Universidade de Aveiro
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-toxicologia e Análise de Risco, realizada sob a orientação científica da Doutora Ana Marta dos Santos Mendes Gonçalves, Investigadora do Centro de Ciências do Mar e do Ambiente, Departamento de Ciências da Vida da Universidade de Coimbra e do Departamento de Biologia & CESAM da Universidade de Aveiro, e do Doutor Fernando José Mendes Gonçalves, Professor Catedrático do Departamento de Biologia & CESAM da Universidade de Aveiro.

o júri

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palavras-chave

Ácidos gordos, hidratos de carbono, biomarcadores, toxicidade, sulfato de cobre, tebuconazole, microalgas, macrófitas, dáfnia.

resumo

O uso de pesticidas é considerado uma atividade comum e até mesmo uma necessidade em alguma regiões, fundamentalmente devido ao crescimento da população humana e, consequentemente, ao aumento da procura de alimento. O uso descontrolado de pesticidas é uma ameaça aos ecossistemas, afetando a biodiversidade. Uma das preocupações mais relevantes sobre a contaminação de pesticidas é os seus impactos em organismos não-alvo. Os ecossistemas de água doce nas proximidades de áreas agrícolas são especialmente vulneráveis, uma vez que esses contaminantes podem alcançá-los facilmente pelo escoamento de água e podem, portanto, afetar processos biológicos em diferentes níveis: molecular, individual e populacional.

O sulfato de cobre e o Tebuconazole são dois pesticidas amplamente usados na agricultura para eliminar ou controlar fungos e patogénios semelhantes a fungos. Devido ao seu uso generalizado e persistência moderada, os pesticidas são frequentemente detetados em sistemas aquáticos próximos de áreas agrícolas. Este estudo tem como objetivo determinar os os efeitos toxicológicos e bioquímicos do sulfato de cobre e do Tebuconazole em três espécies standard de água doce: *Raphidocelis subcapitata, Lemna minor* e *Daphnia magna*, a duas temperaturas distintas de 20ºC e 25ºC para uma prespectiva de cenário de potencial alteração climática. Para compreensão sobre os efeitos de ambos os compostos, foram realizados ensaios de exposição e análises bioquímicas, especificamente, perfis de ácido gordos e de hidratos de carbono.

Os resultados mostram que *D. magna* foi a espécie mais sensível à exposição de sulfato de cobre (valores de EC_{50} de 0,13 e 0,12 $mg.L^{-1}$, a 20ºC e 25ºC, respetivamente), enquanto a exposição ao Tebuconazole induziu mais efeitos em *L. minor* (valores de EC_{50} de 0,78 e 1,40 $mg.L^{-1}$, a 20ºC e 25ºC, respetivamente). Em geral, as microalgas mostraram ser mais afetadas a ambos os contaminantes a 25ºC, enquanto que as macrófitas e as dáfnias mostraram ser mais sensíveis a 20ºC.

Os perfis de ácidos gordos e de hidratos de carbono das três espécies variaram signficativamente, apresentado diferentes respostas de acordo com o pesticida e a temperatura a que os indivíduos estavam expostos. Estas alterações bioquímicas podem ter repercussões no valor nutricional destes organismos. Como importantes fontes de alimento e bases das cadeias alimentares de água doce, alterações nas populações de *R. subcapitata*, *L. minor* e também de *D. magna* pode induzir diversos impactos no ecossistema. Os ácidos gordos e os hidratos de carbono mostraram ser ferramentas relevantes na deteção dos impactos de contaminantes, fornecendo informacão adicional e útil para ensaios ecotoxicológicos, e , por isso, bons bioindicadores da presença destes compostos em sistemas aquáticos.

keywords

Fatty acids, polyssacharides, biomarkers, toxicity, copper sulphate, tebuconazole, microalgae, macrophytes, daphniids.

abstract

Pesticides usage is considered a common activity and even a necessity in some regions, due fundamentally to the growth of the human population worldwide and, consequently, the increasing of food demand. The uncontrolled use of pesticides is a threat to ecosystems, affecting the biodiversity. One of the most relevant concerns about pesticides' contamination is their impacts on non-target organisms. Freshwater ecosystems in the proximity of agricultural areas are especially vulnerable, since these contaminants can easily reach them by water run-off, and therefore can affect biological processes at different levels: molecular, individual and population.

Copper sulphate and Tebuconazole are two widely used pesticides in agriculture to eliminate or control fungi and fungus-like pathogens. Due to their widespread use and moderate persistence, pesticides are frequently detected in aquatic systems near agricultural areas. This study aims to determine the toxicological and biochemical effects of copper sulphate and Tebuconazole in three standard freshwater species: *Raphidocelis subcapitata, Lemna minor* and *Daphnia magna*, at two different temperatures of 20ºC and 25ºC for a potential climate change scenario perspective. To understand the effects of both compounds, exposure assays and biochemical analyzes were performed, especially, fatty acids and carbohydrates profiles.

Results show that *D. magna* was the most sensitive species to copper sulphate exposure (EC_{50} values of 0.13 and 0.12 $mg.L^{-1}$, at 20ºC and 25ºC, respectively), while Tebuconazole exposure induced more effects on *L. minor* (EC_{50} values of 0.78 and 1.40 $mg.L^{-1}$, at 20ºC and 25ºC, respectively). In general, the microalgae presented to be more affected to both contaminants at 25°C, whereas the macrophytes and daphniids showed to be more sensitive at 20°C.

The fatty acid and carbohydrates profiles of the three species significantly changed, presenting different responses according to the pesticide and temperature to which the individuals were exposed. These biochemical alterations can have an impact on the nutritional value of these organisms. As important food sources and basis of freshwater food webs, changes in the populations of *R. subcapitata*, *L. minor* and also *D. magna* may induce severe impacts in the whole ecosystem. Fatty acid and carbohydrates biomarkers showed to be relevant tools to detect contaminants impacts, providing additional and useful information to ecotoxicological studies, and, therefore, good bioindicator of the presence of these compounds in aquatic systems.

Table of Contents

List of Figures

List of Tables

List of Tables of the appendix

List of Abbreviations

AChE	Acetylcholinesterase
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ALA	α-Linolenic acid (C18:3n9,12,15)
Ara	Arabinose
ARA	Arachidonic acid (C20:4n6)
CHO	Carbohydrates
CTL	Control treatments
Cyp 51	Cytochrome P450 - dependent sterol 14α-demethylase
DDT	Dichlorodiphenyltrichloroethane
DGAV	Direcção-Geral De Alimentação e Veterinária
DHA	Docosahexaenoic acid (C22:6n3)
DMI	Demethylation inhibiting
EC	Effect concentration
ECx	Effective concentration required to obtain a x% of the effect (e.g., x = 10, 20 and 50)
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid (C20:5n3)
FA	Fatty acids
FAMEs	Fatty acid methyl esters
Fuc	Fucose
Gal	Galactose
GC-FID	Gas chromatography with Flame-Ionization detection
GC-MS	Gas-chromatography - mass spectrometry
Glu	Glucose
HUFA	Highly unsaturated fatty acids
n-MDS	Non-metric multi-dimensional scaling
Man	Mannose
MBL	Marine Biological Laboratory
MUFA	Monounsaturated fatty acids
NOEC	No observed effect concentration
OECD	Organization for Economic Co-operation and Development
POPs	Persistent organic pollutants
PUFA	Polyunsaturated fatty acids
Rha	Rhamnose
Rib	Ribose
ROS	Reactive oxygen species
SFA	Saturated fatty acids
TEB	Tebuconazole
UFA	Unsaturated fatty acids
WHO	World Health Organization
Xyl	Xylose

Chapter I - INTRODUCTION

I.1. Pesticides

A pesticide is a chemical agent (a substance or a mixture) whose purpose is to prevent, destroy, or control the proliferation of undesired organisms commonly called "pests". Fundamentally, the targets of this group of chemicals are organisms that can transmit infectious diseases between humans or from animals to humans (mostly insects), plant and animal species that can harm the production, processing, storage, transport, and/or marketing of food and other agricultural products, and also endo and ectoparasites [1].

Most of the available pesticides are mixtures of several chemicals, mostly composed of active ingredients and inert ingredients in appropriate proportions to produce the desired effect. The active ingredients correspond to the component responsible for pesticide function and these are usually the chemical that give the pesticide's name. The other components of the pesticides, called inert ingredients, can serve as carriers, diluents, binders, dispersants, or even extend the life of the active ingredients [2].

The first references to the use of pesticides date back to ancient times. Some relevant historical references deserving of mention are, for example, the use of sulphur by the Greeks (1 000 b. C.); the use of arsenicals by the Chinese (900 a. C.); and the use of tobacco as a contact insecticide for plant lice (1763) [3]. Until the nineteenth century (around the 1870s), organic pesticides were the most used, but with the discovery of antifungal properties of copper compounds (with the development of the Bordeaux mixture, made of hydrated lime and copper sulphate), of inorganic sulphur and organic mercury, new compounds started to appear in the market [4].

The mid-1930s was a period of significant advances in the fields of chemistry, and the predominance of organic or inorganic pesticides was replaced by the recently created organic synthetic pesticides that included organochlorines, organophosphates, carbamates, and pyrethroids [3]. Although the early natural pesticides exhibited risks for the environment and human population, the new synthetic pesticides brought a new range of problems, from impacts in aquatic, aerial, and terrestrial ecosystems, to consequences in non-target populations, loss of biodiversity, and even disorders in human health [5-6].

However, despite the toxicity of the new pesticides, the cultural, economic and health contexts of the human population enable the development of the substances. Due to hunger and vector-borne diseases that were common at the time, the risks associated with the use of synthetic pesticides were neglected to improve the quality and quantity of food supply [5].

Agriculture crops are currently damaged by approximately 9 000 species of insects and mites, 50 000 species of plant pathogens, and 8 000 species of weeds (an estimated total of 70 000 different pest species) [7]. Pests can have many and different impacts on crop production and some examples are the reduction of plant biomass and number, the decrease of carbon uptake, due to a reduction of the number of chloroplasts, and the induction of senescence and abscission of leaves [8].

Nowadays, China is the country that presents the highest pesticide consumption and production [9] (1.5 million tons of pesticide consumption in 2018 [10]), followed by the USA (0.5 million tons of pesticide consumption in both 2011 and 2012 [11]), France, Brazil, and Japan [12]. Globally, 2 million tons of pesticides are applied per year [13], a number that, nonetheless, has been declining since 2007, due to the decay of insecticides usage and the population's awareness of the risk of pesticides [14].

I.1.1. Toxicological classification of Pesticides

Currently, the number of different pesticides is colossal, especially when it comes to man-made, synthetic pesticides. Taking into account that there are significant differences among different pesticides, namely concerning physical, chemical, and identity properties, a classification system is fundamental for a better understanding of each group of pesticides. The most used methods of classification consist in pesticide organization into different classes according to their (i) mode of action, (ii) function and target organisms, and (iii) other more specific characteristics [15].

I.1.1.1. Classification based on the mode of action of pesticides

The mode of action of a pesticide is how it influences the target organisms and this type of classification is based on that type of characteristic [2]. It can also be named

"classification based on modes of entry" [6] because the mode of action of a pesticide is directly related to the path it takes to enter or come in contact with the targets. According to this classification, pesticides can be non-systemic (contact), systemic, stomach poisons/toxicants, fumigants, or repellents [2-6].

Non-systemic or contact pesticides do not need to penetrate the tissues of target organisms to produce effect, as they are not transported through the organism vascular system. In opposition, systemic pesticides have to be absorbed and transported to specific tissues of the target organisms (for example, 2, 4-Dichlorophenoxyacetic acid and glyphosate).

Stomach poisons/toxicants and fumigants are pesticides for exclusive use on animals. They enter the target pests through their mouths and digestive system or through the air and their respiratory system. Repellents are the type of pesticides that instead of killing the targets, only keep them away or interfere with their ability to locate crop.

I.1.1.2. Classification based on pesticide function or target organisms

In this classification method, pesticides are classified according to their function (control a particular organism) or just by their target pests (Table 1). In some cases, a pesticide can act on more than one type of pests, being classified within more than one category, which is the case of Albicarb (a pesticide that can control mites, insects, and nematodes, and for this reason, it can be an acaricide, insecticide or nematicide, respectively) [6].

I.1.1.3. Other ways of classification

Besides the classification types explored above, there are some other possible ways to classify pesticides. The most commonly used are, for example, the classification based on the chemical composition, on the source of origin, on the activity spectrum, on the mode of formulation and on the toxicity levels (Table S1, in the appendix).

Table 1. Pesticides classification based on their target organisms [6].

Pesticides	Target organisms
Acaricides	Mites
Algaecides	Algae
Avicides	Birds
Bactericides	Bacteria
Desiccants	Plants (by drying their tissues)
Fungicides	Fungi (including blights, mildews, molds, and rusts)
Herbicides	Weed (and other undesirable plants)
Insecticides	Insects and other arthropods
Lampricides	Larvae of lampreys
Larvicides	Larvae
Molluscicides	Molluscs
Moth balls	Moth larvae and molds
Nematicides	Nematodes
Ovicides	Eggs of insects and mites
Piscicides	Fishes
Rodenticides	Mice and other rodents
Silvicides	Woody vegetation
Termiticides	Termites
Virucides	Viruses

I.1.2. Pesticides used in Portugal

The growing awareness by the European Union and the European Parliament concerning the impacts of pesticides in the environment and human health has led to the development of policy measures for a reduction of their use. The Directive 91/414/CEE was the first being published in 1991, with the purpose to control plant protection products on the market.

In Portugal, as in other member states of the European Union and other developed countries, the control of plant protection products on the market is preceded by a technical-scientific assessment that includes risk assessment for humans, the environment and non-target species. According to the DGAV (Direção-Geral De Alimentação e Veterinária)[16], the

Regulation (EC) No. 1107/2009 of the European Parliament is the technical-regulatory framework which presents the guidelines and procedures for the launch of plant protection products on the market, whose application came into force on the 14th of June, 2011.

Following the current regulations, the DGAV published a list of plant protection products with an authorized sale on the 1st of January, 2016, in Portugal. This list covers a total of 1054 plant protection products based on 367 active substances [16]. The latest data published regarding the sale of these substances in Portugal is from 2014, where there was an increase in sales, reaching a volume of 2 772 tons, which represented an increase of 27% compared to the previous year [17]. However, when compared to 2009, it represents a decrease of about 6% [17]. In the same report, it was highlighted that fungicides were the most sold products, reaching a total of 8 249 440 kg (Fig. 1).

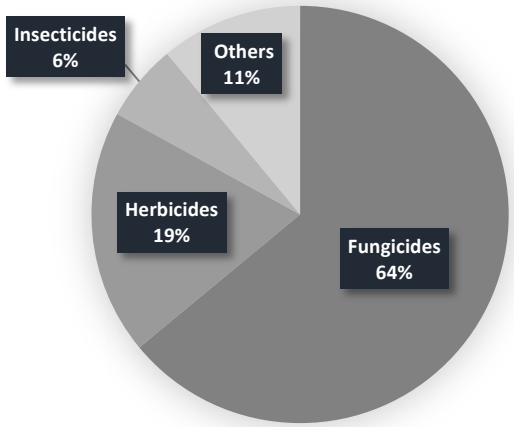

Figure 1. Sales of plant protection products from 2014, divided by function, in Portugal [17].

I.2. Effects of pesticides

I.2.1. Impacts on the environment

Pesticides may be applied in agriculture crops through several techniques, from manual spraying to the use of vehicles and airplanes for larger areas' coverage. Even when applied in a very small area, it is almost impossible to predict the affected area by the pesticide. Once it is released into the environment, it may have many possible fates, as it can spread in the air, be absorbed into the soil, dissolved in water, and eventually, as a

consequence, reach larger areas, far away from the place where the pesticide was first applied [18-19].

To be able to determine pesticide action, its fate, and its impacts on the environment, it is imperative to understand the pesticide's characteristics, such as water solubility, tendency to be adsorbed into the soil, and environmental persistence. For example, highly persistent pesticides may be biologically available for longer periods and have a more extensive distribution (reaching more areas and longer distances from the source), resulting in contamination of groundwater, surface water, soil, air, and food chains of several ecosystems. Besides the environmental contamination, another serious and very important problem associated with pesticides is that target species are not the only organisms affected by their toxic action: non-target species (especially species that are similar to the targets) are also impacted, which causes great damage to biodiversity and ecosystems' health.

I.2.1.1. Air and soil ecosystems

Pesticide distribution into the environment generally occurs through atmospheric, water and soil transport. The proportion of pesticide that can be distributed into different places is dependent on the formulation of the pesticide, method and rate of the application, as well as topography, amount and type of vegetation and groundcover, and weather conditions [20].

A pesticide can be present in air under three possible forms, according to its way of penetrating the atmosphere. It can assume the form of spray droplets (application drift), vapours (post-application vapour loss), and sorbed to wind-eroded sediment (wind erosion of treated soil) [19]. Once in the atmosphere, photodegradation of pesticides (breakdown or transformation of pesticides by sunlight, causing a rupture of chemical bonds [2]) may occur, meaning that not only the original pesticide can be transported, but also their photodegradation products. The problem with these particles in the atmosphere is that they can become a considerable source of exposure to animals and plants (mostly non-target), and (re)contaminate the surface (surface water, groundwater and soils) through dry deposition and precipitation [20].

Regarding the soils' impacts, pesticides can accumulate and induce alterations in soil properties and soil microflora, specifically in microbial diversity, biochemical reactions and enzymatic activity of organisms of the soil, leading to a disturbance in soil ecosystem and loss of soil fertility [21].

Depending on which kind of pesticide is available in soils, the microbial biomass and activity can have different responses to its presence. For example, bacterial populations of the affected soil may grow if they can use the pesticide as a source of energy and nutrients, or they may die if the pesticide is toxic for the respective population [22]. Other examples of possible scenarios are the reduction of microbial biomass with an increase of functional diversity of soil microbial communities [23] or even the inhibition of certain group of microorganisms while others grow by releasing them from the competition [21].

Pesticides can also interfere with the ability of soil microorganisms to perform biochemical transformations of several elements (nitrogen, phosphorus, sulphur, and carbon), and also with the enzymatic activity of soils, which includes all its content on free enzymes, immobilized extracellular enzymes, and enzymes within microbial cells [24-26].

I.2.1.2. Water (surface and groundwater) ecosystems

A major concern about pesticide contamination is the potential impact in water bodies, due to its significant threat to aquatic ecosystems and drinking water resources. Regarding surface water, which includes streams, rivers, lakes, reservoirs and oceans, pesticide contamination may happen as a result of surface run-offs, wastewater discharges, atmospheric deposition and spills [19-20]. The amount of pesticide present in surface run-offs is directly affected by the type of pesticide application, the slope of the area where the pesticide was applied and also by the period between the pesticide application and the occurrence of the climate event previous to the formation of the run-offs [27]. Additionally, the pesticide concentration in surface run-offs is also dependent on the physical-chemical properties of the pesticide, such as vapour pressure, water solubility (a measurement of the amount of pesticide that will dissolve in a known amount of water, indicating the pesticide capacity to be washed off the crop, leach into the soil or moved with surface runoff [20])

and soil sorption characteristics, and its environmental stability (resistance to hydrolysis, photodegradation and others chemical reactions) [19].

Groundwater bodies can also reveal the presence of pesticides, which may enter usually through the leaching process of the treated fields, washing sites or waste disposal areas [20]. The infiltration into the soil of pesticides dissolved in water can happen through a slower transporting process (more dependent of pesticides and soil characteristics) or a more rapid movement of water via preferential pathways such as insect burrows, soil fractures and cavities left by decaying plant roots [19]. This type of contamination directly affects the soil microbial communities and some plant species of the treated site, but the major problem associated with pesticides in groundwater concerns scenarios where the water within field margins reaches bigger aquifers, potential suppliers of wildlife and human communities.

I.2.1.3. Non-target organisms and loss of biodiversity

As it was previously mentioned, pesticides are by definition poisonous substances and do not affect only their targets, but also some organisms that may come in contact with the contaminants. All taxonomic levels can be affected, from bacteria, plants and algae to complex animals, and the more similar to the specific target in physiological functions, the higher the probability of non-target species experiencing severe negative impacts [28].

The response of non-targets organisms depends on species sensitivity to a particular pesticide, on the toxicity of different pesticides to a species, as well as the pesticide dose that the organism comes in contact with. In addition, species sensitivity may vary in accordance with sex, age, nutritional background, stress, health status, and the microenvironment which the organism inhabits [28]. Some of the most studied effects of pesticides on non-targets species are the reduction of species numbers, alteration of habitat with species reduction, changes in growth, behaviour, reproduction, food quality and quantity, resistance, disease susceptibility, and biological magnification [29].

Most pesticides have typical biological interactions with organisms, as other pharmacological products. For example, they can accumulate or increase in any level of a food chain (biomagnification or bioconcentration), and their toxicity can be enhanced by

another compound that would normally be considered nontoxic or relatively nontoxic (synergism) [28]. Generally, the pesticide application tends to reduce significantly the number of individuals of some species in biotic communities, which may lead to the consequent loss of some species. A relevant reduction in the number of species in a community creates instability not only within the community but also in the population, since it alters the normal balance of the community structure [29].

The effects of pesticides on non-target species has been regarded of great concern within the scientific community, but also by the general public, and it served as inspiration for the famous best-seller "Silent Spring", written by Rachel Carson in 1962. The book describes the impacts of pesticides on the environment, particularly in birds, pointing DDT as the cause for the thickness of eggshells to decrease, resulting in reproductive problems and death. Carson was able to raise awareness in the general public by accusing the chemical industry of spreading disinformation and public officials of accepting it, which led to a change in the United States' national pesticide policy and the ban of DDT for agricultural uses, a legislation that was applied across the world not much later.

I.2.2. Impacts on human health

Nowadays, the human population is constantly exposed to pesticides, due to their widespread use and consistent presence in the environment. Workers of the pesticide chemical industry (responsible for the manufacturing, formulation and packaging of pesticides) and farmers represent the group with the higher risk of exposure. Due to the different techniques and equipment required for pesticide application, they can be exposed through several sources and patterns, being the skin the main route of exposure [30].

Concerning the general population, contact with pesticides can occur by inhalation, dermal exposure, and oral exposure. A person can ingest food with some residual trace of pesticides or even heavily contaminated, and can also be exposed through environmental contamination, when living close to pesticide treatment zones (especially rural areas). In some cases, mostly in developing countries, insecticides are sprayed in the streets to control some pests involved in the transmission of diseases, such as in malaria control. Another

example of possible human exposure is through materials such as leather objects and wooden furniture, but these are more rare scenarios [30].

Developed countries have a major concern about this health problem, and to protect their population, governments manage a constant risk assessment of pesticides by determining the level of exposure in the general population and workers, through the comparison with the appropriate health-based limits. Besides the risk assessment, systematic plans for the measurement of pesticide residues in food have also been implemented, from the crops to the production and distribution chain [18].

Human responses to pesticides can be expressed by acute or chronic effects, depending on the period of exposure [6, 30]. Acute poisoning generally occurs from a single exposure, and its effects appear in a short time after contact with the pesticide. On the other hand, chronic exposure is related to small doses repeated over a period of time, which could be years or even decades. Symptoms from chronic poisoning are not immediately noticed and its origin is more difficult to determine than with acute symptoms. Some of both types of symptoms can be found in Table 2 [30].

Continued and repeated exposure to sub-lethal quantities of pesticides is related to the emergence of chronic illnesses in humans, the leading cause of human death in the world. The role of pesticides exposure on the incidences of human chronic diseases is not certain, but the number of studies that establish a link between them has been increasing, in particular regarding the nervous, reproductive, renal, cardiovascular, and respiratory systems [31].

Table 2. Some symptoms of acute and chronic exposure in humans [6].

Acute exposure	*Low-level exposure*	*High-level exposure*
	Irritation of the nose, throat, eyes or skin	Vomiting
	Headache	Blurring of vision
	Dizziness	Rapid pulse
	Nauseas	Mental confusion
	Diarrhea	Inability to breath
	Sweating	Loss of reflexes
	Weakness or fatigue	Uncontrollable muscular twitching
	Insomnia	Death
Chronic exposure	*Diseases*	
	Cancer (Childhood and adult brain cancer, renal cell cancer, lymphocytic leukemia, prostate cancer)	
	Neuro degenerative diseases including Parkinson disease, Alzheimer disease	
	Cardiovascular disease including artery disease	
	Diabetes (type 2)	
	Reproductive disorders	
	Birth defects	
	Hormonal imbalances including infertility and breast pain	
	Respiratory diseases (Asthma, Chronic obstructive pulmonary disease)	

I.3. Fungicides

As previously mentioned, fungicides are the most used and sold pesticides in Portugal [17], and despite the environmental risks associated with them, especially in freshwater ecosystems, they have not received the necessary attention by authorities, when compared with other pesticides, such as insecticides and herbicides [32].

These are the main reasons for the choice of fungicides as the pesticide category to be studied in the present work. Fungicides can be a substance, a formulation or even an organism used for control and treatment of fungal diseases mostly during production, storage, or distribution of an agricultural commodity or food, and in ornamental plants [33]. Their application has a significant impact on crops' yield and quality, and they are often considered indispensable to secure global food supply [34]. Their main goals are to control

the fungal infection in the course of the establishment and growth of a plant and to enhance productivity by decreasing the effects of infection [35].

The first fungicides being produced were inorganic compounds based on sulphur or metal ions, like copper, tin, mercury and cadmium. Although copper and sulphur fungicides are still widely used, nowadays organic synthetic compounds, often with a specific biological action, are the most commonly used [33, 35].

According to their mode of action and effects, most of the fungicides are systemic or non-systemic [35]. Systemic fungicides are mostly used to control and fight infections, once they can be absorbed and transported by the plant to the fungus, without damage the plant tissues. On the other hand, non-systemic fungicides protect the plant from infection on leaf surface and stems. A fungicide can also be considered eradicant, curative, protectant, or even a combination [33]. Fungicides are eradicant when they act in the later stages of the fungal life cycle, curative when they act in the early but post-penetrative effects of fungal action, and protectant if they can prevent the infection by acting in spore germination, germination development, and growth. The classification regarding their biochemical mechanism of action and chemical structures are the most frequently used (Table 3).

Table 3. Some of the most common classes of fungicides, chemical structures and the respective mechanisms of action [35].

Fungicide class	Chemical structure	Mechanism of action
Triazoles	Heterocyclic compounds with a 5-membered ring of two carbon and three nitrogen atoms	Sterol biosynthesis inhibition
Phenylpyrroles	Isomeric phenyl derivatives of aromatic heterocyclic compounds, with a ring of four carbon and one nitrogen atoms	Micellic growth inhibition Glucose phosphorylation reduction Cell membranes disruption
Strobilurins	Derivatives of β-methoxyacrylic acid	Inhibition of respiratory chain in mitochondria, by blocking electron transport chain
Benzimidazoles	Heterocyclic aromatic compound, with the fusion of benzene and imidazole	Ergosterol synthesis inhibition
Morpholines (cinnamic acid derivatives)	Heterocyclic compound, with amine and ether as functional groups	Sterol biosynthesis inhibition Mycelium formation suppression

The diverse classes of fungicides have different features due to their chemical variations. However, in general, fungicides are moderately lipophilic and exhibit moderate to high adsorption potential to organic carbon, which makes possible for them to be adsorbed to sediments and organic surfaces. Despite their lipophilic behaviour, fungicides can also have moderate to high mobility in the soil or pore water matrix and be moderate to highly persistent in soil and water, while their water solubility often varies among and within compound classes [36].

Copper sulphate and Tebuconazole are two widely used fungicides in agriculture whose function is to eliminate or control fungi and fungus-like pathogens. Due to their widespread use and moderate persistence, they are frequently detected in aquatic systems near agricultural areas.

I.3.1. Copper sulphate

Copper (II) sulphate pentahydrate ($CuSO_4$. $5H_2O$ [37]) (Fig. 2), commonly known as "blue vitriol" or "blue stone", is widely used as a fungicide, algaecide, herbicide, antimicrobial or as a molluscicide, for both agricultural and non-agricultural purposes. It is a powerful oxidizing agent, corrosive to mucous membranes and it can be acidic (with pH 4) in concentrated solutions [38]. Copper sulphate can be also served as an additive for fertilizers and foods, and has several applications in industry (such as textiles, leather, wood, batteries, ink, petroleum, paint, and metal) [39].

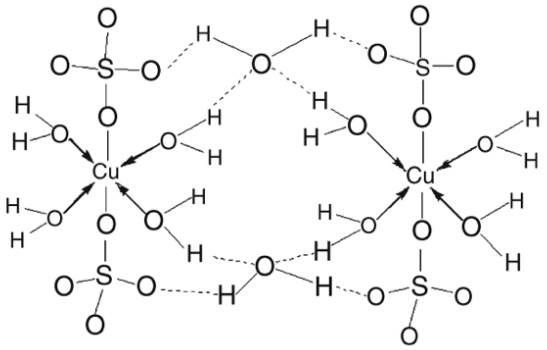

Figure 2. Crystal structure of copper sulphate pentahydrate [40].

The copper (II) ion is the active ingredient of the formulated product [41], which is considered an essential element that can be easily found in the environment. It is vital in

several chemical reactions in living organisms, due to its role as cofactor of many enzymes, for instance, the superoxide dismutase, whose function is to defend the organism against reactive oxygen species (ROS) [42]. In opposition, copper can also inhibit growth and interfere with several cellular processes (photosynthesis, respiration, enzyme activity, pigment and protein synthesis and cell division), if it is present at high concentrations [43].

I.3.1.1. Target organisms and mode of action

Pesticides, with copper (II) ion as their active ingredient, are registered for use in agricultural crops (for example, in the production of root and tubers, leafy vegetables, bulb vegetables, fruiting vegetables and legumes) and ornamental crops (such as flowering/non-flowering plants and trees). Furthermore, they may have aquatic applications, which includes the control of algae, aquatic weed, molluscicide and aquatic macroinvertebrate, and also antimicrobial applications, such as a wood preservative, mildewcide, water treatment, bactericide, and as anti-fouling [37].

Depending on the target, copper can have different modes of action. Regarding its application as a fungicide or as an algaecide, the presence of copper ion can lead to cell leakage, by the induction of non-specific denaturing of proteins, once it can bind with various groups, such sulphidal groups, imidazoles, carboxyls and phosphate (thiol) groups. When it is applied such as a molluscicide, copper can disrupt peroxidase enzymes, which results in the alteration of the surface epithelia functioning of molluscs [37].

I.3.1.2. Environmental toxicity

In Europe, the copper concentration in topsoils is generally low, except for agriculture areas with pesticide treatments. Areas with an intensive application of copper-based pesticides (mostly fungicides) represent a high risk to the environment and human health. Portugal follows the European mean-values for copper concentrations in topsoils, presenting, generally low copper concentrations in topsoils and the risk associated only with treated agricultural areas [44].

Copper accumulation in soil has an impact on both plant growth and species richness, where the most sensitive are earthworms, followed by bacteria, nematodes and fungi, which

induce the loss of soil biota. As a consequence, natural soil reactions are negatively disturbed, for instance, soil bioturbation, aggregate formation and stabilization, and the decomposition and mineralization processes [45].

In addition to soil contamination, copper can also be found in high levels in surface waters near the treated areas. Water contamination is related to the formation of runoff water and eroded sediments with large amounts of copper from contaminated soils, which can reach surface waters due to weather events, such as heavy rainfall [46-47].

Concerning aquatic systems, copper toxicity has already been explored for many freshwater organisms, for example, primary producers [48-49], cladocerans [48], mussels [50], shredders [51-52], bivalves [53] and even some fish species [48, 54]. The exposure to high levels of copper is associated with the disturbance of reproduction and growth rates [55], feeding mechanisms [56] and also with the increasing of the organism susceptibility to diseases and histopathological abnormalities [57].

Besides these impacts, the negative influence of high concentrations of copper in several important reactions of the affected organisms have also been widely reported. For instance, copper can interrupt processes like DNA replication, transcription and repair, once it is able to bind with DNA molecules [58] and proteins involved in these processes [59]. The presence of great amounts of copper is also related to impacts in the metabolism of fatty acid and protein synthesis [53, 60]. However, copper toxicity is mostly associated with oxidative stress, by catalyzing the production of ROS, generally hydroxyl radical, which may lead to damage on many molecules, including DNA [61]. Furthermore, copper can also bind with reduced glutathione, depleting a key antioxidant and therefore indirectly increasing oxidative stress [62].

I.3.2. Tebuconazole

Tebuconazole (TEB) (Fig. 3) is a systemic triazole fungicide, used for agricultural purpose of diseases control, mostly of soil-borne and foliar fungal pathogens [63-64]. It is a relatively new fungicide, but intensively used worldwide and one of the most frequently applied in the European Union, due to its high effectiveness [65-66].

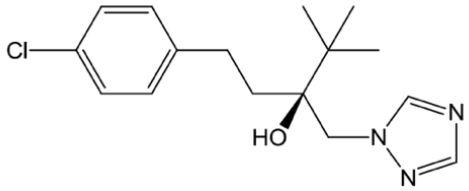

Figure 3. Chemical structure of Tebuconazole [64].

The triazole fungicide group of which TEB belongs, are known for their interaction with the enzyme 14-α-demethylase, which inhibits ergosterol biosynthesis in eukaryotes [67]. When it is pure, it presents a colourless crystalline powder with no characteristic odour, and in its commercial form, it is a white to beige powder with a slight characteristic odour [68].

I.3.2.1. Target organisms and mode of action

TEB is considered to be a broad-spectrum triazole fungicide, once it has the ability to control several fungus infections on several crops, such as fruits (grapes, apple, pear), vegetables, cereals (wheat, barley, oat, rye), and oilseed rape worldwide [63, 65]. It is mostly used against fungal infections, for instance, mildew, brown rot blossom, twig blight, dry rot, leaf spots, and as a growth regulator [66]. *Sclerotium rolfsii* Sacc., responsible for southern stem rot, and *Penicillium digitatum*, the cause of green mould decay, are some examples of fungi pathogens that can be controlled with TEB application [67, 72].

Its mode of action is based on the interaction with the enzyme 14-α-demethylase, an important enzyme in the sterol biosynthetic pathways in eukaryotes. The disruption in ergosterol biosynthesis by the presence of TEB can lead to an accumulation of toxic intermediate sterols in the fungal cell membrane, which may cause the increase of membrane permeability and the consequent inhibition of fungal growth [73]. For this reason, TEB belongs to a group of triazole fungicides called DMI (demethylation inhibiting) or sterol biosynthesis inhibitors. Through the interaction of its N-4 substituents of the azole ring with the heme iron of the cytochrome P450 - dependent sterol 14α-demethylase (cyp 51), this type of fungicides can inhibit protein activity [74-75].

I.3.2.2. Environmental toxicity

Due to the intensive use of TEB worldwide, this fungicide is commonly found in soils and water bodies near treated fields. In soils, the amount of TEB absorbed after being applied varies between 28 and 74%, mainly concentrated in the topsoil layer, and it presents a half-life period of 40 to 170 days (depending on the amount applied) [76]. TEB exhibits a high affinity for soil organic matter, which makes its degradation significantly influenced by the organic C content [77], i.e. low organic C content promotes the microbial degradation of TEB and the decreasing of its sorption.

Since TEB is not frequently detected in deeper soil layers, it is unlikely to cause groundwater contamination. However, it is considered one of the most usual fungicides identified in numerous freshwater ecosystems in Europe, even after wastewater treatment [77-78]. In aquatic ecosystems, TEB is recognised for affecting important ecosystems functions. The most threatened communities of freshwater environments are the aquatic fungi, once TEB can present significant toxic effects at a concentration as low as 1 $\mu g.L^{-1}$ [79]. The fungal activities influenced by the presence of TEB are, for instance, the litter decomposition [80] and the ergosterol biosynthesis. At concentrations as low as 2 $\mu g.L^{-1}$, this fungicide can inhibit respiration and photosynthesis in heterotrophic and photoautotrophic biofilm and plankton communities [81].

Lethal effects of TEB and similar compounds in invertebrates and other aquatic animals has been approached by several authors. Nevertheless, some studies [82-84] have shown that this type of fungicides can have sublethal effects at very low concentrations, such as altered food processing, lowered energy reserves, reduced growth and reproduction. In both cases, lethal and sublethal effects of TEB in aquatic organisms are not yet well known.

I.4. Pesticides on freshwater environments

Pesticides use in crops is directly related to surface water contamination, usually through surface runoffs containing pesticides that may reach water bodies near the treated area, as it was approached in previously chapters. The presence of pesticides in surface

water, such as rivers and natural or artificial lakes, may have negative impacts on freshwater flora and fauna, and also on human health, if the contaminated water is used for public consumption.

Climatic events are the origin for the surface runoffs formation, the main cause of surface water contamination with pesticides. According to their intensity and timing, runoffs can be produced by a critical event, if the event induces the formation of a runoff with a volume of 50% or more, if it reaches at least 1 cm of rain, and if occurs within 2 weeks after pesticide application. Taking into account the pesticide losses, runoffs can be originated by a catastrophic event, which happens when there is a loss of 2% or more of pesticide applied amount. There is a third possible scenario for runoffs formation, which it occurs when it rains right after the pesticide application; even if the runoff volume is low, it will have a high concentration of pesticides [27]. Moreover, and according to the Intergovernmental Panel of Climate Change [85] intense and often scenarios are predicted to occur in the next years, with the raise of temperature being one of the extreme weather events referred.

The occurrence of pesticides in freshwater environments is much likely to cause adverse effects on non-target organisms. Due to the intensive and worldwide use of pesticides, it is extremely important to study their effects and impacts in these communities, being one of the main concerns of pesticide contamination the possible bioaccumulation in primary producers, and the consequent transference along the freshwater food chains [86]. On other hand, the more information there is about the food web dynamics, the better will be the prediction of the consequences caused by the presence of pesticides, the characterization of trophic interactions and even the understanding of organisms' ecology impacts [87].

I.4.1. Biochemical analysis

In the last decades, the number of environmental studies that assess biochemical, physiological and histological changes in organisms to measure the exposure or the effects of contaminants have significantly increased [88]. These effects can manifest at any level of biological organization, from a molecular and biochemical response to an individual, population, community or ecosystem response [89]. Some studies [53], [87], [90]–[94] have

shown that the organism's health and fitness can be analysed in a more efficient and detailed way with the use of specific biomarkers, related to the determination of the impacts on essential biochemical pathways.

Fatty acids (FA), as a lipid and nutrient, have crucial roles in many vital functions of aquatic organisms, mainly on neural levels of biochemical and physiological response. For instance, they are a source of energy, being used as energy supply in all metabolic systems; they are also one of the main constituents of cell membranes (being responsible for several functions on cells' activity and permeability) and regulate the activity of many enzymes and inflammatory processes [94-95]. FA are considered one of the most important molecules transferred from primary producers to higher trophic levels without change, which make FA a good biochemical marker for understanding predator-prey relationships, providing crucial information on the ecosystem health [86, 92].

The earliest studies using FA profiles alterations as a bioindicator for the determination of the effects of chemical stressors are reported in the 1980s (e.g., [97]). Guitart et al. [98] was one of the first studies about pesticides' impacts in aquatic organisms assessing FA composition. It was observed a relationship between the pesticides' concentrations and the FA composition in dolphin tissues, indicating this analysis as promising biomarker of toxic exposure.

FA are carboxylic acids that can be classified in two possible ways. They can be divided into four groups according to the length of their chains: short-chain FA (less than 6 carbons), medium-chain FA (6 to 12 carbons), long-chain FA (13 to 21 carbons) and very-long-chain FA (more than 22 carbons). Based on the fact that FA may contain in their aliphatic chain different numbers of double bonds at different positions, resulting in large FA classes of geometric and structural isomers, they can be classified as saturated fatty acids (SFA) when they do not have any double bond, or unsaturated fatty acids (UFA). UFA can be sub-divided in: monounsaturated fatty acids (MUFA) with a single double bond, polyunsaturated fatty acids (PUFA) with two or three double bonds, and highly unsaturated fatty acids (HUFA) with four or more double bonds [96].

HUFA are classified as essential fatty acids (EFA), due to their fundamental role in the health and function of all animals at all trophic levels. Additionally, animals are able to

produce some EFAs, but not at rates fast enough to satisfy their needs, for that reason, they need to obtain them through food. Some examples of EFA are arachidonic acid (C20:4n6, ARA), eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA) [87].

On the other hand, the presence of contaminants can induce modifications in the pattern of physiological energy allocation of an organism, due to the cost of the metabolic processes necessary to eliminate the contaminant [99]. Carbohydrates (CHO) are considered the most important energy source, but also occur as functional and structural components of cells.

Depending on their chemical structure and their degree of polymerization, carbohydrates can be classified as: monosaccharides, disaccharides, oligosaccharides, and polysaccharides [100]. In this work, it will be given particular attention to polysaccharides, based on the study of the monosaccharides that constitute them. Monosaccharides can be divided into several categories, according to their chain length. For instance, pentoses (e.g., ribose - Rib) and hexoses (e.g., glucose - Glu) are considered as the more nutritionally important, used as building blocks of several polysaccharides and as cell fuel [100].

I.4.2. Tested organisms

For a better understanding of the influence of pesticides in food chains from freshwater environments, ecotoxicological assays were performed in three species used as standard species in ecotoxicological assays: the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor* and the cladoceran *Daphnia magna*.

These organisms are an important biotic component of almost all freshwater communities, they play a key role in energy and nutrient fluxes and, due to their place at the base of many food chains, they are functionally important in transferring environmental contaminants to higher trophic levels. The three species selected herein have been used as biomarkers to assess contamination in freshwater ecosystems, not only due to their wide distribution and position on food chains but also because they are highly sensitive to toxins and easily maintained in the laboratory, have a short generation time and are considered relatively simple organisms to perform any kind of toxicity tests.

Raphidocelis subcapitata and *Lemna minor* are two dominant primary producers in freshwater food chains. Any disruption in these two species, from molecular modifications to population variations, would probably induce effects at higher trophic levels, since they are a fundamental link to consumers in food webs. *Daphnia magna* is one of the most common cladocerans in freshwater ecosystems and a primary consumer of most food webs. Similarly to what happens with producers, primary consumers present an important ecological position in the aquatic food web, as they are the link between primary producers and higher level of consumers, being usually also used as early indicators of environmental contaminations.

I.5. Objectives

Taking into account the exponential growth of the human population worldwide and their demand for a constant increase in food production, a raise in fertilizers and pesticides usage is predicted. The extensive and worldwide use of these compounds in agriculture activities and other anthropogenic activities is responsible for the ecosystem contamination, being the impact on non-target organisms of particular concern.

Freshwater environments are especially vulnerable to this type of contamination, namely the water courses near agriculture fields treated with pesticides. The contaminants can easily reach them by surface run-off and, therefore, affect several biological processes at different biological organization levels, from molecular and biochemical to individual or ecosystem scales. Thus, this study aims to determine the potential toxicological and biochemical effects of copper sulphate and TEB in three freshwater species.

Raphidocelis subcapitata, *Lemna minor* and *Daphnia magna* were exposed to different concentrations of each toxicant, at two different temperatures (20°C and 25°C), for a potential climate change scenario perspective, through the assessment of impacts in the species' FA and CHO profiles.

The main goals of this study are: to determine the most sensitive species to each contaminant, and therefore the most sensitive trophic level (primary producers or primary consumers); to observe if the two fungicides have a different level of toxicity for each tested

species; to define the most favourable temperature in the presence or absence of the contaminants; to link the toxicological responses of the exposed organisms to biochemical responses, and also to evaluate if this biochemical approach can provide more and essential information concerning the contamination of freshwater ecosystems.

Chapter II - MATERIALS AND METHODS

II.1. Tested compounds

II.1.1. Copper sulphate pentahydrate

Copper sulphate impacts were assessed through the organisms' exposure to copper (II) sulphate pentahydrate for analysis EMSURE®, an inorganic salt, with high solubility in water. Its crystal form displays a blue colour, however, once it loses water when exposed to dry air, it becomes opaque white powder [40]. Some information of the product used in the toxicity bioassays can be found in Table 4.

II.1.2. Tebuconazole

Tebuconazole PESTANAL® (analytical standard), or 1-(4-Chlorophenyl)-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)-3-pentanol, was also used in the toxicity bioassays performed in the study. Some information of this compound is presented in Table 4.

Table 4. Information of copper (II) sulphate pentahydrate and tebuconazole used in the bioassays.

	Copper sulphate pentahydrate	*TEB*
Formula	$CuSO_4 . 5H_2O$	$C_{16}H_{22}ClN_3O$
CAS-No	7758-99-8	107534-96-3
Molecular weight (g.mol^{-1})	249.68	307.82
Assay	99.0 – 100.5% (iodometric)	99.5% (HPLC)
Form	solid	neat

II.2. Culture conditions of the tested species

The cultures of *Raphidocelis subcapitata*, *Lemna minor* and *Daphnia magna* used in the tests were maintained by the research team under laboratory conditions. All cultures were kept under a photoperiod of $16h^{L}:8h^{D}$ and $20 \pm 2°C$.

The green microalgae cultures *R. subcapitata* were kept in sterilized Woods Hole MBL (Marine Biological Laboratory) medium [101]. Both MBL medium preparation and material handling were performed under aseptic conditions [102]. Over a period of 5-7 days, the cultures reach an exponential growth phase, being harvested and inoculated into fresh medium to renew microalgae cultures. Microalgae cultures were maintained under aeration conditions.

Cultures of the macrophyte *L. minor* were maintained according to the OECD guideline 221 [103], with Steinberg medium in 500 ml Erlenmeyer vessels, at 20 ± 2°C with a $16h^{L}$:$8h^{D}$ photoperiod. Once a week, the youngest plants were removed to new vessels containing fresh medium, under aseptic conditions. In the renewal process, contaminated (e.g., microalgae) macrophytes were eliminated.

Daphnia magna monoclonal cultures had been reared in the laboratory for several generations, in ASTM hardwater medium [104] enriched with a standard organic additive *Ascophyllum nodosum* seaweed extract [105]. The medium renewal occurred every other day, and thenceforth the organisms were fed with *R. subcapitata* at a rate of 3.00×10^{5} cells.mL^{-1}.

II.3. Toxicity bioassays

In all tests, the organisms were exposed to two contaminants: copper (II) sulphate pentahydrate and TEB, at two different temperatures of 20°C and 25°C ± 2°C.

II.3.1. Growth Inhibition test of Raphidocelis subcapitata

The growth inhibition tests of the green microalgae *R. subcapitata* following exposure to the copper sulphate and TEB was assessed using a static bioassay conducted according to OECD 201 guideline [102], with adaptation to 24-well microplate use (e.g. [106]). The algae were exposed for 96h under continuous light supply at 20°C, to a range of concentrations between 4.74 and 17 $mg.L^{-1}$, and 7 and 23 $mg.L^{-1}$ for copper (II) sulphate pentahydrate and TEB, respectively. The concentration range for 25°C were 7.52 and 20

mg.L^{-1}, and 2.79 to 10 mg.L^{-1}, for copper (II) sulphate pentahydrate and TEB, respectively. Three replicates were established, per treatment, and each replicate well was filled with 900 µL of test solution plus 100 µL of microalgae inoculum adjusted so the final nominal cell density at the beginning of the test could be 10^4 cell.mL^{-1}. The test microplates thoroughly mixed twice a day by repetitive pipetting to promote gas exchange and avoid cell clumping. At the end of the bioassay, the cell density was quantified spectrophotometrically at 440 nm based on previous established calibrated curve. The biomass yield (cell.mL^{-1}) was calculated between the final and initial cell density as well as the growth rate (daily logarithmic increase in yield).

II.3.2. Growth Inhibition test of Lemna minor

The growth inhibition with *L. minor* was performed following the OECD guideline 221 [103], adapted to the use of 6 well plates [107]. The macrophytes were exposed in triplicate, at 20°C, for concentrations between 0.8 and 5 mg.L^{-1}, and 0.31 and 8 mg.L^{-1}, for copper (II) sulphate pentahydrate and TEB, respectively. At 25°C, the concentrations tested ranged between 1 and 4.8 mg.L^{-1}, and 0.31 and 8 mg.L^{-1}, for copper (II) sulphate pentahydrate and TEB, respectively. Each well was filled with 10 mL of test solution and added three colonies of three fronds each. At the beginning of the test, three colonies of three fronds each in triplicate were oven-dried for 24 h at 60°C to obtain the initial dry weight. The test plates were incubated for 7 days, in continuous illumination, in each mentioned test temperature. At the end of each test, fronds in each well were counted and oven-dried. Yield and specific growth rates based on both frond number and dry weight records were calculated to perform data analysis.

II.3.3. Acute immobilization test of Daphnia magna

For *Daphnia magna* nominal concentrations of copper (II) sulphate pentahydrate ranging from 0.08 to 0.38 mg L^{-1} and a geometric factor of 1.25, for TEB treatments with concentrations from 8.18 to 19.29 mg.L^{-1} and a geometric factor of 1.1. The acute immobilization test of *D. magna* was performed following the OECD 208 guideline [108],

where it was tested the effects of copper sulphate and TEB in newborn daphnids for a 48 h exposure period, with a $16h^{L}$:$8h^{D}$ photoperiod, for both temperatures. For every treatment, it was established four replicates, each containing 10 mL of control (ASTM medium) or test solutions and five organisms with less than 24h selected randomly from the third to the fifth brood. Copper sulphate test solutions were prepared by mixing the contaminants with ASTM medium. TEB test solutions used ethanol at 0.1% as solvent, promoting the TEB solubility following the guidelines [108]. During the exposure period, no food or organic additives were supplemented and, at the 24h and at the end of the 48h period, each vial was monitored for immobilized daphnids.

II.4. Toxicity bioassays for biochemical analysis

II.4.1. Raphidocelis subcapitata

Each Erlenmeyer filled with MBL medium and respective aerating systems was autoclaved 30 min to prevent any source of contamination. Four 2 L Erlenmeyers, per treatment, were spiked with the respective NOEC, EC_{10} and EC_{20} contaminant concentration (Table 6), in exception of the control Erlenmeyer's. Copper (II) sulphate pentahydrate concentrations were from 1.35 to 5.40 $mg.L^{-1}$ and from 1.73 to 3.50 $mg.L^{-1}$ at 20°C and 25°C, respectively; whereas range TEB concentrations were from 3.89 to 10.84 $mg.L^{-1}$ at 20°C, and from 4.26 to 7.79 $mg.L^{-1}$ at 25°C. Each replicate was inoculated with *R. subcapitata* bulk culture, in exponential growth, maintained in the same culture conditions described in section above. The set up were maintained aerated with air flow at respective temperatures, 20°C and 25°C, with continuous light supply for five days. After the exposure was finished the green microalgae were retrieved, from each treatment independently, by centrifugation at 4000 rpm, for 4 min, at room temperature, using 50 mL falcon flasks. The concentrated microalgae weighting between 1.3-1.6 g of fresh biomass was carefully transferred to 15 mL glass centrifuge tubes. Each flask constitutes one replicate and 3 replicates per treatment were stored at -80°C until further analysis.

II.4.2. Lemna minor

Five 500 mL Erlenmeyer with approximately 200 mL of Steinberg medium also sterilized, per treatment, were spiked with the respective NOEC, EC_{10} and EC_{20} contaminant concentration (Table 6), in exception of the control Erlenmeyer's. Copper (II) sulphate pentahydrate concentrations were from 0.40 to 0.76 $mg.L^{-1}$ and from 1.61 to 1.93 $mg.L^{-1}$ at 20°C and 25°C, respectively; whereas the TEB range concentration was from 0.05 to 0.21 $mg.L^{-1}$ at 20°C, and from 0.05 to 0.30 $mg.L^{-1}$ at 25°C. Each replicate was inoculated with *L. minor* fronds from the bulk culture, maintained in same culture conditions described in section above. The set up was maintained at respective temperature, 20°C and 25°C, with continuous light supply for seven days. The Erlenmeyers were covered during the test to minimize the evaporation and contamination.

At the end of the 7 days of exposure, the macrophytes from each treatment were recovered by gently drained excess of water and the fresh biomass between 1.1 and 1.4 g was distributed to each 15 ml glass tube, in triplicates, and stored at -80°C until analysis.

II.4.3. Daphnia magna

For daphnids was conducted for 15 days on semi-static test design, with a renewal of test solutions every other day. The test was composed by one control plus three contaminated treatments for each test substance (copper (II) sulphate pentahydrate and TEB), in two different temperatures of 20°C and 25°C ± 2°C and photoperiod of $16h^{L}$:$8h^{D}$. Concentrations of each test solutions were obtained through the dilution of the contaminants in ASTM medium, according to the NOEC, EC_{10} and EC_{20} values of the acute immobilization test described above (Table 6). Copper (II) sulphate pentahydrate concentrations were from 0.07 to 0.10 $mg.L^{-1}$ and from 0.07 to 0.09 $mg.L^{-1}$ at 20°C and 25°C, respectively; whereas the TEB range of concentrations were from 14.39 to 17.37 $mg.L^{-1}$ at 20°C, and from 25.27 to 27.96 $mg.L^{-1}$ at 25°C.

For each treatment, six replicates were established, to which were added forty organisms with less than 24h from the third to the fifth brood, in glass vials with 400 mL of test solution or ASTM medium (in control). The test was developed with a total of sixteen treatments and 3 840 exposed organisms daily fed with *R. subcapitata* in the same ratio as

in maintenance conditions and transferred to freshly prepared test solutions every other day.

During the test period, the daphnids were observed for mortality and offspring production, being recorded the number of living and dead neonates, and undeveloped embryos and eggs. The neonates were collected on the N1, N2, N3, N4 and N5 generation (when available). The offspring from the first generation (N1) was the only with enough number of organisms to proceed to biochemical analyses, thus they were collected and stored at -80°C in micro centrifuge tubes in groups of 200 neonates for replica and three replicas for treatment. At the end of the test, the parental daphnids (mothers) were also collected and stored at -80°C, 68 mothers per replica and three replicas per treatment, until further analyses.

II.5. Biochemical analyses

The biochemical analyses, namely fatty acids and carbohydrate analyses, were conducted for all tested organisms. Three replicates of each treatment of the three species studied, exposed to different concentrations of each contaminant at both temperatures were analysed.

II.5.1. Fatty acid analyses

The total lipids extraction and methylation to FA methyl esters (FAMEs) followed the methodology described by Gonçalves et al. [87]. FAMEs identification was performed by a gas chromatograph with a mass spectrometer (GS-MS), using Agilent Technologies 6890N Network (Santa Clara. CA), equipped with a DB-FFAP column (30 m x 0.25 mm x 0.1 µm). The injector port transported 1.5 µl of sample per run, it was at a temperature of 250°C and had a glass liner with 4.0 mm internal diameter. The mass selective detector (Agilent 5973 Network) was programmed in 70 eV electron impact mode, reading the m/z 40-500 spectrum in 1s cycles in full read mode. The oven was programmed to an initial temperature of 80°C, increasing with a linear rate of 25°C.min^{-1}, when the temperature of 160°C was

reached, it started a linear increase of 2°C.min^{-1} up to 190°C and then an increasing of 40°C.min^{-1} to a final temperature of 230°C, maintained for 5 min. Helium was the carrier gas used in the analysis. The injector ion source was maintained at 220°C, while the transfer line was at 280°C.

FAME peaks' integration was achieved through the software available in the equipment, and the identification of each peak was performed using the Supelco 37 component FAME mix (Sigma-Aldrich, Steinheim, Germany), by comparing the retention time and mass spectrum of each FAME. The quantification was determined following the procedure described by Gonçalves et al. [87], using nonadecanoic acid (C19:0) as an internal standard.

II.5.2. Carbohydrate analyses

Polysaccharide extraction from the tested organisms was done using the remaining lower phase after centrifugation in the protocol [87] for the FA extraction mentioned above. Sugar analysis performed was based in monosaccharides quantification, which was determined after sulphuric acid hydrolysis, derivatization to alditol acetates [110], and analysis by gas chromatography with Flame-Ionization detection (GC-FID). Alditol acetate derivatives were separated with dichloromethane and analysed by GC with an FID detector and equipped with a 30 m column DB-225 (J&W Scientific, Folsom, CA) with internal diameter and film thickness of 0.25 mm and 0.15 µm, respectively. The initial temperature was established to 200°C, increasing with a linear rate of 40°C.min^{-1} up to 220°C, when it was maintained for 7 min and then started a new linear increasing of 20°C.min^{-1} until a final temperature of 230°C, maintained for 1 min. The carrier gas was Hydrogen, at a flow rate of 1.7 mL.min^{-1}. In this analysis, 2-desoxiglucose was the internal standard used.

II.6. Statistical analyses

The data from the preliminary exposure tests were used to estimate the concentrations for the following tests. A Probit analysis was performed [111], using IBM SPSS Statistics software, to determine the concentrations which would cause 50, 20 and 10% of

growth inhibition (in both producers) or immobilization (in the cladocerans), i.e., the EC_{50}, EC_{20} and EC_{10} values with the corresponding 95% confidence intervals.

In order to determine and evaluate the differences in FA and sugar profiles across treatments and the variation of FA and sugar composition of the organisms exposed of copper sulphate and TEB at two different temperatures, analyses of variance (ANOVAs) and multivariate statistical analyses were performed in data from both biochemical analyses.

Significant differences in the FA and sugar profiles regarding the two contaminants at both temperatures were analysed through a nested ANOVA, followed by a two-way ANOVA for more detailed information on the influence of temperature on each exposure test, and also a one-way ANOVA with a Tukey's range test to discriminate significant differences between toxicant concentrations and the control treatment. All ANOVAs were carried out in the FA and sugar data from the respective analyses, using R software, with a designated level of significance of 0.05 for all data analysed.

Multivariate statistical analyses were accomplished through non-metric multidimensional scaling (n-MDS) plots, using PRIMER-5 software. Data from all assays performed in the tested species were transformed through the expression Log (x+1) and converted into similarity triangular matrices using the Bray-Curtis similarity as a resemblance measure. One-way analysis of similarity (ANOSIM) was used to test differences in FA and sugar profiles across the different tests performed for all tested species, with a level of significance of 0.05.

Chapter III - RESULTS

III.1. Toxicity bioassays

All ecotoxicological tests fulfilled the validity requirements established on their respective guidelines [101, 102, 108, 109].

Regarding the results of the copper sulphate toxicity (Table 6), the most sensitive species to the exposure of the contaminants (i.e., the species with the lower EC_{50} values) was *D. magna*, in both tested temperatures, with EC_{50} values of 0.13 and 0.12 $mg.L^{-1}$, at 20 and 25°C respectively. In opposition, the most resistant species was *R. subcapitata*, with the higher EC_{50} values of 17.64 $mg.L^{-1}$ at 20°C and 6.40 $mg.L^{-1}$ at 25°C.

In the TEB toxicity tests (Table 6), *L. minor* was the most sensitive species, with EC_{50} values of 0.78 $mg.L^{-1}$ at 20°C and 1.40 $mg.L^{-1}$ at 25°C. On the other hand, both *R. subcapitata* and *D. magna* had a highly resistance to TEB exposure, at both temperatures. The microalgae presented an EC_{50} value of 26.03 $mg.L^{-1}$ at 20°C and 13.05 $mg.L^{-1}$ at 25°C, while the daphniids had a EC_{50} value of 20.34 and 13.21 $mg.L^{-1}$ at 20°C and 25°C, respectively.

Table 5. NOEC, EC_{10}, EC_{20} and EC_{50} values ($mg.L^{-1}$) and the respective 95% confidence limits (in brackets) for *R. subcapitata*, *L. minor* and *D. magna* after the preliminary exposure to copper sulphate and TEB at 20°C and 25°C.

		NOEC	EC_{10}	EC_{20}	EC_{50}
Copper Sulphate ($mg.L^{-1}$)					
R. subcapitata	20°C	1.35	2.70 (1.79-3.60)	5.40 (4.24-6.55)	17.64 (15.38-19.90)
	25°C	1.73	2.46 (1.04-3.88)	3.50 (2.48-4.52)	6.40 (2.16-10.63)
L. minor	20°C	0.40	0.55 (0.39-0.71)	0.76 (0.6-0.92)	1.32 (1.16-1.49)
	25°C	1.61	1.76 (1.43-2.10)	1.93 (1.67-2.21)	2.28 (2.08-2.47)
D. magna	20°C	0.07	0.08 (0.06-0.09)	0.10 (0.08-0.11)	0.13 (0.12-0.14)
	25°C	0.03	0.08 (0.04-0.10)	0.09 (0.05-0.11)	0.12 (0.09-0.15)
TEB ($mg.L^{-1}$)					
R. subcapitata	20°C	3.89	6.49 (3.00-9.98)	10.84 (7.53-14.14)	26.03 (17.19-34.86)
	25°C	4.26	5.75 (3.72-7.79)	7.79 (6.30-9.28)	13.05 (9.98-16.10)
L. minor	20°C	0.05	0.10 (0.04-0.15)	0.21 (0.12-0.30)	0.78 (0.59-0.96)
	25°C	0.05	0.12 (0.02-0.21)	0.30 (0.12-0.47)	1.40 (0.94-1.86)
D. magna	20°C	14.39	15.81 (7.35-18.07)	17.37 (11.50-19.28)	20.34 (18.00-22.95)
	25°C	8.18	9.95 (8.55-10.89)	11.07 (9.99-11.82)	13.21 (12.55-13.85)

Approaching the toxicity of both fungicides, in general, copper sulphate appeared to be more harmful than TEB, due to its higher impacts in two of the three tested species. Copper sulphate exposure had harmful effects in particular to *D. magna* organisms, which presented the lowest EC_{50} values, at the two temperatures tested.

From an exclusively toxicological perspective, the temperature variation only presented slightly impacts on the growth and immobilization of the tested species. The microalgae were more sensitive at 25°C with copper sulphate exposure and more sensitive at 20°C with TEB exposure. The macrophytes' sensitiveness decreased with the temperature rise, being more resistant to both fungicides at the highest tested temperature. In contrast, the temperature rising induced an increase of the daphniids' sensitiveness, for both copper sulphate and TEB exposures.

III.2. Fatty Acid profiles

Data from the FA analysis performed in *R. subcapitata*, *L. minor* and *D. magna* (offspring and mothers) organisms showed the FA profiles of the three species, with its content expressed in $mg.g^{-1}$ for *R. subcapitata* and *L. minor*, and in $mg.org^{-1}$ for *D. magna*. The FA profiles were accomplished through an analysis on the total FA, SFA, MUFA, PUFA and HUFA content.

III.2.1. FA profile of Raphidocelis subcapitata

Figures 4 and 5 represent the effects of copper sulphate and TEB exposure in the total FA content and also in all FA classes of *R. subcapitata*, for both tested temperatures, and, in the appendix, more detailed information can be found on the impacts in these organisms (Tables S2 – S6).

Impacts of the tested fungicides on the total FA content of microalgae (Fig. 4) indicated that these organisms had their FA content affected by the presence of both contaminants and also regarding the test temperature. At 25°C, the *R. subcapitata* organisms had significant alterations in their total FA content in all contaminated treatments, to both copper sulphate and TEB exposure. While, at 20°C, only NOEC and EC_{20}

treatments from both pesticides presented significant differences compared to control samples. Additionally, it was observed that the lower concentration of both pesticides (NOEC), at the two tested temperatures, was the one that induced higher effects on the microalgae's FA.

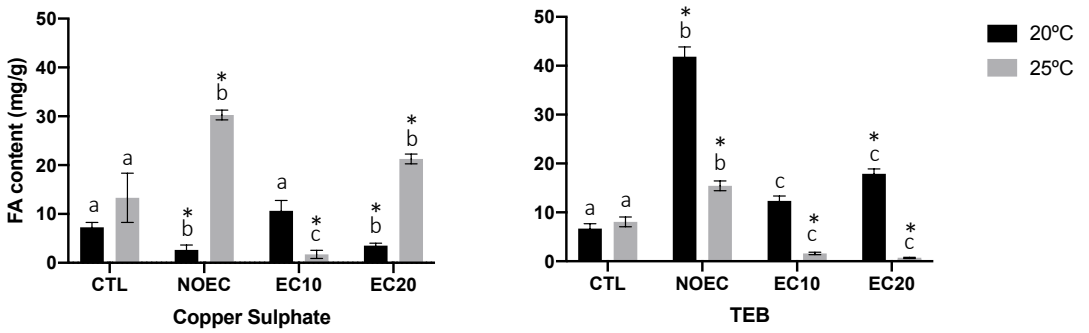

Figure 4. Effects of copper sulphate (on the left) and TEB (on the right) on the total FA content (mg.g^{-1}) of *Raphidocelis subcapitata*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

The temperature influence, as an isolated variable, is evaluated through the control samples. In these producers, the total FA content of the controls at the two temperatures did not present a great variation among themselves, at 20°C their concentrations were from 6.67 to 7.25 mg.g^{-1}, and at 25°C were slightly higher from 8.05 to 13.32 mg.g^{-1}. Nevertheless, the most abundant FA was different according to the temperature of exposure, being C18:3n9,12,15 (α-Linolenic acid or ALA) at 20°C (2.64-3.02 mg.g^{-1}), and C24:1n9 (Nervonic acid) at 25°C (3.03-3.85 mg.g^{-1}).

Taking into account the response of *R. subcapitata* organisms to the two different compounds, their response in the total FA content was not significantly different among copper sulphate and TEB exposure. Almost all FA classes presented the same response, with the exception of SFA, whose content had different variations in accordance with the present fungicide (F=5.66 and p-value < 0.05).

In copper sulphate exposure tests, these organisms displayed significant differences (p-values < 0.05) in their total FA content between the tested temperatures, as well as in SFA and HUFA (F=11.08, F=21.61 and F=14.54 for total FA, SFA and HUFA, respectively). The organisms exposed to copper sulphate at 20°C had significant changes (p-value < 0.05) when

compared to controls on their total FA content and also on all FA classes (F=22.44, F=43.87, F=27.28, F=16.54 and F=8.72 for total FA, SFA, MUFA, PUFA and HUFA, respectively). At this temperature (Fig. 5), FA content decrease on NOEC and EC_{20} copper concentrations, while in the concentration of EC_{10}, an increase was observed. On the other hand, the organisms from the test performed at 25°C were also significantly different (p-values < 0.05) from controls in their total FA and FA classes (F=27.65, F=199.70, F=61.93 and F=87.58 for total FA, SFA, PUFA and HUFA, respectively), except for MUFA content. In opposition to what happened in samples from 20°C, the temperature of 25°C induced an increase of almost all FA classes in NOEC and EC_{20} treatments, and a decrease at EC_{10} (Fig.5).

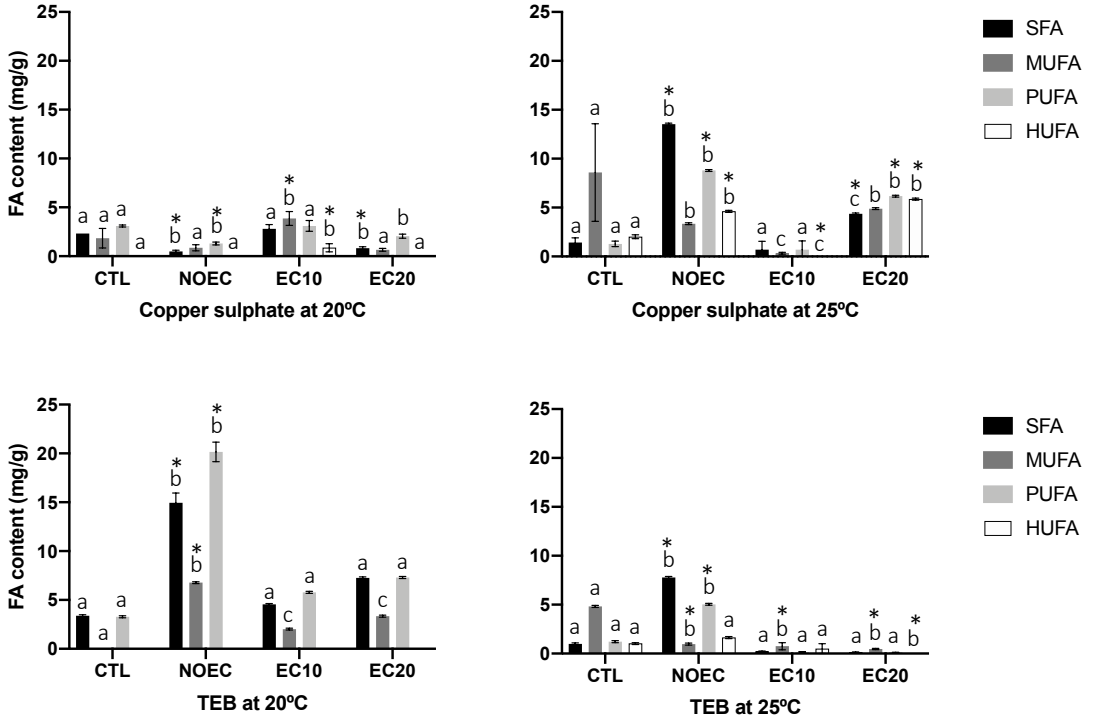

Figure 5. Saturated fatty acids (SFA; black), Monounsaturated fatty acids (MUFA; dark grey), Poliunsaturated fatty acids (PUFA; light grey) and Highly unsaturated fatty acids (HUFA; white) variations ($mg.g^{-1}$) on the treatments from the exposure assays with *Raphidocelis subcapitata*, at 20°C (on the left side) and 25°C (on the right side). The treatments' results with copper sulphate are displayed above, and the ones with TEB are below. Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

TEB exposure tests in *R. subcapitata* also presented significant differences (p-value < 0.05) when comparing data from tests performed at different temperatures (F=18.97,

F=81.85, F=27.79 and F=15.88 for total FA, SFA, PUFA and HUFA, respectively). In this case, only the MUFA class did not present a different variation according to the test temperature. Considering each test individually and comparing contaminated treatments with controls, both tests at 20°C and 25°C presented significant differences in total FA and all FA classes found in the respective organisms. Microalgae from TEB exposure at 20°C presented a significant increase in all FA classes found in them (F=23694, F=2607, F=783.80 and F=3478 for total FA, SFA, MUFA and PUFA, respectively; and p-value < 0.05 in all cases), mostly in NOEC concentration (Fig.5). At 25°C, the organisms had their SFA and PUFA content significantly increased in NOEC treatments, while MUFA and HUFA decreased with the increasing of TEB concentrations (F=3310, F=2528, F=144.30, F=497.70 and F=12.77 for total FA, SFA, MUFA, PUFA and HUFA, respectively; and p-value < 0.05 in all cases) (Fig.5).

EFA content of this species was severely affected, even disappearing completely in almost all treatments from the tests performed at 20°C. At the temperature of 25°C, both copper sulphate and TEB exposure induced a significant increase of EPA (F=64.97 and F=13.09 for copper sulphate and TEB exposure, respectively; with both p-values<0.05), while DHA content decreased (F=64.61 and F=25.44 for copper sulphate and TEB exposure, respectively; with both p-values < 0.05).

Figure 6 represents the samples data's distribution of the FA content of *R. subcapitata* in a n-MDS plot (stress=0.04). The responses of these organisms to both contaminants and temperatures were very different, even within the same test. No clear distinction between the influence of the contaminant or the temperature was found in the four tests. For this reason, it is not possible to observe evident separations of the four tests performed with the microalgae through the n-MDS plot.

The observations from Figure 6 are confirmed in the ANOSIM analysis performed with the same data. *R. subcapitata* organisms displayed different responses to the exposure of the two contaminants at both temperatures (Global R=0.18 and p-value=0.04). However, pairwise tests indicated that data did not present good segregation, due to the lower R-values registered. Only one pair of tests showed to be significantly different (p-value < 0.05): copper sulphate at 25°C/TEB at 20°C (R=0.32).

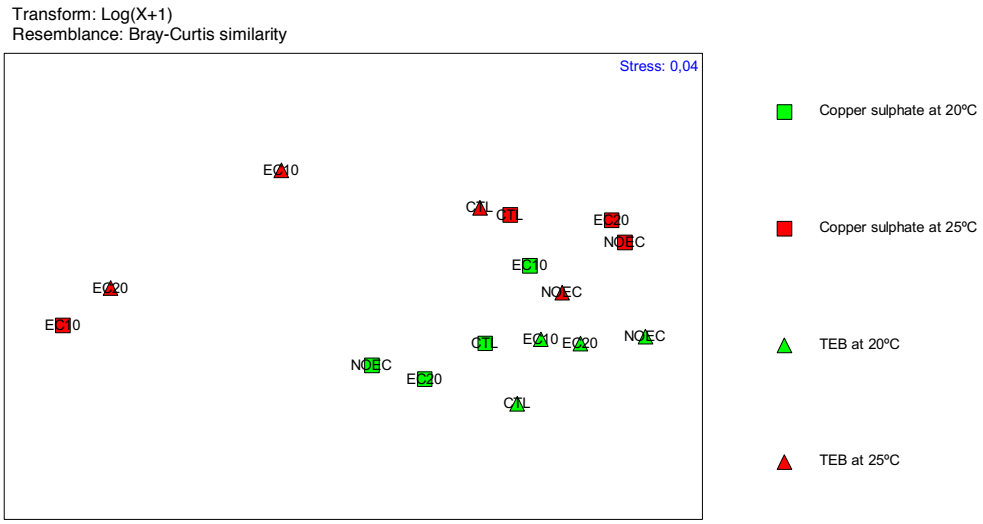

Figure 6. Two-dimensional n-MDS ordination plot of FA content of *Raphidocelis subcapitata* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

III.2.2. FA profile of Lemna minor

A global view of the influence of the two contaminants in the total FA content and their classes is demonstrated in Figures 7 and 8, respectively, and more detailed information is available in the appendix (Tables S12 – S16).

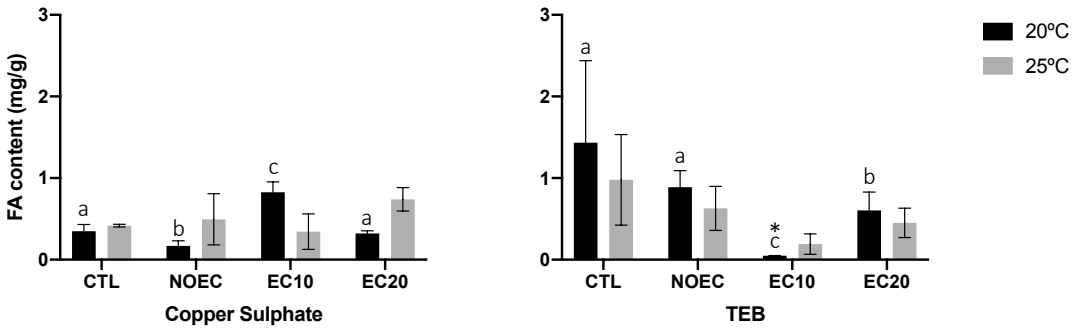

Figure 7. Effects of copper sulphate (on the left) and TEB (on the right) on the total FA content (mg.g^{-1}) of *Lemna minor*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

In general, *L. minor* did not present significant differences in the total FA content between both contaminants, as well as among the two tested temperatures (Fig. 7). Both copper sulphate and TEB did not promote significant variation in the total FA of the *L. minor* organisms, except for the EC_{10} treatment in TEB tests at 20°C. In copper sulphate tests, the total FA content only presented slight variations among the tested concentrations - an increase in EC_{10} treatment at 20°C, and in NOEC and EC_{20} treatments at 25°C (Fig. 7) were registered, for example; in the presence of TEB, both temperatures had a decrease of total FA, especially in the EC_{10} treatment (Fig. 7).

In control conditions, the FA profiles of *L. minor* exhibit variations depending on different temperatures. For instance, the organisms from the control treatments of copper sulphate test at 20°C had PUFA as the most abundant FA class, with 46.50% of the total FA content, and ALA as the FA with the highest concentration (1.41 $mg.g^{-1}$). In contrast, in the control treatments from the copper sulphate test at 25°C, the most abundant FA class was MUFA, with 80.95%, and the FA with the highest concentration was C24:1n9 (2.44 $mg.g^{-1}$).

The ANOVAs run with data from all tests indicates how the total FA content, FA classes, the most abundant FA (C24:1n9) and EFA were affected. Similar to the total FA, the MUFA, PUFA and HUFA content did not present significant variations between contaminants, their concentration varied in a similar way in the presence of copper sulphate or TEB (Fig. 8). SFA was the only FA class where the organisms showed different levels depending on their exposure to the different contaminants ($F=2.45$ and p-value < 0.05).

In these organisms, neither ARA or EPA were found in almost all treatments, not even in the control samples, at either both temperatures. On the other hand, the concentration of DHA was considered significantly different between the two contaminants ($F=6.25$ and p-value < 0.05).

Concerning the two temperatures of 20°C and 25°C, the FA content of MUFA and PUFA from copper sulphate tests presented a significant variation, according to the ANOVAs performed ($F=8.50$ and $F=13.27$, respectively, and p-value < 0.05 for all cases). In TEB exposure tests, significant differences among both temperatures were found in PUFA and HUFA and specifically in the DHA concentration ($F=15.83$, $F=8.35$ and $F=7.66$, respectively,

and p-value<0.05 for both cases). PUFA was the most affected by the temperature discrepancy, showing variations independent of which contaminant was present (Fig. 8).

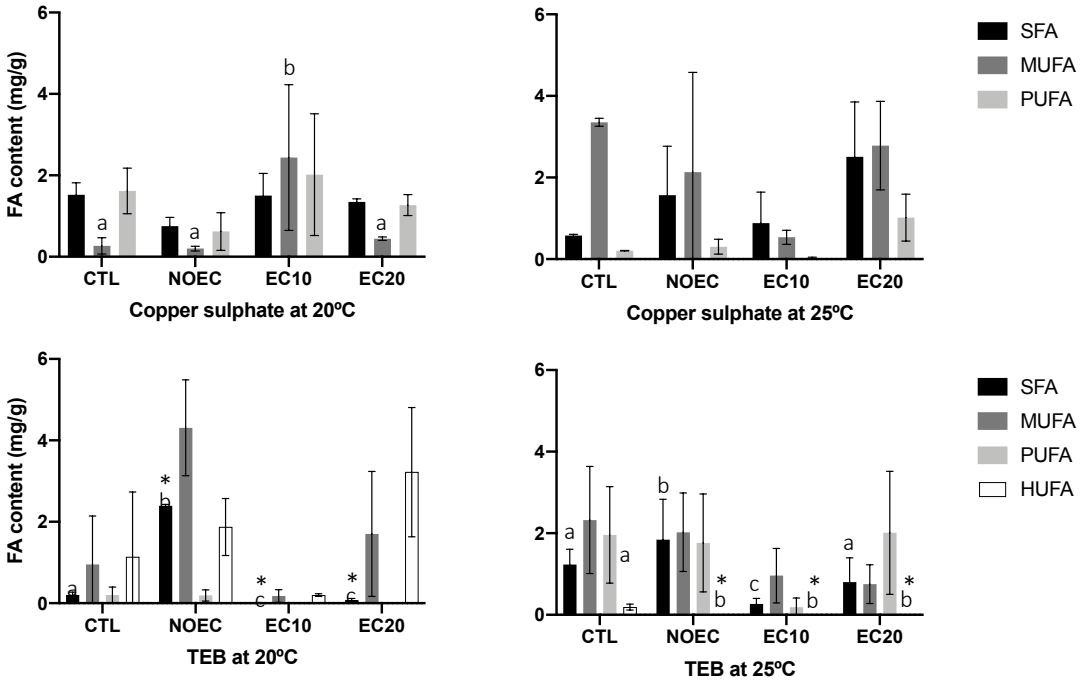

Figure 8. Saturated fatty acids (SFA; black), Monounsaturated fatty acids (MUFA; dark grey), Poliunsaturated fatty acids (PUFA; light grey) and Highly unsaturated fatty acids (HUFA; white) variations ($mg.g^{-1}$) on the treatments from the exposure assays with *Lemna minor*, at 20°C (on the left side) and 25°C (on the right side). The treatments' results with copper sulphate are displayed above, and the ones with TEB are below. Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

In this species, the contaminant that had more impact on the FA content was TEB, in particular in SFA and HUFA (Fig. 8). Some of the TEB tested concentrations had significant effects on these FA, and the largest impact was observed at 20°C. At this temperature, the total FA content was significantly lower between control and the test concentration of EC_{10}, while significant differences in the SFA concentration (F=2097 and p-value < 0.05) were also registered, increasing with the NOEC treatment, and decreasing in EC_{10} and EC_{20}. At the temperature of 25°C, significant effects were only observed in HUFA, and consequently in DHA concentration (F=26.86 for HUFA and F=26.16 for DHA, with a p-value < 0.05 in both

cases). DHA displayed significant differences in all treatments from TEB exposure at 25°C in comparison to the control because it was only present in the absence of TEB.

The responses of the different FA were diverse among the two contaminants and the tested temperatures (Fig. 8). In the copper sulphate tests, although the ANOVAs did not show significant changes, the FAs were affected. For instance, in the concentration of EC_{10} at 20°C there was an increase in all FA classes and consequently also in the total FA content, but the opposite was observed at 25°C, with a decrease in the FA content.

Figure 9 presents the samples' distribution from the four tests through a n-MDS analysis, according to the FA content found in the *L. minor* organisms for each respective treatment (stress=0.04). There is an evident separation between the treatment with the EC_{10} concentration of TEB exposure at 20°C (green triangle) from the others. This treatment is mentioned above as the only one with significant differences in the total FA content. Besides that, also the other samples from the TEB exposure test at 20°C appear to be separated, highlighting the different response of *L. minor* organisms in this test.

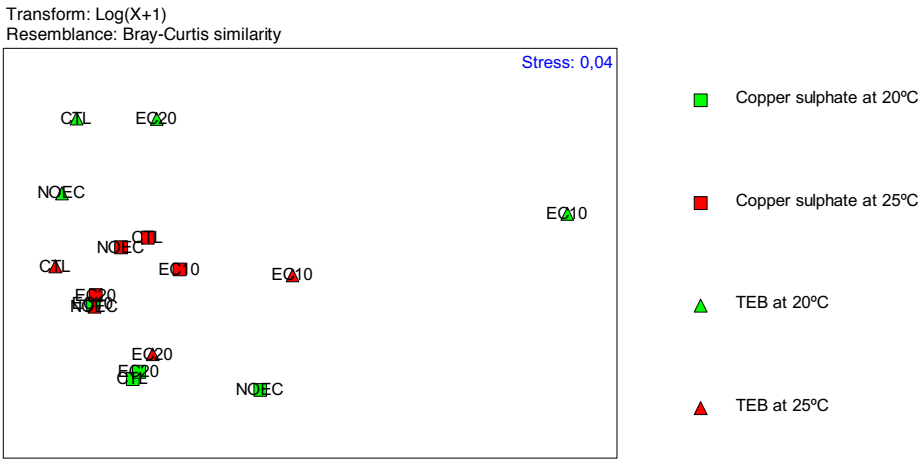

Figure 9. Two-dimensional n-MDS ordination plot of FA content of *Lemna minor* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

The ANOSIM analysis performed in the *L. minor* data indicated a clear separation of the samples (Global R=0.35 and p-value=0.001). According to the pairwise differences, the tests with significant differences (p-value < 0.05 in all cases) between them were: copper sulphate at 20°C/at 25°C (R=0.57), copper sulphate at 20°C/TEB at 20°C (R=0.51), and copper sulphate at 25°C/TEB at 20°C (R=0.51).

The n-MDS (Fig. 9) and ANOSIM analyses suggested that there is not a group of samples (or a test) with a great difference in the total FA content. However, both contaminants and both temperatures presented different impacts in *L. minor* organisms, once almost all tests presented a significate difference among them, showing good segregation.

III.2.3. Daphnid FA profile

Daphnia magna was evaluated in two different life stage: offspring (from first generation) and mothers. The offspring had a shorter time of exposure to the tested contaminants, as they were collected on the sixth day of the experiment (the same day that the mothers deposited their first eggs) and were in contact with the contaminants for only a few hours.

III.2.3.1. Offspring's FA profile

Figures 10 and 11 present the impacts of both contaminants in the total FA content and also in all FA classes, for both tested temperatures. At the appendix more detailed information can be found about the impacts in these organisms (Tables S22 – S26).

FA profile of offspring of *D. magna* was influenced by the presence of contaminants but also by the temperature variation. The total FA content changes significantly due to the presence of copper sulphate or TEB, but also in accordance with the test temperature (Fig. 10). In general, total FA was found in lower concentrations in copper sulphate treatments, and also in the temperature of 20°C. For this reason, the test with copper sulphate at 20°C was the one with the lowest number of total FA registered. Although the concentration of FA per neonate was low (4-55 $\mu g.org^{-1}$), it is possible to observe that both total FA and FA classes were affected in the performed tests.

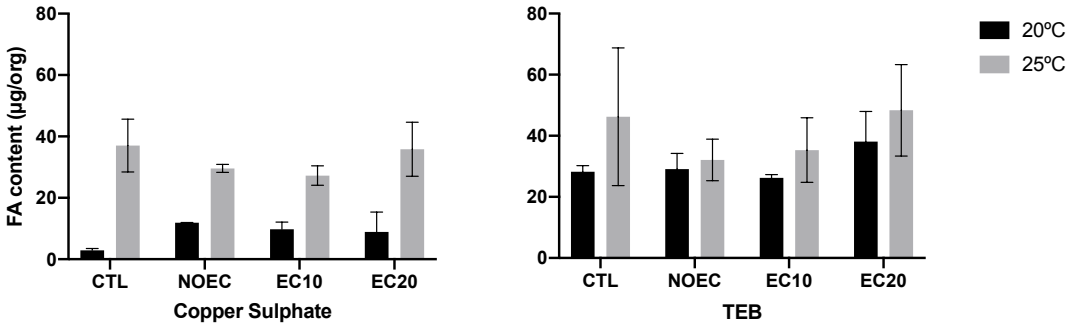

Figure 10. Effects of copper sulphate (on the left) and TEB (on the right) on the total FA content (µg.org^{-1}) of offspring of *Daphnia magna*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars.

Through the analysis of the control treatments, the temperature influence becomes clearer to recognise. In the case of the neonates of *D. magna*, all treatments performed without the presence of both contaminants demonstrated that temperature variation had a large impact in the total FA content. For instance, in the controls for the TEB tests, the total FA was 33.88 ug.org^{-1} at 20°C and 46.24 ug.org^{-1} at 25°C. SFA was the most abundant (42.50-80.50%), despite being the class less diversified, with the most abundant FA be C21:0 (1.55-29.80 ug.org^{-1}) in all control treatments.

Significant differences on the influence of contaminants were found in the total FA and also in all FA classes (F=32.92, F=7.71, F=80.06, F=106.61 and F=9.53 for total FA, SFA, MUFA, PUFA and HUFA, respectively; p-value < 0.05 in all cases). FA classes followed similar behaviours as the total FA content mentioned previously, i.e., the copper sulphate treatments presented lower numbers than TEB treatments at the same temperatures (Fig. 11).

The temperature of 20°C had also an effect of decreasing the concentrations on the total FA content and some FA classes, for both tested contaminants. In the case of copper sulphate, all FA classes (as well as the total FA) had significant differences between the two tested temperatures (F=82.41, F=64.88, F=23.69, F=183.23 and F=165.19, for total FA, SFA, MUFA, PUFA and HUFA, respectively; p-value < 0.05 in all cases), so that treatments with copper sulphate at 20°C were identified as having the lowest values of FA. In TEB tests, the temperature of 20°C presented significant lower values in comparison to 25°C, but not in all

FA classes. In the presence of TEB, only PUFA and HUFA (as well as the total FA) presented significant differences and higher concentrations in the treatments performed at 25°C (F=5.27, F=13.74 and F=18.08, for total FA, PUFA and HUFA, respectively; p-value < 0.05 in all cases).

Despite the copper sulphate test at 20°C be distinguished as the test with lower numbers of FA, it is also considered as the one with more impact in offspring of *D. magna*. In this test, significant variations were found in relation to control treatments on the PUFA and HUFA content (F=5.73 and F=4.71, respectively; p-value < 0.05 in both cases), from the NOEC and EC_{10} tested concentrations. In copper sulphate test at 25°C, no significant differences were found in any concentration tested and the controls, and the same was observed for TEB at 20°C treatments. The other test where significant differences regarding the controls were found was TEB at 25°C, but that was only verified for MUFA content (F=41.43 and p-value < 0.05) in NOEC, EC_{10} and EC_{20} concentrations.

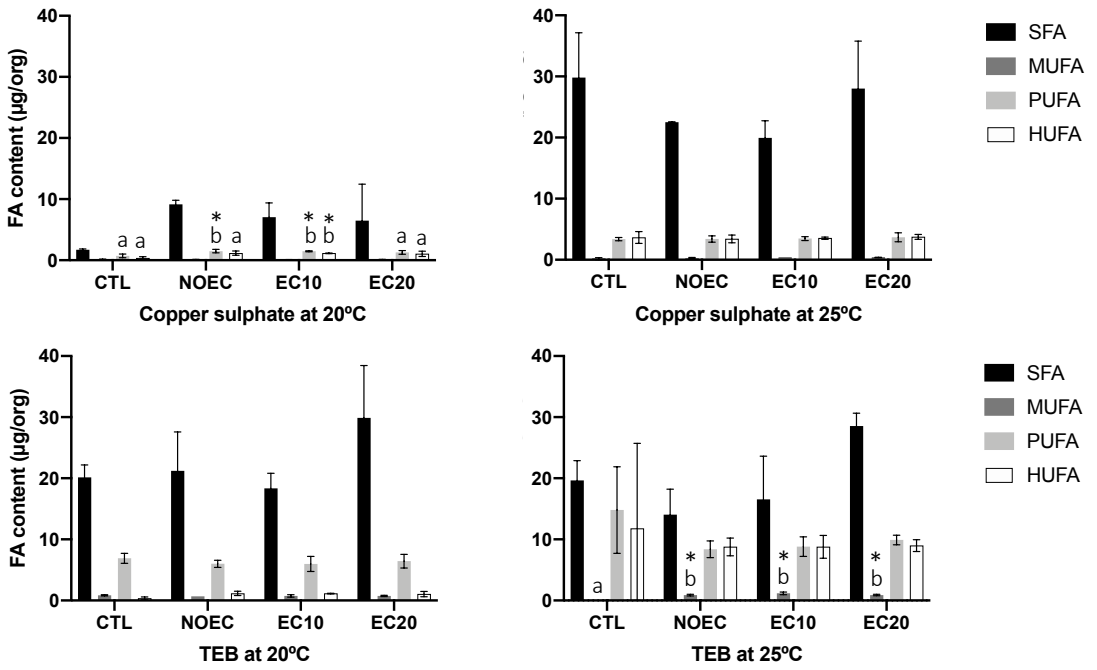

Figure 11. Saturated fatty acids (SFA; black), Monounsaturated fatty acids (MUFA; dark grey), Poliunsaturated fatty acids (PUFA; light grey) and Highly unsaturated fatty acids (HUFA; white) variations (µg.org^{-1}) on offspring from the exposure assays with *Daphnia magna*, at 20°C (on the left side) and 25°C (on the right side). The treatments' results with copper sulphate are displayed above, and the ones with TEB are below. Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

The analysis on the most abundant FA in the offspring of *D. magna* (C21:0) indicated that this particular FA had significantly different concentrations (p-value < 0.05) among the treatments from the exposure to the two contaminants (F=26.21). C21:0 exhibited a different pattern in the two tested temperature, similar to what was mentioned above; however, it was not classified as significantly different. At 20°C, the presence of copper sulphate induced a rise in the concentrations while TEB exposure did not cause changes. On the other hand, at 25°C, it was registered a decrease of C21:0 concentration in all contaminated treatments, in comparison to the control samples.

Regarding the EFA, in general, these organisms appeared not to have DHA in their composition. Both ARA and EPA presented significant differences (p-value < 0.05) among the two contaminants (F=80.83 and F=209.30, respectively) and between the two tested temperatures for the copper sulphate (F=66.48 and F=165.19, respectively) and for TEB exposure (F=6.12 and F=4.62, respectively). Copper sulphate exposure at 20°C was the only test where it was observed a significantly variation of ARA and EPA content in the different tested concentrations (F=5.29 and F=4.71, respectively; p-value < 0.05 in both cases). At these conditions, when compared with control samples, ARA content of offspring of *D. magna* was significant different in NOEC and EC_{10} treatments, and EPA in EC_{10}.

The n-MDS analysis of the distribution of all samples regarding the similarity of FA content from the offspring of *D. magna* can be found in Figure 12 (stress=0.01). Copper sulphate test at 20°C (green squares) was the test whose neonates' FA content from all treatment was more different regarding the results from the other tests, due to the bigger distance verified between samples from the particular test and the others.

The following ANOSIM analysis of *D. magna* neonates showed good segregation of the data (Global R=0.64 and p-value=0.001). Through the analysis of the pairwise test, significant differences (p-value < 0.05 in all cases) were recorded in the following pairs organized in decreasing order of the greatest distance between them (highest R-value): copper sulphate at 20°C/TEB at 25°C (R=0.87), copper sulphate at 25°C/TEB at 25°C (R=0.78), copper sulphate at 20°C/at 25°C (R=0.73), and copper sulphate at 20°C/TEB at 20°C (R=0.71).

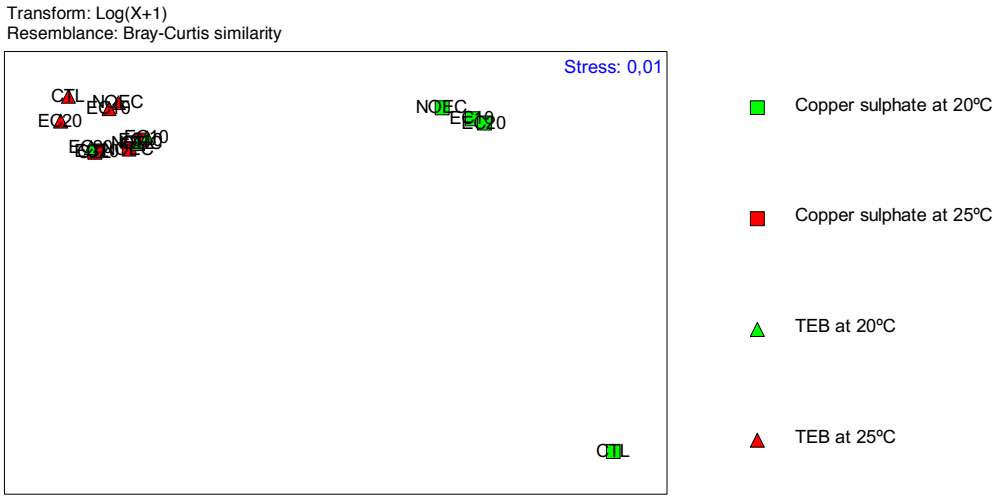

Figure 12. Two-dimensional n-MDS ordination plot of FA content of offspring of *Daphnia magna* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

The samples' distribution accomplished by these analyses indicated a more distinct test regarding the others, but at least one more pair of groups also presented a significant separation.

III.2.3.1. Mothers' FA profile

The FA profile of mothers of *D. magna* was highly marked by the differences among the contaminants and the two tested temperatures. Both total FA and all FA classes content were significant different in all four tests, but most of the tested concentrations of both contaminants did not show considerable variations in the FA values for these animals (Fig. 13 and 14). In the appendix is displayed more detailed information about mothers of *D. magna* to the contaminants' impacts (Tables S27 – S31).

In opposition to what was observed in the total FA content in the neonates of *D. magna*, the TEB treatments presented lower concentrations than copper sulphate treatments, in both tested temperatures (Fig. 13). However, the temperature with the lower FA values was the same as in the offspring tests, the temperature of 20°C. Hereupon, the TEB test at 20°C was the one with the lowest values registered.

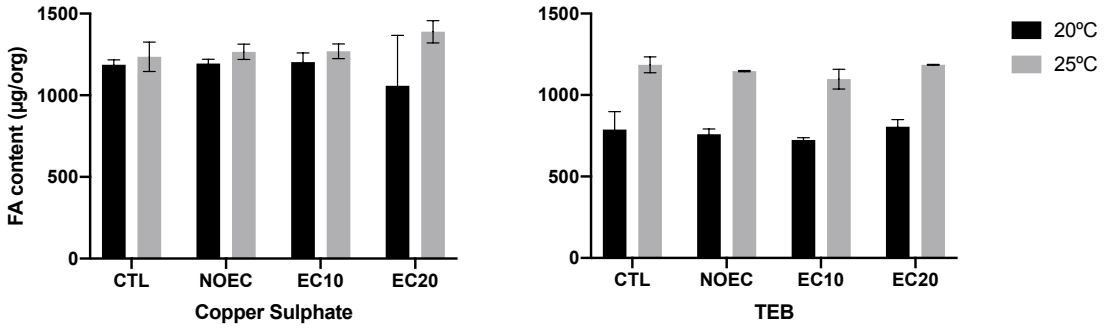

Figure 13. Effects of copper sulphate (on the left) and TEB (on the right) on the total FA content (µg.org^{-1}) of the mothers of *Daphnia magna*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars.

In the control treatments, the tests at 20°C presented lower FA values, for example, the copper sulphate test at 25°C had 1235 µg.org^{-1} of total FA and 1187 µg.org^{-1} at 20°C. For all controls at the two tested temperatures, PUFA present the highest percentage of abundance (41-46%), and the most abundant FA was EPA (239.30-464.30 µg.org^{-1}).

Figure 14 shows that the total FA and all FA classes had values significantly different between the two contaminants (F=85.35, F=12.48, F=135.23, F=87.41 and F=81.74, for total FA, SFA, MUFA, PUFA and HUFA, respectively; p-value < 0.05 in all cases). The presence of TEB induced lower values of FA in both temperatures, according to the copper sulphate tests at the respective temperature.

Regarding the copper sulphate tests, the different temperatures had a significant impact in the total FA and almost all FA classes (F=4.66, F=6.48, F=24.45 and F=16.00, for total FA, SFA, MUFA and PUFA, respectively; p-value < 0.05 in all cases). HUFA concentration was the only that did not significantly vary among 20°C and 25°C. On the other hand, in the TEB test, the temperature had an even larger impact, with a higher increase of the total FA and almost every FA classes at 25°C (F=272.31, F=142.58, F=971.10 and F=206.12, for total

FA, MUFA, PUFA and HUFA, respectively; p-value < 0.05 in all cases), with the exception of SFA that showed not be significant affected by temperature variation.

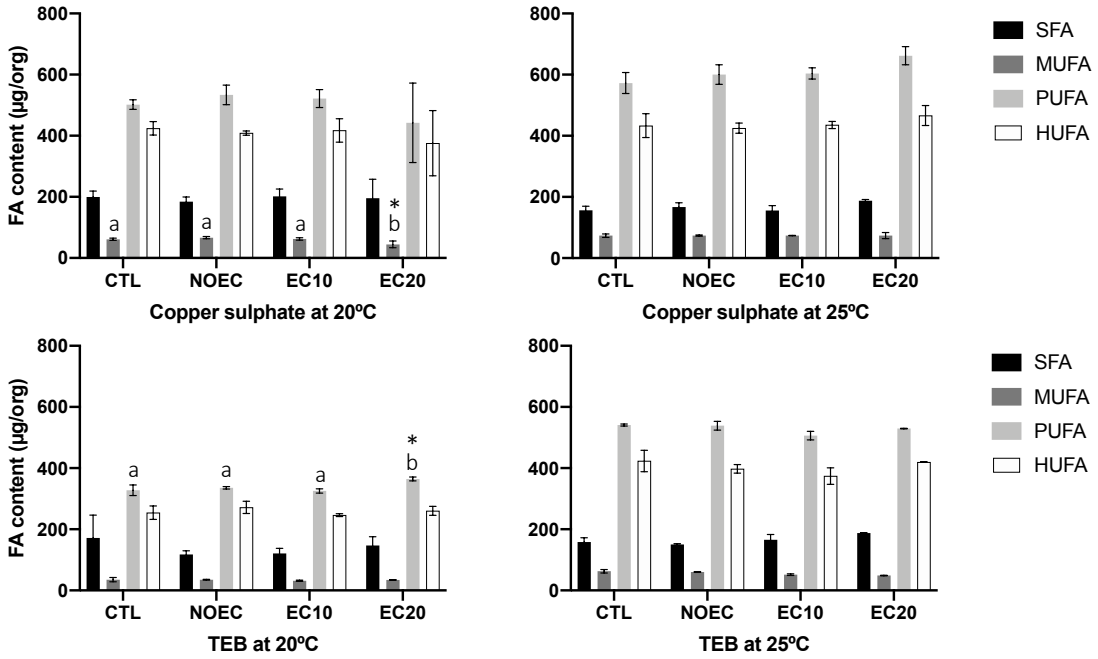

Figure 14. Saturated fatty acids (SFA; black), Monounsaturated fatty acids (MUFA; dark grey), Poliunsaturated fatty acids (PUFA; light grey) and Highly unsaturated fatty acids (HUFA; white) variations (µg.org^{-1}) on the mothers from the exposure assays with *Daphnia magna*, at 20°C (on the left side) and 25°C (on the right side). The treatments' results with copper sulphate are displayed above, and the ones with TEB are below. Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

Mothers of *D. magna* only presented significant variations in two treatments in relation to the controls, in the presence of EC_{20} concentration of copper sulphate and TEB, both at 20°C. In the treatment with copper sulphate with a concentration of EC_{20}, it was only observed a significant decrease at MUFA concentration per organism when compared with the control treatment (F=6.93 and p-value < 0.05), but the others FA classes also presented a similar decrease. In opposition to the temperature of 20°C, the copper sulphate test at 25°C presented a slight increase in all FA classes' content in EC_{20} treatment.

In the other treatment where a significant change in a FA class was registered, TEB concentration of EC_{20} at 20°C caused an increase in the PUFA content of the *D. magna* mothers (F=9.59 and p-value < 0.05). However, despite presenting a substantial impact by

temperature discrepancy, the TEB test did not show any more variations (significant or slight) between the different tested concentrations in both temperatures. The results may suggest that the TEB exposure improve the sensitiveness of mothers of *D. magna* to temperature variation, at least more than copper sulphate exposure.

Mothers of *D. magna* presented high levels of EFA in their composition, mostly since EPA was their most abundant FA. EPA and ARA were found to be significantly different (p-value < 0.05) among the two tested contaminants (F=52.16 and F=87.85, respectively), as well as between the two temperatures in the presence of TEB (F=76.70 and F=205.46, respectively). Despite not presenting a significant variation between the two contaminants, DHA had different concentrations in accordance with the tested temperature, in both copper sulphate and TEB presence (F=12.45 and F=27.76, respectively; and p-value < 0.05 in both cases). Among the treatments with different pesticides concentrations, it was only found significant changes (p-value < 0.05) in ARA content from copper sulphate concentration of EC_{20} at 25°C (F=6.28) and DHA content from TEB concentration of EC_{10} at the same temperature of 25°C (F=9.07).

Figure 15 represents the samples data distribution of the FA content of mothers of *D. magna* in a n-MDS analysis (stress=0.01). In this case, the organisms exposed to TEB at 20°C (green triangles) were the ones with the most different response, when compared to the other groups. The exposure to copper sulphate at 25°C (red squares) also presented a clear separation from the remaining tests.

The observations in Figure 15 are confirmed in the ANOSIM analysis performing in the same data. In general, mothers of *D. magna* exhibited diverse responses to the exposure of the two contaminants in both temperatures (Global R=0.69 and p-value=0.001). In the analysis of pairwise differences, almost all tests were significantly different (p-values < 0.05) and presented high R values, showing good segregation: TEB at 20°C/copper sulphate at 25°C (R=1), TEB at 20°C/copper sulphate at 20°C (R=1), TEB at 20°/TEB at 25°C (R=1), copper sulphate at 25°C/TEB at 25°C (R=0.66) and copper sulphate at 20°C/copper sulphate at 25°C (R=0.51).

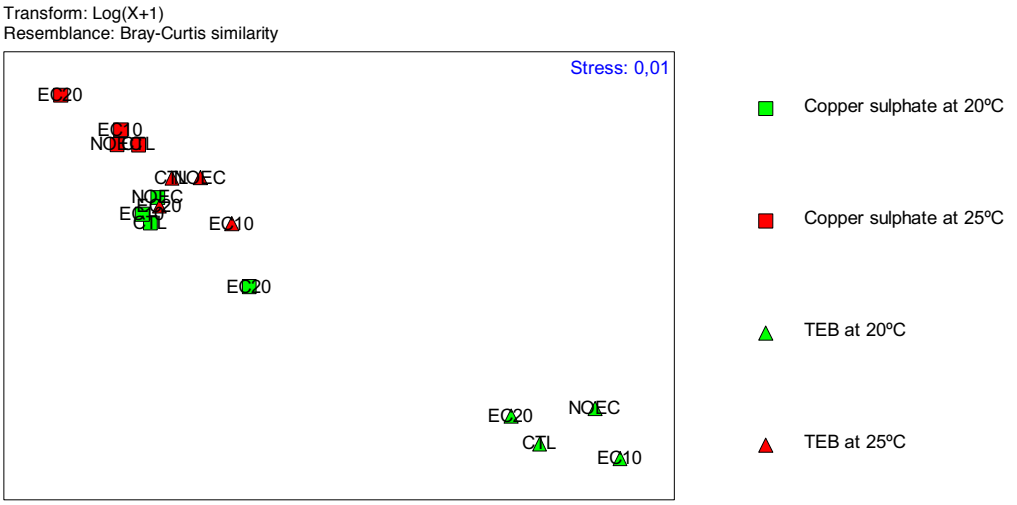

Figure 15. Two-dimensional n-MDS ordination plot of FA content of mothers of *Daphnia magna* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

III.3. Carbohydrate profiles

Data from the sugar analysis performed in *R. subcapitata* and *L. minor* organisms displayed the CHO (evaluated concerning the monosaccharides' profile) content of the species, with its content on Rha (Rhamnose), Fuc (Fucose), Ara (Arabinose), Man (Mannose), Gal (Galactose), Glu (Glucose), Rib (Ribose) and Xyl (Xylose).

This analysis was also performed in the offspring and mothers of *D. magna*. The CHO profile of the mothers only revealed the presence of Glu at an extremely high relative abundance, which may have disguised the spikes of other monosaccharides in the chromatograms. On the other hand, CHO content of neonates of *D. magna* was very low, with no monosaccharide spike detected by the equipment. In both cases, the influence of pesticide exposure and temperature variation was not evident. For this reason, the CHO profiles of *D. magna*, from both offspring and mothers, are not herein analysed.

III.3.1. Carbohydrate profiles of Raphidocelis subcapitata

The microalgae biochemical response to the impact of copper sulphate and TEB exposure at two different temperatures, regarding their total CHO content, is represented in Figure 16. More detailed information about the effects of both compounds and temperature variation on each monosaccharide of the microalgae can be consulted in the appendix (Tables S7 – S11).

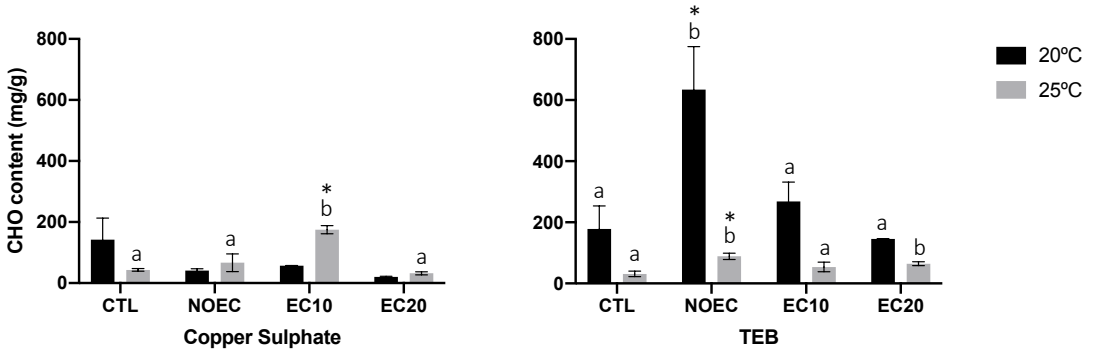

Figure 16. Effects of copper sulphate (on the left) and TEB (on the right) on the total CHO content (mg.g^{-1}) of *Raphidocelis subcapitata*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

In this species, TEB affected more significantly the total CHO content (Fig. 16), in particular in the lowest tested concentration (NOEC), at 20°C and 25°C. In both temperatures an increase in all contaminated treatments was observed regarding the control samples, especially at 20°C. On the other hand, copper sulphate exposure induced a slight decrease of total CHO content at 20°C and an increase at 25°C, significant increase only registered in EC_{10} copper sulphate concentration.

CHO profiles of the microalgae were highly different in their content according to the test temperature that they were subjected to. The temperature influence was verified even in the control samples, where the contaminants' toxicity action was not present. Controls presented a considerably higher CHO content at 20°C (142.64-178.20 mg.g^{-1}) than at 25°C (31.16-43.27 mg.g^{-1}). The most abundant monosaccharide was Glu in almost all control

samples, exhibiting a concentration of 73.25 $mg.g^{-1}$ in copper sulphate test at 20°C and 19.51 $mg.g^{-1}$ at 25°C.

Comparing the data from the two pesticides, significant differences (p-value < 0.05) between the exposures of copper sulphate and TEB were found in the total CHO content and also in all monosaccharides of *R. subcapitata* (F=14.73, F=24.83, F=29.15, F=17.10, F=8.85 and F=5.47 for total CHO, Ara, Man, Gal, Rib and Xyl, respectively), with the only exception of Glu. Despite being the most abundant monosaccharide, Glu presented a similar response in both copper sulphate and TEB exposures.

Raphidocelis subcapitata response to copper exposure did not vary significantly between the two tested temperatures, neither in total CHO content nor in any monosaccharide found in the organisms. Furthermore, alterations in the CHO content were registered in the contaminated treatments regarding the controls, in each temperature. The temperature of 25°C induced more significant impacts from the microalgae exposed to copper sulphate than 20°C. At this temperature, significant differences (p-value < 0.05) were observed in the total CHO and all monosaccharides, especially in EC_{10} concentration (F=52.43, F=404.50, F=36.35, F=204.40, F=23.94 and F=9.21E+30 for total CHO, Ara, Man, Gal, Glu and Xyl, respectively). At 20°C, copper sulphate exhibited significant differences in the total CHO content, but in some monosaccharides such as Ara, Man and Gal, all contaminated treatments were significantly different from the controls (F=268.70, F=50.63 and F=52.53 for Ara, Man and Gal, respectively; and p-value < 0.05 in all cases).

TEB exposure promoted a different response from these organisms according to the test temperature. Significant differences (p-value < 0.05) among the two tested temperatures were found in the total CHO content and also in all monosaccharides (F=25.34, F=42.60, F=24.62, F=1.07 and F=5.78 for total CHO, Man, Gal, Glu and Rib, respectively).

Even though the organisms response was differently regarding to the test temperature. The affected monosaccharides were the same at the two temperatures, as well as the TEB concentrations. Both temperatures significantly influenced (p-value < 0.05) the total CHO content (F=15.12 and F=15.41 for 20°C and 25°C, respectively), and the most affected sugars were Gal (F=13.50 and F=17.35 for 20°C and 25°C, respectively), Glu

(F=38.40 and F=8.12 for 20°C and 25°C, respectively) and Rib (F=41.40 and F=29.04 for 20°C and 25°C, respectively).

The n-MDS analysis of the distribution of all samples regarding the similarity of CHO content from the *R. subcapitata* can be found in Figure 17 (stress=0.07). Samples from TEB exposure test at 20°C (green squares) appear to be the only ones that are separated from the others tests' samples, indicating that it was in this test that the organisms presented a more different response.

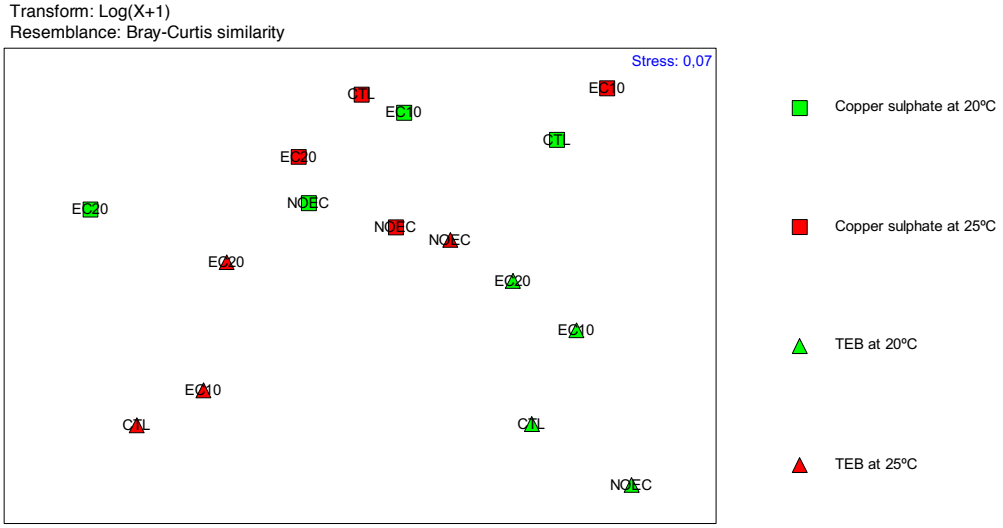

Figure 17. Two-dimensional n-MDS ordination plot of CHO content of *Raphidocelis subcapitata* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

In the ANOSIM analysis performed with the same data (Global R=0.38 and p-value=0.004) confirmed the particular separation of samples from TEB exposure test at 20°C observed in the n-MDS plot. Through the pairwise tests performed, significant differences (p-values < 0.05) were registered in the following pairs: copper sulphate at 20°C/TEB at 20°C (R=0.62), copper sulphate at 25°C/TEB at 20°C (R=0.65) and TEB at 20°C/TEB at 25°C (R=0.69).

III.3.2. Carbohydrate profiles of Lemna minor

The influence of both tested contaminants and temperatures on the total CHO content of *L. minor* organisms is represented in Figure 18, and more detailed information

concerning this influence on each single monosaccharide of the macrophytes can be found in the appendix (Tables S17 – S21).

The total CHO content of *L. minor* (Fig. 18) presented more significant differences on the presence of copper sulphate at both tested temperatures of 20°C and 25°C, in comparison to the control samples. Copper sulphate exposure induced a substantial increase of total CHO content at 20°C, while at 25°C it was registered a slightly (but significant) increase in NOEC and EC_{10} concentrations and a decrease in EC_{20} concentration. In the presence of this compound, the *L. minor* response was more evident at 20°C.

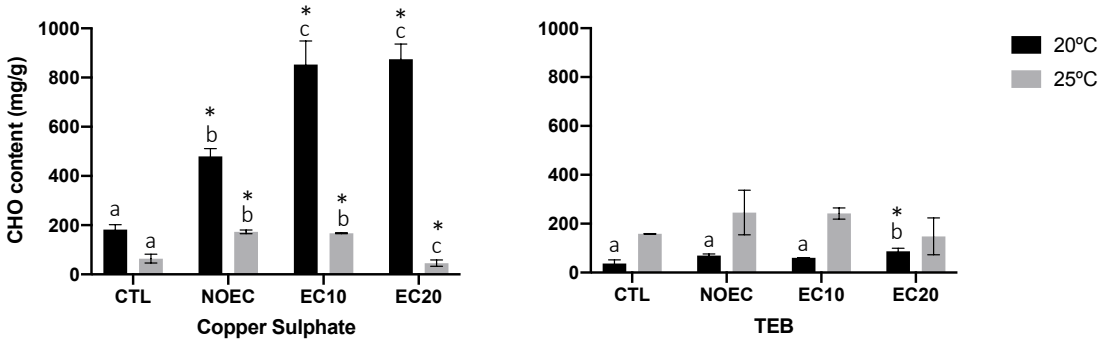

Figure 18. Effects of copper sulphate (on the left) and TEB (on the right) on the total CHO content ($mg.g^{-1}$) of *Lemna minor*, at two different temperatures of 20°C (black) and 25°C (grey). Each column represents the mean-values of all replicas of each treatment (controls and contaminated samples) with their respective standard error bars. The letters above the columns point to significant differences (p-value < 0.05) among the treatments and * indicates that the respective tested concentration was significantly different to the control samples from the same test.

TEB exposure did not induce so many significant differences in the total CHO content of the macrophytes as copper sulphate exposure (Fig. 18). The only treatment with significant variation regarding the control samples was the higher TEB tested concentration (EC_{20}) at 20°C. In opposition to copper sulphate tests, the CHO profiles from the TEB exposure tests exhibit a higher abundance of CHO content at 25°C, where it was also registered a slight increase of CHO content in NOEC and EC_{10} treatments.

CHO profile of *Lemna minor* presented a great variation through samples from the different tests, even in the control samples. For instance, in control from copper sulphate exposure, the total CHO content varied from 182.24 $mg.g^{-1}$ at 20°C to 63.88 $mg.g^{-1}$ at 25°C.

The most abundant monosaccharide was also different regarding the test temperature, being Glu more abundant in control samples at 20°C, and Ara or Gal at 25°C.

Similar to the total CHO content, also almost all the single monosaccharide analyzed present significant differences among the two contaminants' exposure (F=25.86, F=13.39, F=23.78, F=9.22, F=18.60 and F=7.79 for total CHO, Fuc, Ara, Man, Glu and Rib, respectively; and p-value < 0.05 in all cases), with the exceptions of Rha and Gal content.

Concerning the copper sulphate exposure, in these tests was observed more significant variations in CHO profile of *L. minor*, as it was mentioned above. Only Man did not present a significant difference between the two temperatures. All others, as well as the total CHO content, showed different concentrations according to the tested temperature in copper sulphate tests (F=45.94, F=11.19, F=19.03, F=15.56, F=17.08, F=33.97 and F=5.84 for total CHO, Rha, Fuc, Ara, Gal, Glu and Rib, respectively; and p-value < 0.05 in all cases).

The macrophytes from the treatments with copper sulphate at 20°C showed significant differences to the control samples in almost all single monosaccharides, especially in the EC_{20} treatments, except for Man (F=85.40, F=8.91e+30, F=32211, F=51.95, F=42.55 and F=88.58 for total CHO, Rha, Fuc, Ara, Gal and Glu, respectively; and p-value < 0.05 in all cases). On the other hand, at 25°C, significant differences were found for all monosaccharides (F=97.92, F=82.03, F=6.55e+30, F=111.30, F=14.25, F=1312, F=26.61 and F=5.96e+30 for total CHO, Rha, Fuc, Ara, Man, Gal, Glu and Rib, respectively; and p-value < 0.05 in all cases). At 20°C, the most affected sugar was Rha, while at 25°C was Fuc and Rib.

TEB exposure tests performed with *L. minor* did not show as many significant differences also in themonosaccharides as copper sulphate tests did. Besides the total CHO content, differences among the two tested temperatures were registered in Ara, Gal and Glu (F=50.34, F=35.43, F=68.78 and F=21.77 for the total CHO, Ara, Gal and Glu, respectively; and p-value < 0.05 in all cases).

Through the analysis of the CHO profile of *L. minor* from TEB exposure tests at both temperatures, it was observed that significant variations in the CHO content only happened at 20°C, mostly in the higher tested TEB concentration. In this case, monosaccharides and also the total CHO content presented significant differences between control and contaminated treatments (F=10.31, F=99.44, F=15.92, F=934.70, F=8.48 and F=5.40 for the

total CHO, Rha, Ara, Man, Gal and Glu, respectively; and p-value < 0.05 in all cases). In TEB presence at 20°C, Man was the most affected sugar, and at 25°C, although it was not registered significant differences among treatments, Rha and Man were the most affected.

The samples data distribution of the CHO content of *L. minor*, accomplished with a n-MDS analysis, is represented in Figure 19 (stress=0.07). Exclusively through the direct observation of the n-MDS plot, a clear separation of the four tests is not verified. TEB exposure (red and green triangles) appear to be more concentrated in the centre, while copper sulphates samples (red and green squares) are found more dispersed by the margins.

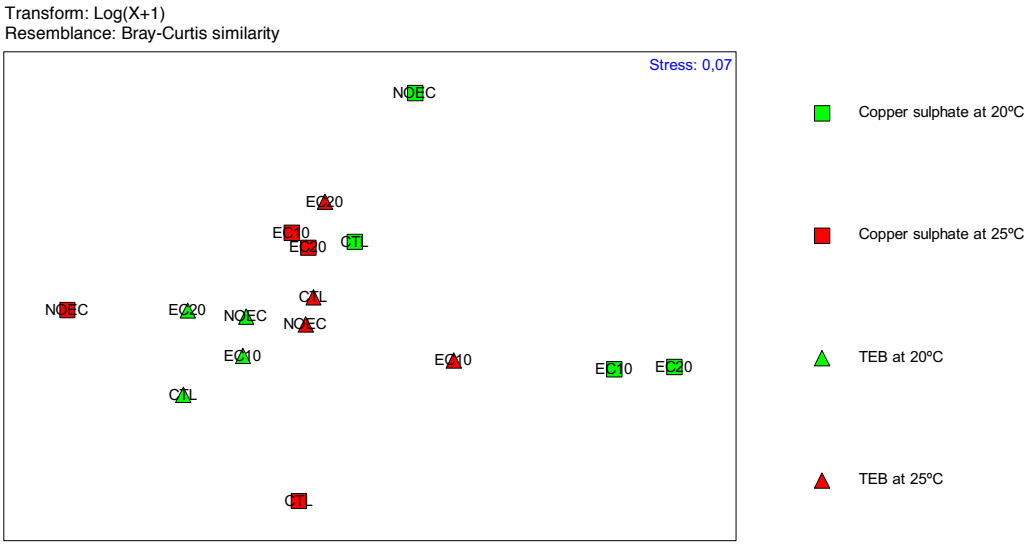

Figure 19. Two-dimensional n-MDS ordination plot of CHO content of *Lemna minor* in the four exposure tests: copper sulphate at 20°C (green squares) and 25°C (red squares), and TEB at 20°C (green triangles) and 25°C (red triangles).

The ANOSIM analysis performed with the CHO data of the macrophytes displayed a more evident separation among the samples from the tests with the two conpounds in both tested temperatures (Global R=0.31 and p-value=0.007). In comparing pairwise differences, the data did not present good segregation, once significant differences (p-value < 0.05) were only found between two pair of tests: copper sulphate at 20°C/TEB at 20°C (R=0.71) and TEB at 20°C/TEB at 25°C (R=0.58).

Chapter IV - DISCUSSION

The exposure tests and the biochemical analysis performed in this study highlighted the most toxic fungicide (copper sulphate or TEB) for the tested species, at a toxicological level, and the impact of the contaminants on the CHO and FA content, for a biochemical perspective. Furthermore, the impact of the temperature variation was evaluated, in both presence and absence of the contaminants, by performing the exposure assays at two different temperatures: 20°C and 25°C.

According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [112], changes in the global climate are expected already in this century, mainly in temperature and precipitation. In this report on climate change, it is indicated that the annual temperature of Southern Europe (Mediterranean area) may increase up to approximately 4°C until the end of the century, and, during summer months, this increase may even reach 5.50°C. Some projected models [113] also suggest that, in southern Europe, the increase in temperature may be followed by a decrease in precipitation and, consequently, in water run-off, which can decrease about 10-30% by the year 2050. Due to these future projections, this particular area is considered a climate change "hotspot" [114] and one of the Europe areas that could be more affected [115], with an alarming increase in temperature and consecutive dry days.

Changes in the air temperature and precipitation are estimated to have several negative impacts on the hydrology of aquatic systems, for instance, in the amount of water, seasonal fluctuations in water availability, and water temperature. In some way, these variations will have a direct influence in the distribution and diversity of freshwater species, namely through its effects in the growth, behaviour, physical traits, reproduction or even in a combination of these factors in the affected organisms.

The response of freshwater species to a temperature rise can be very diverse, highly dependent on the sensitiveness of a species. Some studies [116-117] pointed to faster growth, smaller size at maturity, higher respiration rates (where oxygen may become a limiting factor), declination of fecundity and higher juvenile mortality rates, as possible consequences of higher temperatures for some species. On an ecological level, these climate changes are estimated to interfere with the distribution of species and, consequently, the overall aquatic biodiversity [118].

Additionally, the temperature rise is also associated with an increase in contaminants' toxicity. Oxygen levels in water tend to decrease when the temperature increases, once their solubility has an inverse relationship with water temperature. For this reason, at higher temperatures, a decrease of the biologically available (dissolved) oxygen (DO) supply is observed, which may induce higher ventilation rates of the aquatic organisms [119]. This increase on ventilation rates may be associated with an enhancing of toxicants uptake by organisms (which increases the accumulation of these contaminants), confirming the relation between warmer temperatures and the increase of contaminants' toxicity.

Besides the increase of ventilation rates, the contaminants' toxicity may also happen through an intensification of the production of free radicals and biotransformation products more toxic than the origin compounds, caused by the temperature rising [119].

IV.1. Copper sulphate and Tebuconazole impacts at a toxicological level

There is a wide variety of pesticides toxicity studies on aquatic species, namely with copper and copper sulphate exposure. TEB exposure is not so frequently evaluated as copper-based pesticides, possibly due to TEB having been more recently introduced in the market. Records of copper sulphate use in agriculture date back to the middle of the 18th century, while the use of TEB has only been around since the late 1980s [120].

The EC values calculated from the results of preliminary (or acute) exposure tests indicated copper sulphate exposure as more toxic than TEB for *R. subcapitata* and *D. magna* organisms. In these two species, the EC_{50} values were lower in the presence of copper sulphate, i.e., compared to TEB, a lower concentration of copper sulphate is required to present effects in 50% of the exposed individuals. The only tested species with a higher sensitiveness to TEB exposure was *L. minor*.

Raphidocelis subcapitata was the most influenced by the temperature variation among all tested species, as organism's resistance to the presence of both pesticides declined in the higher tested temperature. The microalgae organisms were found to be

more sensitive to copper sulphate exposure, and it was in the presence of this fungicide that the temperature rise had more impact. For this reason, the test with more effect observed was with copper sulphate at 25°C, with an EC_{50} value of 6.40 $mg.L^{-1}$. When compared to the other tested species, the microalgae presented the higher EC_{50} values observed in copper sulphate presence at both temperatures and in TEB at 20°C.

Other studies about the effects of copper sulphate and TEB on *R. subcapitata* have very different results among them. For instance, Murray-Gulde et al. [121] achieved an EC_{50} value of 21.10 $mg.L^{-1}$ in a 96h assay at 20°C with copper sulphate exposure, while Oliveira-Filho et al. [48] performed a 96h exposure test with copper sulphate at 24°C, resulting in an EC_{50} value of 0.344 $mg.L^{-1}$. This discrepancy in values was also verified in TEB exposure studies, where EC_{50} values similar to the results from this study were found [122], but there are also published results related to much higher EC_{50} values for the microalgae [123].

The other primary producer tested in this study, *L. minor*, presented a different response to the increase of temperature. The influence of temperature on the macrophytes' growth was not so evident as in the case of *R. subcapitata*, i.e., the EC_{50} values from both temperatures did not show a considerable difference. However, the temperature of 25°C induced a slight development of *L. minor* organisms' resistance to contaminant exposure.

The macrophyte EC values for copper sulphate and TEB exposure were slightly higher than the ones available in the literature [124–126], which may be cause mostly due to the different exposure time and test conditions. As mentioned above, these organisms were the only ones with a higher sensitivity to TEB than copper sulphate, and the test with more impact was with TEB at 20°C (EC_{50} value of 0.78 $mg.L^{-1}$).

Lemna minor is considered one of the most sensitive aquatic species to the exposure of TEB and other triazoles, once these types of fungicides are known for being potent inhibitors of elongation growth in vascular plants. The plant hormone responsible for the stimulation of cell elongation in *L. minor* is gibberellin, whose synthesis is blocked by triazoles and, consequently, TEB's action [127]. The microalgae tested, *R. subcapitata*, was not sensitive as *L. minor* to TEB exposure, because they do not rely on the gibberellin system [127].

The influence of a possible climate change scenario on the toxicity of pesticides in freshwater organisms was also evaluated in Silva et al. [128]. In this study, the authors explored the copper sulphate exposure in *R. subcapitata* and *L. minor* organisms at three different temperatures: 15, 20 and 25°C, and they concluded that the effects of water temperature rising on copper toxicity to primary producers is highly dependent of the exposure species. Similar to the results from the present study, in the microalgae organisms, more pronounced effects were observed under higher temperatures and a significant interaction between the contaminants' impacts and the temperature variation was more evident.

Daphnids' results were deeply marked by the difference between the exposures to both fungicides. This species was the most sensitive to copper sulphate at both tested temperatures (EC_{50} value of 0.13 and 0.12 $mg.L^{-1}$ at 20°C and 25°C, respectively), and, in opposition, was one of the most resistant to TEB exposure at 25°C. The difference observed between the EC_{50} values from the exposure to both contaminants is the largest among all tested species. This phenomenon was also observed in other studies were the EC_{50} values for copper sulphate exposure were less than 1 $mg.L^{-1}$ [129], while TEB exposure presented EC_{50} values higher than 10 $mg.L^{-1}$ [130].

Furthermore, *D. magna* organisms also presented a substantial impact from the temperature rising on their mortality rate. In general, this influence was not as evident as in the microalgae, once these organisms only displayed an increase in their resistance at the highest temperature in TEB's presence.

This great diversity of results on fungicides' impacts in the available literature was found for the three tested species. The high number of protocols for toxic exposure assays may be the main cause for the very different results found in several studies. For instance, these protocols can differ in the method of evaluation, cultivation medium, exposure time, temperature, among others, which can provide different responses of the tested individuals. Thus, the exclusive use of a standard protocol for a more reliable comparison of results is extremely important.

IV.2. Copper sulphate and Tebuconazole impacts at a biochemical level

Lipid metabolism disturbance has been reported in the literature as an effect of pesticides exposure in aquatic organisms, and FA biomarkers are being used as powerful tools in ecotoxicological studies as early-warning indicators of contaminants' detection in aquatic ecosystems (e.g., [131-132]). Lipid dynamics is considered by some authors [133] as a contributor cause for contaminants bioaccumulation in aquatic ecosystems, and for this reason, the lipid flow among the whole trophic food web (from primary producers to the top carnivores) is an important tool for ecotoxicological studies on both population dynamics and contaminants' bioaccumulation [134].

In parallel, sugar analyses are also viewed as good bioindicators of environmental stress. Carbohydrates play an important role in supplying organisms with metabolic energy, being considered one of the most important molecules in living organisms, as well as lipids. The available literature (e.g., [135-137]) has indicated that several species use carbohydrates to maintain homeostasis when they are exposed to some kind of stress, parasites, toxic chemicals or any environmental change.

IV.2.1. Changes in the tested primary producer species

The two studied primary producers had contrasting responses to pesticides exposure and temperature variations, in both toxicological and biochemical levels. As mentioned above, copper sulphate and the temperature of 25°C were more harmful to *R. subcapitata*, while TEB exposure and the temperature of 20°C caused more damage in *L. minor*.

Some studies also with the purpose to evaluate the impact of contaminants at a biochemical level have shown that organisms in stress tend to firstly use carbohydrates as the main sources of metabolic energy, mostly glucose, which are then level out as fatty acids are consumed [138]. Even if the sugar content is not affected, it does not mean that the contaminant to which the organisms are exposed to is not causing damage, since they may be using FA as an energy source in the metabolic process of toxicant’s elimination.

Additionally, the greater the alteration in FA content induced by a contaminant, the greater its impacts and toxicity on these organisms (e.g., [86, 90]), once its presence may be affecting in several different ways the metabolism of the contaminated organisms, causing instability and compromising the organisms' integrity.

Both tested fungicides induced significant changes in the CHO and FA profiles of *R. subcapitata*. Decreases in sugar concentrations were observed in all tested concentrations of copper sulphate at 20°C and in the highest copper sulphate concentration (EC_{20}) at 25°C. On the other hand, the most relevant decreases in the FA content of these organisms were registered in NOEC and EC_{20} treatments of copper sulphate test at 20°C and EC_{10} concentration at 25°C. TEB exposure test only presented decreases in total FA content in EC_{10} and EC_{20} treatments at 25°C.

Among all tested species, *R. subcapitata* was considered the most resistant to copper sulphate and TEB exposure at a toxicological level. However, from a biochemical point of view, the microalgae organisms were the most affected by fungicides' exposure, exhibiting the larger modifications in their biochemical content. Although fungicides did not exhibit a larger influence on the microalgae's growth, compared to the other species studied, their presence significantly altered microalgae contribute as a fundamental source of essential molecules (such as saccharides and lipids) to the top layers of freshwater food webs.

R. subcapitata was also considered the most affected species by temperature variation. The temperature influence was not only registered in the toxicological assays but also through the analysis of the biochemical content. Both FA and CHO content were in lower concentrations at the temperature of 25°C, in the contaminated treatments as well in controls. Being this temperature considered as the most harmful for *R. subcapitata* organisms.

The FA profiles of *L. minor* had a similar response to the pesticides' impacts in growth inhibition. The fungicide that caused the greatest decreases on the FA content was TEB, especially at 20°C, which was also demonstrated in the results from the acute exposure test, pointing TEB as the most toxic tested fungicide for this species. However, on the other side, CHO profiles showed to be more modified by the presence of copper sulphate than TEB.

Identical to toxicological results, the macrophytes' FA and CHO profiles indicated that the fungicides' toxicity is highly related to the temperature variation. The influence of temperature was immediately evident in the control samples, where the most abundant FA class and monosaccharide were different according to the tested temperature. Furthermore, the toxicity of the tested pesticides was also dependent on the temperature of the assays.

Regarding the FA content, TEB appears to be more toxic than copper sulphate at both temperatures. Decreases in copper sulphate tests were only observed in NOEC treatment at 20°C, while TEB exposure induced decreases in all tested concentrations at both tested temperatures. In the two tested fungicides' exposures, the temperature of 20°C was the one where higher decreases were registered. For this reason, the temperature of 25°C has proved to be more ideal for organisms than 20°C, a conclusion also found in other studies performed with the same species [139-140].

As mentioned above, the sugar content of *L. minor* presented larger variations regarding the control samples with copper sulphate exposure at a temperature of 20°C, but it was at 25°C that a decrease happened, in the highest copper sulphate concentration. A possible explication could be that copper sulphate toxicity on *L. minor* required the use of saccharides in their metabolic process of elimination in an initial phase exposure. *Lemna minor* organisms may have developed tolerance to copper exposure and partially recover from the stress condition, as Lal et al. [138] have referred in their work.

Despite the two tested producers presented higher sensitiveness in different temperatures (*R. subcapitata* was more sensitive at 25°C, while *L. minor* was at 20°C), both exhibit the same most abundant single FA, regardless of the exposure temperature. At 20°C, the most abundant FA was ALA (PUFA class) and, at 25°C was C24:1n9 (MUFA class). However, the two species presented very different total FA values, being much higher in the microalgae organisms. For producers, some single FA and FA classes are extremely important, especially considering these organisms role as energy sources and basis from all freshwater food webs. In general, producers present PUFA as the FA class responsible for structural function, whereas SFA and MUFA mostly constitute the storage lipid fraction [141].

ALA is one of the most relevant in this point of view, once it serves as substrate for the process of elongation and desaturation that some animals use to synthesize EPA, DHA and others long-chain PUFA [142]. This particular FA displayed the same response in both producers regarding the temperature variation, decreasing their concentration at 25°C. Since *R. subcapitata* was more sensitive at this temperature, ALA decrease was a predictable result, but for *L. minor*, the expected opposite was not verified. Despite the macrophytes' resistance to toxicants increase with the temperature rise, their richness as food source decreases.

Several pesticides are considered inhibitors of the FA desaturation process, through the direct inhibition of the desaturase enzyme in primary producers. For instance, Cohen et al. [143] related the decrease of ALA content in the glycolipids of higher plants and algae to the inhibition of (n − 3) desaturation system induced by a pesticide presence. As ALA is a possible precursor of long-chain PUFA (or EFA), a decrease in its concentration leads to lower levels of EFA.

EPA and DHA were the only EFA found in the FA profiles from the two producers. In the microalgae's profiles, these two FA were only found at 25°C, and their concentration increase with copper sulphate and decrease with TEB exposures. While in the macrophytes' profiles, they were only found in the presence of TEB at 20°C.

A decrease in EFA content is not always the observed biochemical response of producers to pesticides exposure. Some studies (e.g., [153-154]) had also demonstrated that EFA concentration can be increased after exposure to some pesticides. Sicko-Goad et al. [144] associated this increase to the fact that some EFA are probably substituting ALA, which may evidence a photosynthetic dysfunction. On the other hand, Henderson et al. [145] indicated that galactolipids and phospholipids may participate as substrates in desaturations, promoting the synthesis of HUFA and PUFA.

IV.2.2. Changes in the tested primary consumer

Some authors [146-147] demonstrated in their works that contaminants may exert impacts on *D. magna* by inducing changes in the biochemical content of their food. The organisms used in this study were daily fed with *R. subcapitata*. This microalgae species was

also exposed to both fungicides in a different test, their FA content was significantly changed by copper sulphate and TEB exposures, but the contaminated organisms were not used to fed the daphniids. Considering the assumption formulated by Neves et al. [148], the microalgae used as food during the chronic exposure tests of daphniids did not remain enough time in the contaminated water column to produce changes in their biochemical content. For this reason, all changes on the biochemical content of *D. magna* may be directly related to the fungicides' exposure and/or temperature variation.

The study of *D. magna* was approached regarding two life-stages, as mentioned previously, the offspring (neonates from the first generation) and the mothers. The offspring were considered more sensitive individuals and only had an exposure period to contaminants of a few hours. However, the fact that neonates and mothers are organisms with a very different biochemical content in the control samples is the main reason for their different response to the presence of contaminants and also to the temperature variation. *Daphnia magna* neonates presented lower FA concentrations per organisms and also less diversified FA profiles, and their changes were related to the two tested temperatures. Decreases of FA content in the neonates were only registered at the temperature of 25°C, in both copper sulphate and TEB exposures.

Concerning the mothers' FA profiles, the influence of pesticides in these organisms were in accordance with their acute response in the toxicological assays previously analysed. Copper sulphate was the tested fungicide who caused more variations in the FA content, being for this reason considered more toxic for the species. The TEB concentrations used in the chronic exposure test did not present significant differences when compared to the control samples, as it was expected through the EC_{50} values calculated from the acute exposure data.

In *D. magna* mothers, the two tested temperatures induced different responses. The biochemical analysis indicated that the temperature impact was more evident in TEB exposure tests with lower values of total FA content at 20°C, which was in accordance with the toxicological assays. Additionally, in the biochemical analysis was possible to observe the interference of the two different tested temperatures with the contaminants' exposure,

namely in copper sulphate testes. The highest tested concentration of copper sulphate presented a decrease of the FA content, but only in the treatment from the test at 20°C.

Daphnia magna mothers, even without the presence of both fungicides, exhibit lower FA concentrations at 20°C, indicating that the temperature of 25°C is better from a biochemical perspective for these organisms, confirming the results from the acute assays. In the case of the neonates, despite the temperature of 25°C induced greater decreases in FA content from both copper sulphate and TEB exposures, organisms from control samples also presented lower FA values at 20°C. The effects of temperature rise on *D. magna* are not yet clear, with or without the presence of a contaminant. Despite the results from this study pointed to the higher tested temperature as more favourable for the species, the impacts of climate change and global warming on daphniids population dynamics and ecosystem functioning are complex and difficult to predict, due to the presence of antagonistic and synergistic drivers [149].

Copper sulphate exposure has been shown by literature to induce several damages to daphniids populations (e.g., [150]). Despite being studied by several authors, there is no certainty that its toxicity on freshwater animals (as well as most toxics) is entirely caused by the contaminant. According to Agazt et al. [151], some contaminants can cause effects on aquatic species through acclimation of the organism to stress invoked by feeding inhibition during exposure. Considering that these animals cannot synthesize their own FA and their only energy source is from food (algae), a feeding depression can also decrease their FA content, crucial components on the metabolic process of toxicant's elimination.

EFA are almost exclusively synthesized by primary producers, while animals only can convert EFA and other PUFA from one form to another, through elongation and desaturation processes. These FA are known to be essential elements on several processes in animals, regulating cell membrane properties or even serving as precursors for important animal hormones [152]. Daphniids are able to produce EPA and DHA conversion of ALA, but this ability is not enough to support high growth and reproduction rates in zooplankters [153].

EPA was the only EFA presented in *D. magna* offspring and the most abundant in the mothers. In the neonates, EPA content increased following the increase of both fungicides' concentrations. Concerning the mothers, both copper sulphate and TEB exposures induced

an increase in EPA content, but the DHA was only increased in the presence of copper sulphate. The studies of zooplankton species' FA profiles responses to toxicants show an increased PUFA and, consequently, EFA level after exposure [154-155]. This increase may be due to the effective processes of regulation/detoxification in order to prevent PUFA and EFA peroxidation.

Chapter V - CONCLUSION

The exponential growth of the human population worldwide, and the consequent consumption of more food, exerts a growing pressure on the food industries, with a faster and more efficient production being increasingly necessary. For this reason, pesticides usage is still necessary to respond to the increasing human need for food, despite the constant warnings made by the scientific community concerning their toxicity to both environment and human health. The uncontrolled use of pesticides has already proven to be very dangerous for ecosystems, affecting all global biodiversity, even in remote areas where it was thought that there would be no human impact.

The purpose of this study was to evaluate the impact of two fungicides - copper sulphate and TEB - in non-target freshwater species, through a toxicological and also a biochemical perspective. The presence of toxicants in aquatic systems is related to a decline in the organisms' value as food [156], and consequently to a disturbance in the entire trophic food web and the good health status of the ecosystems.

Several authors have reported that a decrease in sugar and FA content as a direct influenced of contaminants exposure (e.g., [157-159]). It is suggested that modifications in these biochemical indicators could be used as a useful tool to assess the effects of toxicants on metabolic pathways, to prevent potential stressful situations and to manage and implement mitigation measurements.

The results from this study indicated that TEB was more toxic for *L. minor*, while copper sulphate was more toxic for *R. subcapitata* and *D. magna*. The growth of both producers and the immobilization of daphniids, as well as their biochemical content changed according to the temperature variation. In general, *R. subcapitata* presented more damages at 25°C, whereas *L. minor* and *D. magna* organisms at 20°C. The impacts of temperature variation linked to climate change and global warming are very complex and highly dependent on the population dynamics and their relations with the whole ecosystem. In this study, warmer temperatures were considered to be more suitable for some species, but, except for the presence of the two tested fungicides, no additional interaction with biotic and abiotic factors was considered.

The observed alterations in the biochemical content of the three studied species from both fungicides exposure and temperature variation can result in modifications on the

nutritional value of these organisms. As very important food sources and basis from freshwater food webs, changes in the populations of *R. subcapitata*, *L. minor* and also *D. magna* may induce severe impacts in the whole ecosystem.

Moreover, this work highlights the importance of FA and CHO biomarkers as relevant tools to detect xenobiotic impacts, in this case in non-target species, providing additional information and useful tools to ecotoxicological studies. Analysis on the biochemical profiles should be used as early-warning indicators on the detection of contaminants in aquatic systems, once they provide physiological data about metabolic changes, before the occurrence of populational effects. Further research may combine these biomarkers with other physiological indicators, such as protein content, to obtain better insight on the effects of toxicants in non-target species.

Further work on the results obtained in the present study could be pursued, to provide more information on the impacts of contaminants in non-target organisms, in the light of the current pesticide usage and climate change scenarios. For instance, it would be interesting to explore the impacts of these fungicides on organisms of higher levels in the food chain. In addition to the analysis with a direct exposition, such as the ones performed here, it would be relevant to carry out exposure tests whose food source was previously exposed to contaminants. Since the results from the analysis performed in this study proved to be effective in determining impacts of pesticides in non-target species, providing a more accurate recognition of the effects of fungicides on non-target species, further research could be carried out with a wider range of pesticides, as well as of species.

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APPENDIX

Table S1. Other ways of pesticide classification, with the groups of the classification of each type on the left side. [2], [6]

Classification based on the chemical composition	For example: organochlorines, organophosphates, carbamates and pyrethroids.
Classification based on the source of origin	Chemical pesticides Biochemical pesticides Plant incorporated protectants Microbial pesticides
Classification based on the activity spectrum	Broad-spectrum pesticides Selective pesticides
Classification based on the mode of formulation	Emulifiable concentrates Wettable Powders Granules Baits Dust Ultra low volume liquid
Classification based on the toxicity levels (developed by the World Health Organization – WHO) [160]	*Class Ia* = Extremely hazardous *Class Ib* = Highly hazardous *Class II* = Moderately hazardous *Class III* = Slightly hazardous *Class IV* = Unlikely to present acute hazard in normal use

Table S2. Fatty acid abundance mean-values (mg.g^{-1}) of *Raphidocelis subcapitata* organisms exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark orange) and 25°C (light orange). Total content of SFA, MUFA, PUFA and HUFA are expressed in % for each contaminant.

Test species	*Raphidocelis subcapitata*															
Contaminant	Copper Sulphate								TEB							
Temperature	20°C				25°C				20°C				25°C			
Concentration	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}
C16:0	2.32	0.42	2.47	0.62	1.22	10.2	0.39	4.36	1.44	14.69	4.54	6.86	0.98	6.14	0.21	0.12
C17:0	0.00	0.06	0.33	0.21	0.19	0.00	0.06	0.00	1.29	0.00	0.00	0.08	0.00	0.17	0.00	0.00
C18:0	0.00	0.00	0.00	0.00	0.00	3.33	0.24	0.00	0.66	0.26	0.00	0.32	0.01	1.46	0.01	0.01
TOTAL% SFA	**32.00**	**18.41**	**26.33**	**23.54**	**10.64**	**44.67**	**39.65**	**20.48**	**50.81**	**35.70**	**36.83**	**40.56**	**12.38**	**50.44**	**13.18**	**18.41**
C16:1n9	0.11	0.00	0.13	0.00	0.28	0.31	0.00	0.13	0.00	0.53	0.00	0.15	0.03	0.00	0.05	0.02
C17:1n10	0.21	0.59	0.28	0.65	0.29	0.92	0.00	0.43	0.00	1.26	0.30	0.47	0.07	0.47	0.08	0.04
C18:1n9	0.99	0.00	0.94	0.00	1.97	0.00	0.22	0.07	0.00	3.47	1.23	2.65	0.54	0.00	0.03	0.01
C22:1n9	0.30	0.00	1.20	0.00	2.20	0.00	0.02	0.00	0.00	0.63	0.21	0.00	1.15	0.00	0.12	0.18
C24:1n9	0.23	0.27	1.32	0.00	3.85	2.11	0.10	4.26	0.00	0.89	0.27	0.08	3.03	0.50	0.47	0.22
TOTAL% MUFA	**25.38**	**32.55**	**36.37**	**18.44**	**64.51**	**11.06**	**19.53**	**23.00**	**0.00**	**16.18**	**16.35**	**18.69**	**59.85**	**6.28**	**46.53**	**67.23**
C18:2n9,12	0.45	0.26	0.34	0.38	0.16	0.00	0.07	0.95	0.26	1.54	0.55	0.70	0.25	0.70	0.04	0.03
ALA	2.64	1.04	2.75	1.67	1.13	8.79	0.64	5.22	3.02	18.61	5.23	6.60	0.96	4.34	0.12	0.07
TOTAL% PUFA	**42.61**	**49.04**	**29.13**	**58.02**	**9.69**	**29.04**	**40.82**	**28.99**	**49.19**	**48.13**	**46.82**	**40.75**	**15.08**	**32.66**	**10.11**	**14.36**
EPA	0.00	0.00	0.00	0.00	0.00	3.02	0.00	3.46	0.00	0.00	0.00	0.00	0.00	1.20	0.30	0.00
DHA	0.00	0.00	0.87	0.00	2.02	1.59	0.00	2.40	0.00	0.00	0.00	0.00	1.02	0.44	0.19	0.00
TOTAL% HUFA	**0.00**	**0.00**	**8.17**	**0.00**	**15.16**	**15.23**	**0.00**	**27.53**	**0.00**	**0.00**	**0.00**	**0.00**	**12.7**	**10.63**	**30.18**	**0.00**
TOTAL FA	7.25	2.66	10.63	3.54	13.32	30.29	1.73	21.28	6.67	41.87	12.34	17.9	8.05	15.43	1.61	0.70

Table S3. Summary of nested ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Raphidocelis subcapitata*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in FA data of *Raphidocelis subcapitata*		
DATA	Source of variation	FA scores		
		MS	*F*	*p*
Analysis of total FA	*Contaminant*	11.70	0.26	0.62
	Contaminant : Temperature	738.50	8.10	0.01*
	Contaminant : Temperature : Concentration	1047.60	3.28	0.03*
	Residuals	45.60		
Analysis of SFA	*Contaminant*	12.47	5.66	0.03*
	Contaminant : Temperature	49.97	22.70	5.74e-05*
	Contaminant : Temperature : Concentration	33.37	15.16	2.54e-05*
	Residuals	2.20		
Analysis of MUFA	*Contaminant*	4.31	0.43	0.52
	Contaminant : Temperature	14.42	14.42	0.27
	Contaminant : Temperature : Concentration	5.74	5.74	0.77
	Residuals	10.04		
Analysis of PUFA	*Contaminant*	15.14	2.37	0.15
	Contaminant : Temperature	77.77	12.18	1.05e-03*
	Contaminant : Temperature : Concentration	24.60	24.60	0.01*
	Residuals	6.39		
Analysis of HUFA	*Contaminant*	3.70	2.85	0.12
	Contaminant : Temperature	8.75	6.73	0.01*
	Contaminant : Temperature : Concentration	2.20	1.69	0.20
	Residuals	1.30		
Analysis of C24:1n9	*Contaminant*	2.57	1.09	0.32
	Contaminant : Temperature	6.66	2.83	0.10
	Contaminant : Temperature : Concentration	1.32	0.56	0.78
	Residuals	2.36		
Analysis of ALA	*Contaminant*	13.17	2.41	0.14
	Contaminant : Temperature	67.45	12.35	9.88e-04*
	Contaminant : Temperature : Concentration	21.63	3.96	0.02*
	Residuals	5.46		
Analysis of EPA	*Contaminant*	0.47	1.11	0.31
	Contaminant : Temperature	2.11	4.97	0.03*
	Contaminant : Temperature : Concentration	1.40	3.32	0.03*
	Residuals	0.42		
Analysis of DHA	*Contaminant*	1.54	3.46	0.09
	Contaminant : Temperature	2.27	5.11	0.02*
	Contaminant : Temperature : Concentration	0.29	0.65	0.71
	Residuals	0.44		

Table S4. Summary of two-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Raphidocelis subcapitata*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in FA data of *Raphidocelis subcapitata*					
DATA	Source of variation	FA scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Temperature*	431.40	11.08	7.64e-03*	696.70	18.97	1.15e-03*
	Concentration	145.60	3.74	0.04*	433.80	11.81	9.17e-04*
	Residuals	38.90			36.70		
Analysis of SFA	*Temperature*	45.89	21.61	9.09e-04*	114.74	81.85	1.99e-06*
	Concentration	46.59	21.94	6.14E-05*	73.69	52.57	8.26e-07*
	Residuals	2.12			1.40		
Analysis of MUFA	*Temperature*	24.26	2.29	0.16	8.34	1.49	0.25
	Concentration	6.48	0.61	0.66	4.27	0.76	0.54
	Residuals	10.61			5.59		
Analysis of PUFA	*Temperature*	13.76	4.24	0.07	227.87	27.79	2.63e-04*
	Concentration	15.35	4.73	0.02*	94.53	11.53	1.01e.03*
	Residuals	3.24			8.20		
Analysis of HUFA	*Temperature*	29.83	14.35	3.55e-03*	2.56	15.88	2.14e.03*
	Concentration	4.13	1.99	0.17	0.49	3.03	0.07
	Residuals	2.08			0.16		
Analysis of C24:1n9	*Temperature*	21.18	6.45	0.03*	2.66	3.80	0.08
	Concentration	1.54	0.47	0.76	1.56	2.23	0.14
	Residuals	32.84			0.70		
Analysis of ALA	*Temperature*	14.74	8.26	0.02*	199.89	28.05	2.54e-04*
	Concentration	21.05	11.80	8.38e-04*	77.00	10.80	1.32e-03*
	Residuals	1.78			7.13		
Analysis of EPA	*Temperature*	12.88	13.28	4.51e-03*	0.52	5.39	0.04*
	Concentration	4.16	4.29	0.03*	0.30	3.05	0.07
	Residuals	0.97			0.10		
Analysis of DHA	*Temperature*	6.62	10.43	0.01*	6.62	10.43	0.01*
	Concentration	0.34	0.54	0.71	0.34	0.54	0.71
	Residuals	0.63			0.63		

Table S5. Summary of one-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Raphidocelis subcapitata*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

		One-way ANOVAs in FA data of *Raphidocelis subcapitata*					
DATA	Source of variation	FA scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	26.79	22.44	5.78e-03*	282.00	27.65	3.91e-03*
	Residuals	1.19			10.20		
Analysis of SFA	*Concentration*	2.52	43.87	1.62e-03*	66.16	199.70	8.23e-05*
	Residuals	0.06			0.33		
Analysis of MUFA	*Concentration*	4.30	27.28	4.01e-03*	23.04	1.87	0.28
	Residuals	0.16			12.34		
Analysis of PUFA	*Concentration*	1.52	16.54	0.01*	29.01	61.93	8.27e-04*
	Residuals	0.09			0.47		
Analysis of HUFA	*Concentration*	0.38	8.47	0.03*	11.82	87.58	4.19e-04*
	Residuals	0.04			0.14		
Analysis of C24:1n9	*Concentration*	0.69	15.16	0.01*	7.86	2.38	0.21
	Residuals	0.05			3.30		
Analysis of ALA	*Concentration*	1.33	19.38	0.01*	32.04	78.63	5.18e-04*
	Residuals	0.07			0.41		
Analysis of EPA	*Concentration*				8.61	64.97	7.53e-04*
	Residuals				0.13		
Analysis of DHA	*Concentration*	0.38	8.47	0.03*	2.09	64.61	7.61e-04*
	Residuals	0.04			0.03		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	473.90	23694	5.94e-09*	94.48	3310	3.04e-07*
	Residuals	0.00			0.03		
Analysis of SFA	*Concentration*	52.15	2607	4.90e-07*	26.65	2528	5.21e-07*
	Residuals	0.02			0.01		
Analysis of MUFA	*Concentration*	16.65	783.80	5.41e-06*	8.00	144.30	1.57e-04*
	Residuals	0.02			0.06		
Analysis of PUFA	*Concentration*	113.05	3478	2.75e-07*	11.47	497.70	1.34e-05*
	Residuals	0.03			0.02		
Analysis of HUFA	*Concentration*				0.98	12.77	0.02*
	Residuals				0.08		
Analysis of C24:1n9	*Concentration*	0.25	25.21	4.65e-03*	3.81	111.70	2.60e-04*
	Residuals	0.01			0.03		
Analysis of ALA	*Concentration*	95.23	3628	2.53e-07*	7.85	470.20	1.5e-05*
	Residuals	0.03			0.02		
Analysis of EPA	*Concentration*				0.59	13.09	0.02*
	Residuals				0.05		
Analysis of DHA	*Concentration*				0.49	25.44	0.01*
	Residuals				0.02		

Table S6. Summary ANOSIM analysis on the total fatty acid content of *Raphidocelis subcapitata*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in data of *Raphidocelis subcapitata*		
Global Test		
Global R		0.18
Significance level (p value)		0.04*
Pairwise Tests	**R statistic**	**p-value**
Copper sulphate at 20°C, Copper sulphate at 25°C	0.24	0.11
Copper sulphate at 20°C, TEB at 20°C	0.29	0.06
Copper sulphate at 20°C, TEB at 25°C	0.19	0.23
Copper sulphate at 25°C, TEB at 20°C	0.32	0.03*
Copper sulphate at 25°C, TEB at 25°C	-0.10	0.57
TEB at 20°C, TEB at 25°C	0.33	0.06

Table S7. Carbohydrates abundance mean-values (mg.g^{-1}) of *Raphidocelis subcapitata* organisms exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark orange) and 25°C (light orange).

Test species	*Raphidocelis subcapitata*															
Contaminant	Copper Sulphate								TEB							
Temperature	20°C				25°C				20°C				25°C			
Concentration	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}
Ara	17.34	0.00	6.99	0.00	4.35	0.00	17.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Man	28.88	7.47	13.87	4.83	13.51	21.24	25.39	9.00	83.81	203.17	117.22	58.00	7.60	22.92	6.87	10.78
Gal	23.17	4.91	6.87	0.00	5.90	10.49	22.54	9.74	49.80	289.92	124.49	47.34	0.00	28.61	0.00	0.00
Glu	73.25	28.58	28.97	15.19	19.51	34.96	103.49	13.39	21.96	61.37	26.79	40.03	11.71	37.28	40.10	53.65
Rib	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	22.64	77.88	0.00	0.00	11.85	0.00	6.80	0.00
Xyl	0.00	0.00	0.00	0.00	0.00	0.00	6.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL	**142.64**	**40.96**	**56.70**	**20.02**	**43.27**	**66.69**	**174.78**	**32.13**	**178.20**	**634.34**	**268.50**	**145.37**	**31.16**	**88.81**	**53.77**	**64.43**

Table S8. Summary of nested ANOVAs on the carbohydrates (total CHO, Ara, Man, Gal, Glu, Rib and Xyl) profiles of *Raphidocelis subcapitata*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in CHO data of *Raphidocelis subcapitata*		
DATA	Source of variation	CHO scores		
		MS	*F*	*p*
Analysis of total CHO	*Contaminant*	158330	14.733	0.000647*
	Contaminant : Temperature	190429	17.720	1.06e-05*
	Contaminant : Temperature : Concentration	35537	3.307	0.013761*
	Residuals	10747		
Analysis of Ara	*Contaminant*	268.82	24.825	2.91e-05*
	Contaminant : Temperature	4.11	0.379	0.688
	Contaminant : Temperature : Concentration	86.88	8.023	4.29e-05*
	Residuals	10.83		
Analysis of Man	*Contaminant*	27149	29.151	9.32e-06*
	Contaminant : Temperature	31257	33.562	3.67e-08*
	Contaminant : Temperature : Concentration	2616	2.809	0.0288*
	Residuals	931		
Analysis of Gal	*Contaminant*	41277	17.102	0.000292*
	Contaminant : Temperature	45690	18.930	6.3e-06*
	Contaminant : Temperature : Concentration	8633	3.577	0.009320*
	Residuals	2414		
Analysis of Glu	*Contaminant*	8.4	0.015	0.9022
	Contaminant : Temperature	75.4	0.138	0.8720
	Contaminant : Temperature : Concentration	1773.7	3.237	0.0152*
	Residuals	547.9		
Analysis of Rib	*Contaminant*	2549.3	8.852	0.00597*
	Contaminant : Temperature	1319.8	4.583	0.01897*
	Contaminant : Temperature : Concentration	703.9	2.444	0.05008
	Residuals	288.0		
Analysis of Xyl	*Contaminant*	4.592	5.468	0.0267*
	Contaminant : Temperature	3.490	4.155	0.0263*
	Contaminant : Temperature : Concentration	6.551	7.800	5.4e-05*
	Residuals	0.840		

Table S9. Summary of two-way ANOVAs on the carbohydrates (total CHO, Ara, Man, Gal, Glu, Rib and Xyl) profiles of *Raphidocelis subcapitata*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in CHO data of *Raphidocelis subcapitata*					
DATA	Source of variation	CHO scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Temperature*	151	0.061	0.8091	380708	25.335	0.000148*
	Concentration	6510	2.618	0.0952	78957	5.254	0.011186*
	Residuals	2487			15027		
Analysis of Ara	*Temperature*	8.21	0.354	0.56191			
	Concentration	174.38	7.523	0.00362*			
	Residuals	23.18					
Analysis of Man	*Temperature*	23.87	0.489	0.4969	62490	42.603	9.57e-06*
	Concentration	165.99	3.398	0.0505	6212	4.235	0.0235*
	Residuals	48.86			1467		
Analysis of Gal	*Temperature*	29.09	0.515	0.486	91350	24.622	0.00017*
	Concentration	79.94	1.416	0.283	20916	5.638	0.00861*
	Residuals	56.44			3710		
Analysis of Glu	*Temperature*	5	0.005	0.947	145.8	1.074	0.31648
	Concentration	2090	1.926	0.175	1192.0	8.778	0.00133*
	Residuals	1085			135.8		
Analysis of Rib	*Temperature*				2639.6	5.782	0.0296*
	Concentration				1812.9	3.971	0.0288*
	Residuals				456.5		
Analysis of Xyl	*Temperature*	6.980	2.991	0.1074			
	Concentration	10.827	10.827	0.0204*			
	Residuals	2.334					

Table S10. Summary of two-way ANOVAs on the carbohydrates (total CHO, Ara, Man, Gal, Glu, Rib and Xyl) profiles of *Raphidocelis subcapitata* , with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

		One-way ANOVAs in CHO data of *Raphidocelis subcapitata*					
DATA	Source of variation	CHO scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Concentration*	5799	4.677	0.0851	9472	52.43	0.000107*
	Residuals	1240			181		
Analysis of Ara	*Concentration*	134.5	268.7	4.57e-05*	139.14	404.5	2.6e-07*
	Residuals	0.5			0.34		
Analysis of Man	*Concentration*	231.93	50.63	0.00122*	132.38	36.35	0.000304*
	Residuals	4.58			3.64		
Analysis of Gal	*Concentration*	201.93	52.53	0.00114*	116.35	204.4	1.98e-06*
	Residuals	3.84			0.57		
Analysis of Glu	*Concentration*	1283	1.242	0.405	3813	23.94	0.000973*
	Residuals	1033			159		
Analysis of Xyl	*Concentration*				20.94	9.208e+30	<2e-16*
	Residuals				0.00		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Concentration*	134391	15.12	0.00333*	1700.7	15.41	0.00317*
	Residuals	8890			110.4		
Analysis of Man	*Concentration*	10295	6.82	0.0232*	155.54	4.074	0.0677
	Residuals	1510			38.18		
Analysis of Gal	*Concentration*	33818	13.5	0.00447*	573.1	17.35	0.00232*
	Residuals	2505			33.0		
Analysis of Glu	*Concentration*	843.4	38.4	0.00026*	789.1	8.115	0.0156*
	Residuals	22.0			97.2		
Analysis of Rib	*Concentration*	3814	41.4	0.00021*	90.74	29.04	0.000571*
	Residuals	92			3.12		

Table S11. Summary ANOSIM analysis on the total carbohydrates content of *Raphidocelis subcapitata*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in CHO data of *Raphidocelis subcapitata*		
Global Test		
Global R		0.378
Significance level (p-value)		0.004*
Pairwise Tests	**R statistic**	**p-value**
Copper sulphate at 20°C, Copper sulphate at 25°C	-0.188	0.914
Copper sulphate at 20°C, TEB at 20°C	0.625	0.029*
Copper sulphate at 20°C, TEB at 25°C	0.156	0.2
Copper sulphate at 25°C, TEB at 20°C	0.646	0.029*
Copper sulphate at 25°C, TEB at 25°C	0.292	0.143
TEB at 20°C, TEB at 25°C	0.688	0.029*

Table S12. Fatty acid abundance mean-values ($mg.g^{-1}$) of *Lemna minor* organisms exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark green) and 25°C (light green). Total content of SFA, MUFA, PUFA and HUFA are expressed in % for each contaminant.

Test species	*Lemna minor*															
Contaminant	Copper Sulphate								TEB							
Temperature	20°C				25°C				20°C				25°C			
Concentration	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}
C16:0	0.00	0.18	0.72	0.31	0.56	0.58	1.32	0.60	0.03	0.15	0.01	0.05	1.06	1.84	0.19	0.55
C17:0	0.59	0.05	1.02	0.14	0.02	0.00	0.00	0.20	0.00	2.04	0.00	0.00	0.16	0.00	0.21	0.23
C18:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.12	0.01	0.04	0.00	0.00	0.00	0.00
C20:0	0.00	0.00	0.00	0.00	0.00	0.99	0.00	1.04	0.12	0.11	0.00	0.00	0.00	0.00	0.00	0.00
C22:0	0.93	0.48	0.00	0.94	0.00	0.00	0.00	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30
TOTAL% SFA	**43.81**	**41.82**	**21.05**	**43.33**	**13.99**	**31.91**	**38.72**	**42.32**	**1.56**	**27.34**	**3.23**	**1.55**	**12.54**	**29.32**	**20.89**	**23.86**
C16:1n9	0.12	0.00	0.11	0.00	0.06	0.11	0.12	0.23	0.00	0.03	0.00	0.02	0.18	0.26	0.23	0.09
C17:1n10	0.21	0.16	0.26	0.45	0.13	0.30	0.00	0.56	0.07	0.12	0.00	0.04	0.52	0.62	0.80	0.86
C18:1n9	0.00	0.00	0.07	0.00	0.03	0.06	0.05	0.07	0.00	0.00	0.00	0.00	0.20	0.11	0.06	0.03
C22:1n13	0.00	0.00	0.54	0.00	0.69	0.88	0.63	0.73	0.00	0.00	0.00	0.00	1.20	0.41	0.24	0.00
C24:1n9	0.00	0.00	2.68	0.00	2.44	1.75	1.10	1.39	6.89	4.16	0.27	2.48	3.61	1.29	0.00	0.00
TOTAL% MUFA	**9.68**	**9.62**	**44.21**	**13.88**	**80.95**	**63.32**	**55.82**	**40.28**	**48.57**	**48.51**	**56.04**	**42.09**	**58.33**	**42.60**	**69.30**	**21.80**
C18:2n9,12	0.20	0.10	0.30	0.18	0.21	0.23	0.19	0.23	0.00	0.01	0.00	0.03	0.56	0.73	0.19	0.24
C18:3n6,9,12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.14	0.00	0.00	0.00	0.12	0.00	0.00	0.12
ALA	1.41	0.72	2.57	1.19	0.00	0.00	0.00	0.95	0.11	0.26	0.00	0.00	1.98	1.03	0.00	2.09
TOTAL% PUFA	**46.50**	**48.56**	**34.74**	**42.79**	**5.06**	**4.76**	**5.46**	**17.41**	**1.74**	**3.13**	**0.00**	**0.44**	**27.20**	**28.08**	**9.81**	**54.34**
EPA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.49	0.00	0.00	0.00	0.00
DHA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6.90	1.87	0.17	2.89	0.19	0.00	0.00	0.00
TOTAL% HUFA	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**48.12**	**21.02**	**40.73**	**55.92**	**1.94**	**0.00**	**0.00**	**0.00**
TOTAL FA	3.48	1.69	8.27	3.21	4.14	4.92	3.41	7.37	14.34	8.88	0.48	6.04	9.78	6.28	1.91	4.52

Table S13. Summary of nested ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in FA data of *Lemna minor*		
DATA	Source of variation	FA scores		
		MS	*F*	*p*
Analysis of total FA	*Contaminant*	5.81	0.698	0.802912
	Contaminant : Temperature	11.42	1.371	0.254772
	Contaminant : Temperature : Concentration	74.60	8.955	0.000513*
	Residuals	8.33		
Analysis of SFA	*Contaminant*	0.803	2.453	0.0208*
	Contaminant : Temperature	0.700	2.139	0.1584
	Contaminant : Temperature : Concentration	4.261	13.012	5.06e-05*
	Residuals	0.327		
Analysis of MUFA	*Contaminant*	2.965	0.393	0.9854
	Contaminant : Temperature	3.002	0.397	0.5352
	Contaminant : Temperature : Concentration	24.555	3.251	0.0422*
	Residuals	7.553		
Analysis of PUFA	*Contaminant*	0.710	0.862	0.639966
	Contaminant : Temperature	13.615	16.530	0.000555*
	Contaminant : Temperature : Concentration	1.687	2.049	0.137806
	Residuals	0.824		
Analysis of HUFA	*Contaminant*	0.70	0.148	0.99999
	Contaminant : Temperature	38.11	8.056	0.00985*
	Contaminant : Temperature : Concentration	4.50	0.950	0.43433
	Residuals	4.73		
Analysis of C24:1n9	*Contaminant*	6.784	1.579	0.2162
	Contaminant : Temperature	12.901	3.003	0.0610
	Contaminant : Temperature : Concentration	10.180	2.369	0.0472*
	Residuals	4.297		
Analysis of ALA	*Contaminant*	0.040	0.068	0.795580
	Contaminant : Temperature	6.731	11.558	0.000109*
	Contaminant : Temperature : Concentration	0.845	1.451	0.219723
	Residuals	0.582		
Analysis of EPA	*Contaminant*	0.021014	2.400	0.1292
	Contaminant : Temperature	0.025538	2.916	0.0657
	Contaminant : Temperature : Concentration	0.011987	1.369	0.2507
	Residuals	0.008757		
Analysis of DHA	*Contaminant*	15.669	6.245	0.0166*
	Contaminant : Temperature	17.687	7.049	0.00239*
	Contaminant : Temperature : Concentration	2.216	0.883	0.51603
	Residuals	2.509		

Table S14. Summary of two-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in FA data of *Lemna minor*					
DATA	Source of variation	FA scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Temperature*	4.971	0.872	0.362	11.42	1.463	0.239968
	Concentration	2.060	0.361	0.782	78.25	10.021	0.000265*
	Residuals	5.699			7.81		
Analysis of SFA	*Temperature*	0.0646	0.092	0.765	0.700	2.509	0.128
	Concentration	0.9636	1.366	0.283	4.598	16.471	9.82e-06*
	Residuals	0.7052			0.279		
Analysis of MUFA	*Temperature*	19.929	8.503	0.00886*	3.002	0.400	0.5340
	Concentration	0.714	0.305	0.82167	24.879	3.314	0.0398*
	Residuals	2.344			7.507		
Analysis of PUFA	*Temperature*	6.193	13.273	0.00173*	13.62	15.825	0.000685*
	Concentration	0.657	1.408	0.27137	1.43	1.663	0.205447
	Residuals	0.467			0.86		
Analysis of HUFA	*Temperature*				38.11	8.35	0.00877*
	Concentration				5.66	1.24	0.32013
	Residuals				4.56		
Analysis of C24:1n9	*Temperature*	7.365	6.032	0.0238*	18.44	2.645	0.1188
	Concentration	1.100	0.901	0.4589	20.03	2.874	0.0605
	Residuals	1.221			6.97		
Analysis of ALA	*Temperature*	6.601	16.248	0.000714*	6.861	9.010	0.0068*
	Concentration	0.534	1.314	0.298878	1.018	1.337	0.2892
	Residuals	0.406			0.761		
Analysis of EPA	*Temperature*				0.05108	3.285	0.0842
	Concentration				0.03190	2.052	0.1374
	Residuals				0.01555		
Analysis of DHA	*Temperature*				35.37	7.662	0.0115*
	Concentration				5.57	1.207	0.3317
	Residuals				4.62		

Table S15. Summary of two-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA, HUFA, C24:1n9, ALA, EPA and DHA) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

		One-way ANOVAs in FA data of *Lemna minor*					
DATA	Source of variation	FA scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	9.954	5.634	0.0226*	4.019	0.551	0.662
	Residuals	1.767			7.300		
Analysis of SFA	*Concentration*	0.3916	3.591	0.0658	2.1980	2.299	0.154
	Residuals	0.1091			0.9561		
Analysis of MUFA	*Concentration*	3.480	4.283	0.0444*	1.435	0.452	0.154
	Residuals	0.813			3.178		
Analysis of PUFA	*Concentration*	1.0344	1.462	0.296	0.3939	3.535	0.068
	Residuals	0.7075			0.1114		
Analysis of C24:1n9	*Concentration*	2.3890	3.102	0.0891	1.422	1.277	0.346
	Residuals	0.7702			1.113		
Analysis of ALA	*Concentration*	0.7907	1.172	0.379	0.29885	3.651	0.0635
	Residuals	0.6747			0.08186		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	53.51	9.123	0.00583*	29.13	2.804	0.0945
	Residuals	5.87			10.39		
Analysis of SFA	*Concentration*	3.955	2097	6.4e-12*	1.3630	3.696	0.0504
	Residuals	0.002			0.3688		
Analysis of MUFA	*Concentration*	13.81	1.204	0.369	14.192	2.51	0.118
	Residuals	11.47			5.654		
Analysis of PUFA	*Concentration*	0.03388	2.406	0.143	2.448	1.654	0.239
	Residuals	0.01408			1.480		
Analysis of HUFA	*Concentration*	10.63	1.052	0.421	0.028233	26.86	4.23e-05*
	Residuals	10.11			0.001051		
Analysis of C24:1n9	*Concentration*	13.17	1.135	0.391	9.506	2.083	0.166
	Residuals	11.60			4.564		
Analysis of ALA	*Concentration*	0.022433	2.888	0.102	1.810	1.343	0.315
	Residuals	0.007769			1.348		
Analysis of EPA	*Concentration*	0.07736	3.256	0.0808			
	Residuals	0.02376					
Analysis of DHA	*Concentration*	10.34	1.002	0.44	0.028233	26.86	4.23e-05*
	Residuals	10.32			0.001051		

Table S16. Summary ANOSIM analysis on the total fatty acid content of *Lemna minor*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in FA data of *Lemna minor*		
Global Test		
Global R		0.351
Significance level (p-value)		0.001*
Pairwise Tests	**R statistic**	**p-value**
Copper sulphate at 20°C, Copper sulphate at 25°C	0.573	0.029*
Copper sulphate at 20°C, TEB at 20°C	0.51	0.029*
Copper sulphate at 20°C, TEB at 25°C	-0.021	0.515
Copper sulphate at 25°C, TEB at 20°C	0.51	0.029*
Copper sulphate at 25°C, TEB at 25°C	0.26	0.026*
TEB at 20°C, TEB at 25°C	0.333	0.057

Table S17. Carbohydrates abundance mean-values (mg.g^{-1}) of *Lemna minor* organisms exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark green) and 25°C (light green).

Test species	*Lemna minor*															
Contaminant	Copper Sulphate								TEB							
Temperature	20°C				25°C				20°C				25°C			
Concentration	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}
Rha	0,97	0,00	0,00	0,00	1,21	2,74	0,00	11,70	0,75	1,06	1,36	0,97	0,00	4,10	0,00	4,09
Fuc	2,00	151,48	202,05	0,00	9,66	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	17,69	0,00	0,00
Ara	11,36	57,37	58,79	63,68	25,70	29,36	27,00	8,45	2,97	7,49	3,37	5,54	25,15	23,54	35,01	22,06
Man	8,80	0,64	0,00	9,47	0,00	4,40	2,45	11,15	0,00	0,00	4,62	0,76	0,00	0,00	6,29	0,00
Gal	40,91	75,17	144,42	180,58	4,88	76,97	75,83	7,81	10,79	23,12	12,67	36,41	76,95	110,67	105,75	70,66
Glu	116,20	195,23	445,80	619,68	20,61	59,25	62,13	6,49	22,36	37,76	38,70	43,35	56,35	89,76	94,89	51,47
Rib	0,00	0,00	0,00	0,00	1,81	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
TOTAL	**182,24**	**479,89**	**853,05**	**874,91**	**63,88**	**172,72**	**167,42**	**45,60**	**36,87**	**69,43**	**60,72**	**87,03**	**158,45**	**245,75**	**241,94**	**148,28**

Table S18. Summary of nested ANOVAs on the carbohydrates (total CHO, Rha, Fuc, Ara, Man, Gal, Glu and Rib) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in CHO data of *Lemna minor*		
DATA	Source of variation	CHO scores		
		MS	*F*	*p*
Analysis of total CHO	*Contaminant*	444768	25.860	1.24e-05*
	Contaminant : Temperature	660393	38.397	1.49e-09*
	Contaminant : Temperature : Concentration	69325	4.031	0.00358*
	Residuals	17199		
Analysis of Rha	*Contaminant*	6.06	1.612	0.212578
	Contaminant : Temperature	40.20	10.688	0.000238*
	Contaminant : Temperature : Concentration	32.51	8.644	8.54e-06*
	Residuals	3.76		
Analysis of Fuc	*Contaminant*	22993	13.388	0.000827*
	Contaminant : Temperature	25715	14.973	2e-05*
	Contaminant : Temperature : Concentration	5095	2.967	0.018973*
	Residuals	1717		
Analysis of Ara	*Contaminant*	3671	23.777	2.33e-05*
	Contaminant : Temperature	2930	18.978	2.62e-06*
	Contaminant : Temperature : Concentration	360	2.331	0.0534
	Residuals	154		
Analysis of Man	*Contaminant*	102.72	9.218	0.004503*
	Contaminant : Temperature	1.34	0.120	0.886923
	Contaminant : Temperature : Concentration	63.07	5.660	0.000343*
	Residuals	11.14		
Analysis of Gal	*Contaminant*	2235	2.455	0.1261
	Contaminant : Temperature	25219	27.706	6.13e-08*
	Contaminant : Temperature : Concentration	4053	4.453	0.0019*
	Residuals	910		
Analysis of Glu	*Contaminant*	153534	18.603	0.000125*
	Contaminant : Temperature	232669	28.192	5.08e-08*
	Contaminant : Temperature : Concentration	22910	2.776	0.025835*
	Residuals	8253		
Analysis of Rib	*Contaminant*	0.6260	7.787	0.00846*
	Contaminant : Temperature	0.5868	7.300	0.00224*
	Contaminant : Temperature : Concentration	0.7581	0.7581	3.6e-06*
	Residuals	0.0804	0.0804	

Table S19. Summary of two-way ANOVAs on the carbohydrates (total CHO, Rha, Fuc, Ara, Man, Gal, Glu and Rib) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in CHO data of *Lemna minor*					
DATA	Source of variation	CHO scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Temperature*	1213887	45.94	2.38e-06*	106900	50.344	1.8e-06*
	Concentration	164087	6.21	0.00439*	4659	2.194	0.126
	Residuals	26422			2123		
Analysis of Rha	*Temperature*	76.39	11.118	0.00369*	4.009	1.730	0.2059
	Concentration	46.77	6.807	0.00291*	7.778	3.356	0.0435*
	Residuals	6.87			2.317		
Analysis of Fuc	*Temperature*	51301	19.029	0.000376*	128.0	0.982	0.336
	Concentration	13197	4.895	0.011647*	114.6	0.879	0.472
	Residuals	2696			130.4		
Analysis of Ara	*Temperature*	3231	15.556	0.000951*	2629.6	35.431	1.58e-05*
	Concentration	813	3.912	0.025897*	42.0	0.566	0.645
	Residuals	208			74.2		
Analysis of Man	*Temperature*	0.23	0.012	0.9137	2.45	0.945	0.345
	Concentration	88.88	4.667	0.0139*	38.29	14.750	5.52e-05*
	Residuals	19.04			2.60		
Analysis of Gal	*Temperature*	22419	17.080	0.000625*	28019	68.784	2.23e-07*
	Concentration	7969	6.072	0.004846*	573	1.406	0.275
	Residuals	1313			407		
Analysis of Glu	*Temperature*	456614	33.97	1.61e-05*	8724	21.767	0.000222*
	Concentration	58199	4.33	0.0183*	981	2.447	0.099084
	Residuals	13442			401		
Analysis of Rib	*Temperature*	1.1737	5.842	0.02649*			
	Concentration	1.2485	6.214	0.00438*			
	Residuals	0.2009					

Table S20. Summary of two-way ANOVAs on the carbohydrates (total CHO, Rha, Fuc, Ara, Man, Gal, Glu and Rib) profiles of *Lemna minor*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

		One-way ANOVAs in CHO data of *Lemna minor*					
DATA	Source of variation	CHO scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Concentration*	300557	85.4	7.07e-06*	13482	97.92	1.2e-06*
	Residuals	3519			138		
Analysis of Rha	*Concentration*	0.687	8.91e+30	<2e-16*	84.57	82.03	2.39e-06*
	Residuals	0.000			1.03		
Analysis of Fuc	*Concentration*	29301	32211	7.53e-15*	69.97	6.553e+30	<2e-16*
	Residuals	1			0.00		
Analysis of Ara	*Concentration*	1701.0	51.95	3.77e-05*	274.77	111.3	7.32e-07*
	Residuals	32.7			2.47		
Analysis of Man	*Concentration*	69.99	3.16	0.095	68.62	14.25	0.00142*
	Residuals	22.15			4.82		
Analysis of Gal	*Concentration*	10355	42.55	7.31e-05*	4912	1312	4.16e-11*
	Residuals	243			4		
Analysis of Glu	*Concentration*	132796	88.58	6.24e-06*	2325.9	26.61	0.000163*
	Residuals	1499			87.4		
Analysis of Rib	*Concentration*				2.454	5.959e+30	<2e-16*
	Residuals				0.000		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total CHO	*Concentration*	1302.9	10.31	0.00581*	7325	2.2	0.176
	Residuals	126.3			3329		
Analysis of Rha	*Concentration*	0.15359	99.44	4.21e-06*	15.241	6.451	0.02*
	Residuals	0.00154			2.362		
Analysis of Fuc	*Concentration*				227.6	0.848	0.51
	Residuals				268.2		
Analysis of Ara	*Concentration*	12.365	15.92	0.00165*	95.79	0.634	0.616
	Residuals	0.777			151.13		
Analysis of Man	*Concentration*	10.800	934.7	1.79e-09*	28.75	5.001	0.0367*
	Residuals	0.012			5.75		
Analysis of Gal	*Concentration*	391.7	8.479	0.00991*	1065.1	1.888	0.22
	Residuals	46.2			564.3		
Analysis of Glu	*Concentration*	245.92	5.401	0.0307*	1330.5	1.978	0.206
	Residuals	45.53			672.6		

Table S21. Summary ANOSIM analysis on the total carbohydrates content of *Lemna minor*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in CHO data of *Lemna minor*		
Global Test		
Global R		0.309
Significance level (p-value)		0.007*
Pairwise Tests	R statistic	p-value
Copper sulphate at 20°C, Copper sulphate at 25°C	0.333	0.086
Copper sulphate at 20°C, TEB at 20°C	0.708	0.029*
Copper sulphate at 20°C, TEB at 25°C	0.24	0.2
Copper sulphate at 25°C, TEB at 20°C	0.177	0.171
Copper sulphate at 25°C, TEB at 25°C	0.021	0.343
TEB at 20°C, TEB at 25°C	0.583	0.029*

Table S22. Fatty acid abundance mean-values (mg.g^{-1}) of *Daphnia magna* offspring exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark blue) and 25°C (light blue). Total content of SFA, MUFA, PUFA and HUFA are expressed in % for each contaminant.

Test species	*Daphnia magna*															
Age	Offspring															
Contaminant	Copper Sulphate								TEB							
Temperature	20°C				25°C				20°C				25°C			
Concentration	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}	CTL	NOEC	EC_{10}	EC_{20}
C20:0	0,06	0,00	0,10	0,05	0,00	0,00	0,00	0,00	0,51	0,44	0,36	0,59	0,00	0,40	0,40	0,52
C21:0	1,55	8,93	6,71	6,29	29,80	22,40	19,50	28,00	18,40	20,00	17,30	28,30	13,30	12,80	15,30	27,10
C22:0	0,05	0,21	0,21	0,11	0,00	0,12	0,30	0,00	0,85	0,80	0,69	1,02	6,35	0,83	0,77	0,96
TOTAL% SFA	**58,04**	**76,74**	**72,22**	**72,88**	**80,52**	**76,08**	**73,01**	**78,19**	**58,32**	**62,11**	**58,20**	**66,72**	**42,50**	**43,76**	**46,74**	**59,07**
C15:1n10	0,02	0,00	0,01	0,02	0,00	0,00	0,00	0,00	0,22	0,09	0,13	0,07	0,00	0,23	0,31	0,16
C17:1n10	0,02	0,00	0,01	0,01	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,00
C18:1n9	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	0,00	0,00
C20:1n11	0,10	0,12	0,07	0,05	0,18	0,27	0,31	0,38	0,63	0,59	0,63	0,68	0,00	0,65	0,83	0,74
TOTAL% MUFA	**4,90**	**1,01**	**0,93**	**0,90**	**0,49**	**0,91**	**1,14**	**1,06**	**2,51**	**1,99**	**2,41**	**1,67**	**0,00**	**2,74**	**3,35**	**1,86**
C18:2n9,12	0,07	0,11	0,13	0,13	0,14	0,24	0,28	0,26	0,70	0,69	0,68	0,70	0,00	0,87	0,94	0,89
C18:3n6,9,12	0,06	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,84	0,14	0,06	0,13	1,87	0,09	0,07	0,31
ALA	0,09	0,41	0,43	0,27	1,42	1,07	1,00	1,25	0,29	0,00	0,11	0,16	1,98	0,31	0,39	0,34
C20:2n11,13	0,21	0,35	0,33	0,31	0,83	1,02	1,12	1,09	2,19	2,19	2,20	2,42	4,57	3,56	3,77	3,89
C20:3n7,10,13	0,06	0,00	0,00	0,01	0,00	0,00	0,00	0,00	0,50	0,44	0,29	0,18	0,00	0,55	0,62	1,14
ARA	0,21	0,62	0,58	0,55	1,00	1,07	1,08	1,08	2,39	2,55	2,65	2,85	6,38	3,00	3,02	3,33
TOTAL% PUFA	**24,48**	**12,51**	**15,12**	**14,46**	**9,16**	**11,49**	**12,83**	**10,28**	**20,40**	**17,57**	**19,00**	**14,37**	**32,01**	**26,14**	**25,00**	**20,46**
EPA	0,36	1,16	1,14	1,04	3,64	3,41	3,53	3,75	6,36	6,27	6,43	7,73	5,55	8,77	8,78	9,00
DHA	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	6,24	0,00	0,00	0,00
TOTAL% HUFA	**12,59**	**9,74**	**11,73**	**11,75**	**9,84**	**11,52**	**13,02**	**10,47**	**18,77**	**18,33**	**20,39**	**17,24**	**25,50**	**27,35**	**24,91**	**18,60**
TOTAL FA	2,86	11,91	9,72	8,85	37,01	29,60	27,12	35,81	33,88	34,20	31,53	44,83	46,24	32,06	35,24	48,38

Table S23. Summary of nested ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of offspring of *Daphnia magna*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in FA data of *Daphnia magna* offspring		
DATA	Source of variation	FA scores		
		MS	*F*	*p*
Analysis of total FA	*Contaminant*	0.0026111	32.921	1.56e-06*
	Contaminant : Temperature	0.0019743	24.891	1.63e-07*
	Contaminant : Temperature : Concentration	0.0001236	1.559	0.188
	Residuals	0.0000793		
Analysis of SFA	*Contaminant*	0.0003256	7.709	0.00867*
	Contaminant : Temperature	0.0010600	25.098	1.5e-07*
	Contaminant : Temperature : Concentration	0.0001073	2.541	0.03733*
	Residuals	0.0000422		
Analysis of MUFA	*Contaminant*	3.505e-06	80.060	1.11e-10*
	Contaminant : Temperature	9.300e-08	2.128	0.13386
	Contaminant : Temperature : Concentration	2.060e-07	4.710	0.00124*
	Residuals	4.400e-08		
Analysis of PUFA	*Contaminant*	0.0004166	106.613	2.62e-12*
	Contaminant : Temperature	0.0000671	17.162	5.83e-06*
	Contaminant : Temperature : Concentration	0.0000102	2.617	0.0329*
	Residuals	0.0000039		
Analysis of HUFA	*Contaminant*	1.161e-04	9.531	0.00388*
	Contaminant : Temperature	2.272e-04	18.660	2.75e-05*
	Contaminant : Temperature : Concentration	1.190e-06	0.098	0.99616
	Residuals	1.218e-05		
Analysis of C21:0	*Contaminant*	0.0001427	26.206	9.47e-08*
	Contaminant : Temperature	0.0010911	2.767	0.0257*
	Contaminant : Temperature : Concentration	0.0001152		
	Residuals	0.0000416		
Analysis of ARA	*Contaminant*	7.12e-05	80.830	9.85e-11*
	Contaminant : Temperature	6.38e-06	7.248	0.00226*
	Contaminant : Temperature : Concentration	2.24e-06	2.538	0.03752*
	Residuals	8.80e-07		
Analysis of EPA	*Contaminant*	2.955e-04	209.302	<2e-16*
	Contaminant : Temperature	2.587e-05	18.324	3.25e-06*
	Contaminant : Temperature : Concentration	3.440e-06	2.436	0.0444*
	Residuals	1.410e-06		
Analysis of DHA	*Contaminant*	6.983e-06	0.939	0.339
	Contaminant : Temperature	7.300e-06	0.981	0.385
	Contaminant : Temperature : Concentration	8.903e-06	1.197	0.330
	Residuals	7.439e-06		

Table S24. Summary of two-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of offspring of *Daphnia magna*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in data FA of *Daphnia magna* offspring					
DATA	Source of variation	FA scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Temperature*	0.003337	82.410	6.27e-08*	0.0006120	5.267	0.0333*
	Concentration	0.000015	0.378	0.77	0.0002184	1.879	0.1674
	Residuals	0.000040			0.0001162		
Analysis of SFA	*Temperature*	0.0020860	64.882	3.32e-07*	3.405e-05	0.651	0.4296
	Concentration	0.0000144	0.447	0.723	1.939e-04	3.709	0.0296*
	Residuals	0.0000322			5.227e-05		
Analysis of MUFA	*Temperature*	1.827e-07	23.69	0.000145*	3.600e-09	0.040	0.8428
	Concentration	4.930e-09	0.64	0.599862	3.189e-07	3.542	0.0343*
	Residuals	7.710e-09			9.000e-08		
Analysis of PUFA	*Temperature*	2.856e-05	183.233	1.56e-10*	1.056e-04	13.736	0.0015*
	Concentration	2.600e-07	1.667	0.212	1.753e-05	2.281	0.1120
	Residuals	1.560e-07			7.680e-06		
Analysis of HUFA	*Temperature*	3.992e-05	165.188	3.5e-10*	0.0004145	18.078	0.00043*
	Concentration	1.800e-07	0.751	0.537	0.0000017	0.076	0.972399
	Residuals	2.400e-07			0.0000229		
Analysis of C21:0	*Temperature*	0.0021057	64.309	0.684	7.652e-05	1.500	0.2357
	Concentration	0.0000165	0.504		2.048e-04	4.014	0.0227*
	Residuals	0.0000327			5.103e-05		
Analysis of ARA	*Temperature*	1.837e-06	66.476	2.82e-07*	1.093e-05	6.135	0.0228*
	Concentration	7.200e-08	2.604	0.0855	3.527e-06	1.979	0.1513
	Residuals	2.760e-08			1.782e-06		
Analysis of EPA	*Temperature*	3.992e-05	165.188	3.5e-10*	1.182e-05	4.617	0.0448*
	Concentration	1.800e-07	0.751	0.537	6.059e-06	2.367	0.1030
	Residuals	2.400e-07			2.560e-06		
Analysis of DHA	*Temperature*				1.46e-05	1	0.330
	Concentration				1.46e-05	1	0.414
	Residuals				1.46e-05		

Table S25. Summary of one-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of offspring of *Daphnia magna*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

		One-way ANOVAs in FA data of *Daphnia magna* offspring					
DATA	Source of variation	FA scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	4.003e-05	2.93	0.109	6.447e-05	1.388	0.324
	Residuals	1.366e-05			4.645e-05		
Analysis of SFA	*Concentration*	2.631e-05	2.242	0.171	6.156e-05	1.767	0.241
	Residuals	1.174e-05			3.484e-05		
Analysis of MUFA	*Concentration*	1.888e-09	0.897	0.489	2.020e-08	2.179	0.178
	Residuals	2.104e-08			9.269e-09		
Analysis of PUFA	*Concentration*	3.829e-07	5.729	0.0267*	5.198e-08	0.22	0.88
	Residuals	6.680e-08			2.367e-07		
Analysis of HUFA	*Concentration*	4.104e-07	4.708	0.042*	5.250e-08	0.138	0.934
	Residuals	8.720e-08			3.792e-07		
Analysis of C21:0	*Concentration*	2.549e-05	2.075	0.192	6.603e-05	1.882	0.221
	Residuals	1.229e-05			3.508e-05		
Analysis of ARA	*Concentration*	1.016e-07	5.287	0.0323*	3.900e-09	0.116	0.948
	Residuals	1.922e-08			3.352e-08		
Analysis of EPA	*Concentration*	4.104e-07	4.708	0.042*	5.250e-08	0.138	0.934
	Residuals	8.720e-08			3.792e-07		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	8.300e-05	2.564	0.128	0.0001917	0.862	0.499
	Residuals	3.237e-05			0.0002225		
Analysis of SFA	*Concentration*	8.280e-05	2.249	0.16	1.198e-04	1.426	0.305
	Residuals	3.683e-05			8.404e-05		
Analysis of MUFA	*Concentration*	1.333e-08	0.835	0.512	7.828e-07	41.43	3.21e-05*
	Residuals	1.597e-08			1.890e-08		
Analysis of PUFA	*Concentration*	7.165e-07	0.759	0.548	2.621e-05	1.902	0.208
	Residuals	9.441e-07			1.378e-05		
Analysis of HUFA	*Concentration*	2.552e-06	1.652	0.253	6.530e-06	0.13	0.939
	Residuals	1.545e-06			5.016e-05		
Analysis of C21:0	*Concentration*	7.769e-05	2.161	0.171	1.354e-04	1.648	0.254
	Residuals	3.595e-05			8.215e-05		
Analysis of ARA	*Concentration*	1.329e-07	1.455	0.298	8.050e-06	3.361	0.0757
	Residuals	9.135e-08			2.395e-06		
Analysis of EPA	*Concentration*	1.774e-06	3.61	0.065	8.178e-06	1.981	0.195
	Residuals	4.915e-07			4.128e-06		
Analysis of DHA	*Concentration*				2.92e-05	1	0.441
	Residuals				2.92e-05		

Table S26. Summary ANOSIM analysis on the total fatty acid content of offspring of *Daphnia magna*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in FA data of *Daphnia magna* offspring		
Global Test		
Global R		0.639
Significance level (p-value)		0.001*
Pairwise Tests	R statistic	p-value
Copper sulphate at 20°C, Copper sulphate at 25°C	0.729	0.029*
Copper sulphate at 20°C, TEB at 20°C	0.708	0.029*
Copper sulphate at 20°C, TEB at 25°C	0.865	0.029*
Copper sulphate at 25°C, TEB at 20°C	0.323	0.171
Copper sulphate at 25°C, TEB at 25°C	0.781	0.029*
TEB at 20°C, TEB at 25°C	0.635	0.057

Table S27. Fatty acid abundance mean-values (mg.g^{-1}) of mothers of *Daphnia magna* exposed to copper sulphate (on the left) and TEB (on the right), at 20°C (dark yellow) and 25°C (light yellow). Total content of SFA, MUFA, PUFA and HUFA are expressed in % for each contaminant.

Test species	*Daphnia magna*															
Age	Mothers															
Contaminant	**Copper Sulphate**								**TEB**							
Temperature	20°C				25°C				20°C				25°C			
Concentration	**CTL**	**NOEC**	**EC_{10}**	**EC_{20}**	**CTL**	**NOEC**	**EC_{10}**	**EC_{20}**	**CTL**	**NOEC**	**EC_{10}**	**EC_{20}**	**CTL**	**NOEC**	**EC_{10}**	**EC_{20}**
C10:0	7,66	12,17	10,98	6,38	0,00	7,04	0,53	1,51	7,29	3,45	3,34	4,13	7,18	4,21	3,87	0,00
C11:0	1,73	2,36	1,63	0,00	0,00	0,00	0,00	0,00	1,02	0,00	0,00	0,25	0,43	0,00	0,00	0,00
C12:0	0,98	1,19	1,78	0,60	0,00	5,94	3,70	6,36	0,94	1,12	1,14	1,47	1,89	1,63	0,70	0,00
C13:0	9,02	12,12	15,98	6,75	7,02	9,15	5,22	11,85	8,69	3,67	3,93	5,46	6,28	4,54	3,65	2,70
C14:0	0,94	0,00	0,23	0,00	0,00	1,07	0,40	1,21	0,34	0,00	0,00	0,71	0,40	0,55	0,57	0,00
C15:0	16,83	23,88	21,02	20,94	7,68	9,77	8,74	10,23	15,78	10,15	13,17	15,29	14,52	17,19	15,94	23,06
C16:0	4,97	0,80	1,92	6,66	6,10	1,17	5,47	6,46	4,97	0,57	0,00	0,00	0,92	1,34	1,71	0,00
C17:0	3,61	4,33	3,77	2,29	1,54	3,78	3,16	3,45	2,00	1,44	0,88	0,47	2,53	2,15	1,96	1,87
C18:0	1,80	3,21	1,53	0,65	5,68	1,75	3,93	2,10	0,00	1,26	0,33	0,97	1,19	0,66	0,00	0,00
C20:0	51,04	18,86	29,22	26,81	19,65	23,39	19,68	26,89	19,01	20,02	12,91	11,18	29,22	25,36	30,84	24,10
C21:0	44,61	58,33	62,75	77,11	61,85	55,52	52,32	60,37	80,52	38,71	54,23	77,49	41,64	45,73	59,38	82,05
C22:0	56,21	47,38	50,82	47,30	46,59	48,56	52,60	57,15	30,97	37,35	30,77	29,64	52,34	47,07	46,79	54,01
TOTAL% SFA	**16,80**	**15,47**	**16,76**	**18,47**	**12,64**	**13,20**	**12,27**	**13,50**	**21,76**	**15,50**	**16,67**	**18,25**	**13,37**	**13,12**	**15,07**	**15,84**
C15:1n10	10,01	7,22	8,48	4,74	0,00	11,20	6,55	6,49	5,57	6,98	5,79	5,87	12,04	11,52	13,98	12,73
C16:1n9	4,43	4,22	3,95	2,47	9,96	3,25	7,34	3,63	2,62	1,22	1,74	1,84	3,80	2,25	2,14	2,40
C17:1n10	4,52	3,67	3,80	3,49	2,68	4,20	3,64	4,95	3,03	2,69	2,46	2,68	5,08	4,69	4,86	4,87
C18:1n9	0,93	2,08	1,06	0,00	0,00	0,00	0,00	0,00	1,47	0,00	0,00	0,59	0,88	2,92	0,00	0,00
C20:1n11	40,28	47,19	43,98	33,90	51,88	54,17	53,16	58,44	20,18	21,69	21,67	23,30	39,01	38,98	30,70	28,68
C22:1n13	0,95	2,25	1,07	0,00	8,89	0,89	3,20	0,00	2,28	2,27	0,00	0,00	2,11	0,00	0,00	0,00
TOTAL% MUFA	**5,15**	**5,58**	**5,18**	**4,21**	**5,94**	**5,82**	**5,82**	**5,29**	**4,46**	**4,59**	**4,37**	**4,25**	**5,31**	**5,26**	**4,71**	**4,11**
C18:2n9,12	46,20	42,81	43,46	38,44	38,98	58,19	57,27	52,15	31,00	33,98	30,83	31,83	50,82	53,61	51,57	56,11
C18:3n6,9,12	20,96	16,72	18,98	16,61	17,81	22,48	10,25	23,55	14,62	15,04	14,15	22,04	27,33	20,59	19,01	18,35
ALA	8,48	8,81	7,04	8,29	15,43	5,85	9,87	4,15	4,96	3,15	2,53	1,65	4,64	4,03	4,50	3,73
C20:2n11,13	226,6	249,3	244,4	200,9	311,3	314,3	345,6	350,6	147,4	142,7	144,6	150,7	246,4	259,1	247,5	240,0
C20:3n7,10,13	27,82	32,81	30,85	27,72	29,02	34,34	15,15	40,01	20,21	22,93	22,84	35,85	43,82	45,38	42,70	54,88
ARA	171,8	183,2	177,0	150,6	160,1	165,1	165,8	191,4	109,3	117,6	110,4	122,3	168,0	156,0	141,1	156,5
TOTAL% PUFA	**42,29**	**44,70**	**43,37**	**41,82**	**46,35**	**47,41**	**47,59**	**47,65**	**41,53**	**44,16**	**44,94**	**45,22**	**45,62**	**46,97**	**46,13**	**44,66**
EPA	407,6	384,1	394,7	355,6	421,6	410,1	424,3	448,4	239,3	260,1	233,9	247,5	392,9	375,7	359,7	403,9
DHA	16,66	24,85	22,62	19,93	11,53	14,87	11,16	17,73	14,96	11,52	12,34	12,64	30,59	21,77	14,40	15,77
TOTAL% HUFA	**35,75**	**34,25**	**34,69**	**35,49**	**35,07**	**33,57**	**34,32**	**33,56**	**32,25**	**35,75**	**34,02**	**32,28**	**35,71**	**34,65**	**34,09**	**35,40**
TOTAL FA	1187	1194	1203	1058	1235	1266	1269	1389	788,4	759,6	724,0	805,9	1186	1147	1098	1186

Table S28. Summary of nested ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of the mothers of *Daphnia magna*, with the degrees of freedom (d.f.), variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Nested ANOVAs in FA data of *Daphnia magna* mothers		
DATA	Source of variation	FA scores		
		MS	*F*	*p*
Analysis of total FA	*Contaminant*	0.8950	85.345	1.52e-10*
	Contaminant : Temperature	0.3954	37.703	3.85e-09*
	Contaminant : Temperature : Concentration	0.0042	0.397	0.875
	Residuals	0.0105		
Analysis of SFA	*Contaminant*	0.011695	12.477	0.00128*
	Contaminant : Temperature	0.003600	3.841	0.03199*
	Contaminant : Temperature : Concentration	0.000677	0.722	0.63495
	Residuals	0.000937		
Analysis of MUFA	*Contaminant*	0.005070	135.230	4.99e-13*
	Contaminant : Temperature	0.001958	52.214	8.39e-11*
	Contaminant : Temperature : Concentration	0.000098	2.619	0.0352*
	Residuals	0.000037		
Analysis of PUFA	*Contaminant*	0.18441	87.407	1.15e-10*
	Contaminant : Temperature	0.11872	56.268	3.33e-11*
	Contaminant : Temperature : Concentration	0.00107	0.507	0.799
	Residuals	0.00211		
Analysis of HUFA	*Contaminant*	0.11376	81.739	2.52e-10*
	Contaminant : Temperature	0.05389	38.724	2.85e-09*
	Contaminant : Temperature : Concentration	0.00055	0.395	0.877
	Residuals	0.00139		
Analysis of ARA	*Contaminant*	0.015309	52.161	3.33e-08*
	Contaminant : Temperature	0.004228	14.405	3.46e-05*
	Contaminant : Temperature : Concentration	0.000143	0.488	0.812
	Residuals	0.000293		
Analysis of EPA	*Contaminant*	0.11164	87.850	1.08e-10*
	Contaminant : Temperature	0.04876	38.371	3.16e-09*
	Contaminant : Temperature : Concentration	0.00048	0.378	0.888
	Residuals	0.00127		
Analysis of DHA	*Contaminant*	0.0000100	0.527	0.4732
	Contaminant : Temperature	0.0003681	19.399	3.03e-06*
	Contaminant : Temperature : Concentration	0.0000633	3.336	0.0115*
	Residuals	0.0000190		

Table S29. Summary of two-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of the mothers of *Daphnia magna*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences between treatments, whenever *p*-values < 0.05 were recorded.

		Two-way ANOVAs in FA data of *Daphnia magna* mothers					
DATA	Source of variation	FA scores					
		Copper sulphate			*TEB*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Temperature*	0.08088	4.661	0.0454*	0.7099	272.308	5.01e-11*
	Concentration	0.00160	0.092	0.9632	0.0072	2.772	0.0777
	Residuals	0.01735			0.0026		
Analysis of SFA	*Temperature*	0.004743	6.48	0.0209*	0.002457	2.146	0.164
	Concentration	0.000191	0.26	0.8529	0.001289	1.125	0.370
	Residuals	0.000732			0.001145		
Analysis of MUFA	*Temperature*	0.0012181	24.45	0.000123*	0.002697	142.575	4.63e-09*
	Concentration	0.0001644	3.30	0.045671*	0.000055	2.903	0.0694
	Residuals	0.0000498			0.000019		
Analysis of PUFA	*Temperature*	0.06022	15.998	0.000928*	0.17721	971.104	4.74e-15*
	Concentration	0.00138	0.367	0.777691	0.00102	5.585	0.00892*
	Residuals	0.00376			0.00018		
Analysis of HUFA	*Temperature*	0.005325	2.441	0.137	0.10246	206.124	3.6e-10*
	Concentration	0.000280	0.128	0.942	0.00082	1.643	0.222
	Residuals	0.002182			0.00050		
Analysis of ARA	*Temperature*	0.0000147	0.032	0.859	0.008441	76.701	2.78e-07*
	Concentration	0.0000944	0.207	0.890	0.000194	1.759	0.198
	Residuals	0.0004551			0.000110		
Analysis of EPA	*Temperature*	0.008110	4.066	0.0598	0.08941	205.455	3.68e-10*
	Concentration	0.000477	0.239	0.8677	0.00056	1.284	
	Residuals	0.001995			0.00044		
Analysis of DHA	*Temperature*	2.917e-04	12.447	0.00258*	0.0004444	27.762	9.45e-05*
	Concentration	3.536e-05	1.509	0.24821	0.0000808	5.046	0.0129*
	Residuals	2.344e-05			0.0000160		

Table S30. Summary of one-way ANOVAs on the fatty acid (total FA, SFA, MUFA, PUFA and HUFA) profiles of the mothers of *Daphnia magna*, with the variance (MS), *F* test and corresponding *p*-value. * stands for significant differences among the contaminated samples and the controls, whenever *p*-values < 0.05 were recorded.

One-way ANOVAs in FA data of *Daphnia magna* mothers							
DATA	Source of variation	FA scores					
		Copper sulphate at 20°C			*Copper sulphate at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	0.01403	0.562	0.655	0.010206	2.244	0.184
	Residuals	0.02498			0.004548		
Analysis of SFA	*Concentration*	0.0001625	0.129	0.94	0.0004794	2.779	0.133
	Residuals	0.0012569			0.0001725		
Analysis of MUFA	*Concentration*	2.811e-04	6.931	0.0129*	1.050e-07	0.004	1
	Residuals	4.056e-05			2.873e-05		
Analysis of PUFA	*Concentration*	0.004911	1.033	0.429	0.003232	3.427	0.093
	Residuals	0.004756			0.000943		
Analysis of HUFA	*Concentration*	0.001398	0.418	0.745	0.0007268	0.906	0.492
	Residuals	0.003342			0.0008022		
Analysis of ARA	*Concentration*	0.0006018	1.073	0.413	4.37e-04	6.283	0.0279*
	Residuals	0.0005607			6.96e-05		
Analysis of EPA	*Concentration*	0.001470	0.498	0.694	0.0005980	0.65	0.611
	Residuals	0.002953			0.0009194		
Analysis of DHA	*Concentration*	3.740e-05	1.193	0.372	2.119e-05	1.631	0.279
	Residuals	3.135e-05			1.300e-05		
		TEB at 20°C			*TEB at 25°C*		
		MS	*F*	*p*	MS	*F*	*p*
Analysis of total FA	*Concentration*	0.003853	1.023	0.432	0.003513	1.643	0.314
	Residuals	0.003767			0.002139		
Analysis of SFA	*Concentration*	0.001910	1.123	0.396	0.0003308	1.867	0.276
	Residuals	0.001701			0.0001771		
Analysis of MUFA	*Concentration*	7.587e-06	0.58	0.644	8.324e-05	4.666	0.0854
	Residuals	1.308e-05			1.784e-05		
Analysis of PUFA	*Concentration*	0.0009751	9.589	0.00501*	0.0005449	5.175	0.0731
	Residuals	0.0001017			0.0001053		
Analysis of HUFA	*Concentration*	0.0003403	1.206	0.368	0.0010859	1.288	0.393
	Residuals	0.0002822			0.0008428		
Analysis of ARA	*Concentration*	1.144e.04	2.151	0.172	0.0002915	1.982	0.259
	Residuals	5.318e-05			0.0001471		
Analysis of EPA	*Concentration*	0.0003876	1.428	0.305	0.0006363	0.86	0.531
	Residuals	0.0002715			0.0007401		
Analysis of DHA	*Concentration*	6.526e-06	1.531	0.28	1.246e-04	9.073	0.0294*
	Residuals	4.263e-06			1.373e-05		

Table S31. Summary ANOSIM analysis on the total fatty acid content of mothers of *Daphnia magna*, through a global test and pairwise tests of the four tests (copper sulphate exposure at 20°C and 25°C, and TEB exposure at 20°C and 25°C). * stands for significant differences between the analysed tests, whenever *p*-values < 0.05 were recorded.

ANOSIM analysis in FA data of *Daphnia magna* mothers		
Global Test		
Global R		0.694
Significance level (p-value)		0.001*
Pairwise Tests	**R statistic**	**p-value**
Copper sulphate at 20°C, Copper sulphate at 25°C	0.51	0.029*
Copper sulphate at 20°C, TEB at 20°C	1	0.029*
Copper sulphate at 20°C, TEB at 25°C	0.083	0.286
Copper sulphate at 25°C, TEB at 20°C	1	0.029*
Copper sulphate at 25°C, TEB at 25°C	0.656	0.029*
TEB at 20°C, TEB at 25°C	1	0.029*