Universidade de Aveiro Departamento de Biologia

Ano 2019

Patrícia Alexandra Franco Roque

Impacts of contaminants in freshwater macrophytes

O impacto de contaminantes em espécies de macrófitas de água-doce

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em *Toxicologia e Ecotoxicologia*, realizada sob a orientação científica da Doutora Ana Marta Mendes Gonçalves, investigadora auxiliar do Centro de Ciências do Mar e do Ambiente (MARE), Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia da Universidade de Coimbra, e do Professor Doutor Fernando Gonçalves do Departamento de Biologia da Universidade de Aveiro.

Aos meus pais, namorado e avós.

"It seems to me that the natural world is the greatest source of excitement; the greatest source of visual beauty; the greatest source of intellectual interest. It is the greatest source of so much in life that makes life worth living."

David Attenborough

o júri

presidente

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agradecimentos

Com este trabalho chega também o fim de mais uma importante etapa da minha vida académica, que só foi possível graças a pessoas importantes, às quais seguem o meu agradecimento.

Ao Professor Doutor Fernando Gonçalves pela minha integração na equipa, pelo espaço cedido para trabalhar e por todo o apoio prestado na realização deste trabalho.

À Doutora Ana Marta Gonçalves pela confiança que depositou em mim para a orientação deste trabalho, pelo esforço e dedicação impostos sobre o mesmo e pela paciência em corrigir os meus erros. Erros esses que fazem parte de tudo na vida e só assim se segue em frente e se melhora a qualidade de trabalho.

À Doutora Ana Patrícia Cuco e à Andreia Filipa Mesquita por todo o apoio e ensinamentos prestados na parte prática em laboratório e tratamento estatístico associado.

À Doutora Cláudia Nunes e ao Professor Doutor Manuel Coimbra pelo acesso aos laboratórios no Departamento de Química para a realização de uma parte deste trabalho.

A toda a equipa LEADER pela simpatia, boa disposição, integração e apoio que me favoreceram em todo o tempo que passei no laboratório.

Um agradecimento para toda a minha família e amigos por estarem sempre lá nos piores e nos melhores momentos, e que mesmo estando longe de casa, fazem de tudo para fazerem parte do meu dia-a-dia. E por fim, um agradecimento especial ao meu namorado por ser o meu apoio e a pessoa mais envolvida na minha vida em qualquer lugar.

palavras-chave

Herbicidas, Contaminação, Macrófitas, Ecossistema de Água-doce, Perfis bioquímicos, Ecotoxicologia.

resumo

Nas últimas décadas, o uso de pesticidas tem sido intensificado nomeadamente nas regiões mediterrânicas, que em muitos casos excede os limites permitidos pela União Europeia. O uso intensivo de químicos nos campos agrícolas em redor dos sistemas aquáticos pode ter consequências para os ecossistemas e comunidades. O Primextra Gold TZ é um dos herbicidas mais utilizados em campos de milho no estuário do Mondego, de acordo com a informação das cooperativas agrícolas locais. Os principais ingredientes ativos do herbicida são a Terbutilazina e o S-Metolacloro. Adicionalmente, formulações baseadas em cobre, como o sulfato de cobre, também são frequentemente utilizadas em práticas agrícolas, sendo um dos principais constituintes desses produtos. Assim, este trabalho pretende avaliar os efeitos tóxicos e bioquímicos destes químicos (Terbutilazina, S-Metolacloro e Cobre) em espécies não-alvo *Lemna minor* e *Lemna gibba*, duas macrófitas conhecidas como espécies padrão de ensaios ecotoxicológicos. Num primeiro passo, os organismos foram expostos aos químicos sob condições laboratoriais, seguindo-se uma análise bioquímica às potenciais alterações nos perfis em ácidos gordos, polissacarídeos e açúcares livres. Os resultados mostraram que a *L. minor* é mais sensível que a *L. gibba* ao S-Metolacloro ($EC50_{Lm}$ = 43,10 µg/L (30,89-55,30) e $EC50_{Lg}$ 86,81 µg/L (40,51-133,12), respetivamente), e ao Cobre ($EC50_{Lm}$ =199,20 µg/L (149,28-249,13) e $EC50_{Lg}$ =504,0 µg/L (308,73-699,90), respetivamente). Contudo, uma tendência oposta foi observada quando as macrófitas estão expostas a Terbutilazina, em que *L. minor* (EC50=93,43 µg/L (75,35-111,51)) demonstra menor sensibilidade à ação deste químico do que a *L. gibba* (EC50=43,89 µg/L (38,63- 49,15). Além disso, a composição de lípidos decresceu, principalmente polinsaturados (PUFA), e o conteúdo de hidratos de carbono também se alterou com a exposição de ambas as espécies aos três compostos individualmente. Assim, biomarcadores bioquímicos são ferramentas e sinais importantes em estudos ecotoxicológicos e, podem ser usados como indicadores de aviso-prévio da presença de contaminantes nos ecossistemas e na determinação do potencial efeito nas comunidades aquáticas.

keywords Herbicides, Contamination, Macrophytes, Freshwater ecosystem, Biochemical profiles, Ecotoxicology.

abstract In the last decades, the use of pesticides has been intensified mainly in European Mediterranean regions, and in some cases exceeding the limits of regular legislations established by the European Union. The intensive use of chemicals on agricultural fields surrounding aquatic systems may comport consequences to the ecosystems and communities. Primextra® Gold TZ is one of the most used herbicide in corn crop fields of the Mondego valley, according to the information from agricultural cooperatives. The main active ingredients of the herbicide are S-Metolachlor and Terbuthylazine. Moreover, copper based formulations, such as copper sulphate, are also quite used in agriculture practices being one of the main constituents of these products. Thus, this work pretended to evaluate the toxic and biochemical effects of these chemicals (S-Metolachlor, Terbuthylazine and copper) in the non-target species *Lemna minor* and *Lemna gibba*, two freshwater macrophytes, reported as standard species in ecotoxicological bioassays. In a first step, the organisms were exposed to the contaminants under laboratory conditions, after which the biochemical analysis to determine changes on the fatty acids, polysaccharides and free sugars profiles. The results showed *L. minor* is more sensitive than *L. gibba* to S-Metolachlor ($EC50_{Lm}$ = 43.10 µg/L (30.89-55.30) and $EC50_{Lg}$ 86.81 µg/L (40.51-133.12), respectively), and to Copper ($EC50_{Lm}$ =199.20 µg/L (149.28-249.13) and $EC50_{Lg}$ =504.31 µg/L (308.73- 699.90), respectively). However, the opposite trend was observed when macrophytes were exposed to Terbuthylazine, with *L. minor* (EC50=93.43 µg/L (75.35-111.51)) demonstrating less sensitivity to this chemical action than *L. gibba* (EC50=43.89 µg/L (38.63- 49.15). Furthermore, the composition on lipids decreased, especially polyunsaturated fatty acids (PUFA), and carbohydrates contents also changed with the exposure to all compounds for both species. Therefore, biochemical biomarkers revealed to be important tools and endpoints in ecotoxicological studies and may be used as early-warning indicators of the presence of contaminants at the ecosystems and on the determination of potential effects in aquatic communities.

Index

List of Abbreviations

SM- S-Metolachlor

TBZ- Terbuthylazine

Cu-Copper

FA- Fatty acid

SFA- Saturated Fatty Acid

MUFA- Monounsaturated Fatty Acid

HUFA- Highly Unsaturated Fatty Acid

EFA- Essential Fatty Acid

PUFA- Polyunsaturated Fatty Acid

FAME- Fatty Acid Methyl Ester

EC- Effective Concentration

NOEC- No Observable Effect Concentration

FS-Free Sugars

GC-MS- Gas Chromatography- Mass Spectrometer

GC-FID- Gas Chromatography- Flame Ionization Detector

SIMPER- Similarity Percentage analysis

ANOSIM- Analysis of Similarity

Rha- Rhamnose

Fuc- Fucose

Rib-Ribose

Ara-Arabinose

Xyl- Xylose

Man-Mannose

Gal-Galactose

Glc-Glucose

Introduction

1. Freshwater ecosystems under anthropogenic stress

Water is the most important element in Earth, available in different states (liquid, solid, or gas), and occupy a great volume of the planet. From all available water, only 2.5% is freshwater, of which 0.26% is liquid water present in lakes, reservoirs and rivers (Carpenter *et al*., 2011). As a good source of resources, freshwater ecosystems are surrounded by agricultural areas, industries, numerous civil constructions and a high human population density taking advantage of them. As a consequence, the flux of freshwater is altered by several factors in a large scale. Those factors include climate change, hydrologic flow modification, land-use change, harvest, aquatic invasive species, and chemical inputs (Carpenter *et al.*, 2011).

Chemical contamination has increased overtime related with the overuse of chemicals, sometimes above legislation levels, to raise food production according to the needs of human population growth. The pollution is also related with industrial and domestic activities (e.g. discharges of waste water and atmospheric pollution) (Carpenter *et al*., 2011; Matos *et al.*, 2007) releasing a great variety of chemicals directly or indirectly to aquatic ecosystems, some of them with a great toxicity. Contamination degrades the water quality of the large water bodies, reducing the water supply and increasing the costs of treating that water for human use (Carpenter *et al.*, 1998).

Chemical pollutants may cause toxic effects in the entire ecosystem, mainly in the aquatic organisms, with exposure situations causing chronic and acute responses (Amin & Hashem, 2012). Still, little is known about the ecosystems' response to this changes and there is more to explore about this subject, once such chemicals can accumulate in nature (Filimonova *et al.*, 2016) and compromise the function of the aquatic ecosystem.

Chemical formulations can cause several consequences for biodiversity and the functioning of aquatic ecosystems, which in turn will cause implications on economic and social systems that depend upon them. Agriculture and urban activities have an important role in nutrients cycle, providing Phosphorus (P) and Nitrogen (N) to the environment. Still, when these nutrients exceed favourable levels (by nonpoint sources) they compromise aquatic ecosystems, where the nutrients input results, frequently, in eutrophication processes, by increasing phytoplankton and macrophytes biomass, depleting oxygen

reserves from water and the final death of aquatic species, reducing considerably the water quality (Smith, 1998).

Estuaries are important ecosystems for human-beings, unique by their biodiversity and resources, providing important services to local populations, and is a place to navigate, fish and harvest seafood, and a great location for industries, houses and farmlands, representing one of the most inhabited habitats in the world (McLusky & Elliott, 2004; O'Gorman *et al.*, 2012). The most suitable definition for Estuary was given by Fairbridge (1980) as "an inlet of the sea reaching into a river valley as far as the upper limit of tidal rise, usually being divisible into three sectors: a) a marine or lower estuary, in free connections with the open sea; b) a middle estuary subject to strong salt and freshwater mixing; and c) an upper or fluvial estuary, characterized by freshwater but subject to strong tidal action. The limits between these sectors are variable and subject to constant changes in the river discharges" (McLusky & Elliott, 2004).

Estuaries have been constantly occupied by human population and, consequently, have been one of the most suitable aquatic ecosystems to contamination/pollution, once they are exposed to chemical discharges by anthropogenic activities both in land and water, e.g. fishing activities, directly and indirectly affecting the water quality and the living organisms (Cardoso *et al.*, 2008; Filimonova *et al*., 2016; Gonçalves *et al*., 2016).

The intensive use of pollutants in agriculture, e.g. fertilizers and pesticides, can flow by water discharges and generate an eutrophication process as described above (Cardoso *et al*., 2008; Gonçalves *et al*., 2016). Although, the value of the present pesticide concentration is still uncertain according to the scientific literature (Rodrigues *et al.*, 2018), since they derive from nonpoint sources and it becomes difficult to measure and control mixtures. Furthermore, those concentration levels can pose a risk not only for the aquatic biota but also for human health. According to this, since 1998 there are Pesticide-Monitoring programs to recover the quality of aquatic systems near ecologically valuable coastal wetlands (Galhano *et al.*, 2011). Pesticide contamination has become a problem for many years because most farmers use concentrations above those permitted by law, e.g. in the case of Portugal's surface waters and ground waters it exceeds the $0.1 \mu g L^{-1}$ EU limit in areas occupied by intensive agriculture (e.g. Mondego River Estuary) (Cruzeiro *et al*., 2016; Galhano *et al.*, 2011).

In Mondego River Estuary, the agricultural areas are mainly occupied by corn crops fields that according to agricultural cooperatives Primextra® Gold TZ is the most used herbicide in these fields, and copper sulphate is widely used in several agrochemical formulations: fungicides, herbicides, molluscides, and others (Filimonova *et al.*, 2018a; Filimonova *et al.*, 2018b). Primextra® Gold TZ acts by absorption from leafs and roots, preventing the weeds' growth and kills before weed emerging or shortly after its emergence (Gonçalves *et al.*, 2016). Primextra® Gold TZ is composed by two active ingredients (a.i.), S-Metolachlor (29%) and Terbuthylazine (17,4%) (Syngenta, 2017), plus co-adjuvants, and is a selective and systemic herbicide used to control weeds that grow up annually in corn fields, primarily grasses and *Cyperus esculentus* (Filimonova *et al.*, 2016a; Gonçalves *et al.*, 2016; Neves *et al.*, 2015). Active ingredients together with coadjuvants (supposedly inert) are present in commercial formulations and can potentiate the effects of the isolated active ingredients, so it is important to address the predictive risk assessment and access the effects of both active ingredients (Gonçalves *et al.*, 2016).

Metolachlor is part of the family of chloroacetamides, acting by inhibition of several biological processes (mainly photosynthesis) and is an important herbicide used in many commercial formulations (Neves *et al.*, 2015). It was first introduced in market with a structure containing R- and S-enantiomers (optical isomers from molecules acting as biochemical receptors), however, later formulations were composed mainly with S-isomers because of the greatest effectiveness (Liu & Xiong, 2009; Neves *et al.*, 2015). S-Metolachlor (SM), 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-[(1S)- 2-methoxy-1-methylethyl] acetamide, is classified as an inhibitor of very long chain fatty acids (VLCFAs) formation, because it inhibits the activity of elongase enzyme, responsible for the expression of FAE1 gene for VLCFAs elongation. It interferes with normal cell development and inhibits both cell division and cell enlargement (Liu & Xiong, 2009). S-Metolachlor can also affect other important pathways leading plants to oxidative stress (Neves *et al.*, 2015). According to its mode of action, S-Metolachlor is suggested to affect lipid (Fatty Acid-FA) profiles of aquatic species (Robert *et al.*, 2007).

Terbuthylazine (TBZ), N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine, is part of the group of chloro-s-triazines and acts primarily as an inhibitor of photosynthesis at photosystem II (Filimonova et al., 2016a). This a.i. besides being an important herbicide, can also act as algaecide and microbicide (Želježić *et al.*, 2018). This substance has

substituted atrazine, previously banned in EU countries and has become a concern after several studies, suggesting it has serious problems for the environment as does S-Metolachlor, with effects in the development and growth of some aquatic organisms and in the oxidative stress (Želježić *et al.*, 2018).

Copper (Cu) is one of the main constituents of pollutants used in agriculture fields, found mainly as Copper Sulphate in fungicides formulations. It belongs to transitional essential metals' group, and is important for vital functions of every organism, acting as a cofactor of many enzymes, however it can become toxic when in high concentrations (acute exposure) or at low concentrations (chronic exposure) (Filimonova *et al.*, 2016; Mesquita *et al.*, 2018). The excess of Cu affects different metabolic and biochemical processes such as photosynthesis, chlorophyll synthesis, fatty acid metabolism, carbohydrate synthesis, respiration, cell division and pigment synthesis (Filimonova *et al.*, 2016a; Ritter *et al.*, 2008). Cu is also known for its action on enzyme activity and reactive oxygen species (ROS) accumulation, by shifting the balance of free radicals in photosynthetic organisms, with consequences for photosystems (Ritter *et al*., 2008). The presence of copper in water systems may alter the FA profiles of the organisms.

2. Fatty acid and carbohydrates analysis as biomarkers of environmental contamination

Ecosystems are naturally influenced by biotic and abiotic stress factors such as food supply, climate fluctuations, radiation and ecological relationships between species (Markert *et al*., 2003). This condition is necessary for evolution, however, stressors have reach a new dimension trough human activities with the release of substances not naturally occurring in the environment (xenobiotics), many of them very harmful for all ecosystems *(*Markert *et al.*, 2003). Over the past years, the bioindicators and biomarkers approach gained more interest from toxicologists and international regulatory agencies, in the way they can be successful tools to assess, evaluate and document the effects of environmental stressors.

A bioindicator is defined as an organism (a sentinel or ecologically important species) or a community of organisms containing information relative to the quality of the environment; at the sublevel of organization measurable biological parameters, called

biomarkers (molecular and biochemical responses), can provide a rapidly response of environmental influence and the action of pollutants, in qualitative and quantitative terms (e.g. enzyme or substrate induction of cytochrome P450) (Adams & Greeley, 2000; Filimonova *et al.*,2016b; Markert *et al.*,2003). Moreover, the application of biomarkers to determine the effects of stressors in organisms' biochemical pathways are intended as early warning bio-indicators of stress (Gutiérrez *et al*., 2019a; Gutiérrez *et al.*, 2019b; Mesquita *et al.*, 2018)

Nutrients are essential for human and animal life, being the most important macromolecules of cells, they make up more than 99% of the total living cells as organic and inorganic forms within four general classes of macromolecules: nucleic acids, proteins, polysaccharides and lipids (Engelking, 2015).

All aquatic organisms need nutrients for their metabolism activities and vital functions, either by food (primary and secondary consumers) or by their own metabolism (primary producers). The essential nutrients (e.g. Essential Fatty Acids-EFAs) have proven to be useful trophic markers (Kelly & Scheibling, 2012), and more studies are needed to clarify the useful of such biochemical markers in ecotoxicological studies to evaluate chemical risk assessment.

2.1. Lipids

The definition of lipids is based on the solubility properties and their structure, once they are defined as apolar compounds, insoluble in water. Additionally, lipids are fatty acids and their derivates or substances related to them in terms of function or synthesis (Fuchs *et al*., 2011; Gurr *et al.*, 2002). The term "Lipid" means "fat", in Greek word *lipos*, yet they can be a fat molecule with that grass texture or being an oil, called triglycerides (e.g. fatty acid esters) (Engelking, 2015; Gurr *et al*., 2002).

Lipids are very important at several metabolism functions and cell's integrity, as a source of energy (triglycerides) and for production and permeability of cell membranes (cholesterol, glycolipids and phospholipids), influencing the traffic of cell's compounds and the activity of membrane proteins, as precursors to other biomolecules (fatty acids) and gene transcription factors, insolation barriers (neutral fat stores), prevention of infections, and in

forms of vitamins (A, D, E and K) and hormones (Engelking, 2015; Mesquita *et al.*, 2018; Neves *et al.*, 2015). They also play a significant role in phagocytosis by segregation of signalling microdomains for generation of second messengers and remodelling the actin cytoskeleton and directing membrane traffic (Yeung & Grinstein, 2007).

Lipids are classified into several main classes as saturated (SFA) and unsaturated (UFA) fatty acids, triglycerides, lipoproteins (i.e. chylomicrons (CMs), very low density (VLDL), low density (LDL), intermediate density (IDL), and high density lipoproteins (HDL)), phospholipids and glycolipids, steroids (e.g. cholesterol) and eicosanoids (Engelking, 2015).

Fatty acids (FA) are naturally occurring in nature, with multiple structures on a range of 8 to over 80 carbon atoms, present in all organisms (Kenar *et al.*, 2017) and are mostly found in a bound state as lipid components (Bielawska *et al.*, 2010).

The principal structure of FA varies according to the numbers of carbon atoms and functional groups, with carbon-carbon double bonds resulting on a *cis*- or a *trans*- geometry (Figure 1). Once they have such diversity, they are the main components of food and may have several industrial applications on feedstocks, but also in chemical industry in soaps, detergents, coatings, cosmetics and others (Kenar *et al.*, 2017). UFA can have isomers either positional or geometrically. It is called positional isomers when there are different positions for double bonds in the carbon chain, for example, a C16:0 monounsaturated fatty acid may have positional isomeric forms with double bonds at C7 or C9. Geometric isomerism is the capability to configuration the double bond to *cis* or *trans* forms: the *cis* form refers to the same side of the two hydrogen substituents with the molecule, while in opposite sides it has a *trans* form (Figure 1) (Gurr *et al.*, 2002).

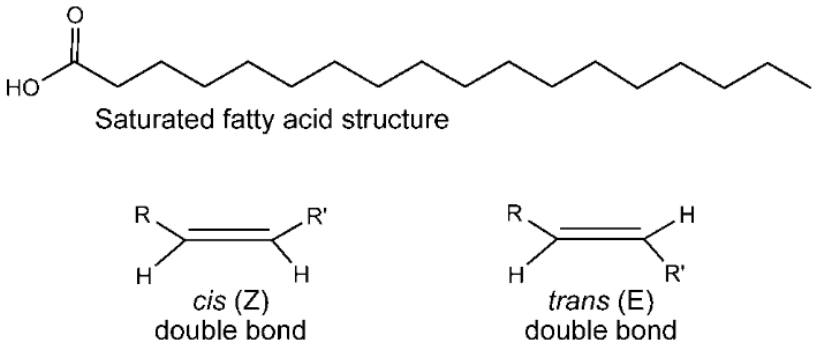

Figure 1. (a) General structure of a saturated fatty acid; (b) functional groups with the *cis*- and *trans*-geometry (Adapted from Kenar et al., 2017).

A profile of FAs contains SFA, those without double bonds, and UFA, with one or more double bonds in the molecular structure. According to the unsaturation level, UFA are divided into monounsaturated FAs (MUFA) with single double bond and polyunsaturated FAs (PUFA) with two or more double bonds. Among PUFAs, there are highly unsaturated FAs (HUFA), which are also termed as Essential FAs (EFA) as well, since most of them cannot be synthesized *de novo* in animals (Filimonova *et al.*, 2016a).

UFA are very vulnerable to the action of xenobiotics and their oxidizing agents (reactive oxygen and nitrogen species), and transitional metals (iron and copper) whose action evolves lipid peroxidation (Bielawska *et al.*, 2010). As a consequence, peroxidation products are susceptible to alter physical and biological properties of cell's membrane, including depolarization, proteins transport and membrane enzymes' activity (Dutta-Roy, 2000).

The profiles and distribution of FA within fats and oils obtained from plants and animals are influenced by their source and raise conditions (Kenar *et al.*, 2017), so the extraction method depends on the type of tissue and the type of lipids desired. Lipids occur in nature as triglycerides within plants and animals, and to recover the desired FA, they have to undergo several processes, including hydrolysis to separate the fatty acids from glycerol (Kenar *et al.*, 2017).

Recently there have been an improvement on the numerous analytical techniques for FA quantification, and the most used nowadays include: thin-layer chromatography (TLC),

electrophoresis, liquid chromatography (LC), high-performance liquid chromatography (HPLC) and gas chromatography (GC), coupled frequently to detectors: flame ionization, spectrophotometric, mass spectrometry (MS) and nuclear magnetic resonance (Fuchs *et al.*, 2011; Wei & Zeng, 2011).

FA are, in most cases, transferred from primary producers to higher trophic levels without change, thus, FA analysis is a well-established tool for studying trophic interactions in aquatic habitats, make them good trophic-markers and biochemical markers (Filimonova *et al.*, 2016a; Kelly & Scheibling, 2012).

2.2. Carbohydrates

Carbohydrates are macronutrients only composed by carbon, hydrogen and oxygen and are known as polyhydroxy aldehydes, ketones, alcohols, acids, or their simple derivates and polymers linked to an acetal type; and they are divided into three main groups, according to the chemical structures: monosaccharides, oligosaccharides and polysaccharides (FAO, 1998). Their classification is based on their molecular size (degree of polymerization-DP), their linkage (α or non-α) and their individual monomers' character (Cummings & Stephen, 2007). Simple Sugars include the Monosaccharides and the disaccharides, composed by two molecules of monosaccharides; Oligosaccharides comprise malto-oligosaccharides and others; and Polysaccharides include starch and non-starch polysaccharides (Table 1). Monosaccharides liking together form a great number of disaccharides, oligosaccharides and polysaccharides.

Polysaccharides are also known as complex carbohydrates by some nutritionists, and they include the starch, the principal carbohydrates in most diets, consisting only by glucose (Fig. 2) molecules. On the other hand, the major components of non-starch polysaccharides from plant cell wall include cellulose, hemicellulose and pectin and others like gums, mucilages and hydrocolloids, with a large number of monosaccharides (glycose) biding together by glycosidic linkages (Cummings & Stephen, 2007).

Table 1: Main groups of Carbohydrates and their respective subgroups and components' examples (Cummings & Stephen, 2007; FAO, 1998). DP- Degree of Polymerization or number of single sugar.

Class (DP)	Sub-group	Components
Simple Sugars (1-2)	Monosaccharides	Glucose, galactose, fructose
	Disaccharides	Sucrose, lactose, maltose, trehalose
Oligosaccharides (3-9)	Malto-oligosaccharides (α-glucans)	Maltodextrins
	Other oligosaccharides (non-α glucans)	Raffinose, stachyose, fructo and galacto oligosaccharides, polydextrose, inulin
Polysaccharides (>9)	Starch (α-glucans) and other polysaccharides	Amylose, amylopectin, modified starches,cellulose, hemicellulose, pectins, hydrocolloids, arabinoxylans, β-glucan, glucomannans, plant gums and mucilages

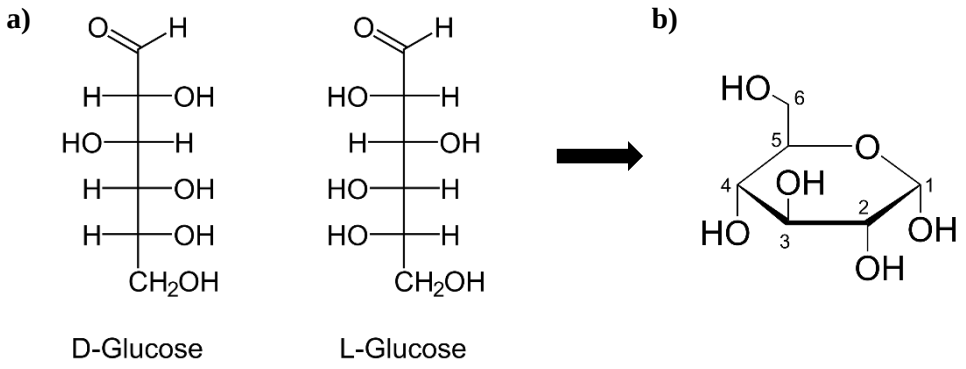

Figure 2: The monosaccharide Glucose in the two stereoisomers forms (D-glucose and L-Glucose) (a), and in its cyclic form (b).

Plants can produce several starch α-glucans that are present at cytoplasm as insoluble granules, acting as an energy storage reserve (Stick, 2008). These granules are composed by a linear polymer (amylose) with a great number of α -1,4-linked D-glucose residues, and a highly branched polymer (amylopectin) with a-1,4 and a-1,6 D-glucose residues linkages (Cummings & Stephen, 2007).

Carbohydrates, in general, are of great interest by pharmaceutical and food industries because of their natural structures and high energy values. Sugars are naturally abundant in

honey and fruits for diet. Food industries also produce processed sugars for other proposes. Polysaccharides and their derivatives are also extensively used as gelling agents, stabilizers, thickeners and disintegrators (Hu & Goff, 2018).

The analysis to determine and quantify free sugars and polysaccharides can be analysed by different techniques that include: i) Exclusion Chromatography (SEC) where molecular weight and respective polymer distribution can be measured (Hu & Goff, 2018); ii) Fourier Transform Infrared Spectroscopy (FTIR) to evaluate the degree of esterification of the polysaccharides (Coimbra *et al.*, 1996); iii) fractionation of polysaccharides by Chromatography, ultrafiltration and gradient non-solvent precipitation (based on differential solubility) (Hu & Goff, 2018) and iv) Gas Chromatography (GC) coupled to a detector (e.g. GC-FID).

3. *Lemna* - Biological characterization

Primary producers are of high importance in ecotoxicological studies due to their key position in trophic food chains, making a link with the higher trophic levels. Duckweeds belong to *Lemnaceae* family of monocotyledons, as primary producers of aquatic ecosystems and sometimes mistaken with algae, making a controversy between some taxonomists (Hasan & Chakrabarti, 2009). This family consists of four genera: *Lemna*, *Spirodela*, *Wolffia* and *Wolffiella*. *Lemna* represents the largest genera, but with a complexity group of very similar species. Duckweeds are easy to find in surface waters, since they are floating macrophytes and are widely distributed in almost every region worldwide, with exception of waterless deserts and polar regions (Skillicorn *et al.*, 1993). Additionally, they are abundant in the tropical and subtropical countries (Chakrabarti *et al.*, 2018), with a great performance of growth in warm temperature and sunny days (Skillicorn *et al.*, 1993), although some species of *Lemna* (e.g. *L. minor* and *L. gibba*) can still grow in cold temperatures (Hasan & Chakrabarti, 2009).

Lemna sp. reproduce by vegetative propagation, multiplying their fronds from 1 to 20 daughter fronds during its lifetime, with an intermediate size between 6 to 8 mm, for about 10 days or several weeks, depending on the species (Skillicorn *et al.*, 1993).There are some environmental requirements necessary for a healthy growth, such as a great quality of

water with a temperature between 17.5º and 30º (although sometimes they show a relative growth in lower temperatures), a pH tolerance ranging from 5 to 9, light intensity and water nutrients richness (Nitrogen, Phosphorous, Potassium, and others). To *L. minor* and *L. gibba* the electrolyte conductivity from 400-500 µS/cm apparently have some effect in their growth rate (Hasan & Chakrabarti, 2009).

Duckweeds are part of several aquatic species diet, such as herbivorous fish (e.g. grass carp), and recently they have been produced in a large scale by industries for feed production for aquaculture, since they have high nutritional values (including essential fatty acids) and an easy production, (Hasan & Chakrabarti, 2009). Such nutritional values account with higher contents of fibre, protein (about 12%), lipids (from 1.8 to 9.2%) and carbohydrates (about 51.2%) involved in biomass growth, when in good conditions (Hasan & Chakrabarti, 2009; Zhao *et al.*, 2014). Moreover, growth depends on carbon assimilation by photosynthesis, whereas sugars (starch) are synthetized and stored in vacuoles during the day and by night they are used for plant growth, thus varying with photoperiod *(Pagliuso et al.*, 2018).

Lemna sp. contain polysaccharides, phenolic compounds and proteins in their cell walls, with hemicelluloses (xyloglucan, arabinoxylans, mannans, β-glucans and others) attached to cellulose, submerged in a matrix containing also homogalacturonans, rhamnogalacturonans and other polysaccharides (Carpita & Gibeaut, 1993). Fatty acid profiles reveal high proportions of UFA, representing 72.6% of Fatty Acid Methyl Esters (FAMEs), including EFA (linoleic and α-linolenic acid, C18:2n6 and C18:3n3 respectively) (Zhao *et al.*, 2014).

Disturbances in *Lemna* growth rate can be related to toxic exposures, which highlights this group as good bioindicator for ecotoxicological bioassays, and since they absorb contaminants they are also good candidates for water remediation (Panfili *et al.*, 2019).

4. The main issue and objectives

Human population has increased overtime, exceeding 7.60 billion people nowadays, with a tendency to extend even more (Countrymeters, 2019). With the exponential world population growth, it has become a challenge to gather the nutritional needs of the population, both quantity and quality requirements, as we are facing the problem of aquatic pollution. To overcome such problem, it is necessary to know how pollution affects the aquatic systems and the aquatic communities and thus, the food quality along the trophic food web, and look at the effect on nutritional contents. According to this, we can project further conclusions on how to control food requirements and how to restrict chemicals formulations or replace them with biodegradable products.

In this work two non-target organisms from freshwater were carefully selected: *Lemna minor* and *Lemna gibba*. Both are macrophytes and have been extensively studied in ecotoxicological studies, being the subject of the standards and easy to work with. The two species were selected also because of the possible genetic variability in the response to stress pollutants (OECD, 2006), whereas there are insufficient data on this subject.

The accumulation of herbicides in the aquatic ecosystem poses a stress condition to non-target species, such as Duckweeds, and its subsequent propagation through the trophic chain is one of the major environmental concerns about herbicide contamination and bioconcentration. Moreover, chemicals are mostly present in the environment as mixtures, whereby individual effects must be determined to further studies on combined effects.

Less information is found in literature about the toxicity and biochemical effects of the two active ingredients of the herbicide Primextra® Gold TZ and copper in freshwater primary producers, particularly on *Lemna* spp. In this context, we hypothesized that herbicides are toxic to *Lemna* sp. and affect their biochemical components. This study aims to 1) assess the single toxicity effect of the three compounds (Terbuthylazine, S-Metolachlor and Copper) in two freshwater macrophytes (*Lemna minor* and *Lemna gibba*), and 2) determine potential changes on the fatty acids' and carbohydrates' profiles of both species, and the consequences for the trophic chain after stress chemical exposure. This work thus provides an integrated approach towards a more realistic assessment on the overall impacts of S-Metolachlor, Terbuthylazine and Copper on sensitive bioindicators.

Materials and Methods

1. Chemicals and test solutions

Terbuthylazine N^2-*tert*-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine, S-Metholaclor (2-Chloro-N-(2-ethyl-6-methylphenyl)-N-[(1S)-2-methoxy-1-methylethyl] acetamide) and Copper sulphate pentahydrate ($CuSO_4.5H_2O$) were obtained from Sigma-Aldrich (Germany) and from these products, stock solutions were prepared in our laboratory before each assay.

The stock solution containing TBZ had to be prepared 2 or 3 days before each assay to dissolve completely the powder in the test medium. The stock solutions containing SM and Cu were prepared a few hours before each assay. The dissolution of the active ingredients conducted only with a stirrer.

2. Test organisms

Cultures of macrophytes *Lemna minor* and *Lemna gibba* (Figure 2 (a) and (b), respectively) were maintained in Steinberg medium for both inhibition (toxicity) and biochemical tests (OECD, 2006), at 20 °C with a photoperiod of 16 h^L:8 h^D, with renewal scheduled once a week.

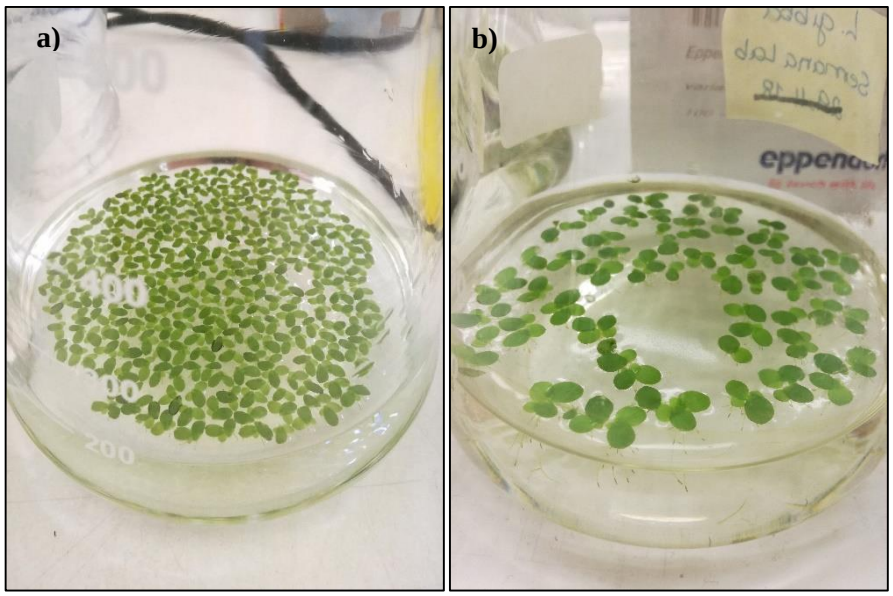

Figure 2: Cultures of *Lemna minor* (a) and *Lemna gibba* (b).

3. Toxicity bioassays

Growth inhibition tests with *Lemna minor* and *Lemna gibba* were produced according to OECD guideline 221 (OECD, 2006). According to the principle of the test, *Lemna* cultures grow as monoculture with different concentrations of the test solution in a period of seven days.

The two macrophytes species were exposed to distinct range of concentrations of each compound, since after preliminary experiments was found no statistical significance associated with some concentrations. It was adjusted the range of concentrations for each bioassay. *L. minor* was exposed to a range of i) TBZ from 21.8 to 1500 µg/L (dilution factor (DF)= 1.6), ii) SM from 2.5 to 2 µg/L (DF=2.1), and iii) Cu from 5.7 to 674.6 µg/L (DF=1.46). *L. gibba* was exposed to a range of i) TBZ from 10.6 to 300 µg/L (DF=1.45), ii) SM from 1.3 to 5000 µg/L (DF=2.5) and iii) Cu from 21.9 to 1502 µg/L (DF= 1.6).

Tests were carried out in disposable 6-well microplates and incubated in a chamber at 23 ± 1°C, under continuous light, with 3 replicates per chemical treatment and 6 replicates for the control treatment (blank Steinberg medium). Each replicate well was inoculated with 3 healthy colonies of 2 fronds of *L. gibba* (Figure 3) and 3 fronds of *L. minor* at the beginning of the test. Six extra replicates were collected from the culture for determination of the average fresh and dry weight, at the beginning of the test. At the end of the test, the fronds number and fresh weight of each replicate was registered and then dried at 60ºC during more than 3 days. After that, the dried weight was registered for final dry weight records. *Lemna* biomass yield for each individual treatment was calculated on the basis of either frond number and fresh weight, as the difference between records at the end and at the beginning of the test.

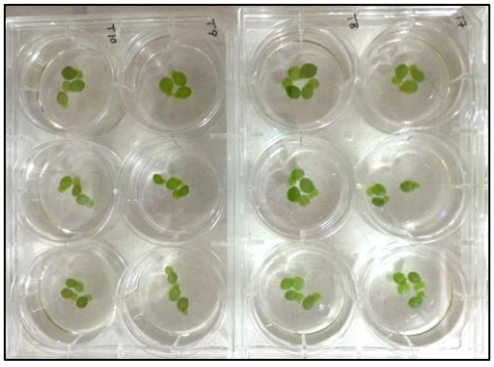

Figure 3: Replicates of *Lemna gibba* toxicity bioassay in microplates at the beginning of the test.

4. Biochemical analysis

For biochemical analysis of FA and carbohydrates profiles, tests were performed based on the concentrations estimated for EC_{10}, EC_{20}, after the toxicity data analysis. Tests were conducted similarly to the growth inhibition tests. The single exception was the use of 200ml erlenmeyers for each 6 replicates of three test solutions (C1, C2 and C3) and control, with a total of 10 ml of test solution in each Erlenmeyer (Figure 4). At the beginning of the test, each replicate was inoculated with 9 groups of 2 healthy fronds for *Lemna gibba* bioassay and 9 groups of 3 healthy fronds for *Lemna minor* experiment. The incubation conditions were the same as growth inhibition tests. After a period of 7 days, the frond number was counted, then stored in Eppendorfs and fresh weight was registered for biomass records. At the beginning of the test, three replicates of the control fronds were registered in fresh weigh as well, to evaluate the differences in biomass weight. At the end all samples were stored at -80ºC until further biochemical analysis.

The following steps were performed through protocols established from our laboratories and are briefly resumed bellow.

Figure 4: Biochemical bioassay in the incubation chamber during the period of the biochemical experiment.

4.1. FA Extraction and methylation protocol to FAMEs

For total lipids analysis, samples were first extracted and methylated to fatty acid methyl esters (FAMEs) by modified one-step derivatisation method of Abdulkadir & Tsuchiya (2008), by replaced boron trifluoride-methanol (BF_3-methanol) reagent to 2.5% H_2SO_4-methanol solution. BF3-methanol was replaced since it can cause artefacts or loss of PUFAs (Eder, 1995). In this step, the internal standard methyl nonadecanoate (C19:0) was added with a concentration of 1 mg/ml of n-hexane on our samples allowed to a later quantification (Fluka 74208). Then, the tubes were shaken 3 times with a vortex and centrifuged (Eppendorf Centrifuge 5810R) for 20 min at 2000 rpm. From the two phases formed, the upper layer containing the FAMEs was stored to FA analysis. The lower layer was also transferred (between 200 to 800 µl) to clean sample vials using a pipette to be later prepared for free sugars analysis. The final data was corrected according to the volumes collected in each step and the internal standard added.

Separation and quantification of FAMEs were performed on an Agilent GC-MS (Agilent Technologies 6890 Network GC System (Santa Clara, CA), with an Agilent 5973 Inert Mass Selective Detector, equipped with a DB-FFAP column with 30m length, 0.25mm internal diameter and 0.1 µm film thickness using helium as carrier gas. The detector was used to scan the *m/z* range of 40-500 in 1 s cycle at 70 eV electron impact mode and in full scan mode acquisition. The oven temperature was 80 ºC at the beginning, turning to 160ºC, increasing 25 ºC min^{-1}, then reaching 190ºC at 2 ºC min^{-1}, and finally the maximum temperature of 230ºC increasing from 40 ºC min^{-1}. The GC-MS had Helium as carrier gas with a flow rate of 4.4 ml min^{-1} and a column head pressure of 2.66 psi. The injector and transfer line were maintained at 220 ºC and 280 ºC, respectively. We injected 0.6 µl of each sample, by manual sampling, and FAMEs' profiles were given by the equipment's software as chromatograms with peaks at each retention time. FAMEs were identified individually in the software and data were collected, calculated according to Gonçalves et al. (2012) and then statistically analysed.

4.2. Free Sugars and polysaccharides preparation and analysis

All sugar analyses were performed to determine the monosaccharides' composition of each sample. Free sugars and Polysaccharides were prepared based on Coimbra and co-workers (1996) and determined by gas chromatography coupled to a flame ionization detector (GC-FID) as alditol acetates. In a first step, a portion of the lower layer of the vials was to polysaccharides released by hydrolysis performed with 1M H_2SO_4 at 100ºC for 2.5h, and other portion was to Free Sugars (FS) preparation, which does not include the hydrolysis part, only the following steps are common for both Free Sugars and polysaccharides. The acid hydrolysate was cooled and an internal standard 2-deoxy-D-glucose (1 mg/ml- Sigma-Aldrich) was added. A neutralization with 25% NH_3 was conducted and then carbohydrates were reduced with 15% $NaBH_4$ in 3 M NH_3 during 1h at 30ºC. Finally, the reaction ends with the addition of glacial acetic acid to eliminate the excess of borohydride anions ($BH4^-$).

Acetylation started with 0.3 ml of the reduced solution with 1-methylimidazole and acetic anhydride for 30 min at 30°C. The solution containing the acetylated sugars is then washed three times with water and dichloromethane and a fourth time only with water. The final solution was evaporated and vacuum dried.

The alditol acetates evaporated were dissolved in anhydrous acetone before the analysis by Perkin-Elmer - Clarus 400 gas chromatography equipment with an Auto sampler, coupled with a flame ionization detector (GC-FID). The GC-FID was equipped with a DB-225 GC column with 30 m length, 0.25mm internal diameter and 0.15 µm of film thickness. The initial oven temperature was 200ºC, followed a linear increase of 40ºC min^{-1} until 220ºC, where it stained for 7 min, and finally an increase with a rate of 20ºC min^{-1} until final temperature of 230ºC maintaining this temperature for 1 min. The flow rate of Hydrogen as carried gas was set at 1.7 mL/min. Sugars' quantification was performed by comparison of each sugar chromatographic peak to the peak obtained for the standard used.

5. Statistical analysis

Data collected as Yield (frond number) and fresh weight were used to calculate the average specific growth rate as the logarithmic increase in growth variable (in this case our variable was fresh weight) according to the guidelines (OECD, 2006):

$$\mu_{j-i} = \frac{ln(Nj) - ln(Ni)}{t}$$

where μ_{j-i} represents the average specific growth rate from day 1(j) until day 7(i), *N* is the variable measured at both days, and *t* is the time period of the test.

The Effective concentrations (EC_{10}, EC_{20} and EC_{50}) were determined as standard ecotoxicological benchmarks useful for environmental risk assessment purposes (EFSA, 2013), by a non-linear regression, using the least squares method to fit the data from Yield (frond number) to the logistic equation in IBM SPSS statistics 22 software. A one-way ANOVA was also applied followed the post-hoc Dunnet's test ($p>0.05$) to determine the significant differences between the treatments and the Control for toxicity data and biochemical profiles.

Fatty acids and sugars profiles were examined by multivariate statistical analyses carried out using PRIMER-6 software (Clarke & Gorley, 2006). In a first step, a pre-treatment with a transformation to Logarithm (X+1) was done to all data. The variation in FA composition was taken through non-metric multidimensional scaling (n-MDS) plots and hierarchical clustering based on group average distance linkage, from Bray-Curtis resemblance measures, by converting data to similarity triangular matrices (Clarke & Warwick, 2001). Additionally, a One-way analysis of similarity (ANOSIM) was performed to test differences in fatty acids or sugars profiles for each species and substance. Finally, a Similarity Percentage analysis routine (SIMPER) was running to see the contribution of individual FA and Sugars to similarities and dissimilarities within and between groups.

Results

1. Toxicity Bioassays

The growth rate of both macrophytes species declined after the exposure of all toxicants (Figures 5 and 6). One-way ANOVA followed by the multiple comparison post-hoc Dunnet's test showed almost all treatments of *Lemna gibba* and *Lemna minor* exposed to Terbuthylazine (TBZ) were significantly different from the control (Figure 6A and 5A, respectively). For S-Metolachlor (SM) exposures the significant differences from the control for both species (Figures 5B and 6B) occurred after the middle values of concentrations; and after the exposure to Copper (Cu) also half of the treatments were significantly different for *Lemna minor* (Figure 5C), and for *Lemna gibba* (Figure 6C).

The Effective Concentration (EC) values determined for both species showed *L. minor* to be more sensitive than *L. gibba* to SM ($EC50_{Lm}$ = 43.10 µg/L (30.89-55.30) and $EC50_{Lg}$ 86.81 µg/L (40.47-133.12), respectively), and to Cu ($EC50_{Lm}$ =199.20 µg/L (149.28-249.13) and $EC50_{Lg}$ =504.31 µg/L (308.73- 699.90), respectively) (Table 2). On the other hand, TBZ revealed to be more toxic to *Lemna gibba* rather than *Lemna minor* ($EC50_{Lg}$=43.89 µg/L (38.63- 49.15); $EC50_{Lm}$=93.43 µg/L (75.35-111.51), respectively).

Table 2: Effective concentrations (EC_{10}, EC_{20} and EC_{50}) in µg/L with respective 95% confidence limits (in brackets). Effective concentrations were obtained from a non-linear regression using specific functions for EC_{10}, EC_{20} and EC_{50}.

Species	Terbuthylazine (µg/L)	S-Metolachlor (µg/L)	Copper (µg/L)
Lemna minor	EC_{10} =16.30 (9.17-23.43) EC_{20} =31.05 (20.82-41.27) EC_{50} =93.43 (75.35-111.51)	EC_{10} = 2.49 (0.94-4.04) EC_{20} = 6.44 (3.31-9.58) EC_{50} = 43.10 (30.89-55.30)	EC_{10} = 29.28 (10.25-49.32) EC_{20} = 59.42 (31.53-87.30) EC_{50} = 199.20 (149.28-249.13)
Lemna gibba	EC_{10} = 14.40 (10.47-18.33) EC_{20} = 21.73 (17.27-26.18) EC_{50} = 43.89 (38.63- 49.15)	EC_{10} = 1.82 (-0.45-4.08) EC_{20} = 7.57 (0.50-14.64) EC_{50} = 86.81 (40.47-133.12)	EC_{10} = 51.78 (-2.23- 105.79) EC_{20} =119.95 (30.35- 209.55) EC_{50} =504.31 (308.73- 699.90)

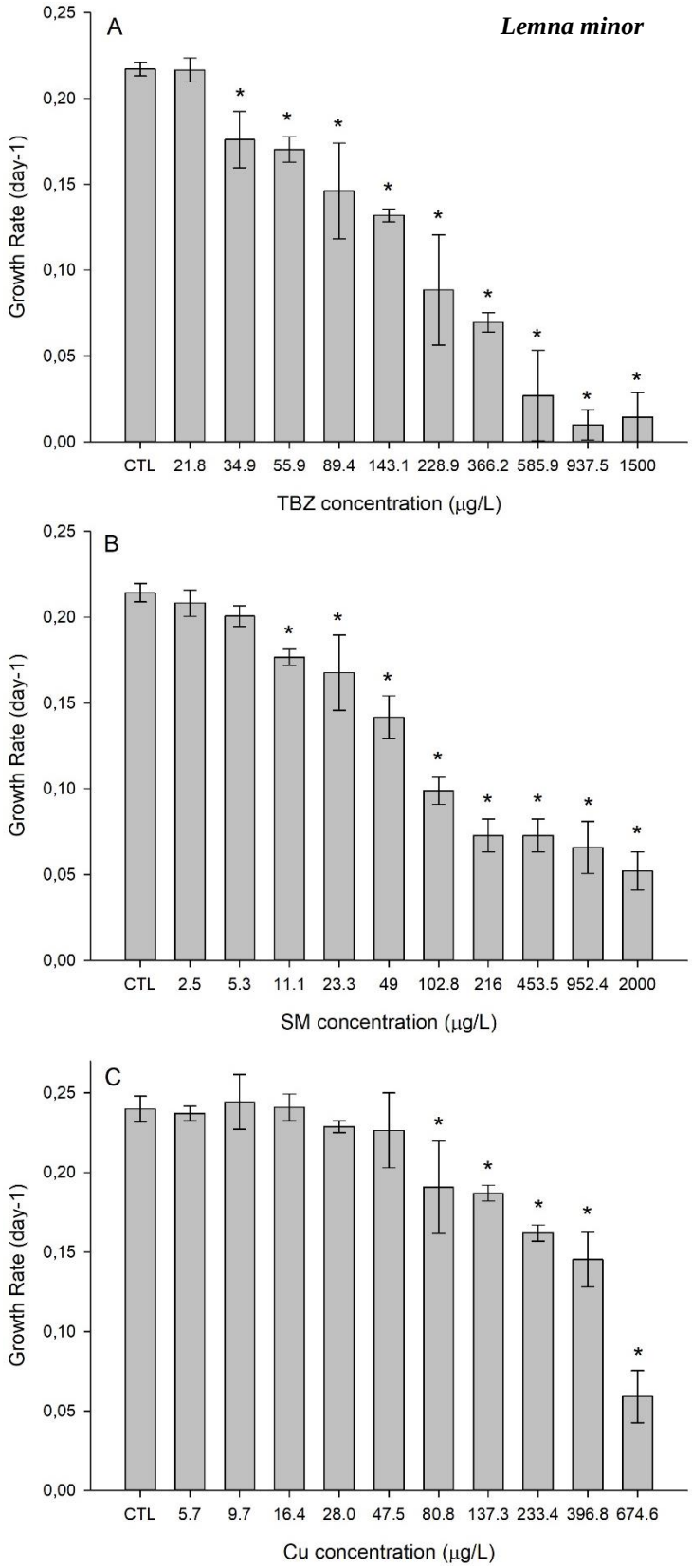

Figure 5: Growth Rate *per* day of *L. minor* after 7-day exposure to a) Terbuthylazine-TBZ, b) S-Metolachlor-SM and c) Copper-Cu treatments, where CTL is the negative control treatment. ('*' indicates a significant difference of the treatments to the control at $p < 0.05$)

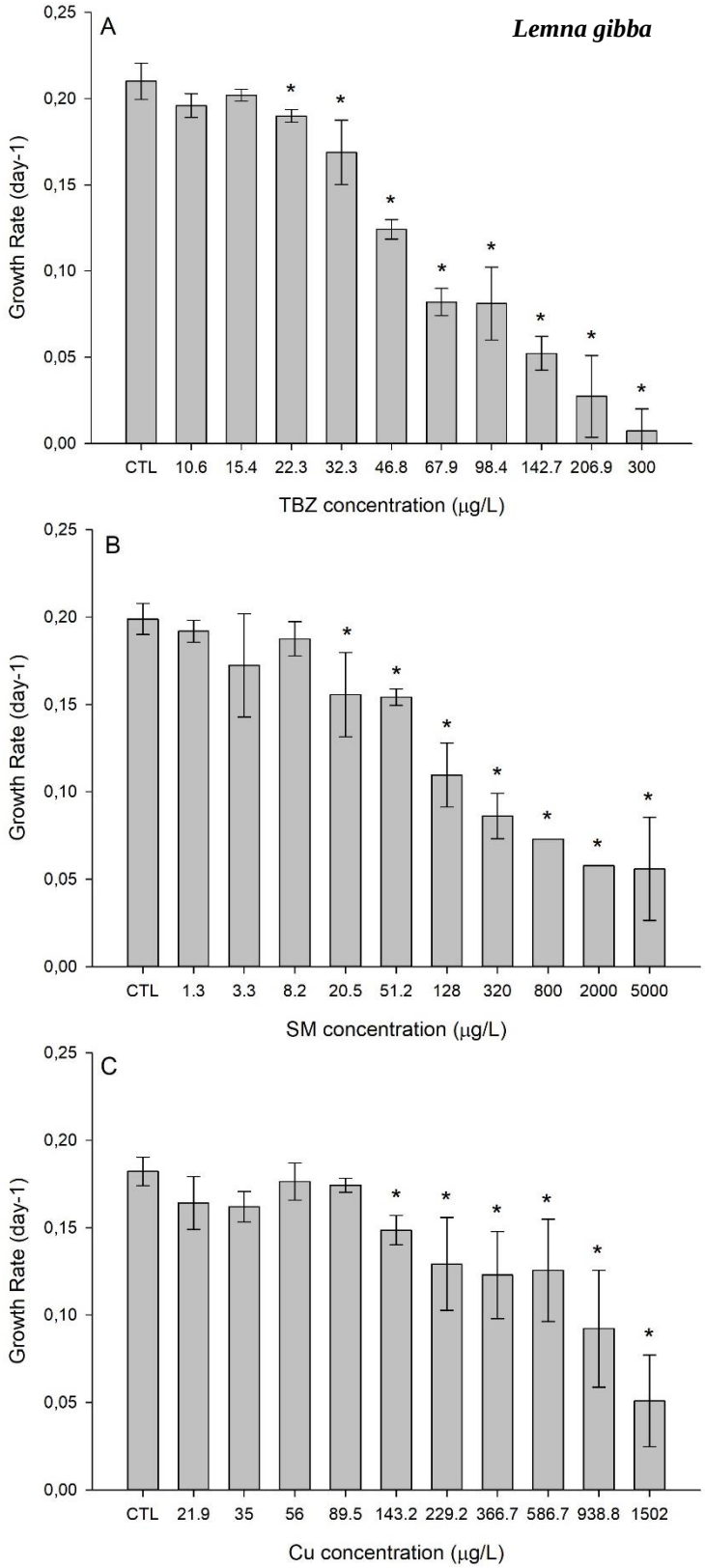

Figure 6: Growth Rate *per* day of *L. gibba* after 7-day exposure to a) Terbuthylazine-TBZ, b) S-Metolachlor-SM and c) Copper-Cu treatments, where CTL is the negative control treatment. ('*' indicates a significant difference of the treatments to the control at $p < 0.05$)

2. Fatty acids' profiles

The fatty acid compositions (mg/g of biomass) of *Lemna minor* and *Lemna gibba* is summarized in Table 3. The FA diversity was the same for both *Lemna* species in all exposed treatments and in control. However, in terms of quantifications, the smaller species *L. minor* revealed higher FA contents than *L. gibba*. We also found two new PUFAs, C16:2n3 and C16:3n3, not found in literature for these species. The quantification of these PUFA was calculated with a Retention Factor (RF) of 0.000000130, based on RF value for C16:0 (RF=0.000000135). After TBZ exposure, the FA composition of *L. gibba* decreased compared with control according to total quantifications of SFA, MUFA, but PUFA increased. Yet no statistically differences were found between the treatments and control by the one-way ANOVA followed the post-hoc Dunnet's test at $p<0.05$. After S-Metolachlor treatment, *L. gibba* decreased all FA contents, specially the PUFA's, but no significant differences were found as well ($p<0.05$). The exposure with Cu only decreased the PUFA group, and slightly increased the SFA and MUFA, although there was no statistically difference between treatments and control ($p<0.05$). For *L. minor* the exposure to TBZ showed a great decrease of FA contents, although there was no statistically differences with control. After SM exposure, *L. minor* decreased drastically the SFA content with C20:0 statistically different from control at 2µg/L ($p<0.05$). The Cu exposure also decreased the FA contents with C16:0, C18:1n9 and C18:2n6 statistically different from control at 255.3µg/L.

In general, PUFA (C16:2n3, C16:3n3, C18:2n6, C18:3n3 and C18:3n6) was the dominant group in both profiles, followed by SFA and MUFA. PUFA represent the essential omegas -3 and -6 present in both macrophytes and are important for all organisms' diet. The most affected group was PUFA in the situations of *L. minor* exposed to the organic compounds and *L. gibba* exposed to SM and Cu.

Table 3: Abundance of fatty acids (saturated fatty acids-SFA; monounsaturated fatty acids-MUFA and polyunsaturated fatty acids-PUFA; in mg/g of biomass) in the profiles of *L. minor* and *L. gibba* after exposure of increase concentrations (CTL, C1, C2 and C3) of Terbuthylazine (TBZ), S-Metolachlor (SM) and Copper (Cu).

Lemna minor

		TBZ								**SM**							
		CTL	**±SE**	**9.0 µg/L**	**±SE**	**16 µg/L**	**±SE**	**31 µg/L**	**±SE**	**CTL**	**±SE**	**2 µg/L**	**±SE**	**3 µg/L**	**±SE**	**6.5 µg/L**	**±SE**
SFA	**C12:0**	0.43	0.19	6.29	5.07	0.21	0.06	0.16	0.08	37.79	37.64	0.16	0.01	0.14	0.03	0.17	0.03
	C14:0	3.47	0.71	6.09	4.37	1.85	0.62	1.27	0.34	23.57	21.69	1.99	0.24	1.53	0.20	1.57	0.25
	C15:0	0.54	0.15	1.79	1.33	0.42	0.14	0.27	0.08	0.61	0.18	0.66	0.08	0.46	0.07	0.60	0.16
	C16:0	48.78	11.13	43.66	11.13	46.31	16.88	27.05	6.92	76.94	30.30	63.65	5.88	43.32	4.75	51.17	4.11
	C18:0	3.03	0.81	5.88	2.94	5.03	2.61	2.27	0.92	6.04	3.85	2.89	0.14	2.04	0.72	3.29	0.69
	C20:0	0.88	0.19	1.44	0.88	0.53	0.16	0.45	0.13	0.55	0.05	0.80	0.10	0.40	0.01	0.49	0.04
	C22:0	1.87	0.67	2.40	1.26	0.60	0.39	0.97	0.71	1.22	0.03	0.97	0.15	0.75	0.09	1.35	0.25
	C24:0	3.55	1.63	5.64	2.31	2.77	1.48	1.96	0.71	3.05	1.55	4.71	0.45	3.68	0.76	4.36	0.78
	Total SFA	**62.55**	**10.17**	**73.19**	**21.18**	**57.72**	**20.83**	**34.40**	**9.90**	**149.78**	**92.19**	**75.84**	**5.86**	**52.32**	**6.42**	**63.00**	**6.25**
MUFA	**C16:1n9**	2.00	0.50	4.41	2.94	1.61	0.54	0.84	0.23	7.51	6.05	1.84	0.20	1.41	0.17	1.65	0.03
	C18:1n9	3.52	0.39	2.94	0.61	2.78	0.96	1.82	0.51	4.24	0.95	3.83	0.63	2.94	0.38	3.19	0.48
	Total MUFA	**5.52**	**0.87**	**7.35**	**3.06**	**4.39**	**1.49**	**2.66**	**0.73**	**11.76**	**6.97**	**5.67**	**0.81**	**4.35**	**0.55**	**4.84**	**0.49**
PUFA	**C16:2n3**	0.73	0.20	2.29	0.94	0.93	0.20	1.55	0.52	3.40	2.52	1.23	0.32	0.80	0.05	0.90	0.17
	C16:3n3	2.01	0.40	1.87	0.59	1.64	0.77	0.97	0.48	1.69	0.53	3.14	0.58	2.17	0.16	2.69	0.50
	C18:2n6	25.55	2.61	24.54	5.82	23.88	7.97	19.14	8.00	30.75	4.96	35.24	2.61	24.41	2.72	26.73	4.55
	C18:3n3	117.21	12.06	109.84	25.65	116.98	40.14	73.19	18.22	146.84	42.55	143.91	4.49	103.34	15.84	118.01	14.64
	C18:3n6	0.26	0.26	0.248	0.25	0.22	0.18			1.37	0.26	1.11	0.58	0.99	0.06	1.03	0.19
	Total PUFA	**145.76**	**15.26**	**138.79**	**31.59**	**143.66**	**48.76**	**94.85**	**26.17**	**184.05**	**49.55**	**184.63**	**7.26**	**131.72**	**18.56**	**149.37**	**19.70**
N		**15**		**15**		**15**		**14**		**15**		**15**		**15**		**15**	

Lemna minor

		Cu							
		CTL	**±SE**	**6 µg/L**	**±SE**	**30 µg/L**	**±SE**	**65 µg/L**	**±SE**
SFA	**C12:0**	1.18	1.06	0.15	0.01	0.14	0.02	0.15	0.02
	C14:0	1.14	0.25	0.98	0.16	0.78	0.06	0.72	0.06
	C15:0	0.36	0.01	0.37	0.05	0.34	0.02	0.32	0.04
	C16:0	33.23	0.16	33.03	4.10	26.88	0.47	22.44	0.97
	C18:0	2.17	0.21	2.14	0.33	1.76	0.24	1.93	0.14
	C20:0	0.36	0.01	0.51	0.06	0.35	0.11	0.36	0.01
	C22:0	0.31	0.07	0.48	0.10	0.37	0.05	0.40	0.32
	C24:0	2.27	0.52	2.54	0.67	1.69	0.34	0.91	0.24
	Total SFA	**41.01**	**0.84**	**40.19**	**4.72**	**32.30**	**0.95**	**27.23**	**1.15**
MUFA	**C16:1n9**	0.76	0.01	0.78	0.10	0.64	0.04	0.95	0.30
	C18:1n9	2.50	0.11	2.71	0.41	2.01	0.06	1.56	0.10
	Total MUFA	**3.27**	**0.10**	**3.49**	**0.51**	**2.65**	**0.04**	**2.51**	**0.32**
PUFA	**C16:2n3**	0.56	0.06	0.88	0.29	0.54	0.09	1.14	0.08
	C16:3n3	1.42	0.09	1.25	0.11	0.93	0.10	0.62	0.08
	C18:2n6	20.33	0.25	21.15	2.69	17.49	0.24	13.23	0.46
	C18:3n3	46.20	22.86	68.20	7.72	52.25	1.44	36.73	0.84
	C18:3n6	0.51	0.26	0.75	0.09	0.71	0.07		
	Total PUFA	**69.02**	**23.23**	**92.22**	**10.37**	**71.92**	**1.21**	**51.72**	**1.10**
N		**15**		**15**		**15**		**14**	

Lemna gibba

		TBZ								SM							
		CTL	**±SE**	**10µg/L**	**±SE**	**15µg/L**	**±SE**	**22µg/L**	**±SE**	**CTL**	**±SE**	**1.3µg/L**	**±SE**	**1.8µg/L**	**±SE**	**7.6µg/L**	**±SE**
SFA	**C12:0**	0.06	0.00	0.06	0.01	30.58	30.52	0.05	0.01	0.12	0.03	0.10	0.02	0.10	0.00	0.10	0.01
	C14:0	0.50	0.17	0.59	0.15	26.72	24.11	0.37	0.05	0.99	0.13	0.89	0.02	0.81	0.09	0.81	0.07
	C15:0	0.13	0.04	0.15	0.04	0.11	0.06	0.08	0.01	0.21	0.02	0.23	0.03	0.24	0.01	0.48	0.05
	C16:0	21.76	7.30	27.68	7.09	41.34	12.21	15.72	0.81	32.33	2.76	31.84	2.75	33.63	0.46	26.63	3.34
	C18:0	1.10	0.26	1.12	0.24	19.62	18.42	0.75	0.02	1.56	0.07	1.76	0.23	1.79	0.07	1.77	0.35
	C20:0	0.25	0.45	0.34	0.13	15.39	15.08	0.21	0.02	0.31	0.04	0.35	0.05	0.36	0.05	0.37	0.05
	C22:0	0.42	0.12	0.62	0.23	20.89	20.20	0.40	0.02	0.53	0.04	0.92	0.41	0.60	0.08	0.51	0.13
	C24:0	1.21	0.32	1.95	0.26	31.94	29.63	1.01	0.17	1.89	0.08	2.25	0.20	2.00	0.26	0.95	0.20
	Total SFA	**25.41**	**8.17**	**32.51**	**8.08**	**186.59**	**149.65**	**18.59**	**0.68**	**37.93**	**2.99**	**38.36**	**3.24**	**39.54**	**0.84**	**31.62**	**4.15**
MUFA	**C16:1n9**	0.79	0.23	1.01	0.26	9.11	8.13	0.68	0.05	1.26	0.13	1.14	0.10	1.17	0.04	0.98	0.07
	C18:1n9	1.20	0.40	1.51	0.38	0.93	0.48	0.68	0.03	1.41	0.11	1.30	0.13	1.47	0.10	0.96	0.12
	Total MUFA	**1.99**	**0.62**	**2.52**	**0.64**	**10.05**	**7.67**	**1.36**	**0.07**	**2.67**	**0.24**	**2.44**	**0.23**	**2.63**	**0.14**	**1.95**	**0.18**
PUFA	**C16:2n3**	0.28	0.16	0.26	0.08	1.16	0.76	0.21	0.01	0.60	0.07	0.48	0.02	0.58	0.03	0.43	0.04
	C16:3n3	0.80	0.43	0.69	0.23	1.03	0.13	0.58	0.05	2.23	0.27	1.86	0.10	2.05	0.08	1.51	0.18
	C18:2n6	7.93	2.36	10.66	2.86	7.13	3.52	5.86	0.22	11.26	0.66	10.28	0.48	11.26	0.23	8.19	0.99
	C18:3n6	20.72	10.07	53.53	13.00	54.62	5.63	28.80	1.14	58.42	4.50	54.21	5.08	57.33	1.34	40.48	4.47
	C18:3n3	0.24	0.19	0.23	0.16	5.10	4.91			0.75	0.05	0.68	0.08	0.77	0.07	0.66	0.03
	Total PUFA	**29.96**	**6.97**	**65.37**	**15.93**	**69.03**	**6.66**	**35.45**	**1.42**	**73.25**	**5.55**	**67.52**	**5.54**	**71.99**	**1.74**	**51.26**	**5.65**
N		**15**		**15**		**15**		**14**		**15**		**15**		**15**		**15**	

Lemna gibba

		Cu							
		CTL	**±SE**	**25µg/L**	**±SE**	**50µg/L**	**±SE**	**115µg/L**	**±SE**
SFA	**C12:0**	0.07	0.01	0.15	0.01	0.14	0.02	0.10	0.02
	C14:0	0.56	0.13	1.12	0.13	0.84	0.12	0,72	0.17
	C15:0	0.14	0.03	0.29	0.04	0.23	0.03	0.16	0.02
	C16:0	23.54	4.41	41.96	3.86	29.04	3.56	23.60	3.62
	C18:0	1.21	0.27	1.92	0.14	1.35	0.28	1.31	0.22
	C20:0	0.29	0.03	0.43	0.05	0.50	0.03	0.59	0.12
	C22:0	0.57	0.12	0.64	0.08	0.84	0.14	0.56	0.06
	C24:0	2.05	0.35	2.41	0.13	2.55	0.54	2.12	0.43
	Total SFA	**28.43**	**5.10**	**48.92**	**4.17**	**35.49**	**3.93**	**29.16**	**4.54**
MUFA	**C16:1n9**	0.86	0.14	1.59	0.21	1.25	0.17	1.02	0.13
	C18:1n9	1.21	0.26	2.29	0.20	1.51	0.22	1.42	0.25
	Total MUFA	**2.07**	**0.40**	**3.88**	**0.41**	**2.76**	**0.39**	**2.44**	**0.38**
PUFA	**C16:2n3**	0.55	0.08	0.77	0.09	0.49	0.09	0.35	0.08
	C16:3n3	1.68	0.21	2.26	0.17	1.49	0.29	0.83	0.13
	C18:2n6	8.94	1.43	14.11	1.46	9.71	1.43	8.10	1.31
	C18:3n6	44.34	7.64	61.83	6.85	38.75	6.77	26.50	4.08
	C18:3n3	0.65	0.07	1.22	0.21	0.79	0.15	0.72	0.07
	Total PUFA	**56.16**	**9.43**	**80.18**	**8.69**	**51.23**	**8.70**	**36.50**	**5.67**
N		**15**		**15**		**15**		**15**	

3. Sugar profiles

The Free sugars' analysis showed a different pattern between the organic (TBZ and SM) and the inorganic (Cu) compounds for the species *L. gibba*, where a general decrease in the contaminated samples with TBZ (Figure 7A) and a general maintenance of levels for SM exposure is observed (Figure 7B) compared to the control. In the opposite trend, Cu was able to increase the levels of all sugars in almost all treatments (Figure 7C), compared to the control. For *L. minor* the pattern was similar with *L. gibba*, showing the same response with the sugars increase when exposed to terbuthylazine (Figure 8A), but when exposed to SM it was clear the increase of sugar contents compared to the control (Figure 8B) and Cu exposures (Figure 8C). Curiously, both species present high quantities of Xylose, Mannose, Galactose and Glucose as main monosaccharides in their biomass. Although there were clear differences between sugars contents after all exposures, no statistical differences were found in Free sugars' profiles by one-way ANOVA for all treatments at $p<0.05$.

Polysaccharides' analysis revealed *L. gibba* with an increment in sugars exposed to TBZ (Figure 9A), with Rhamnose statistically different from the control at 22 µg/L ($p<0.05$), and to Copper (Figure 9C), with all monosaccharides statistically different from control at 451.7 µg/L, except Ribose. After SM exposure, the *L. gibba* polysaccharides showed not much variance between treatments and the control (Figure 9B). Indeed, all treatments from SM exposure showed significant differences in Rhamnose content at 7.6 µg/L of SM ($p<0.05$). On the other hand, *L. minor* showed some variances when exposed to Terbuthylazine, with the sugars' decrease in almost all samples, yet an increase in Rhamnose and Fucose contents at 31 µg/L (Figure 10A). After S-Metolachlor exposure there was a maintenance of sugars at maximum concentration, but a clear increase with lower concentrations (Figure 10B). The one-way ANOVA showed significant differences after S-Metolachlor exposure with Rhamnose different from the control at 3 µg/L, and Ribose, Arabinose, Xylose and Galactose different from the control at 2 µg/L ($p<0.05$). The exposure with copper showed an increase in all sugars contents but none of them had statistical differences with the control (Figure 10C).

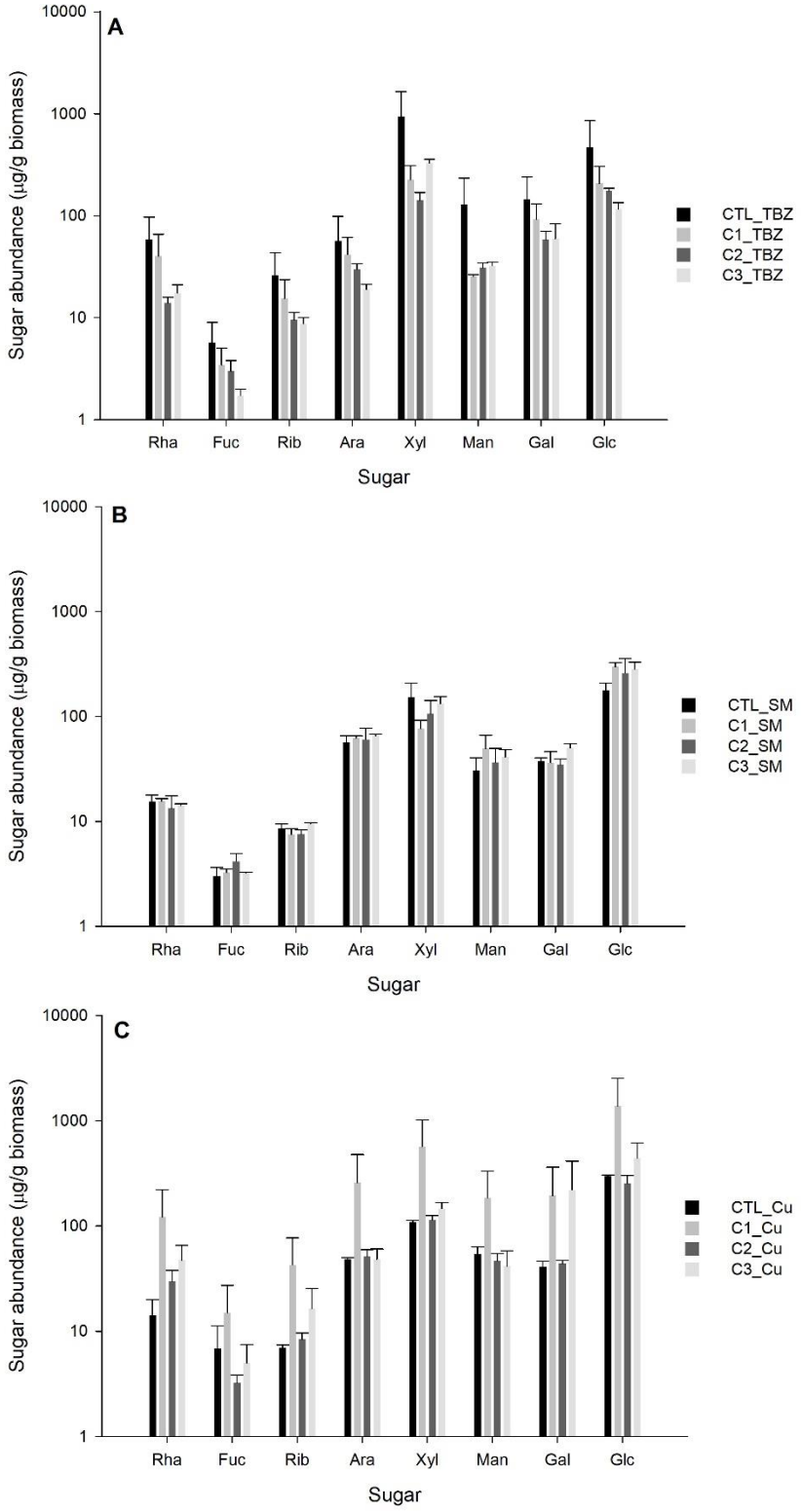

Figure 7: Quantification of Free Sugars in *Lemna gibba* exposed to (A) Terbuthylazine (Control-CTL; C1=10µg/L; C2=15µg/L; C3=22µg/L), (B) S-Metholaclor (CTL; C1=1.3µg/L; C2=1.8µg/L; C3=7.6µg/L) and (C) Copper (CTL; C1=25µg/L; C2=50µg/L; C3=115µg/L). Bars represent the Mean and standard error of samples per treatment. Rha- Rhamnose, Fuc- Fucose, Rib- Ribose; Ara- Arabinose, Xyl- Xylose, Man- Mannose, Gal- Galactose, Glc- Glucose.

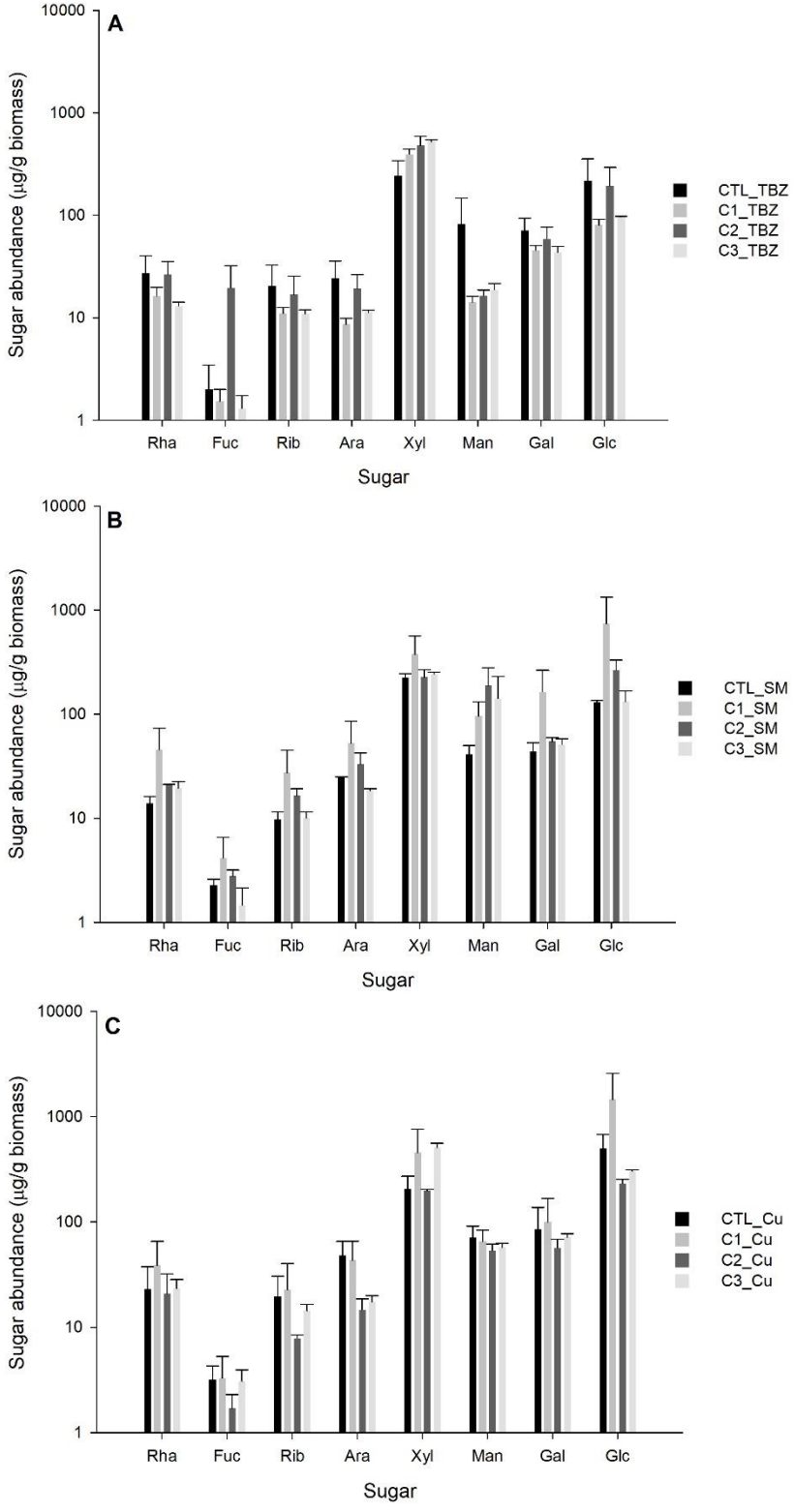

Figure 8: Quantification of Free Sugars in *Lemna minor* exposed to (A) Terbuthylazine (Control-CTL; C1=9 µg/L; C2=16 µg/L; C3=31 µg/L), (B) S-Metholaclor (CTL; C1=2µg/L; C2=3µg/L; C3=6.5µg/L) and (C) Copper (CTL; C1=6µg/L; C2=30µg/L; C3=65µg/L). Bars represent the Mean and standard error of samples per treatment. Rha- Rhamnose, Fuc- Fucose, Rib- Ribose; Ara- Arabinose, Xyl- Xylose, Man- Mannose, Gal- Galactose, Glc- Glucose.

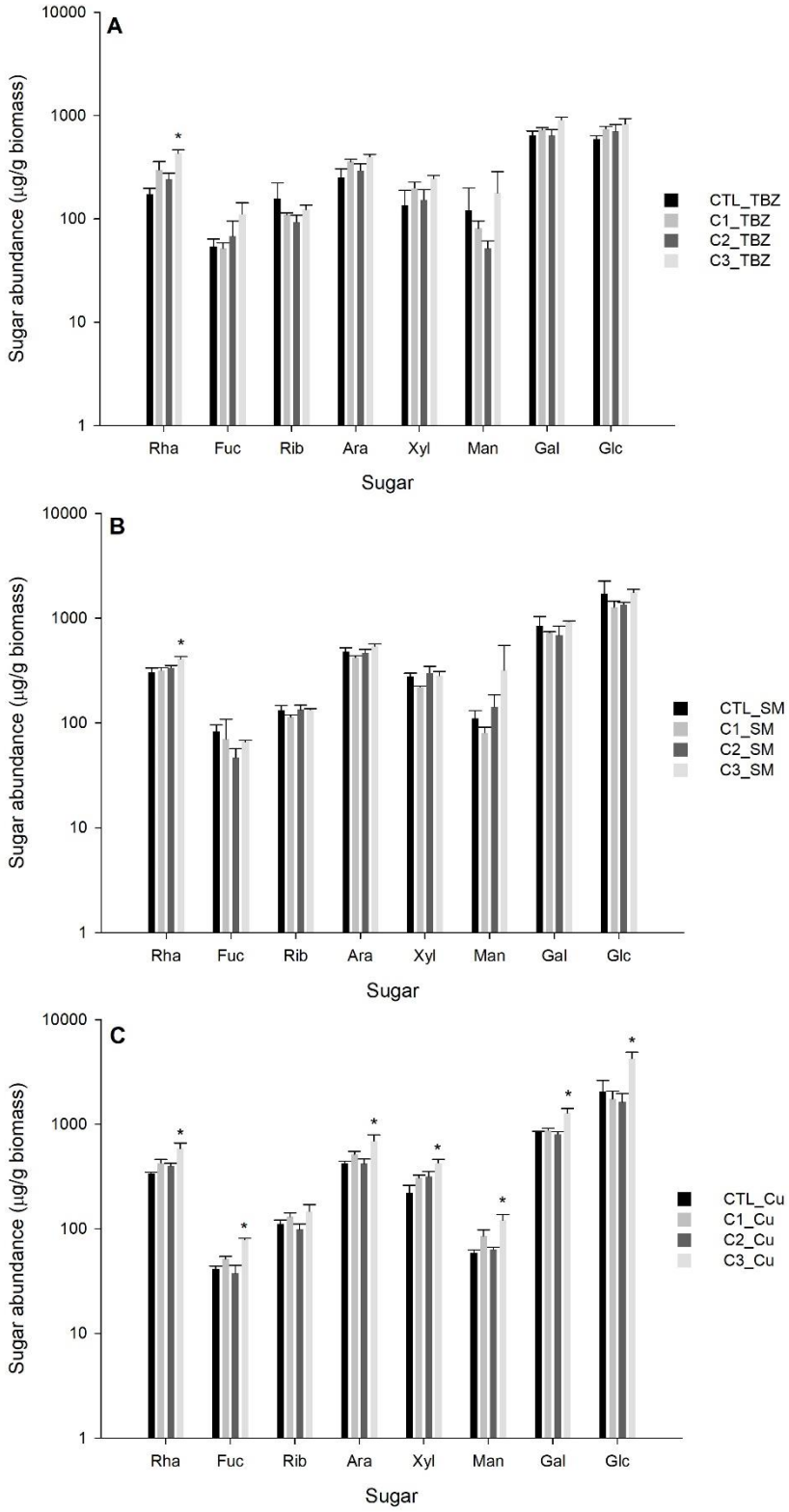

Figure 9: Quantification of Polysaccharides based on monosaccharides' composition in *Lemna gibba* exposed to (A) Terbuthylazine (Control-CTL; C1=10µg/L; C2=15µg/L; C3=22µg/L), (B) S-Metholaclor (CTL; C1=1.3µg/L; C2=1.8µg/L; C3=7.6µg/L) and (C) Copper (CTL; C1=25µg/L; C2=50µg/L; C3=115µg/L). Bars represent the mean and standard error of samples per treatment. Rha- Rhamnose, Fuc- Fucose, Rib- Ribose; Ara- Arabinose, Xyl- Xylose, Man- Mannose, Gal- Galactose, Glc- Glucose. '*' on top of bars indicate statistical significant difference with control ($p<0.05$).

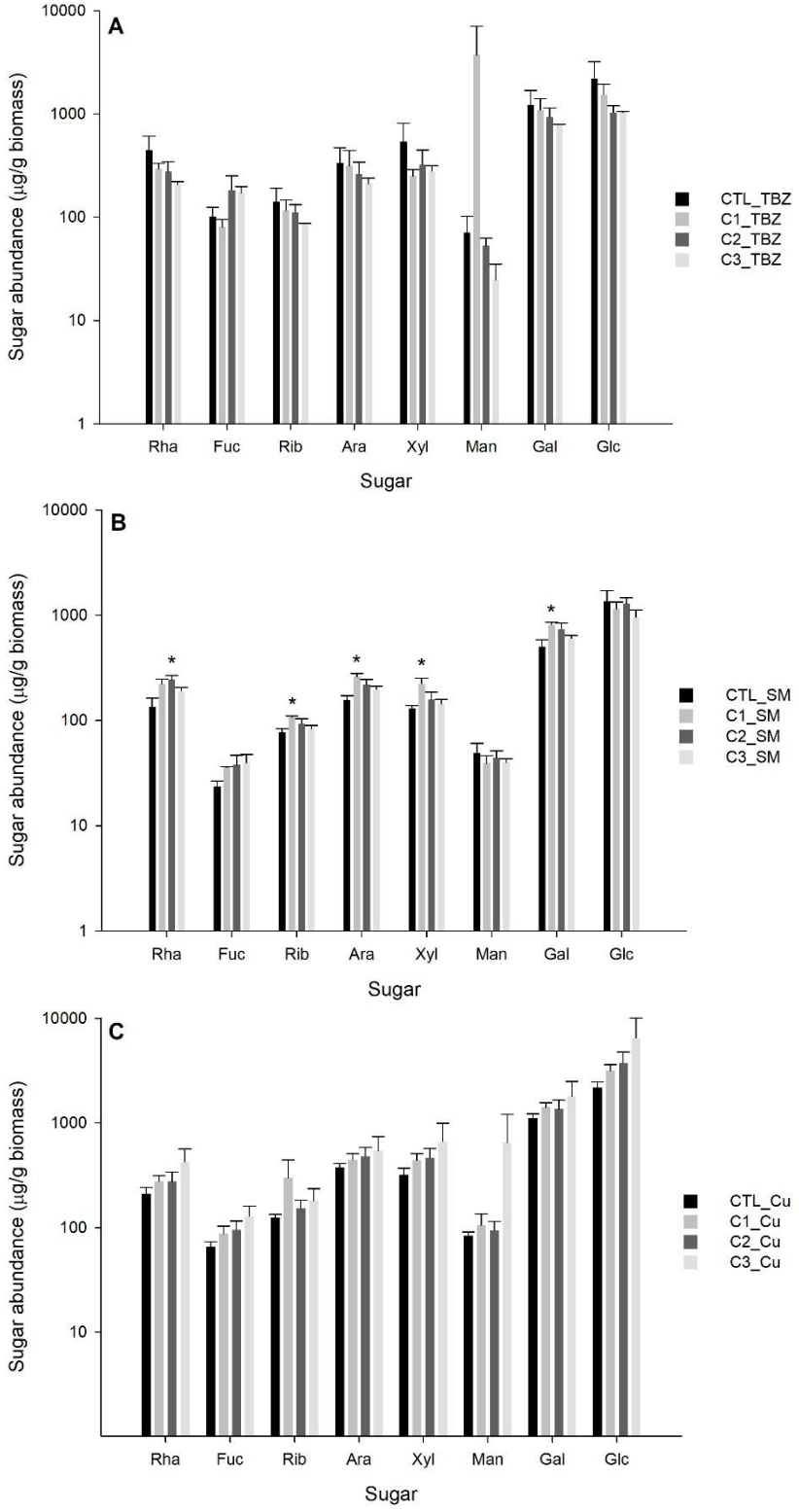

Figure 10: Quantification of Polysaccharides based on monosaccharides' composition in *Lemna minor* exposed to (A) Terbuthylazine (Control-CTL; C1=9 µg/L; C2=16 µg/L; C3=31 µg/L), (B) S-Metholaclor (CTL; C1=2µg/L; C2=3µg/L; C3=6.5µg/L) and (C) Copper (CTL; C1=6µg/L; C2=30µg/L; C3=65µg/L). Bars represent the mean and standard error of samples per treatment. Rha- Rhamnose, Fuc- Fucose, Rib- Ribose; Ara- Arabinose, Xyl- Xylose, Man- Mannose, Gal- Galactose, Glc- Glucose. '*' on top of bars indicate statistical significant difference with control ($p<0.05$).

4. Multivariate analysis

The ordination diagram (nMDS) and cluster analysis identified three principal groups for species according to FA's samples exposed to TBZ, SM and Cu (Figures 11 and 12). Samples account with one Control (CTL) and three different concentrations (C1, C2 and C3) for each contaminant exposure. Treatments of *Lemna gibba* were distributed in the groups A (C2 of TBZ), B (CTL, C1, C2 and C3 of Cu; CTL and C1 of TBZ; C1, C2 and C3 of SM) and C (C3 of TBZ). Treatments of *Lemna minor* were distributed also in three groups: A (C3 of Cu and C3 of TBZ), B (CTL, C1 and C2 of Cu; C1, C2 and C3 of SM; CTL and C2 of TBZ), and C (CTL of SM; C1 of TBZ). SIMPER analysis showed a similarity of 97.63 for group B of *L. gibba* composed by samples exposed to the three chemical compounds. SIMPER analysis of *L. minor* reported a similarity average of 97.53, 96.96 and 95.85 for groups A, B and C, respectively with major contributions of C18:3n3, C16:0 and C18:2n6 for such similarity within each group (Table 4). The dissimilarities between *L. gibba* groups were greater between Groups A and C (Av. Dissimilarity= 18.35), curiously responding to different treatments of TBZ samples, which means these samples had great differences specially due to some SFA contributions (C12:0, C20:0, C22:0 and C14:0), and to the PUFA C18:3n6. The least dissimilar groups for this species, or the most similar groups, are B and C with an average dissimilarity of 7.72, putting Cu and SM samples very similar, with mainly PUFA contributing to such dissimilarities. On the other hand, for *L. minor* species, the most dissimilar groups are C and A with 10.86 of average dissimilarity, where the highest FA contributions within groups are C18:3n6, C12:0, C14:0 and C16:1n9 (Table 4). The smallest dissimilarity was between C and B groups accounting with 6.28 Av. Dissimilarity with the highest contributions of C12:0 (27.39%), C14:0 (13.52%) and C16:1n9 (10.08%).

The ANOSIM analysis showed no significant differences between groups found for *Lemna gibba* where Global Test R=0.998 failed at $p<0.015$ and Pairwise Tests failed at $p<0.091$ (R=1 between groups A and B; R=0.996 between groups B and C). The ANOSIM analysis of *L. minor* also proved no significant differences between groups from Global test (R= 0.95; $p<0.002$), and in Pairwise tests it also showed no significant differences between groups B and C (R=0.927), and between B and A (R= 0.957) at $p<0.022$, and within groups C and A (R=1) at $p<0.333$. These tests were according to SIMPER analysis where the dissimilarities between groups were pretty small, given the fact that samples are very similar in terms of Fatty acids. However, we expected that representative samples of a single

treatment were closer to each other, than separated as we can see on both nMDS, which might be explained by the differences in FA's abundance.

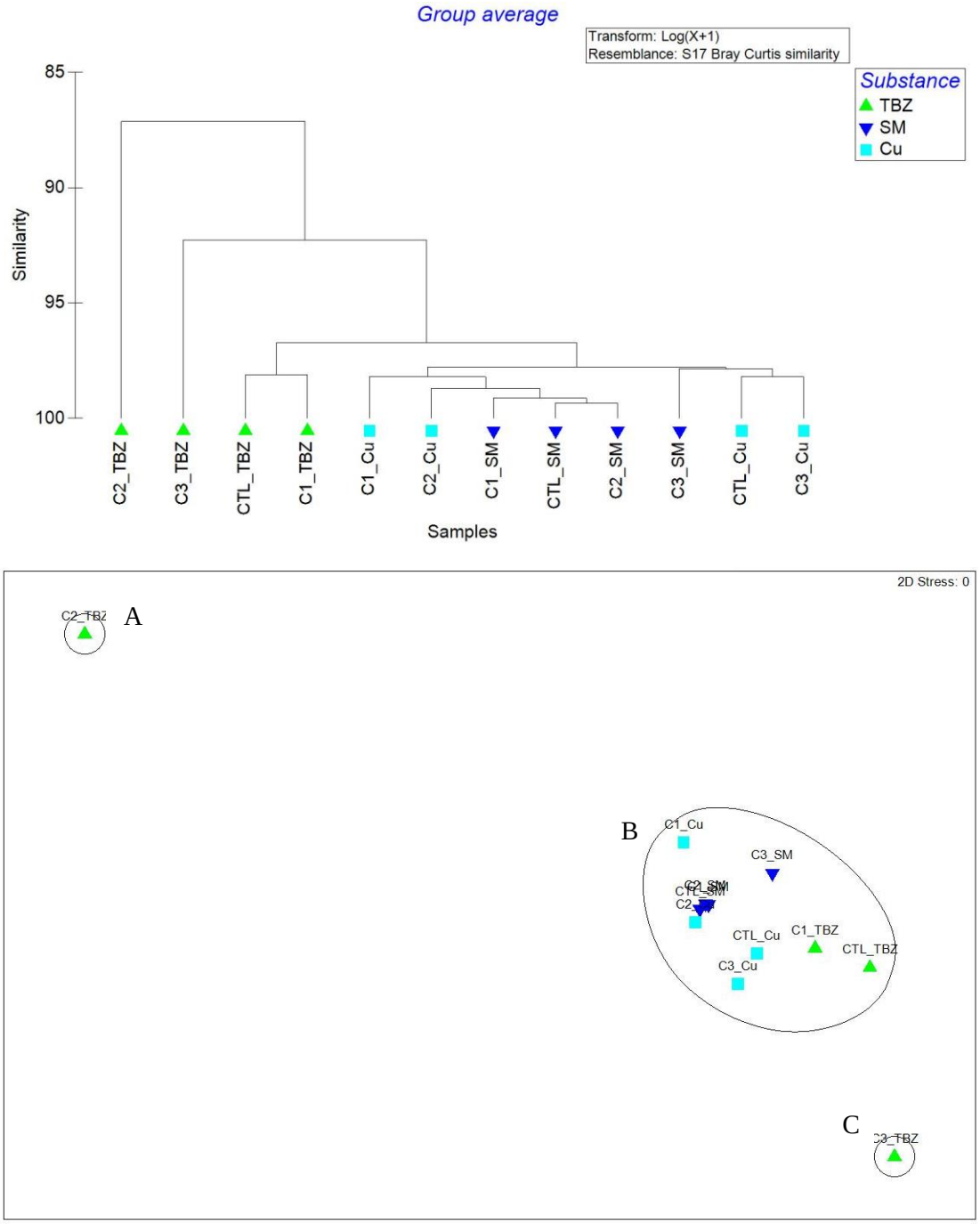

Figure 11: Representation of the affinity groups of FA between samples from TBZ, SM and Cu exposure to *Lemna gibba*. The cluster and nMDS were obtained by the group average linkage method and Bray Curtis resemblance after a transform of Log (X+1). Samples: *L. gibba* CTL=0µg/L; C1_TBZ=10µg/L;

C2_TBZ=15µg/L; C3_TBZ=22µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=25µg/L; C2_Cu=50µg/L; C3_Cu=115µg/L.

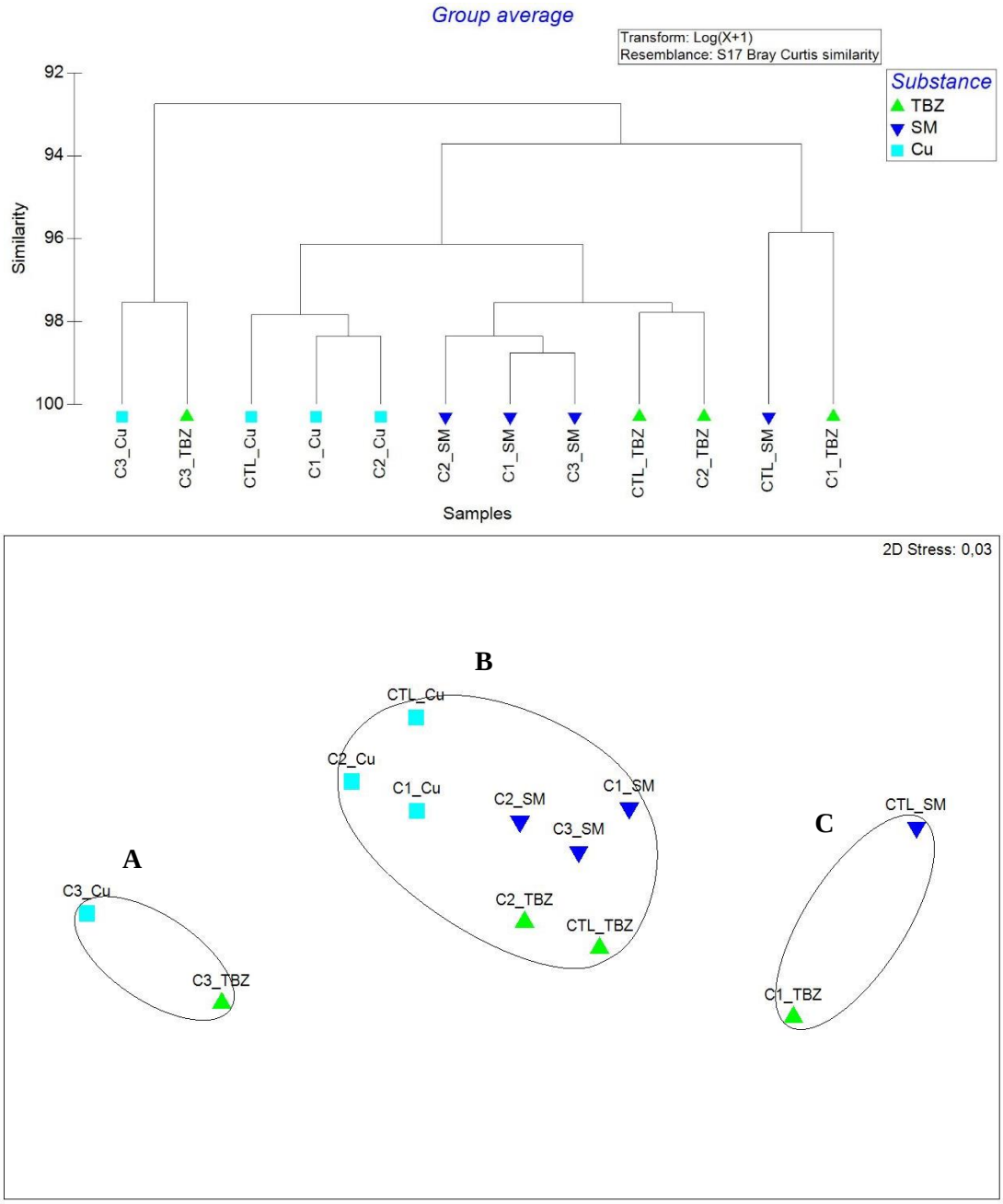

Figure 12: Representation of the affinity groups of FA between samples from TBZ, SM and Cu exposure to *Lemna minor*. The cluster and nMDS were obtained by the group average linkage method and Bray Curtis resemblance after a transform of Log (X+1). Samples: *L. minor* CTL=0µg/L; C1_TBZ=9µg/L; C2_TBZ=16µg/L; C3_TBZ=31µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=6µg/L; C2_Cu=30µg/L; C3_Cu=65µg/L.

Table 4: SIMPER analysis with average similarities and dissimilarities between FA samples groups of *Lemna gibba* and *Lemna minor*, related with n-MDS analysis. Av. Abund= average abundance of the FA in the group; Av. Sim/ Av.Diss= average similarity/ dissimilarity; Sim/SD or Diss/SD= ratio between average similarity/ dissimilarity and the standard deviation associated; Contrib% = percentage contribution of each FA for the average similarity/dissimilarity; Cum%= cumulative value of FA contribution for average similarity/ dissimilary.

Lemna gibba

Groups	Similarity	FA	Av. Abund	Av. Sim	Sim/SD	Contrib%	Cum.%
B	97.63	C18:3n3	10.67	9.71	39.47	9.94	9.94
		C16:0	10.26	9.41	75.29	9.64	19.58
		C18:2n6	9.20	9.20	61.92	8.64	28.22
		C24:0	7.53	6.83	25.61	7.00	35.21
		C18:0	7.29	6.64	65.34	6.80	42.02
A	Less than 2 samples in group						
C	Less than 2 samples in group						

	Dissimilarity	FA	Av. Abund		Av. Diss.	Diss/SD	Contrib%	Cum.%
B & A	12.31	C12:0	4.57	10.33	2.38	13.94	19.35	19.35
		C20:0	5.91	9.64	1.55	13.21	12.55	31.90
		C14:0	6.64	10.19	1.47	11.50	11.96	43.87
		C22:0	6.41	9.95	1.46	13.25	11.90	55.77
		C24:0	7.53	10.37	1.18	8.46	9.55	65.32
B & C	7.72	C18:3n6	6.41	0.00	3.20	14.69	41.38	41.38
		C15:0	5.35	4.44	0.46	2.42	5.90	47.28
		C16:3n3	7.26	6.36	0.45	2.04	5.77	53.05
		C16:2n3	6.12	5.34	0.39	2.31	5.02	58.07
		C14:0	6.64	5.90	0.36	2.91	4.70	62.77
A & C	18.35	C18:3n3	8.54	0.00	3.77	Undefined!	20.55	20.55
		C12:0	10.33	3.87	2.85	Undefined!	15.54	36.09
		C20:0	9.64	5.33	1.90	Undefined!	10.38	46.47
		C14:0	10.19	5.90	1.89	Undefined!	10.33	56.80
		C22:0	9.95	5.99	1.75	Undefined!	9.52	66.32

Lemna minor

Groups	Similarity	FA	Av. Abund	Av. Sim	Sim/SD	Contrib%	Cum.%
A	97.53	C18:3n3	10.86	10.15		10.41	10.41
		C16:0	10.11	9.67		9.92	20.32
		C18:2n6	9.68	9.16		9.39	29.72

		C18:0	7.65		7.31		7.49	37.21
		C18:1n9	7.43		7.10		7.28	44.49
B	96.96	C18:3n3	11.40		9.67	46.14	9.97	9.97
		C16:0	10.64		9.08	92.15	9.37	19.34
		C18:2n6	10.08		8.63	84.21	8.91	28.24
		C18:1n9	7.97		6.80	85.52	7.02	35.26
		C24:0	8.02		6.77	49.00	6.98	42.24
C	95.85	C18:3n3	11.75		9.01		9.40	9.40
		C16:0	10.97		8.29		8.65	18.05
		C18:2n6	10.22		7.85		8.19	26.24
		C12:0	9.64		6.79		7.08	33.33
		C14:0	9.39		6.77		7.06	40.38

	Dissimilarity	**FA**	**Av. Abund**		**Av. Diss.**	**Diss/SD**	**Contrib%**	**Cum.%**
C & A	10.86	C18:3n6	6.37	0.00	2.74	6.86	25.22	25.22
		C12:0	9.64	5.03	1.98	4.63	18.26	43.48
		C14:0	9.39	6.86	1.09	3.02	10.01	53.49
		C16:1n9	8.66	6.80	0.80	6.33	7.36	60.86
		C15:0	6.95	5.69	0.54	1.96	5.01	65.87
C & B	6.28	C12:0	9.64	5.44	1.72	3.62	27.39	27.39
		C14:0	3.39	7.32	0.85	2.49	13.52	40.91
		C16:1n9	8.66	7.12	0.63	2.90	10.08	50.99
		C16:2n3	7.93	6.68	0.51	3.67	8.18	59.17
		C22:0	7.45	6.56	0.40	1.56	6.29	65.46
B & A	6.34	C18:3n6	6.40	0.00	2.92	10.39	46.10	46.10
		C24:0	8.02	7.20	0.38	1.72	6.04	52.14
		C16:3n3	7.48	6.65	0.38	1.90	6.02	58.16
		C18:3n3	11.40	10.86	0.30	1.64	4.67	62.82
		C22:0	6.56	6.44	0.28	1.38	4.37	67.19

L. gibba and *L. minor* ordination diagrams (nMDS) with Free Sugars (FS) (Figure 13) identified two principal groups with an internal similarity between samples of more than 90%. For *L. gibba* Group A is composed by samples C1, C2 and C3 of TBZ; CTL, C1, C2 and C3 of SM; and CTL, C2 and C3 of Cu exposure and Group B is composed by samples CTL of TBZ and C1 of Cu. For *L. minor* the Group A is composed by the samples C1, C2 and C3 of TBZ, and group B by the samples CTL, C1, C2 and C3 of SM; CTL, C1, C2 and C3 of Cu; and CTL of TBZ.

The SIMPER analysis report of FS for both species is present at Table 5. The average similarity within group A of *L. gibba* was 94.41, accounting with the highest contribution of Glucose (19.30%), Xylose (17.36%), Galactose (13.76%) and Arabinose (13.36%). Group B had a similarity of 92.48 within samples, whereas contributions account more with Xylose (17.77%), Glucose (17.22%), Galactose (13.96%) and Mannose (13.65%). The dissimilarity between groups A and B was 14.51 with Xylose, Rhamnose and Mannose contributing to such differences.

In the case of *L. minor* exposed to the three chemicals, SIMPER analysis revealed an average similarity of 93.16 for group A and 93.42 for group B, with highest contributions of Xylose, Glucose, Mannose and Galactose sugars for the similarities between samples (Table 5). The average dissimilarity between groups was 10.33, with Mannose, Glucose, Fucose and Arabinose contributing with 25.57%, 17.94%, 13.67% and 13.31%, respectively. The multivariate analysis results for Free sugars show differences between samples in agreement with their sugars contents (Figures 7 and 8), hence there is no clear distinction between samples of the same treatment and others. The results also suggest that the random separations between samples may be due to the fact that samples are not statistically different from the control.

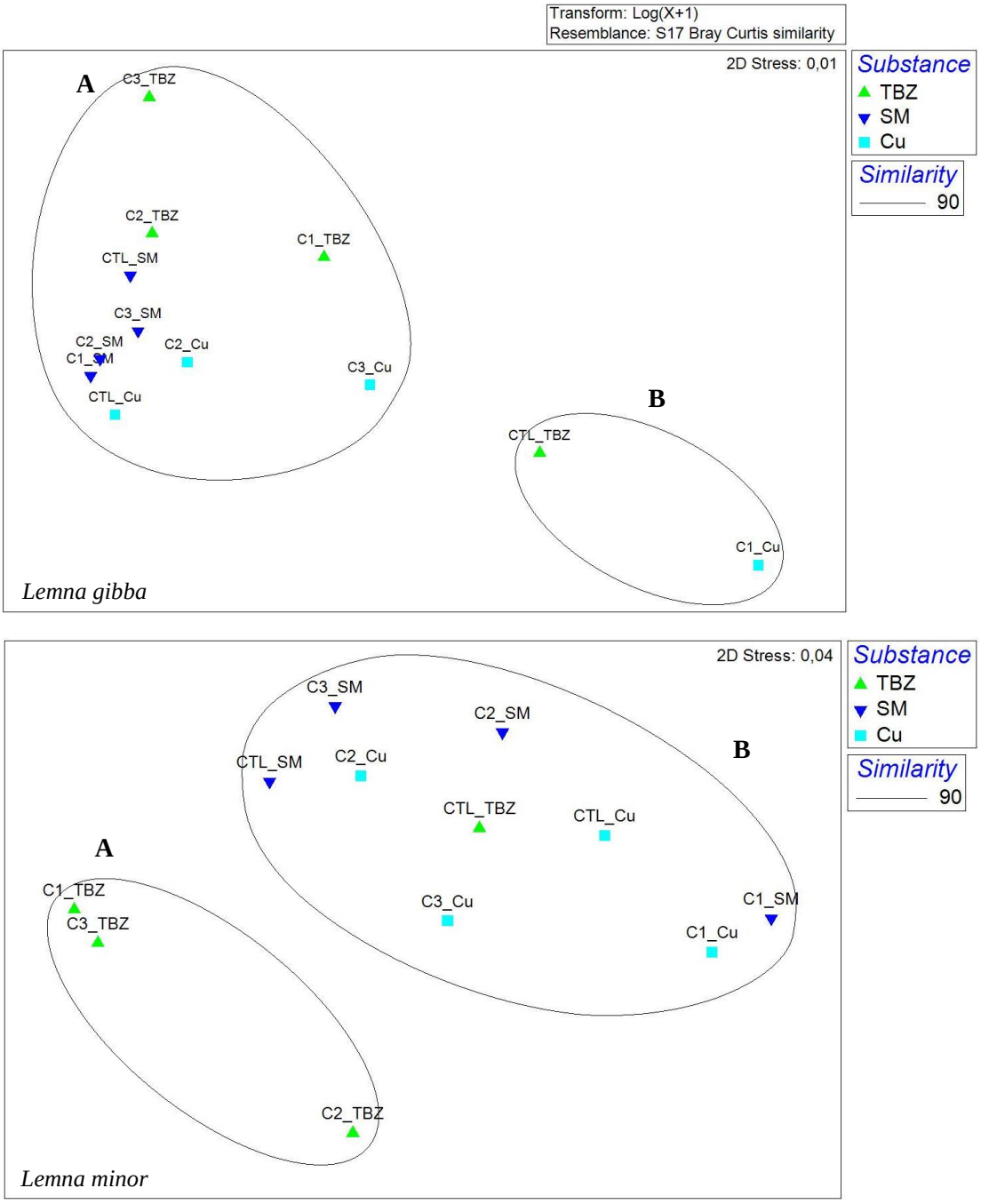

Figure 13: Representation of affinity groups between samples in ordination diagrams (nMDS) of Bray-Curtis similarity matrixes after Log (X+1) transformation of Free Sugars' samples from *Lemna gibba* and *Lemna minor*. Samples: *L.gibba* CTL=0µg/L; C1_TBZ=10µg/L; C2_TBZ=15µg/L; C3_TBZ=22µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=25µg/L; C2_Cu=50µg/L; C3_Cu=115µg/L; *L.minor* CTL=0µg/L; C1_TBZ=9µg/L; C2_TBZ=16µg/L; C3_TBZ=31µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=6µg/L; C2_Cu=30µg/L; C3_Cu=65µg/L.

Table 5: SIMPER analysis with average similarity and dissimilarity between the Free Sugars' samples groups of *Lemna gibba* and *Lemna minor*, related with n-MDS analysis. Av. Abund= average abundance of the sugar in the group; Av. Sim/ Av.Diss= average similarity/ dissimilarity; Sim/SD or Diss/SD= ratio between average similarity/ dissimilarity and the standard deviation associated; Contrib%= percentage contribution of each sugar for the average similarity/dissimilarity; Cum%= cumulative value of sugars contribution for average similarity/ dissimilarity.

Lemna gibba

Groups	Similarity	Sugar	Av. Abund		Av. Sim	Sim/SD	Contrib%	Cum.%
B	92.48	Xyl	6.60		16.44		17.77	17.77
		Glc	6.69		15.93		17.22	34.99
		Gal	5.13		12.91		13.96	48.95
		Man	5.05		12.63		13.65	62.61
		Rha	4.45		10.58		11.44	74.05
A	94.41	Glc	5.47		18.22	18.99	19.30	19.30
		Xyl	4.96		16.39	17.85	17.36	36.66
		Gal	4.04		12.99	19.88	13.76	50.42
		Ara	3.85		12.62	9.81	13.36	63.78
		Man	3.66		12.20	16.91	12.92	76.71
	Dissimilarity	**Sugar**	**Av. Abund**		**Av. Diss.**	**Diss/SD**	**Contrib%**	**Cum.%**
B & A	14.51	Xyl	6.60	4.96	2.44	3.20	16.85	16.85
		Rha	4.45	3.04	2.09	2.50	14.40	31.24
		Man	5.05	3.66	2.05	5.16	14.16	45.40
		Glc	6.69	5.47	1.79	1.90	12.32	57.72
		Rib	3.54	2.35	1.76	3.47	12.13	69.85

Lemna minor

Groups	Similarity	Sugar	Av. Abund	Av. Sim	Sim/SD	Contrib%	Cum.%
B	93.42	Xyl	6.14	17.72	20.48	18.96	18.96
		Glc	4.75	17.28	17.86	18.50	37.46
		Man	3.91	13.36	15.15	14.31	51.76
		Gal	2.85	13.20	25.53	14.13	65.89
		Ara	2.93	10.05	12.86	10.76	76.65
A	93.16	Xyl	6.14	22.11	22.58	23.73	23.73
		Glc	4.75	16.30	23.14	17.50	41.23
		Gal	3.91	13.93	19.91	14.96	56.19
		Man	2.85	10.11	23.14	10.86	67.04
		Rha	2.93	9.90	19.91	10.63	77.67

	Dissimilarity	Sugar	Av. Abund		Av. Diss.	Diss/SD	Contrib%	Cum.%
B & A	10.33	Man	4.39	2.85	2.64	3.23	25.57	25.57
		Glc	5.78	4.75	1.85	1.45	17.94	43.51
		Fuc	1.27	1.60	1.41	1.25	13.67	57.18
		Ara	3.37	2.60	1.38	1.66	13.31	70.49
		Xyl	5.64	6.14	0.93	1.69	9.02	79.51

The Polysaccharides' multidimensional diagrams (nMDS) revealed a separation of three principle groups for *L. gibba* with 96.5 of internal similarity and for *L. minor* with 94 (Figure 14). Samples of *L. gibba* were divided into the groups: A, composed by the samples CTL and C2 of TBZ; B composed by the samples C1 and C3 of TBZ; CTL, C1, C2 and C3 of SM; CTL, C1 and C2 of Cu; and C by the sample C3 of Cu exposure.

SIMPER analysis report between groups formed by clustering arrangement are given in Table 6, and it shows the groups of *L. gibba* with 97.54 and 97.16 of average similarity for groups A and B, respectively. Group C could not be tested since it has only one sample. The similarities are giving mostly due to the monosaccharides Galactose (A=16.01; B=15.79), Glucose (A=15.10; B=15.57) and Arabinose (A=13.69; B=13.49) contributions. The greatest dissimilarity is between groups B and C, where differences are mainly between the monosaccharides Glucose (28.62%), Xylose (16.49%) and Rhamnose (15.88%) for both groups. Indeed, this was expected since group C is constituted by the last treatment exposed to Cu, where it had significant differences by the one-way ANOVA compared to the control (Figure 9C).

By the other hand, considering *L. minor* treatments, SIMPER analysis showed great similarities within groups B (Av. Sim=96.53) and C (Av. Sim=96.60), but could not test group A, since it is composed by a single sample (Table 6). The similarities within each group of Polysaccharides are due to Glucose (16.93%), Galactose (15.39%) and Xylose (12.97%) in group B, and due to Glucose (17.14%), Galactose (15.88%) and Arabinose (12.98%) in group C. The greatest dissimilarity between polysaccharides samples was between groups A and C (Av. Diss= 8.15) where the monosaccharides Mannose and Fucose contributed with 60.91% and 11.31%, respectively. This great contribution of Mannose is due to the great increment of such sugar in the first treatment of *L. minor* with TBZ (C1=9 µg/L). As with Free Sugars, the results of Polysaccharides are expected in a random layout

given the differences in sugars contents, which do not show a tendency to increase or decrease in a linear manner.

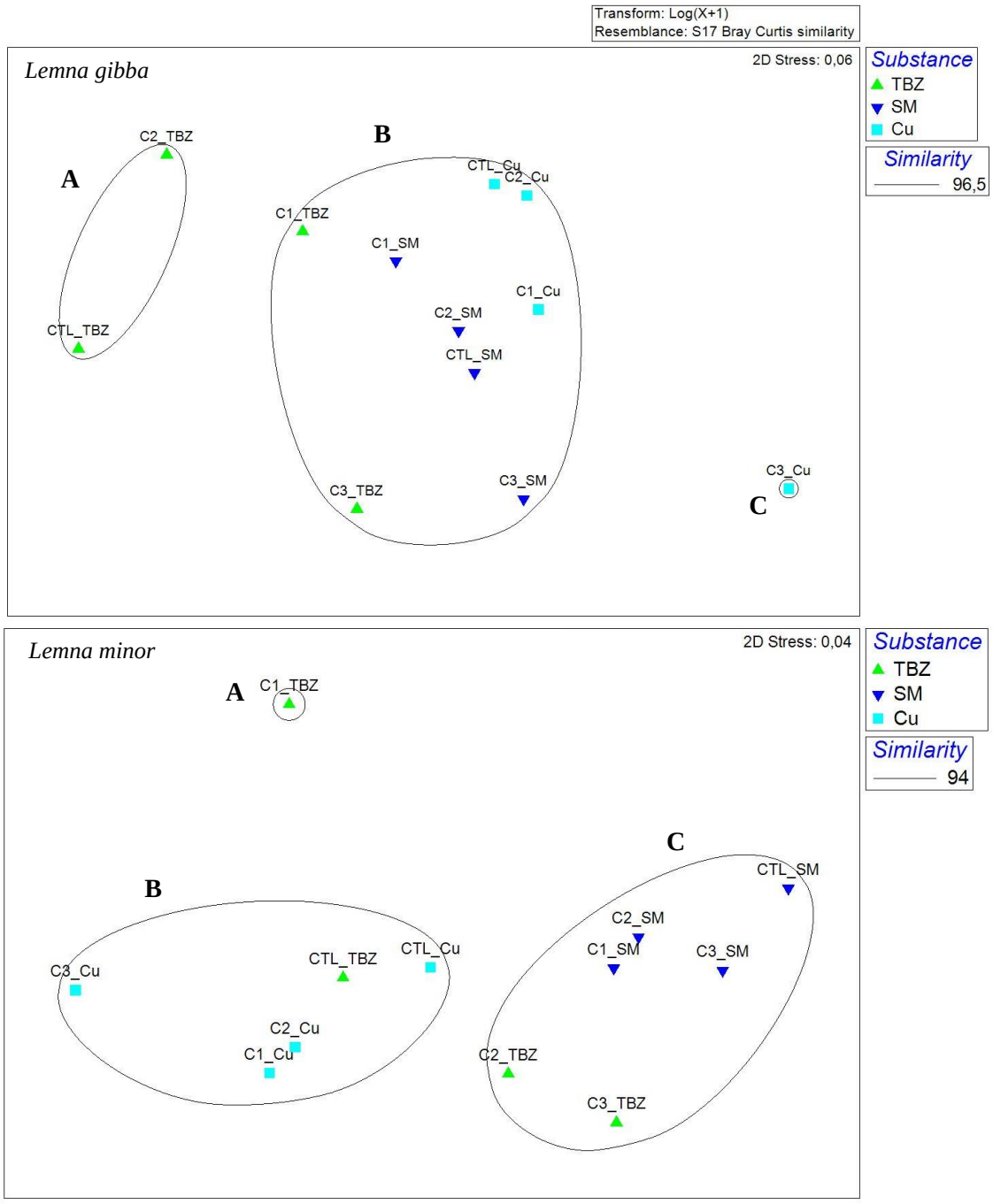

Figure 14: Representation of affinity groups between samples in ordination diagrams (nMDS) of Bray-Curtis similarity matrixes after Log (X+1) transformation of Polysaccharides' samples from *Lemna gibba* and *Lemna minor*. Samples: *L.gibba* CTL=0µg/L; C1_TBZ=10µg/L; C2_TBZ=15µg/L; C3_TBZ=22µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=25µg/L; C2_Cu=50µg/L; C3_Cu=115µg/L; *L.minor* CTL=0µg/L; C1_TBZ=9µg/L; C2_TBZ=16µg/L; C3_TBZ=31µg/L; C1_SM=1.3µg/L; C2_SM=1.8µg/L; C3_SM=7.6µg/L; C1_Cu=6µg/L; C2_Cu=30µg/L; C3_Cu=65µg/L.

Table 6: SIMPER analysis with average similarities and dissimilarities between Polysaccharides' samples groups of *Lemna gibba* and *Lemna minor*, related with n-MDS analysis. Av. Abund= average abundance of the sugar in the group; Av. Sim/ Av.Diss= average similarity/ dissimilarity; Sim/SD or Diss/SD= ratio between average similarity/ dissimilarity and the standard deviation associated; Contrib%= percentage contribution of each sugar for the average similarity/dissimilarity; Cum%= cumulative value of sugars contribution for average similarity/ dissimilarity.

Lemna gibba

Groups	Similarity	Sugar	Av. Abund	Av. Sim	Sim/SD	Contrib%	Cum.%
B	97.16	Gal	6.47	15.34		15.79	15.79
		Glc	6.47	15.13		15.57	31.36
		Ara	5.61	13.10		13.49	44.85
		Rha	5.33	12.26		12.62	57.47
		Xyl	4.98	11.65		11.99	69.46
A	97.54	Glc	7.24	15.61	22.52	16.01	16.01
		Gal	6.70	14.73	64.94	15.10	31.11
		Ara	6.10	13.36	72.97	13.69	44.80
		Rha	5.89	12.87	55.22	12.44	57.99
		Xyl	5.57	12.13	50.25	10.78	70.43
C	Less than 2 samples in group						

	Dissimilarity	Sugar	Av. Abund		Av. Diss.	Diss/SD	Contrib%	Cum.%
B & A	4.30	Glc	6.47	7.24	0.87	2.15	20.31	20.31
		Xyl	4.98	5.57	0.68	3.41	15.72	36.03
		Man	4.38	4.69	0.67	1.36	15.59	51.62
		Rha	5.33	5.89	0.63	2.53	14.69	66.31
		Ara	5.61	6.10	0.56	3.31	13.04	79.34
B & C	7.23	Glc	6.47	8.35	2.07	15.09	28.62	28.62
		Xyl	4.98	6.06	1.19	12.43	16.49	45.11
		Rha	5.33	6.38	1.15	4.59	15.88	61.00
		Ara	5.61	6.54	1.03	8.32	14.25	75.24
		Gal	6.47	7.16	0.76	167.71	10.45	85.69
A & C	4.31	Glc	7.24	8.35	1.19	3.09	27.69	27.69
		Xyl	5.57	6.06	0.53	2.85	12.22	39.91
		Rha	5.89	6.38	0.53	3.22	12.19	52.10
		Gal	6.70	7.16	0.49	4.19	11.33	63.44
		Man	4.69	4.80	0.48	1.67	11.22	74.66

Lemna minor

Groups	Similarity	Sugar	Av. Abund	Av. Sim	Sim/SD	Contrib%	Cum.%
B	96.53	Glc	8.09	16.34	42.58	16.93	16.93

		Gal	7.22		14.86	47.79	15.39	32.32
		Xyl	6.16		12.52	38.48	12.97	45.29
		Ara	6.07		12.43	39.85	12.87	58.16
		Rha	5.75		11.61	32.59	12.03	70.19
A			Less than 2 samples in group					
C	96.60	Glc	7.03		16.56	34.24	17.14	17.14
		Gal	6.57		15.34	56.02	15.88	33.03
		Ara	5.38		12.54	48.42	12.98	46.01
		Rha	5.34		12.38	33.66	12.81	58.82
		Xyl	5.29		12.07	32.53	12.50	71.31

	Dissimilarity	**Sugar**	**Av. Abund**		**Av. Diss.**	**Diss/SD**	**Contrib%**	**Cum.%**
B & A	6.33	Man	4.88	8.22	3.48	3.52	54.95	54.95
		Glc	8.09	7.34	0.77	1.74	12.12	67.07
		Xyl	6.16	5.53	0.65	2.42	10.23	77.30
		Rib	5.15	4.77	0.39	1.13	6.17	83.47
		Ara	6.07	5.75	0.33	1.69	5.18	88.65
B & C	7.05	Man	4.88	3.73	1.27	1.41	18.03	18.03
		Glc	8.09	7.03	1.17	2.64	16.62	34.65
		Fuc	4.55	4.09	0.99	2.69	13.98	48.63
		Xyl	6.16	5.29	0.97	2.05	13.72	62.36
		Ara	6.07	5.38	0.77	2.76	10.92	73.27
A & C	8.15	Man	8.22	3.73	4.96	17.04	60.91	60.91
		Fuc	4.40	4.09	0.92	4.31	11.31	72.22
		Gal	7.00	6.57	0.47	1.84	5.78	78.00
		Ara	5.75	5.38	0.41	1.87	5.08	83.08
		Xyl	5.53	5.29	0.39	1.50	4.84	87.92

Discussion

In this study, the Fatty acid and Carbohydrate profiles of two freshwater macrophytes highlighted biochemical changes after exposed to two organic and one inorganic contaminants. The results confirmed that herbicides are toxic and affect biochemical composition of lipids and carbohydrates, with strong decreases in such profiles, thus decreasing nutritional values of *Lemna minor* and *Lemna gibba*.

The complete growth inhibition (no Yield increase) is achieved at higher concentrations of all contaminants for both species with statistical significance, although *L. gibba* show more tolerance to S-Metolachlor (SM) and Copper (Cu), with higher effective

concentrations than *L. minor*. According to Queirós *et al.* (2018) the EC_{50} for *L. minor* and *L. gibba* after Terbuthylazine (TBZ) exposure are 81.29 μg/L and 22.07 μg/L, respectively, revealing *L. gibba* is more sensitive to TBZ than *L.minor* in accordance to our results. Other studies performed with the green microalgae *Scenedesmus vacuolatus* (Copin *et al.*, 2016; Vallotton *et al.*, 2008) and *Chlorella pyrenoidosa* (Liu & Xiong, 2009) exhibited an EC_{50} equal to 3000 μg/L and 106 μg/L, respectively, after 48h exposure to SM. Comparing to our results, these microalgae show higher tolerance to the chemical than *L. minor* and *L. gibba*. Obermeier *et al.* (2015) exposed *L. minor* during 7 days to pethoxamide, a chloroacetamide (chemical family of S-Metolachlor), and reported a 15% growth inhibition at 2.5 μg/L, matching with our EC_{10} value for the same species exposed to SM. Although there is some information to compare with our results, none of them include interspecific differences. Indeed, our work is a remarkable study comparing toxicity benchmarks between two species of *Lemna* exposed to SM.

The toxic effects of the organic compounds are more evident, in terms of growth inhibition, than the inorganic one. This might be explained by the fact that Cu is an essential metal for the metabolism, when in small concentrations it may be required by the macrophytes, not promoting a harmful effect, but in high concentrations it produces growth inhibition and biochemical effects. Moreover, Cu is one of the most reported compounds found in the literature. Gopalapillai and Hale (2016) studied the toxicity effect of Cu in *L. minor* and report an EC_{50} value of 70.7 (50.0–99.8) μg/L, much different from our EC_{50} value of 199.20 (149.28-249.13) μg/L at frond inhibition growth. On the other hand, we could find some similarities with the study from Obermier and co-workers (2015) where 21% growth inhibition was achieved with 50 μg/L of Cu in *L. minor*, similar to our EC_{20} responding to 20% frond inhibition (EC_{20}=59.42 (31.53-87.30) μg/L). That study also compiles information about pigmentation loss and phytoremediation capacity of *L. minor* to metals and organic xenobiotics. We were able to see that both macrophytes lost pigmentation at the end of the bioassays, but the decline in the number of fronds with increasing Cu concentration was less evident, perhaps because *Lemna* spp. is able to absorb and resist to low Cu concentrations (Obermeier *et al.*, 2015).

According to several authors, Primextra® Gold TZ commercial formulation and their active ingredients promote alterations on lipid composition of other aquatic organisms, most of them from other trophic level (Filimonova *et al.*, 2016a; Gonçalves *et al.*, 2016, 2017;

Gutiérrez *et al.*, 2019; Mesquita *et al.*, 2018). Fatty acids (FA) and Carbohydrates alterations were expected since it has already been related that the active ingredients (S-Metolachlor and Terbuthylazine) are able to interfere with FA synthesis, due to its mode of action in plants and microalgaes. S-Metolachlor blocks the enzymatic activity of elongase, inhibiting the expression of FAE1 gene, responsible for very long chain fatty acids (VLCFA) elongation (Liu & Xiong, 2009; Robert *et al.*, 2007), whereas Terbuthylazine inhibits the photosystem II activity, altering the cellular energy balance for other metabolic functions (Filimonova *et al.*, 2016a). Additionally, all the chemicals (TBZ, SM and Cu) can also promote oxidative stress in plant cells (Neves *et al*., 2015; Ritter *et al*., 2008; Želježić *et al*., 2018), thus producing Reactive Oxygen Species (ROS) and promote lipid peroxidation, affecting the structural fatty acids important for the cells integrity. The biochemical alterations after Cu exposure were also expected, once this inorganic compound affects several mechanisms' pathways in photosynthetic organisms, such as photosynthesis, fatty acid metabolism and carbohydrate synthesis (Filimonova *et al.*, 2016a; Ritter *et al.*, 2008).

In this work, it was clear a decrease in the macrophytes FA contents at higher concentrations, since these chemicals have proven to cause several effects in photosynthetic organisms (plants and microalgae), rather than in animals. Yet, there was the exception of PUFA from the *L. gibba* exposure to TBZ at all treatments, and SFA and MUFA from the same species exposed to Cu at all treatments, that showed an increase content compared to the control. After Cu exposure *L. gibba* increased all FA content except the PUFA from the last treatment of 115µg/L. The trend associated to the increase of SFA and MUFA and a decrease in PUFA, may be the fact that *L. gibba* might not be able to complete elongation processes to synthetize PUFA, once this process is too energetically costly (Gonçalves *et al.*, 2017). Indeed, transitional metals (e.g. Cu) and other xenobiotics make UFA (MUFA and PUFA) more vulnerable to lipid peroxidation (Bielawska *et al.*, 2010), and thus it was expected to see a decrease in these class of FA, although we had some exceptions.

Literature studies found FA composition of *Lemna* sp. mainly constituted by the SFAs Lauric acid (C12:0), Myristic acid (C14:0), Palmitic acid (C16:0), Stearic acid (C18:0) and Eicosanoic acid (C20:0); and by the UFAs: Palmitoleic acid (16:1n9), Vaccenic acid (C18:1n7), Oleic acid (C18:1n9), Linoleic acid (C18:2n6), α-Linolenic acid (C18:3n3), γ-Linolenic acid (C18:3n6), Eicosadienoic acid (C20:2n6), Eicosatrienoic acid (C20:3n3) and Eicosapentaenoic acid (20:5n3) with high proportions of PUFA regarding the other groups

of FA composition (Chakrabarti *et al*., 2018; Zhao *et al.*, 2014). Our results also showed the same pattern for both *Lemna* species, although we have not found some FA, some of them the essential ones, similarly to other authors. The main PUFA detected for both species α-linoleic acid (C18:3n3) and linoleic acid (C18:2n6) were the highest FA content in all profiles, and the ones more affected by contamination. Those essential fatty acids are very important in the diet since some of them cannot be synthetized by animals, and due to their benefits in preventing cardiovascular diseases. α-Linoleic acid (ALA) is also an important precursor for animals production of other essential fatty acids, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), important for organs' health (Kim *et al.*, 2014), so the content decrease given by contaminants pose a risk for the nutritional composition transferred for other trophic levels. Therefore, FA profiles of freshwater organisms prove to be good bioindicators of chemical stress conditions at the aquatic ecosystems.

By our knowledge, no studies have evaluated the alterations in carbohydrate profiles by this herbicides in freshwater species, except the single work conducted by our research team, Gutiérrez *et al.* (2019a), exhibiting the changes in carbohydrate contents on the benthic clam *Scrobicularia plana* after exposure to SM and TBZ. According to that study, the main sugar present in *S. plana* was Glucose that may be explained by glycogen represents about 90% of the sugar supplies in the bivalves' species. Still, the present study found a great number of sugars present in *Lemna minor* and *Lemna gibba* as expected. Our results showed a great abundance of the Free Sugars Xylose, Mannose, Galactose and Glucose at control samples of both species, meaning both species have similar carbohydrate profiles. However, the Free Sugars profiles show interspecific differences after exposure to the contaminants: *L. gibba* exposed to TBZ showed a decreased of all sugars content, while *L. minor* was able to increase Xylose content at 31µg/L treatment.

The high amounts of the monosaccharide glucose in polysaccharides' profiles may be expected given the presence of starch, as plant energy reserve, and by the structural molecules of cellulose and hemicelluloses that compose the skeleton of the cell walls. Additionally, the high amounts of the Arabinose, Galactose, Rhamnose and Xylose in the two polysaccharides' profiles suggest the presence of high quantities of pectins present in cell wall, namely the pectin lemnan found by Ovodova *et al.* (2000) in *Lemna minor*. This pectin performs an enhancement of inflammatory responses (Ovodova *et al*., 2000; Popov *et al.*, 2006), make it an important polysaccharide. Zhao *et al.* (2014) also found that the

profile of the cell wall of *L. minor* was mainly constituted by a great amount of the pectin galacturonan (with small amounts of xylogalacturonan, rhamnogalacturonans) and a small portion of hemicellulose consisting of xyloglucan and xylan, being consisting with our amounts of sugars specially after the stress conditions, enhancing a protective response to the medium in the presence of contaminants.

The small increases of Polysaccharides in *L. gibba* profile after exposure to TBZ may suggest a defence mechanism by the production of polysaccharides from the cell wall, to maintain cells homeostasis. However, for *L. minor* the results after TBZ exposure were not consistent with those of *L. gibba*, showing a decrease in all sugars. Yet, we could see a similar pattern between both species after Cu exposure, whereas plants increased all sugar contents at the highest treatment (C3). Even though we could see small changes in the Carbohydrate contents, more studies are needed to understand the sugars mechanisms behind stress conditions, once there is no sufficient studies to prove carbohydrates from aquatic organisms as bioindicators of aquatic ecosystems' health. The increase of some monosaccharides' contents after contaminants exposure may be a strategic response to chemical stress, by some kind of mechanism.

Indeed, xenobiotics are able to induce the production of reactive oxygen species (ROS) by cells. Couée *et al*.(2006) reviewed that soluble sugars can play an important anti-oxidant defence, where high glucose content can function as signal pathway useful for plants, since it is a precursor sugar for the synthesis of amino-acids involved in glutathione blockage, which means it is involved in the oxidative stress defence. Moreover, the pesticides TBZ, SM and the metal Cu are known to interfere with photosystem II and other metabolism activities that produce ROS (Filimonova *et al.*, 2016a; Neves *et al*., 2015). Thus the present study also suggests that sugars increase in Duckweeds can generate an antioxidant response as defence mechanism under stress conditions. However, further studies in this subject are needed to understand better these mechanisms since they are not very clear and the sugars content here present are not very linear with these conclusions, and also the application of other biomarkers should be implemented to better understand the mechanisms involved.

The efforts from the work of the research team where this study was conducted have already established biochemical stress responses as good biomarkers for aquatic

contamination. Those studies include bivalves, crustaceans and microalgae exposed to the herbicide Primextra® Gold TZ, and to its active ingredients, individually, and to inorganic compounds, such as Cu. Thus, this work contributes to the compilation of information on toxic effects of organic and inorganic compounds mainly present in the Mondego estuary, and highlights the Fatty acids and Carbohydrates as good biomarkers of environment and food quality.

Conclusions

Giving the fact that *Lemna* sp. is a great macrophyte for remediation processes, it was expected that both aquatic plant species absorbed the contaminants and, therefore, produced toxic responses and biochemical alterations. Although there were significant toxic responses, not all treatments were statistically different from the control in Copper exposures. Exposure to S-Metolachlor and Terbuthylazine, on the other hand, obtained more significant differences. Indeed, Copper could be more aggressive giving its inorganic form, but according to our findings the organic compounds were more toxic to plants than the inorganic one. However, when the organic compounds are present in the same formulation (Primextra® Gold TZ) they show a synergistic effect, being more aggressive together than isolated. This subject should be studied in the future to further compare the results of the isolated compounds and their combination not only for *Lemna minor* and *Lemna gibba*, but for other aquatic species.

The presence of Terbuthylazine, S-Metolachlor and Copper decreased the fatty acid contents and increased some sugars as a result of the stress conditions. Indeed, this study highlights the PUFA decrease and Glucose content increase as good biomarkers for the detection of Terbuthylazine, S-Metholaclor and Cu in aquatic systems. Plants may increase the glucose content to potentiate an anti-oxidant response to prevent the oxidative damage by reactive oxygen species, although studies must be conducted to better understand the defence mechanisms. Even so, these small increases occurred at lower concentrations of the pollutants (concentrations given for EC_{10} and EC_{20} values), and greater amounts may damage such mechanisms and cause oxidative damage, enhancing cell death and consequently the death of plants. Despite the amount of the compounds here studied are still in lower concentrations at the environment, it is believed that there is a tendency to increase

in time with the increasing usage of pesticides and fertilizers, which may be concerning for aquatic fauna and flora, and thus producing more toxic responses and deaths on aquatic organisms.

This study is in agreement with the main objectives, but still more efforts are needed to understand the effect of several contaminants present in the aquatic environment, to improve existing legislation and make agrochemical products more environmentally friendly. Moreover, this study shows the biochemical biomarkers as important tools and endpoints in ecotoxicological studies and the potential to be used as early-warning indicators of the presence of these contaminants at the aquatic ecosystems and on the determination of potential effects in their communities and thus on food quality.

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