. Its biosynthesis is inhibited when exposed to the reviewed pollutants, along with alterations in the processes of FA desaturation and elongations.

It is notorious in literature a lot of research attention spent to study the response of FA profiles in micro- and macro algae and fishes to pollutants impact. Little attention has been given to the alterations in FA composition of primary consumers in the presence of metals and/or pesticides. Noteworthy, most studies are focused on the FA profile's response to a single pollutant exposure (pesticide or metal). Only few studies about the mixture effects are available. Moreover, the mixtures referred to the contaminants only from one chemical group, i.e. two metals or group of pesticides, not the combination of different groups of chemicals.

Therefore, investigations on the species biochemical response (e.g. FA profiles) to combined exposure of different pollutants (e.g. pesticide and metal) is a potential research contributing to foresee future changes in the health of aquatic communities and in the energy flow through the related ecosystem and thus on trophic food webs and food quality. In addition, it has been stated by this review that future studies with primary consumers (i.e. zooplankton species) can contribute to obtain more comprehensive results to estimate the impacts of pollutants in the marine and estuarine environment.

These findings were taken into consideration in our further research.

Chapter 3 – Ecotoxicological and biochemical effects caused by individual exposure to a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species

Changes in the FA profiles of primary producer and primary consumers are observed when exposed to Primextra® Gold TZ and the metal copper, with an increase of saturated FA and a decrease of unsaturated FA contents. FA composition of zooplanktonic species responded with higher sensitivity to the individual exposures of both contaminants with *Artemia* sp. being the most sensitive to the applied stressors. Noteworthy, after copper exposure essential FA content were considerably reduced to the trace amounts in the brine shrimp nauplii (15 times) and totally disappeared in the copepod *A. tonsa* exposed to the highest concentration of the contaminant compared to the uncontaminated treatment. FA profile of phytoplankton species revealed higher tolerance to the individual exposures of the herbicide and the metal: slight changes were observed in SFA, MUFA and PUFA content along the concentration gradient, with slight decrease in diatom`s essential FA (1.3 and 1.2 times, respectively, compared to the control treatment).

Our results suggested that: (1) the herbicide Primextra® Gold TZ or other pesticides mainly composed by copper imply a threat to the estuarine and marine systems, being toxic to the investigated planktonic species; (2) alteration in species FA composition, specifically, the decrease in the amount essential FA, influence the health status of the ecosystem on the base of food web, i.e. primary producer – primary consumer, being more severe for the species from higher trophic level; (3) changes in FA profiles of organisms may be used as an early-warning indicator of anthropogenic stressors for the assessment of the health status of aquatic species.

Still, it is crucial to achieve the toxic and biochemical impact of both pollutants in natural systems, i.e. mixture effects, as normally the compounds occur. This statement was taken in consideration to the further works developed in this study.

Chapter 4 - Ecotoxicological and biochemical mixture effects of a herbicide and a metal on marine and estuarine phytoplankton and zooplankton species

A higher tolerance of the diatom *T. weissflogii* and a greater sensitivity of the copepod *A. tonsa* to the applied mixture of copper and Primextra® in terms of both, abundance and FA composition, was stated in this chapter.

Mixture effects revealed that the Cu-Pr mixture acted significantly synergistic on the copepod survival (relatively to the independent action model), while acted significantly antagonistic on the diatom growth (relatively to the concentration addition model).

The interactive mixture revealed a non-interactive effect on saturated FA of the diatom species. However, a synergistic effect was observed for each group of unsaturated FA: MUFA, PUFA and HUFA, including the essential FA: DHA and EPA, mostly at the intermediate levels of contamination. The opposite trend was observed in FA response of the copepod species. Individual and mixture exposure to copper and Primextra® have great effect on the FA composition of the copepod *A. tonsa* with the most harmful effect on the essential FA after exposure to the metal-herbicide mixture.

The overall observations suggest that applied metal-herbicide mixture may cause potential worse effect on the higher trophic levels than on primary producers that may lead to a decrease in available biomass and energy flow through the ecosystem and therefore its healthy status. Moreover, the observed synergism at the low effect levels on the survival of the copepod species indicated a potential ecotoxicological risk associated with a higher possibility of the co-occurrence of these chemicals at environmentally relevant concentrations.

Finally, the obtained results may contribute to future ecological risk assessment of potentially hazardous metal-herbicide mixtures on non-target species.

Furthermore, predict scenarios may be built applying appropriate statistical models which was used in the following work.

Chapter 5 – Effects of a herbicide-metal mixture on the food quality of marine and estuarine primary producers and primary consumers

In this chapter was concluded that the effects of the metal-herbicide mixture on the food quality of phytoplankton and zooplankton species were non-additive, i.e. the results of the generalized linear models fitted to the experimentally observed responses of biochemical composition (total FA, essential FA, protein and TBARS contents) to the single substances and the mixture, revealed that non-additive effects allow a better prediction of the mixture effect of the herbicide Primextra® and the metal copper on planktonic food quality, compared to a model that assumes purely additive effects. Furthermore, the model shows nutritionally important biochemical parameters of the copepod *A. tonsa* are generally more sensitive to the chemical stressors than those of the diatom *T. weissflogii*.

In addition, the chemical mixture affect the essential FA of the diatom in an additive way, but affect the essential FA of the copepod non-additively.

Generalized linear models revealed that effects of the copper-Primextra® mixture are non-additive not only for the essential FA content of the copepod but as well of the diatom species and that the food quality of the species from higher trophic level, i.e. primary consumer *A. tonsa* are more sensitive to the chemical stressors than of the primary producer *T. weissflogii*, when species are exposed to the equal levels of contamination.

Therefore, there is the possibility that food quality decreases when moving up the food web, which would have important implications for the food quality of the secondary consumers and subsequently for the human diet.

A joint approach applied in this study, i.e. controlled laboratory experiments (toxicity tests and microcosm bioassays), involving single and mixture exposures to chemical stressors with the further modelling of the obtained data was the first necessary step for the investigation of the effective concentrations of each compound on the key study species and their nutritionally biochemical parameters, improved our understanding of their mechanisms of action and gave valuable information concerning their adverse effect on the food quality of the species from higher trophic level (i.e. primary consumers).

Thus, the results of our study proved the earlier proposed research hypothesis: phtyo- and zooplankton species show different biochemical responses to the anthropogenic stressors, which are more severe for the species from the higher trophic level (i.e. for primary consumers) at the highest concentrations of the mixture exposure (i.e. for the essential FA of the copepod species) and therefore more sensitive for their food quality.

Therefore, the expected results were confirmed only for the primary consumer *Acartia tonsa*: (1) at the highest concentrations of the metal copper, the herbicide Primextra® and their equitoxic mixture FA concentrations, including the essential FA, decrease in the copepod species, however slightly increase in the diatom species; (2) a mixture of contaminants has stronger effect to *A. tonsa* than their individual exposure, i.e. synergism relatively to the independent action model is revealed, whereas for the *T. weissflogii* antagonism relatively to the concentration addition model is observed.

This could be explained based on the differences in the internal cell processes, in the morphology of the cell structure of both the diatom and copepod species and the modes of action of applied anthropogenic stressors.

Thus, the presented cell wall in the diatom cell structure plays an important role in the defense responses against potential stressors (Keegstra, 2010). Moreover, it is constituting by silica, which is known to give greater resistance to the herbicide transport (Ferreira et al., 2007).

In addition, diatom species may induce morphological and chemical defenses as a result of exposure to various xenobiotics (Debenest et al., 2010), i.e. an increase of the cell wall surface and its roughness in the presence of metals (i.e. copper) and herbicides (Rijstenbil et al., 1994), an induction of the formation of abnormal frustules and their disrupted orientation (Debenest et al., 2008). It may increase adsorption capacity of the contaminants by the diatom cell walls (Rijstenbil et al., 1994) and lead to more difficult penetration of the herbicide and metal molecules into diatom cell membranes, promoting the additional resistance of *T. weissflogii* to the applied copper and Primextra® treatments.

In addition, plant cells are able to synthesize PUFA and HUFA *de novo* (Brett and Müller-Navarra, 1997), whereas planktonic calanoid copepods have limited ability of FA bioconversion since they have a lack of the necessary enzymes to produce their significant amounts (De Troch et al., 2012; Tang and Taal, 2005).

A stronger effect of chemical mixture for the copepod species may be explained by the possible formation of the copper-terbuthylazine complexes, which are difficult to interact with the diatom cell wall and easy for a direct diffusion through the copepod cell membrane (Ahsanullah and Florence, 1984; Lyapchenko et al., 2004; Undabeytia et al., 1996; Wang et al., 2015; WHO, 2003).

The overall results have important consequences since copepods are the preferred natural prey of most marine fishes during the larval phase (Olivotto et al., 2012) and the essential FA are critical for the juvenile fish`s growth, disease resistance, and general well being (Brett et al., 2009). Larvae of many marine fishes are believed to require HUFA of the ω3 series such as eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3). Their deficiency can cause a general decrease in larval health, poor growth, low feeding efficiency, anemia and high mortality and negatively affects fecundity, fertilization and hatching rates of the adult fish populations (Olivotto et al., 2012; Rainuzzo et al., 1997). Aquatic organisms have been and continue to be our primary source of readily available EFA (Arts et al., 2001), which have proven their effects in preventing and mitigating cardiovascular diseases, ontogenesis, atherosclerosis, neural disorders, and, potentially, some cancers, as well as autoimmune diseases (Gladyshev et al., 2009). Therefore, the presence of EFA 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.

Although the Mondego estuary is still considered to be low contaminated by the metal copper and the active ingredients of the herbicide Primextra®, S-metolachlor and TBA (Cruzeiro et al., 2016; Vasconcelos et al., 2011), there is a tendency for such an adverse effect on the food quality of planktonic species to occur, as a result of the continuous intensive agriculture practices applied near this ecosystem.

This is also true for other aquatic basins, in the EU and worldwide, with already registered high concentration levels of the investigated chemical stressors, resulting from the continuous anthropogenic impacts to those aquatic ecosystems (Brix et al., 2006; Gerecke et al., 2002; Konstantinou et al., 2006; Lekkas et al., 2004; Mai et al., 2012; Nwani et al., 2014; Stoate et al., 2009).

Our applied approach along with the obtained results, may serve as a reference point for further studies concerning the determination of the effects on the food quality of species from higher trophic levels (i.e. secondary consumers), especially of those with high economic value in the Mondego region.

Further research should be conducted to determine the effects of the studied stressors and others on nutritional biochemical parameters (e.g. fatty acid profiles, carbohydrates, amino acids and protein contents) of organisms from different trophic levels, relating it with contaminant`s concentrations in the Mondego estuary. All this information significantly contributes to an objective environmental decision-making by stakeholders, politicians and researchers in order to prevent further and harmful impacts on this estuarine basin, and on the human diet and health. These studies are crucial to be developed to estuarine systems threatened by anthropogenic stressors in order to evaluate the health status of the systems and the quality food of their resources and thus, to predict the impacts along the trophic food web and, at last, in human beings. Based on this information, acting plans may be implemented to prevent or reduce the impact of stressors and thus contributing to healthy aquatic systems.

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ANNEXES

Table A1. Summary of nominal and measured concentrations of copper and Primextra® and the treatment combinations used, where Cu – copper, Pr - Primextra® Gold TZ, NA – not applicable.

Treatment type/species	Nominal values, mg/L		Measured values, mg/L		Relative growth/ relative survival, %
	Cu	Pr	Cu	Pr	
Diatom *T. weissflogii*					
Metal copper single dose treatments					
	0.0049	NA	0.0133	NA	82
	0.0107	NA	0.0260	NA	80
	0.0224	NA	0.0420	NA	72
	0.0488	NA	0.0519	NA	72
	0.1073	NA	0.1056	NA	4
	0.2444	NA	0.2089	NA	0
	0.2247	NA	0.2051	NA	0
Herbicide Primextra® single dose treatments					
	NA	0.0003	NA	0.0021	95
	NA	0.0008	NA	0.0060	90
	NA	0.0016	NA	0.0179	78
	NA	0.0035	NA	0.0256	61
	NA	0.0076	NA	0.0555	29
	NA	0.0159	NA	0.1057	13
	NA	0.0159	NA	0.1456	13
Equitoxic mixture treatments					
	0.0049	0.0003	0.0103	0.0042	73
	0.0107	0.0008	0.0176	0.0098	68
	0.0224	0.0016	0.0274	0.0190	67
	0.0488	0.0035	0.0499	0.0238	68
	0.1073	0.0076	0.0926	0.0529	13
	0.2444	0.0159	0.1593	0.1326	2
	0.2247	0.0159	0.1349	0.1146	2
Copepod *A. tonsa*					
Metal copper single dose treatments					
	0.0024	NA	0.0076	NA	100
	0.0054	NA	0.0067	NA	100
	0.0112	NA	0.0135	NA	93
	0.0244	NA	0.0291	NA	73
	0.0537	NA	0.0575	NA	60
	0.1124	NA	0.1234	NA	40
	0.1125	NA	0.1290	NA	40

Table A1. Continued.

Herbicide Primextra® single dose treatments					
	NA	0.0651	NA	0.2392	100
	NA	0.1432	NA	0.3197	100
	NA	0.2995	NA	0.5834	93
	NA	0.6510	NA	1.3496	93
	NA	1.4322	NA	2.7714	87
	NA	2.9946	NA	5.6160	47
	NA	2.9979	NA	5.3702	7
Equitoxic mixture treatments					
	0.0024	0.0651	0.0060	0.1591	93
	0.0054	0.1432	0.0090	0.3135	80
	0.0112	0.2995	0.0149	0.6191	80
	0.0244	0.6510	0.0259	1.1968	47
	0.0537	1.4322	0.0559	2.6178	40
	0.1124	2.9946	0.1136	5.6933	0
	0.1125	2.9979	0.1098	5.5879	0

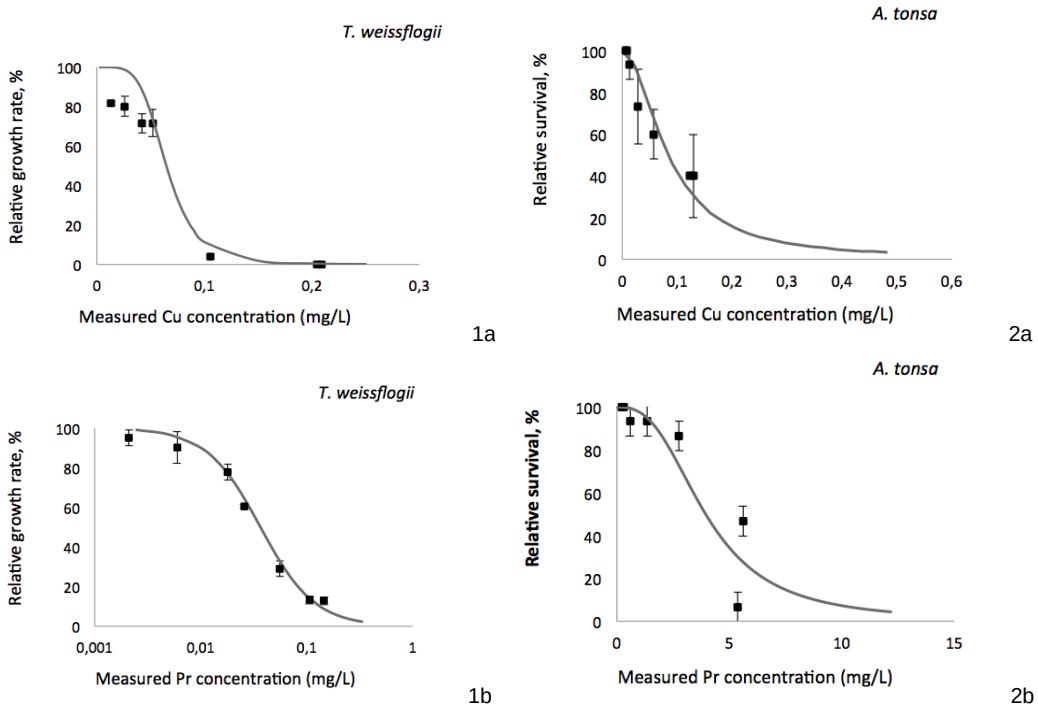

Fig. A1. Single stressor concentration response curves: single measured copper concentration (a) and single measured Primextra® concentration (b) for relative growth rate (%, symbols) of *T. weissflogii* (1) and relative survival (%, symbols) of *A. tonsa* (2). Error bars indicate standard errors. Standard error values for some relative growth rate or relative survival data points are smaller than the symbol size.

Table A2. Estimated model parameters for the mixture reference models CA and IA fitted to Cu-Pr mixture (96h-) growth rate data of diatom *T. weissflogii* and to (48h-) survival data of copepod *A. tonsa* (single and mixture data)

Species	Parameters	CA-non interaction		CA mixture interacions		IA-non interaction		IA mixture interacions	
		Pr	Cu	Pr	Cu	Pr	Cu	Pr	Cu
T. weissflogii	EC_{50} (mg/L)	0.04	0.07	0.04	0.06	0.04	0.07	0.03	0.06
	β	1.24	2.74	1.26	3.49	1.30	3.83	1.11	3.34
	a	-		2.06		-		0.70	
	AIC	171.11		167.64		167.35		168.87	
	SSE	2639.46		2034.54		2207.36		2157.67	
	F-test	p=0.04				p=0.55			
A. tonsa	EC_{50} (mg/L)	4.17	0.07	4.36	0.09	3.80	0.07	4.13	0.09
	β	4.05	1.04	4.42	1.23	2.93	0.99	3.94	1.23
	a	-		-1.30		-		-2.31	
	AIC	164.81		164.41		168.46		161.93	
	SSE	1955.74		1744.80		2327.43		1549.91	
	F-test	p=0.18				p=0.01			

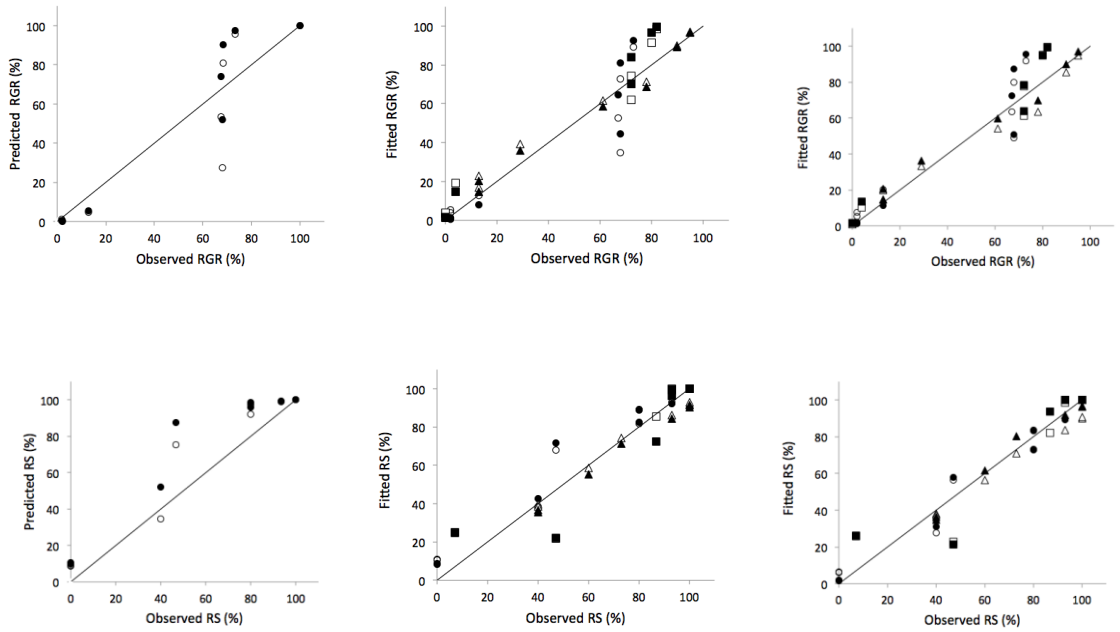

Fig. A2. Observed relative growth rate (%) of diatom species versus the predicted or fitted relative growth rate (upper panels) and observed relative survival (%) of copepod species versus the predicted or fitted relative survival for the mixture reference models concentration addition (open symbols) and independent action (closed symbols) after exposure to equitoxic mixture of copper and Primextra®. Left panel shows model predictions based on parameters of the mixture contaminant exposure, middle panels shows models fitted to all data (single and mixture treatment), right panel shows models extended with interactive effects parameter fitted to all data.
RGR = relative growth rate; RS = relative survival; square symbols – single copper exposure; triangle symbols – single Primextra® exposure; circle symbols – mixture exposure.

Table A3. FA profiles (absolute concentration in 10^{-9} µg FA/1 cell) of marine diatom *T. weissflogii* after exposure to copper (II) sulphate pentahydrate, Primextra® Gold TZ and their equitoxic mixture. CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3

FA profile		Copper (II) sulphate pentahydrate				Primextra® Gold TZ				Equitoxic mixture of copper and Primextra®			
		CTL	C1	C2	C3	CTL	C1	C2	C3	CTL	C1	C2	C3
SFA	C 14:0	1263	2148	1983	1674	1217	1314	1836	1334	1381	1981	1586	1346
	C 15:0	143	259	236	191	142	147	216	154	164	221	173	144
	C 16:0	3467	4595	4393	3671	3016	3285	4079	3007	4349	5246	4134	3614
	C 17:0	165	252	262	235	158	184	253	183	220	278	230	203
	C 18:0	3118	1910	2068	2208	2107	2741	2744	2103	4486	4240	3736	3657
	C 20:0	54	26	32	29	31	42	45	27	80	75	68	59
	C 22:0	0	0	0	0	0	0	0	0	0	0	0	0
	C 24:0	141	182	183	178	129	133	184	131	162	167	134	125
	Total SFA	**8352**	**9372**	**9157**	**8187**	**6799**	**7846**	**9356**	**6940**	**10841**	**12208**	**10060**	**9148**
MUFA	C16:1ω9	234	297	299	282	246	272	385	284	274	330	314	303
	C16:1ω7	1439	3812	3396	2667	1565	1617	2320	1675	1597	3094	2243	1856
	C16:1ω5	121	172	172	134	130	127	186	132	129	150	114	83
	C 18:1ω9 trans	98	99	93	87	89	66	95	69	103	108	86	93
	C 18:1ω7 trans	71	49	55	47	71	76	112	83	93	62	63	49
	C 20:1ω9	0	0	0	0	0	0	0	0	0	0	0	0
	C 22:1ω9	0	0	0	0	0	0	0	0	0	0	0	0
	Total MUFA	**1963**	**4429**	**4015**	**3217**	**2102**	**2159**	**3099**	**2243**	**2196**	**3744**	**2820**	**2383**
PUFA	C 16:2ω6	10	17	16	14	11	12	18	13	11	19	16	15
	C 16:2ω4	54	108	106	95	56	59	84	61	53	106	83	74
	C 16:3ω3	172	265	265	234	186	203	279	214	195	292	259	230
	C 18:2ω6 trans	122	167	155	165	128	145	181	126	147	190	177	201
	C 18:2ω6 cis	0	0	0	0	0	0	0	0	0	0	0	0
	C 18:3ω6	0	0	0	0	0	0	0	0	0	0	0	0
	C 18:3ω3	0	0	0	0	0	0	0	0	0	0	0	0
	Total PUFA	**358**	**556**	**543**	**507**	**382**	**419**	**562**	**413**	**407**	**606**	**536**	**520**

Table A3. Continued

HUFA	C 20:4ω6 (ARA)	0	0	0	0	0	0	0	0	0	0	0	0
	C 20:5ω3 (EPA)	2967	5242	5331	4689	3164	3253	4440	3323	3513	4977	4063	3523
	C 22:6ω3 (DHA)	640	982	1042	875	666	678	961	696	784	909	732	627
	Total HUFA	**3607**	**6224**	**6373**	**5563**	**3830**	**3931**	**5401**	**4019**	**4296**	**5886**	**4795**	**4149**
	Total FA	**14279**	**20581**	**20088**	**17475**	**13113**	**14354**	**18418**	**13615**	**17740**	**22445**	**18212**	**16200**

Table A4. FA profiles (absolute concentration in 10^{-4} µg FA/1 individual) of estuarine copepod *A. tonsa* after exposure to copper (II) sulphate pentahydrate, Primextra® Gold TZ and their equitoxic mixture. CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3

FA profile		**Copper (II) sulphate pentahydrate**				**Primextra® Gold TZ**				**Equitoxic mixture of copper and Primextra®**			
		CTL	C1	C2	C3	CTL	C1	C2	C3	CTL	C1	C2	C3
SFA	C 14:0	1216	1146	744	807	599	599	549	315	1037	617	403	226
	C 15:0	119	108	68	68	111	60	54	36	126	70	44	28
	C 16:0	3563	3295	2282	2752	3843	2946	2208	1994	2309	1642	1490	1452
	C 17:0	41	37	28	40	66	56	36	38	32	26	27	31
	C 18:0	2003	1756	1462	2162	3796	3595	2203	2561	1338	1393	1585	2015
	C 20:0	47	43	31	47	79	69	41	45	32	27	29	36
	C 22:0	45	49	33	33	49	32	25	20	36	21	12	8
	C 24:0	108	116	81	79	91	52	55	39	112	69	48	28
	Total SFA	**7143**	**6549**	**4728**	**5988**	**8635**	**7409**	**5172**	**5049**	**5023**	**3866**	**3638**	**3824**
MUFA	C16:1ω9	53	53	31	13	43	21	18	0	85	43	25	19
	C16:1ω7	2152	2174	1190	1287	1522	736	718	306	1878	932	608	308
	C16:1ω5	55	59	32	32	55	27	27	0	62	39	25	0
	C 18:1ω9 trans	93	101	68	88	152	105	80	51	111	74	68	71
	C 18:1ω7 trans	314	342	184	191	447	193	205	110	340	181	120	73
	C 20:1ω9	24	28	15	10	33	14	14	0	24	0	0	0
	C 22:1ω9	50	60	32	34	81	36	33	0	67	35	9	0
	Total MUFA	**2741**	**2817**	**1553**	**1655**	**2332**	**1133**	**1095**	**466**	**2568**	**1304**	**855**	**471**
PUFA	C 16:2ω6	6	6	3	2	4	0	1	0	10	3	1	0
	C 16:2ω4	26	23	12	10	18	6	7	2	32	14	7	2

Table A4. Continued.

	C 16:3ω3	77	72	38	19	48	12	15	4	120	45	19	2
	C 18:2ω6 trans	96	102	63	87	144	99	70	55	94	66	65	64
	C 18:2ω6 cis	20	22	0	0	22	0	0	0	31	15	0	0
	C 18:3ω6	38	39	18	28	34	16	14	0	36	0	0	0
	C 18:3ω3	0	0	0	0	10	0	0	0	27	0	0	0
	Total PUFA	**263**	**263**	**135**	**146**	**280**	**134**	**107**	**61**	**349**	**144**	**91**	**68**
HUFA	C 20:4ω6 (ARA)	89	109	62	88	130	89	79	46	70	85	49	18
	C 20:5ω3 (EPA)	2131	2049	1131	901	2057	925	1032	522	2845	1659	976	406
	C 22:6ω3 (DHA)	898	862	518	564	1131	664	710	512	1446	971	622	413
	Total HUFA	**3118**	**3020**	**1711**	**1553**	**3318**	**1678**	**1822**	**1080**	**4362**	**2714**	**1647**	**838**
	Total FA	**13265**	**12649**	**8127**	**9342**	**14565**	**10354**	**8195**	**6657**	**12301**	**8028**	**6231**	**5200**

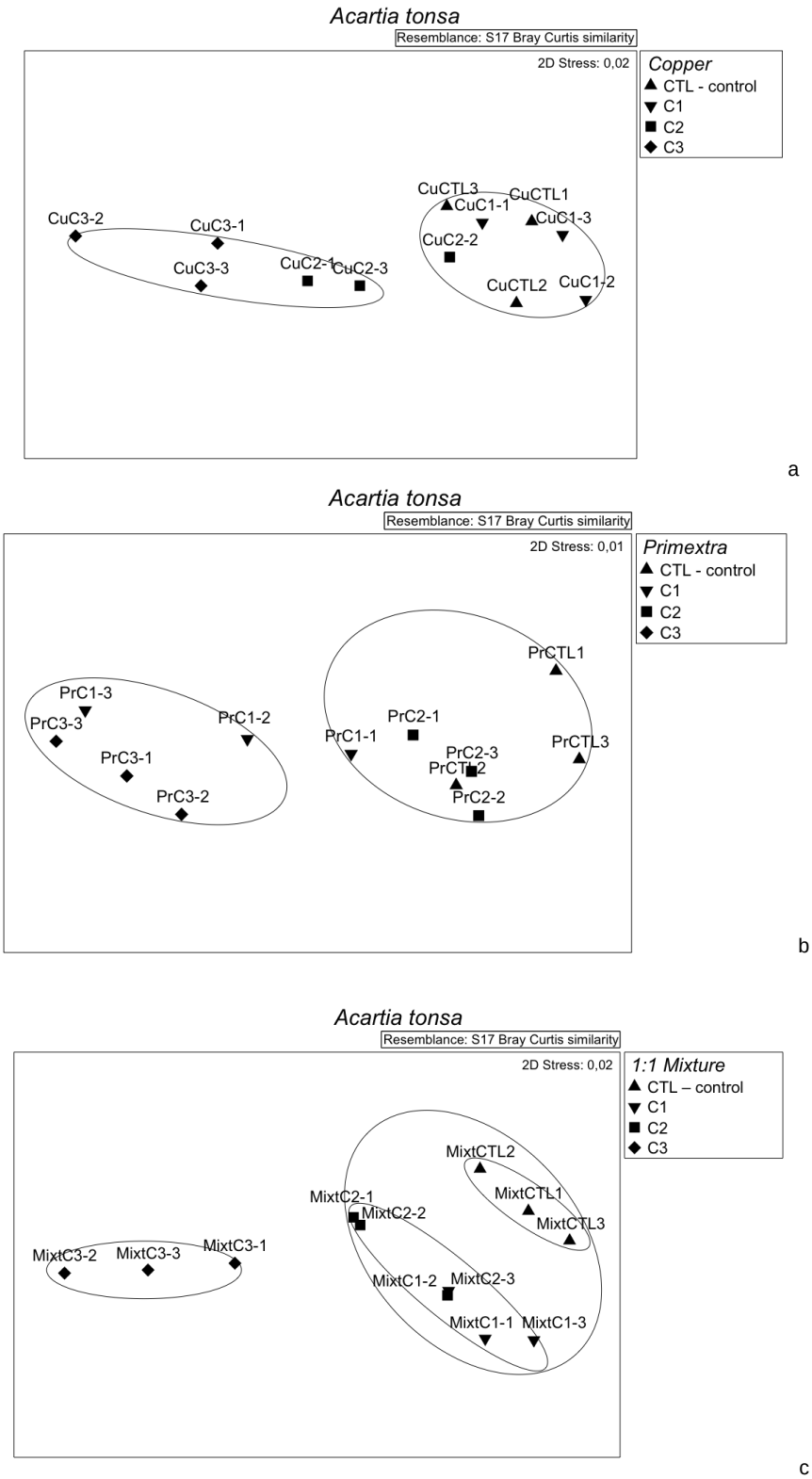

Fig. A3. – Two-dimensional non-metric MDS ordination plots of copepod FA profiles exposed to copper (II) sulphate pentahydrate – Cu (a) and to the herbicide Primextra® Gold TZ – Pr (b) and their equitoxic mixture – Mixt (c): CTL, C1, C2, C3 – treatments, referring to the uncontaminated treatment (not inverted triangles) and the low, the intermediate and the high levels of contaminants, where CTL<C1<C2<C3

Table A5. Summary of two-way ANOVA for FA profiles (log10) of diatom *T. weissflogii* with three levels of contamination (C1<C2<C3). Significant p-values at the 95% significance level are shown in bold. If significant copper × Primextra® interactions were detected, the observed effect in the mixture treatment (Eq. (8)), the predicted effect based on the Independent Action model (Eq. (7)) and the interaction type are also provided.

FA	Contamination level	p	Oberved Effect, $E_{CuPr\ Observed}$	Predicted Effect, $E_{CuPr\ Predicted}$	Interaction type
SFA	C1	0,092			non-interactive
	C2	0,952			non-interactive
	C3	0,147			non-interactive
MUFA	C1	**0,050**	0,01	-0,01	synergism
	C2	**0,000**	-0,01	-0,07	synergism
	C3	0,173			non-interactive
PUFA	C1	0,971			non-interactive
	C2	**0,015**	0,01	-0,03	synergism
	C3	0,996			non-interactive
HUFA	C1	0,520			non-interactive
	C2	**0,001**	-0,01	-0,05	synergism
	C3	0,220			non-interactive
14:0	C1	0,251			non-interactive
	C2	**0,002**	-0,01	-0,05	synergism
	C3	0,312			non-interactive
16:0	C1	0,194			non-interactive
	C2	0,370			non-interactive
	C3	0,366			non-interactive
18:0	C1	**0,009**	0,048	0,010	synergism
	C2	**0,042**	0,037	-0,014	synergism
	C3	**0,012**	0,036	0,065	antagonism
16:1ω7	C1	**0,024**	0,01	-0,01	synergism
	C2	**0,000**	-0,01	-0,08	synergism
	C3	0,116			non-interactive
20:5ω3	C1	0,493			non-interactive
(EPA)	C2	**0,001**	-0,01	-0,05	synergism
	C3	0,214			non-interactive
22:6ω3	C1	0,635			non-interactive
(DHA)	C2	**0,000**	-0,01	-0,04	synergism
	C3	0,241			non-interactive

Table A6. FA profiles (absolute concentration µg FA/1 cell) of marine diatom *T. weissflogii* after exposure to copper (II) sulphate pentahydrate copper (CuSO4.5H2O) represented as "CuSP", Primextra® Gold TZ indicated as "Pr" and their equitoxic mixture (mg/L).

CuSP	Pr	14:0	15:0	16:0	16:1ω9	16:1ω7	16:1ω5	17:0
0	0	0.00000121870062	0.00000015885304	0.00000395148548	0.00000022798325	0.00000132510402	0.00000011033481	0.00000017336475
0	0	0.00000119506579	0.00000012630131	0.00000313803809	0.00000021230702	0.00000138495421	0.00000011327295	0.00000014582419
0	0	0.00000137458870	0.00000014252632	0.00000331146606	0.00000026093519	0.00000160781321	0.00000013810727	0.00000017726715
0.1793	0	0.00000231447398	0.00000029186829	0.00000497475923	0.00000034453968	0.00000392496696	0.00000019893912	0.00000028529901
0.1793	0	0.00000213020800	0.00000024892711	0.00000435908364	0.00000027815124	0.00000391243711	0.00000015855001	0.00000023791719
0.1793	0	0.00000200025697	0.00000023472507	0.00000445154217	0.00000026794845	0.00000359875460	0.00000015922645	0.00000023378971
0.2159	0	0.00000201307063	0.00000023462980	0.00000419435598	0.00000033001407	0.00000336670562	0.00000016974824	0.00000027239206
0.2159	0	0.00000166333160	0.00000020579104	0.00000369709260	0.00000024992729	0.00000272139293	0.00000015803645	0.00000022148587
0.2159	0	0.00000227110050	0.00000026705965	0.00000528757282	0.00000031826188	0.00000410073365	0.00000018691298	0.00000029346814
0.2962	0	0.00000125082053	0.00000014102725	0.00000307117900	0.00000023174834	0.00000171862808	0.00000010054954	0.00000019670430
0.2962	0	0.00000213623663	0.00000024710498	0.00000454293476	0.00000034187789	0.00000368697482	0.00000016512963	0.00000028810986
0.2962	0	0.00000163570130	0.00000018347205	0.00000339913751	0.00000027168616	0.00000259676136	0.00000013533239	0.00000021973912
0	0	0.00000120933369	0.00000012975605	0.00000308229932	0.00000026216314	0.00000148551240	0.00000012455665	0.00000017256965
0	0	0.00000121097046	0.00000014847677	0.00000297099601	0.00000023707933	0.00000160293606	0.00000013340316	0.00000015848467
0	0	0.00000123079069	0.00000014642015	0.00000299387535	0.00000023794265	0.00000160769405	0.00000013311151	0.00000014428622
0	0.0033	0.00000143377767	0.00000016695312	0.00000343004033	0.00000029858913	0.00000183016239	0.00000014427155	0.00000021311288
0	0.0033	0.00000116155442	0.00000011273715	0.00000293060943	0.00000024848618	0.00000135522195	0.00000010951638	0.00000016788037
0	0.0033	0.00000134581760	0.00000016018049	0.00000349509577	0.00000026915890	0.00000166676783	0.00000012796283	0.00000017146955
0	0.0039	0.00000138546711	0.00000016182349	0.00000327164657	0.00000029121920	0.00000182837337	0.00000014471785	0.00000019273914
0	0.0039	0.00000227389621	0.00000027479555	0.00000502285443	0.00000047508921	0.00000273958063	0.00000021853803	0.00000031010883
0	0.0039	0.00000184857561	0.00000021122430	0.00000394162433	0.00000038804100	0.00000239341246	0.00000019451880	0.00000025534022
0	0.0054	0.00000116329078	0.00000013608031	0.00000297343685	0.00000024159403	0.00000144710796	0.00000011607466	0.00000015924431
0	0.0054	0.00000096639874	0.00000011353460	0.00000219077455	0.00000020591072	0.00000121843804	0.00000009622328	0.00000013556290
0	0.0054	0.00000187264387	0.00000021102663	0.00000385789910	0.00000040473358	0.00000236051975	0.00000018230997	0.00000025434477
0	0	0.00000127548401	0.00000013024565	0.00000370180679	0.00000026487310	0.00000142090466	0.00000012267188	0.00000020935750
0	0	0.00000141938616	0.00000017749113	0.00000459360919	0.00000028372914	0.00000156250926	0.00000012793412	0.00000023088578
0	0	0.00000144776164	0.00000018545889	0.00000475163170	0.00000027211204	0.00000180687939	0.00000013718108	0.00000022094532
0.1793	0.0033	0.00000212478992	0.00000023971071	0.00000579433734	0.00000036399951	0.00000328958477	0.00000015961695	0.00000031435748
0.1793	0.0033	0.00000187686239	0.00000020740871	0.00000492452719	0.00000031267918	0.00000290512825	0.00000014234483	0.00000025704576
0.1793	0.0033	0.00000194067102	0.00000021456930	0.00000501962492	0.00000031229047	0.00000308703662	0.00000014874261	0.00000026255968
0.2159	0.0039	0.00000183951959	0.00000020496274	0.00000499262905	0.00000035128680	0.00000280053977	0.00000013255144	0.00000027494852
0.2159	0.0039	0.00000143352798	0.00000015910514	0.00000370955486	0.00000029925515	0.00000189850887	0.00000011302253	0.00000021853058
0.2159	0.0039	0.00000148462406	0.00000015462297	0.00000369912067	0.00000029077891	0.00000203037476	0.00000009708359	0.00000019531334
0.2962	0.0054	0.00000138343066	0.00000014517204	0.00000383262049	0.00000031509178	0.00000185289328	0.00000008191088	0.00000021709986
0.2962	0.0054	0.00000144858465	0.00000015523730	0.00000373128634	0.00000032912213	0.00000201192091	0.00000009350073	0.00000022045017
0.2962	0.0054	0.00000120501519	0.00000013224176	0.00000327852526	0.00000026487588	0.00000170188522	0.00000007226397	0.00000017064777

Table A6 (Continued)

CuSP	Pr	16:2ω6	16:2ω4	18:0	16:3ω3	18:1ω9 tr	18:1ω7	18:2ω6 tr
0	0	0.00000000826494	0.00000004885496	0.00000399882239	0.00000015623891	0.00000013142342	0.00000007436621	0.00000013589968
0	0	0.00000001012199	0.00000005195865	0.00000274916758	0.00000016787031	0.00000008047568	0.00000006683616	0.00000011489921
0	0	0.00000001039719	0.00000006089697	0.00000260746307	0.00000019251852	0.00000008096734	0.00000007309805	0.00000011507593
0.1793	0	0.00000001869213	0.00000011502962	0.00000223215812	0.00000028016501	0.00000012346236	0.00000005775140	0.00000019418796
0.1793	0	0.00000001686935	0.00000011046002	0.00000155499814	0.00000027183799	0.00000007493964	0.00000004033914	0.00000016016126
0.1793	0	0.00000001429096	0.00000009724662	0.00000194169813	0.00000024155160	0.00000009762491	0.00000004874805	0.00000014784643
0.2159	0	0.00000001740655	0.00000011482749	0.00000177588131	0.00000028257067	0.00000009782836	0.00000005675114	0.00000018230586
0.2159	0	0.00000001385377	0.00000009119323	0.00000183685260	0.00000023561370	0.00000007085487	0.00000005726168	0.00000010873904
0.2159	0	0.00000001635497	0.00000011329366	0.00000259112301	0.00000027770311	0.00000011004984	0.00000005060108	0.00000017398498
0.2962	0	0.00000001091441	0.00000007115590	0.00000256529385	0.00000018815167	0.00000009068986	0.00000004095764	0.00000014231150
0.2962	0	0.00000001648982	0.00000012300167	0.00000224591984	0.00000028621809	0.00000009666770	0.00000005479720	0.00000020164968
0.2962	0	0.00000001311519	0.00000009153748	0.00000181407002	0.00000022678653	0.00000007424548	0.00000004586753	0.00000015036634
0	0	0.00000001017316	0.00000005639081	0.00000243261325	0.00000019160878	0.00000008578777	0.00000007719975	0.00000013490262
0	0	0.00000001154447	0.00000005641846	0.00000197845393	0.00000018282176	0.00000009841125	0.00000006738055	0.00000013047203
0	0	0.00000001161970	0.00000005593728	0.00000190932468	0.00000018500029	0.00000008358695	0.00000006777615	0.00000012000547
0	0.0033	0.00000001253585	0.00000006597788	0.00000270860971	0.00000022864734	0.00000007788928	0.00000008765168	0.00000015461667
0	0.0033	0.00000001183726	0.00000005128541	0.00000254622361	0.00000018234988	0.00000005323198	0.00000007217903	0.00000013606104
0	0.0033	0.00000001261825	0.00000005889325	0.00000296873627	0.00000019775739	0.00000006709142	0.00000006844947	0.00000014403492
0	0.0039	0.00000001184373	0.00000006279904	0.00000225592076	0.00000021774913	0.00000007792250	0.00000008737693	0.00000013059542
0	0.0039	0.00000002294460	0.00000010259203	0.00000359533814	0.00000033293480	0.00000012009382	0.00000013574008	0.00000023509240
0	0.0039	0.00000001925411	0.00000008561057	0.00000238050299	0.00000028625899	0.00000008848154	0.00000011320515	0.00000017734306
0	0.0054	0.00000000973477	0.00000005137526	0.00000235833585	0.00000017863131	0.00000006504419	0.00000006921827	0.00000010669010
0	0.0054	0.00000000993813	0.00000004361179	0.00000158698673	0.00000015858910	0.00000004983374	0.00000005565969	0.00000008875506
0	0.0054	0.00000001881183	0.00000008744832	0.00000236473083	0.00000030430621	0.00000009330843	0.00000012379178	0.00000018215260
0	0	0.00000001195218	0.00000004770727	0.00000360362902	0.00000018179214	0.00000008971070	0.00000010262995	0.00000013709220
0	0	0.00000001090550	0.00000005302453	0.00000510465722	0.00000019668396	0.00000011124885	0.00000009439665	0.00000015686774
0	0	0.00000001115478	0.00000005928945	0.00000474865434	0.00000020695021	0.00000010900130	0.00000008210009	0.00000014639812
0.1793	0.0033	0.00000002059502	0.00000011302467	0.00000482488456	0.00000031491379	0.00000012565596	0.00000006700015	0.00000021235500
0.1793	0.0033	0.00000001808065	0.00000009802737	0.00000397826450	0.00000027481093	0.00000009210709	0.00000005084248	0.00000017767821
0.1793	0.0033	0.00000001868382	0.00000010740155	0.00000391728631	0.00000028521389	0.00000010741884	0.00000006835599	0.00000017850259
0.2159	0.0039	0.00000001922924	0.00000009865649	0.00000446652175	0.00000028861026	0.00000009973775	0.00000005914517	0.00000018884309
0.2159	0.0039	0.00000001289956	0.00000007096908	0.00000335400107	0.00000024155914	0.00000008386494	0.00000005310889	0.00000016416595
0.2159	0.0039	0.00000001663824	0.00000008075145	0.00000338837413	0.00000024824178	0.00000007471955	0.00000007625765	0.00000017809998
0.2962	0.0054	0.00000001552548	0.00000007111812	0.00000404357947	0.00000023205482	0.00000009394153	0.00000004796476	0.00000022025613
0.2962	0.0054	0.00000001750940	0.00000008558397	0.00000364463357	0.00000024730681	0.00000009653954	0.00000004482166	0.00000020819839
0.2962	0.0054	0.00000001190921	0.00000006630379	0.00000328247389	0.00000020999443	0.00000008719437	0.00000005449077	0.00000017439891

Table A6. Continued.

CuSP	Pr	18:2ω6 cis	20:0	18:3ω6	18:3ω3	20:1ω9	22:0	20:4ω6 (ARA)
0	0	0	0.00000007155332	0	0	0	0	0
0	0	0	0.00000004404560	0	0	0	0	0
0	0	0	0.00000004673266	0	0	0	0	0
0.1793	0	0	0.00000003641363	0	0	0	0	0
0.1793	0	0	0.00000001470698	0	0	0	0	0
0.1793	0	0	0.00000002691173	0	0	0	0	0
0.2159	0	0	0.00000002739931	0	0	0	0	0
0.2159	0	0	0.00000002518585	0	0	0	0	0
0.2159	0	0	0.00000004443907	0	0	0	0	0
0.2962	0	0	0.00000003727116	0	0	0	0	0
0.2962	0	0	0.00000003313733	0	0	0	0	0
0.2962	0	0	0.00000001769170	0	0	0	0	0
0	0	0	0.00000003658477	0	0	0	0	0
0	0	0	0.00000002862002	0	0	0	0	0
0	0	0	0.00000002725291	0	0	0	0	0
0	0.0033	0	0.00000004239044	0	0	0	0	0
0	0.0033	0	0.00000003947459	0	0	0	0	0
0	0.0033	0	0.00000004370580	0	0	0	0	0
0	0.0039	0	0.00000003202444	0	0	0	0	0
0	0.0039	0	0.00000006588334	0	0	0	0	0
0	0.0039	0	0.00000003683539	0	0	0	0	0
0	0.0054	0	0.00000002990308	0	0	0	0	0
0	0.0054	0	0.00000001506233	0	0	0	0	0
0	0.0054	0	0.00000003669062	0	0	0	0	0
0	0	0	0.00000006162013	0	0	0	0	0
0	0	0	0.00000009535440	0	0	0	0	0
0	0	0	0.00000008158545	0	0	0	0	0
0.1793	0.0033	0	0.00000008353870	0	0	0	0	0
0.1793	0.0033	0	0.00000007072832	0	0	0	0	0
0.1793	0.0033	0	0.00000007209764	0	0	0	0	0
0.2159	0.0039	0	0.00000008351281	0	0	0	0	0
0.2159	0.0039	0	0.00000005734188	0	0	0	0	0
0.2159	0.0039	0	0.00000006322220	0	0	0	0	0
0.2962	0.0054	0	0.00000006608857	0	0	0	0	0
0.2962	0.0054	0	0.00000006017805	0	0	0	0	0
0.2962	0.0054	0	0.00000005069308	0	0	0	0	0

Table A6. Continued.

CuSP	Pr	22:1ω9	20:5ω3 (EPA)	24:0	22:6ω3 (DHA)
0	0	0	0.00000273658658	0.00000013681369	0.00000060245406
0	0	0	0.00000283683903	0.00000012185998	0.00000061931730
0	0	0	0.00000332707704	0.00000016568344	0.00000069880754
0.1793	0	0	0.00000529981889	0.00000019120395	0.00000102151223
0.1793	0	0	0.00000549841716	0.00000018346569	0.00000100868238
0.1793	0	0	0.00000492738019	0.00000017188481	0.00000091673694
0.2159	0	0	0.00000558108945	0.00000022619757	0.00000111903894
0.2159	0	0	0.00000456810093	0.00000015630488	0.00000091094580
0.2159	0	0	0.00000584388796	0.00000016630667	0.00000109549925
0.2962	0	0	0.00000363609070	0.00000015254175	0.00000069976747
0.2962	0	0	0.00000595510731	0.00000022375824	0.00000108628041
0.2962	0	0	0.00000447456876	0.00000015908126	0.00000083844809
0	0	0	0.00000330376815	0.00000013377086	0.00000069483838
0	0	0	0.00000309369468	0.00000012355035	0.00000064676290
0	0	0	0.00000309435223	0.00000013000232	0.00000065622119
0	0.0033	0	0.00000360350086	0.00000014346654	0.00000077264775
0	0.0033	0	0.00000298781884	0.00000012129226	0.00000060023327
0	0.0033	0	0.00000316744897	0.00000013407521	0.00000066136142
0	0.0039	0	0.00000351224907	0.00000013002186	0.00000073551177
0	0.0039	0	0.00000514035194	0.00000022791894	0.00000111885511
0	0.0039	0	0.00000466661878	0.00000019323336	0.00000103009881
0	0.0054	0	0.00000279046801	0.00000010821953	0.00000056377990
0	0.0054	0	0.00000236643958	0.00000008655175	0.00000046721243
0	0.0054	0	0.00000481127853	0.00000019817443	0.00000105686873
0	0	0	0.00000338611729	0.00000017160947	0.00000077370090
0	0	0	0.00000354037843	0.00000015477609	0.00000079181237
0	0	0	0.00000361122242	0.00000015831088	0.00000078595274
0.1793	0.0033	0	0.00000544237437	0.00000018462445	0.00000102266697
0.1793	0.0033	0	0.00000466278946	0.00000014239804	0.00000084340762
0.1793	0.0033	0	0.00000482710114	0.00000017374048	0.00000086109973
0.2159	0.0039	0	0.00000474704192	0.00000014767443	0.00000082928369
0.2159	0.0039	0	0.00000368584237	0.00000012130251	0.00000070124167
0.2159	0.0039	0	0.00000375665738	0.00000013282758	0.00000066626347
0.2962	0.0054	0	0.00000365732738	0.00000012686574	0.00000065195710
0.2962	0.0054	0	0.00000383613100	0.00000013437832	0.00000068270624
0.2962	0.0054	0	0.00000307484630	0.00000011475124	0.00000054504160

Table A7. FA profiles (absolute concentration in µg FA/1 individual) of estuarine copepod *A. tonsa* after exposure to copper (II) sulphate pentahydrate copper (CuSO4.5H2O) represented as "CuSP", Primextra® Gold TZ indicated as "Pr" and their equitoxic mixture (mg/L).

CuSP	Pr	14:0	15:0	16:0	16:1ω9	16:1ω7	16:1ω5	17:0
0	0	0.107629995	0.010408023	0.298499689	0.004781932	0.177456804	0.004908585	0.003498610
0	0	0.112691320	0.010720129	0.329120067	0.004130750	0.203645313	0.004895583	0.003305152
0	0	0.144394499	0.014608679	0.441372781	0.007029412	0.264564619	0.006710385	0.005471146
0.018	0	0.100729764	0.009418683	0.290935645	0.004944680	0.168267553	0.004907151	0.003491068
0.018	0	0.122907278	0.011507254	0.353456592	0.004942820	0.253642398	0.006529145	0.003606228
0.018	0	0.120039807	0.011386005	0.344051645	0.005964333	0.230171528	0.006321324	0.003879421
0.030	0	0.073246348	0.006947635	0.236195859	0.002755227	0.118023030	0.003287829	0.002820512
0.030	0	0.075991608	0.006953591	0.223767626	0.003316262	0.127142470	0.003258806	0.002619817
0.030	0	0.073871983	0.006495083	0.224573923	0.003178781	0.111842053	0.003116576	0.002880586
0.082	0	0.089318203	0.007467581	0.298209015	0.001761733	0.147023723	0.003362944	0.004039827
0.082	0	0.066267369	0.005920181	0.235891271	0	0.096987428	0.002415874	0.003588175
0.082	0	0.086654286	0.006983666	0.291428008	0.002051503	0.142111629	0.003900613	0.004451654
0	0	0.009088706	0.014979092	0.476211910	0.005816014	0.210547182	0.007791059	0.007567385
0	0	0.063271501	0.007106566	0.289113118	0.002473973	0.079409105	0.002718814	0.005657038
0	0	0.107386862	0.011291975	0.387495566	0.004648809	0.166532557	0.005896661	0.006681802
0	0.120	0.074913628	0.007074929	0.319793797	0.002126951	0.098981314	0.003612842	0.005780606
0	0.120	0.058768298	0.006185647	0.284112662	0.002690831	0.073501315	0.002875371	0.005535883
0	0.120	0.045982234	0.004695643	0.279881370	0.001509919	0.048214219	0.001714174	0.005459547
0	0.203	0.059102335	0.005419883	0.238188174	0.001710874	0.078806822	0.002938910	0.003929004
0	0.203	0.046231538	0.005154874	0.187161183	0.001900672	0.056610310	0.002377138	0.003238053
0	0.203	0.059338689	0.005722914	0.236986711	0.001742177	0.079909888	0.002735945	0.003740809
0	0.555	0.032874173	0.003909416	0.212394597	0	0.034282988	0	0.004030349
0	0.555	0.029380475	0.003726996	0.168424873	0	0.025496744	0	0.003182943
0	0.555	0.032110599	0.003300358	0.217378490	0	0.032000302	0	0.004277099
0	0	0.117854577	0.013616239	0.251671392	0.009854525	0.199688199	0.007365682	0.003591698
0	0	0.076340753	0.010181013	0.199371610	0.006615982	0.148478862	0.004277336	0.002717776
0	0	0.116974578	0.013922752	0.241702080	0.009011273	0.215336234	0.007039171	0.003301088
0.018	0.120	0.060808619	0.007436528	0.161201354	0.004209571	0.092835992	0.003712241	0.002554723
0.018	0.120	0.052012805	0.006158951	0.157081175	0.004569747	0.075276211	0.003415362	0.002733805
0.018	0.120	0.072310381	0.007444904	0.174383606	0.004070099	0.111456275	0.004534389	0.002558725
0.030	0.203	0.036501084	0.003997998	0.154213966	0.002243582	0.053452426	0.002312061	0.003035298
0.030	0.203	0.026773251	0.003624883	0.110706701	0.002109211	0.039991567	0.001723498	0.002180659
0.030	0.203	0.057512223	0.005654336	0.182159345	0.003066751	0.088946199	0.003429727	0.003009606
0.082	0.555	0.024173454	0.002528251	0.136196902	0.001542266	0.038791654	0	0.002864977
0.082	0.555	0.020971629	0.003007875	0.154275180	0.002279596	0.022800381	0	0.003314224
0.082	0.555	0.022572542	0.002768063	0.145236041	0.001910931	0.030796018	0	0.003089600

Table A7. Continued

CuSP	Pr	16:2ω6	16:2ω4	18:0	16:3ω3	18:1ω9 tr	18:1ω7	18:2ω6 tr
0	0	0.000519355	0.002290842	0.154781634	0.006869638	0.007888716	0.028020415	0.007855513
0	0	0.000455784	0.002191497	0.159909602	0.005987661	0.009201918	0.028621834	0.008781449
0	0	0.000787740	0.003408205	0.286335958	0.010187483	0.010725125	0.037645409	0.012235773
0.018	0	0.000593363	0.001843002	0.175763973	0.006236996	0.007944101	0.028307190	0.008457305
0.018	0	0.000596729	0.002445736	0.164671390	0.007055533	0.012015871	0.037822830	0.011090876
0.018	0	0.000684814	0.002483781	0.186339703	0.008173094	0.010459129	0.036577908	0.010938969
0.030	0	0.000323550	0.001105291	0.156740888	0.003472087	0.009300631	0.018249780	0.006862602
0.030	0	0.000377800	0.001238119	0.132968026	0.004128768	0.005870644	0.019622839	0.006200072
0.030	0	0.000330133	0.001194864	0.148912211	0.003924385	0.005327608	0.017438495	0.005764124
0.082	0	0.000280660	0.001082462	0.223593151	0.002059857	0.010181535	0.022244167	0.009698495
0.082	0	0.000232480	0.000852762	0.198024210	0.001784228	0.006090221	0.013447512	0.006028024
0.082	0	0.000207359	0.000922693	0.227028943	0.001733404	0.010250902	0.021494453	0.010288263
0	0	0.000459258	0.002458170	0.424403901	0.006168564	0.021337640	0.058943310	0.018157451
0	0	0.000221394	0.000873763	0.340269996	0.002859230	0.008523738	0.024368907	0.009875852
0	0	0.000553014	0.001978586	0.374027641	0.005467774	0.015794501	0.050671020	0.015272048
0	0.120	0	0.000966283	0.367610827	0.002106114	0.012222954	0.025963987	0.012041801
0	0.120	0	0.000569650	0.334265607	0.000974970	0.011087361	0.019086494	0.009349770
0	0.120	0	0.000335314	0.376751354	0.000649684	0.008333466	0.012904955	0.008328456
0	0.203	0	0.000644065	0.240860489	0.001253375	0.009466114	0.020720317	0.007803634
0	0.203	0.000136715	0.000597012	0.187660364	0.001776987	0.005641270	0.018367799	0.005558969
0	0.203	0.000145076	0.000749215	0.232323702	0.001434579	0.009031336	0.022355283	0.007615904
0	0.555	0	0.000234841	0.272996391	0.000262560	0.005654582	0.012387289	0.006104512
0	0.555	0	0.000251013	0.203099968	0.000554914	0.003967001	0.009863970	0.004336017
0	0.555	0	0.000214213	0.292291800	0.000240459	0.005592186	0.010602276	0.006079066
0	0	0.000937231	0.003231977	0.153716120	0.013052364	0.013589833	0.032601924	0.011438406
0	0	0.000818981	0.002519652	0.125613941	0.009263285	0.007864907	0.028424062	0.006985316
0	0	0.001154669	0.003757864	0.122165421	0.013742609	0.011811690	0.041037886	0.009921680
0.018	0.120	0.000317368	0.001434390	0.140059358	0.004541390	0.006970247	0.017704932	0.006879732
0.018	0.120	0.000285852	0.001173556	0.147969905	0.003913844	0.006828344	0.015951670	0.005910977
0.018	0.120	0.000374345	0.001611973	0.129823584	0.005116084	0.008542092	0.020579374	0.007133917
0.030	0.203	0	0.000586651	0.182271469	0.001750700	0.006179597	0.011114972	0.006225376
0.030	0.203	0	0.000432890	0.120696801	0.001131449	0.004757685	0.008265526	0.004387302
0.030	0.203	0.000164434	0.000988107	0.172424907	0.002671734	0.009441599	0.016534257	0.008994645
0.082	0.555	0	0.000229109	0.171518539	0.000259520	0.006684190	0.008957606	0.006478172
0.082	0.555	0	0.000119527	0.231556117	0.000187816	0.007534501	0.005651130	0.006263330
0.082	0.555	0	0.000174318	0.201537328	0.000223668	0.007109346	0.007304368	0.006370751

Table A7. Continued

CuSP	Pr	18:2ω6 cis	20:0	18:3ω6	18:3ω3	20:1ω9	22:0	20:4ω6 (ARA)
0	0	0.001748624	0.003508891	0.003376205	0	0.001919592	0.003576476	0.007639881
0	0	0.001666283	0.003689174	0.003738661	0	0.002193315	0.003985731	0.008632264
0	0	0.002527189	0.006879199	0.004293531	0	0.003008431	0.006004857	0.010466982
0.018	0	0.001605187	0.004123417	0.003103724	0	0.002467646	0.004656928	0.008652177
0.018	0	0.002589627	0.004238213	0.004754496	0	0.002889027	0.004488951	0.012488046
0.018	0	0.002474114	0.004621237	0.003852963	0	0.003161829	0.005605878	0.011492929
0.030	0	0	0.003379592	0.001915137	0	0.001563111	0.003072003	0.006761319
0.030	0	0	0.002792562	0.001931344	0	0.001480846	0.003135220	0.005720904
0.030	0	0	0.002994860	0.001696788	0	0.001382829	0.003802985	0.006072543
0.082	0	0	0.005006035	0.003318298	0	0.001488023	0.003438316	0.010141400
0.082	0	0	0.004067634	0.002109566	0	0	0.002902083	0.005439019
0.082	0	0	0.004880694	0.003071854	0	0.001640675	0.003697099	0.010895846
0	0	0.002567025	0.008971293	0.005300060	0.001802451	0.004666128	0.005744397	0.021170643
0	0	0.001797325	0.006639807	0.001325787	0	0.001683816	0.003583318	0.005181576
0	0	0.002232248	0.008160879	0.003624172	0.001074239	0.003677513	0.005455388	0.012545530
0	0.120	0	0.007343458	0.002229612	0	0.002041021	0.003859680	0.009863934
0	0.120	0	0.006364465	0.001805250	0	0.001384189	0.002979808	0.011534467
0	0.120	0	0.007081942	0.000701291	0	0.000838910	0.002877763	0.005387515
0	0.203	0	0.004497390	0.001638607	0	0.001391152	0.002381156	0.009435684
0	0.203	0	0.003398946	0.000810221	0	0.001138467	0.002358948	0.005410337
0	0.203	0	0.004448396	0.001822261	0	0.001578997	0.002828580	0.008897357
0	0.555	0	0.004844325	0	0	0	0.002319894	0.005844180
0	0.555	0	0.003435689	0	0	0	0.001679629	0.003614686
0	0.555	0	0.005359707	0	0	0	0.002055495	0.004487147
0	0	0.002856204	0.003307307	0.004130791	0.003094996	0.002374313	0.003608997	0.008459225
0	0	0.002578638	0.003120136	0.002086485	0.001844957	0.001921267	0.003121396	0.004098460
0	0	0.003720195	0.003180889	0.004558504	0.003028608	0.003030272	0.004105262	0.008556412
0.018	0.120	0.001562092	0.002862812	0	0	0	0.002321424	0.015652565
0.018	0.120	0.001478897	0.002976461	0	0	0	0.001705705	0.003085843
0.018	0.120	0.001601617	0.002362070	0	0	0	0.002216309	0.006641635
0.030	0.203	0	0.003206555	0	0	0	0.001522570	0.003822355
0.030	0.203	0	0.001930413	0	0	0	0	0.002678590
0.030	0.203	0	0.003437434	0	0	0	0.002027272	0.008248635
0.082	0.555	0	0.003126269	0	0	0	0.001551812	0.003642380
0.082	0.555	0	0.004106849	0	0	0	0	0
0.082	0.555	0	0.003616559	0	0	0	0.000775906	0.001821190

Table A7. Continued

CuSP	Pr	22:1ω9	20:5ω3 (EPA)	24:0	22:6ω3 (DHA)
0	0	0.004625950	0.187127075	0.010302062	0.087285103
0	0	0.003652962	0.182623929	0.010171387	0.078525971
0	0	0.006698199	0.269461098	0.011943518	0.103709600
0.018	0	0.005726802	0.175613681	0.011155484	0.079273181
0.018	0	0.006321739	0.212050124	0.011364206	0.086913516
0.018	0	0.005861623	0.226933959	0.012206966	0.092555556
0.030	0	0.003577349	0.102537027	0.007920171	0.049767267
0.030	0	0.002582948	0.125000687	0.007955089	0.055080631
0.030	0	0.003555876	0.111765358	0.008473930	0.050586877
0.082	0	0.003698945	0.103858715	0.007917800	0.063589730
0.082	0	0.002951393	0.067001855	0.006106016	0.043805093
0.082	0	0.003435609	0.099548637	0.009555711	0.061667436
0	0	0.009585903	0.265225662	0.011040489	0.126407426
0	0	0.004914787	0.112894264	0.006437965	0.078472627
0	0	0.009743255	0.239096059	0.009809546	0.134275255
0	0.120	0.005283991	0.126212121	0.006471672	0.082026541
0	0.120	0.004005709	0.087107287	0.004902872	0.062626338
0	0.120	0.001511201	0.064139138	0.004132479	0.054426334
0	0.203	0.003816580	0.096846857	0.005296587	0.064506997
0	0.203	0.002353691	0.096618824	0.005324186	0.070758221
0	0.203	0.003847570	0.116200345	0.005876826	0.077871372
0	0.555	0	0.057034241	0.003823263	0.055431086
0	0.555	0	0.052146961	0.004169155	0.051850425
0	0.555	0	0.047363423	0.003759546	0.046377818
0	0	0.006254791	0.301006291	0.012818457	0.134093140
0	0	0.005641744	0.214698659	0.008247673	0.122429678
0	0	0.008111588	0.337872518	0.012563496	0.177237013
0.018	0.120	0.003271068	0.165385778	0.006877918	0.104252479
0.018	0.120	0.003644204	0.145516940	0.006126528	0.087056456
0.018	0.120	0.003587119	0.186685105	0.007794514	0.099887464
0.030	0.203	0	0.091938931	0.004816951	0.062521857
0.030	0.203	0	0.066646433	0.003177318	0.044456286
0.030	0.203	0.002806158	0.134117173	0.006456997	0.079737540
0.082	0.555	0	0.049081767	0.003128389	0.049024401
0.082	0.555	0	0.032152304	0.002402236	0.033638404
0.082	0.555	0	0.040617035	0.002765312	0.041331402

Table A8. Protein and TBARS contents of diatom *T. weissflogii* and copepod *A. tonsa* after exposure to copper (II) sulphate pentahydrate copper (CuSO4.5H2O) represented as "CuSP", Primextra® Gold TZ indicated as "Pr" and their equitoxic mixture (mg/L).

Diatom *T. weissflogii*				Copepod *A. tonsa*			
CuSP, mg/L	**Pr, mg/L**	**Protein, mg/mL**	**TBARS, nmol.mg-1 protein**	**CuSP, mg/L**	**Pr, mg/L**	**Protein, mg/mL**	**TBARS, nmol.mg-1 protein**
0	0	0.27	5.29	0	0	1.04	18.58
0	0	0.24	5.26	0	0	1.06	20.15
0	0	0.26	5.16	0	0	1.05	18.71
0	0	0.25	5.75	0	0	1.02	26.14
0	0	0.26	5.57	0	0	1.12	23.24
0.1793	0	0.19	5.50	0.018	0	1.24	20.34
0.1793	0	0.23	6.68	0.018	0	1.18	23.46
0.1793	0	0.14	6.42	0.018	0	1.09	23.36
0.1793	0	0.23	4.78	0.018	0	1.06	24.74
0.1793	0	0.27	5.75	0.018	0	1.02	25.14
0.2159	0	0.28	6.26	0.030	0	0.85	18.08
0.2159	0	0.32	5.58	0.030	0	0.93	24.33
0.2159	0	0.27	6.52	0.030	0	1.15	23.73
0.2159	0	0.25	6.07	0.030	0	1.11	25.54
0.2159	0	0.24	6.54	0.030	0	1.03	23.82
0.2962	0	0.27	5.85	0.082	0	0.87	19.39
0.2962	0	0.31	6.43	0.082	0	1.04	28.89
0.2962	0	0.30	7.66	0.082	0	0.91	24.40
0.2962	0	0.25	7.76	0.082	0	0.91	29.39
0.2962	0	0.25	7.01	0.082	0	1.04	32.80
0	0	0.24	5.95	0	0	0.85	16.52
0	0	0.28	5.80	0	0	0.86	22.61
0	0	0.29	5.72	0	0	0.94	19.45
0	0	0.29	5.72	0	0	1.12	16.46
0	0	0.31	5.16	0	0	1.09	19.88
0	0.0033	0.30	5.99	0	0.120	0.92	25.78
0	0.0033	0.27	5.84	0	0.120	0.94	34.71
0	0.0033	0.30	5.25	0	0.120	0.92	29.81
0	0.0033	0.29	4.78	0	0.120	0.98	29.34
0	0.0033	0.31	4.86	0	0.120	0.97	25.72
0	0.0039	0.32	5.78	0	0.203	0.81	24.37
0	0.0039	0.30	5.24	0	0.203	0.89	22.82
0	0.0039	0.29	4.98	0	0.203	0.81	18.89
0	0.0039	0.29	5.28	0	0.203	1.02	18.05
0	0.0039	0.28	5.24	0	0.203	0.85	18.04
0	0.0054	0.24	5.98	0	0.555	1.11	13.97
0	0.0054	0.23	6.07	0	0.555	0.82	14.84
0	0.0054	0.21	6.45	0	0.555	0.86	17.69
0	0.0054	0.22	6.89	0	0.555	0.74	17.01
0	0.0054	0.20	5.13	0	0.555	0.96	15.40
0	0	0.25	5.46	0	0	0.98	16.15
0	0	0.27	5.75	0	0	0.89	14.68
0	0	0.31	5.09	0	0	1.17	19.51
0	0	0.29	5.80	0	0	0.78	17.02
0	0	0.31	5.90	0	0	1.08	20.31
0.1793	0.0033	0.34	4.25	0.018	0.120	1.10	14.13
0.1793	0.0033	0.25	5.26	0.018	0.120	1.09	16.23
0.1793	0.0033	0.29	4.83	0.018	0.120	1.08	18.39
0.1793	0.0033	0.29	5.01	0.018	0.120	1.04	14.57
0.1793	0.0033	0.35	4.52	0.018	0.120	1.03	11.21
0.2159	0.0039	0.28	8.10	0.030	0.203	0.88	16.95
0.2159	0.0039	0.23	9.49	0.030	0.203	0.92	11.40
0.2159	0.0039	0.23	8.77	0.030	0.203	0.93	14.42
0.2159	0.0039	0.28	9.41	0.030	0.203	0.90	14.75
0.2159	0.0039	0.28	8.43	0.030	0.203	1.03	18.37
0.2962	0.0054	0.29	6.84	0.082	0.555	0.79	7.11
0.2962	0.0054	0.47	7.12	0.082	0.555	1.00	8.03
0.2962	0.0054	0.52	4.69	0.082	0.555	1.15	7.87
0.2962	0.0054	0.50	3.65	0.082	0.555	0.91	8.04
0.2962	0.0054	0.52	4.39	0.082	0.555	0.91	6.83

Table A9. FA profiles (absolute concentration µg FA/1 cell) of primary producer *T. weissflogii* after exposure to copper (II) sulphate pentahydrate copper (CuSO4.5H2O) represented as "CuSP", Primextra® Gold TZ indicated as "Pr" and their equitoxic mixture in a values equal to the values used for the exposure of primary consumer *A. tonsa* (mg/L).

CuSP	Pr	14:0	15:0	16:0	16:1ω9	16:1ω7	16:1ω5	17:0
0	0	0.00000181507494	0.00000031478782	0.00000806369374	0.00000011852931	0.00000504544999	0.00000009397174	0.00000025134286
0	0	0.00000152930264	0.00000025224313	0.00000484370980	0.00000008977052	0.00000460192154	0.00000009259687	0.00000018027263
0	0	0.00000143949063	0.00000023991620	0.00000556511410	0.00000007898265	0.00000422976820	0.00000008634924	0.00000018180621
0.018	0	0.00000191905358	0.00000030532597	0.00000746149203	0.00000007704172	0.00000620571603	0.00000009128537	0.00000021523990
0.018	0	0.00000160255745	0.00000028200997	0.00000556524914	0.00000008351377	0.00000519381523	0.00000008190371	0.00000019690110
0.018	0	0.00000159173846	0.00000025908823	0.00000574016949	0.00000008703691	0.00000489021518	0.00000008396787	0.00000018304655
0.030	0	0.00000136620389	0.00000021165707	0.00000595205975	0.00000006371614	0.00000420815265	0.00000006735976	0.00000015355614
0.030	0	0.00000133530418	0.00000022119034	0.00000459333036	0.00000009168128	0.00000391349929	0.00000006462486	0.00000014843727
0.030	0	0.00000002417306	0.00000021659395	0.00000426569931	0.00000008829175	0.00000391541788	0.00000008104703	0.00000017768335
0.082	0	0.00000149653418	0.00000023293792	0.00000549101536	0.00000011857321	0.00000436686765	0.00000006962326	0.00000020980070
0.082	0	0.00000200782505	0.00000032961127	0.00000630791930	0.00000012316159	0.00000634971341	0.00000009080649	0.00000023663217
0.082	0	0.00000001882876	0.00000032260193	0.00000730580661	0.00000013331763	0.00000731262227	0.00000010231193	0.00000028146449
0	0	0.00000167979435	0.00000034471741	0.00000464084833	0.00000046067073	0.00000373508127	0.00000010311569	0.00000026424500
0	0	0.00000161793128	0.00000026749762	0.00000460318506	0.00000013525935	0.00000384025124	0.00000010433978	0.00000022552167
0	0	0.00000192734968	0.00000030017641	0.00000555891226	0.00000012902110	0.00000449289609	0.00000012134479	0.00000023274436
0	0.120	0.00000141228766	0.00000023483214	0.00000521654201	0.00000027338347	0.00000261804014	0.00000011567184	0.00000022969535
0	0.120	0.00000106233934	0.00000017973732	0.00000460522246	0.00000019427082	0.00000207321072	0.00000008484784	0.00000015274633
0	0.120	0.00000098930777	0.00000017540363	0.00000372012261	0.00000019352280	0.00000198207682	0.00000008497477	0.00000016338564
0	0.203	0.00000143360779	0.00000023656520	0.00000673585437	0.00000030581554	0.00000331014995	0.00000012635700	0.00000025879615
0	0.203	0.00000110046118	0.00000018653635	0.00000477877227	0.00000020841939	0.00000208475180	0.00000006909526	0.00000017360278
0	0.203	0.00000085249212	0.00000015091666	0.00000409937358	0.00000023595699	0.00000123875822	0.00000063483450	0.00000015347841
0	0.555	0.00000113793548	0.00000007783740	0.00000473856909	0.00000022374410	0.00000217245635	0.00000012962611	0.00000022525257
0	0.555	0.00000079876358	0.00000017691727	0.00000390365742	0.00000013205040	0.00000141295747	0.00000005814607	0.00000013588113
0	0.555	0.00000082642042	0.00000019769042	0.00000505235627	0.00000012424514	0.00000138520552	0.00000007396783	0.00000016894414
0	0	0.00000125956725	0.00000022524712	0.00000534772327	0.00000022915127	0.00000259472182	0.00000006964338	0.00000022859538
0	0	0.00000076241190	0.00000013941153	0.00000333517945	0.00000004598567	0.00000167845738	0.00000003721279	0.00000013193095
0	0	0.00000111399539	0.00000020463067	0.00000360010297	0.00000015659155	0.00000259672046	0.00000006803286	0.00000019871469
0.018	0.120	0.00000095087157	0.00000016506079	0.00000492941272	0.00000022030049	0.00000257794952	0.00000005602186	0.00000018372374
0.018	0.120	0.00000119686379	0.00000018883167	0.00000639503733	0.00000023000029	0.00000249181003	0.00000007314963	0.00000024259919
0.018	0.120	0.00000086435806	0.00000012845434	0.00000595255344	0.00000014851050	0.00000194245345	0.00000004216303	0.00000019618400
0.030	0.203	0.00000079402858	0.00000012404944	0.00000564995099	0.00000013354503	0.00000172955127	0.00000003171220	0.00000017964336
0.030	0.203	0.00000099866076	0.00000016449169	0.00000491205810	0.00000021109581	0.00000251382451	0.00000005370639	0.00000019806145
0.030	0.203	0.00000137497371	0.00000020377440	0.00000622755063	0.00000029388020	0.00000411899985	0.00000007656913	0.00000026084444
0.082	0.555	0.00000107237840	0.00000017593325	0.00000562891819	0.00000017583637	0.00000262620447	0.00000006516353	0.00000023226269
0.082	0.555	0.00000108719144	0.00000014713854	0.00000602252752	0.00000019121074	0.00000327630326	0.00000006582430	0.00000023141789
0.082	0.555	0.00000095234919	0.00000013270831	0.00000461457366	0.00000015301912	0.00000245855068	0.00000005374210	0.00000018551930

Table A9

CuSP	Pr	16:2ω6	16:2ω4	18:0	16:3ω3	18:1ω9 tr	18:1ω7	18:2ω6 tr
0	0	0.00000001348874	0.00000008706137	0.00000658364086	0.00000020944896	0.00000027844078	0.00000273049599	0.00000021617956
0	0	0.00000001151209	0.00000008641861	0.00000205147312	0.00000022387311	0.00000017355055	0.00000294415812	0.00000016799119
0	0	0.00000001425074	0.00000007774943	0.00000376930207	0.00000020456011	0.00000017445407	0.00000285800868	0.00000018169969
0.018	0	0.00000001094580	0.00000008895310	0.00000463563398	0.00000021910701	0.00000019721938	0.00000216863020	0.00000017707735
0.018	0	0.00000001237552	0.00000007866226	0.00000298367519	0.00000019969347	0.00000017163318	0.00000257988710	0.00000015751490
0.018	0	0.00000001031293	0.00000008293111	0.00000316994359	0.00000019698322	0.00000016741019	0.00000251418193	0.00000015580456
0.030	0	0.00000001092806	0.00000006097902	0.00000400422946	0.00000016185413	0.00000015762835	0.00000210829995	0.00000012747214
0.030	0	0.00000000946020	0.00000007210240	0.00000298187329	0.00000018557803	0.00000017102144	0.00000220896172	0.00000012464975
0.030	0	0.00000001478087	0.00000007975426	0.00000252767455	0.00000019230794	0.00000014606382	0.00000300737932	0.00000012488025
0.082	0	0.00000001566633	0.00000008074235	0.00000402471967	0.00000019423690	0.00000016937805	0.00000269725949	0.00000018963222
0.082	0	0.00000002097853	0.00000010490326	0.00000314497169	0.00000024774368	0.00000020919631	0.00000305865490	0.00000016719789
0.082	0	0.00000002236392	0.00000011608360	0.00000375011205	0.00000026924197	0.00000021095097	0.00000386460187	0.00000024758597
0	0	0.00000002116532	0.00000009540555	0.00000117178747	0.00000029424278	0.00000089095993	0.00000428081259	0.00000026935653
0	0	0.00000001716188	0.00000010393225	0.00000229419601	0.00000028583690	0.00000028907809	0.00000278371122	0.00000019640929
0	0	0.00000002170413	0.00000011015624	0.00000268776435	0.00000027081320	0.00000023720589	0.00000238257422	0.00000021014255
0	0.120	0.00000001235076	0.00000003504880	0.00000407464575	0.00000018592651	0.00000036229424	0.00000633311850	0.00000028829862
0	0.120	0.00000000722842	0.00000002314393	0.00000419624680	0.00000012650407	0.00000023639132	0.00000543296597	0.00000022459011
0	0.120	0.00000001048745	0.00000002785015	0.00000309517420	0.00000014028260	0.00000015999931	0.00000407426042	0.00000017204537
0	0.203	0.00000000947785	0.00000003039762	0.00000682785737	0.00000018430425	0.00000035069130	0.00000811369401	0.00000032272864
0	0.203	0.00000000652357	0.00000002463630	0.00000477558336	0.00000013917201	0.00000020407341	0.00000420273678	0.00000024670045
0	0.203	0.00000000670610	0.00000001690000	0.00000435234308	0.00000011012003	0.00000018337374	0.00000160054552	0.00000017202282
0	0.555	0.00000000789977	0.00000002356836	0.00000488577319	0.00000011696469	0.00000027304378	0.00000661421346	0.00000027135839
0	0.555	0.00000000321597	0.00000001392116	0.00000454918329	0.00000008652461	0.00000016724395	0.00000378444716	0.00000023055732
0	0.555	0.00000000310130	0.00000001164985	0.00000604607576	0.00000006644823	0.00000023328454	0.00000489807571	0.00000024003515
0	0	0.00000001474093	0.00000005653140	0.00000500876887	0.00000016685383	0.00000026571866	0.00000148832237	0.00000019087133
0	0	0.00000000692187	0.00000003111308	0.00000374353195	0.00000010192658	0.00000012122468	0.00000071386228	0.00000010792889
0	0	0.00000001554493	0.00000007110944	0.00000272526861	0.00000022861278	0.00000012718040	0.00000101448153	0.00000015550517
0.018	0.120	0.00000000792219	0.00000002859844	0.00000585716883	0.00000012245050	0.00000020528326	0.00000188211795	0.00000020672304
0.018	0.120	0.00000000686603	0.00000003175580	0.00000889744510	0.00000012834259	0.00000022102380	0.00000205566188	0.00000027413002
0.018	0.120	0.00000000507677	0.00000002003524	0.00000890164170	0.00000008431479	0.00000017691351	0.00000171649274	0.00000021990077
0.030	0.203	0.00000000445102	0.00000001455567	0.00000862316282	0.00000007286966	0.00000020141570	0.00000101163126	0.00000019960513
0.030	0.203	0.00000000547554	0.00000002553474	0.00000603488212	0.00000012475096	0.00000018384761	0.00000151660703	0.00000019369359
0.030	0.203	0.00000001000959	0.00000003626697	0.00000698528956	0.00000016254882	0.00000021842545	0.00000243204123	0.00000024683181
0.082	0.555	0.00000000690587	0.00000002115469	0.00000756601922	0.00000009556922	0.00000017149456	0.00000117862574	0.00000019710673
0.082	0.555	0.00000000615685	0.00000001766877	0.00000762594068	0.00000009523312	0.00000017226752	0.00000147817250	0.00000018814838
0.082	0.555	0.00000000491893	0.00000001561028	0.00000579879278	0.00000009145341	0.00000013413054	0.00000117206490	0.00000015461413

CuSP	Pr	18:2ω6 cis	20:0	18:3ω6	18:3ω3	20:1ω9	22:0	20:4ω6 (ARA)
0	0	0	0.00000011944393	0	0	0	0	0
0	0	0	0.00000003725479	0	0	0	0	0
0	0	0	0.00000005618068	0	0	0	0	0
0.018	0	0	0.00000008224494	0	0	0	0	0
0.018	0	0	0.00000005759203	0	0	0	0	0
0.018	0	0	0.00000006235954	0	0	0	0	0
0.030	0	0	0.00000005681062	0	0	0	0	0
0.030	0	0	0.00000005486291	0	0	0	0	0
0.030	0	0	0.00000005429889	0	0	0	0	0
0.082	0	0	0.00000008280622	0	0	0	0	0
0.082	0	0	0.00000006304342	0	0	0	0	0
0.082	0	0	0.00000009083464	0	0	0	0	0
0	0	0	0.00000003637877	0	0	0	0	0
0	0	0	0.00000004295308	0	0	0	0	0
0	0	0	0.00000006315826	0	0	0	0	0
0	0.120	0	0.00000008096577	0	0	0	0	0
0	0.120	0	0.00000007731546	0	0	0	0	0
0	0.120	0	0.00000006055286	0	0	0	0	0
0	0.203	0	0.00000014185528	0	0	0	0	0
0	0.203	0	0.00000008608719	0	0	0	0	0
0	0.203	0	0.00000008772936	0	0	0	0	0
0	0.555	0	0.00000011731585	0	0	0	0	0
0	0.555	0	0.00000008626868	0	0	0	0	0
0	0.555	0	0.00000012184531	0	0	0	0	0
0	0	0	0.00000009448266	0	0	0	0	0
0	0	0	0.00000007072552	0	0	0	0	0
0	0	0	0.00000004939651	0	0	0	0	0
0.018	0.120	0	0.00000011246485	0	0	0	0	0
0.018	0.120	0	0.00000017957848	0	0	0	0	0
0.018	0.120	0	0.00000018143291	0	0	0	0	0
0.030	0.203	0	0.00000016494399	0	0	0	0	0
0.030	0.203	0	0.00000010675977	0	0	0	0	0
0.030	0.203	0	0.00000014254417	0	0	0	0	0
0.082	0.555	0	0.00000015207887	0	0	0	0	0
0.082	0.555	0	0.00000015279531	0	0	0	0	0
0.082	0.555	0	0.00000011057521	0	0	0	0	0

CuSP	Pr	22:1ω9	20:5ω3 (EPA)	24:0	22:6ω3 (DHA)
0	0	0	0.00000299115301	0.00000009570669	0.00000122415449
0	0	0	0.00000284023310	0.00000010670103	0.00000122359030
0	0	0	0.00000258134081	0.00000007407306	0.00000115590619
0.018	0	0	0.00000310119696	0.00000008654413	0.00000104480923
0.018	0	0	0.00000277578496	0.00000005600815	0.00000099734779
0.018	0	0	0.00000263853216	0.00000008539783	0.00000095164400
0.030	0	0	0.00000205290748	0.00000005811521	0.00000064758087
0.030	0	0	0.00000235462687	0.00000005504361	0.00000076217795
0.030	0	0	0.00000216627573	0.00000007991006	0.00000077448283
0.082	0	0	0.00000236176932	0.00000008709079	0.00000074031362
0.082	0	0	0.00000334990235	0.00000011097701	0.00000106914533
0.082	0	0	0.00000389970882	0.00000011172646	0.00000134297289
0	0	0	0.00000352427969	0.00000015295161	0.00000240096704
0	0	0	0.00000352504831	0.00000013308112	0.00000212373910
0	0	0	0.00000329340647	0.00000015290034	0.00000213976451
0	0.120	0	0.00000219259999	0.00000013383640	0.00000396451696
0	0.120	0	0.00000128244678	0.00000007707960	0.00000182316449
0	0.120	0	0.00000139733395	0.00000010803753	0.00000173347549
0	0.203	0	0.00000221927678	0.00000014448495	0.00000256612331
0	0.203	0	0.00000153687768	0.00000008647961	0.00000172246723
0	0.203	0	0.00000148501629	0.00000009399939	0.00000083523743
0	0.555	0	0.00000188457423	0.00000012873106	0.00000321793287
0	0.555	0	0.00000111248731	0.00000006709039	0.00000160401221
0	0.555	0	0.00000094837420	0.00000008092841	0.00000157108499
0	0	0	0.00000212021445	0.00000008596104	0.00000081760456
0	0	0	0.00000136888867	0.00000006911977	0.00000050100419
0	0	0	0.00000006486368	0.00000259836763	0.00000117667056
0.018	0.120	0	0.00000151598333	0.00000006226278	0.00000077806415
0.018	0.120	0	0.00000154186952	0.00000007866059	0.00000068242922
0.018	0.120	0	0.00000117966712	0.00000002908040	0.00000062265474
0.030	0.203	0	0.00000084121632	0.00000000363221	0.00000038731890
0.030	0.203	0	0.00000155469185	0.00000002626877	0.00000074905956
0.030	0.203	0	0.00000205503668	0.00000011545931	0.00000099766448
0.082	0.555	0	0.00000161408929	0.00000008395437	0.00000076385940
0.082	0.555	0	0.00000150791917	0.00000008344557	0.00000059755298
0.082	0.555	0	0.00000145643715	0.00000006200522	0.00000059812326

Table A10. Fatty acid data used for the determination of the correlation between trophic levels, where SFA / PUFA are saturated / polyunsaturated fatty acids of the primary producer *T. weissflogii* (absolute concentration µg FA/1 cell) and the primary consumer *A. tonsa* (absolute concentration µg FA/1 individual) after exposure to the equal levels of contaminants.

SFA (*T. weissflogii)*	PUFA (*T. weissflogii)*	SFA (*A. tonsa*)	PUFA (*A. tonsa*)
0.00001724369083	0.00002656293592	0.59220537898153	0.30471223676524
0.00000900095715	0.00001788254475	0.63359256132940	0.29260349779253
0.00001132588295	0.00001970996574	0.91701063682409	0.41707760185559
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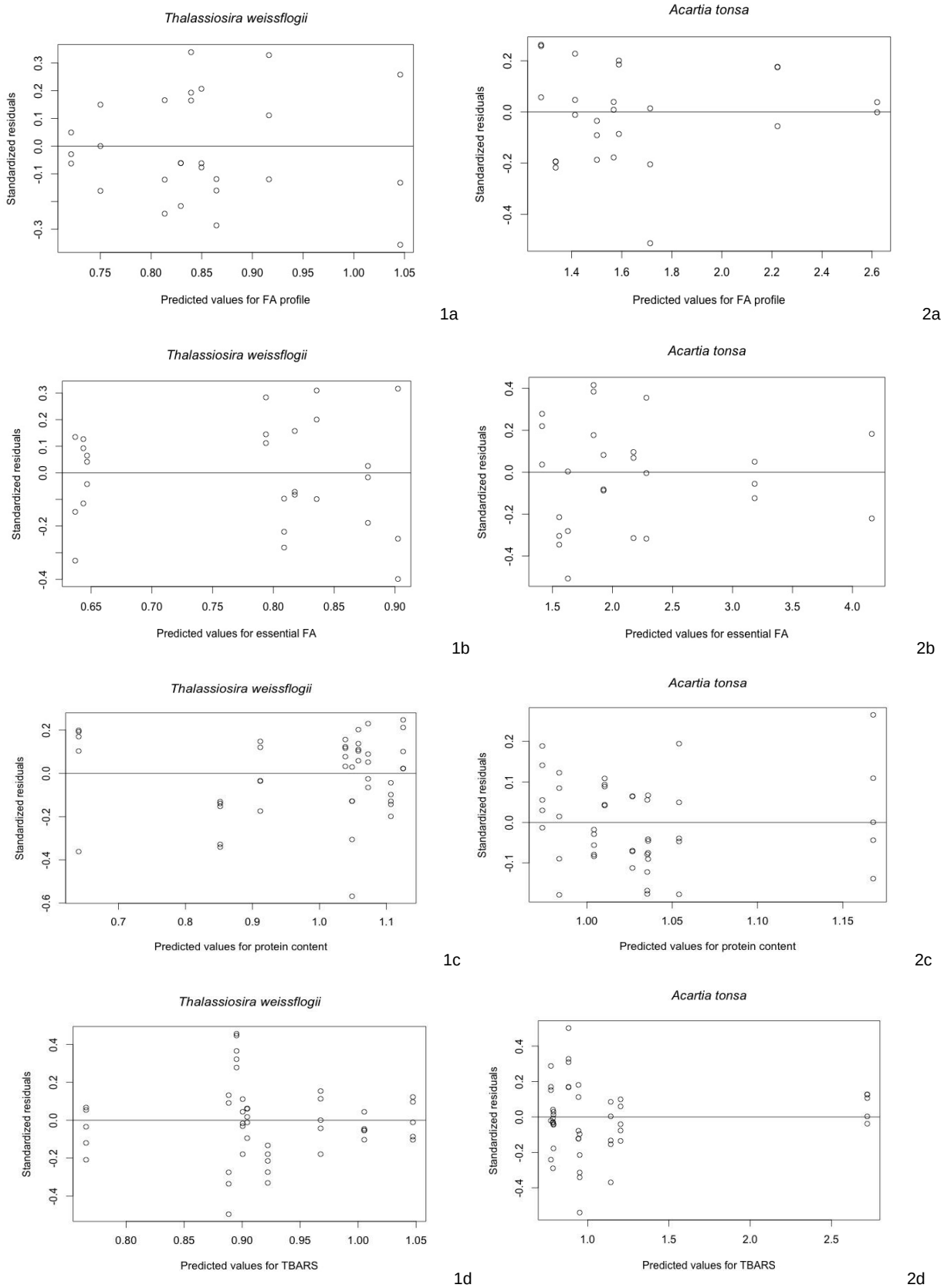

Fig. A4. Plots indicating the homogeneity of model residuals (model 1): standardized residuals versus predicted values for total FA profile (a), essential FA (b), protein (c) and TBARS (d) contents of diatom *T. weissflogii* (1) and copepod *A. tonsa* (2).

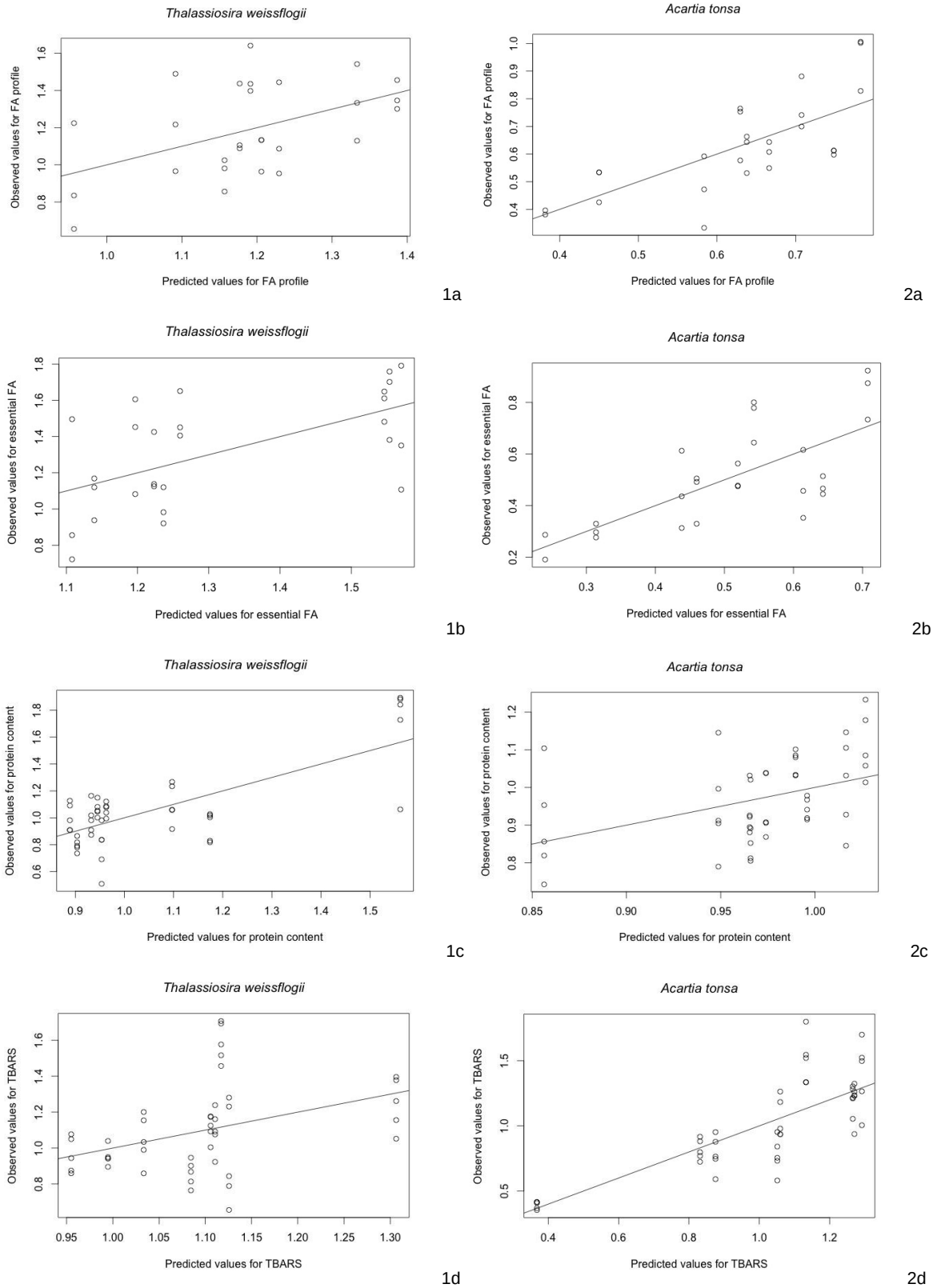

Fig. A5. Plots indicating the goodness of the model fit (model 1): observed values versus predicted values for total FA profile (a), essential FA (b), protein (c) and TBARS (d) contents of diatom *T. weissflogii* (1) and copepod *A. tonsa* (2).

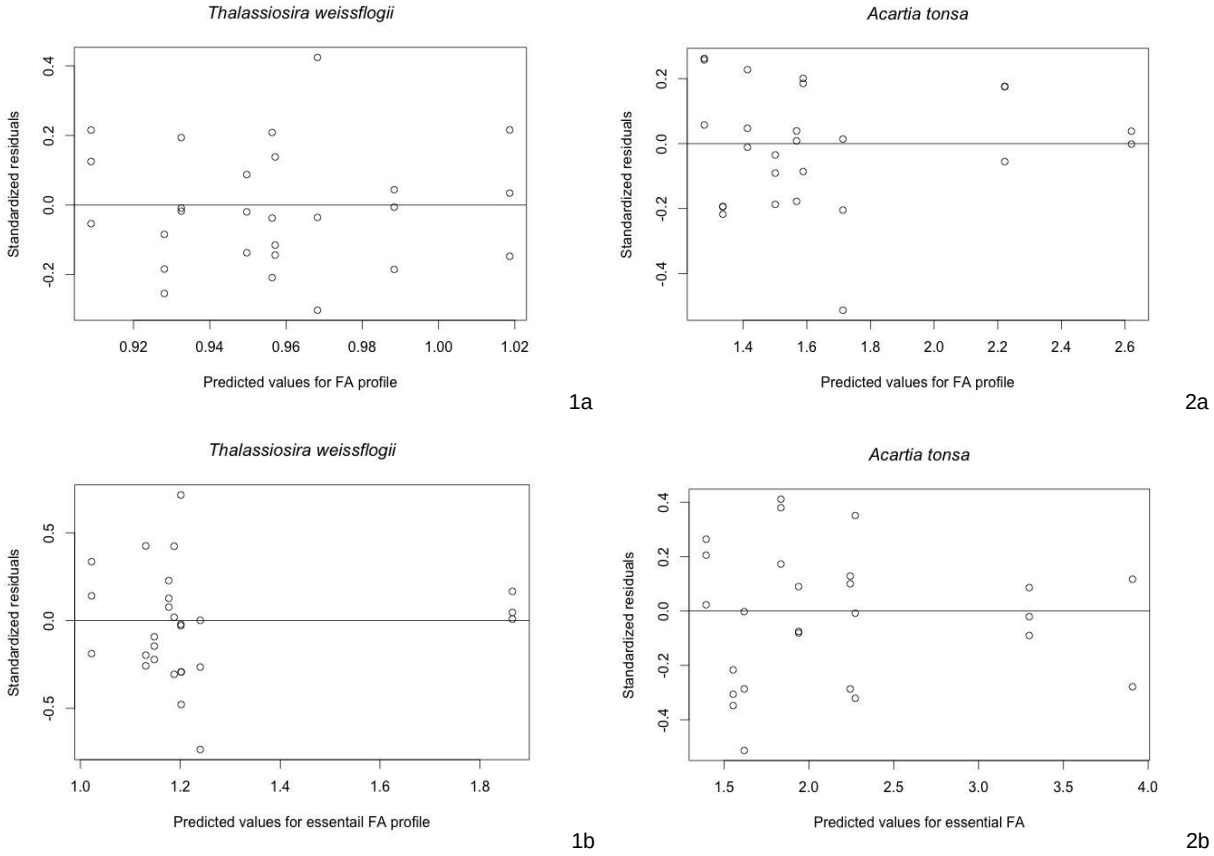

Fig. A6. Plots indicating the homogeneity of model residuals (model 1): standardized residuals versus predicted values for total FA profile (a), essential FA (b) of primary producer *T. weissflogii* (1) and primary consumer *A. tonsa* (2).

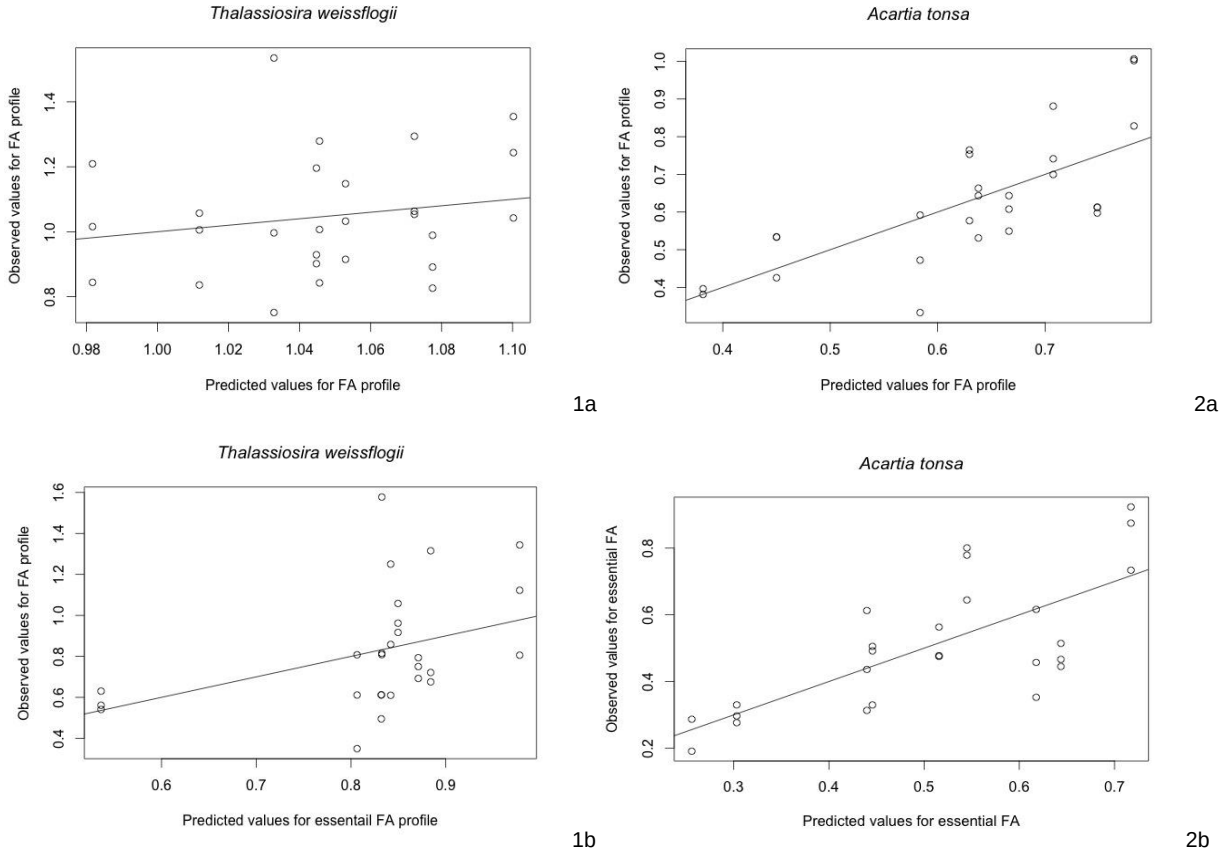

Fig. A7. Plots indicating the goodness of the model fit (model 1): observed values versus predicted values for total FA profile (a), essential FA (b) of primary producer *T. weissflogii* (1) and primary consumer *A. tonsa* (2).

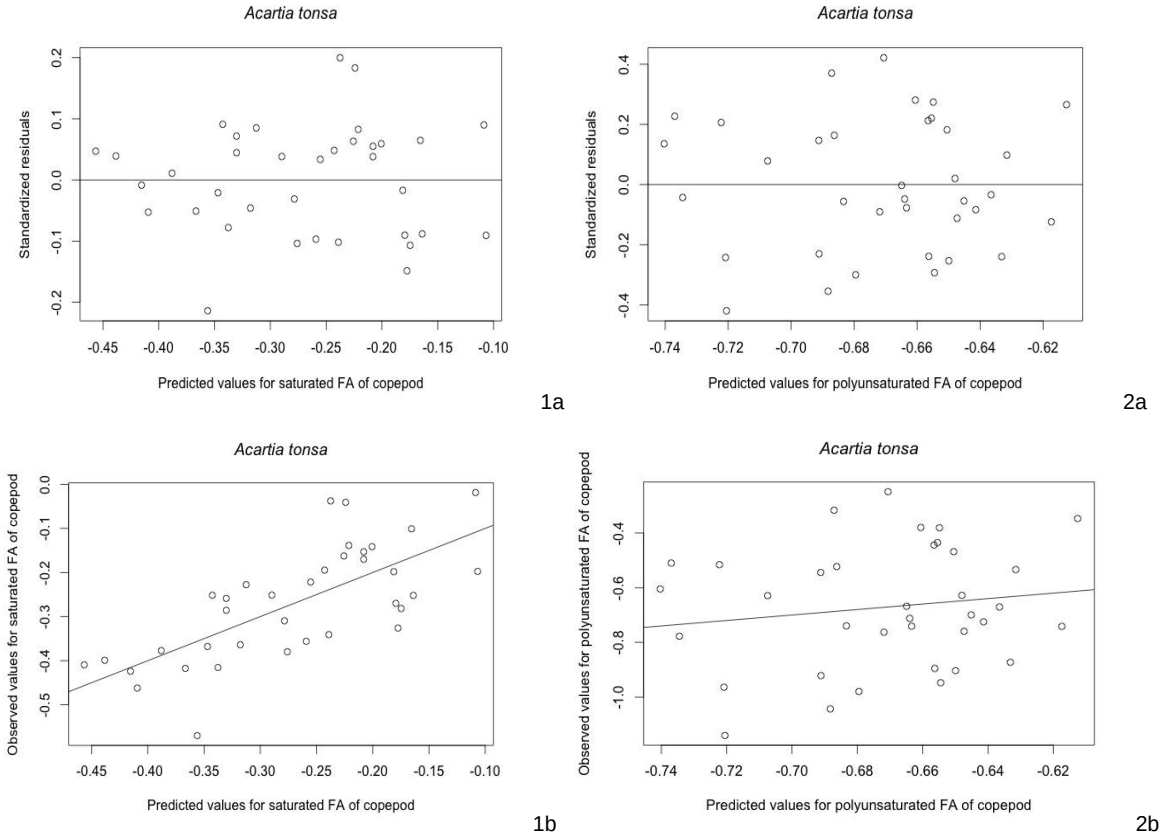

Fig. A8. Plots indicating the homogeneity of model residuals and the goodness of the model fit (model 2): standardized residuals versus predicted values (a) and observed values versus predicted values (b) respectively for saturated FA (1) and polyunsaturated FA (2) of primary consumer *A. tonsa.*