Universidade de Aveiro
Ano 2020

GILBERTO DIAS DE ALKIMIN

Efeitos de drogas farmacêuticas em espécies de água doce - uso integrado de ferramentas bioquímicas, fisiológicas e populacionais

Effects of pharmaceutical drugs in freshwater species – integrated use of biochemical, physiological and populational tools

Universidade de Aveiro
Ano 2020

GILBERTO DIAS DE ALKIMIN

Efeitos de drogas farmacêuticas em espécies de água doce - uso integrado de ferramentas bioquímicas, fisiológicas e populacionais

Effects of pharmaceutical drugs in freshwater species – integrated use of biochemical, physiological and populational tools

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica do Doutor Bruno André Fernandes de Jesus da Silva Nunes, Equiparado a Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e co-orientação do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e o Doutor Carlos Barata Martí, Investigador do Consejo Superior de Investigaciones Científicas (Barcelona/Espanha)

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma bolsa de doutoramento atribuída a Gilberto Dias de Alkimin (PD/BD/127922/2016).

Dedico este trabalho aos meus avós, Hilda e Waltides (*in memoriam*), que foram a base de tudo e, mesmo quando nada sabiam, se preocupavam em saber “como vão as coisas, fi?”. Se dedicavam, ofereciam carinho, amor e atenção.

o júri

Presidente: Doutor **João Carlos de Oliveira Matias**
Professor Catedrático, Universidade de Aveiro

Vogais: Doutor **Evaldo Luis Gaeta Espindola**
Professor Titular, Universidade de São Paulo

Doutor **Michiel Adriaan Daam**
Professor Auxiliar, Universidade Nova de Lisboa

Doutora **Isabel Maria Cunha Antunes Lopes**
Investigadora Principal, Universidade de Aveiro

Doutora **Ana Paula de Campos Mucha**
Investigadora Auxiliar, Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Doutor **Bruno André Fernandes de Jesus da Silva** (Orientador)
Equiparado a Investigador Auxiliar, Universidade de Aveiro

agradecimentos

Primeiro tenho que agradecer às forças da Natureza por me fazerem chegar até aqui, me dando força, me mantendo são e iluminando meu caminho. Agora, não em uma ordem de importância, mas sim mais cronológica...

À minha mãe, pessoa mais importante da minha vida. Mãe, obrigado. Te amo!

À minha família, em especial irmãs e sobrinhes, por mesmo longe, se fazerem perto e não me deixarem sentir só.

Ao Alex, primo, gratidão por me aturar, escutar minhas lamúrias, meus desabafos e mesmo assim conseguir me fazer rir e prosseguir.

Ao Bruno Nunes, prof, obrigado por ter me aberto as portas da sua equipe quando não me conhecia e ter acreditado no meu potencial, mesmo quando eu mesmo não acreditava. Obrigado pelos ensinamentos, pelas chamadas de atenção nos momentos necessários, mas também por me ouvir e ser um ombro amigo, por ver o meu choro e compartilhar risadas. Fica aqui minha gratidão por troca toda de experiência e aprendizado.

Ao Prof Amadeu Soares por me aceitar em seu grupo de pesquisa e todo apoio durante esses anos.

Não posso deixar de agradecer ao Willian, a Cintia e a Cleo. Palavras não descrevem a gratidão pela forma como me receberam e me acolheram quando cheguei em Portugal, fazendo me sentir em família. E ao Willian, por continuar, até quando esteve em Portugal a compartilhar momentos de risada, tristeza, viagens, alegrias e gordices entre ida e vindas entre Porto-Coimbra-Aveiro.

À Giu(liana), por ser aquele ombro amigo que me ensinou, me escutou e me ajudou, fazendo aquele início ser menos difícil. Gratidão!

À Anabela, por tantas conversas, risadas, tantos almoços compartilhados. Pelos desabafos e por me fazer sentir bem, obrigado!

Ao Abel, melhor técnico de sempre hehehe mesmo falando não tão bem do meu bolo hahaha Muito obrigado Abel.

Agradecimentos (cont.)

Ao David e ao Dionísio, obrigado por dividirem essa caminhada junto comigo, por aprendermos e crescermos juntos. Por compartilharmos ideias, horas e horas no laboratório trocando testes, contando daphnias, preenchendo placas e acabarmos a madrugada no Autocarro. E agora incluo a Ana e a Filipa pelos momentos de docinhos e gordices pra aliviar e adoçar o dia.

Ao David (de novo), por me apresentar a Vanessa. Aos dois, especialmente ao longo desse último ano, por serem o meu lar. Por serem, mesmo em tempos tão sombrios como o desta pandemia os meus ombros direito e esquerdo e assim sendo, o apoio um do outro para que ninguém surtasse e ninguém desistisse. O que foi feito com muito carinho e respeito....comida também, bebida... Aos dois, fica aqui minha eterna gratidão! (Não pensem que eu esqueci do "meu" portuguesinho e seja isso onde eu estiver).

À Sofia, por ter se tornado uma amiga para as mais diferentes horas, seja cinema, um jantar qualquer e até mesmo viagens a Barcelona, um tanto quanto peculiares hahaha. Cafés, conversas (sérias e sem sentido) e risadas. Voa garota, o mundo é teu!

A todos brasileiros que aqui passaram e, de alguma maneira me fizeram lembrar de casa. Me trouxeram o calor do hemisfério Sul e tornaram esse ciclo mais leve e cheio de carinho, companheirismo e divertimento. Em especial ao Eduardo, Mayara, Raquel, Gabi, Lê, Évila, Léo, Diego, Bruno e George, que me fizeram criar grandes memórias....obrigado galera!

A toda malta da Biologia da UA, Rafa, Hadden, Rosário, Rita, Diana, Max, Bea e tantos outros (são muitos). Mas que, de alguma forma, me fizeram sentir acolhido, pertencer a um grupo, ser amigo e ser querido. Tornaram esse ciclo mais leve e cheio de cores e risadas. Obrigado!

Ao Emanuel, independentemente de qualquer coisa, muito obrigado por, de alguma maneira, ter feito parte deste meu ciclo.

Aos amigos da Bio/09 que mesmo com a distâncias sempre se fizeram presentes e confirmaram que amizade não é estar e sim ser. Obrigado pessoal.

Agradecimentos (cont.)

Ao Benildo e ao Edilzo, por serem aqueles amigos que me faziam rir sempre e em momentos inesperados e que tornaram tudo mais leve e divertido.

Ao Carlos Barata, por abrir as portas do seu laboratório em Barcelona e compartilhar seu conhecimento. Obrigado!

Ao pessoal do IDAE-CSIC, representados pela Marta, pela paciência e ensinamentos. Meu muito obrigado.

Aos amigos de Barcelona, Cláudia, Olga, Irene, Inma, Rúben, Justo, Guilie obrigado por me receberem de braços abertos, me fazer sentir acolhido, se esforçarem pra entender o meu “espanhol” jajaja. Obrigado por cada risada, cada aventura e cada momento compartilhado! Muchas grácias chicos!

Aqueles e aquelas, aqui não citados, que, de forma direta e/ou indireta contribuíram para a realização desse trabalho. Muito obrigado.

À FCT pela bolsa de doutoramento em Portugal e no estrangeiro (Espanha).

palavras-chave

Lemna minor, *Lemna gibba*, *Daphnia magna*, ecotoxicologia, anti-inflamatório não-esteroidal, clorpromazina.

resumo

Os riscos potenciais apresentados pelos produtos farmacêuticos podem resultar em efeitos adversos em espécies ambientalmente expostas, que precisam ser estudadas. Assim, o principal objetivo desta tese foi compreender o efeito de duas classes de compostos farmacêuticos (anti-inflamatórios não esteróides e neuroativos) em espécies de organismos aquáticos de diferentes níveis tróficos, produtores (*Lemna* spp.) e um consumidor de primeira ordem (microcrustáceo - *D. magna*). Sendo assim, algumas questões foram elaboradas para abordar esta questão: os parâmetros não padronizados são adequados para estudar os efeitos de compostos farmacêuticos em espécies de macrófitas? O tempo de exposição é um fator que pode influenciar o tipo de resposta biológica aos produtos farmacêuticos, avaliados em espécies de macrófitas? Que tipo de interações e respostas podem ser obtidas após a co-exposição de macrófitas a dois medicamentos, sendo um deles também um composto endógeno? Um composto farmacêutico pode provocar os mesmos efeitos (tipo e extensão) em organismos de diferentes níveis tróficos? Como endpoints funcionais de *D. magna* respondem ao estresse químico (farmacêutico) e natural (kairomona de peixe), isolado e em combinação? Esses objetivos foram desenvolvidos ao longo de cinco capítulos, usando abordagens ecotoxicológicas padrão e não padronizadas, do indivíduo ao nível populacional, através do uso integrado de ferramentas fisiológicas, bioquímicas e populacionais. Os resultados obtidos mostraram que o uso de organismos do mesmo gênero (mas de espécies diferentes) em testes ecotoxicológicos de produtos farmacêuticos pode gerar resultados diferentes quanto à sua toxicidade; concluímos que parâmetros ecotoxicológicos não padronizados, baseados em plantas aquáticas, são ferramentas adequadas e promissoras para avaliação ecotoxicológica de produtos farmacêuticos. Também foi possível observar que o tempo é um fator que contribui para a toxicidade de fármacos em plantas aquáticas, modulando não apenas a extensão da resposta tóxica, mas principalmente o tipo dos efeitos tóxicos. Um contaminante que também é um composto endógeno produzido pelas plantas pode modular as respostas das espécies de macrófitas submetidas a um stress. Em geral, os medicamentos mostraram uma toxicidade eminentemente espécie-específica, cujos efeitos foram alterados de acordo com o tipo de medicamento, seus níveis, duração da exposição e co-ocorrência de outras substâncias bioativas no meio. E, finalmente, os resultados apresentaram a capacidade de interação entre um estressor antropogênico e natural, influenciando nas respostas dos microcrustáceos aos mesmos. Em conclusão, as drogas farmacêuticas têm se mostrado muito tóxicas para os organismos aquáticos; o uso de plantas aquáticas em avaliações ecotoxicológicas é, portanto, uma ferramenta valiosa, cujo uso requer a proposta de novas metodologias e diretrizes de teste ou a revisão de métodos padrão já em vigor, considerando suas vantagens gerais como organismos-teste. Além disso, a crescente relevância ecológica dos dados obtidos em testes baseados em crustáceos deve abranger a co-ocorrência de estressores naturais, cujos efeitos vão muito além da toxicidade por si só, alterando as características de vida dos organismos expostos, alterando assim as propriedades da população dos organismos expostos. Novos desenhos e abordagens experimentais precisam ser desenvolvidos e aplicados para tentar entender os efeitos toxicológicos de um ambiente complexo como ecossistemas aquáticos, principalmente quando se considera o uso de espécies de plantas aquáticas e crustáceos de água doce como organismos-teste.

keywords

Lemna minor, *Lemna gibba*, *Daphnia magna*, ecotoxicology, non-steroidal anti-inflammatory drug, chlorpromazine.

abstract

Potential risks posed by pharmaceuticals, may result in adverse effects in environmentally exposed species, which need to be studied. Thus, the main objective of this thesis was to understand the effect of two classes of pharmaceutical compounds (neuroactive, and non-steroidal anti-inflammatory drugs) on species of aquatic organisms from different trophic levels, namely, producers (*Lemna* species) and a consumer of first order (microcrustaceans - *Daphnia magna*). Same specific questions were posed to address this issue: Are non-standard endpoints suitable to study pharmaceutical effects in macrophyte species? Is time of exposure a factor that can influence the type of biological response to pharmaceuticals, measured in macrophyte species? What type of interactions and responses might be obtained after co-exposing macrophytes to two drugs, being one of them also an endogenous compound?, Can a pharmaceutical compound provoke the same effects (type and extent) in organisms from different trophic levels? How *D. magna* functional endpoints respond to the chemical (pharmaceutical) and natural stress (fish kairomone), isolated and in combination? These objectives were addressed along five chapters, by using standard and non-standard ecotoxicological approaches from the individual to the populational level through an integrated use of physiological, biochemical and populational tools. The obtained results showed that the use of organisms of the same genus (but from different species) in ecotoxicological testing of chemicals may result in different results as to their toxicity; we concluded that plant-based, non-standard ecotoxicological parameters are suitable and promising tools for ecotoxicological evaluation of pharmaceuticals. It was also possible to observe that time is a factor that contributes to the toxicity of pharmaceutical drugs in aquatic plants, by modulating not only the extent of the toxic response, but especially the type of the toxic effects. A contaminant that also is an endogenous compound produced by plants can modulate the challenged macrophyte species responses. In general, pharmaceutical drugs showed an eminently species-specific toxicity, whose effects were altered according to the type of drug, its levels, duration of exposure, and co-occurrence of other bioactive substances in the media. And finally, the results presented the capacity of interaction between an anthropogenic and natural stressor influencing in microcrustaceans responses to these stressors. In conclusion, pharmaceutical drugs have been shown be very toxic to aquatic organisms; the use of aquatic plants in ecotoxicological assessments is thus a valuable tool, whose use requires the proposal of novel methodologies and test guidelines, or the revision of already in place standard methods, considering their overall advantages as test organisms. In addition, increased ecological relevance of data obtained from crustacean-based tests must encompass the co-occurrence of natural stressors, whose effects go well beyond toxicity by itself, by changing the life traits of exposed organisms, thereby altering population features of exposed organisms. New experimental designs and approaches need be developed and applied to try understanding the toxicological effects of a complex environment as aquatic ecosystems, principally, when considering the use aquatic plant species, and freshwater crustaceans as test organisms.

Table of contents

Figure list

Chapter 2

Chapter 3

Chapter 4

Chapter 6

List of tables

Chapter 4

Chapter 1

General Introduction

1.1 Pharmaceutical compounds

A diverse array of synthetic organic compounds are used by the modern society in vast quantities for wide a range of purposes, including the production and preservation of food, industrial manufacturing processes, as well as for human and animal healthcare (Huschek et al., 2004; Lapworth et al., 2012). In fact, every day, industries, agriculture facilities and the general population are using water and releasing many compounds, including pharmaceutical drugs, in wastewater. Indeed, agriculture practices, industrial and hospital discharges, and the human excretion of pharmaceuticals play an important role in pollution of surface and wastewater, in general (Deblonde et al., 2011; WHO, 2012). The scenario of worldwide contamination by pharmaceutical drugs is an important issue since over 100,000 tonnes of pharmaceutical products are consumed globally every year. Approximately 24% of this amount in Europe alone (EEB, 2018). Thus, pharmaceuticals are considered emerging pseudo-persistent pollutants since they are continuously being released into the environment by WWTP or other sources, thereby affecting the exposed biota at multiple levels (Bu et al., 2016). Consequently, global concern about their potential adverse impacts on both human health, and the environment is growing (Bielen et al., 2017), thereby requiring the development of new regulatory actions (Küster and Adjer, 2014), and building a large body of evidence (namely, comprehensive ecotoxicological data of the most used pharmaceutical classes) supporting such options.

This can happen since, conventional wastewater treatment plants (WWTPs) are designed to deal with bulk substances that occur in wastewater regularly and in large quantities (primarily organic matter and the nutrients, nitrogen and phosphorus) (OECD, 2019). Moreover, new technologies and novel treatment systems have been recently studied and developed to help WWTPs removing pharmaceutical drugs from their influents, to produce a cleaner effluent; however, even state-of-the art technologies are not completely effective for the removal of drug residues from wastewater. Among these methodologies, one may find techniques such as conventional activated sludge (Oppenheimer et al. 2007; Nielsen et al. 2013), biological processes, hydrodynamic cavitation and UV treatment (Zupanc et al., 2013), intermittent electrocoagulation (Ensano et al., 2017), membrane bioreactors (MBRs) (Clara et al., 2005; Radjenović et al., 2009; Kim et al., 2014; Verlicchi et al., 2010; Kovalova et al., 2012), and moving

bed biofilm reactors (MBBRs) (Escola Casas et al., 2015). In addition, treatments such as advanced oxidation processes (AOPs) that rely on ozone and UV radiation (Rojas et al., 2013; Beijer et al., 2017; Roccaro, 2018) can be used; these treatments, using reactive oxygen species and nanomaterials (activated or not by a light source, UV/Visible/Sunlight – photocatalytic process; Yaqoob et al., 2020) for the treatment of wastewater, are able to deal with almost all classes of organic compounds (Cincinelli et al., 2015). The efficiency of some of these new methods of wastewater treatment was analyzed by Angeles et al., (2020), after testing the effectiveness of a number of conventional and more advanced treatment methods, for the removal of pharmaceuticals from municipal WWTPs, including a highly advanced pilot plant. The authors concluded that each technology has its own advantages, and any of them were capable to equally remove the different pharmaceutical compounds, being their overall efficacy is far from complete.

The low removal rates of pharmaceutical drugs at WWTPs occurs since drugs are an entirely different group of chemicals; they are single compounds with a unique behaviour in the treatment plant (considering their structures, chemical stability, biological effects, and toxicological profile), and they represent only a minor part of the wastewater organic load (Larsen et al., 2004). Due to these features, and to their physiological properties and physical chemical characteristics, the majority of pharmaceuticals are not removed during water treatment (Kaleniecka and Zarzycki, 2015). Taking into account their widespread use, inability of WWTPs to treat them, and consequently discharge into the aquatic environment, the review by aus der Beek et al. (2016) concludes that the most different classes of drugs are detected in the aquatic environment (different compartments) worldwide in the order of ng/L to mg/L. In fact, incomplete removal favors the continuous discharge of pharmaceuticals into the environment, which may result in a chronic exposure of aquatic organisms to these compounds and/or their bioactive metabolites and transformation products (Lolić et al., 2015). In general, the WWTPs, as already mentioned, are not designed to treat pharmaceutical drugs and, in addition, the treatment phases of each WWTPs can be different, resulting in a different pharmaceutical rate of treatment/removal which varies from one installation to another. According to Angeles and co-workers (2020) the pharmaceutical removal depends on WWTPs characteristic, being these drugs not

completely removed and the treatment efficiency can range from negative values to 100%, but mostly in a lower percentage. Negative values for removal efficiencies are reported due to the increase of concentrations for target pharmaceuticals in WWTP effluents when compared to the loads of similar compounds found in the influents (Zorita et al., 2009). Non-steroidal anti-inflammatory (NSAIDs), for example, can be removed from -174 to 99% with a mean value of 34.4% (Quintana et al., 2005; Behera et al., 2011; Kosma et al., 2014; Sari et al., 2014; Kermia et al., 2016; Sun et al., 2016), however, as already mentioned, the removal capacity depends on the applied treatment. Even though NSAIDs are sometimes efficiently removed during wastewater treatment, they are also ubiquitous and present at substantial concentrations in water bodies receiving effluents from WWTPs (Yankelani et al., 2019). In addition, neuroactive drugs, follow the same ttrend described for NSAIDs, in general they have a remove rating from -30 to 100% with mean values around 25% (Metcalfe et al., 2003; Radjenovic et al., 2007; Subedi et al., 2013, 2015; Villar-Navarro et al., 2018).

The public and scientific awareness for this reality was incremented along time. Two decades ago, studies on the topic of pharmaceuticals in the environment, and of their ecotoxicological effects in general, and aquatic ecosystems in particularly, were rare (Brooks, 2018). For example, a recent Web of Science search with the terms ‘aquatic’ and ‘pharmaceutical’ yielded just six published documents until 1998; this number grew to 636 in 2019 alone; and, during less than 3 months in 2020, this number already reached 128. The new advances in analytical methods allowed the detection and quantification of minute amounts of these chemicals in natural waters (Snyder et al., 2010). These technologies include, for example, gas chromatography with mass spectrometry (GC-MS) and GC with tandem MS ($GC\text{-}MS^2$), or liquid chromatography with MS (LC-MS), and LC with tandem MS ($LC\text{-}MS_2$), that are more sensible than LC with ultraviolet (UV) or fluorimetric detection (Fatta et al., 2007).

The increase in the number of studies concerning this broad issue of the environmental contamination by drugs is important since these compounds exhibit a large set of features that turn them into particularly environmental troublesome compounds. Drugs have been designed and synthetized to be biologically active and to cause very specific effects in target organism, according with their pharmaceutical class, nature, and chemical characteristics. However, potential risks posed by exposure to

pharmaceuticals, may result in adverse physiological consequences in exposed species, which are likely to be considered as ecotoxicological effects. The scientific literature now shows a profuse number of data on the ecotoxicological effects of drugs, ranging from acute and chronic toxicity, genotoxicity, carcinogenicity, enzyme inhibition, oxidative stress, epigenetic alterations, behavioral changes, and others (Alkimin et al., 2019a; Antunes et al., 2013; Araújo et al., 2019; David and Pancharatna, 2009; De Felice et al., 2019; Fontes et al., 2018; Hong et al., 2007; Imhof et al., 2017; Li et al., 2008; Oliveira et al., 2015; Silva et al., 2019; Wang et al., 2019). Other effects of pharmacological are mediated by the interference with the hormonal and immune systems of biota (Desforges et al., 2016; Zelikoff, 1998; Ji et al., 2013; Windsor et al., 2018; Koagouw and Ciocan, 2019). Another field of extreme importance related to the presence of antibiotics is resistance development of microorganisms towards therapeutic compounds (Komolafe, 2003; Davies and Davies, 2010; Sayadi et al., 2010; Li and Webster, 2018). Certain pharmaceutical substances can also accumulate in the organism' tissues and be transferred to higher trophic levels along food chains, which makes them extremely hazardous to health or life of organisms, including humans (Kaleniecka and Zarzycki, 2015; Lagesson et al., 2016; Xie et al., 2017; Nunes et al., 2020).

Thus, evaluating pharmaceutical ecotoxicology is challenging due to uncertainties about appropriate dosages, to be able to understand the most likely toxicological effects, durations of exposure, selection of sensitive taxa, sensitivity of developmental stages, and toxicological endpoints (Kostich and Lazorchak, 2014). In this regards the knowledge of the pharmacokinetic properties in the species actually existing in the wild is vital (Randak, 2009) to try understanding the effects. In general, aquatic organisms are non-target species, and no data exist in the literature for these organisms. Furthermore, the understanding of comparative biological traits among species is critical when selecting a model organism to be used in further bioassays and to quantify responses during ecotoxicology studies with pharmaceuticals and other acting chemicals that act through extremely specific toxicity mechanisms (Brooks, 2018).

The pharmaceuticals compounds that have been found in the aquatic compartment belong to a large number of pharmaco-therapeutic classes, including contraceptives, β-blockers, antiparasitics, antibiotics, antifungals, antiepileptic, anti-inflammatory and

psychotropics; drugs from the last two mentioned classes were selected to be studied in this thesis.

1.1.1 Neuroactive drugs

The first major breakthroughs in the development of effective neuroactive drugs came in the years following the Second World War (Weissman, 2015), when the chemical-based antipsychotic therapy was first studied. This led to the successful development and practical proposal of drugs to be used in antipsychotic therapy. This class of pharmaceutical drugs are primarily used to manage psychosis. The word “psychosis” is used to describe conditions that affect the mind, and in which there has been some loss of contact with reality, often including delusions (false, fixed beliefs) or hallucinations (hearing or seeing things that are not really there) (NIH, 2016a). It can be a symptom of a physical condition, such as drug abuse, or a mental disorder including schizophrenia, bipolar disorder, or “psychotic depression” (NIH, 2016a). This last condition is a depressive state, which in general corresponds to a constant mindset of sadness; this is a modern concept of affective disorders, associated with psychosis disorders, such as delusions and/or hallucinations (Paykel, 2008).

The introduction of effective anti-psychotics, to treat schizophrenia revolutionized the therapeutics of human mental illnesses. The major step forward, as mentioned, was in the schizophrenia treatment, which is a chronic and severe mental disorder that affects how a person thinks, feels, and behaves (NIH, 2016b). The proposed antipsychotic therapy aimed to control mostly the acute symptoms of psychotic episodes, which consists in clearly psychotic symptoms such as delusions, hallucinations, disordered speech, disorganized behavior, flat affect, avolition and alogia. This is a mental disorder without cure, thereby requiring the research and development of new and safe treatments, namely the use of drugs (Parekh, 2017).

However, there are other classes of psychotropic drugs, which may be classified according to many features. Their division can be based on the pharmacological domains, that are based on the neurotransmitter involved in the modulation of the altered response caused by the drug. Based on this criterion, there are 11 domains,

namely drugs that act on acetylcholine, dopamine, GABA, glutamate, histamine, ion channels, lithium-mimetic, melatonin, norepinephrine, opioid, and serotonin (Rao and Andrade, 2016).

Chlorpromazine, discovered in 1950, was the first marketed neuroleptic in 1952 used to treat schizophrenia (Martson, 2013), being included in the older or first-generation antipsychotic medications that are also called conventional "typical" antipsychotics or “neuroleptics”. Other substances of this class include haloperidol, perphenazine, and fluphenazine (NIH, 2016a), which in general share the same mode of action (dopamine receptors antagonists) (Guzmam, 2019) and, in some cases, potential to cause extrapyramidal side effects and tardive dyskinesia, in special the last three (Guzmam, 2019; Jibson, 2020).

In 2017, an estimated 46.6 million adults aged 18 or older in the United States suffered from mental illness; this number represented 18.9% of all U.S. adults (NIH, 2019) and, in 2018 a total of 611,780,251 prescriptions were made for psychiatric medications in the U.S., at a cost of over $29 billion (Grohol, 2019). In a broader perspective, according to the Organization for the Economic Co-operation and Development (OECD), in 2018, Portugal was the second country that consumed more drugs acting on the nervous system, totalizing 326.2 defined daily dosage per 1000 inhabitants per day, just behind Iceland with 396.9 dosages, and before Sweden with 304.6 dosages; in Canada this number was of 230.2 dosages, and in Asia (South Korea) values to 2017 was equal 112.4 dosages (OECD, 2019). We may conclude that the consumption of these drugs is extremely high in comparison with those used, for example, for diabetes and treat conditions of the respiratory system (OECD, 2019).

As a direct consequence of this wide use, these drugs are frequently found in the environment, especially in aquatic ecosystems. In a review, Cunha et al. (2017) observed that some psychotropic drugs are detected in different water matrices, including wastewater treatment effluents and freshwater in concentrations ranging from 0.14 to 840,000 ng/L. In addition, drugs of this class were detected ranging 0.14 and 2.81 ng/L in UK drink water (Peng et al., 2019). In the Lis river (Portugal) and in the influents and effluents of two wastewater treatment plants located along this river, the psychiatric drugs were the second group that more contributed to the total mass load of

pharmaceuticals entering the river, just behind to non-steroidal anti-inflammatory drugs/analgesics (Paíga et al., 2016). Among psychotropic drugs, antipsychotics (e.g. haloperidol, perphenazine, fluphenazine, clozapine, olanzapine, quetiapine, chlorpromazine and others), have been found in different aquatic matrices, in concentrations around 1 to 500 ng/L. However antipsychotics drugs have been detected in concentrations up to 13,200 ng/L (Roberts and Bersuder, 2006; Yuan et al., 2013; Logarinho et al., 2016; Reichert et al., 2019) and their detection varied according to the hour of the day and with the day of week (Reichert et al., 2019). In addition, the biodegradability of pharmaceuticals from this group can be very variable and depend on water parameters, namely pH, dissolved oxygen, and/or light intensity (Felmeister and Disher, 1964; Trautwein and Kümmerer, 2012; Hermann et al., 2016). These features favor the widespread presence of this class of pharmaceutical drugs in the aquatic environment.

Among antipsychotic drugs, chlorpromazine (CPZ), a dimethylamine derivative of phenothiazine, with the chemical formula of 2-chloro-10-[3-(dimethylamino) propyl] phenothiazine monohydrochloride was selected for study. It was synthesized in December 1951 in the laboratories of Rhône-Poulenc and became available after prescription in France in November 1952 (Ban, 2007). This drug was also approved by Food and Drug Administration in 1954 (Kane and Correll, 2010). CPZ was the first antipsychotic drug in the market and was followed by a large number of other antipsychotics. However, so far, no antipsychotic has been shown to be significantly more effective than chlorpromazine in treating schizophrenia with the notable exception of clozapine (Haddad et al., 2016). Nevertheless, it remains on the World Health Organization list of essential medicines (WHO, 2019). In the USA, the market for CPZ (hydrochloride) tablets is estimated to be approximately $207 million in annual sales for the 12 months ended July 2018 (Bridgewater, 2018).

The main mode of action of CPZ occurs through blocking the dopamine receptors (Ban, 2007). However, it can also block histamine, adreno, muscarinic, and 5-hydroxytryptamine receptors (Sepulveda et al., 1994), decreasing neural activity and exerting a sedative and antiemetic activity (Man and Marwaha, 2019). CPZ main usage is to treat schizophrenia, however, it can be used in other psychiatric conditions for the control of anxiety and tension (Borges et al., 2011), as well to control nausea and

vomiting, persistent singultus (chronic hiccups), relief of apprehension before surgery (Man and Marwaha, 2019).

CPZ, in mammals, is metabolized in the liver and kidneys by cytochrome P450 isoenzymes CYP2D6 (major pathway), CYP1A2 and CYP3A4; 20% of CPZ and its metabolites are excreted unconjugated in the urine as unchanged drug, demonomethylchlorpromazine, dedimethylchlorpromazine, their sulfoxide metabolites, and chlorpromazine-N-oxide. The remaining 80% consists of conjugated metabolites, principally O-glucuronides and small amounts of ethereal sulfates of the mono- and dihydroxy-derivatives of CPZ and their sulfoxide metabolites; finally, approximately 37% of the administered dose of CPZ is excreted in urine (DrugBank, 2020).

In the aquatic environments, CPZ data are scare, however, it was found in amounts up to 100 ng/L in the Baltic Sea (UNESCO and HELCOM, 2017), and in hospital and municipal wastewater treatment plants in Beijing (China) in concentrations reaching 364 ng/L (Yuan et al., 2013); Roberts and Bersuder (2006) detected this pharmaceutical in concentrations up to 9 ng/L in surface water and sewage effluent samples. In addition, this pharmaceutical drug made part of the OSPAR list, which included drugs posing possible concern to aquatic environment (McEneff et al., 2015). However, the toxicological effects of this pharmaceutical to non-target organisms, especially aquatic species is very limited, restricted to *Daphnia magna* (Oliveira et al., 2015), *Lemna minor* and *L. gibba* (Alkimin et al., 2019b), and fish (*Carassius auratus* - Li et al., 2008).

1.1.2 Non-steroidal anti-inflammatory drugs

The ability to treat fever and inflammation dates back to approximately 400 b.C. This treatment started with the early use of decoctions or preparations of plants containing salicylates, following the Egyptian Ebers papyrus recommendation for the application of a decoction of the dried leaves of myrtle to the abdomen and back, to counteract rheumatic pains from the womb (Vane and Botting, 1998). The search for anti-inflammatory effects goes to a time when the Greek physician Hippocrates prescribed an extract from willow bark and leaves (Rao and Knaus, 2008). Later in the 17th

century, the active ingredient of willow bark salicin was identified in Europe, and The Kolbe company in Germany started mass producing salicylic acid in 1860 (Rao and Knaus, 2008) thereby sowing the seeds to modern therapeutics with non-steroidal anti-inflammatory drugs (NSAIDs).

Over the years, the NSAIDs usage systematically increased. In general, NSAIDs are anti-inflammatory, analgesic, and antipyretic agents, being typically used chronically to reduce pain, decrease stiffness, and improve function in patients with osteoarthritis, rheumatoid arthritis, and other forms of arthritis (Simon, 2013). Thus, NSAIDs are one of the most prescribed classes of medication for pain and inflammation, besides their antipyretic activity. In Italy, their consumption is around more than 100 tons/year (OsMEd, 2013) while, in Portugal, acetylsalicylic acid (Aspirin®) alone is consumed annually in more than one hundred billion tablets, with a production of around 40,000 tons per year (Freches, 2017).

Despite the diversity of their chemical structures, these drugs share the same therapeutic properties (Vane and Botting, 1998; Laufer, 2007; Ghlichloo and Gerriets, 2020). The major mechanism of action of NSAIDs is the inhibition of cyclooxygenases (COX; COX-1 and COX-2) which are involved in the production of prostaglandins (PGs) and thromboxanes (derivatives of arachidonic acid), which are in turn inflammatory mediators (Brooks and Day, 1991). COX-1 is expressed in normal cells, and is responsible for the production of PGs and thromboxane A2, which control a diversity of functions, including mucosal barrier in gastrointestinal tract, platelet aggregation, renal homeostasis and other physiological functions.On the other hand, COX-2 is involved in the production of PGs that are related with inflammation, pain and fever, PGs that are induced in inflammatory cells (Cashman, 1996; Rainsford, 2007). Thus, COX-2 inhibition most likely represents the desired effect of NSAIDs' anti-inflammatory, antipyretic and analgesic response; while COX-1 inhibition plays a major role in the undesired side effects, such as gastrointestinal and renal toxicities. (Wongrakpanich et al., 2018). Some NSAIDs block both COX-1 and COX-2, being designated nonselective NSAIDs. This NSAIDs group includes acetylsalicylic acid, ibuprofen, diclofenac, fenoprofen, indomethacin, naproxen and other (Risser et al., 2009; FDA, 2018).The group of selective NSAIDs (blocking only COX-2), is a smaller group and includes drugs such as celecoxib, valdecoxib and rofecoxib (Wright, 2002; FDA, 2018).

However, there is still much that is not known about how these COX proteins work (Jin, 2015).

In environmental terms, NSAIDs is among the most frequently detected pharmaceutical drugs. Drugs from this class were the most detected in River Lis in Portugal (Paíge et al., 2016) and diclofenac, ibuprofen, naproxen, paracetamol and acetylsalicylic acid, are included in the short list of drugs whose presence was determined worldwide (eastern Europe; Latin America and Caribbean; western Europe; Asia-Pacific and Africa, der Beek et al. 2015)

NSAIDs have been found in all aquatic compartments, ground, drink, sea and fresh-water, and also in influents and effluents of waste water treatments plants in concentrations of ng/L to µg/L (Pereira et al., 2016; Bound and Voulvoulis, 2006; Robert and Thomas, 2006; Heberer, 2002; Jux et al., 2002; Weigel et al., 2002 Ternes, 1998; Paíga et al., 2016; Farré et al., 2001; Metcalfe et al., 2003).

Among the most commonly used NSAIDs, paracetamol (also known as acetaminophen – APAP) is a non-opioid, antipyretic, and analgesic drug, first used clinically in 1893. Paracetamol is now probably the most commonly used drug worldwide, available as an over the counter preparation, used in almost all ages, and forming Step 1 of the World Health Organization analgesic ladder (Chiam et al., 2015; Sharma et al., 2014). Paracetamol is widely regarded as cheap, safe and effective (Fleming, 2015). Consequently, 22.5 million of prescriptions (around 200 million packets) of paracetamol are sold annually, accounting, for two-thirds of the UK market for over-the-counter painkillers. Despite its differences in both action mechanisms and profile of side effects, APAP is considered a weak PGs inhibitor (Graham and Scott, 2005) and often included among the classifications group of the NSAIDs drugs (Ward and Alexander-Williams, 1999). However, after more than 100 years, the exact mechanism of action of APAP remains to be determined. There is evidence for a number of central mechanisms, including effects on prostaglandin production, and on serotonergic, opioid, nitric oxide (NO), and cannabinoid pathways, and it is likely that a combination of interrelated pathways are in fact involved in the pharmacological activity of APAP (Sharma et al., 2014). It is likely that APAP has a multifactorial mechanism of action,

which may include the activation of different pain pathways, hence the difficulty in elucidating its precise mechanism of action (Chiam et al., 2015).

Primarily, APAP is metabolized in the liver by phase I and II enzymes. The metabolism of paracetamol occurs through glucuronidation (52-57%), sulfation (30–44%) and oxidation (5%) (Prescott and Critchley, 1983). The first two processes yield inactive, nontoxic final products which are eventually excreted by the kidneys. However, the hepatic cytochrome P450 enzyme system, in the oxidation pathway, metabolizes APAP, forming catechol and NAPQI (N-acetyl-p-benzo-quinone imine) (Nelson and Bruschi, 2007) that is responsible for the already described APAP hepatotoxicity. In addition, high levels of APAP lead to a reduction of the glutathione reserves, which may fall to less than 30% of the normal (Mitchell et al., 1973; Agrawal and Khazaeni, 2020). As a consequence, the NAPQI concentration in liver increases, preventing thus ATP biosynthesis, and leading to DNA and RNA damages, binding to proteins and subcellular structures, causing cell death and consequently necrosis (Ben-Shachar et al. 2012). APAP also induces accumulation of intracellular peroxide (due to glutathione depletion) leading to an increase in damages by reactive species of oxygen (ROS) via Fenton mechanism, causing oxidative stress (Hinson et al. 2010).

In the aquatic environments, APAP has been considered a priority pharmaceutical (Voogt et al., 2009), based on literature review and a series of criteria (including toxicity, persistence, environmental fate, etc.) (Giri et al., 1993; Hinson, et al., 2010; Hinz and Brune, 2012; Nunes et al., 2014; Woang and Graundis, 2017; McCrae et al., 2018; Żur et al., 2018). Paracetamol was already detected in different aquatic systems as ground, fresh and saltwater. Its presence was also determined in drinking water. In Brazilian costal zones APAP was detected in levels around 20 ng/L (Pereira et al., 2016), while in Las Palmas de Gran Canaria (Spain) around 290 ng/L (Afonso-Olivares et al., 2013). In UK surface waters of small rivers the maximum detected concentration was 555 ng/L (Bound and Voulvoulis, 2006) and up to 65 ng/L in Elbe River (Wiegel et al., 2004). It was also detected at levels of 0.036 µg/L and 6.5 µg/L, respectively, in public and private supplies wells in Massachusetts (Zimmerman et al., 2005) and 0.38 µg/L in groundwater samples (Barnes et al., 2008). Finally, the presence of paracetamol also occurred in amounts up to 65 µg/L in wastewater (Robert and Thomas, 2006).

The toxicity of paracetamol has been demonstrated across different hierarchical biological levels and in distinct organisms. Its effects were reported at the molecular level in *Mytilus edulis* in genes from gonads related with the endocrine system and hormonal production (Koagouw and Ciocan, 2019), at the biochemical level in the clams *Venerupis decussata* and *Venerupis philippinarum* (Antunes et al., 2013), in the crustacean *Daphnia magna* (Oliveira et al., 2015; Daniel et al., 2019) and in the mollusk *Phorcus lineatus* (Almeida and Nunes, 2019). APAP also caused alterations in embryonic zebrafish larvae development (David and Pancharatna, 2009; Nogueira et al., 2019) and also was responsible for behavioral impairment in zebrafish larvae (Nogueira et al., 2019). In addition to physiological disturbances, APAP affected the chlorophyll content in *Lemna minor* and *L. gibba*, (Alkimin et al., 2019b).

Diclofenac sodium (DCF) is a potent NSAID, which belongs to the class of phenylacetic acid derivatives comprising phenyl acetic group, secondary amino group, and phenyl ring having two ortho chloro groups (Menassé et al., 1978). The name "diclofenac" has been derived from its chemical name 2-(2,6-dichloroanilino) phenylacetic acid (Hafeez et al., 2018). Since its synthesis in a commercial form in 1973 by Rudolf Pfister and Alferd Sallmann (Sallmann, 1986), the use of this pharmaceutical was allowed in 120 countries for the last 40 years, being ranked in the 30th position among the list of top 200 drugs worldwide. For example, in 2017, DFC reached more than 8.5 million prescriptions in the USA alone, being in the top 100 most prescribed pharmaceutical drugs, with a top 3 positions among all NSAIDs, just behind meloxicam and naproxen (ClinCalc, 2020a).

DCF is widely used to reduce inflammation and to relieve pain in conditions such as arthritis or acute injury (McGettigan and Henry, 2013). It is also active as antiuricosuric (Lonappan et al., 2016) and is also important for the relief of post-operative pain (caused by inflammation) and some moderate muscular pain disorders (Hafeez et al., 2018). DCF is highly persistent in the environment and difficult to be degraded by WWTP, thus it was included in the priority list developed by Voogt et al. (2009) of pharmaceuticals relevant for the water cycle and was classified as class 1 – high-priority pharmaceuticals which forced the water industries to search for technologies to remove DCF from wastewater, since this is one of the most used worldwide pharmaceuticals, being also detected in the environment (soil and water) (Lonappan et al., 2016). DCF

was also included in its First Watch List of the Water Framework Directive, in order to obtain more information on its occurrence and effects in the environment (EU 2015/495, European Commission; Bonnefille et al., 2018). Although DCF has been proved to be very effective, this drug also exhibits limitations and adverse effects toward gastrointestinal irritation, ulceration, and hepatotoxicity.

In mammals, the metabolism of DCF mainly takes place in the liver and involves two major pathways, namely oxidative metabolism and conjugation with glucuronic acid. The oxidative metabolism (ring hydroxylation) of DCF is catalyzed by enzymes of the cytochrome P450 family (CYP2C9 and CYP3A4 – Tang, 2003); this results in the formation of 4-hydroxydiclofenac and 5-hydroxydiclofenac as the major metabolites (Boelsterli et al., 2003). The above-mentioned metabolites of DCF can be further oxidized to a highly reactive benzoquinone imine with great potential for redox cycling and hence oxidative stress; these metabolites are excreted from the body through the renal route (Tang, 2003). DCF is also conjugated to glucuronic acid, this results in the formation of an acyl glucuronide which is a potentially reactive metabolite. The electrophilic carboxylic carbon on the acyl glucuronide can be further metabolized to 4-hydroxydiclofenac acyl-glucuronide, and subsequently to a benzoquinone imine, which can cause oxidative stress to cells by redox cycling (Vickers, 2009). The acyl glucuronides are largely excreted from the body through the biliary system (Tang, 2003).

Considering DCF excretion and other usages/discharges, it can be detected in different environmental compartments, such as seawater, in concentrations up to 19.4 ng/L (Pereira et al., 2016). DCF concentrations between 0.25 and 5.45 μg/L were reported in sewage treatment plants effluents in France, Italy and Greece (Andreozzi et al. 2003). However, this pharmaceutical was also detected in drinking water in the USA (1.2 ng/L; Benotti et al., 2009). In addition, concentrations between 0.06 and 15 μg/L were found in German rivers (Heberer, 2002; Jux et al., 2002; Weigel et al., 2002); and, its presence was reported in levels of 195 ng/L in UK estuaries (Thomas and Hilton, 2004).

In addition, due to its wide detection and characteristics such as persistence and low degradability (Vieno and Sillanpää 2014), DCF has been shown to be toxic to a wide variety of organisms, especially aquatic species. It can cause biochemical (Alkimin et

al., 2019b) and physiological alterations, altering the pigments content (Alkimin et al., 2019a) in macrophyte species. DCF was also responsible for shell malformation and molecular alterations in embryos of the marine bivalve *Mytilus galloprovincialis* (Balbi et al., 2018). In Japanese medaka (*Oryzias latipes*) it had the potential to cause cellular toxicity, p53-related genotoxicity and estrogenic effects (Hong et al., 2007), and also alterations in the feeding performance (Nassef et al., 2010). DCF was toxic to the mussel *Perna perna*, leading to decreased lysosomal membrane stability and increased cyclooxygenase (COX) activity, oxidative stress, and DNA damage (Fontes et al., 2018). This pharmaceutical, in *Danio rerio* delayed the hatching time in embryos (Hallare et al., 2004) and also caused oxidative stress (Felice et al., 2012)

Another NSAID compound whose ecotoxicity assessment should be prioritized is salicylic acid (SA), which is the active metabolite of acetylsalicylic acid (ASA) after being deacetylated in the body (Nunes, 2019) and then further metabolized. It is a simple phenolic compound synthesized in a wide range of prokaryotic and eukaryotic organisms, including plants. It has been used as therapeutic drug for a long time. In fact, in ancient Egypt (~3,500 years ago) the Ebers papyrus already mentioned the use of leaves of myrtle (after decoction) to treat pain. Years later, Hippocrates recommended the juices of the poplar tree to treat eye diseases and those of willow bark to relieve the pain of childbirth and to reduce fever; all of these forms contain salicylates (Vane and Botting, 1998).

Johann A. Buchner, a German scientist, purified salicyl alcohol glucoside (a SA derivative called salicin) from willow bark in 1828. The Italian chemist Raffaele Piria, in 1838, converted salicin into an acidic aromatic compound that he named salicylic acid. Finally, in 1859 Hermann Kolbe chemically synthesized SA (Jin et al., 2017). This ready supply of SA led to its extended use as an external antiseptic, as an antipyretic, and in the treatment of rheumatism (Vane and Botting, 1998). Once synthesized/consumed, SA may undergo a few biologically relevant chemical modifications including glucosylation, methylation, and amino acid conjugation; most modifications render SA inactive, while at the same time they allow fine-tuning of its accumulation, function, and/or mobility in the organisms (Dempsey et al., 2011). SA is metabolized and excreted mostly conjugated (with both glycine and glucuronic acid), and as gentisic acid (Davison 1971).

Nowadays it is estimated that 40,000 tons of ASA are consumed per year, corresponding to about 120 billion tablets (Warner and Mitchell 2002). Consequently, SA is largely detected in the aquatic environment. In Belgium costal zones, SA was detected at concentrations up to 855 ng/L (Willi et al., 2010), with a mean concentration of 1157 ng/L in influent samples, 169 ng/L in secondary treated effluent samples, and 120 ng/L in tertiary treatment effluent samples from municipal sewage treatment plants in Western Greece (Stamatis and Konstantinou, 2013). In addition, amounts of up to 4.1 µg/L were detected in German sewage treatment plants (Ternes, 1998). In Portugal, the detection frequency of SA was extremely high, as shown by Paíga et al. (2016), who determined levels up to 1.3 µg/L in a freshwater system in the central region of the country (River Lis) and also in Portugal groundwater collected in cemetery areas SA ranged from 33.7 to 71 ng/L (Paíga and Delerue-Matos, 2016).

Ecotoxicological effects of SA have been already determined in several species. SA is capable of triggering the antioxidant defense system, and cause histological alterations in the *Salmo trutta fario* gills (Nunes et al., 2015). It is also responsible for biochemical alterations in Polychaeta species *Hediste diversicolor* (Nunes, 2019) and *Danio rerio* (Zivna et al., 2016). Biochemical alterations in *Lemna minor* (Alkimin et al., 2019a), were also detected. SA affects growth and reproduction in *Daphnia magna* and *D. longispina* (Marques et al., 2004). Also, in *D. magna,* SA induces oxidative stress and DNA damage (Gómez-Oliván et al., 2014). Alterations in glycogen content in *Mytilus* sp. (digestive gland) but not in defense mechanism (such as antioxidant defense system) (Piedade et al., 2020) were detected upon exposure to SA. It also induced metabolic, neurotoxic and oxidative stress in *M. galloprovincialis* (Freitas et al., 2019)

Ketoprofen (2-[3-benzoylphenyl] propionic acid - KTF) is an aryl carboxylic acid derivative NSAID. It was synthesized at the Rhone-Poulenc Research Center in Paris in 1967 and introduced into the French and English markets in 1973 (Avouac and Teule, 1988). It was approved for clinical use in USA, especially in cases of osteoarthritis and rheumatoid arthritis, in January 1986 (Kantor, 1986). KTF is generally prescribed for arthritis-related inflammatory pains or severe dental pain; it has antipyretic and analgesic effects, and it is rarely used for postoperative pain (Gaskell et al., 2017). Efficacy and safety of this pharmaceutical drug has been demonstrated by several evidences that underline how KTF is highly effective and compares favorably to other

NSAIDs, such as DCF or ibuprofen (Sarzi-Puttini et al., 2010), however it can cause renal failure (Pazmiño and Pazmiño, 1988) and liver injury (LiverTox, 2012). In 2015, in England, there were about 25,400 prescriptions for KTF (PACT 2016), while in 2017 this value was of 147,971 in the USA alone (ClinCalc, 2020b).

This drug, in humans, is extensively and rapidly metabolized by the liver, mainly via conjugation with glucuronic acid (Gierse et al., 1999) and approximately 80% of the administered dose is excreted in the urine in a 24-hour period after the administration (Prášková et al., 2011); however, there is no information about these parameters for aquatic organisms.

KTF is reported in the aquatic environment in levels up to 0.3 µg/L and up to 0.87 µg/L in Catalonia (Spain) rivers and effluents respectively (Farré et al., 2001); its presence was determined at levels around 0.2 µg/L in a German municipal sewage treatment plant (Ternes, 1998), and of 0.18 µg/L in Swiss waste water treatment plant (Tixier et al, 2003). In addition, KTF levels were also measured in effluents of Canadian sewage treatment plants, reaching 7 µg/L (Metcalfe et al., 2003). KTF was reported at concentrations of up to 2 µg/L in Finish land influents (Lindqvist et al., 2005). KTF ecotoxicological information is very limited and restricted to few fish species such as the *Danio rerio* (Diniz et al., 2015; Rangasamy et al., 2018) and *Cyprinus carpio* (Prášková et al., 2013).

In addition to the above mentioned, environmental stressors are factors whose influence results in the impairment of productivity, reproductive success, and ecosystem development; to some degree, stressors affect all organisms, as well as their populations, communities, and ecoscapes (landscapes and seascapes; Freedman, 2004). Stressors may be natural in origin, being associated with such environmental influences as competition (Knillmann et al., 2012), predation (Alkimin et al., 2020a), disease, and other interactions among organisms (Cuco et al., 2017); some also involve constraints related to climate or to inadequate or excessive nutrients, moisture, or space (Choi et al., 2016), and disturbances such as wildfires (Nunes et al., 2017) and windstorms (Freedman, 2004). Even endogenous natural compounds, such as hormonal (e.g. salicylic acid in plants) substances, can, sometimes, trigger toxicological responses (Rao et al., 1997; Dat et al., 2000). In addition, another important factor that can have a

significant inpact on ecotoxicological assessments and approaches is the duration of the exposure, which can directly influence the obtained toxicological response (Ares, 2003; Alkimin et al., 2019a). Finally, the above-mentioned stressors may be interacting with environmental contaminanst (including pharmaceutical compounds), changing/interfering at the toxicological profile, causing, for instance antagonistic and/or synergistic effects (Holmstrup et al., 2010; Hooper et al., 2012).

1.2 Macrophytes and their use in ecotoxicology

Macrophytes are primary producers and provide habitats and refuges for periphyton, zooplankton, other invertebrate species, fish and frogs, and also play key functions in biogeochemical cycles (e.g. organic carbon production and phosphorous mobilization) directly influencing hydrology and sediment dynamic of freshwater ecosystems through their effects on water flow (Bornette and Puijalon, 2009). True macrophytes contribute to maintain key functions and related biodiversity in freshwater ecosystems, but also through the services they provide directly to man (Hillman, 1961); characteristics that turn this species into an ideal candidate for aquatic toxicity tests (Wang, 1986).

Common duckweeds (*Lemna* species) are floating aquatic C3 monocotyledons preferring still waters and known to be the smallest flowering angiosperms. They are characterized by rapid growth, lack of a well-developed and sediment-interacting adventitious root system, and lack of stems.

For almost a century (Ashby, 1929) these species have been used as an experimental organism in plant physiology and as a tool to assess phytotoxicity, principally of herbicidal compounds, since synthetic pesticides emerged as part of modern agriculture (Blackman, 1951). They have even been used to assess the toxicity of pharmaceuticals (Greenwood and Nelder, 1964) almost 60 years prior to these compounds garnering broader scientific attention.

An in depth understanding of contaminant dynamics in aquatic ecosystems requires a thorough examination of the pollutant mobilization by (and toxicity to) plants (Greenberg et al., 1992). Along the existing standardized protocols and guidelines for aquatic plant ecotoxicological studies (Brain and Solomon 2007; ASTM 2004; OECD

2002), the most conventionally monitored endpoints are typically biomass (fresh and dry), frond numbers, yield and growth rate. Nevertheless, it is also necessary to investigate additional (non-standard) endpoints as biochemical and physiological traits, in an attempt to deeply understand the effects of contaminants in aquatic plants. However, it is necessary to overcome the barriers regarding the acceptance of this alternative. The most commonly used macrophyte test organism is *L. minor* and, to a lesser degree, *L. gibba* (Hanson, 2013).

1.3 *Daphnia* species as model organisms and their ecotoxicological usage

Daphnia (water fleas) are planktonic crustaceans that belong to the Phyllopoda class (sometimes called Branchiopoda), and to the Cladocera order, whose bodies are enclosed by an uncalcified shell known as the carapace. It has a double wall, between which hemolymph flows and which is part of the body cavity; the carapace is largely made of chitin, a polysaccharide (Ebert, 2005). In general, and in favorable conditions, (i.e. laboratory conditions) daphnids reproduce by parthenogenesis, in which females asexually produce genetically diploid identical female offspring. However, in the wild, when facing adverse environmental conditions, daphnids may alter their reproductive features, by adopting sexual reproduction. This results in the production of haploid eggs that are then enclosed in a protective shell (ephippia) and need to undergo a diapause period before female offspring may hatch from them (Ebert, 2005). Because the parthenogenetic reproduction system provides rapid expansion of daphnid populations, a daphnid population consists almost entirely of females when resources are abundant (Tatarazako and Oda, 2007)

Populations of *Daphnia* spp. can be found in freshwater environments ranging from huge lakes to small temporary pools, and seasonally flooded depressions (Stollewerk, 2010). They are filter feeders, and so they play a key role in freshwater ecology. Their moving legs generate a water current that directs protozoa, algae, bacteria, and organic detritus to the mouth. Therefore, their activities facilitate nutrient cycling in the water (Tatarazako and Oda, 2007). *Daphnia* are in turn consumed by a number of larger aquatic predators and therefore constitute an important component of many freshwater ecosystems (Meaden, 2014). The genus *Daphnia* includes more than 100 known species

of freshwater plankton organisms found around the world; as European representatives of this genera we have (but are not limited to), *D. magna*, *D. cucullata* and *D. longispina* (Ebert, 2005).

These microcrustaceans, specially, *D. magna*, are frequently used as a test animal for the biological assay of many materials data from the early 30's. The pioneering works by Naumann (1933) established this trend, followed by other authors, such as Anderson (1944; 1946) that used this organism to determine toxicity thresholds of various substances found in industrial wastes and different sodium salts. *Daphnia* species have been studied extensively because of their importance to aquatic ecosystems, showing a striking ability to contend with environmental challenges (Stollewerk, 2010). Taking this into account, a diversity of ecotoxicological testing protocols and guidelines were developed (Adema, 1978; ABNT, 2016; OECD, 2004, 2012; ASTM, 1984, 2012; EPA, 2016; Environment Canada 1990, 2000) mostly of then limited to acute (lethality) and chronic (reproduction) assays. Thus, one of the most internationally used bioassays for toxicity screening of chemicals and for toxicity monitoring of effluents and contaminated waters is the acute toxicity test with daphnid crustaceans, and in particular the one based on *D. magna* (Baird et al., 1989; Soares et al., 1992; Gilhermino et al., 2000; Barata et al., 2006; Persoone et al., 2009).

Despite their immense importance, both historically and scientifically, these tests have been revised, and new alternatives are being proposed. The scientific community nowadays already overcome the mandatory use of those standard but limiting endpoints, such as mortality/immobility, and reproductive performance as mandatory parameters. In fact, these endpoints are not capable of signaling most subtle changes, which are of extreme ecological importance. In this regard non-standard responses have been uses such as genes (Soetaert et al., 2006; Connon et al., 2008; Zheng et al., 2020), biochemical (De Felice et al., 2019, 2020; Dionísio et al., 2020), physiological (Araújo et al., 2019), morphological (Imhof et al., 2017; Grzesiuk et al., 2020), behavioral (Alkimin et al., 2020b; Wang et al., 2019) and transgenerational ones (Araújo et al., 2019; Silva et al., 2019).

1.4 Aims and general structure

The main objective of this thesis was to understand the effect of two classes of pharmaceutical compounds on species of aquatic organisms from different trophic levels, namely producers (*Lemna* species) and consumers of first order (microcrustacean - *D. magna*). Same specific questions were posed to address this issue:

-Are non-standard endpoints suitable to study pharmaceutical effects in macrophyte species?

- Is time of exposure a factor that can influence the type of pharmaceutical response, measured in macrophyte species?

- What type of interaction and response might be obtained after co-exposing macrophytes to two drugs, being one of them also an endogenous compound?

- Can a pharmaceutical compound provoke the same effects (type and extent) in organisms from different trophic levels?

- How *D. magna* functional endpoints respond to the chemical and natural stress, isolated and in combination?

To achieve the proposed aim and answer the questions, the overall structure of this thesis was divided into the following chapters:

Chapter 1 - General introduction, providing general information of the topic of pharmaceuticals in the environment, the studied classes and specific compounds and the organisms used in this study.

Chapter 2 - Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. In general the international guidelines recommend 7 days as duration of exposure for *Lemna*-based bioasays, focusing on growth, yield and weight as evaluated endpoint. This chapter brings new perspectives to this type of tests, being shorter (4 days) and evaluating alternative non-standard endpoints, such as biochemical and physiological traits, which work as early signals reflecting the toxic response of aquatic plants to pharmaceutical compounds.

Chapter 3 - Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is time a factor? In addition to the previous chapter, this one brings an evaluation of the already mentioned non-standard endpoints over the time to pharmaceutical exposure, in an attempt to understand the influence of this parameter in the pharmaceutical toxicity in this aquatic species.

Chapter 4 – Can salicylic acid modulate biochemical, physiological and population alterations in a macrophyte species under chemical stress by diclofenac? This chapter brings the complex response of the macrophyte species *Lemna* spp. when challenged by the co-occurrence of two drugs, one being a natural plant hormone.

Chapter 5 – Evaluation of ketoprofen toxicity in two freshwater species: effects on biochemical, physiological and population endpoints. Using endpoints of different hierarchical biological levelse (biochemical, physiological and populational), this chapter evaluated the ability of a NSAID to provoke toxicological effects in organisms from different trophic levels.

Chapter 6 - *Daphnia magna* responses to fish kairomone and chlorpromazine exposures. This chapter increased the realism of the response, by combining exposure to a drug, to a natural stressor (such as fish kairomones, which are signaling chemicals that indicate the presence of predators to potential preys) Thus, this chapter was an insight to the real world complexity, by addressing how these compounds can affect functional endpoints in an macroinvertebrate species (*D. magna*), isolated and in mixture.

Finally, **Chapter 7 – Final remarks.** This last chapter brings a general discussion and the main conclusions drawn from the results acquired in this study.

1.5 References

ABNT (Assossiação Brasileira de Normas Técnicas). 2016. NBR12713 de 05/2016 - Ecotoxicologia aquática - Toxicidade aguda - Método de ensaio com *Daphnia* spp (Crustacea, Cladocera). 27p.

Adema, D. M. M. 1978. *Daphnia magna* as a test animal in acute and chronic toxicity tests. Hydrobiologia, 59, 125-134

Afonso-Olivares, C., Torres-Padrón, M. E., Sosa-Ferrera, Z., Santana-Rodríguez, J. J. 2013. Assessment of the Presence of Pharmaceutical Compounds in Seawater Samples from Coastal Area of Gran Canaria Island (Spain). Antibiotics (Basel, Switzerland), 2(2), 274–287. https://doi.org/10.3390/antibiotics2020274

Agrawal, S., Khazaeni, B. 2019. Acetaminophen Toxicity. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available: <https://www.ncbi.nlm.nih.gov/books/NBK441917/>.

Alkimin, G. D., Daniel, D., Dionísio, R., Soares, A. M. V. M., Barata, C., Nunes, B. 2019a. Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is time a factor? Environmental Research, 177, 108609. doi:10.1016/j.envres.2019.108609

Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M., Barata, C., Nunes, B. 2019b. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Sci Total Environ 657, 926–937

Alkimin, G. D., Nunes, B., Soares, A. M. V. M., Bellot, M., Gómez-Canela, C., Barata, C. 2020a. *Daphnia magna* responses to fish kairomone and chlorpromazine exposures. Chemico-biological Interaction. 109123. doi.org/10.1016/j.cbi.2020.109123

Alkimin, G. D., Paisio, C., Agostini, E., Nunes, B. 2020b. Phytoremediation processes of domestic and textile effluents: evaluation of the efficacy and toxicological effects in *Lemna minor* and *Daphnia magna*. Environ Sci Pollut Res 27, 4423–4441. https://doi.org/10.1007/s11356-019-07098-3

Almeida, F., Nunes, B. 2019. Effects of acetaminophen in oxidative stress and neurotoxicity biomarkers of the gastropod *Phorcus lineatus*. Environ Sci Pollut Res 26, 9823–9831. https://doi.org/10.1007/s11356-019-04349-1

Anderson, B. G. 1944. The Toxicity Thresholds of Various Substances Found in Industrial Wastes as Determined by the Use of *Daphnia magna.* Sewage Works Journal, 16, 1156-1165

Anderson, B. G. 1946. The Toxicity Thresholds of Various Sodium Salts Determined by the Use of *Daphnia magna*. Sewage Works Journal, 18, 82-87

Andreozzi, R., Raffaele, M., Nicklas, P. 2003. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere, 50(10), 1319–1330. doi:10.1016/s0045-6535(02)00769-5

Angeles, L. F., Mullen, R. A., Huang, I. J., Wilson, C., Khunjar, W., Sirotkin, H. I., McElroy, A. E., Aga, D. S. 2020. Assessing pharmaceutical removal and reduction in toxicity provided by advanced wastewater treatment systems. Environmental Science: Water Research & Technology. doi:10.1039/c9ew00559e

Antunes, S. C., Freitas, R., Figueira, E. et al. 2013. Biochemical effects of acetaminophen in aquatic species: edible clams *Venerupis decussata* and *Venerupis philippinarum*. Environ Sci Pollut Res 20, 6658–6666. https://doi.org/10.1007/s11356-013-1784-9

Araujo, G. S., Pinheiro, C., Pestana, J. L. T., Soares, A. M. V. M., Abessa, D. M. S., Loureiro, S. 2019. Toxicity of lead and mancozeb differs in two monophyletic *Daphnia* species. Ecotoxicology and Environmental Safety, 178, 230–238. doi:10.1016/j.ecoenv.2019.03.095

Ares, J. 2003. Time and space issues in ecotoxicology: Population models, landscape pattern analysis, and long-range environmental chemistry. Environmental Toxicology and Chemistry, 22(5), 945–957. doi:10.1002/etc.5620220501

Ashby, E. 1929. The interaction of factors in the growth of *Lemna*. III. The interrelationship of duration and intensity of light. Ann Bot 43:333–354

ASTM - American Society for Testing and Materials. 2004. E1415-91 Standard guide for conducting static toxicity tests with *Lemna gibba* G3. ASTM International, West Conshohocken, PA, USA

ASTM - American Society of Testing and Materials. 1984. Standard Practice for Conducting Static Acute Toxicity Test on Wastewaters with *Daphnia*, 1987, in Annual Book of ASTM Standards; Water and Environmental Technology, Vol. 11.01; D–4229.

ASTM - American Society of Testing and Materials. 1998. Standard Guide for Conducting Daphnia magna Life-Cycle Toxicity Tests, in Annual Book of ASTM Standards, Water and Environmental Technology, 11.04; E 1193–97.

Aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A. 2016. Pharmaceuticals in the environment-Global occurrences and perspectives. Environmental Toxicology and Chemistry, 35(4), 823–835. doi:10.1002/etc.3339

Avouac, B., Teule, M. 1988. Ketoprofen: The European Experience. The Journal of Clinical Pharmacology, 28, S2–S7. doi:10.1002/j.1552-4604.1988.tb05972.x

Baird, D. J., Barber, I., Bradley, M. et al. 1989. The *Daphnia* bioassay: a critique. Hydrobiologia 188, 403–406. https://doi.org/10.1007/BF00027806

Balbi, T., Montagna, M., Fabbri, R., Carbone, C., Franzellitti, S., Fabbri, E., Canesi, L. 2018. Diclofenac affects early embryo development in the marine bivalve *Mytilus galloprovincialis*. Science of The Total Environment, 642, 601–609. doi:10.1016/j.scitotenv.2018.06.125

Ban, T. A. 2007. Fifty years chlorpromazine: a historical perspective. Neuropsychiatric disease and treatment, 3(4), 495–500.

Barata, C., Baird, D. J., Nogueira, A. J. A., Soares, A. M. V. M., Riva, M. C. 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquatic Toxicology, 78(1), 1–14. doi:10.1016/j.aquatox.2006.01.013

Barnes K. K., Kolpin, D. W., Furlong, E. T., Zaugg, S. D., Meyer, M. T., Barber, L. B. 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States –I) Groundwater. Science of the Total Environment. 402: 192-200.

Behera, S. K., Kim, H. W., Oh, J.-E., Park, H.-S. 2011. Occurrence and removal of antibiotics, hormones and several other pharmaceuticals in wastewater treatment plants of the largest industrial city of Korea. Science of the Total Environment, 409(20), 4351–4360. doi:10.1016/j.scitotenv.2011.07.015

Beijer, K., Björlenius, B., Shaik, S., Lindberg, R. H., Brunström, B., Brandt, I. 2017. Removal of pharmaceuticals and unspecified contaminants in sewage treatment effluents by activated carbon filtration and ozonation: Evaluation using biomarker responses and chemical analysis. Chemosphere, 176, 342–351. doi:10.1016/j.chemosphere.2017.02.127

Ben-Shachar, R., Chen, Y., Luo, S., Hartman, C., Reed, M., Nijhout, H. F. 2012. The biochemistry of acetaminophen hepatotoxicity and rescue: a mathematical model. Theor Biol Med Model 9(1):55. https://doi.org/10.1186/1742-4682-9-55

Bielen, A., Šimatović, A., Kosić-Vukšić, J., Senta, I., Ahel, M., Babić, S., … Udiković-Kolić, N. 2017. Negative environmental impacts of antibiotic-contaminated effluents from pharmaceutical industries. Water Research, 126, 79–87. doi:10.1016/j.watres.2017.09.019

Blackman, G. E. 1951. Studies in the principles of phytotoxicity. I. The assessment of relative toxicity. Jour Exp Bot 3:1–27

Boelsterli, U. 2003. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. Toxicology and Applied Pharmacology, 192(3), 307–322. doi:10.1016/s0041-008x(03)00368-5

Bonnefille, B., Gomez, E., Courant, F., Escande, A., Fenet, H. 2018. Diclofenac in the marine environment: A review of its occurrence and effects. Marine Pollution Bulletin, 131, 496–506. doi:10.1016/j.marpolbul.2018.04.053

Borges, N. C., Rezende, V. M., Santana, J. M., Moreira, R. P., Moreira, R. F., Moreno, P., … Moreno, R. A. 2011. Chlorpromazine quantification in human plasma by UPLC–electrospray ionization tandem mass spectrometry. Application to a comparative pharmacokinetic study. Journal of Chromatography B, 879(31), 3728–3734. doi:10.1016/j.jchromb.2011.10.020

Bornette, G., Puijalon, S. 2009. Macrophytes: Ecology of Aquatic Plants. Encyclopedia of Life Sciences. doi:10.1002/9780470015902.a0020475

Bound, J. P., Voulvoulis, N. 2006. Predicted and measured concentrations for selected pharmaceuticals in UK rivers: Implications for risk assessment. Water Research, 40(15), 2885–2892. doi:10.1016/j.watres.2006.05.036

Brain, R. A., Solomon, K. R. 2007. A protocol for conducting 7-day daily renewal tests with Lemna gibba. Nature Protocols, 2(4), 979–987. doi:10.1038/nprot.2007.146

Bridgewater, N. J. 2018. Amneal Announces FDA Approval and Launch of Generic Chlorpromazine Hydrochloride Tablets USP and Approval of Methylergonovine Maleate Tablets USP. Available: <https://www.marketwatch.com/press-release/amneal-announces-fda-approval-and-launch-of-generic-chlorpromazine-hydrochloride-tablets-usp-and-approval-of-methylergonovine-maleate-tablets-usp-2018-09-12>

Brooks, B. W. 2018. Urbanization, environment and pharmaceuticals: advancing comparative physiology, pharmacology and toxicology. Conservation Physiology, 6(1). doi:10.1093/conphys/cox079

Brooks, P. M., Day, R. O. 1991. Nonsteroidal Antiinflammatory Drugs — Differences and Similarities. New England Journal of Medicine, 324(24), 1716–1725. doi:10.1056/nejm199106133242407

Bu, Q., Shi, X., Yu, G., Huang, J., Wang, B. 2016. Assessing the persistence of pharmaceuticals in the aquatic environment: Challenges and needs. Emerging Contaminants, 2(3), 145–147. doi:10.1016/j.emcon.2016.05.003

Cashman, J. N. 1996. The mechanisms of action of NSAIDs in analgesia. Drugs, 52 Suppl 5: 13-23

Chiam, E., Weinberg, L., Bellomo, R. 2015. Paracetamol: a review with specific focus on the haemodynamic effects of intravenous administration. Heart, lung and vessels, 7(2), 121–132.

Choi, J.-Y., Kim, S.-K., La, G.-H., Chang, K.-H., Kim, D.-K., Jeong, K.-Y. et al. 2016. Effects of algal food quality on sexual reproduction of *Daphnia magna*. Ecology and Evolution, 6(9), 2817–2832. doi:10.1002/ece3.2058

Cincinelli, A., Martellini, T., Coppini, E., Fibbi, D., Katsoyiannis, A. 2015. Nanotechnologies for Removal of Pharmaceuticals and Personal Care Products from Water and Wastewater. A Review. Journal of Nanoscience and Nanotechnology, 15(5), 3333–3347. doi:10.1166/jnn.2015.10036

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H. 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Res 39(19):4797–4807

ClinCalc. 2020a. Diclofenac: Drug Usage Statistics, United States, 2007 – 2017. Available: <https://clincalc.com/DrugStats/Drugs/Diclofenac>.

ClinCalc. 2020b. Ketoprofen - Drug Usage Statistics, United States, 2007 – 2017. Available: <https://clincalc.com/DrugStats/Drugs/Ketoprofen>.

Connon, R., Hooper, H. L., Sibly, R. M., Lim, F.-L., Heckmann, L.-H., Moore, D. J., … Callaghan, A. 2008. Linking Molecular and Population Stress Responses in *Daphnia magna* exposed to cadmium. Environmental Science & Technology, 42(6), 2181–2188. doi:10.1021/es702469b

Cuco, A. P., Abrantes, N., Gonçalves, F., Wolinska, J., Castro, B. B. 2017. Interplay between fungicides and parasites: Tebuconazole, but not copper, suppresses infection in a *Daphnia*-Metschnikowia experimental model. PLOS ONE, 12(2), e0172589. doi:10.1371/journal.pone.0172589

Cunha, D. L., de Araujo, F. G., Marques, M. 2017. Psychoactive drugs: occurrence in aquatic environment, analytical methods, and ecotoxicity—a review. Environ Sci Pollut Res 24, 24076–24091. https://doi.org/10.1007/s11356-017-0170-4

Daniel, D., Dionísio, R., Alkimin, G. D., Nunes, B. 2019. Acute and chronic effects of paracetamol exposure on Daphnia magna: how oxidative effects may modulate responses at distinct levels of organization in a model species. Environ Sci Pollut Res 26, 3320–3329 https://doi.org/10.1007/s11356-018-3788-y

Dat, J. F., Lopez-Delgado, H., Foyer, C. H., Scott, I. M. 2000. Effects of Salicylic Acid on Oxidative Stress and Thermotolerance in Tobacco. Journal of Plant Physiology, 156(5-6), 659–665. doi:10.1016/s0176-1617(00)80228-x

David, A., Pancharatna, K. 2009. Effects of acetaminophen (paracetamol) in the embryonic development of zebrafish, *Danio rerio*. Journal of Applied Toxicology, 29(7), 597–602. doi:10.1002/jat.1446

Davies, J., Davies, D. 2010. Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews, 74(3), 417–433. doi:10.1128/mmbr.00016-10

Davison, C. 1971. Salicylate metabolism in man. Ann NY Acad Sci 179:249–268

De Felice, B., Copia, L., Guida, M. 2012. Gene expression profiling in zebrafish embryos exposed to diclofenac, an environmental toxicant. Molecular Biology Reports, 39(3), 2119–2128. doi:10.1007/s11033-011-0959-z

De Felice, B., Mondellini, S., Salgueiro-González, N., Castiglioni, S., Parolini, M. 2020. Methamphetamine exposure modulated oxidative status and altered the reproductive output in *Daphnia magna*. Science of The Total Environment, 721, 137728. doi:10.1016/j.scitotenv.2020.137728

De Felice, B., Salgueiro-González, N., Castiglioni, S., Saino, N., Parolini, M. 2019. Biochemical and behavioral effects induced by cocaine exposure to *Daphnia magna*. Science of The Total Environment. doi:10.1016/j.scitotenv.2019.06.383

Deblonde, T., Cossu-Leguille, C., Hartemann, P. 2011. Emerging pollutants in wastewater: A review of the literature. International Journal of Hygiene and Environmental Health, 214(6), 442–448. doi:10.1016/j.ijheh.2011.08.002

Dempsey, D. A., Vlot, A. C., Wildermuth, M. C., Klessig, D. F. 2011. Salicylic Acid biosynthesis and metabolism. The arabidopsis book, 9, e0156. https://doi.org/10.1199/tab.0156

Desforges, J.-P. W., Sonne, C., Levin, M., Siebert, U., De Guise, S., Dietz, R. 2016. Immunotoxic effects of environmental pollutants in marine mammals. Environment International, 86, 126–139. doi:10.1016/j.envint.2015.10.007

Diniz, M. S., Salgado, R., Pereira, V. J., Carvalho, G., Oehmen, A., Reis, M. A. M., Noronha, J. P. 2015. Ecotoxicity of ketoprofen, diclofenac, atenolol and their photolysis byproducts in zebrafish (*Danio rerio*). Science of The Total Environment, 505, 282–289. doi:10.1016/j.scitotenv.2014.09.103

Dionísio, R., Daniel, D., de Alkimin, G. D., Nunes, B. 2020. Multi-parametric analysis of ciprofloxacin toxicity at ecologically relevant levels: short- and long-term effects on *Daphnia magna*. Environmental Toxicology and Pharmacology, 103295. doi:10.1016/j.etap.2019.103295

DrugBank. 2020. Chlorpromazine. Available: <https://www.drugbank.ca/drugs/DB00477>.

Ebert D. 2005. Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia* [Internet]. Bethesda (MD): National Center for Biotechnology Information (US). Chapter 2, Introduction to Daphnia Biology. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/

EEB - European Environmental Bureau. 2018. The Problem of Pharmaceutical Pollution. Available: < https://eeb.org/the-problem-of-pharmaceutical-pollution/>.

Ensano, B., Borea, L., Naddeo, V., Belgiorno, V., de Luna, M., & Ballesteros, F. 2017. Removal of Pharmaceuticals from Wastewater by Intermittent Electrocoagulation. Water, 9(2), 85. doi:10.3390/w9020085

Environment Canada (EC). 1990. Biological Test Method: Acute Lethality Test Using *Daphnia* spp. Conservation and Protection, Ottawa, Report EPS/RM/11, Amended in May 1996 and December 2000.

Environment Canada (EC). 2000. Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*, Environmental Technology Centre, Ottawa, Report EPS/RM/14 Second Edition.

EPA - United States Environmental Protection Agency. 2016. Ecological Effects Test Guidelines OCSPP 850.1300: Daphnid Chronic Toxicity Test.

Escola Casas, M., Chhetri, R. K., Ooi, G., Hansen, K. M. S., Litty, K., Christensson, M., Kragelund, C., Andersen, H. R., Bester, K. 2015. Biodegradation of pharmaceuticals in hospital wastewater by staged moving bed biofilm reactors (MBBR). Water Res 83:293–302

Farré, M., Ferrer, I., Ginebreda, A., Figueras, M., Olivella, L., Tirapu, L. et al. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. Journal of Chromatography A, 938(1-2), 187–197. doi:10.1016/s0021-9673(01)01154-2

Fatta, D., Achilleos, A., Nikolaou, A., Meriç, S. 2007. Analytical methods for tracing pharmaceutical residues in water and wastewater. TrAC Trends in Analytical Chemistry, 26(6), 515–533. doi:10.1016/j.trac.2007.02.001

FDA – U.S. Food and Drug Administration. 2018. COX-2 Selective (includes Bextra, Celebrex, and Vioxx) and Non-Selective Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) [online]

Felmeister, A., Discher, C. A. 1964. Photodegradation of Chlorpromazine Hydrochloride. Journal of Pharmaceutical Sciences, 53(7), 756–762. doi:10.1002/jps.2600530712

Fleming, N. 2015. Does paracetamol do you more harm than good? The Guardian. Available <https://www.theguardian.com/lifeandstyle/2015/may/25/does-paracetamol-do-you-more-harm-than-good>

Fontes, M. K., Gusso-Choueri, P. K., Maranho, L. A., Abessa, D. M. de S., Mazur, W. A., de Campos, B. G., … Pereira, C. D. S. 2018. A tiered approach to assess effects of diclofenac on the brown mussel Perna perna: A contribution to characterize the hazard. Water Research, 132, 361–370. doi:10.1016/j.watres.2017.12.077

Freches, S. 2017. Medicamento que bate todos os recordes e até já foi à Lua faz 120 anos. Available <https://www.dn.pt/sociedade/medicamento-que-bate-todos-os-recordes-e-ate-ja-foi-a-lua-faz-120-anos-8830107.html>.

Freedman, B. 2004. Environmental Science--a Canadian Perspective, Third Edition. TestGen 5.0, QuizMaster 3.0 [electronic Resource]

Freitas, R., Silvestro, S., Coppola, F., Meucci, V., Battaglia, F., Intorre, L., … Faggio, C. 2019. Biochemical and physiological responses induced in Mytilus galloprovincialis after a chronic exposure to Salicylic Acid. Aquatic Toxicology, 105258. doi:10.1016/j.aquatox.2019.105258

Gaskell, H., Derry, S., Wiffen, P. J., Moore, R. A. 2017. Single dose oral ketoprofen or dexketoprofen for acute postoperative pain in adults. Cochrane Database of Systematic Reviews. doi:10.1002/14651858.cd007355.pub3

Ghlichloo, I., Gerriets, V. 2020. Nonsteroidal Anti-inflammatory Drugs (NSAIDs). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available: <https://www.ncbi.nlm.nih.gov/books/NBK547742/>.

Ghlichloo, I., Gerriets, V. 2020. Nonsteroidal Anti-inflammatory Drugs (NSAIDs). In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available: <https://www.ncbi.nlm.nih.gov/books/NBK547742/>.

Gierse, J. K., Koboldt, C. M., Walker, M. C, Seibert, K., Isakson, P. C. 1999. Kinetic basis for selective inhibition of cyclooxygenases. Biochem J 339: 607–614.6

Giri, A. K. (1993). The genetic toxicology of paracetamol and aspirin: A review. Mutation Research/Reviews in Genetic Toxicology, 296(3), 199–210. doi:10.1016/0165-1110(93)90011-b

Gómez-Oliván, L. M., Galar-Martínez, M., Islas-Flores, H., García-Medina, S., SanJuan-Reyes, N. 2014. DNA damage and oxidative stress induced by acetylsalicylic acid in *Daphnia magna*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 164, 21–26. doi:10.1016/j.cbpc.2014.04.004

Graham, G. G., Scott, K. F. 2005. Mechanism of Action of Paracetamol. American Journal of Therapeutics, 12(1), 46–55. doi:10.1097/00045391-200501000-00008

Greenberg, B. M., Huang, X. Dixon, D. G. 1992. Applications of the aquatic higher plant *Lemna gibba* for ecotoxicological assessment. Journal of Aquatic Ecosystem Health 1, 147–155. https://doi.org/10.1007/BF00044046

Greenwood, J., Nelder, J. A. 1964. Effect of drugs on growth of *Lemna minor* C. Ann Bot 28:711–715

Grohol, J. M. 2019. Top Psychiatric Medications for 2018. Available: <https://psychcentral.com/blog/top-25-psychiatric-medications-for-2018/>

Grzesiuk, M., Pijanowska, J., Markowska, M., Bednarska, A. 2020. Morphological deformation of *Daphnia magna* embryos caused by prolonged exposure to ibuprofen. Environmental Pollution, 114135. doi:10.1016/j.envpol.2020.114135

Guzmam, F. 2019. First-Generation Antipsychotics: An Introduction. Psycopharmacology Institute. [online]. Available: <https://psychopharmacologyinstitute.com/publication/first-generation-antipsychotics-an-introduction-2110>.

Haddad, P., Kirk, R., Green, R. 2016. Chlorpromazine, the first antipsychotic medication: history, controversy and legacy. British Association for Psychopharmacology. Available: <https://www.bap.org.uk/articles/chlorpromazine-the-first-antipsychotic/>.

Hafeez, F., Zahoor, A. F., Ahmad, S., Ahmad, M., Faiz, S. 2018. Recent progress in the synthesis of diclofenac based NSAIDs analogs/derivatives. Synthetic Communications, 1–26. doi:10.1080/00397911.2018.1515367

Hallare, A. V., Köhler, H.-R., Triebskorn, R. 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere, 56(7), 659–666. doi:10.1016/j.chemosphere.2004.04.007

Hanson, M. L. 2013. Aquatic Macrophytes in Ecotoxicology. Encyclopedia of Aquatic Ecotoxicology, 89–98. doi:10.1007/978-94-007-5704-2_9

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters, 131(1-2), 5–17. doi:10.1016/s0378-4274(02)00041-3

Herrmann, M., Menz, J., Gassmann, M., Olsson, O., Kümmerer, K. 2016. Experimental and in silico assessment of fate and effects of the antipsychotic drug quetiapine and its bio- and phototransformation products in aquatic environments. Environmental Pollution, 218, 66–76. doi:10.1016/j.envpol.2016.08.040

Hillman, W. S. 1961. The Lemnaceae, or duckweeds – a review of the descriptive and experimental literature. Bot Rev 27:221–287

Hinson, J. A., Roberts, D. W., James, L. P. 2010. Mechanisms of acetaminophen-induced liver necrosis. Handb Exp Pharmacol 196:369–405. https://doi.org/10.1007/978-3-642-00663-0_12

Hinson, J. A., Roberts, D. W., James, L. P. 2010. Mechanisms of acetaminophen-induced liver necrosis. Handb Exp Pharmacol. (196): 369-405.

Hinz, B., Brune, K. 2012. Paracetamol and cyclooxygenase inhibition: is there a cause for concern? Ann Rheum Dis 71(1): 20-25.

Holmstrup, M., Bindesbøl, A.-M., Oostingh, G. J., Duschl, A., Scheil, V., Köhler, H.-R. et al. 2010. Interactions between effects of environmental chemicals and natural stressors: A review. Science of The Total Environment, 408(18), 3746–3762. doi:10.1016/j.scitotenv.2009.10.067

Hong, H. N., Kim, H. N., Park, K. S., Lee, S.-K., Gu, M. B. 2007. Analysis of the effects diclofenac has on Japanese medaka (*Oryzias latipes*) using real-time PCR. Chemosphere, 67(11), 2115–2121. doi:10.1016/j.chemosphere.2006.12.090

Hooper, M. J., Ankley, G. T., Cristol, D. A., Maryoung, L. A., Noyes, P. D., Pinkerton, K. E. 2012. Interactions between chemical and climate stressors: A role for mechanistic toxicology in assessing climate change risks. Environmental Toxicology and Chemistry, 32(1), 32–48. doi:10.1002/etc.2043

Huschek, G., Hansen, P. D., Maurer, H. H., Krengel, D., Kayser, A. 2004. Environmental risk assessment of medicinal products for human use according to European Commission recommendations. Environmental Toxicology, 19(3), 226–240. doi:10.1002/tox.20015

Imhof, H. K., Rusek, J., Thiel, M., Wolinska, J., Laforsch, C. 2017. Do microplastic particles affect *Daphnia magna* at the morphological, life history and molecular level? PLOS ONE, 12(11), e0187590. doi:10.1371/journal.pone.0187590

Ji, K., Liu, X., Lee, S., Kang, S., Kho, Y., Giesy, J. P., Choi, K. 2013. Effects of non-steroidal anti-inflammatory drugs on hormones and genes of the hypothalamic-pituitary-gonad axis, and reproduction of zebrafish. Journal of Hazardous Materials, 254-255, 242–251. doi:10.1016/j.jhazmat.2013.03.036

Jibson, M. D. 2020. First-generation antipsychotic medications: Pharmacology, administration, and comparative side effects. UpToDate [online]. Available: <https://www.uptodate.com/contents/first-generation-antipsychotic-medications-pharmacology-administration-and-comparative-side-effects>.

Jin J. 2015. Nonsteroidal Anti-inflammatory Drugs. JAMA. 314(10):1084. doi:10.1001/jama.2015.9936

Jin, J. B., Cai, B., Zhou, J.-M. 2017. Salicylic acid. Hormone Metabolism and Signaling in Plants, 273–289. doi:10.1016/b978-0-12-811562-6.00008-6

Jux, U., Baginski, R. M., Arnold, H.-G., Krönke, M., Seng, P. N. 2002. Detection of pharmaceutical contaminations of river, pond, and tap water from Cologne (Germany) and surroundings. International Journal of Hygiene and Environmental Health, 205(5), 393–398. doi:10.1078/1438-4639-00166

Kaleniecka, A., Zarzycki, P. K. 2015. Pharmaceuticals in the aquatic environment: sources, effects, treatment methods. Arch Physiother Glob Res. 19 (3), 39-52

Kane, J. M., Correll, C. U. 2010. Past and present progress in the pharmacologic treatment of schizophrenia. J. Clin. Psychiatry, 9, 1115-1124

Kantor, T. G. 1986. Ketoprofen: A Review of Its Pharmacologic and Clinical Properties. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 6(3), 93–102. doi:10.1002/j.1875-9114.1986.tb03459.x

Kermia, A. E. B., Fouial-Djebbar, D., Trari, M. 2016. Occurrence, fate and removal efficiencies of pharmaceuticals in wastewater treatment plants (WWTPs) discharging in the coastal environment of Algiers. Comptes Rendus Chimie, 19(8), 963–970. doi:10.1016/j.crci.2016.05.005

Kim, M., Guerra, P., Shah, A., Parsa, M., Alaee, M., Smyth, S. 2014. Pharmaceuticals and personal care products removal in a membrane bioreactor wastewater treatment plant. Water Sci Technol 69(11):2221–2229

Knillmann, S., Stampfli, N. C., Beketov, M. A., Liess, M. 2012. Intraspecific competition increases toxicant effects in outdoor pond microcosms. Ecotoxicology, 21(7), 1857–1866. doi:10.1007/s10646-012-0919-y

Koagouw, W., Ciocan, C. 2019. Effects of short-term exposure of paracetamol in the gonads of blue mussels *Mytilus edulis*. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-019-06861-w

Komolafe O. O. 2003. Antibiotic resistance in bacteria - an emerging public health problem. Malawi medical journal, 15(2), 63–67. https://doi.org/10.4314/mmj.v15i2.10780

Kosma, C. I., Lambropoulou, D. A., Albanis, T. A. 2014. Investigation of PPCPs in wastewater treatment plants in Greece: Occurrence, removal and environmental risk assessment. Science of The Total Environment, 466-467, 421–438. doi:10.1016/j.scitotenv.2013.07.044

Kostich, M. S., Lazorchak, J. M. 2008. Risks to aquatic organisms posed by human pharmaceutical use. Science of The Total Environment, 389(2-3), 329–339. doi:10.1016/j.scitotenv.2007.09.008

Kovalova, L., Siegrist, H., Singer, H., Wittmer, A., McArdell, C. 2012. Hospital wastewater treatment by membrane bioreactor: performance and efficiency for organic micropollutant elimination. Environ Sci Technol 46(3):1536–1545

Küster, A., Adler, N. 2014. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 369(1656), 20130587. https://doi.org/10.1098/rstb.2013.0587

Lagesson, A., Fahlman, J., Brodin, T., Fick, J., Jonsson, M., Byström, P., Klaminder, J. 2016. Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment. Science of The Total Environment, 568, 208–215. doi:10.1016/j.scitotenv.2016.05.206

Lapworth, D. J., Baran, N., Stuart, M. E., Ward, R. S. 2012. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. Environmental Pollution, 163, 287–303. doi:10.1016/j.envpol.2011.12.034

Larsen, T. A., Lienert, J., Joss, A., Siegrist, H. 2004. How to avoid pharmaceuticals in the aquatic environment. Journal of Biotechnology, 113(1-3), 295–304. doi:10.1016/j.jbiotec.2004.03.033

Laufer, S. 2007. NSAIDs, Chemical Structure and Molecular Mode of Action. In: Schmidt R., Willis W. (eds) Encyclopedia of Pain. Springer, Berlin, Heidelberg. DOIhttps://doi.org/10.1007/978-3-540-29805-2

Laufer, S. 2007. NSAIDs, Chemical Structure and Molecular Mode of Action. In: Schmidt R., Willis W. (eds) Encyclopedia of Pain. Springer, Berlin, Heidelberg. DOIhttps://doi.org/10.1007/978-3-540-29805-2

Li, B., Webster, T. J. 2018. Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. Journal of orthopaedic research: official publication of the Orthopaedic Research Society, 36(1), 22–32. https://doi.org/10.1002/jor.23656

Li, Q., Zhou, N., Zhang, Y. 2008. Luo Toxic effects of chlorpromazine on *Carassius auratus* and its oxidative stress J. Environ. Sci. Health B, 43, 638-643

Li, Z. H., Randak, T. 2009. Residual pharmaceutically active compounds (PhACs) in aquatic environment – status, toxicity and kinetics: a review. Veterinarni Medicina, 54 (7): 295–314

Lindqvist, N., Tuhkanen, T., Kronberg, L. 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Research, 39(11), 2219–2228. doi:10.1016/j.watres.2005.04.003

LiverTox: Clinical and Research Information on Drug-Induced Liver Injury 2012. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Ketoprofen. [Updated 2018 Apr 25]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK548678/

Logarinho, F., Rosado, T., Lourenço, C., Barroso, M., Araujo, A. R. T. S., & Gallardo, E. 2016. Determination of antipsychotic drugs in hospital and wastewater treatment plant samples by gas chromatography/tandem mass spectrometry. Journal of Chromatography B, 1038, 127–135. doi:10.1016/j.jchromb.2016.10.031

Lonappan, L., Brar, S. K., Das, R. K., Verma, M., Surampalli, R. Y. 2016. Diclofenac and its transformation products: Environmental occurrence and toxicity - A review. Environment International, 96, 127–138. doi:10.1016/j.envint.2016.09.014

Mann, S. K., Marwaha, R. 2019. Chlorpromazine. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available: <https://www.ncbi.nlm.nih.gov/books/NBK553079/>.

Marques, C. R., Abrantes, N., Gonçalves, F. 2004. Life-history traits of standard and autochthonous cladocerans: II. Acute and chronic effects of acetylsalicylic acid metabolites. Environmental Toxicology, 19(5), 527–540. doi:10.1002/tox.20060

Marston, H. 2013. A Brief History of Psychiatric Drug Development. British Association for Psychopharmacology. Available: <https://www.bap.org.uk/articles/a-brief-history-of-psychiatric-drug-development/>.

McCrae, J. C., Morrison, E. E., MacIntyre, I. M., Dear, J. W., Webb, D. J. 2018. Long-term adverse effects of paracetamol - a review. British Journal of Clinical Pharmacology. doi:10.1111/bcp.13656

McEneff, G., Schmidt, W., Quinn, B., 2015. EPA Report Nº 142: Pharmaceuticals in the Aquatic Environment: A Short Summary of Current Knowledge and the Potential Impacts on Aquatic Biota and Humans. Wexford, Ireland. 52 p.

McGettigan, P., Henry, D. 2013. Use of Non-Steroidal Anti-Inflammatory Drugs That Elevate Cardiovascular Risk: An Examination of Sales and Essential Medicines Lists in Low-, Middle-, and High-Income Countries. PLoS Medicine, 10(2), e1001388. doi:10.1371/journal.pmed.1001388

Meaden, R. 2014. Developments in *Daphnia*. BMC Biology. Available: <https://blogs.biomedcentral.com/on-biology/2014/03/25/developments-in-daphnia/>.

Menassé, R., Hedwall, P. R., Kraetz, J., Pericin, C., Riesterer, L., Sallmann, A., … Jaques, R. 1978. Pharmacological Properties of Diclofenac Sodium and Its Metabolites. Scandinavian Journal of Rheumatology, 7(sup22), 5–16. doi:10.3109/03009747809097211

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., Hirsch, R. 2003. Occurrence of Neutral and Acidic Drugs in The Effluents Of Canadian Sewage Treatment Plants. Environmental Toxicology and Chemistry, 22(12), 2872. doi:10.1897/02-469 doi:10.1016/j.watres.2005.04.003

Mitchell, J. R., Jollow, D. J., Potter, W. Z., Davis, D. C., Gillette, J. R., Brodie, B. B. 1973. Acetaminophen-Induced Hepatic Necrosis. I. Role of Drug Metabolism. Journal of Pharmacology and Experimental Therapeutics. 187(1), 185-194

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I. J., Shimasaki, Y., … Honjo, T. 2010. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). Chemosphere, 80(9), 1095–1100. doi:10.1016/j.chemosphere.2010.04.073

Naumann, E., 1933. *Daphnia magna* Straus als Versuchstier. K. Fysiografiska Sällskapet i Lund. Färhandlingar, 3, 15-25.

Nelson, S. D., Bruschi, S. A. 2007. Mechanisms of acetaminophen induced liver disease. In: Kaplowitz N., Deleve, L. D. (Eds.), drug induced liver disease, second ed. (pp 287–326. New York: Informa Healthcare.

Nielsen, U., Hastrup, C., Klausen, M. M., Pedersen, B. M., Kristensen, G. H., Jansen, J. L. C., Bak, S. N., Tuerk, J. 2013. Removal of APIs and bacteria from hospital wastewater by MBR plus O 3, O3 + H2O2, PAC or ClO2. Water Sci Technol 67(4):854–862

NIH – National Institute of Mental Health 2016a. Mental Health Medications. Available: <https://www.nimh.nih.gov/health/topics/mental-health-medications/index.shtml>.

NIH – National Institute of Mental Health 2016b. Schizophrenia. Available: <https://www.nimh.nih.gov/health/topics/schizophrenia/index.shtml>.

NIH – National Institute of Mental Health 2019. Mental Illness. Available: <https://www.nimh.nih.gov/health/statistics/mental-illness.shtml>.

Nogueira, A. F., Pinto, G., Correia, B., Nunes, B. 2019) Embryonic development, locomotor behavior, biochemical, and epigenetic effects of the pharmaceutical drugs paracetamol and ciprofloxacin in larvae and embryos of *Danio rerio* when exposed to environmental realistic levels of both drugs. Environmental Toxicology. doi:10.1002/tox.22819

Nunes B., Silva V., Campos I., Pereira J.L., Pereira P., Keizer J.J., Goncalves F., Abrantes N. 2017. Off-site impacts of wildfires on aquatic systems - Biomarker responses of the mosquitofish *Gambusia holbrooki*. Science of The Total Environment, 581 305-313.

Nunes, B. 2019. Acute ecotoxicological effects of salicylic acid on the Polychaeta species *Hediste diversicolor*: evidences of low to moderate pro-oxidative effects. Environ Sci Pollut Res 26, 7873–7882. https://doi.org/10.1007/s11356-018-04085-y

Nunes, B., Antunes, S. C., Santos, J., Martins, L., Castro, B. B. 2014. Toxic potential of paracetamol to freshwater organisms: A headache to environmental regulators? Ecotoxicology and Environmental Safety, 107, 178–185. doi:10.1016/j.ecoenv.2014.05.027

Nunes, B., Campos, J.C., Gomes, R. et al. 2015. Ecotoxicological effects of salicylic acid in the freshwater fish *Salmo trutta fario*: antioxidant mechanisms and histological alterations. Environ Sci Pollut Res 22, 667–678. https://doi.org/10.1007/s11356-014-3337-2

Nunes, B., Daniel, D., Canelas, G. G., Barros, J., Correia, A. T. 2020. Toxic effects of environmentally realistic concentrations of diclofenac in organisms from two distinct trophic levels, *Hediste diversicolor* and *Solea senegalensis*. Comparative

Biochemistry and Physiology Part C: Toxicology & Pharmacology, 108722. doi:10.1016/j.cbpc.2020.108722

OECD - Organisation for Economic Co-operation and Development. 2019, Pharmaceutical Residues in Freshwater: Hazards and Policy Responses, OECD Studies on Water, OECD Publishing, Paris.

OECD - Organisation for Economic Co-operation and Development. 2019. Pharmaceutical Market. Available: <https://stats.oecd.org/Index.aspx?DataSetCode=HEALTH_PHMC>.

OECD - Organisation for Economic Cooperation and Development. 2004. OECD Guideline for Testing of Chemicals. *Daphnia* sp., Acute Immobilisation Test, Test Method 202, adopted April 13, 2004, Paris, France

OECD - Organisation for Economic Cooperation and Development. 2012. OECD Guidelines for Testing of Chemicals. *Daphnia magna* Reproduction Test Method 211. Paris, France. 25p.

OECD- Organisation for Economic Co-operation and Development. 2006. Test No. 221: *Lemna* sp. Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264016194-en.

Oliveira, L. L. D., Antunes, S. C., Gonçalves, F., Rocha, O., Nunes, B. 2015. Evaluation of ecotoxicological effects of drugs on *Daphnia magna* using different enzymatic biomarkers. Ecotoxicology and Environmental Safety, 119, 123–131. doi:10.1016/j.ecoenv.2015.04.028

Oppenheimer, J., Stephenson, R., Burbano, A., Liu, L. 2007. Characterizing the passage of personal care products through wastewater treatment processes. Water Environ Res 79(13):2564–2577

OsMed - Gruppo di lavoro dell'Osservatorio Nazionale sull'Impiego dei Medicinali L'uso dei farmaci in Italia. 2013. Rapporto Nazionale Anno p.406

PACT - Prescribing and Medicines Team Health and Social Care Information Centre. 2016. Prescription Cost Analysis, England 2015. Leeds (UK): Health and Social Care Information Centre. ISBN: 978-1-78386-680-9

Paíga, P., Delerue-Matos, C. 2016. Determination of pharmaceuticals in groundwater collected in five cemeteries' areas (Portugal). Science of The Total Environment, 569-570, 16–22. doi:10.1016/j.scitotenv.2016.06.090

Paíga, P., Santos, L. H. M. L. M. L., Ramos, S., Jorge, S., Silva, J. G., Delerue-Matos, C. 2016. Presence of pharmaceuticals in the Lis river (Portugal): sources, fate and seasonal variation. Sci Total Environ 573:164–177

Parekh, R. 2017. What Is Schizophrenia?. American Psychiatric Association. Available: <https://www.psychiatry.org/patients-families/schizophrenia/what-is-schizophrenia>.

Paykel E. S. 2008. Basic concepts of depression. Dialogues in clinical neuroscience, 10(3), 279–289.

Pazmiño, P. A., Pazmiño, P. B. 1988. Ketoprofen-Induced Irreversible Renal Failure. Nephron 50: 70-71

Peng, Y., Gautam, L., Hall, S. W. 2019. The detection of drugs of abuse and pharmaceuticals in drinking water using solid-phase extraction and liquid chromatography-mass spectrometry. Chemosphere. doi:10.1016/j.chemosphere.2019.02.040

Pereira, C. D. S., Maranho, L. A., Cortez, F. S., Pusceddu, F. H., Santos, A. R., Ribeiro, D. A., … Guimarães, L. L. 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Science of The Total Environment, 548-549, 148–154. doi:10.1016/j.scitotenv.2016.01.051

Persoone, G., Baudo, R., Cotman, M., Blaise, C., Thompson, K. C., Moreira-Santos, M., … Han, T. 2009. Review on the acute *Daphnia magna* toxicity test – Evaluation of the sensitivity and the precision of assays performed with organisms from

laboratory cultures or hatched from dormant eggs. Knowledge and Management of Aquatic Ecosystems, 393, 01. doi:10.1051/kmae/2009012

Piedade, F., Bio, S., Nunes, B. 2020. Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel *Mytilus* spp. Environmental Toxicology and Pharmacology, 103276. doi:10.1016/j.etap.2019.103276

Prášková, E., Štěpánová, S., Chromcová, L., Plhalová, L., Voslářová, E., Pištěková, V., … Svobodová, Z. 2013. The effects of subchronic exposure to ketoprofen on early developmental stages of common carp. Acta Veterinaria Brno, 82(3), 343–347. doi:10.2754/avb201382030343

Prescott, L. F., Critchley, J. A. J. H. 1983. The treatment of acetaminophen poisoning. Annu Rev Pharmacol Toxicol 23:87–101.

Quintana, J., Weiss, S., Reemtsma, T. 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Research, 39(12), 2654–2664. doi:10.1016/j.watres.2005.04.068

Radjenovic J., Petrović M., Barceló D. 2007Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. Anal. Bioanal. Chem. 387, 1365–1377. (doi:10.1007/s00216-006-0883-6).

Radjenović, J., Petrović, M., Barceló, D. 2009. Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. Water Res 43(3):831–841

Rainsford, K. D. 2007. Anti-inflammatory drugs in the 21st century. Subcell Biochem, 42: 3-27

Rangasamy, B., Hemalatha, D., Shobana, C., Nataraj, B., Ramesh, M. 2018. Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to anti-inflammatory drug ketoprofen. Chemosphere. doi:10.1016/j.chemosphere.2018.09.013

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C. B. 1997. Influence of Salicylic Acid on H_2O_2 Production, Oxidative Stress, and H_2O_2-Metabolizing Enzymes Salicylic Acid-Mediated Oxidative Damage Requires H_2O_2. Plant Physiology, 115(1), 137-149.

Rao, P. N. P., Knaus, E. E., 2008. Evolution of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Cyclooxygenase (COX) Inhibition and Beyond. J Pharm Pharmaceut Sci 11 (2): 81s-110s,

Rao, T. S. S, Andrade, C. 2016. Classification of psychotropic drugs: Problems, solutions, and more problems. Indian journal of psychiatry, 58(2), 111–113. https://doi.org/10.4103/0019-5545.183771

Reichert, J. F., Souza, D. M., Martins, A. F. 2019. Antipsychotic drugs in hospital wastewater and a preliminary risk assessment. Ecotoxicol Environ Saf 170:559–567. https://doi.org/10.1016/j.ecoenv.2018.12.021

Risse, A., Donovan, D., Heintzman, J., Page, T. 2009. NSAID Prescribing Precautions. Am Fam Physician. 80(12):1371-1378.

Robert, P. H., Thomas, K. V. 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Sci Total Environ 356(1–3):143–153

Roberts, P. H.; Bersuder, P. 2006. Analysis of OSPAR priority pharmaceuticals using high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry J. Chromatogr. A, 1134, 143-150

Roccaro, P. 2018. Treatment processes for municipal wastewater reclamation: the challenges of emerging contaminants and direct potable reuse. Curr. Opin. Environ. Sci. Heal. 2, 46–54

Rojas, M. R. et al. 2013. Assessment of the effectiveness of secondary wastewater treatment technologies to remove trace chemicals of emerging concern. Crit. Rev. Environ. Sci. Technol. 43, 1281–1314

Sallmann, A. R. 1986. The history of diclofenac. The American Journal of Medicine, 80(4), 29–33. doi:10.1016/0002-9343(86)90076-8

Sari, S., Ozdemir, G., Yangin-Gomec, C., Zengin, G. E., Topuz, E., Aydin, E.,et al. 2014. Seasonal variation of diclofenac concentration and its relation with wastewater characteristics at two municipal wastewater treatment plants in Turkey. Journal of Hazardous Materials, 272, 155–164. doi:10.1016/j.jhazmat.2014.03.015

Sarzi- Puttini, P., Atzeni, F., Lanata, L., Bagnasco, M., Colombo, M., Fischer, F., & D'Imporzano, M. 2010. Pain and ketoprofen: what is its role in clinical practice? Reumatismo, 62(3). doi:10.4081/reumatismo.2010.172

Sayadi, M. H., Trivedy, R. K, Pathak, R. K. 2010. Pollution of Pharmaceuticals in Environment. Jr. of Industrial Pollution Control 26(1), 89-94

Sepulveda, M. L., Baker, J. and Lummis, S. C. R. 1994. Chlorpromazine and Q X 222 block 5-HT3 receptors in N1E-115 neuroblastoma cells. Neuropharmacol, 33: 493–499.

Sharma, C. V., Mehta, V. 2014. Paracetamol: mechanisms and updates. Continuing Education in Anaesthesia Critical Care & Pain, 14(4), 153–158. doi:10.1093/bjaceaccp/mkt049

Silva A.R.R., Santos C.S.A., Ferreira N.G.C., Morgado R., Cardoso D.N., Cruz A., Mendo S., Soares A.M.V.M., Loureiro S. 2019. Multigenerational effects of carbendazim in Daphnia magna: From a subcellular to a population level. Environmental Toxicology And Chemistry, 38(2), 412-422.

Simon, L. S. 2013. Nonsteroidal anti-inflammatory drugs and their risk: a story still in development. Arthritis Res Ther 15, https://doi.org/10.1186/ar4173

Snyder, S., Lue-Hing, C., Cotruvo, J., Drewes, J. E., Eaton, A., Pleus, r. C., Schlenk, D.2010. Pharmaceuticals in the Water Environment. NACWA report. 38p.

Soares, A. M. V. M., Baird, D. J., Calow, P. 1992. Interclonal variation in the performance of *Daphnia magna* straus in chronic bioassays. Environmental Toxicology and Chemistry, 11(10), 1477–1483. doi:10.1002/etc.5620111013

Soetaert, A., Moens, L. N., Van der Ven, K., Van Leemput, K., Naudts, B., Blust, R., De Coen, W. M. 2006. Molecular impact of propiconazole on *Daphnia magna* using a reproduction-related cDNA array. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 142(1-2), 66–76. doi:10.1016/j.cbpc.2005.10.009

Stamatis, N. K., Konstantinou, I. K. 2013. Occurrence and removal of emerging pharmaceutical, personal care compounds and caffeine tracer in municipal sewage treatment plant in Western Greece. Journal of Environmental Science and Health, Part B, 48(9), 800–813. doi:10.1080/03601234.2013.781359

Stollewerk, A. 2010. The water flea Daphnia - a 'new' model system for ecology and evolution?. J Biol 9, 21. https://doi.org/10.1186/jbiol212

Subedi, B., Balakrishna, K., Sinha, R. K., Yamashita, N., Balasubramanian, V. G., Kannan, K. 2015. Mass loading and removal of pharmaceuticals and personal care products, including psychoactive and illicit drugs and artificial sweeteners, in five sewage treatment plants in India. Journal of Environmental Chemical Engineering, 3(4), 2882–2891. doi:10.1016/j.jece.2015.09.031

Subedi, B., Lee, S., Moon, H.-B., Kannan, K. 2013. Psychoactive Pharmaceuticals in Sludge and Their Emission from Wastewater Treatment Facilities in Korea. Environmental Science & Technology, 47(23), 13321–13329. doi:10.1021/es404129r

Sun, Q., Li, M., Ma, C., Chen, X., Xie, X., Yu, C.-P. 2016. Seasonal and spatial variations of PPCP occurrence, removal and mass loading in three wastewater treatment plants located in different urbanization areas in Xiamen, China. Environmental Pollution, 208, 371–381. doi:10.1016/j.envpol.2015.10.003

Tang, W. 2003. The Metabolism of Diclofenac - Enzymology and Toxicology Perspectives. Current Drug Metabolism, 4(4), 319–329. doi:10.2174/1389200033489398

Tatarazako, N., Oda, S. 2007. The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. Ecotoxicology 16, 197–203. https://doi.org/10.1007/s10646-006-0120-2

Ternes, T. A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research, 32(11), 3245–3260. doi:10.1016/s0043-1354(98)00099-2

Thomas, K. V., Hilton, M. J. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Marine Pollution Bulletin, 49(5-6), 436–444. doi:10.1016/j.marpolbul.2004.02.028

Tixier, C., Singer, H. P., Oellers, S., Müller, S. R. 2003. Occurrence and Fate of Carbamazepine, Clofibric Acid, Diclofenac, Ibuprofen, Ketoprofen, and Naproxen in Surface Waters. Environmental Science & Technology, 37(6), 1061–1068. doi:10.1021/es025834r

Trautwein, C., Kümmerer, K. 2012. Degradation of the tricyclic antipsychotic drug chlorpromazine under environmental conditions, identification of its main aquatic biotic and abiotic transformation products by LC–MSn and their effects on environmental bacteria. Journal of Chromatography B, 889-890, 24–38. doi:10.1016/j.jchromb.2012.01.022

UNESCO and HELCOM. 2017. Pharmaceuticals in the aquatic environment of the Baltic Sea region – A status report. UNESCO Emerging Pollutants in Water Series – No. 1, UNESCO Publishing, Paris.

Vane, J. R., Botting, R. M. 1998. Mechanism of action of nonsteroidal anti-inflammatory drugs. Am J Med, 104: 2S-8S

Verlicchi, P., Galletti, A., Masotti, L. 2010. Management of hospital wastewaters: the case of the effluent of a large hospital situated in a small town. Water Sci Technol 61(10):2507–2519

Vickers, A. E. M. 2009. Tissue slices for the evaluation of metabolism-based toxicity with the example of diclofenac. Chemico-Biological Interactions, 179(1), 9–16. doi:10.1016/j.cbi.2008.10.010

Vieno, N., Sillanpää, M. 2014. Fate of diclofenac in municipal wastewater treatment plant — A review. Environment International, 69, 28–39. doi:10.1016/j.envint.2014.03.021

Villar-Navarro, E., Baena-Nogueras, R. M., Paniw, M., Perales, J. A., & Lara-Martín, P. A. 2018. Removal of pharmaceuticals in urban wastewater: High rate algae pond (HRAP) based technologies as an alternative to activated sludge based processes. Water Research, 139, 19–29. doi:10.1016/j.watres.2018.03.072

Voogt, P., Janex-Habibi, M.-L., Sacher, F., Puijker, L., Mons, M. 2009. Development of a common priority list of pharmaceuticals relevant for the water cycle. Water Science and Technology, 59(1), 39–46. doi:10.2166/wst.2009.764

Wang, P., Huang, B., Chen, Z., Lv, X., Qian, W., Zhu, X., … Cai, Z. 2019. Behavioural and chronic toxicity of fullerene to Daphnia magna: Mechanisms revealed by transcriptomic analysis. Environmental Pollution, 113181. doi:10.1016/j.envpol.2019.113181

Wang, W. 1986. Toxicity tests of aquatic pollutants by using common duckweed. Environmental Pollution Series B, Chemical and Physical, 11(1), 1–14. doi:10.1016/0143-148x(86)90028-5

Ward, B., Alexander-Williams, J. M. 1999. Paracetamol revisited: A review of the pharmacokinetics and pharmacodynamics. Acute Pain, 2(3), 139–149. doi:10.1016/s1366-0071(99)80006-0

Warner, T. D., Mitchell, J. A. 2002. Cyclooxygenase-3 (COX-3): filling in the gaps toward COX continuum? Proc Natl Acad Sci U S A 99(21):13371–13373

Weigel, S., Kuhlmann, J., Hühnerfuss, H. 2002. Drugs and personal care products as ubiquitous pollutants: occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. Science of The Total Environment, 295(1-3), 131–141. doi:10.1016/s0048-9697(02)00064-5

Weissman, S. H. 2015. Changes in the Practice of Psychiatry Since World War II. Psychiatry advisor. Available: <https://www.psychiatryadvisor.com/home/practice-management/changes-in-the-practice-of-psychiatry-since-world-war-ii/>.

WHO - World Health Organization. 2012. Pharmaceuticals in drinking-water. WHO Library Cataloguing-in-Publication ata

WHO – World Health Organization. 2019 The selection and use of essential medicines: report of the WHO Expert Committee on Selection and Use of Essential Medicines (including the 21st WHO Model List of Essential Medicines and the 7th WHO Model List of Essential Medicines for Children). Geneva: World Health Organization; (WHO Technical Report Series, No. 1021). Licence: CC BY-NC-SA 3.0 IGO.

Wiegel, S., Aulinger, A., Brockmeyer, R., Harms, H., Loffler, J., Reincke, H., Schmidt, R., Stachel, B., von Tumpling, W., Wanke, A. 2004. Pharmaceuticals in the river Elbe and its tributaries. Chemosphere 57:107–126

Wille, K., Noppe, H., Verheyden, K. et al. 2010. Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater. Anal Bioanal Chem 397, 1797–1808. https://doi.org/10.1007/s00216-010-3702-z

Windsor, F. M., Ormerod, S. J., Tyler, C. R. 2018. Endocrine disruption in aquatic systems: up-scaling research to address ecological consequences. Biological Reviews, 93(1), 626–641. doi:10.1111/brv.12360

Wong, A., Graudins, A. 2017. Risk prediction of hepatotoxicity in paracetamol poisoning, Clinical Toxicology, 55:8, 879-892, DOI: 10.1080/15563650.2017.1317349

Wongrakpanich, S., Wongrakpanich, A., Melhado, K., Rangaswami, J. 2018. A Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The Elderly. Aging and disease, 9(1), 143–150. https://doi.org/10.14336/AD.2017.0306

Wright, J. M. 2002. The double-edged sword of COX-2 selective NSAIDs. Canadian Medical Association journal. 167(10), 1131–1137.

Xie, Z., Lu, G., Yan, Z., Liu, J., Wang, P., Wang, Y. 2017. Bioaccumulation and trophic transfer of pharmaceuticals in food webs from a large freshwater lake. Environmental Pollution, 222, 356–366. doi:10.1016/j.envpol.2016.12.026

Yamkelani Mlunguza, N., Ncube, S., Nokwethemba Mahlambi, P., Chimuka, L., Mzukisi Madikizela, L. 2019. Adsorbents and removal strategies of non-steroidal anti-inflammatory drugs from contaminated water bodies. Journal of Environmental Chemical Engineering, 103142. doi:10.1016/j.jece.2019.103142

Yuan, S., Jiang, X., Xia, X., Zhang, H., Zheng, S., 2013. Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere 90, 2520–2525.

Zelikoff, J. T. 1998. Biomarkers of immunotoxicity in fish and other non-mammalian sentinel species: predictive value for mammals? Toxicology, 129(1), 63–71. doi:10.1016/s0300-483x(98)00064-x

Zhao, L., Pickering, G. 2010. Paracetamol metabolism and related genetic differences. Drug Metabolism Reviews, 43(1), 41–52. doi:10.3109/03602532.2010.527984

Zheng, Y., Yuan, J., Gu, Z., Yang, G., Li, T., Chen, J. 2020. Transcriptome alterations in female Daphnia (*Daphnia magna*) exposed to 17β-estradiol. Environmental Pollution, 261, 114208. doi:10.1016/j.envpol.2020.114208

Zimmerman, M. J. 2005. Occurrence of organic wastewater contaminants, pharmaceuticals, and personal care products in selected water supplies, Cape Cod, Massachusetts, June 2004: U.S. Geological Survey Open-File Report 2005-1206, 16 p.

Zivna, D., Blahova, J., Siroka, Z., Plhalova, L., Marsalek, P., Doubkova, V., … Svobodova, Z. 2016. The Effects of Salicylic Acid on Juvenile Zebrafish *Danio rerio* Under Flow-Through Conditions. Bulletin of Environmental Contamination and Toxicology, 97(3), 323–330. doi:10.1007/s00128-016-1877-5

Zorita, S. Mårtensson, L., Mathiasson, L. 2009.Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. Sci. Total Environ., 407, 2760-2770, 10.1016/j.scitotenv.2008.12.030

Zupanc, M., Kosjek, T., Petkovšek, M., Dular, M., Kompare, B., Širok, B., … Heath, E. 2013. Removal of pharmaceuticals from wastewater by biological processes, hydrodynamic cavitation and UV treatment. Ultrasonics Sonochemistry, 20(4), 1104–1112. doi:10.1016/j.ultsonch.2012.12.003

Żur, J., Piński, A., Marchlewicz, A. et al. 2018. Organic micropollutants paracetamol and ibuprofen—toxicity, biodegradation, and genetic background of their utilization by bacteria. Environ Sci Pollut Res 25, 21498–21524https://doi.org/10.1007/s11356-018-2517-x

Chapter 2

Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species Lemna minor and Lemna gibba

Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M., Barata, C., Nunes, B. 2019. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Science of The Total Environment, 657, 926–937. doi:10.1016/j.scitotenv.2018.12.002

Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*

Alkimin GD[1,2], Daniel D[1], Frankenbach S[1,2], Serôdio J[1,2], Soares AMVM[1,2], Barata C[3], Nunes, B[1,2*]

[1] Department of Biology, Aveiro University, Campus de Santiago, 3810-193 Aveiro, Portugal

[2] Centre for Environmental and Marine Studies (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[3]Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

2.1 Abstract

In the last years the environmental presence of pharmaceuticals has gained increasing attention. Research data show that these compounds can cause toxicological effects in different species of fish, mollusks and macroinvertebrates. However, the literature is scarce in terms of ecotoxicity data especially focusing on plants as test organisms. Ecotoxicological plant-based tests following the standard OEDC guideline 221 (OECD, 2006) are strongly restricted due to the recommended endpoints: growth and yield of plants. It is necessary to develop and validate alternative macrophyte-based tests (non-standard endpoints), more sensible and providing additional information about the chemical contamination effects in plants. To attain this purpose, species from the *Lemna* genus were selected. Thus, the aim of this paper was to analyze the toxic effects of pharmaceuticals in non-standard endpoints on two macrophyte species, *Lemna minor* and *Lemna gibba*. To this purpose an acute assay (96 h) was performed with *L. minor* and *L. gibba* exposed to chlorpromazine (CPZ), paracetamol (APAP), and diclofenac

(DCF), in the following concentration ranges: 0 to 20 µg/L, 0 to 125 µg/L, and 0 to 100 µg/L, respectively. The analyzed endpoints were: levels of chlorophyll a and b, total chlorophyll, carotenoids, and anthocyanins; chlorophyll fluorescence; and catalase activity. In general, higher concentrations of the tested pharmaceuticals caused significant effects on both *Lemna* species in terms of the different endpoints analyzed. In conclusion, acute exposures to CPZ, APAP, and DCF differently affected the defensive system of the tested species; among chlorophylls, chlorophyll b content was more affected, but pharmaceutical exposure was not able to cause alterations on chlorophyll fluorescence.

Keywords: chlorpromazine; paracetamol; diclofenac; aquatic plants; macrophytes; pharmaceutical drugs

2.2 Introduction

During the past three decades, the presence of pharmaceuticals in the environment has been recognized as an indisputable reality, gaining increased attention. The already existing scientific literature demonstrates that pharmaceuticals enter the environment, especially the aquatic compartment, and can exert adverse effects on exposed biota (Küster and Adler, 2014). Even though being usually present in low amounts in the wild, pharmaceuticals attain levels that may be causative of deleterious effects on aquatic non target organisms (Corcoran et al., 2010).

Among pharmaceuticals that have been reported to occur in the aquatic environment, one assuming an important role due to its intrinsic toxicity, is paracetamol (APAP) (N-acetyl-p-aminophenol, also known as acetaminophen) (Nunes et al., 2014). This is a very common over-the-counter analgesic used for fever, headaches, and minor pain (Wu et al., 2012), and was included in the priority list developed by Voogt et al. (2009) of pharmaceuticals relevant for the water cycle.

Other important drug commonly found in the wild is diclofenac (DCF), a non-steroidal anti-inflammatory drug (NSAID), widely used to reduce inflammation and to relieve pain in conditions such as arthritis or acute injury. It also works as antiuricosuric (Lonapan et al., 2016). In addition, this substance is another pharmaceutical included by

Voogt et al. (2009) in the already mentioned list, and was classified as class 1 – high-priority pharmaceuticals (Lonapan et al., 2016).

Chlorpromazine (CPZ) was the first modern antipsychotic drug released in 1952 and approved by the Food and Drug Administration in 1954 (Kane and Correll, 2010). It is used to control excitement, agitation, aggression and other psychomotor disturbances in schizophrenic patients, and is sometimes used in other psychiatric conditions for the control of anxiety and tension (Borges et al., 2008). Given its wide use, it has been recognized as an important emerging pollutant in the aquatic environment (Li et al., 2008).

The toxicity of these pharmaceuticals towards aquatic organisms (fauna and flora) may occur chronically as a result of exposure of aquatic biota to wastewater residues mostly, and over their entire life cycle (Fent et al., 2006). Researches show that these pharmaceutical compounds can cause ecotoxicological effects in different species such as fish (Munro, 1986; Guiloski et al., 2017), mollusks (Brandão et al., 2014; Nunes et al., 2017), and macroinvertebrates (Du et al., 2016; Oliveira et al., 2015). However, the literature is scarce in terms of ecotoxicity data, especially focusing on plants as test organisms. Among potentially exposed wild organism, macrophyte species occupy a very important key position in aquatic environments, since they provide food, shelter and substrate to various aquatic organisms (Carpenter and Lodge, 1986). However, the ultimate consequences of chemical impacts on plant communities are largely unknown and should be explored (Coutris et al., 2011).

Ecotoxicological plant-based tests following the standard OEDC guideline 221 (OECD, 2006) are strongly restricted due to the recommended endpoints: growth and yield of plants. To have a better understanding of ecotoxicological consequences of drug exposure in the aquatic environment when challenged by such chemical contamination, more effects that reflect the impairment of other pathways are strongly required. It is necessary to develop and validate alternative macrophyte-based tests, more sensible and providing more information about the chemical contamination effects on plants. To attain this purpose, the present article described a series of experiments based on the use of two species of the *Lemna* genus, which present several advantages as test species. Organisms from this genus are characterized by small size (requiring little space in the

laboratory), rapid growth (toxicity can be detected within a relatively short time), and the establishment of clonal lineages (allowing genetically identical plants to be used in tests; Cedergreen et al., 2009).

Among the species from this genus, the two most used in ecotoxicological testing are *Lemna minor* and *Lemna gibba.* Besides presenting all the above mentioned advantages, these two species are strongly recommended by international guidelines to be used for the purpose of ecotoxicity screening (ISO, 2005; OECD, 2006), being routinely used in this research area (Lakatos et al., 1993; Brain and Solomon, 2007; Scherr et al., 2008). In addition, both species occupy the same ecological niche, thus being putative competitors (Oláh et al., 2010).

Therefore, the aim of this paper was to analyze the toxic effects of pharmaceuticals on non-standard endpoints (pigments, chlorophyll fluorescence and biochemical biomarker) on two macrophyte species, *L. minor* and *L. gibba*.

2.3 Material and Methods

2.3.1 Chemicals

All pharmaceutical drugs were purchased from Sigma Aldrich, with purities >98%: acetaminophen (CAS: 32113- 41-0), chlorpromazine hydrochloride (CAS: 69-09-0), diclofenac sodium (CAS: 15307-79-6) and all other chemicals used in this study have analytical purity.

2.3.2. *Lemna* spp. culture

Plants of the species *L. minor* and *L. gibba* were obtained from laboratory axenic cultures, established at the Centre for Environmental and Marine Studies (CESAM) at University of Aveiro, reared under controlled conditions (temperature 20 ± 2 °C; photoperiod 16 h^L:8 h^D; light intensity, ~5,500 lux) in modified Steinberg medium, according to the guideline OECD 221 (OECD, 2006). The cultures were maintained in 1

L glass with approximately 200 mL of modified Steinberg medium (OECD, 2006); the total medium was renewed twice a week.

2.3.3 *Lemna* spp. assay

Tests were performed with ranges of nominal concentrations of CPZ, APAP and DCF that were chosen based on the reported environmental concentrations in rivers and waste water effluents (Yuan et al., 2013; Wiegel et al. 2004; Roberts and Thomas, 2006; Hilton and Thomas, 2003), CPZ (0, 0.032, 0.16, 0.8, 4 and 20 µg/L), APAP (0, 0.2, 1, 5, 25 and 125 µg/L) and DCF (0, 0.16, 0.8, 4, 20 and 100 µg/L). Individuals of *Lemna* spp. were exposed in plastic flakes, in six replicates with a final volume of 150 mL of modified Steinberg medium (OECD, 2006) per replicate, adequately supplemented with the pharmaceuticals stock solutions. In control treatment replicates were exposed only to the modified Steinberg medium (OECD, 2006). The assay started with plants covering ~30% of the vessel area. Assays were conducted under controlled conditions (temperature 23 ± 2 °C; photoperiod 24 h^L; light intensity, ~5,500 lx). *Lemna* spp. fronds were exposed for 96 h and the total medium was renewed once (at 48 h) during the test. At the end of the exposure period, the plant biomass of each replicate was divided into two portions; a portion was used for the determination of chlorophyll fluorescence (immediately after the end of the exposure period) and the remaining biomass was placed in Eppendorf microtubes and stored at -80 ºC for the quantification of pigments (chlorophyll a, b and total, carotenoids and anthocyanin) and biochemical endpoint (catalase activity).

2.3.4 Pigments analysis (chlorophylls, carotenoids, and anthocyanins)

Pigments were extracted from fronds of *Lemna* spp. (about 25 mg - fresh weight - FW); For the determination of fresh weight, the fronds of both species were collected with tweezers, placed in Eppendorf microtubes with absorbent paper at the bottom, and centrifuged at 1000 g for 2 min. After this procedure, the samples were removed from the microtubes and weighed in analytical balance.

Total chlorophylls (Chl) and carotenes (car) levels were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979). The analyze was performed in 3.5 mL of dimethyl sulphoxide (DMSO), the extract was put in water at 65ºC during 30 min and allowed to cool overnight at room temperature and dark conditions. The next day, samples were thoroughly vortexed for about 10 s and centrifuged for 5 min at 15000 g at 4 °C. The obtained supernatants were used to quantify the amounts of Chl a, b and total chlorophylls, and carotenoids, through spectrophotometry by measuring absorbances of the extracts at wavelengths of 470, 645, 646, and 663 nm in a spectrophotometer Thermo Scientific Multiskan (Software Ascent 2.6). The extraction solution was used as blank and the calculation of the pigments followed the equations 1, 2 and 3 proposed by Arnon (1949 - shown by Hiscox and Israelstam (1979) to be virtually identical to Chl extracted in DMSO) for a, b and total Chls, respectively, and the equation 4 to calculate the amount of carotenoids (Lichtenthaler, 1987), and results were expressed in milligram per gram of fresh weight (mg/g FW).

$$\text{Chl a} = (12.70 \times A_{663}) - (2.69 \times A_{645}) \quad (1)$$

$$\text{Chl b} = (22.90 \times A_{645}) - (4.68 \times A_{663}) \quad (2)$$

$$\text{Total Chl} = (20.20 \times A_{646}) + (8.02 \times A_{663}) \quad (3)$$

$$\text{Car} = (1000 \times A_{470} - 1.43\text{Chl a} - 35.87\text{Chl b})/205 \quad (4)$$

Anthocyanin (Ant) content was determined according to Close et al. (2004) with modifications described below. Plants (~20 mg) were placed in 2 mL of acidified methanol (methanol: 1%HCl: water, 90:1:1, v/v). The mixture was then immersed in hot water (~95 ºC) for 1.5 min and left for 24 h in the dark at 4 °C. After centrifugation at 15000 g for 5 min, absorbance was measured at 530 and 657 nm in a Thermo Scientific Vis Spectrophotometer 10S TM. Anthocyanin content was determined according to the equation 5 (Mancinelli et al., 1975). The anthocyanin content was calculated using an extinction coefficient of 33,000 mol^{-1} cm^{-1} (Sanchez-Zabala et al., 2013) and results were expressed in number of moles per gram of fresh weight (mol/g FW).

$$\text{Ant} = A_{530} - (0.25 \times A_{657}) \quad (5)$$

2.3.5. Chlorophyll fluorescence

Chlorophyll fluorescence was measured immediately at the end of the exposure (96 h), at room temperature using an imaging chlorophyll fluorimeter (Open FluorCam 800-O/101; Photon System Instruments; Brno, Czech Republic) Minimum fluorescence (Fo), maximum fluorescence (Fm), variable fluorescence (Fv, equivalent to Fm – Fo) and maximum quantum yield of photosystem II (PSII) photochemistry (Fv/Fm) were measured as Chl components and the analyzes were made using the program FluorCam 7. The FluorCAM was composed of two red LED panels (MLS13x13, Photon Systems Instruments, PSI), which provided the measuring non-actinic light (0.1 μmol $m^{-2} \cdot s^{-1}$), while two other red LED panels provided the saturating pulse (7500 μmol $m^{-2} \cdot s^{-1}$; SL3500, PSI). All red panels had an emission peak at 621 nm and a 40nm bandwidth. A user-defined protocol, with a total duration of 2 s, was employed to measure the minimum fluorescence yields (Fo) in the first 1.4 s, and the maximum fluorescence values (Fm) after a saturating pulse of 0.6 s. Measurements were performed after 15 min dark-adaptation.

2.3.6 Biochemical marker - catalase activity determination

Approximately 25 mg of tissue of both species, per replicate, was homogenized in ice-cold phosphate buffer (50 mM, pH 7.0, with 0.1 % Triton X-100) and then the homogenized samples were centrifuged at 15,000 g for 10 min at 4 °C. Each homogenate sample was stored at −80 °C until further enzymatic determination as described below.

Catalase (CAT) activity was determined in a 96 well plate. Spectrophotometric readings were performed in a microplate reader Thermo Scientific Multiskan (Software Ascent 2.6). CAT activity was assayed by the procedure described by Aebi (1984). CAT activity was quantified based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein (mol/min/mg protein).

2.3.7 Protein determination

Protein quantification was performed at 595 nm using the Bradford method (Bradford, 1976), adapted to microplate with bovine γ-globulin as standard (1 mg/mL), in order to express enzymatic activities per mg of protein of the analyzed samples.

2.3.8 Statistical analysis

A one-way analysis of variance (ANOVA) with appropriate post hoc test (Dunnett's or Dunn's test) was conducted. The type of ANOVA (parametric or nonparametric) and post hoc test were chosen depending on whether normality and homogeneity of data were demonstrated by analysis of the residuals with the Shapiro-Wilks test. Test statistics and analysis of normality were conducted using the software SigmaPlot Ver 12.5 and a significance level of 0.05.

2.4. Results

2.4.1. Pigments analysis (chlorophylls, carotenoids and anthocyanins)

In general, CPZ, APAP and DCF exposures resulted in Chl a contents close to those from plants of control treatment (figure 1). Chlorophyll b was a more sensitive endpoint than Chl a. All tested pharmaceuticals caused effects on the Chl b content of *L. minor*, increasing its content. DCF and APA were the most toxic compounds affecting Chl b at 4-5 µg/L and CPZ the least toxic. The Chl b content of *L. gibba* was only affected by CPZ at 0.16 µg/L (figure 2). Total Chl changed significantly ($p<0.05$) at 20 µg/L of DCF on *L. minor* and at 100 µg/L on *L. gibba* (figure 3).

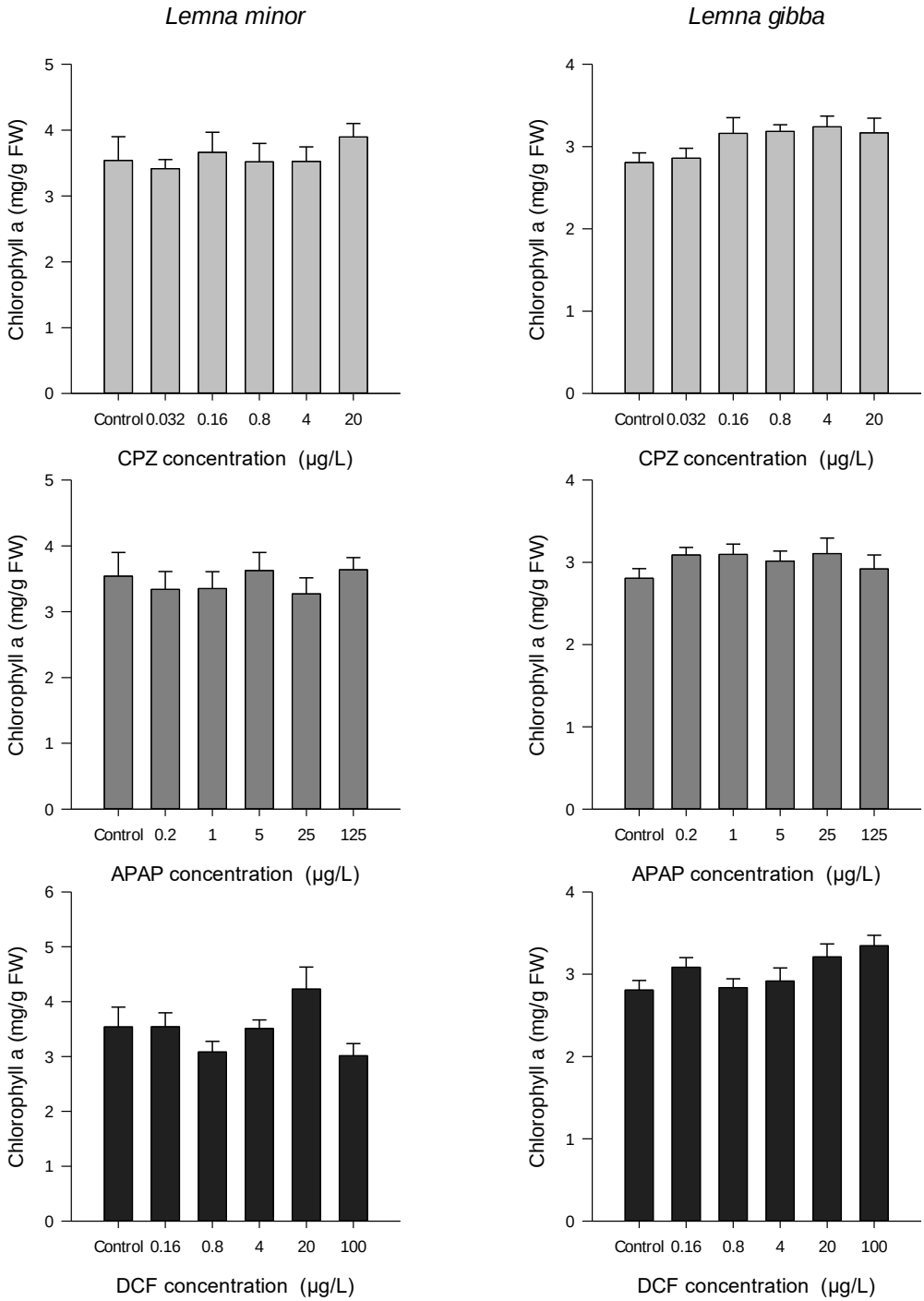

Figure 1. Effects of the pharmaceuticals (CPZ, APAP and DCF) on chlorophyll a of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

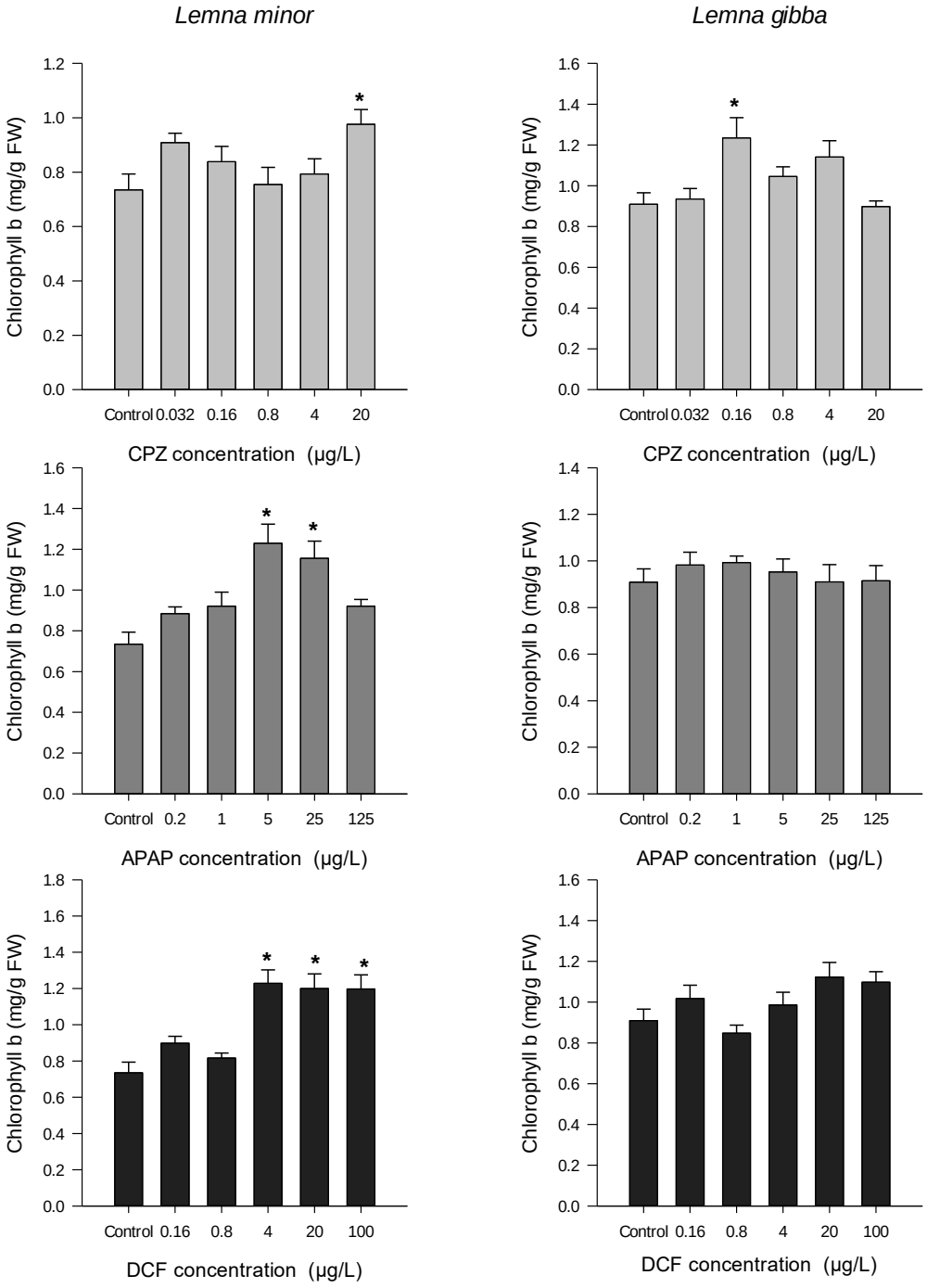

Figure 2. Effects of the pharmaceuticals (CPZ, APAP and DCF) on chlorophyll b of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

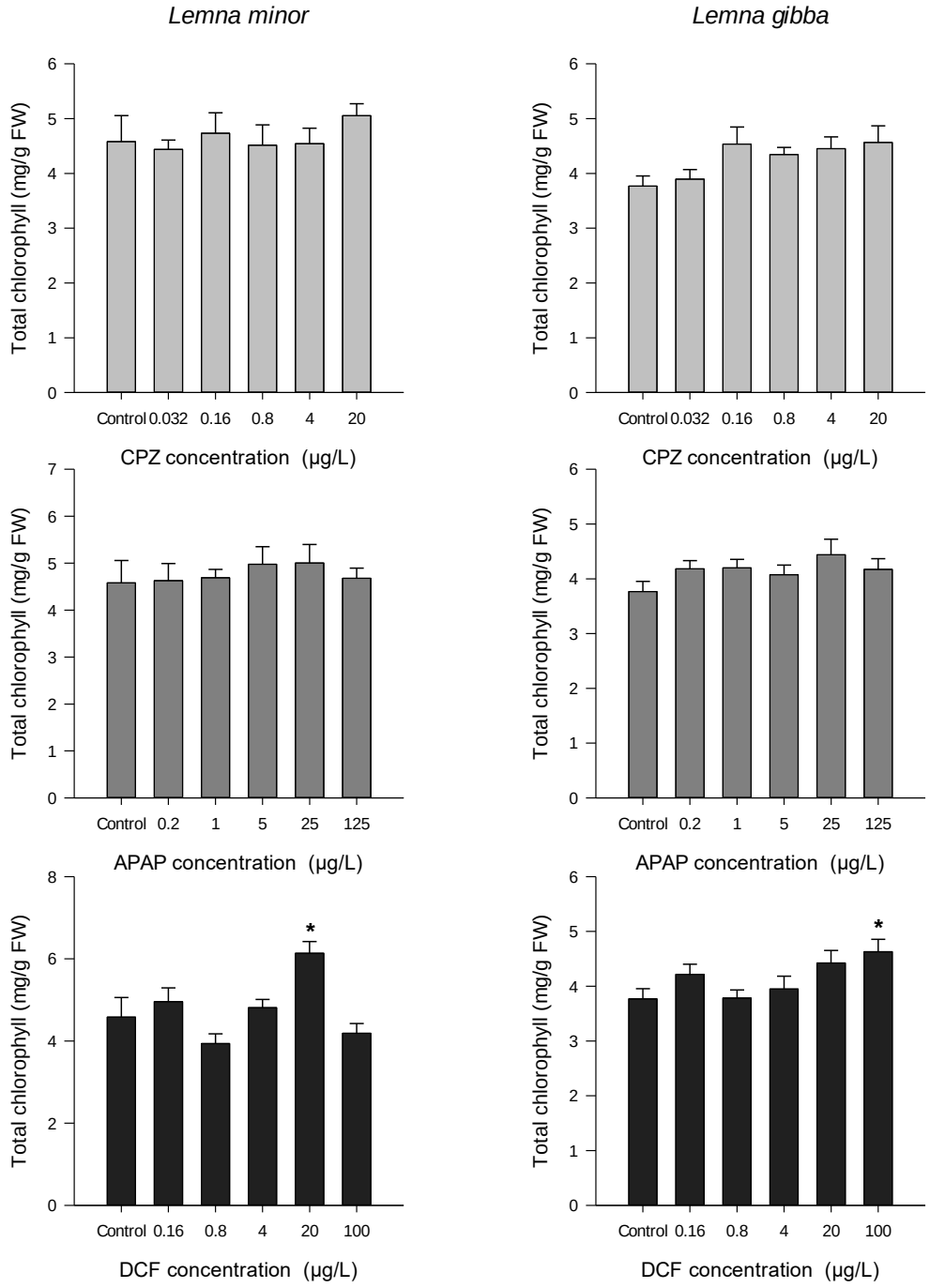

Figure 3. Effects of the pharmaceuticals (CPZ, APAP and DCF) on total chlorophyll of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

The carotenoid content was only significantly affected in *L. gibba* at 20, 100 µg/L of DCF, and at 20 µg/L of CPZ (figure 4). The levels of anthocyanins in *L. gibba* were quite sensitive to the tested pharmaceuticals decreasing significantly ($p<0.05$) in plants exposed to all tested concentrations (figure 5).

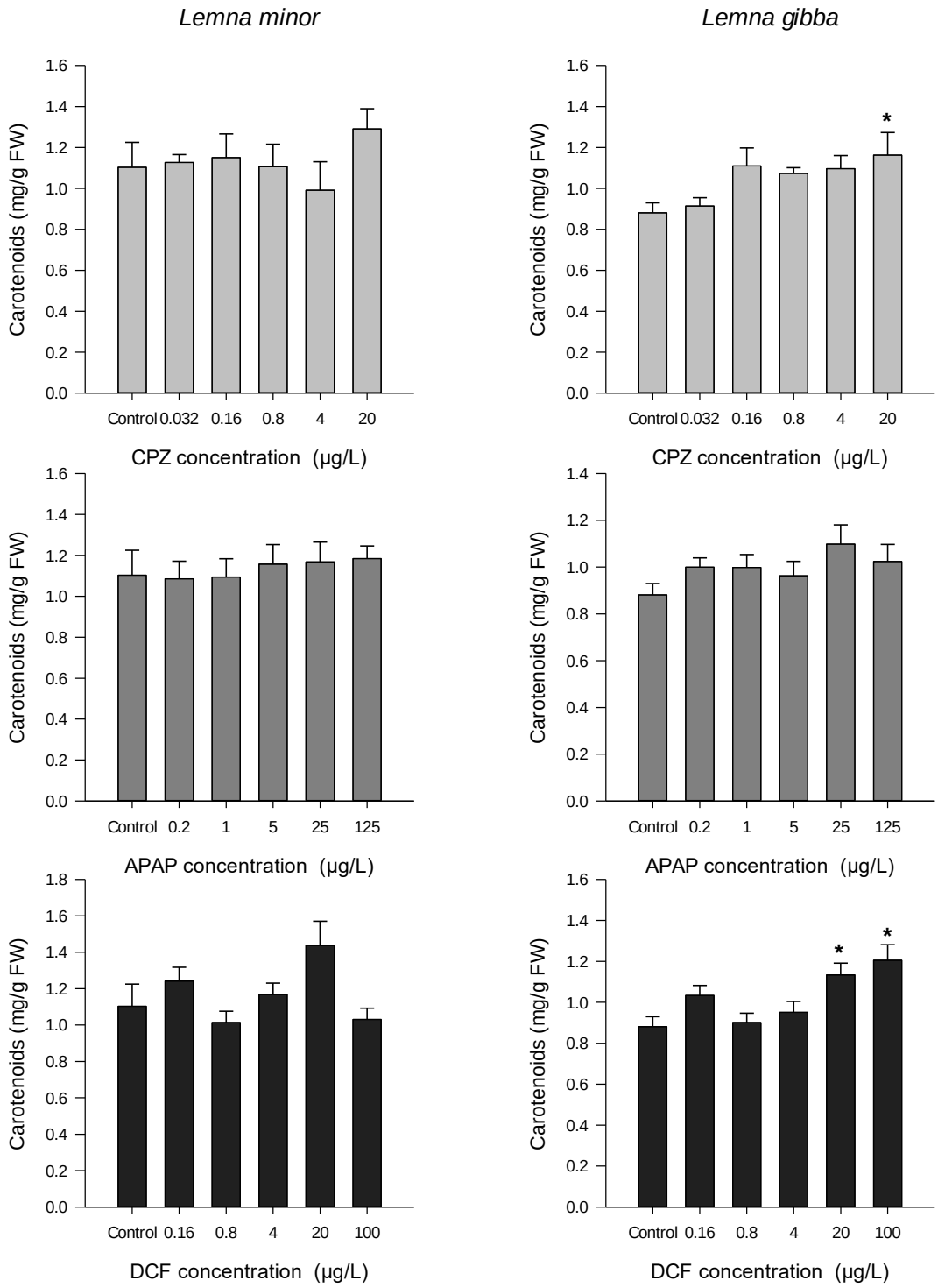

Figure 4. Effects of the pharmaceuticals (CPZ, APAP and DCF) on carotenoids of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

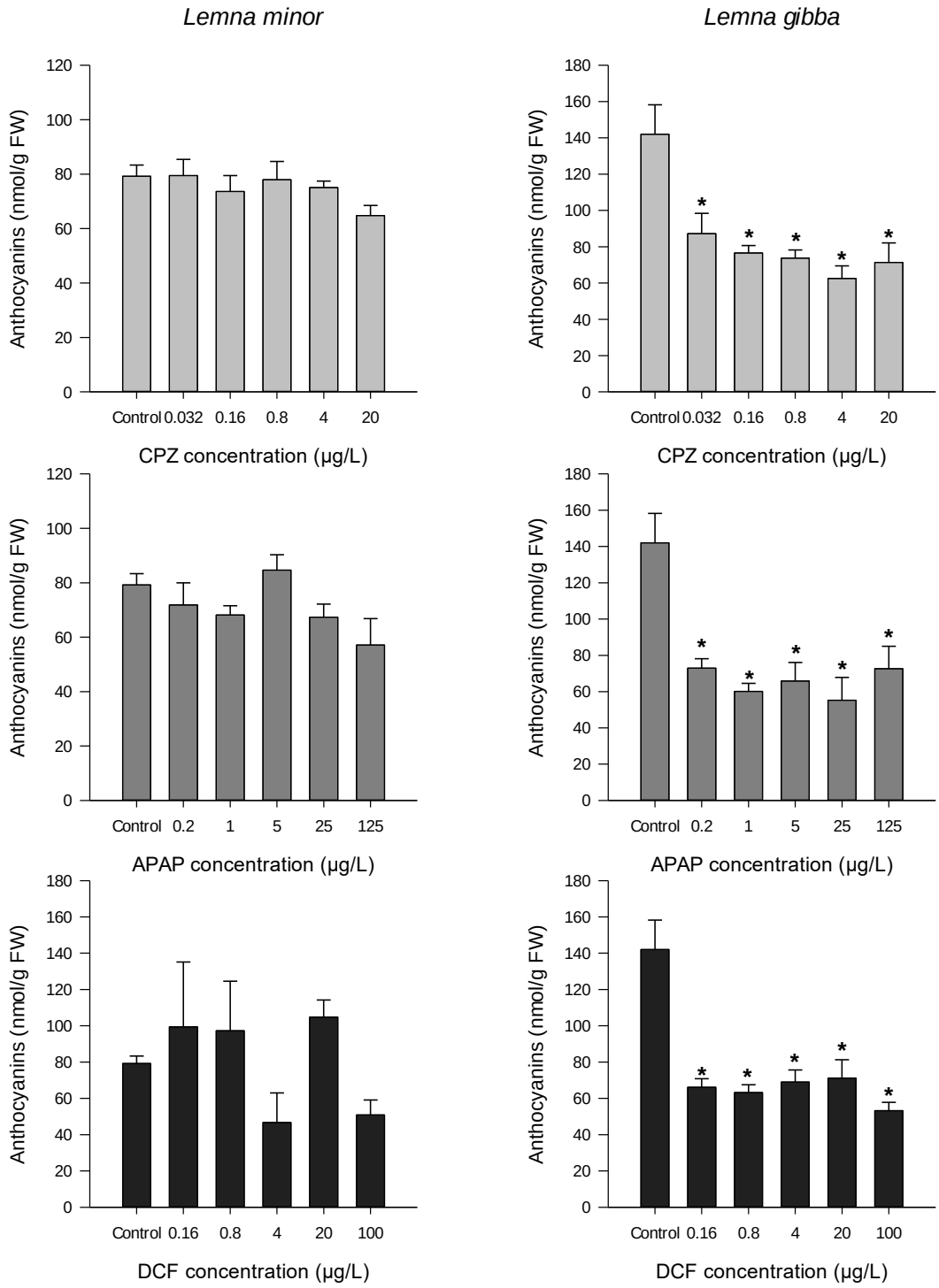

Figure 5. Effects of the pharmaceuticals (CPZ, APAP and DCF) on anthocyanins of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

2.4.2. Chlorophyll fluorescence

Considering Chl fluorescence, the maximal quantum yield of PSII (Fv/Fm) and was not significantly ($p>0.05$) affected by all pharmaceutical concentrations exposure in both *Lemna* species (figure 6).

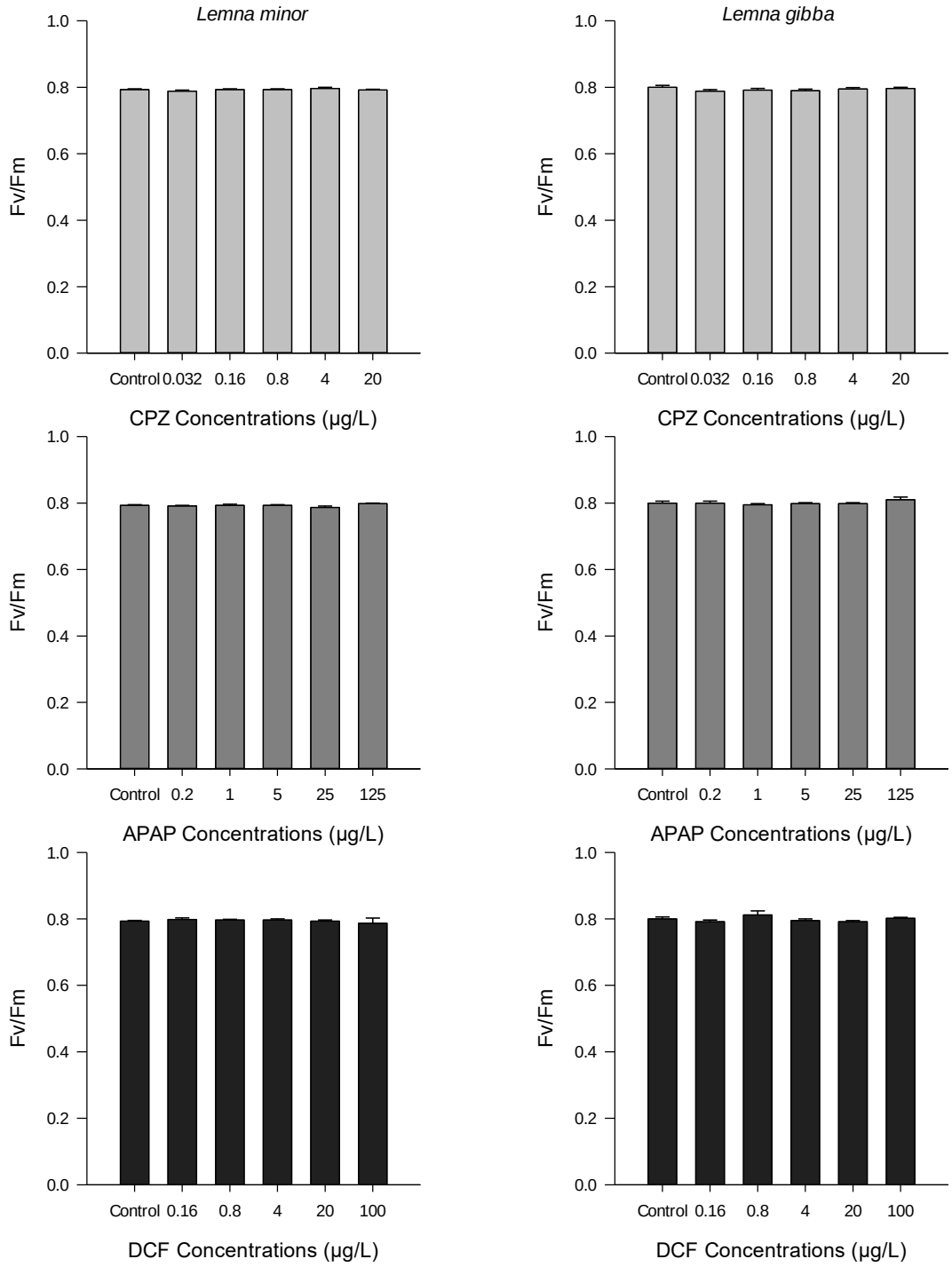

Figure 6. Effects of the pharmaceuticals (CPZ, APAP and DCF) on maximal quantum yield of PSII (Fv/Fm) *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands

for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

2.4.4. Catalase determination

The *L. minor*'s CAT activity was affected by the three tested pharmaceuticals, in general causing an increase in activity compared with the control treatment. Most concentrations of CZP (with the exception of 0.8 µg/L) enhanced CAT activity. APAP and DCF, respectively, caused significant ($p<0.05$) increases of CAT activity at 25 µg/L and 100 µg/L. Catalase activity in *L. gibba* decreased at, 1 µg/L of APAP and increased at 5 and 125 µg/L (figure 7).

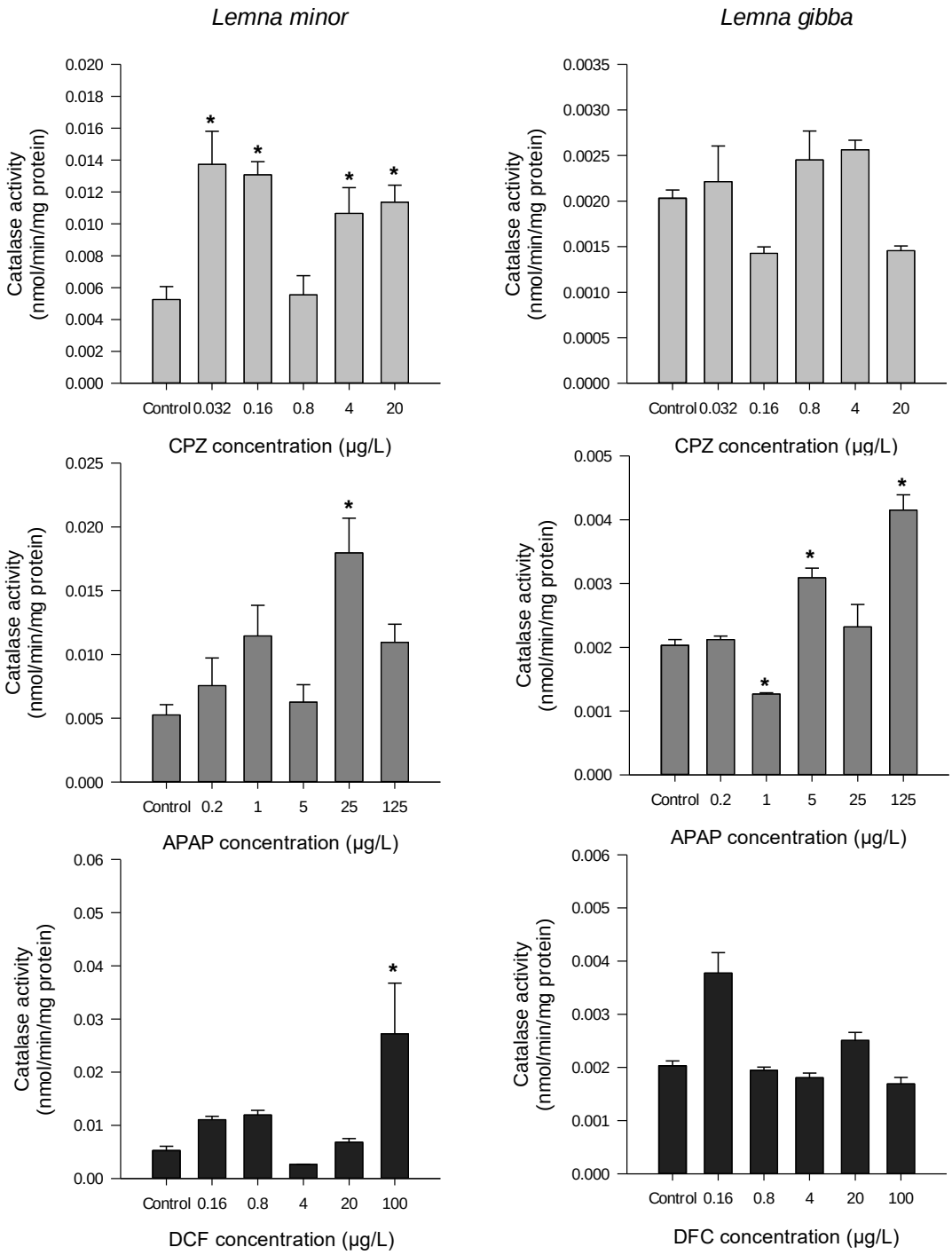

Figure 7. Effects of the pharmaceuticals (CPZ, APAP and DCF) on catalase activity of *L. minor* and *L. gibba*. For each parameter, mean and standard error are shown (N=6). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

2.5 Discussion

2.5.1. Antioxidant responses

Plants can react rapidly to adverse environmental conditions ranging from biochemical processes up to the development of visible symptoms of injury, independent of the primary target of the stress. Anthocyanins are part of the defense against oxidative stress for shoots growing under unfavorable environments (Gould et al., 2002). These pigments show scavenging ability against various artificially generated free radicals (Wang et al., 1997). Often this pigment increase in response to different stresses (Pietrini et al., 2002) to prevent reactive oxygen species (ROS) damage acting as antioxidant. However, the here-obtained results showed that, in general, anthocyanins content decreased in both species after exposure to the three tested pharmaceuticals, suggesting a failure in this defense mechanism causing high oxidative stress, mostly on *L. gibba*, which was the most affected species.

In addition, carotenoid content in plants usually increases in presence of pro-oxidative xenobiotics because of their antioxidant action, providing protection against toxicity (Murthy et al., 2005). This response was more evident for *L. gibba*, species that evidenced more oxidative damage considering its anthocyanins content; in fact, carotenoids levels were significantly increased in plants exposed to the higher concentrations of CPZ and DCF, showing the onset of compensatory mechanism to better cope with the chemical insult by both compounds. Changes in carotenoid levels likely trigger different regulatory mechanisms and result in the activation of a signaling cascade to generate an adaptive response in the cell (Nogueira et al., 2013) and are efficient antioxidants protecting against oxidative damage (Stahal and Sies, 2003). Beside this, carotenoids roles include protection of the reaction centers and the antenna complex (Edge and Truscott, 2018) to avoid damage in photosynthetic apparatus. This pattern suggests that, at the beginning of the toxic process, *L. gibba* has a more efficient defense mechanism against toxicants than *L. minor*. However, the biological meaning of this difference remains unclear, considering the absence of enough physiological data about the two species that support this indication. Nevertheless, it is known that the competition between both duckweed species occurs in the wild, and it was thought to be due only to morphological and physiological characteristics (Wolek, 1974) favoring *L.*

gibba over *L. minor*, rather than enhanced and more successful response to pollutants. This particular feature (response to xenobiotics) must be also considered as an important source of competitive advantage, especially in ecological terms. Empirically, *L. gibba* has a competitive advantage over *L. minor*, being able to dominate the environment and outcompete *L. minor* (Rejmánková, 1975), and this advantage may result not only from morphological and physiological traits, but also from the enhanced response to adverse toxic effects.

Therefore, it is possible to hypothesize that *L. minor* may have relied on another mechanism to defend against the here-tested chemicals stressors. This possibility is raised considering that for all tested pharmaceuticals, CAT activity was significantly increase as a response to xenobiotic exposure on *L. minor*. And it is know that CAT acts also as an anti-oxidant mechanism, which acts later than anthocyanins and carotenoids in the timeline of oxidative stress once normally pigments react first with singlet oxygen, reaction that occur before CAT action (Edge et al., 1997; Triantaphylidès and Havaux, 2009; Havaux, 2013).

In contrast, for *L. gibba* only exposure to APAP caused alterations in CAT activity. This scenario may be associated with higher pro-oxidant APAP capacity (Nunes et al., 2014) compared to CZP and DCF. When interpreting CAT data, it is also important to note that plants, unlike animals, have multiple enzymatic forms (or isozymes) of CAT (Scandalios et al., 1997). The presence of multiple CAT isozymes suggests multiple functions for catalases in a variety of plant tissues at various developmental stages and under constantly changing environments from which plants cannot readily escape (Scandalios et al., 1997). This may act as a confounding for the interpretation of CAT activity in plants.

In summary both species show different defense mechanisms and strategies against xenobiotics, *L. gibba* presents a primary antioxidant defense with the use of pigments, while *L. minor* makes use of enzymatic mechanisms, such as CAT. The relative sensitivities of the different duckweed species are not well understood. Little differences about the sensitivity of the two more widely used species, *L. minor* and *L. gibba* have been reported (Lewis, 1995).

2.5.2. Pigments (Chlorophyll)

This bioassay, which is based on the determination of the Chl content in plants, is highly sensitive to pollution exposure (Fekete-Kertész et al., 2015). The relevance of this parameter is high, since concentrations of Chls provide an indirect, but accurate estimate of plant nutritional status as well (Paul et al., 2017). In addition, previous work suggests that Chl biosynthesis is connected to carotenoids biosynthesis (Ramirez and Tomes, 1964). In fact, our study is in line with the previously mentioned article, since carotenoids contents were significantly increased, and the same occurred with Chl a and b, for both species and for all tested compounds. For plants of the species *L. minor*, this increase in Chl b biosynthesis was significant after exposure to the higher concentrations of CPZ, APAP and DCF. This response is likely to occur, since in some cases, Chl b level is generally a more sensitive endpoint than Chl a content (Brain et al., 2004).

In addition, the total Chl level was just affected by higher concentration of DCF, 20 µg/L to *L. minor* and 100 µg/L to *L. gibba*. This result is in agreement with Nunes et al. (2014) that exposed both tested species to higher concentrations of APAP, without the impairment of total Chl content. Besides, as far we know this is the first report about CPZ effects in *Lemna* species and its metabolism and mode of action in plants is very poor understood. On the other hand, other organic compounds such as toluene, ethylbenzene, xylene (Yan and Zhou, 2011), caffeine, diuron, bisphenol A, and others were able to decrease total Chl content in the macrophyte species *Hydrilla verticillata* and *L. minor* (Fekete-Kertész et al., 2015), evidencing the sensibility of this parameter. This sensibility was evidenced after DCF exposure, that had their metabolism studied in *Typha latifolia*, another macrophyte species (Bartha et al., 2014). The authors concluded that the macrophyte is capable to uptake the pharmaceutical, however the mechanism of toxicity still not completely understood. Thus, assays measuring pigment concentration provide valuable information about associations between toxicants and the photosynthetic apparatus (Brain et al., 2004).”

2.5.3. Chlorophyll fluorescence

Photosynthetic apparatus and alterations in this system can be measured though Chl fluorescence (mostly of Chl a), which is one of the most popular techniques in plant

physiology because of the detailed information about the state of photosystem II (PSII) (Murchie and Lawson, 2013) that may be obtained; however, this endpoint is still not widely applied in ecotoxicology (Raulph et al., 2007). In this study, the photosynthetic parameter (Fv/Fm – maximum quantum yield) was not affected on *L. minor* and *L. gibba* by pharmaceutical exposure (CPZ, APAP and DCF) after 96 h exposure. Nevertheless, it is possible to establish a relationship of modifications in this parameter with changes in the contents of Chl a content, since this is a constituent of photosynthetic reaction centers, and consequently it is an essential photosynthetic pigment (Paul et al., 2017). This idea can be supported by some studies in which there is a relation between the effect on the content of Chl a and alterations in the Chl fluorescence (Di Baccio et al., 2017; Oláh et al., 2010; Ibrahim and Zaafar, 2011; Sitko et al., 2017). In this study, this relationship was also verified, since no changes occurred in terms of Chl a content, and consequently this led to the absence of alterations in Chl fluorescence.

Despite being a sensible parameter that may describe a dose-response relationship for a variety of toxicants (Raulph et al., 2007), the parameter of chlorophyll fluorescence is not always simple to interpret. It is necessary to take into account that toxic interactions are extremely dependent upon the specific mechanism of toxicity of each substance, which is highly variable. There are cases for which the contaminant mode of toxic action is related to PSII, rather than to PSII operating efficiency (Agathokleous et al., 2016). Thus, the more information about the photosynthetic parameters that are compromised by toxicant exposure, the better to understand the actual effect of the studied contaminant.

The three tested pharmaceuticals were able to affect *Lemna* antioxidant defense system, but were unable to cause significant alterations in Chl fluorescence of exposed plants, showing that the defense system was sufficiently effective to prevent deleterious effects on the plants´ physiologies. Among such putative defensive mechanisms, it is possible to suggest the antioxidant activity of carotenoids (whose levels were increased in our study), considering their essential role in photosynthesis and in the protection against photo-oxidative damage (Baroli and Niyogi, 2000).

Finally, data of the functional endpoint Chl fluorescence is a valuable tool to detect subtle effects on phyto-communities (Knauer and Hommen, 2012). In addition, effects on photosynthetic efficiency may occur earlier than those that might be observed concerning growth parameters (Di Baccio et al., 2017) e.g. after a 48 h exposure (Oláh et al., 2010); this is an extremely short period of exposure, for which growth alterations might not be fully determined.

2.5.4 Ecological relevance

In ecological terms, the here-obtained results are of major importance. *L. gibba* has a competitive advantage over *L. minor* (Rejmánková, 1975). Our data support this assumption since *L. gibba* had a better, more effective, and defense system against the tested pharmaceuticals. Considering that both species share the same habitat, this advantage may grant a stronger dissemination and growth of *L. gibba* when compared to *L. minor* populations, when in contaminated environments. On the other hand, our results are extremely significant, since the here tested pharmaceuticals CPZ, APAP and DCF are widely distributed in aquatic environmental, and the selected concentrations are close to already reported environmental levels, thus being realistic. The obtained results were yielded in some cases at such levels, showing that toxic effects are likely to occur in real contaminated sites. The combination of these two factors highlights the ecological importance of this study. In addition, it is possible to say that this is the first study on the effect of CPZ on *Lemna* species. Therefore, this data set reinforces the need for further ecotoxicological studies based on environmental concentrations of contaminants in the aquatic environment to try understand the real effects of these compounds at the environmental level. Further studies with longer exposures are need it. Our work has largely contributed to the generation of data on the ecotoxicological effects of pharmaceuticals in non-target organisms, especially on aquatic plants. It is worth noting that this is still an area that deserves greater attention since the focus of the majority of ecotoxicological studies on aquatic plants is not on pharmaceutical drugs. Finally, it must be also emphasized that non-standard parameters are a suitable and promising tool for ecotoxicological evaluation of pharmaceuticals effects in aquatic plants.

2.6 Conclusions

In conclusion, both duckweed species responded promptly to the tested pharmaceuticals (CPZ, APAP and DCF). *L. gibba* showed to possess a defense system that acted first by mobilizing antioxidant pigments (anthocyanins and carotenoids), and relied finally on the activation of CAT hydrolytic activity. In this species, pigments (a, b and total Chl) were not considerably affected, and Chl fluorescence was not compromised after exposure to the drugs. On the other hand, *L. minor* did not suffer significant alterations in its antioxidant pigments (anthocyanins and carotenoid) levels, and focused its antioxidant defense only on the activation of CAT activity. In terms of pigments, Chl b was the most affected when compared with Chl a and total Chl levels. Finally, Chl fluorescence was not affected by the exposure to the three drugs. It is thus possible to draw three main conclusions: (1) use of organisms of the same genus (but different species) in ecotoxicological testing of chemicals may result in different results as to their toxicity; (2) differences on antioxidant defense system may grant *L. gibba* competitive advantages over *L. minor*, since it is more efficient to cope with the early phases of the toxic process (and presumably with other stress sources); this difference may be of importance considering the potential competition between of these two coexisting species in their natural habitats; (3) plant-based, non-standard ecotoxicological parameters are suitable and promising tools for ecotoxicological evaluation of pharmaceuticals.

2.7 References

Aebi, H., 1984. Catalase in vitro. Methods in Enzymology, 6. 105-121.

Agathokleous, E., Mouzaki-paxinou, A., Saitanis, C. J., Paoletti, E., Manning, W. J., 2016. The first toxicological study of the antiozonant and research tool ethylene diurea (EDU) using a *Lemna minor* L. bioassay: Hints to its mode of action. Environmental Pollution, *213*, 996–1006.

Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant physiology, 24, 1-15.

Baroli, I., Niyogi, K. K., 2000. Molecular genetics of xanthophyll − dependent photoprotection in green algae and plants. Phil. Trans. R. Soc. Lond. B355, 1385-1394.

Borges, N. C., Rezende, V. M., Santana, J. M., Moreira, R. P., Moreira, R. F., Moreno, P., Borges, D. C., Donato, J. L., Moreno, R. A., 2011. Chlorpromazine quantification in human plasma by UPLC–electrospray ionization tandem mass spectrometry. Application to a comparative pharmacokinetic study. J. Chromatogr. B. 879, 3728–3734.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Brain, R. A, Solomon K. R., 2007. A protocol for conducting 7-day daily renewal tests with *Lemna gibba*. Nat Protoc 2, 979-987.

Brain, R. A., Johnson, D. J., Richards, S. M., Sanderson, H., Sibley, P. K., Solomon, K. R., 2004. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. Environmental Toxicology. *23*, 371–382.

Brandão, F. P., Pereira J. L., Gonçalves F., Nunes B., 2014. The Impact of Paracetamol on Selected Biomarkers of the Mollusc Species *Corbicula fluminea*. Environmental Toxicology. 29, 74-83.

Carpenter, S. R., Lodge, D. M., 1986. Effects of submersed macrophytes on ecosystem processes. Aquat. Bot., 26, 341-370.

Cedergreen, N., Arts, G., Davies, J., Knaue, K., 2009. Critical Evaluation of Laboratory Toxicity Testing Methods with Aquatic Macrophytes. In: Lorraine Maltby, Dave Arnold, Gertie Arts, Jo Davies, Fred Heimbach, Christina Pickl, Véronique Poulsen. Aquatic macrophyte risk assessment for pesticides. Wagenigen:SETAC. 135p.

Close, D. C., Beadle, C. L., Battaglia, M., 2004. Foliar anthocyanin accumulation may be useful indicator of hardiness in eucalypt seedlings. For Ecol Manage 198, 169–181.

Corcoran, J., Winter, M. J., Tyler, C. R., 2010. Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. Crit Rev Toxicol. 40, 287-304.

Daughton, C. G., Ternes, T. A., 1999. Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change? Environmental Health Perspectives. 107, 907-938.

Di Baccio, D., Pietrini, F., Bertolotto, P., Pérez, S., Barcelò, D., Zacchini, M., Donati, E., 2017. Response of *Lemna gibba* L. to high and environmentally relevant concentrations of ibuprofen: Removal, metabolism and morpho-physiological traits for biomonitoring of emerging contaminants. Sci Total Environ. 584-585, 363-373.

Du, J., Mei, C. F., Ying, G. G., Xu, M. Y., 2016. Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea *Daphnia magna*. Bull Environ Contam Toxicol 97, 84-90.

Edge, R., Truscott, T. G., 2018. Singlet Oxygen and Free Radical Reactions of Retinoids and Carotenoids — A Review. Antioxidants. 7, 1–16.

Edge, R., Mcgarvey, D. J., Truscott, T. G., 1997. The carotenoids as anti-oxidants - a review. Journal of Photochemistry and Photobiology B: Biology. 41, 189-200.

Fekete-kertész, I., Kunglné-Nagy, Z., Gruiz, K., 2015. Assessing Toxicity of Organic Aquatic Micropollutants Based on the Total Chlorophyll Content of *Lemna minor* as a Sensitive Endpoint. Periodica Polytechnica Chemical Engineering. *59*, 262–271.

Fent, K., Weston, A. A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquatic Toxicology, 76:122-159.

Gould, K. S., Mckelvie, J., Markham, K. R., 2002. Do anthocyanins function as antioxidants in leaves? Imaging of H_2O_2 in red and green leaves after mechanical. Plant, Cell and Environment. 25, 1261–1269.

Guiloski, I. C., Piancini, L. D. S., Dagosti, A. C., Calado, S. L. M., Fávaro, L. F., Boschena, S. L., Cestari, M. M., Cunha, C., Assis, H. C. S., 2017. Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish *Rhamdia quelen*. Ecotoxicology and Environmental Safety. 139, 291-300.

Havaux, M., 2013. Carotenoid oxidation products as stress signals in plants. The Plant Journal. 79, 597–606.

Hilton, M. J., Thomas, K.V., 2003. Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquid chromatography–electrospray tandem mass spectrometry J. Chromatogr. A. 1015, 129-141.

Hiscox, J. D., Israelstam, G. F., 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can J. Bot. 57, 1332-1334.

Ibrahim, M. H., Jaafar, H. Z., Application, M., Growth, I., 2011. Photosynthetic capacity, photochemical efficiency and chlorophyll content of three varieties of *Labisia pumila* Benth. exposed to open field and greenhouse growing conditions. Acta Physiologiae Plantarum. 33, 2179–2185.

ISO. 2005. Water quality - determination of toxic effect of water constituents and waste water to duckweed (*Lemna minor*) - duckweed growth inhibition test. International Standard ISO 20079: Geneva, Switzerland. 23p.

Kahkonen M. P., Heinonen M., 2003. Antioxidant activity of anthocyanins and their aglycons. Journal of Agricultural and Food Chemistry. 51, 628–633.

Kane, J. M., Correll, C. U., 2010. Past and present progress in the pharmacologic treatment of schizophrenia. J Clin Psychiatry. 9, 1115–1124.

Knauer, K., Hommen, U., 2012. Sensitivity, variability, and recovery of functional and structural endpoints of an aquatic community exposed to herbicides. Ecotoxicology and Environmental Safety *78*, 178–183.

Küster, A., Adler, N., 2014. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Philosophical Transactions of the Royal Society B: Biological Sciences, 369, 1656, 20130587.

Lakatos, G., Mszfiros, I., Bohfitka, S., Szab, S., 1993. Application of *Lemna* species in ecotoxicological studies of heavy metals and organic biocides. Science of The Total Environment. 134, 773-78.

Lewis, M. A., 1995. Use of freshwater plants for phytotoxicity testing: A review. Environmental Pollution. 87, 319-336.

Li, T., Zhou, Q., Zhang, N., Luo, Y., 2008. Toxic effects of chlorpromazine on *Carassius auratus* and its oxidative stress. Journal of Environmental Science and Health Part B. 43, 638-643.

Lichtenthaler, H. K., 1987. Chlorophylls and Carotenoids Pigments of Photosynthetic Biomembranes. Methods in Enzymology. 148, 350-382.

Lonappan, L., Brar, S. K., Das, R. K., Verma, M., Surampalli, R. Y., 2016. Diclofenac and its transformation products: Environmental occurrence and toxicity - A review. Environment International. 96, 127–138.

Mancinelli, A. L., Yang, C. P. H., Lindquist, P., Anderson, O. R., Rabino, I., 1975. Photocontrol of anthocyanin synthesis. III. The action of streptomycin. Plant Physiol. 55, 251–257.

Munro A. D., 1986. The effects of apomorphine, d-amphetamine and chlorpromazine on the aggressiveness of isolated *Aequidens pulcher* (Teleostei, Cichlidae). Psychopharmacology (Berl). 88, 124-128.

Murchie, E.H., Lawson, T., 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications, Journal of Experimental Botany. 64, 3983–3998.

Murthy, K. N. C., Vanitha, A., Rajesha, J., Swamy, M. M., Sowmya, P. R., Ravishankar, G. A., 2005. In vivo antioxidant activity of carotenoids from *Dunaliella salina* — a green microalga. Life Science. 76, 1381–1390.

Nogueira, M., Mora, L., En, E. M. A., Bramley, P. M., Fraser, P. D., 2013. Subchromoplast Sequestration of Carotenoids Affects Regulatory Mechanisms in Tomato Lines Expressing Different Carotenoid Gene Combinations. The Plant Cell. 25, 4560–4579.

Nunes B., Nunes J., Soares A. M. V. M., Figueira E., Freitas R., 2017. Toxicological effects of paracetamol on the clam *Ruditapes philippinarum*: exposure vs recovery. Aquatic Toxicology. 192, 198-206.

Nunes, B., Antunes, S. C., Santos, J., Martins, L., Castro, B. B., 2014. Toxic potential of paracetamol to freshwater organisms: A headache to environmental regulators? Ecotoxicology and Environmental Safety. 107, 178-185.

OECD (2006) Lemna sp. growth inhibition test n. 221. OECD–Guideline for Testing of Chemicals, Paris, 22 p.

Oláh, V., Lakatos, G., Bertók, C., Kanalas, P., Szőllősi., E., Kis, J., Mészáros, I., 2010. Short-term chromium (VI) stress induces different photosynthetic responses in two duckweed species, *Lemna gibba* L. and *Lemna minor* L.. Photosynthetica. *48*, 513–520.

Oliveira, L. L. D., Antunes, S. C., Goncalves, F., Rocha, O., Nunes, B., 2015. Evaluation of ecotoxicological effects of drugs on *Daphnia magna* using different enzymatic biomarkers. Ecotoxicology And Environmental Safety. 119, 123-131.

Paul, V., Sharma, L., Kumar, R., Pandey, R., Meena, R. C., 2017. Estimation of Chlorophylls / Photosynthetic Pigments – Their Stability is an Indicator of Crop Plant Tolerance to Abiotic Stresses. Method, 8-14.

Pietrini, F., Iannelli, M. A., Massacci, A., 2002. Anthocyanin accumulation in the illuminated surface of maize leaves enhances protection from photo-inhibitory

risks at low temperature, without further limitation to. Plant, Cell and Environment. 25 1251–1259.

Ralph, P. J., Smith, R. A., Seery, C. R., Smith, R. A., Seery, C. R., 2007. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: Review. Toxicological & Environmental Chemistry. 89, 589-607.

Ramirez, D. A., Tomes, M. L., 1964. Relationship between chlorophyll and carotenoid biosynthesis in dirty-red (green-flesh) mutant in tomato. Bot Gaz. 125, 221-226.

Rejmánková, E., 1975. Comparison of *Lemna gibba* and *Lemna minor* from the production ecological viewpoint. Aquat. Bot. 1, 423--427.

Roberts, P., Thomas, K., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment Sci. Total Environ. 356, 143-153.

Sanchez-Zabala, J., Majada, J., Martín-Rodrigues, N., Gonzalez-Murua, C., Ortega, U., Alonso-Graña, M., Duñabeitia, M. K., 2013. Physiological aspects underlying the improved outplanting performance of *Pinus pinaster* Ait. seedlings associated with ectomycorrhizal inoculation. Mycorrhiza. 23, 627-640.

Scandalios, J. G., Guan, L., Polidoros, A. N., 1997. Catalases in Plants: Gene Structure, Properties, Regulation, and Expression. Oxidative Stress and the Molecular Biology of Antioxidant Defenses. 34, 343-406.

Scherr C, Simon M, Spranger J, Baumgartner S., 2008 Test system stability and natural variability of a *Lemna gibba* L. bioassay. PLoS ONE. 3, e3133.

Sitko, K., Rusinowski, S., Kalaji, H. M., 2017. Photosynthetic Efficiency as Bioindicator of Environmental Pressure in *A. helleri.* Plant Physiology. 175, 290–302.

Stahl, W., Sies, H., 2003. Antioxidant activity of carotenoids. Molecular Aspects of Medicine. *24*, 345–351.

Triantaphylides, C., Havaux, M., 2009. Singlet oxygen in plants: production, detoxification and signaling. Trends in Plant Cell. 14, 219-228.

Voogt, P., Janex-Habibi, M. L., Sacher, F., Puijker, L., Mons, M., 2009. Development of a common priority list of pharmaceuticals relevant for the water cycle Water Sci. Technol. 59, 39-46.

Wang, H., Cao, G., Prior, R. L., 1997. Oxygen Radical Absorbing Capacity of Anthocyanins, J. Agric. Food Chem. *45*, 304–309.

Wiegel, S., Aulinger, A., Brockmeyer, R., Harms, H., Loffler, J., Reincke, H., Schmidt, R., Stachel, B., von Tumpling. W., Wanke, A., 2004. Pharmaceuticals in the river Elbe and its tributaries. Chemosphere. 57, 107–126.

Wolek, J., 1974. A preliminary investigation in interactions (competition, allelopathy) between some species of Lemna, Spirodela and Wolffia, vol. 42. Ber. Geobot. Inst. ETH, Stiftung Rübel, Zürich, pp. 140–162.

Wu, S., Zhang, L.; Chen, J., 2012. Paracetamol in the environment and its degradation by microorganisms. J. Appl Microbiol Biotechnol. 96, 875–884.

Yan, S., Zhou, Q., 2011. Chemosphere Toxic effects of *Hydrilla verticillata* exposed to toluene, ethylbenzene and xylene and safety assessment for protecting aquatic macrophytes. Chemosphere. 85, 1088–1094.

Yuan, S., Jiang, X., Xia, X., Zhang, H., Zheng, S., 2013. Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere. 90, 2520–2525.

Chapter 3

Effects of diclofenac and salicylic acid exposure on Lemna minor: Is time a factor?

Alkimin, G. D., Daniel, D., Dionísio, R., Soares, A. M. V. M., Barata, C., Nunes, B. 2019. Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is time a factor? Environmental Research, 177, 108609. doi:10.1016/j.envres.2019.108609

Effects of diclofenac and salicylic acid exposure on *Lemna minor*: is time a factor?

Alkimin GD[1,2], Daniel D[1], Dionísio R[1], Soares AMVM[1,2], Barata C[3], Nunes, B[1,2*]

[1] Departamento de Biologia da Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

[2] Centro de Estudos do Ambiente e do Mar (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[3]Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

3.1 Abstract

The global occurrence of pharmaceuticals in the aquatic environment has been considered a particularly concerning problem with unknown consequences. Non-steroidal anti-inflammatory drugs (NSAIDs) including diclofenac (DCF) and salicylic acid (SA), are among the most frequently prescribed drugs in the world, being consequently commonly found in the aquatic environment. Prolonged experiments (with duration of exposure that surpass those recommended by already established testing guidelines) are important to obtain ecologically relevant data to address the issue of NSAIDs ecotoxicity, because by being more realistically (namely in terms of levels and durations of exposure), such tests may indicate realistic challenges posed to aquatic organisms. Among the most common test species that are used for assessing environmental quality, plants play a leading role. *Lemna* species are among the most important plants used for ecotoxicity testing. Therefore, the aim of this study was to evaluate the temporal effect of a prolonged exposure of DCF and SA on *Lemna minor*. To attain this purpose, *L. minor* plants were chronically exposed to 0, 4, 20, and 100 µg/L of both pharmaceuticals, and samplings were performed at 6, 10 and 14 days of exposure. The analyzed endpoints were levels of chlorophyll a, b and total, and of carotenoids; and enzymatic biomarkers as catalase, ascorbate peroxidase and glutathione-S-transferases. Diclofenac was responsible for alterations in all analyzed

parameters in different intervals of exposure. Salicylic acid exposure was not capable of causing alterations on pigment contents of *L. minor*, however, enzymatic biomarkers were altered at all sampling intervals. Thus, it is possible to conclude that both pharmaceuticals can cause damage on the tested macrophyte species, biochemical parameters being more sensitive than physiological ones. Additional prolonged experiments are required to understand the chronic effects of different pharmaceuticals in the aquatic environment, especially in plants.

Key-words: Pharmaceuticals; diclofenac; salicylic acid; *Lemna minor*; biochemical analyzes; physiological analyzes.

3.2 Introduction

Human health is one of the most important concerns of modern life, and it has improved substantially during the past decades, in terms of general access to healthcare and availability of pharmaceutical drugs. The role attributed to (among other factors) the usage of large amounts of pharmaceutically active substances (Kunkel and Radke, 2012) is prominent. These biologically active compounds are distributed among several therapeutic classes, which encompass a diverse array of chemical natures and structures with multiple pharmacological modes of action to produce biological effect (Daughton and Ternes, 1999). After administration, some pharmaceuticals are not completely metabolized. The unmetabolized parent drugs and some metabolites are subsequently excreted from the body via urine and feces (Zhang et al., 2008), being treated in municipal wastewater treatment plants (if available) and ultimately enter the aquatic ecosystems. However, drugs may reach the environment via disposal of industrial and agricultural wastes, and accidental spills (Farré et al., 2008). Once in the environment, they can be widely distributed at some time (Farré et al., 2008). From these assumptions, we may consider that the majority of these chemicals are released into the environment, after their ultimate use by humans and/or animals, and they occur in the wild, where they may keep their chemical structure and biological activity. As a result, the number of potential non-target pharmaceutical–receptor interactions, indirect interactions and ecotoxicological effects in the environment is difficult to estimate,

especially in complex matrices and when considering the vast number of living organisms that may interact with such substances (Brain et al., 2004; Farré et al., 2008).

The global occurrence of pharmaceuticals in the aquatic environment has been arising as a problem with unknown consequences (Kunkel and Radke, 2012) because this is mostly an unregulated regulated environmental issue (Pal et al., 2010). Some contaminants can be found in various environmental compartments and/or in areas where they were never used, mainly due to their persistence and long distance transport (Gavrilescu et al., 2015).

The ecological concern stems from the fact that pharmaceutical compounds are known to have biological effects, but only limited information has been obtained to quantitatively assess potential ecotoxicological impacts (Pal et al., 2010). What is not known, however, is whether these chemicals and their transformation products can elicit physiologic effects on biota at the low concentrations (ng-µg/L) at which they occur (Daughton and Ternes, 1999). The consequences of pollutants in aquatic ecosystems are of particular concern, because living organisms from these environments are chronically subjected to potential contaminations with consequences that may affect future generations (Gavrilescu et al., 2015).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most often prescribed drugs in the world, being consequently very common in the aquatic environment, since the early 2000s, and the environmental effects of NSAID have become a growing concern (Bonnefille et al., 2018). Among NSAIDs, one may find diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl) acetic acid; DCF), which is used to reduce inflammation and to relieve pain, such as arthritis or acute injury, and as antiuricosurics. DCF, when released into the environment, is likely to reach aquatic ecosystems and cause harmful effects on resident species. This compound is the most toxic among NSAIDs drugs, and shows also the potential to exert chronic toxicity (Kalenieeka and Zarzycki. 2015), being one of the most commonly found substances within the aquatic environment (Schwaiger et al., 2004). These features lead the European Union to include DCF in its First Watch List of the Water Framework Directive, in order to obtain more information on its occurrence and effects in the environment (EU 2015/495, European Commission; Bonnefille et al., 2018).

Similarly to what happens for other pharmaceuticals, DCF often enters aquatic environments via inputs from wastewater treatment plants and the extent of its degradation depends on the used wastewater treatment technology (Lonappan et al., 2016). However, its inherent characteristics contribute for its environmental presence. The low DCF biodegradability often results in low elimination rates during biological wastewater treatment, and only a minor portion is adsorbed by sludge (Vieno and Sillanpää, 2014) and eliminated. That way, DCF can be detected in different environmental compartments such as drinking water in the USA (1.2 ng/L; Benotti et al., 2008), seawater (subtropical coastal zone - Brazil) in concentrations up to 19.4 ng/L (Pereira et al., 2016), concentrations between 0.06 to 15 µg/L in Germany rivers (Heberer, 2002; Jux et al., 2002; Weigel et al., 2002), levels of 195 ng/L in UK estuaries (Thomas and Hilton, 2004), and concentrations between 0.25 and 5.45 µg/L in sewage treatment plants effluents in France, Italy and Greece, according to Andreozzi et al. (2003) .

Another important NSAID is acetylsalicylic acid (ASA), which exerts its therapeutic action by inhibiting cyclooxygenase enzymes, at inflammatory sites (Gómez-Oliván et al., 2014). ASA has a widespread use given its additional beneficial therapeutic properties, such as analgesic and anti-pyretic action. ASA is rapidly hydrolyzed to salicylic acid (SA), which is its main metabolite and primarily responsible for the pharmacological activity of ASA; SA can be further conjugated to glycine to give rise to salicyluric acid or to glucuronic acid to form glucuronide conjugates, being also excreted as the unchanged SA form (Davidson, 1971; Mullangi et al., 2012). In addition, SA has been found to play a key role in the regulation of plant growth, development, interaction with other organisms and in the responses to environmental stresses (Raskin, 1992). Its massive use is directly responsible for its environmental presence. Salicylic acid has been determined in Canadian influents and effluents in levels of 330 and 3.6 µg/L respectively (Metcalfe et al., 2003). In many municipal wastewater, SA was found at levels up to 4.1 µg/L (Ternes, 1998) and even in coastal zones of Belgium, SA was reported at concentrations up to 0.855 µg/L (Claessens et al., 2013).

A variety of aquatic species and responses are employed to derive environmental quality criteria, to assess toxicity of surface water and effluents, and to inform risk of chemicals

in an ecological framework (Brooks et al., 2015). Among these species, plants are an essential part of an ecosystem, and a balance of plant-animal-bacteria complex is vital to a healthy environment (Wang, 1986). *Lemna* (duckweed) species constitute an extremely important group of plants, which may be found in diverse aquatic environments, including lakes, streams, effluents and sediments (Wang, 1990). Considering the importance of these species, several previous studies stressed out that *Lemna* species could be a promising indicator of aquatic toxicity (Wang, 1986), and that its use could also be applied to ecotoxicological tests, considering its sensitivity towards chemical contamination (Lakatos et al., 1993).

Lemna species have many advantages as test organisms in ecotoxicology. They can easily be cultured in the laboratory, in which they may achieve optimum growth conditions attaining exponential growth (Arts et al., 2008). In addition, *Lemna* species present small size, rapid growth and relative structural simplicity; they can be grown in aseptic cultures, simplifying working conditions with organic compounds. Reproduction is usually vegetative, so that genetic variability can be eliminated by using a single clone for all experiments (Hillman, 1961). *Lemna* spp-based tests may also be complementary to those based on algae (i.e., *Selenastrum*) since test solutions can be renewed, and background contaminant algal cells present in the receiving water or effluent can be removed for frond production or chlorophyll measurements (Taraldsen and Norberg-King, 1990). *Lemna* species may be also used for other measurements of toxicological importance, such as biochemical and enzymatic alterations. Among different *Lemna* species, *Lemna minor* (common duckweed) assumes a leading position, since it is a floating, widespread, fast-growing plant, small and easy to cultivate, characteristics that turn this species into an ideal candidate for aquatic toxicity tests (Wang, 1986).

Prolonged experiments (that exposed test organisms for longer periods, thereby exceeding common and already established exposure durations) are important for the development of ecologically relevant data because by being more realistic (namely in terms of levels and durations of exposure), they simulate more closely polluted environments (García-Gómez et al., 2014) and may help deciphering adaptive and acclimation processes that are likely to occur in the wild (Coutellec and Barata, 2013). Therefore, the aim of this study was to evaluate the temporal effect of a prolonged

exposure of DCF and SA on *Lemna minor* in terms of physiological responses, namely pigments amount and enzymatic biomarkers.

3.3 Material and methods

3.3.1 Chemicals

All pharmaceutical drugs were purchased from Sigma Aldrich, with purities >98%: diclofenac sodium (CAS: 15307-79-6) and salicylic acid (sodium salt form; CAS 54-21-7) and all other chemicals used in this study have analytical purity.

3.3.2 *Lemna minor* culture

The plants to be used during the experiment were obtained from Centre of Environmental and Marine Studies (CESAM) - University of Aveiro - where were raised according to Alkimin et al. (2019).

3.3.3 *Lemna minor* assay

Tests were performed be exposing plants to ranges of concentrations of DCF and SA that were chosen based on the already reported environmental concentration and also on predicted worst case scenarios of contamination. Diclofenac levels from ~1 and 20 ng/L were found in the river Elbe estuary (Weigel et al., 2002); up to 490 ng/L were registered in UK effluents (Hilton and Thomas, 2003); SA has been shown to occur in amounts ranging from 330 to 3.6 µg/L in influents and effluents (Metcalfe et al, 2003), up to 4.1 µg/L in many municipal wastewater (Ternes, 1998), and even in coastal zones at concentrations up to 0.855 µg/L (Claessens et al., 2013). Considering these values, the here-tested nominal concentrations were 0, 4, 20 and 100 µg/L for both pharmaceuticals. *Lemna minor* was exposed to the mentioned levels in eight replicates, in 400 ml plastic flasks with a final volume of 250 mL of modified Steinberg medium (OECD, 2006) per replicate, adequately supplemented with the pharmaceuticals stock solutions (prepared in modified Steinberg medium). In the control treatment, replicates

were exposed only to the modified Steinberg medium. The assay started with plants that covered ~30% of the vessel area. Assays were conducted under controlled conditions (temperature 23 ± 2 °C; photoperiod 24h^{L}; light intensity, ~6000 lux). The total volume of medium was renewed every other day in order to keep the exposure concentrations constant during the experiment. *L. minor* fronds were exposed for a total of 14 days and samples of each treatment were withdrawn at 6, 10 and 14 days of exposure. This total time of exposure was chosen considering that it corresponds to twice the exposure period recommended by OECD guideline 221 (OECD, 2006) for *Lemna* sp. tests. The collected biomass was divided in Eppendorf microtubes and stored at -80 ºC until the performance of analyzes.

3.3.4 Pigments analysis (chlorophylls and carotenoids)

Total, a, and b chlorophylls (TChl; Chl a; Chl b) and carotenoids (Car) amounts were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979) with the modifications proposed by Alkimin et al. (2019). Pigments were extracted from the previously exposed fronds of *Lemna* spp. (about 10 mg per replicate - fresh weight - FW) in 2.5 mL of dimethyl sulphoxide (DMSO). The extract was placed in water at 65 ºC during 30 min and allowed to cool in the dark at room temperature. The next day, samples were thoroughly vortexed for about 10 s and centrifuged for 5 min at 15,000 g at 4°C (ThermoFisher Megafuge 8R). The obtained supernatants were used to quantify the amounts of chlorophylls (a, b, and total), and carotenoids levels, by spectrophotometrically measuring the absorbances of the extracts at the wavelengths of 470, 645, 646, and 663 nm in a spectrophotometer Thermo Scientific Multiskan (ScanIt Software 2.4.4). The levels of the pigments were calculated by using the equations proposed by Arnon (1949) and demonstrated by Hiscox and Israelstam (1979) to be suitable if the extraction was undertaken with DMSO, to calculate the amounts of Chl (a, b, and total; equations 1, 2 and 3 respectively), and Lichtenthaler (1987) to quantify the Car amount (equation 4).

$$\text{Chl a} = (12.70 \times A_{663}) - (2.69 \times A_{645}) \quad (1)$$

$$\text{Chl b} = (22.90 \times A_{645}) - (4.68 \times A_{663}) \quad (2)$$

$$TChl = (20.20 \times A_{646}) + (8.02 \times A_{663}) \quad (3)$$

$$Car = (1000 \times A_{470} - 1.43Chl\ a - 35.87Chl\ b)/205 \quad (4)$$

3.3.5 Enzymatic biomarkers

Tissue samples were processed according to Alkimin et al. (2019). Catalase activity (CAT) was assayed by the procedure described by Aebi (1984), where the activity was quantified based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein. The ascorbate peroxidase activity (APX) followed the method described per Kovacik et al., (2009) where the oxidation of ascorbic acid was followed as a decrease in absorbance monitored at 290 nm for 5 min. The enzymatic activity was calculated using a molar absorption coefficient 2.8 mM^{-1} cm^{-1}, according to Nakato and Asada (1981). For the glutathione-S-transferases (GSTs) activity determination, the procedure described by Habig et al. (1974) was used. These procedure monitors the increment of absorbance of the thioether resulting from the catalysis of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GSTs, at 340 nm. Results were expressed as nanomoles of thioether produced per minute, per milligram of protein. Total soluble protein quantification of samples was performed at 595 nm using the Bradford method (Bradford, 1976), adapted to microplate with bovine γ-globulin as standard. All parameters were performed spectrophotometrically, and the readings were performed in a microplate reader Thermo Scientific Multiskan (Software Ascent 2.6).

3.3.6 Statistical analysis

A one-way analysis of variance (ANOVA) was performed. Statistics tests and analysis of normality (Shapiro-Wilk) and homogeneity (Levene test) were conducted using the software SPSS v25. Analyzes on ranks (Kruskall-Wallis) were performed if even after being transformed, the data did not pass in normality and/or homogeneity tests. A significance level of 0.05 was adopted and post hoc test were applied, Dunn's or Dunnet were chosen according to the data.

3.4 Results

Chlorophyll a was affected by DCF exposure (figure 1A), being its content significantly increased in plants exposed to the concentrations of 20 µg/L, after 6 d of exposure; an increase of this parameter was observed after 10 d of exposure to 4 and 100 µg/L. After an exposure of 14 d, the Chl a content stabilized. On the other hand, SA exposure (figure 1B) was not capable of causing any significant alterations of the pigments levels, for all sampling intervals.

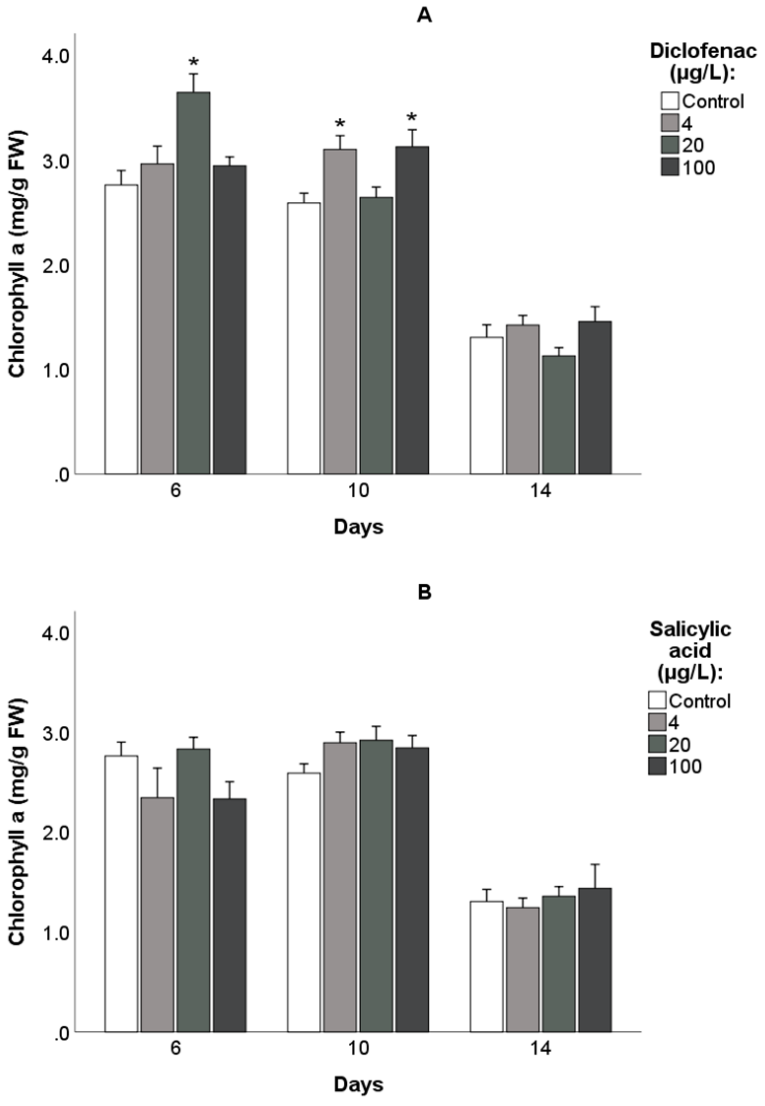

Figure 1. Effects of diclofenac (A) and salicylic acid (B) on chlorophyll a of *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for each species (n=8); FW = fresh weight.

Diclofenac (figure 2A), after 6 d of exposure to 4 and 20 µg/L, caused an increase on Chl b content; after 10 d of exposure, plants exposed to 4 and 100 µg/L of this substance had significant increases in this same parameter. No differences were however reported after 14 d of exposure. On the contrary, SA (figure 2B) did not cause changes on Chl b amount for any sampling interval.

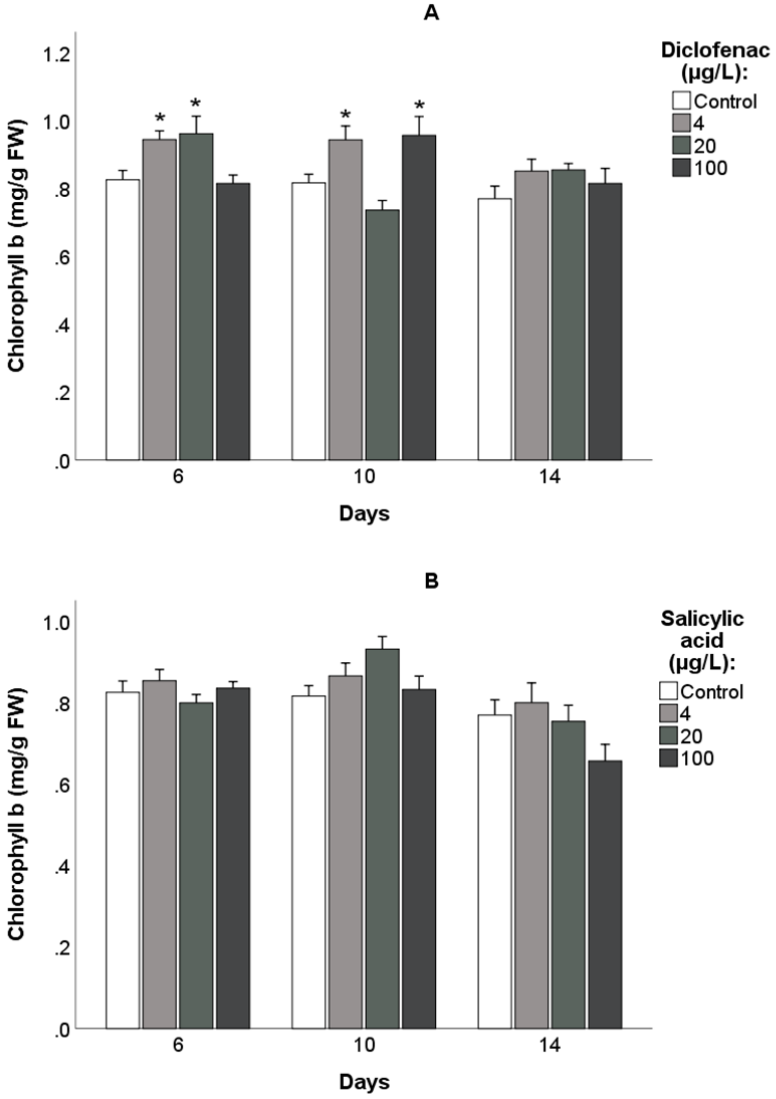

Figure 2. Effects of diclofenac (A) and salicylic acid (B) on chlorophyll b of *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for each species (n=8).

Total Chl amounts were affected after 6 d of exposure to DCF (figure 3A) causing a significant increase on this pigment content in plants exposed to a concentration of 20 µg/L. 10 d of exposure to 4 µg/L of the same drug caused a significant increase, while

14 d of exposure were not capable to cause changes in this parameter, and total Chl values were similar to those registered for control plants. Under no circumstances SA exposure (figure 3B) was capable of causing changes in Total Chl amount.

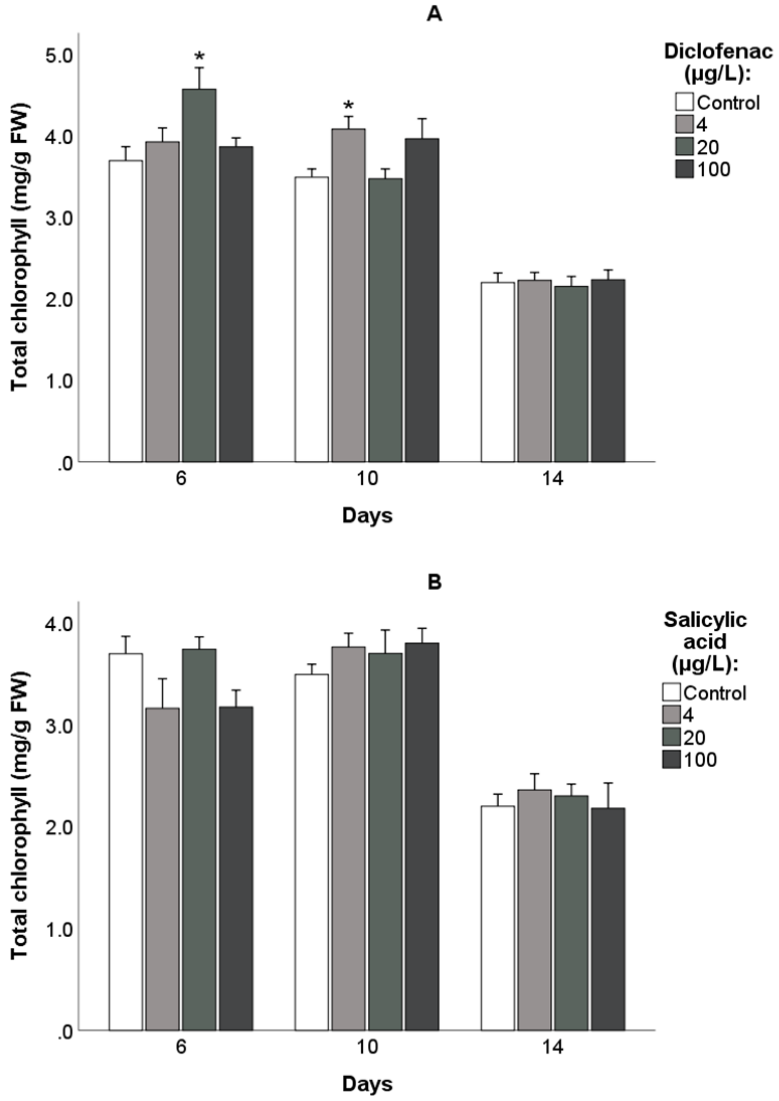

Figure 3. Effects of diclofenac (A) salicylic acid (B) on total chlorophyll of *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for each species (n=8).

Considering DCF exposure (figure 4A), results for Car levels showed an increase after 6 d of exposure to 20 µg/L and for 10 d to a 100 µg/L, the amount of this pigment was reestablished after 14 d of exposure. Carotenoids levels were not altered after SA exposure (figure 4B) for all intervals.

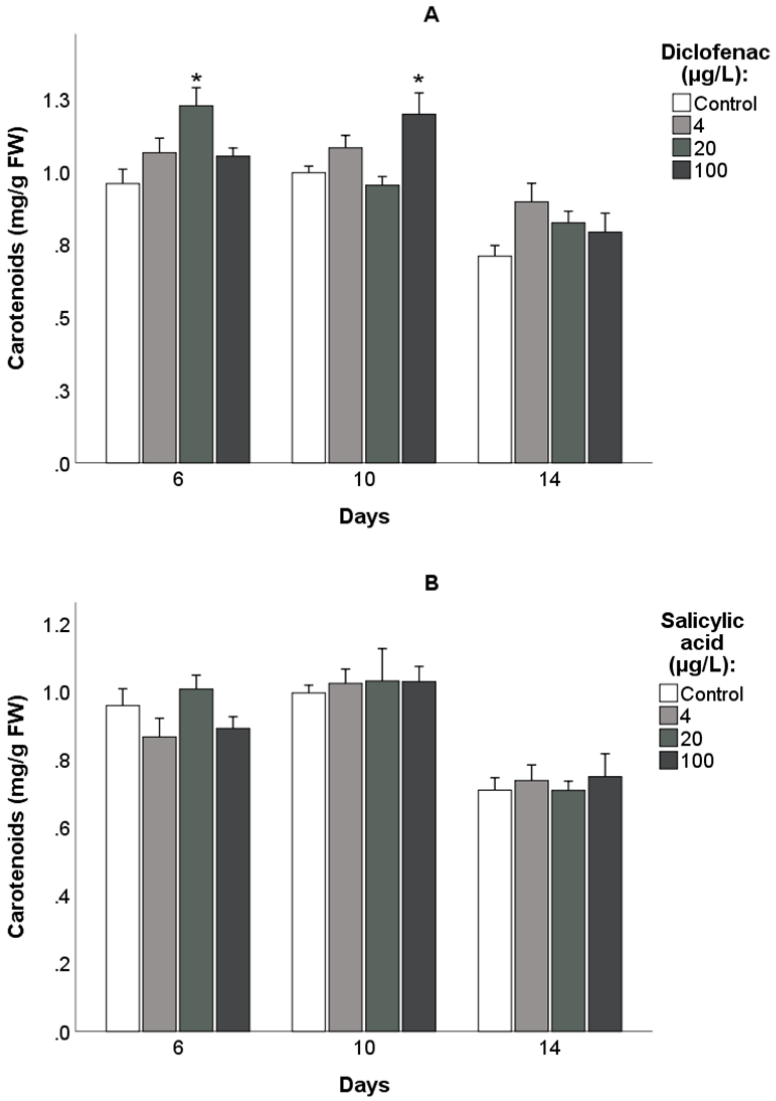

Figure 4. Effects of diclofenac (A) and salicylic acid (B) on carotenoids of *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for each species (n=8).

The first enzymatic marker analyzed was CAT activity. A 6 d exposure to DCF (figure 5A) caused a significant increase in the activity of this biomarker for plants exposed to concentrations of 4 and 100 µg/L; at the 10th d of exposure, a significant increase in CAT activity was also observed for plants exposed to 20 and 100 µg/L; on the other hand, after 14 d of exposure, the activity of catalase declined, being statistically different only for plants exposed to 100 µg/L. The same enzyme presented different behavior when *L. minor* was exposed to SA (figure 5B); after 6 d of exposure only plants exposed to 4 µg/L had a significant increase in their CAT activity; plants exposed for 10 d and to all tested concentrations presented a significant increase of this

parameter; on the 14th d, the CAT values tended to normalize, being similar to those founded in plants from the control treatment.

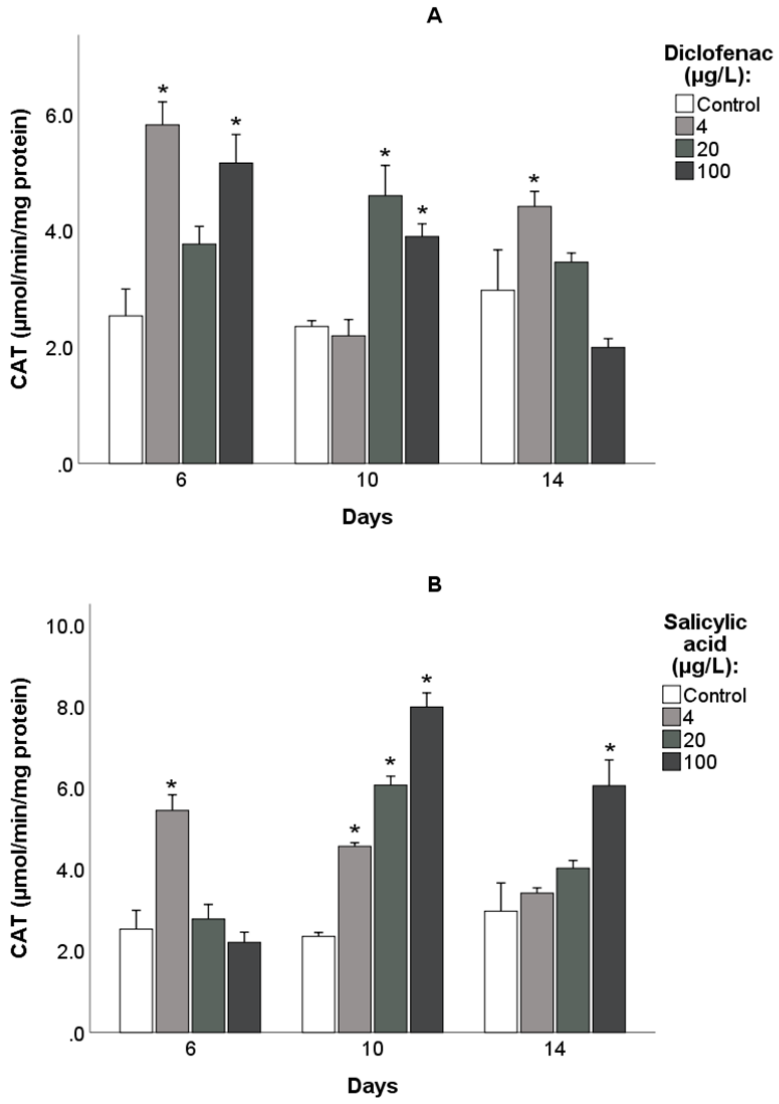

Figure 5. Effects of diclofenac (A) and salicylic acid (B) on catalase activity of *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences ($p<0.05$) in relation to control, for each species (n=8).

Ten days of exposure to DCF (figure 6A) caused a significant increase of APX activity, but only plants subjected to 20 µg/L showed a significant effect; no alterations were reported after 6 or 14 d of exposure. However, SA exposure (figure 6B) allowed obtaining more consistent results; 6 d of exposure was capable to significant decrease APX activity in all tested concentrations, after 10 d of exposure, a significant increase of APX activity was observed for plants exposed to levels of 20 and 100 µg/L; after 14

d of exposure, plants exposed to low SA levels had their APX decreased, while those exposed to higher SA levels had significant increases of APX activity.

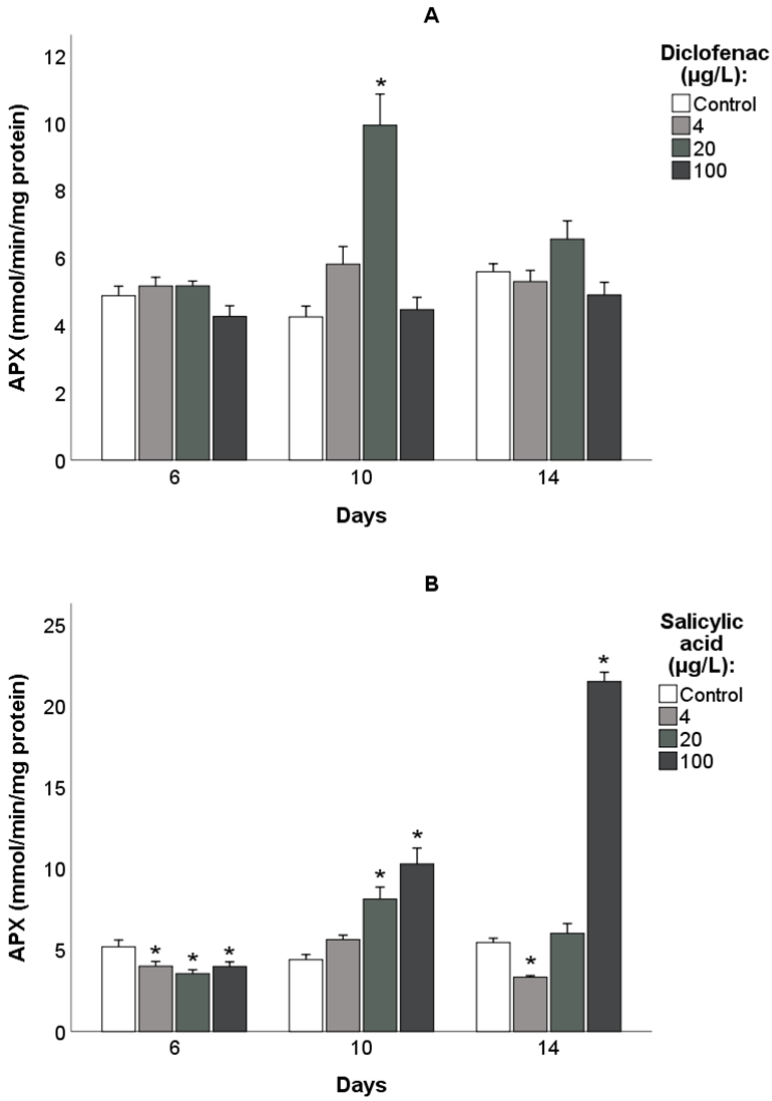

Figure 6. Effects of diclofenac (A) and salicylic acid (B) on ascorbate peroxidase activity on *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences in relation to control, for each species (n=8).

Six days of DCF exposure (figure 7A) did not cause any significant alteration on GSTs activity on *L. minor*; on the other hand, 10 d of exposure caused an increase in GSTs activity, for all tested concentrations, being statistically different for levels of 4 and 100 µg/L; 14 d of exposure to DCF caused a significant decrease of GSTs activity for plants exposed to all concentrations. SA (figure 7B) was able to provoke a significant increase on GSTs activity in plants exposed to 4 and 100 µg/L; GSTs levels returned to normal

after the 10th day of exposure, and a significant decrease in GSTs activity was observed in plants exposed to all SA concentrations after day 14.

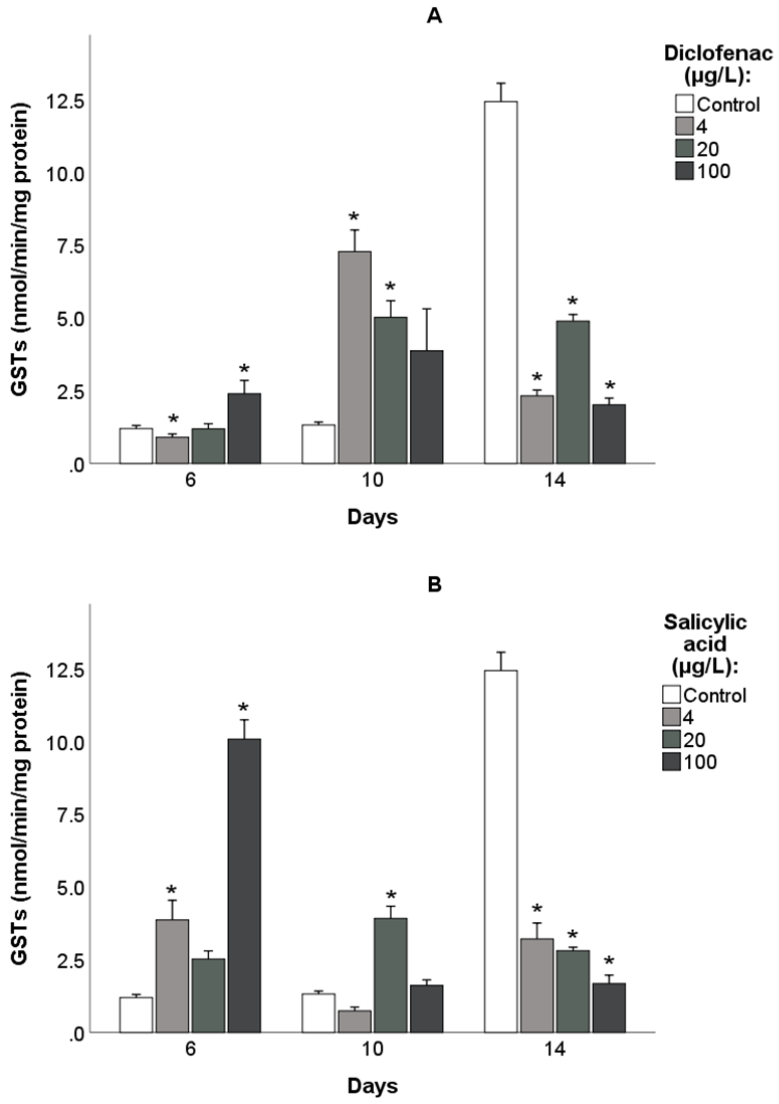

Figure 7. Effects of diclofenac (A) and salicylic acid (B) on glutathione-S-transferases activity on *L. minor*. For each parameter, mean and standard error are shown. * stand for statistical differences in relation to control, for each species (n=8).

3.5 Discussion

3.5.1 Diclofenac

Ecological relevance, toxicological sensitivity (as expressed by the values of toxicity parameters), and discrepancy vs. agreement of results among distinct species are three important criteria for the selection of useful endpoints in macrophyte toxicity tests (Arts

et al., 2008). This is particularly important when considering the use of *Lemna* for ecotoxicity testing purposes. In fact, some *Lemna* species are referred by some studies to be extremely sensitive in terms of their biological response, while being described as tolerant to environmental stressors by others. This apparent contradiction can be explained on the basis that plants may be highly adaptive (Wang, 1990), and it is necessary to test for this adaptive potential along the time course of the exposure. This mean that different responses (in terms of their intensity) may be attained after exposing these species for distinct periods, to the same combination toxicant/levels. This is also important because ecotoxicological relevance of data obtained in short term ecotoxicity tests at relatively high concentrations is difficult (if not impossible) to extrapolate to real conditions in the wild, where sensitive species may be chronically exposed to multiple contaminants (Paul et al., 2017). Under such realistic conditions, the quality of results obtained from short-term, as well as from prolonged exposures, are difficult to interpret, considering the number and complex contributions of potential confounding factors (Kunkel and Radke, 2012). However, prolonged exposure to pollution may induce community tolerance to chemical stress, and may also reduce the intensity (or alter the nature) of the response that may be detected, indicating that the community has been restructured as a response to the continuous presence of the toxicant in question (Eriksson et al., 2015). That is, distinct sampling intervals may yield different results, not only in terms of intensity but also in the type of response to be observed. The here-obtained results underline the need to adopt prolonged exposure modes, during which sampling must be assured at discrete intervals, as a way of assuring that environmentally realistic conditions are used; in addition, it is of fundamental importance to adopt a specific timeframe of exposure, to avoid having a strong modulation in the extent, and more importantly, the type of response.

Exposure to toxicants is one of the most common triggering factors for the production and release of reactive oxygen species (ROS) by the organism (Tripathy and Oelmüller, 2012), despite being a normal metabolic process in all aerobic organism (Perl-Treves and Treves, 2002). These stressful environments induce the generation of ROS such as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and others in plants (Hayat et al., 2010). The production of ROS is not deleterious when the redox homeostasis is not challenged, but it can suffer alterations after exposure to

toxicant (and its metabolism), since this may culminate in an increased stress challenging the organism (Bailey-Serres and Mittler, 2006). When such condition arises, the redox homeostasis is questioned, and antioxidants are necessary to help restore the normal organism functioning (Ahmad et al., 2010). This group of chemicals includes distinct entities, such as carotenoids, a group of natural tetraterpenoid pigments distributed widely in plants, but also common in algae, fungi, and bacteria (Sun et al., 2018). In our study, Car seemed to have been involved in the response to the presence of DCF. Levels of these pigments were altered in plants after a short-term challenge of 6 d of exposure to an intermediate concentration of DCF; a similar response was also reported after 10 d of exposure but to the highest concentration of the same drug. On the contrary, and after a 14 d exposure, Car levels were very similar to those measured in control plants. Carotenoids play an important role in the prevention of several degenerative stress processes in plants owing to their antioxidant function (Sytar et al., 2013). Consequently, carotenoids can be photoprotectants, acting as ROS scavengers (Nisar et al., 2015) and preventing self-oxidation of the photosynthetic systems (Braslavsky and Holzwarth, 2012); these structures are prone to oxidative damage since they may act as antennas, collecting solar radiation in the 400 nm to 500 nm range and transmitting the energy by energy transfer to the chlorophylls or bacteriochlorophylls in the reaction centers with photosynthetic functions (Braslavsky and Holzwarth, 2012). The here-obtained pattern of results seem to show the involvement of carotenoids: after 6 d of exposure to a concentration of 20 µg/L of DCF, the antioxidant defense was dependent upon the antioxidant scavenging activity of Car, without the activation of the antioxidant enzymatic defense pathway, since CAT and APX were not activated. However, under harsher conditions (e.g. the combination of a longer period of exposure and higher amounts of DCF), a full antioxidant response was deployed, that involved the activation of CAT activity. However, this set of effects may suggest that the here adopted conditions (levels and durations of exposures) only elicited transient moderate alterations, that were reverted after longer exposure periods due to adaptive responses of the plants, and were not enough to cause permanent damage in this group of pigments.

On the other hand, the antioxidant system was activated on *L. minor* after DCF exposure, at least partially. Among the different enzymes with antioxidant activity, CAT was more responsive than APX. Despite being distributed among a large number

of locations, APX was not equally responsive when compared to CAT. APX isoenzymes are distributed by at least four distinct cellular compartments: stromal APX and thylakoid membrane-bound APX in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX, and cytosolic APX. A fifth APX isoenzyme can occur as a mitochondrial membrane-bound form (Shigeoka et al., 2002). On the contrary, CAT is mostly found in peroxisomes (Copper, 2000). Despite this difference, in general, APX levels of DCF-exposed plants were kept unchanged, similar to those reported for the control plants. However, Bartha et al. (2014) demonstrated that much higher DCF concentrations (1 mg/L) are capable of increasing this isoenzyme activity after 1 and 7 d of exposure, on the species *Typha latifolia*. After being exposed for 6 and 10 d to DCF, plants had their CAT activity significantly increased. This effect might have been triggered to cope with increased levels of ROS caused by DCF exposure and metabolism; this assumptions is made based on the physiological role of both APX and CAT, which belong to two different classes of H_2O_2 scavenging enzymes; APX is responsible for the fine modulation of ROS for signaling, whereas CAT is responsible for the removal of excess ROS during stress (Mittler, 2012) associated to a very fast turnover rate (Mhamdi et al., 2010). DCF has been shown to be pro-oxidative, causing oxidative stress in different organisms. Exposure to DCF (100 µg/L) resulted in the increase of CAT in gills and liver of *Cyprinus carpio* after 96 h (Nava-Álvarez et al., 2014). Islas-Flores et al. (2013) reported similar results in the same organism, with a CAT activity increase in gills and liver, after exposure to DCF at a level of 7.098 mg/L during different intervals, 12 h and 48 h for gills, and 72 h for liver. Exposure to DCF contaminated sediment (46.7 µg/kg), resulted in a CAT increase in *Hyalella azteca* from 12 to 72 h of exposure (Oviedo-Gómez et al., 2010). In plants, similar results suggesting that DCF metabolism can indeed result in pro-oxidative alterations were found by Alkimin et al. (2019), in a study that reported a significant increase in CAT activity on *L. minor* acutely exposed to 100 µg/L of DCF.

Other pharmaceutical drugs from the same NSAID class, namely ibuprofen, have been involved in similar responses. Dordio et al. (2011) found an increase in CAT activity in *Typha* spp. after being exposed to this drug in varying concentrations (0.5 – 2 mg/L) during 7, 14, and 21 d. The results found in the literature show the putative triggering of antioxidant mechanisms to counteract the challenge posed by the increased amounts of

ROS caused by exposure to this drug, a response that is also common to other taxa. Despite these assumptions based on response patterns described in the literature, our study evidenced that this response might be reverted along time. In fact, after 14 d of exposure to DCF, the CAT activity of exposed plants was decreased in relation to the control, raising the hypothesis that a prolonged exposure caused a long period of chemical stress in the plant, that lead to a permanent condition of failure in the antioxidant defense mechanism. This corresponds to a scenario for which the plant cannot fully respond, being incompetent to handle the excess of formed ROS and thereby suffering oxidative damages caused by them. This alteration, evidenced by the decrease of CAT activity, caused by long-term (35 d) exposure to DCF levels of 60 µg/L was reported in other organisms such as *Tinca tinca* (Stancova et al. (2017); similarly, Saucedo-Vence et al. (2015) reported lower levels of CAT activity in blood and liver of *Cyprinus carpio* after 24 d of exposure to 7.098 mg/L of DCF.

This hypothesis can be also supported by the results concerning the here measured GSTs activity. These enzymatic forms correspond to a phase II metabolism isoenzymes group, responsible for the detoxification of exogenous substances through the ability to conjugate glutathione (GSH) with compounds containing an electrophilic center, in order to modify the substrate into a more water soluble, less toxic complex (Habig et al., 1974). It is known that the majority of GSTs substrates are either xenobiotics or products of oxidative stress (Hayes and Pulford, 1995). In this study, it was possible to observe an increase in GSTs activity in plants exposed for 6 and 10 d to DCF. This tendency might be interpreted as a biological response aiming at transforming DCF into an excretable metabolite, which is a common trend among most living organisms, and plants are no exception. This pathway seems also to be important in plants, considering the versatility of their conjugation machinery, namely their GSTs isoenzymes. In fact, individual gene analysis and genomics studies indicate that plants have 25 or more genes encoding for GSTs (Edwards et al., 2000), showing that similarity of GSTs-mediated responses among a large set of distinct organisms. In addition, the increase of GSTs activity can result from an attempt to eliminate ROS, since this enzyme is part of glutathione-peroxidase cycle, a metabolic pathway that detoxifies hydrogen peroxide (Smirnoff, 2000). However, plants exposed for higher periods (14 d), showed a decrease in this isoenzymes activity, similar to the pattern reported for CAT activity. As

previously cited, Stancova et al. (2017) found a decrease in CAT activity on *T. tinca* after 35 d of exposure to 60 µg/L of DCF and, similarly to our results, the authors reported a decrease in GSTs activity too. This comparable pattern of response emphasizes the possibility of similar biological responses to DCF prolonged exposures, even in different organisms. It is however necessary to stress that this pattern is not always repeated, since GSTs response seems to depend upon different factors, such as time and route of exposure, tested concentration, and type of organism. These assumptions are reinforced considering the following studies. According to Stepanova et al. (2013) GSTs were increased in *C. carpio* larvae after 30 d of exposure to 3 mg/L of DCF. On the other hand, even 15 d of exposure to 250 ng/L of DCF were not capable of causing changes on GSTs activity on gills and digestive gland on *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno, 2014). Finally, in plants (*T. latifolia*), after 3 and 7 d of exposure to 1 mg/L of DCF, an increase on GSTs activity was recorded (Bartha et al., 2014). On the other hand, Kummerová et al. (2016) found a decrease on GSTs activity after 10 d of exposure to 100 µg/L of DCF in *L. minor*. A decrease in GSTs activity, detected in our study, may be a consequence of a long-term damage that was only ascertained after a prolonged exposure period to DCF, most likely by the adverse effect of ROS on this pathway. However, the analysis of the here-observed changes concerning this particular enzymatic activity alone does not allow a clear identification of the specific function that was activated, the mere conjugation with GSH to facilitate excretion of the drug, or an antioxidative defense function (Bartha et al., 2014). At low concentrations of toxicant, the hydroxylation process seem to be sufficient to detoxify the xenobiotic compound in plants, while at higher concentrations, more effective and comprehensive metabolic pathways may be activated (Bartha, 2012). On the other hand, ROS are capable of damaging lipids, DNA, and especially proteins, consequently causing a decrease in enzymatic activity by denaturation (Schieber and Chandel, 2014). Considering that GSTs isoenzymes are a considerable portion of all soluble proteins in plants (e.g. in *Zea mays*, GSTs constitute >1% of the soluble protein; Marrs, 1996), protein denaturation in a moderate extent may significantly compromise the enzymatic activity of affected enzymatic forms. Another factor to consider in the analysis of this denaturation effects is linked to the accumulation of ROS along time, as a result of their natural production by biological systems. This scenario may be aggravated considering the occurrence of a spatially and temporally isolated event such

as the exposure to an oxidant xenobiotic, whose toxicity will depend on the duration of exposure to this additional stress factor (Bailey-Serres and Mittler, 2006). ROS may be produced in cells in general at any time, but their production may increase if cells are exposed to specific stressors, such as pro-oxidants, resulting in a combination of factors that are ultimately responsible for extreme levels of ROS, whose presence will certainly result in increased toxicity (Bailey-Serres and Mittler, 2006). Another option justifying DCF toxic effects may involve its phase II metabolites, such as glucuronide diclofenac and glutathione diclofenac, which have also been identified in mammals. The bioactivation of these conjugates has been implicated in diclofenac-induced toxicity effects, including oxidative stress and liver injury (Tang et al., 1999; Boelsterli, 2003). In plants, the metabolism of xenobiotics follows somewhat similar principles to those described for mammals (phase I - activation, phase II – conjugation and phase III, for plants, incorporation/stabilization) (Huber et al., 2012). In fact, this assumption is reinforced by the finding of metabolites of DCF in the plant species *T. latifolia* similar to those found in humans (such as 4'-OH diclofenac and diclofenac-glucopyranoside) (Huber et al., 2012), being possible to suggest this hypothesis. However, we must not forget that although plants and animals have partially similar detoxification enzymes, and that in both groups of organisms xenobiotic detoxification follows the two-phase model, the exact mechanisms behind the process of xenobiotic detoxification may be very different (Bartha et al., 2014) making it more difficult to explain all mechanisms involved in DCF effects in plants since this drug was designed to interact with the physiology of animals.

Furthermore, the oxidative stress caused by DCF exposure can be related to the capacity of this drug to induce ROS production, and its deleterious consequences, namely by provoking the peroxidation of the membrane lipid bilayer, which results in changes in adverse modifications of its integrity, and the malfunction of membrane-bound proteins and lipids (Hájková et al., 2019). Associated with this capacity, the high log K_{OW} values of DCF (4.51) suggest the possibility of its enhanced accumulation cells, by promptly permeating biological membranes (Corcoll et al., 2014). This might have decisively contributed to the oxidative stress scenario made evident by this study; however, the principal mechanism of DCF toxic action in plants is largely still unknown (Hájková et al., 2019).

Chls are a group of structurally closely related compounds, universally acknowledged to be the indispensable photoreceptors in plant and bacterial photosynthesis (Katz et al., 1978). The biosynthesis of Chl and the development of chloroplasts in higher plants involve a highly controlled series of events, many of which may be subjected to inhibition by exogenously applied chemical agents (Wolf, 1977). As described in the results section, exposure to DCF was capable of causing an increase in Chls (a, b and total) contents after 6 and 10 d of exposure. However, these levels were reestablished near to control amounts after 14 d. DCF uptake and metabolization by plants occur very rapid (Huber et al., 2012), and may cause impact on primary processes of photosynthesis, mainly in the disruption of electron transport chain between photosystems II and I, which reflects in significant changes in "vitality index" (Kummerová et al., 2016). This effect raises the hypothesis that the chlorophyll biosynthesis system is altered, increasing the amount of these pigments, in an attempt to maintain the basic functioning of photosynthesis and consequently absorption of energy to perform the normal metabolic activities of plants. On the other hand, the reestablishment of Chl levels after 14 d may confirm the highly adaptative capacity of plants suggested by Wang (1990) and demonstrated by this study after a prolonged exposure. However, despite phytotoxic effects caused by DCF exposure are evident, information about which metabolic pathways this drug may alter, as well as the potential toxicity of DCF metabolites for plants, are still unclear (Vannini et al., 2018).

In general, this work demonstrated that DCF can cause damage on physiological and biochemical parameters on *L. minor*, however, apparently after a prolonged exposure the plant could cope with the exposure to the drug and physiological parameters were reestablished to values close of unexposed organisms. On the other hand, the biochemical system continues to demonstrate changes, emphasizing that even over time the plant still makes use of defensive barriers/biochemical defense to try maintaining its homeostasis.

3.5.2 Salicylic acid

From the literature, it is difficult to ascertain the real toxic effects caused by exogenous SA exposures since, in general, the exogenous application of this natural plant hormone

might act as a powerful tool in enhancing the growth, productivity and also in combating the adverse effects generated by various abiotic stresses in plants. Consequently, SA is intentionally used in agriculture being a great promise as a management tool for providing tolerance to crops against the aforesaid factors (Joseph et al., 2010). However, it is extremely important to understand the possible adverse effects caused by exogenous sources of SA, in particularly in aquatic organisms, which occur in the final environmental compartment which is the most common destination of this drug after wastewater disposal and agricultural application. Despite being a natural plant hormone (Raskin, 1992), SA can cause adverse effects on biochemical parameters, especially if exposure results from exogenous sources, as shown in this work. Ascorbate peroxidase is one of the most important ROS-scavenging enzymes (Sofo et al., 2015) and works along with other (iso)enzymes, with catalase-like functions (Apel and Hirt, 2004). The literature is well established determining that endogenous SA is normally capable of inhibiting the CAT and APX activities, to increase H_2O_2 concentrations in the cell, to activate its immune system after infection by pathogens (Vlot et al., 2009). This inhibition, as suggested by Durner and Klessig (1996) probably results from peroxidative reactions. On the other hand, according to Rao et al. (1997), exogenous SA treatment is capable of causing the increment of H_2O_2 levels in plants, thus provoking oxidative stress. Additionally, SA inhibits the electron transport system in plant membranes, favoring ROS accumulation; on the contrary, it also activates alternative oxidase (AOX), a specific electron transport route that is part of cytochrome oxidase pathway, retarding this process (Krasavina, 2007). However, the effects of exogenous SA in these enzymes in plants is not extensively described, and a considerable lack of information on this subject still exists, demanding better understanding of the effects of SA on plants. In this work, *L. minor* plants, after being submitted to an exogenous SA source, presented significant alterations on both enzymes cited above (APX and CAT), which may be interpreted as an indication of the activation of the antioxidant defense system. After an initial period of 6 d of exposure to SA, APX activity decrease in all tested concentrations; in addition, plants exposed to the lower concentrations of SA showed an increase of CAT activity, suggesting a prompt activation of this defensive mechanism, faster than APX. On the other hand, the detected APX decrease are in agreement with Vlot el al. (2009), previously cited. As mentioned, the authors suggest that the inhibition of this enzymatic activity are capable to occur to increase H_2O_2

concentrations in the cell, to activate its immune system after infection by pathogens; this may like to occur after a detection/contamination of exogenous concentration of SA, as demonstrated in this study. On the other hand, a 10 d exposure period caused the activation of both enzymes, in an attempt to cope with increased ROS caused by this longer exposure, being this increase dose-dependent. Furthermore, SA-induced redox regulation, to cope with ROS increase, appears not only to involve the here studied mechanisms; in fact, it may also lead to the accumulation of phytohormones, such as ethylene, nitric oxide, and jasmonate (Dat et al. 2003). Finally, data concerning a 14 d exposure showed that CAT activity was similar to the control plants; plants exposed to the highest SA levels had significantly higher levels of APX, suggesting that the activation of this enzymatic form occurred at a later stage. However, exogenous SA enhanced the activities of antioxidant enzymes like APX and superoxide-dismutase (SOD), with a concomitant decline in the activity of CAT in maize plants (Krantev et al., 2008). Similar results were found in this work, after a 14 d exposure of *L. minor* to SA. In summary, as cited above, it seems to exist a contradiction, regarding the physiological role of SA, since its presence might in some circumstances prevent ROS production, but it may also stimulate it, causing oxidative damage in plants. Based on the here obtained results, it is possible to suggest the relationship between both antioxidant enzymes activity after an exogenous SA. From the literature, no data showing that SA may exert adverse damaging effects in aquatic plants are available. In fact, the literature on the toxic effects caused by exposure to exogenous SA towards aquatic plants is extremely scarce; in general, toxicological studies use SA as a preventive factor for possible damage caused by other exogenous stressors, such as chemicals (paraquat; Ananieva et al., 2004), drought effects (Hayat et al., 2008) and low temperatures (Janda et al., 1999).

In contrast, the activity of phase II metabolism (GSTs isoenzymes, in this case), significantly increased its activity after the 6^{th} d of exposure to SA. This response demonstrates that the plants soon recognized the exogenous SA as a chemical challenger, whose presence required the increased efficacy of the GSH conjugation biotransformation route, to form a more soluble and less toxic compound to be eliminated. It is necessary to consider that SA is metabolized by a combination of glycosylation and decarboxylation, as shown to occur in tobacco leaves (Edwards,

1994), and by conjugation with glucuronic acid, reflected by a glucosyltransferase activity increase in rice roots (Silverman et al., 1995). Assuming that these same metabolism pathways might occur in *L. minor*, since there is no such information for the species in question, the increase in GSTs activity and consequently GSH increase may not be related to the biotransformation of the exogenous SA. Therefore, it can be assumed that in this case the GSH conjugation by means of GSTs acts as ROS scavenger. GSH not only participates in the direct detoxification of ROS, it may also protect cells against unfavorable stress effects through the activation of various defense mechanisms due to its involvement in redox signaling (Apel and Hirt 2004; Foyer and Noctor 2005). In this signaling pathway, GSH interacts with ROS, redox molecules [Trxs, glutaredoxins (Grxs)], and plant hormones [salicylic acid (SA), abscisic acid (ABA)] (Szalai et al., 2009), giving rise to a complex, albeit effective, protective mechanisms against toxic effects of ROS. This suggestion can be supported by data from the literature, since exogenous SA application also activated GSH synthesis in *Brassica juncea* and *B. napus* and caused enhanced protection against abiotic stressors, such as drought- and salt-induced oxidative damages (Alam et al., 2013; Hasanuzzaman et al., 2014). On the contrary, after the 10^{th} d of SA exposure, no alterations in GSTs activity were reported. Taking into account the previous assumed hypothesis (GSTs as ROS scavenger), this could be an expected response since at that time interval, CAT and APX activities were already increased, assuming a leading role against oxidative injury. On the contrary, after 14 d of exposure SA was responsible for decreasing GSTs activity, suggesting the possibility that this enzyme could have been directly denaturated by ROS generated after a prolonged SA exposure, similarly to what was observed after DCF exposure. According to Hasanuzzaman et al. (2017) low concentrations of SA caused advantageous effects in abiotic stress tolerance of plants. In contrast, high concentrations of SA showed to exert toxic effects. This contradictory profile makes difficult to described and identify all metabolic pathways, functions and alterations caused by SA exposure, since several factors (e.g., the concentration and application method of SA) are critical to yield distinct effects in different plant species (Hasanuzzaman et al., 2017).

As previously cited, SA is a natural plant hormone, and has direct functions on plant physiology, regulating growth. In addition, in some cases, endogenous SA protects

plants against xenobiotics and stimulates the production of photosynthetic pigments (Hashmi et al., 2012). SA may act as a stressor as well, which among others, negatively affects the photosynthetic processes, especially above a certain threshold concentration (Janda et al., 2014). The effective concentration to attain this effect may highly depend on the plant species, the way of the application, the duration of the treatment, and the environmental conditions (Janda et al., 2014). For example, the same concentration that provided protection against low temperature-induced damage in young maize according to the time and conditions of exposure (Janda et al. 1999), could decrease/protect barley against paraquat effects (Ananieva et al. 2002). Take into account, that in this study, there were no observed effects on pigments levels (Chl a; b and total and Car), these results can be attributed to two different hypothesis: first, the absence of effect on pigments levels may result from an acclimation mechanism to exogenous SA exposure (Zait et al., 2018); or second, the here tested concentrations were not capable of causing damages on pigments analyzed in this specific aquatic species. According to Janda et al. (2014), the effects of exogenous SA depends of the effective concentration acting on plant tissues. In turn, this amount can vary according to the plant species, the application route, the duration of exposure, and the environmental conditions. In addition, Chls represent the central part of the entire metabolism of the green plant system, therefore, any significant change in their levels is likely the reason of significant toxic effects, manifested primarily on growth alterations (Belkadh et al., 2014). However, the importance of SA in the regulation of plant growth, and the clear establishment of SA toxic mechanisms that may adversely influence growth, are areas of further investigation, reinforced by the assumption that even the SA natural biosynthesis is not completed elucidated (Janda and Ruelland, 2015). Even without effects in pigments content, compounds such as SA can affect the gas exchange rates, in maize, and may also influence the processes related to the photosynthetic electron transport by enhancing the non-photochemical fluorescence quenching mechanisms (Janda et al., 2000). On the other hand, the complete elucidation of the mechanism by which SA causes toxic alterations in this pigment group is a task made difficult by the enormous gaps in the knowledge about its metabolism in plants, a scenario that mostly favors speculation. In addition, it is necessary to take into account that the majority of findings about the effects of exogenous SA were obtained for terrestrial plants, especially those used in agricultural practices (such as maize, tomato, pea and others).

These species may substantially differ from aquatic species, since these inhabit different environments and show some specific physiologic processes and adaptations. Finally, SA signalling is complex and over the coming years, further advances will be required (Janda and Ruelland, 2015); despite being a natural plant hormone, exogenous SA can cause biochemical alterations in *L. minor* metabolism that in a longer-term or even in future generations, can challenge the survival and adaptation of this species in different aquatic ecosystems, affected by with the presence of SA as a result of human excretion.

3.6 Conclusions

In general, both tested pharmaceuticals showed to be toxic to *L. minor*. Diclofenac, in all sampling intervals, caused alterations in biochemical parameters, more pronounced in CAT and GSTs, and varying according to the time and tested concentration. DCF exposure provoked significant increases of the amounts of pigments (Chl a, b, total and Car). This response was evident after the two initial samplings intervals (6 and 10 d), but was followed by a long-term adaptive response, made evident by the results obtained for the 14 d sampling. Results obtained after a 14 d exposure to DCF showed that plants were capable to recover to basal levels of physiological traits. In addition, SA exposure, under the here tested conditions (distinct intervals of exposure and concentrations), was capable of increasing CAT and provoking a variation in APX and GSTs, according to time and concentration. These responses, in general, may ultimately compromise survival, by demanding additional adaptive effort to this species. However, it is necessary to undertake more studies about the toxicological effects of DCF and SA, mainly focusing on aquatic plants, considering that these pharmaceuticals are largely found in aquatic environment. Thus, this work shows that time is a factor that contribute to pharmaceutical toxicity in aquatic plants, by modulating not only the extent but especially the type of the toxic effects, that may results from exposure to environmental relevant concentrations of the tested pharmaceuticals. Finally, more prolonged exposure tests are recommended to understand the toxicology of pharmaceuticals in aquatic plants.

3.7 References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 6, 105–121.

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., Sharma, S. 2010. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Critical Reviews in Biotechnology. 30:3 161-175. https://doi.org/10.3109/07388550903524243

Alkimin, G. D., Daniel, D., Frankenbach, S. Serôdio, J., Soares, A. M. V. M., Barata, C. Nunes, B. 2019. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Science of The Total Environment. 657, 926-937. https://doi.org/10.1016/j.scitotenv.2018.12.002

Alam, M.M., Hasanuzzaman, M., Nahar, K., Fujita, M. 2013. Exogenous salicylic acid ameliorates short-term drought stress in mustard (*Brassica juncea* L.) seedlings by upregulating the antioxidant defense and glyoxalase system. Australian Journal of Crop Science. 7: 1053–1063.

Ananieva, E. A., Christov, K. N., Popova, L. P. 2004. Exogenous treatment with Salicylic acid leads to increased antioxidant capacity in leaves of barley plants exposed to Paraquat. Journal of Plant Physiology. 161:3, 319-328. https://doi.org/10.1078/0176-1617-01022

Ananieva, E. A., Alexieva, V. S., Popova, L. P. 2002. Treatment with salicylic acid decreases the effects of paraquat on photosynthesis. J Plant Physiol 159, 685–693

Andreozzi, R., Raffaele, M., Nicklas, P. (2003). Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. Chemosphere. 50:10, 1319–1330.

Apel, K., Hirt, H. 2004. Reactive oxygen species: Metabolism, Oxidative Stress, and Signal Transduction. Annual Review of Plant Biology. 55, 373-399. https://doi.org/10.1146/annurev.arplant.55.031903.141701

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant physiology, 24, 1-15.

Arts, G. H. P., Belgers, J. D. M., Hoekzema, C. H., Thissen, J. T. N. M. 2008. Sensitivity of submersed freshwater macrophytes and endpoints in laboratory

toxicity tests. Environmental Pollution. 153:1, 199–206. https://doi.org/10.1016/j.envpol.2007.07.019

Bailey-Serres, J., Mittler. R. 2006. The Roles of Reactive Oxygen Species in Plant Cells. Plant physiology. 141, 311. https://doi.org/10.1104/pp.104.900191.

Bartha, B., Huber, C., Schröder, P. 2014. Uptake and metabolism of diclofenac in *Typha latifolia* – How plants cope with human pharmaceutical pollution, Plant Science. 227, 12–20.

Bartha, B 2012. Uptake and metabolism of human pharmaceuticals in plants. Identification of metabolites and specification of the defense enzyme systems under pharmaceutical exposure. PhD thesis. Technische Universität München. Lehrstuhl für Ökologischen Landbau und Pflanzenbausysteme.

Belkadhi, A., De Haro, A., Soengas, P., Obregon, S., Cartea, M.E., Chaibia, W., Djebali, W. 2014. Salicylic acid increases tolerance to oxidative stress induced by hydrogen peroxide accumulation in leaves of cadmium-exposed flax (*Linum usitatissimum* L.). Journal of Plant Interactions, 9(1), 647–654.

Benotti, M. J., Trenholm, R. A., Vanderford, B. J., Holady, J. C., Stanford, B. D., Snyder, S. A. 2009. Pharmaceuticals and Endocrine Disrupting Compounds in U. S. Drinking Water. Environ. Sci. Technol. 43:3, 597–603. DOI: 10.1021/es801845a

Boelsterli, U. A. 2003. Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. Toxicology and Applied Pharmacology 192, 307-322.

Bonnefille, B., Gomez, E., Courant, F., Escande, A., Fenet, H. 201). Diclofenac in the marine environment: A review of its occurrence and effects. Marine Pollution Bulletin. 131, 496–506. DOI: 10.1016/j.marpolbul.2018.04.053

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Brain, R. A., Johnson, D. J., Richards, S. M., Hanson, M. L., Sanderson, H., Lam, M. W., Young, C., Mabury, A. S., Sibley, P. K., Solomon, K. R. 2004. Microcosm evaluation of the effects of an eight pharmaceutical mixture to the aquatic macrophytes *Lemna gibba* and *Myriophyllum sibiricum*. Aquatic Toxicology. 70:1, 23–40. https://doi.org/10.1016/j.aquatox.2004.06.011

Braslavsky, S.E., Holzwarth, A.R. 2012. Role of Carotenoids in Photosystem II (PSII) Reaction Centers. Int J Thermophys. 33: 2021. https://doi.org/10.1007/s10765-012-1274-1

Brooks, B. W., Fulton, B. A., Hanson, M. L. 2015. Aquatic toxicology studies with macrophytes and algae should balance experimental pragmatism with environmental realism. Science of the Total Environment. 536:1, 406–407. https://doi.org/10.1016/j.scitotenv.2015.07.085

Claessens, M., Vanhaecke, L., Wille, K., Janssen, C. R. 2013. Emerging contaminants in Belgian marine waters: Single toxicant and mixture risks of pharmaceuticals. Mar Pollut Bull. 71:1-2, 41-50.

Cooper, G. M. The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Peroxisomes. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9930/

Corcoll, N., Acuña, V., Barceló, D., Casellas, M., Guasch, H., B. Huerta, B., Petrovic, M., Ponsatí, L., Rodríguez-Mozaz, S., Sabeter S. 2014. Pollution-induced community tolerance to non-steroidal anti-inflammatory drugs (NSAIDs) in fluvial biofilm communities affected by WWTP effluents. Chemosphere, 112, 185-193

Coutellec, M. A. Barata, C. 2013. Special issue on long-term ecotoxicological effects: an introduction. Ecotoxicology. 22: 763. 763-766 https://doi.org/10.1007/s10646-013-1092-7

Daughton, C. G., Ternes, T. A. 1999. Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change?. Environ Health Perspect. 107:6, 907–938. doi: 10.1289/ehp.99107s6907

Davison, C. 1971. Salicylate Metabolism in Man. Annals New York Academy of Sciences. 249-268.

Dat, J.F., Pellinen, R., Beeckman, T., Van De Cotte, B., Langebartels, C., Kangasjarvi, J., Inze, D., Van Breusegem, F. 2003. Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. Plant J. 33:621–632.

Dordio, A.V., Belo, M., Martins, T.D., Palace, C.A.J., Dias, C.M.B., Picó, Y., Pinto, A.P. 2011. Evaluation of carbamazepine uptake and metabolization by *Typha* spp., a plant with potential use in phytotreatment. Bioresour. Technol. 102, 7827–7834. https://doi.org/10.1080/03067311003782708

Durner, J., Klessig, D.F. 1996. Salicylic acid is a modulator of tobacco and mammalian catalases. J Biol Chem 272: 28492-28501

Edwards, R. 1994. Conjugation and Metabolism of Salicylic Acid in Tobacco. Journal of Plant Physiology, 143(6), 609–614. doi:10.1016/s0176-1617(11)81146-6

Edwards, R., Dixon, D.P, Walbot, V. 2000. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health Trends Plant Sci., 5, 193-198.

Eriksson, K. M., Johansson, C. H., Fihlman, V., Grehn, A., Sanli, K., Andersson, M. X., Blanck, H., Arrhenius, Å., Sircar, T., Backhaus, T. 2015. Long-term effects of the antibacterial agent triclosan on marine periphyton communities. Environ Toxicol Chem. 34:9, 2067-77. doi: 10.1002/etc.3030. Epub 2015 Jun 24.

Farré, M., Pérez, S., Kantiani, L., Barceló, D. 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends in Analytical Chemistry. 27:11, 991–1007. https://doi.org/10.1016/j.trac.2008.09.010

Foyer, C. H., Noctor, G. 2005. Redox homeostasis and antioxidant signalling: a metabolic link between stress perception and physiological responses. Plant Cell. 17, 1866–1875

García-Gómez, C., Esteban, E., Sánchez-Pardo, B., Fernández, M. D. 2014. Assessing the ecotoxicological effects of long-term contaminated mine soils on plants and

earthworms: relevance of soil (total and available) and body concentrations. Ecotoxicology. 23:7, 1195–1209. https://doi.org/10.1007/s10646-014-1262-2

Gavrilescu, M., Demnerová, K., Aamand, J., Agathos, S., Fava, F. 2015. Emerging pollutants in the environment: present and future challenges in biomonitoring, ecological risks and bioremediation. N Biotechnol. *32*:1, p.147–156.

Gómez-Oliván, L. M., Galar-Martínez, M., Islas-Flores, H., García-Medina, S., Sanjuan-Reyes, N. 2014. DNA damage and oxidative stress induced by acetylsalicylic acid in *Daphnia magna*. Comparative Biochemistry and Physiology, Part C. 164, 21–26. doi: 10.1016/j.cbpc.2014.04.004

Gonzalez-Rey, M., Bebianno, M. J. 2014. Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*. Aquatic Toxicology, 148, 221–230. doi:10.1016/j.aquatox.2014.01.011

Habig, W. H., Pabst, M. J., Jakoby, W. B. 1974. Glutathione S-transferases—the first enzymatic step in mercapturic acid formation. J Biol Chem. 249, 7130–7139.

Hájková M., Kummerová, M., Zezulka, S, Babula, P., Váczi, P. 2019. Diclofenac as an environmental threat: Impact on the photosynthetic processes of *Lemna minor* chloroplasts. Chemosphere 224, 892-899

Hasanuzzaman, M., Nahar, K., Bhuiyan, T.F., Anee, T.I., Inafuku, M., Oku, H., Fujita, M. 2017. Salicylic Acid: An All-Rounder in Regulating Abiotic Stress Responses in Plants. In Phytohormones - Signaling Mechanisms and Crosstalk in Plant Development and Stress Responses. DOI: 10.5772/intechopen.68213

Hasanuzzaman, M., Alam, M.M., Nahar, K., Mahmud, J.A., Ahamed, K.U., Fujita, M 2014. Exogenous salicylic acid alleviates salt stress-induced oxidative damage in Brassica napus by enhancing the antioxidant defense and glyoxalase systems. Australian Journal of Crop Science. 8:631–639

Hashmi, N., Khan, M. M. A. 2012. Exogenous salicylic acid stimulates physiological and biochemical changes to improve growth, yield and active constituents of

fennel essential oil. Plant Growth Regulation. 68:2, 281–291. https://doi.org/10.1007/s10725-012-9716-0

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. 2010. Effect of exogenous salicylic acid under changing environment: A review. Environmental and Experimental Botany. 68:1, 14–25. https://doi.org/10.1016/j.envexpbot.2009.08.005

Hayat, S., Hasan, S.A., Fariduddin, Q., Ahmad, A. 2008. Growth of tomato (*Lycopersicon esculentum*) in response to salicylic acid under water stress J. Plant Int., 3:4, 297-304

Hayes, J. D., Pulford, D. J. 1995. The Glutathione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance part I. Critical Reviews in Biochemistry and Molecular Biology. 30:6, 445-520. https://doi.org/10.3109/10409239509083491

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology letters. 131:1-2, 5–17. https://doi.org/10.1016/S0378-4274(02)00041-3

Hillman, W. S. 1961. The Lemnaceae, or Duckweeds: A Review of the Descriptive and Experimental Literature. Botanical review. 27:2, 221–287.

Hilton, M. J., Thomas, K. V. 2003. Determination of selected human pharmaceutical compounds in effluent and surface water samples by high-performance liquidchromatography–electrospray tandem mass spectrometry. J. Chromatogr. A 1015,129–141.

Hiscox, J. D., Israelstam, G. F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can J. Bot. 57, 1332-1334.

Huber, C., Bartha, B., Schröder, P. 2012. Metabolism of diclofenac in plants – Hydroxylation is followed by glucose conjugation. Journal of Hazardous Materials, 243, 250–256. doi:10.1016/j.jhazmat.2012.10.023

Islas-Flores, H., Gómez-Oliván, L. M., Galar-Martínez, M., Colín-Cruz, A., Neri-Cruz, N., García-Medina, S. 2013. Diclofenac-induced oxidative stress in brain, liver, gill and blood of common carp (*Cyprinus carpio*). Ecotoxicology and Environmental Safety, 92, 32–38. doi:10.1016/j.ecoenv.2013.01.025

Janda, M., Ruelland E. 2015. Magical mystery tour: salicylic acid signaling. Env. Exp. Bot., 114, 117-128

Janda, T., Gondor, O.K., Yordanova, R., Szalai, G., Pál, M. 2014 Salicylic acid and photosynthesis: signalling and effects. Acta Physiol Plant 36. 2537. https://doi.org/10.1007/s11738-014-1620-y

Janda, T., Szalai, G., Antunovics, Z., Horvath, E., Paldi, E. 2000. Effect of benzoic acid and aspirin on chilling tolerance and photosynthesis in young maize plants. Maydica 45:29–33.

Janda, T., Szalai, G., Tari. I, Paldi, E. 1999. Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. Planta 208: 175 –180

Joseph, B., Jini, D., Sujatha, S. 2010. Insight into the Role of Exogenous Salicylic Acid on Plants Grown under Salt Environment. Asian Journal of Crop Science, 2, 226-235.

Jux, U., Baginski, R. M., Arnold, H., Kronke, M., Seng, P. N. 2002. Short communication detection of pharmaceutical contaminations of river, pond and tap water from Cologne (Germany) and surroundings. Int J Hyg Environ Health. 205:5, 393-8. DOI: 10.1078/1438-4639-00166

Kaleniecka, A., Zarzycki, P. K. 2015. Pharmaceuticals in the aquatic environment: sources, effects, treatment methods. Arch Physiother Glob Res. 19: 3, 39-52.

Katz, J. J., Norris, J. R., Shipman, L. L., Thurnauer, M. C., Wasielewski, M. R. 1978. Chlorophyll function in the photosynthetic reaction center. Annu Rev Biophys Bioeng. 1978:7, 393-434. DOI: 10.1146/annurev.bb.07.060178.002141

Krantev, A., R. Yordanova, T. Janda, G. Szalai and L. Popova, 2008. Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. J. Plant Physiol., 165: 920-931

Krasavina, M.S. 2007. Effect of salicylic acid on solute transport in plants. In Salicylic Acid, A Plant Hormone. Hayat, S., and Ahmad, A., (eds). Dordrecht, The Netherlands.

Kummerová, M., Zezulka, Š., Babula, P., Tříska, J. 2016. Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant *Lemna minor*. J Hazard Mater. 302, 351–361.

Kunkel, U., Radke, M. 2012. Fate of pharmaceuticals in rivers: Deriving a benchmark dataset at favorable attenuation conditions. Water Research, 46:17, 5551–5565. https://doi.org/10.1016/j.watres.2012.07.033

Lakatos, G., Mszfiros, I., Bohfitka, S., Szab, S. 1993. Application of Lemna species in ecotoxicological studies of heavy metals and organic biocides. Science of The Total Environment. 134:1, 773–778. https://doi.org/10.1016/S0048-9697(05)80081-6

Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids pigments of photosyntheticbiomembranes. Methods Enzymol. 148, 350–382.

Lonappan, L., Brar, S. K., Das, R. K., Verma, M., Surampalli, R.Y. 2016. Diclofenac and itstransformation products: environmental occurrence and toxicity - a review. Environ.Int. 96, 127–138.

Marrs, K. A. 1996. The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Phys. Plant Mol. Biol. 47, 127-158.

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T.A., Hirsch, R. 2003. Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. Environ Toxicol Chem. 22:12, 2872-80.

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F., Noctor, G. 2010. Catalase function in plants: a focus on Arabidopsis mutants as stress-

mimic models, Journal of Experimental Botany 61(15), 4197–4220, https://doi.org/10.1093/jxb/erq282

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends in plant science 7: 405– 410. PMID: 12234732 52.

Mullangi, R., Sharma, K., Srinivas, N. R. 2012. Review of HPLC methods and HPLC methods with mass spectrometric detection for direct determination of aspirin with its metabolite(s) in various biological matrices. Biochemical chromatography. 26:8, 906-941. https://doi.org/10.1002/bmc.2694

Nava-Álvarez, R., Razo-Estrada, A. C., García-Medina, S., Gómez-Olivan, L. M., Galar-Martínez, M. 2014. Oxidative Stress Induced by Mixture of Diclofenac and Acetaminophen on Common Carp (*Cyprinus carpio*). Water, Air, & Soil Pollution, 225(2). doi:10.1007/s11270-014-1873-5

Nisar, N., Li, L., Lu, S., Khin, N.C., Pogson, B.J. 2015. Carotenoid Metabolism in Plants. Send to Mol Plant. 8:1, 68-82. doi: 10.1016/j.molp.2014.12.007

OECD. 2006. Lemna sp. growth inhibition test n. 221. OECD–Guideline for Testing of Chemicals, Paris, 22 p.

Oviedo-Gómez, D.G.C., Galar-Martínez, M., García-Medina, S., Razo-Estrada, C., Gómez-Oliván, L. M. 2010. Diclofenac-enriched artificial sediment induces oxidative stress in *Hyalella azteca*. Environmental Toxicology and Pharmacology, 29(1), 39–43. doi:10.1016/j.etap.2009.09.004

Pal, A., Gin, K. Y., Lin, A. Y., Reinhard, M. 2010. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. Science of the Total Environment. 408, 6062–6069. https://doi.org/10.1016/j.scitotenv.2010.09.026

Paul, V., Sharma, L., Kumar, R., Pandey, R., Meena, R. C. 2017. Estimation of Chlorophylls / Photosynthetic Pigments – Their Stability is an Indicator of Crop Plant Tolerance to Abiotic Stresses. Manual of ICAR Sponsored Training

Programme on “Physiological Techniques to Analyze the Impact of Climate Change on Crop Plants” Division of Plant Physiology, IARI, New Delhi

Pereira, C. D. S., Maranho, L. A., Cortez, F. S., Pusceddu, F. H., Santos, A. R., Ribeiro, D. A., Cesar, A., Guimarães, L. 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Science of the Total Environment. 548-549(1), 148–154.

Perl-Treves, R., Perl, A. 2002. Oxidative stress: An introduction. In: Inzé D, Van Montagu M (eds), Oxidative Stress in Plants, pp.1-32. Taylor & Francis, London.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., Watkins, C. B. 1997. Influence of Salicylic Acid on H_2O_2 Production, Oxidative Stress, and H_2O_2-Metabolizing Enzymes Salicylic Acid-Mediated Oxidative Damage Requires H_2O_2. Plant Physiology, 115(1), 137-149.

Raskin, I. 1992. Salicylate, A New Plant Hormone. Plant Physiol. 99, 799–803.

Saucedo-Vence, K., Dublán-García, O., López-Martínez, L. X., Morachis-Valdes, G., Galar-Martínez, M., Islas-Flores, H., Gómez-Oliván, L. M. 2015. Short and long-term exposure to diclofenac alter oxidative stress status in common carp *Cyprinus carpio*. Ecotoxicology, 24(3), 527–539. doi:10.1007/s10646-014-1401-9

Schieber, M., Chandel, N. S. 2014. ROS function in redox signaling and oxidative stress. Current Biology, 24:10, 453–R462. https://doi.org/10.1016/j.cub.2014.03.034

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H., Negele, R. D. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquatic toxicology. 68:2, 141–150. https://doi.org/10.1016/j.aquatox.2004.03.014

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y. 2002. Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany. 53:372, 1305–1319. https://doi.org/10.1093/jexbot/53.372.1305

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Metraux, J.P., Raskin, I. 1995. Salicylic Acid in Rice (Biosynthesis, Conjugation, and Possible Role). Plant Physiol. 108:2, 633-639.

Smirnoff, N. 2000. Ascorbic acid: metabolism and functions of a multi-facetted molecule. Current Opinion in Plant Biology, 3(3), 229–235.doi:10.1016/s1369-5266(00)80070-9.

Sofo, A., Scopa, A., Nuzzaci, M., Vitti, A. 2015. Ascorbate Peroxidase and Catalase Activities and Their Genetic Regulation in Plants Subjected to Drought and Salinity Stresses, Int. J. Mol. Sci. 16:6, 13561-13578. https://doi.org/10.3390/ijms160613561

Stancova, V., Plhalova, L., Blahova, J., Zivna, D., Bartoskova, M., Siroka, Z., Maršálek, P., Svobodova, Z. 2017. Effects of the pharmaceutical contaminants ibuprofen, diclofenac, and carbamazepine alone, and in combination, on oxidative stress parameters in early life stages of tench (*Tinca tinca*). Veterinární medicína. 68:02, 90–97. https://doi.org/10.17221/125/2016-VETMED

Stepanova, S., Praskova, E., Chromcova, L., Plhalova, L., Prokes, M., Blahova, J., Svobodova, Z. 2013. The effects of diclofenac on early life stages of common carp (*Cyprinus carpio*), 5, 454–460.

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., Li, L. 2018. Carotenoid Metabolism in Plants: The Role of Plastids. Molecular Plant, 11:1, 58–74. doi:10.1016/j.molp.2017.09.010

Szalai, G., Kellős, T., Galiba, G., Kocsy, G. 2009 Glutathione as an Antioxidant and Regulatory Molecule in Plants Under Abiotic Stress Conditions J Plant Growth Regul (2009) 28:1, 66-80. https://doi.org/10.1007/s00344-008-9075-2

Sytar, O., Kumar, A., Latowski, D., Kuczynska, P., Strzałka, K., Prasad, M. N. V. 2013. Heavy metal-induced oxidative damage, defence reactions and detoxification mechanisms in plants Acta Physiol. Plant., 35, 985-999

Tang, W., Stearns, R. A., Bandiera, S. M., Zhang, Y., Raab, C., Braun, M. P., Dean, D. C., Pang, J., Leung, K. H., Doss, G. A., Strauss, J. R., Kwei, G. Y., Rushmore, T. H., Chiu, S. H. L., Baillie, T. A. 1999. Studies on cytochrome P-450-mediated bioactivation of diclofenac in rats and in human hepatocytes: Identification of glutathione conjugated metabolites. Drug Metabolism Disposition 27: 365-372.

Taraldsen, J. E., Norberg-King, T. L. 1990. New method for determining effluent toxicity using duckweed (*Lemna minor*). Environ Toxicol Chem 9:6, 761–767. DOI: 10.1002/etc.5620090610

Ternes, T. A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32:11, 3245-3260.

Thomas, K.V., Hilton, M. J. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Marine Pollution Bulletin, 49, 436–444. https://doi.org/10.1016/j.marpolbul.2004.02.028

Tripathy, B. C., Oelmüller, R. 2012. Reactive oxygen species generation and signaling in plants. Plant Signal Behav. 7:12, 1621–1633. doi: 10.4161/psb.22455

Vannini, A., Paoli, L., Vichi, M., Bačkor, M., Bačkorová, M., Loppi, S. 2018. Toxicity of Diclofenac in the Fern *Azolla filiculoides* and the Lichen *Xanthoria parietina*. Bull Environ Contam Toxicol 100: 430. https://doi.org/10.1007/s00128-017-2266-4

Vieno, N., Sillanpää, M. 2014. Fate of diclofenac in municipal wastewater treatment plant — A review. Environment International. 69, 28-39. https://doi.org/10.1016/j.envint.2014.03.021

Vlot, A. C., Dempsey, D. A., Klessig, D. F. 2009. Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annual Review of Phytopathology, 47:1, 177–206. doi:10.1146/annurev.phyto.050908.1352

Wang, W. 1986. Toxicity Tests of Aquatic Pollutants by Using Common Duckweed. Environmental Pollution Series B, Chemical and Physical. 11:1, 1–14. https://doi.org/10.1016/0143-148X(86)90028-5

Wang, W. 1990. Literature Review on Duckweed Toxicity Testing. Environmental Research. 52:1, 7-22. https://doi.org/10.1016/S0013-9351(05)80147-1

Weigel, S., Kuhlmann, J., Huhnerfuss, H. 2002. Drugs and personal care products as ubiquitous pollutants: occurrence and distribution of clofibric acid, caffeine and DEET in the North Sea. Science of The Total Environment. 295:1–3, 131-141. https://doi.org/10.1016/S0048-9697(02)00064-5

Wolf, F. T. 1978. Effects of chemical agents in inhibition of chlorophyll synthesis and chloroplast development in higher plants. Bot Rev. 43:4, 395–424. https://doi.org/10.1007/BF02860841

Zait, Y., Stein, I., Schwartz, A. 2018. Long-term acclimation to different stress types: revealing tradeoffs between mesophyll and stomatal conductance. BioRxiv. 1-52. https://doi.org/10.1101/340786

Zhang, Y., Geißen, S. U., Gal, C. 2008. Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. Chemosphere, 73:8, 1151–1161. https://doi.org/10.1016/j.chemosphere.2008.07.086

Chapter 4

Can salicylic acid modulate biochemical, physiological and population alterations in a macrophyte species under chemical stress by diclofenac?

Alkimin GD, Soares AMVM, Barata C, Nunes B. 2020. Can salicylic acid modulate biochemical, physiological and population alterations in a macrophyte species under chemical stress by diclofenac?. Science of the total Environment. 139715. doi.org/10.1016/j.scitotenv.2020.139715

Can salicylic acid modulate biochemical, physiological and population alterations in a macrophyte species under chemical stress by diclofenac?

Alkimin GD[1*], Soares AMVM[1], Barata C[2], Nunes B[1],

[1] Centre for Environmental and Marine Studies (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

4.1 Abstract

Salicylic acid (SA) has been shown to affect plant resistance to biotic and abiotic factors. However, a knowledge gap exists between the possible effects of SA alone, mostly in aquatic plants, and its capacity to modulate response/resistance to other chemical stress posed by exposure to pharmaceutical drugs. Thus, the aim of this work was to investigate the capacity of SA of modulating *Lemna minor* responses co-exposed to the pharmaceutical drug, diclofenac - DCF. This assessment was performed by measuring biochemical, physiological and population endpoints. To attain this objective, *L. minor* was exposed for 7 days, to DCF (concentrations of 0.375, 0.75 and 1.5 mg/L), SA (4 µg/L) alone and in combination with SA (4 µg/L). After exposure, the analyzed endpoints were catalase (CAT) and glutathione S-transferases (GSTs) activities, pigments content (chlorophyll a (Chl a), b (Chl b) and total (TChl), carotenoids (Car) and [Chl a]/[Chl b] and [TChl]/[Car] ratios), and growth specific rate, fresh weight and root length. CAT activity was increased in plants exposed to 0.375 mg/L of DCF, and also in those exposed to a combination of 0.75 mg/L DCF + 1.5 mg/L SA. GSTs activity were significantly decreased in plants exposed to all DCF concentrations, but this effect was reverted after simultaneous exposure to SA, being the activity, in the concentration of 1.5 mg/L of DCF + SA, higher than the control organisms. Chlorophylls content were just affected in plants exposed to the highest

concentration of DCF, but Car content and Chl a and b ratio were not affected. However, Car and TChl ratio suffered a decrease when plants were simultaneously exposed to 1.5 mg/L DCF + SA. DCF alone was responsible for a decrease in growth rate, fresh weight and root length in plants exposed to all concentrations, however exposure to SA partially reverted these effects. In conclusion, we may suggest that SA is capable to prevent the toxicity of some compounds in macrophytes, by modulating the toxic response of exposed plants.

Keywords: *Lemna minor*; pharmaceuticals; pigments content; biochemical alterations; plant toxicology.

4.2 Introduction

In 2018, worldwide sales of pharmaceuticals totaled US$1,204.8 billion, this figure rising in the past ten years approximately in 50% (Statista, 2019). Such an enormous and global use of medication, their varying types, and inherent physicochemical properties of drugs significantly contribute to the release of active pharmaceutical ingredients and their metabolites into the environment, including surface waters (Rzymski et al., 2017), where they may exert biological effects. Due to direct discharge, presence and potential effects of these compounds in the environment, this issue has gained attention (Kosjek et al., 2005) and the European Union recognized pharmaceutical residues in the environment as “priority substances”, similarly to other micropollutants (EU, 2013). It is predicted that pharmaceutical pollution of water resources will be a key issue in the future protection of the environment, as well as for human health (Rzymski et al., 2017). This happens since pharmaceuticals are designed to alter physiological functions preferentially in mammalian species, and this is unlike to occur for most other chemicals entering the environment, where biological effects generally occur as an unintended consequence of their principal function (Corcoran et al., 2010).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed pharmaceutical compounds worldwide, being mostly used to tackle inflammation, to relieve pain and pain-related conditions, such as arthritis and other rheumatic diseases (Al-Khateeb et al., 2017), thereby being used in large quantities by humans and animals (Koumaki et al., 2017). According to data published by the

Organization for Economic Co-operation and Development (OECD), Portugal was the second country consuming more anti-inflammatory and anti-rheumatic non-steroidal drugs, in 2018, totalizing 71.7 defined daily dosage per 1000 inhabitants per day, just behind Iceland with 77 dosages (OECD, 2019).

Diclofenac - DCF (2-[(2,6-dichlorophenyl)amino] phenylacetic acid), is one of the most widely prescribed NSAIDs and it is poorly degraded in WWTP; consequently, it is a drug with high environmental detection rates and concentrations namely in aquatic environments (Balbi et al., 2018). This pharmaceutical has been shown to be toxic to different organisms, including zebrafish (*Danio rerio*; Xia et al., 2017), freshwater crustaceans (*Daphnia magna*; Nkoom et al., 2019), in rainbow trout (*Oncorhynchus mykiss*; Mehinto et al., 2010), three spined stickleback (*Gasterosteus aculeatus*; Näslund et al., 2017), mussels (*Mytilus galloprovincialis*; Gonzalez-Rey and Bebiano, 2014), shrimp (*Palaemon serratus*; González-Ortegón, 2013), marine clam (*Ruditapes philippinarum*; Trombini et al., 2019), polychaetas (*Hediste diversicolor*; Gomes et al., 2019), and also to plants such as fern (*Polystichum setiferum*; Feito et al., 2012), and maize (*Zea mays*; Hammad et al., 2018). Thus, this pharmaceutical was included in the Europe Commission Watch List, under the Water Framework Directive, to be monitored in EU surface waters (Loos et al., 2018). Its presence in an extremely short list of pharmaceutical drugs that deserve further monitoring and attention is a clear sign of its environmental significance. Its environmental presence range from 0.7 to almost 5000 ng/L, in rivers from countries such as France, Canada, Germany, Belgium, Slovenia, Finland, Spain, Pakistan and China (Hereber, 2002; Metcalfe et al., 2003; Scheurell et al., 2009; Lindqvist et al., 2005; Rabiet et al., 2006; López-Serna et al., 2013; Ma et al., 2016). DCF was also detected in groundwater in the Mediterranean region up to 2 ng/L (Rabiet et al., 2006), in UK estuary, and in Brazilian seawater, at concentrations of 195 and 19.4 ng/L respectively (Thomas and Hilton, 2004; Pereira et al., 2016); it was also present in groundwater and drinking water, at levels up to 10 ng/L (Hereber, 2002; López-Serna et al., 2013).

Salicylic acid (SA; 2-hydroxybenzoic acid), is the active metabolite of acetylsalicylic acid (ASA). SA is formed after in vivo hydrolysis in humans (James, 1958) and in other mammals, such as rats, dogs, monkeys and rabbits (Bahar and Imai, 2013). SA-conjugated forms are however, enzymatically metabolized in the aquatic environment

by mollusks, fish and β-glucuronidases of bacteria (Schomburg and Salzmann, 1991). SA is responsible for the pharmacological activity of ASA (Davison, 1971), that occurs through the inhibition of cyclooxygenases, which are involved in the production of the inflammatory mediators prostaglandins and thromboxanes (Brooks and Day, 1991). In addition, SA is one of many phenolic compounds that are synthesized by plants as a secondary metabolite (Dempsey and Klessing, 2017). It is physiologically of crucial importance, since it intervenes in key plant processes, including in the biosynthesis of lignin and of pigments (Metráux and Raskin, 1993). SA, for example, is a critical hormone that plays direct or indirect roles in regulating plant growth and development, as well as thermogenesis and disease resistance (Vlot et al., 2009). Its effects have already been clarified, and after being applied on the plants basil (*Ocimum basilicum* L.) and marjoram (*Majorana hortensis*). SA stimulated the growth and oil yield, by enhancing photosynthesis and nutrient uptake (Muthulakshmi et al., 2017). The use of SA can be a potential chemical strategy to ameliorate water deficit effects on *Eucalyptus globulus* (Jesus et al., 2015). In general, exogenous SA has been shown to affect resistance to pathogen-associated (biotic factor) and tolerance to salinity/osmotic stress, heavy metal, chilling, UV radiation, drought and others (abiotic factors) (Hayat et al., 2010; Kahn et al., 2015; Rivas-San Vicente et al., 2011).

However, the possible effects of SA, mostly in aquatic plants, and their capacity to modulate their response when plants are under chemical stress are still largely unknown. Thus, the aim of this work was to investigate the SA capacity to modulate *Lemna minor* responses when exposed to a pharmaceutical drug (DCF) by quantifying physiological (pigments content), biochemical and population endpoints.

4.3 Material and methods

4.3.1 Chemicals

All pharmaceutical drugs were purchased from Sigma Aldrich, with purities >98%: diclofenac sodium (CAS: 15307-79-6) and salicylic acid (sodium salt form; CAS 54-21-7) and all other chemicals used in this study have analytical purity and were obtained from Sigma Aldrich and Bio-Rad®.

4.3.2 Culture and maintenance of test organisms

Lemna minor were cultured and maintained at Department of Biology at Aveiro University according to OECD (2006) and modifications described in Alkimin et al. (2019b). Briefly, plants were reared under controlled conditions (light intensity, ~5500 lux; photoperiod 16hL:8hD, and temperature 20 ± 2 °C) in 1 L glass with approximately 200 mL of modified Steinberg medium, and the total medium was renewed twice a week.

4.3.3 Macrophytes – *Lemna minor*

The macrophyte species were exposed according OECD (2006) recommendations with adaptations. 6-well micro plates were filled with 10 mL of medium and/or stock solution, 2 independent trials were performed. For the first one, ten replicates per condition (with three fronds each) were used, and tests had a duration of 7 days. After the exposure period, root size, average growth specific rate (calculated based on the number of fronds) and the fresh weight (FW) were determined. Length roots were calculated by measuring, from each well, the longest root in three random plants, totalizing 30 roots per each experimental condition. The average specific growth rate was calculated according to OECD (2006) recommendations, and the FW was obtained by weighing the biomass (after being centrifuged for 2 min at 2000 g) from each well in an analytical balance (GH - 252). After that, the total biomass was placed in Eppendorf microtubes and stored at -80 ºC for the subsequent analysis - determination of biochemical endpoints (catalase (CAT), glutathione-S-transferases (GSTs) activities). The second test also involved ten replicates per experimental condition, in a 6-well microplate, being each well filled with 10 mL of medium and/or stock solution. Plants covered approximately 15% of each well, and the exposure had a duration of 7 days. After the exposure, the obtained biomass also was placed in Eppendorf microtubes, and stored at -80 ºC to the pigments analyzes.

For the performance of both tests, the nominal adopted DCF concentrations were based on the EC_{50} of this compound, previously calculated for the test species (7.5 mg/L; Cleuvers, 2003), to assure that plants were exposed to a stressful condition; this level

was divided by 20, 10 and 5, to calculate the remaining levels of DCF (0.375, 0.75 and 1.5 mg/L respectively). The adopted nominal SA concentration (4 µg/L) was based on already reported environmental concentrations that range between 0.855 to 4.1 µg/L (Claessens et al., 2013; Ternes, 1998). Thus, in addition to the described levels of DCF, the experimental conditions comprised a control group (just with modified Steinberg medium), a SA treatment alone (to analyze the isolated effects of this drug), and the combination of each DCF concentration plus 4 µg/L SA. Assays were conducted under controlled conditions (temperature 23 ± 1 °C; continuous light exposure; light intensity, ~84 µmol photons m^{-2} s^{-1}) (OECD, 2006) in a climate chamber (Binder). Test solutions were totally replaced every other day in an attempt to maintain the pharmaceuticals concentrations.

4.3.4 Pigments analysis (chlorophylls and carotenoids)

Total, a, and b chlorophyll (TChl; Chl a; Chl b) and carotenoids (Car) amounts were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979). Pigments were extracted from the previously exposed fronds of *Lemna* spp. (about 10 mg per replicate - fresh weight - FW) with 1.8 mL of dimethyl sulphoxide (DMSO). The extract was placed in water at 65 ºC during 30 min, and allowed to cool in the dark at room temperature. The obtained extracts were used to quantify the amounts of chlorophylls (a, b, and total), and carotenoids levels, through spectrophotometrical measurement of absorbance at the wavelengths of 470, 645, 646, and 663. The calculation of the pigments followed the equations proposed by Arnon (1949) and demonstrated by Hiscox and Israelstam (1979), suitable if the extraction was undertaken with DMSO, to calculate the amounts of Chl (a, b, and total), and Lichtenthaler (1987) to quantify the Car amount. In addition, the ratios [Chl a]/[Chl b] and [Car]/[TChl] were also calculated.

4.3.5 Tissue homogenization – biochemical markers

For the determination of CAT and GSTs activities, samples were macerated in ice-cold phosphate buffer (50 mM, pH 7.0) with 0.1% Triton X-100. Homogenized samples were then centrifuged at 15,000 g at 4 ºC for 10 min and before analysis.

4.3.5.1 Catalase activity determination

Catalase activity was determined in a 96-well microplate, by the procedure described by Aebi (1984), based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein.

4.3.5.2 Glutathione-S-transferases activity determination

The procedure to determine GSTs activity monitored the increment of absorbance at 340 nm of a thioether resulting from the catalysis of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GSTs, as described by Habig et al. (1974), adapted to a 96 well microplate. Results were expressed as millimoles of thioether produced per minute, per milligram of protein.

4.3.5.3 Protein determination

Protein quantification was performed at 595 nm using the Bradford method (Bradford 1976), adapted to microplate with bovine γ-globulin as standard (1mg/ml), in order to express enzymatic activities per mg of protein of the analyzed samples. Finally, all spectrophotometric readings were performed in a microplate reader Thermo Scientific Multiskan (ScanIt Software 2.4.4).

4.3.6 Statistical analysis

Data for all parameters were tested for homogeneity (Brown-Forsythe) and normality (Shapiro-Wilk). For all endpoints, the statistical analysis started by comparing the results obtained for both control and SA treatments (t-test), to verify if SA alone was responsible for any alteration. The second step involved a one-way analysis of variance (ANOVA), followed by post-hoc test (Dunnet's test-or equivalent non-parametric test) to determine differences in relation to the control treatment. Finally, data were compared (t-tests) to detect differences between pairs from each tested concentration (plants exposed to DCF alone, and plants exposed to the same DCF levels + SA). Statistical analysis was performed with SigmaPlot v.14.0. The adopted level of significance was 0.05.

4.4 Results

All analyzed endpoints presented no statistical difference between control plants, and those exposed to SA alone (4 µg/L). Consequently, all comparisons were performed with the control (pure modified Steinberg medium); all p values obtained from the Student t-test are presented in table 1.

Table 1. p values for Student's t-test between respective pairs.

	Control/SA	0.375/0.375+SA	0.75/0.75+SA	1.5/1.5+SA
CAT activity	0.07	0.072	**0.021**	**<0.001**
GSTs activity	0.353	0.438	**<0.001**	**<0.001**
Chl a	0.753	0.101	0.763	0.763
Chl b	0.763	0.123	0.442	**0.035**
TChl	0.742	0.099	0.715	**0.0137**
Car	0.552	0.106	0.096	0.0982
Chl a/b ratio	0.961	0.822	0.0905	0.481
Car/TChl ratio	0.274	0.699	0.157	0.582
Growth rate	0.563	**<0.001**	**<0.001**	**<0.001**
Weight	0.466	**<0.001**	**<0.001**	**<0.001**
Root size	0.824	**<0.001**	**<0.001**	**<0.001**

Diclofenac caused an increase in CAT activity in plants exposed to 0.375 mg/L ($F_{(7, 64)}$ = 13.931; $p<0.001$); however, when plants were exposed to DCF plus SA, the results were the opposite, and plants exposed to 0.375 mg/L did not evidence any effect. Plants

exposed to SA and to 0.75 and 1.5 mg/L of DCF, had their CAT activities increased (Figure 1A). CAT activity was generally higher in plants exposed to DCF+SA. GSTs activity was reduced after DCF exposure in plants exposed to all tested concentrations of this drug ($F_{(7, 37)}$ = 17.098; p<0.001). However, GSTs activities in plants exposed to 1.5 mg/L DCF plus SA, there were higher than those of control treatments. Again, in general terms, GSTs activity was higher when plants were exposed to the mixture DCF + SA (Figure 1B).

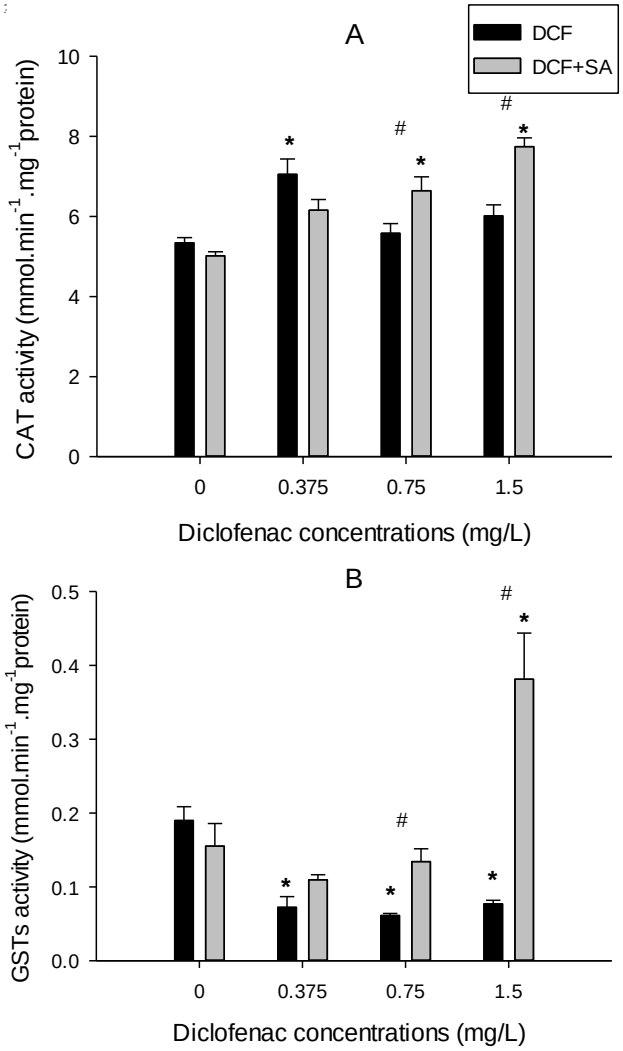

Figure 1. Biomarker responses (Mean ± SE, N=10) of *L. minor* exposed to diclofenac (DCF) and salicylic acid (SA) alone and to both DCF + salicylic acid (SA) A) catalase activity; B) glutathione S-transferases activity. * stands for statistical differences in relation to respective control following ANOVA and post-hoc test (or the equivalent non-parametric test). # stands for statistical differences between pairs of single DCF alone and of DCF plus SA following Student's t-test.

In terms of pigment content, Chl a were only significantly decreased in plants exposed to 1.5 mg/L DCF + SA ($F_{(7, 67)}$ = 3.582; p=0.003; figure 2A). Chlorophyll b content was

higher than control when plants were exposed to 1.5 mg/L of DCF ($F_{(7, 65)}$ = 4.432; $p<0.001$), and this same treatment presented significant ($p<0.05$) differences between pairs (figure 1B). Comparing to control, TChl content increased in plants exposed to 1.5 mg/L, decreased in plants exposed to this same concentration + SA ($F_{(7, 66)}$ = 4.938; $p<0.001$). This treatment showed also significant ($p<0.05$) differences between pairs (Figure 2C). No treatment was capable of causing alterations in Car content (H_{7df} = 11.414; p=0.122), no effects were also observed for the Chl a and b ratio ($F_{(7, 75)}$ = 1.832; p=0.095; Figure 1 C and D respectively). Finally, 1.5 mg/L of DCF + SA caused a decrease in Car and TChl ratio (H_{7df} = 23.492; p=0.001; Figure 2E).

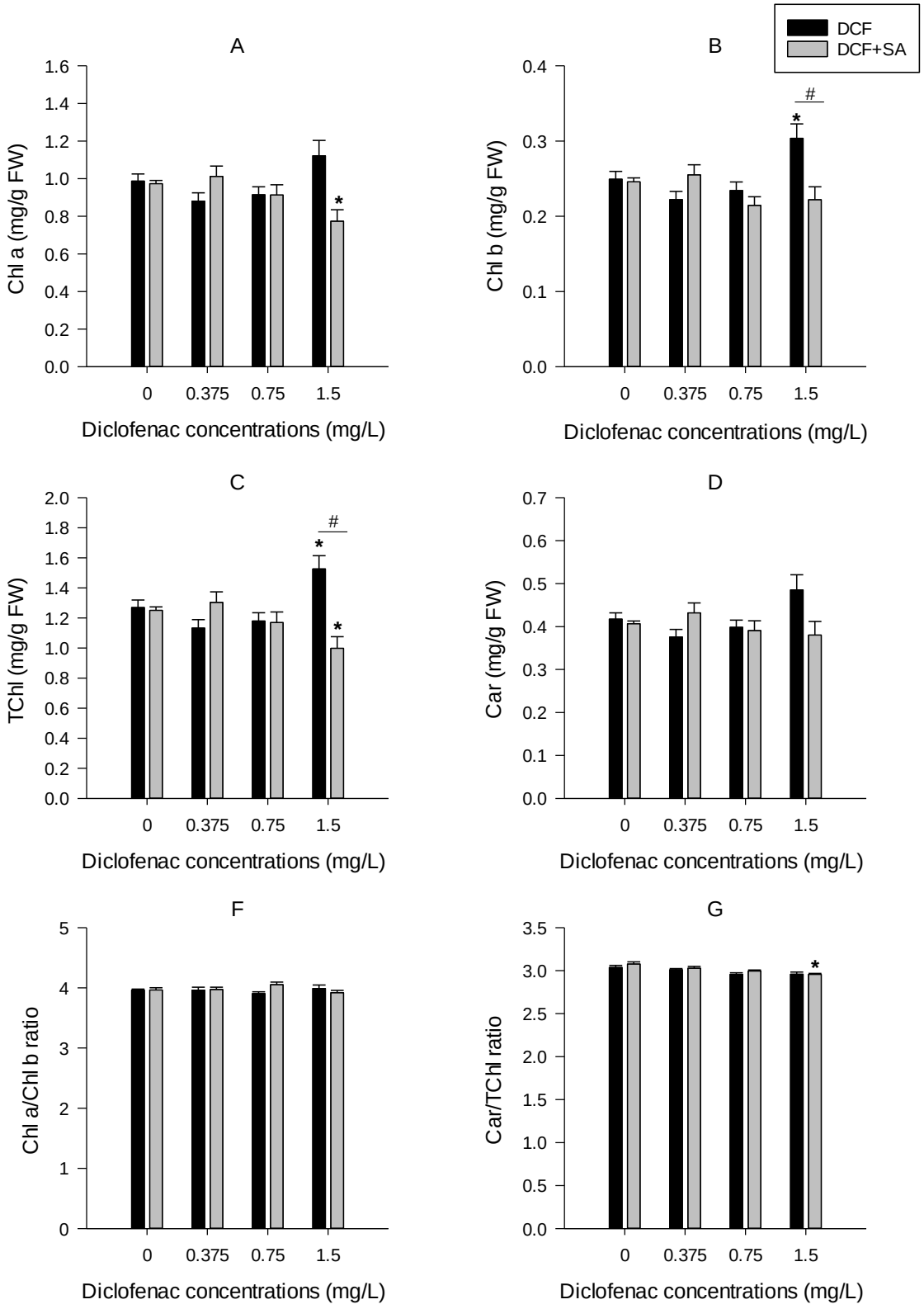

Figure 2 Responses of physiological parameters (pigments content) (Mean ± SE, N=10) of *L. minor* exposed to diclofenac (DCF) and salicylic acid (SA) alone and to both DCF + salicylic acid (SA). A) chlorophyll a content; B) chlorophyll b content; C) total chlorophyll content; D) carotenoids content; E) chlorophyll a and b ratio and F) carotenoids and total chlorophyll ratio. * stands for statistical differences

in relation to respective control following ANOVA and post-hoc test (or the equivalent non-parametric test). # stands for statistical differences between pairs of single DCF alone and of DCF plus SA following Student's t-test.

Average specific growth rate was lower than control in plants exposed to all DCF concentrations, and in plants exposed to 0.75 and 1.5mg/L DCF + SA ($F_{(7, 77)} = 76.259$; $p<0.001$); however, co-exposures of DCF with SA improved the growth rate (Figure 3A). The same pattern of response also was observed for fresh weight ($F_{(7, 69)} = 92.291$; $p<0.001$ – figure 3B) and for root length ($H_{7df} = 64.775$; $p<0.001$ – figure 3C)

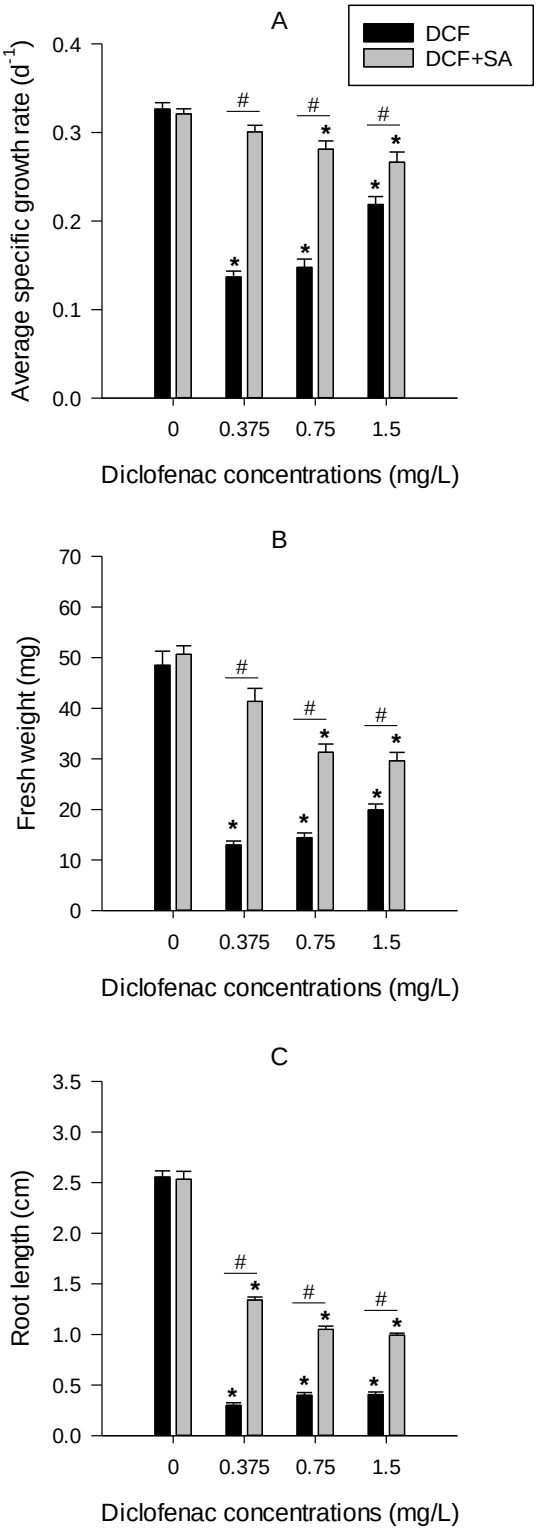

Figure 3. Response of populational and morphometric parameters (Mean ± SE, N=10) of *L. minor* exposed to diclofenac (DCF) and salicylic acid (SA) alone and to both DCF + salicylic acid (SA). A) growth rate (d^{-1}); B) fresh weight (mg) and C) Root length (cm). * stands for statistical differences in relation to respective control following ANOVA and post-hoc test (or the equivalent non-parametric test). # stands for statistical differences between pairs of single DCF alone and of DCF plus SA following Student's t-test.

4.5 Discussion

Abiotic stresses can potentially influence almost all physiological, biochemical, and molecular processes in plants, from the early stage of seed germination to maturity; these effects may eventually cause severe losses in the economic yield of crop plants, especially for terrestrial plants (Khan et al., 2015). If aquatic species are considered, these same effects can cause disturbances in environmental equilibria (Vaughn, 2010). Metabolic pathways that occur in plant organelles are sensitive to changes in environmental conditions, and metabolic imbalances can induce oxidative stress in cells, by promoting the generation and accumulation of reactive oxygen species (ROS), possible causing oxidation of cellular components, hindering metabolic activities and affecting organelle integrity (Suzuki et al., 2011). As a defense against these possible damage, organisms, in general, trigger the antioxidant defense system; this system in plants is mainly constituted by enzymes such as CAT, ascorbate peroxidase (APX), superoxide dismutase (SOD), being these enzymes the major components of this pathway (Bailey-Serres and Mittler, 2006). However, other mechanisms may be also present in plants, including the non-enzymatic pathway, composed by pigments and vitamins, such as Car and α-Tocopherol, respectively. The effectiveness of the antioxidant response in plants depends upon these two components to scavenge ROS (Das and Roychoudhury, 2014).

Our results showed that DCF application, despite having increased CAT activity in plants exposed to all tested concentrations, only caused a significant effect in organisms exposed to the lowest tested level (0.375 mg/L). A possible explanation for this absence of significant effects when plants were exposed to higher levels may reside in a more comprehensive response that may be triggered by such highly challenging conditions. If such defense mechanisms are activated, catalase may be redundant. High levels of DCF, in plants, are known to trigger other antioxidant mechanisms such as SOD, APX and other enzymes (Bartha et al., 2014; Kummerová et al., 2016; Pierattini et al., 2018). SOD is the first barrier in the antioxidant defense system of plants, to cope with ROS generated in pro-oxidative stressful conditions. APX is a widely distributed enzyme, and has a higher affinity for H_2O_2 than CAT; therefore, it is a more efficient scavenger of H_2O_2 when plants are subjected to higher stress (Mittler, 2002; Jaleel et al., 2009; Sharma et al., 2012; Racchi, 2013; Das and Roychoudhury, 2014; Caverszan et al.,

2016). In addition, APX is related with the fine modulation of ROS scavenger activity in plants (Mittler, 2002) and also can be triggered by DCF exposure (Alkimin et al., 2019a). Other defensive mechanisms rely on enzymes such as monodehydroascorbate reductase (MDHAR) that are directly related with the APX activity (Apel and Hirt, 2004). Additional defenses that may be triggered in plants to face oxidative stress include guaiacol peroxidase, which is intracellularly active, but also occurs in the cell wall and extracellularly, being considered as the key enzyme in the removal of H_2O_2 (Das and Roychoudhury, 2014). The combination of these responses facilitates the overall ROS scavenging, thereby modulating the CAT activity. This seems to have been the case of the here-exposed plants. This complex network of antioxidant defenses is evolutionary conserved in plants, and seems to also exist in aquatic species (Sharma and Kaur, 2019). In this case, the efficacy of these defensive mechanisms may have been improved, rendering CAT activity redundant.

On the other hand, plants exposed to higher DCF concentrations (0.75 and 1.5 mg/L) + SA enhanced their CAT activity. This effect agrees with findings of Hayat et al. (2010). The previous authors showed that exogenous application of SA enhanced the activities of antioxidants enzymes. A similar effect was also reported after the application of SA to plants, under stressful conditions (e.g. water deficit). Administration of SA was capable to increase the generation of ROS in leaves of *Arabidopsis thaliana* thus enhancing the effectiveness of the antioxidant system, to prevent ROS mediated tissue damages (Borsani et al., 2001). In addition, the activities of antioxidant enzymes, namely CAT, peroxidase (POX) and SOD, were enhanced when drought stressed plants of the species *Licopersicon esculentum* were sprayed with exogenous SA (Hayat et al., 2008). A similar effect was attained after spraying salinity stressed plants of *Brassica juncea* with SA (Yusuf et al., 2008). The use of molecular approaches has revealed a number of detoxifying and antioxidant genes coding for proteins/enzymes that play crucial roles in defense responses that were upregulated after SA application (Holuigue et al. 2007), being this a strong hypothesis to explain the partial reversion that were here observed. However, the physiological and biochemical mechanisms for SA-induced tolerance are not clearly understood. It has been suggested that SA triggers a cascade of events to provide multiple stress tolerance in plants (Senaratna et al., 2000). Nevertheless Taalat (2019) suggested that SA -ROS interactions are complex.

GSTs are ubiquitous in aerobic organisms and catalyze the conjugation of toxic compounds with glutathione to form non-toxic peptide derivatives. In plants, GSTs have been well studied with respect to their ability to detoxify xenobiotics, with the most observed conjugations involving substitution reactions, through reduced glutathione (GSH) conjugation (Dixon et al., 1998). Bartha (2012) suggested that when plants are subjects to extremely stressful conditions, namely to higher xenobiotic concentrations, plants may trigger more complex systems of defense/xenobiotic biotransformation, not being necessary the activation of GSTs. This assumption is in line with the here-observed data, since a significant decrease in the GSTs activity was reported in DCF-exposed plants. In addition, ROS can induce damages to DNA and RNA, membrane lipid peroxidation, protein oxidation/denaturation with consequent enzyme inhibition (Mittler, 2002; Apel and Hiter, 2004), thus, decreasing the GSTs activity (Galati et al., 2002; Guiloski et al., 2017; Stancova et al., 2017; Owumi and Dim, 2019). SA application caused an increase in the GSTs activity, improving the biotransformation system functionality. Chlorophylls are responsible for capturing light energy, converting it into chemical energy; they rarely occur outside of photosynthetic tissue (Simon, 1997). The numbers of naturally occurring chlorophylls may not yet be fully known (Pareek et al., 2018) and the photosystems in photosynthetic organisms are mainly constituted by chlorophyll a (Chl a) as the major pigment, and of chlorophyll b (Chl b) as an accessory pigment (Lichtenthaler, 1987). Chlorophyll a is present in the light-harvesting antenna pigment complexes and in the reaction center complexes, and functions as an accessory pigment, as well as the primary electron donor in the reaction centers of photosystems I and II (PSI and PSII). Chlorophyll b is present in the light-harvesting antenna complexes, preferentially in the major antenna of PSII, and is absent in the reaction center (Grimm, 2001). In this study, DCF at the highest concentration (1.5 mg/L) + SA caused a decrease in Chl a content. This is an important finding, especially if ones take into account that this same treatment was responsible for the higher enhancement of both CAT and GSTs activities. These data may indicate that, at higher levels of DCF, a high amount of ROS must have occurred, being potentially associated to the here observed decrease of Chl a. This suggestion is supported by the data obtained by Aarti et al. (2006) that demonstrated that in cucumber, oxidative stress generated by ROS accumulation, prevented key steps in the process of chlorophyll biosynthesis, by either directly or indirectly inhibiting the activity of the required

enzymes (Foyer, 2018). However, further studies are necessary to elucidate which step of the biosynthetic pathways is compromised by the NSAID(s), especially DCF. In addition, in this endpoint, the DCF-SA interaction was deleterious to the plant. This effect is of significant importance, since it suggests that exposure for long periods to ecologically realistic conditions, with the co-occurrence of several xenobiotics, may affect the photosynthesis machinery and interfere with plant energy conversion.

There is only a minor difference between the structures of Chl a and Chl b: in the latter, a-CHO group is found in place of CH_3 at the C-7 position (Pareek et al., 2018), being Chl b synthetized from Chl a, with the intervention of chlorophyllide a oxygenase (CAO; Espineda et al., 1999). In fact, previous data show that the biosynthesis of Chl b is regulated by the stability of this enzyme (Masuda and Fujita 2008). It is thus possible to suggest that high concentrations of DCF may interfere with this pathway of Chl b synthesis, by altering the activity of CAO, since the higher DCF concentration was capable to increase que Chl b amount in exposed plants. Despite the general absence of ecotoxicological data supporting this possibility, studies on plant physiology may indicate that this assumption is likely to occur. Espineda et al. (1999) showed the relationship between Chl b deficiency in *Arabidopsis* sp. with impairment of the CAO gene, and Hirashima et al. (2006) demonstrated that overexpression of cyanobacterial CAO lacking the A domain caused the substantial accumulation of Chl b in *Arabidopsis* sp.. However, despite the here presented results, the Chl a and b ratio was not significantly changed, showing that individually observed alterations were not extensive enough to cause any alteration in these pigments ratio. On the other hand, these same results, concerning Chl a and b can support the observed trend for the TChl content. TChl was increased in plants exposed to the highest DCF concentration and decreased in plants only exposed to the same concentration of DFC + SA.

Carotenoids, and their oxidative and enzymatic cleavage products, are crucial for several biological processes in plants, namely, for the assembly of photosystems and light harvesting antenna complexes, for photosynthesis and photoprotection, and for the regulation of growth and development (Hauvax, 2013; Simon, 1997). The levels of these piugments, remained generally unchanged after exposures to DCF, and to DCF + SA, leading to hypothesize that, in these conditions, the antioxidant system was capable of coping with the generated stress, being not necessary the alteration of the natural

levels of this accessory antioxidant. However, the combination of the higher DCF concentration and SA was capable do induce a decrease in TChl and Car ratio. These data indicate that this treatment can be deleterious to the plants, since this ratio is a good indicator of stress in plants and reflect the degree of adaptation of plants to environmental adverse conditions (Hendry and Price, 1993).

Phenotypic variation in plants is often observed and is considered a functional response that can maximize plant fitness in different and variable environments (Mishra, 2019). The roots are the first contact of macrophytes with dissolved contaminants in the water, and root structure can reflect adaptations of plant to the different habitats and conditions, including chemical contamination (Taub and Goldenberg, 1996). In this study, it was observed that DCF was capable of causing a decrease in the root length in plants exposed to all tested concentrations. Two hypothesis may occur due to two causes; first, the high ability of the roots to uptake DCF, being the pharmaceutical detected around 22% of initial exposure concentration in this organ in *Typha latifolia* and the probably oxidative stress suffered by the roots after DCF exposure, confirmed by the increase in 250% of peroxidase after 7 d of exposure (Bartha et al., 2014). In addition, these results can indicate that macrophyte species can be more sensitive to DCF than soil plant species. Ziolkowska et al. (2014) recorded an inhibition of root lengths of three leguminous plants after being exposed tolevels of 17 mg/L (and higher) DCF in soil. On the other hand, SA was able to revert (at least partially) the effects of DCF on this parameter, root length, in all tested concentrations. This effect is not completely described, however, Echevarria-Machado et al. (2007) suggested that SA promotes lateral root initiation, emergence, and growth, possibly due to the effects of cytokinins or auxin. These are phytohormones related with cell division and consequently with plant growth and development and, in general, they work to coordinate these functions.

Similar to here observed, and in agreement with our data, Kummerová et al. (2016), also detected a decrease in the *L. minor* growth after exposure to 100 µg/L of DCF during 10 days, concentration almost four times lower than the lowest concentration used in this study. This trend suggests that DCF is highly capable of causing alterations in macrophyte species. The effects of exogenous SA on growth is controversial, since it depends on the plant species, developmental stage, and also on the tested SA

concentrations (Rivas San-Vicente and Plasencia, 2011). In addition, the SA application was capable to modulate the toxic response to DCF, since plants simultaneously co-exposed to DCF + SA presented higher growth rate than those exposed only to DCF. According to Koo et al. (2020), in general, high levels of SA negatively affect plant development and growth; nevertheless, the application of optimal concentrations of SA showed beneficial effects on these features. The authors still conclude that depending on the experimental conditions, SA distinctly stimulated growth under both normal and different abiotic stress conditions, in different plant species. However, the plants species dealt with in this review were only of terrestrial habitats. Concerning aquatic plants, there is still a huge gap in this field of knowledge. In addition, the here observed results for fresh weight can be a consequence of the other two morphometrical parameters already discussed (root length and growth rate), since the variation of fresh weight followed the same pattern that was described, i.e. a decrease in plants exposed to all DCF concentrations, and an partial reversion of these effects with SA application. This effect confirmed, in another morphometrical parameter, the positive influence of an exogenous SA application, as well as its ability to modulate toxic responses. In general, the reason for the occurrence of some of these processes may be indirect, because SA modulates the synthesis and/or signaling of other hormones such as jasmonic acid, ethylene, and auxin (Yusuf et al., 2013) that are directly involved in plant growth and development.

Finally, considering that the environmental concentrations of DCF are well below that those here tested, it is possible to hypothesize that under likely environmental conditions, SA is capable of conferring protective effects in *L. minor*, namely against this stressor, by modulating the type, nature and extent to the toxic responses. In addition, besides the exposure to ecologically unrealistic DCF concentrations, these results serve as a mechanistic insight about the ability of SA to act as a response modulator when in co-exposure with other contaminants, in plants. Moreover, the here presented mechanistic interaction is a warning and a justification for the difficulty of evaluating effects in more complex matrices, such as the aquatic environment, where several substances coexist. However, the SA protective role and/or modulation of the toxicological response against pharmaceutical compounds in plants (especially aquatic species) has been neglected by the scientific community. The crosstalk between

chemicals such as SA and environmental contaminants has been generally unaddressed, and this conclusion was made possible only by analyzing the number of review articles, published along time. In general, the already published studies are limited to terrestrial plants with commercial interest; in addition, the considered factors include metal stress, heat, chilling, drought and UV-B radiation (Horváth et al., 2007; Hayat et al., 2010; Das and Roychoudhury, 2014; Khan et al., 2015; Koo et al., 2020; Pál et al., 2013), excluding chemical contamination by drugs from this arena.

4.6 Conclusion

In general, parameters such as growth rate, fresh weight, and root length were the most affected by DCF application, being however the most responsive to SA application. Following the results, the biochemical traits also were affected, namely the antioxidant system, since CAT activity was more affected by DCF+SA, than DCF alone. On the other hand, biotransformation system (GSTs activity) was largely affected by DCF, but reverted by SA application. Lastly, the pigments were the less affected parameter by DCF exposure and/or SA application. Finally, in all analyzed organizational levels (biochemical, physiological and population) the SA application was capable to ameliorate (and in some cases even revert) the toxic DCF effects on *L. minor*, having these analyzed parameters returned to the basal values determined in control organisms. Thus, it is extremely important that this research subject gains further attention in the future among the scientific community, to fully understand how SA acts when interacting with exogenous organic compounds, namely pharmaceutical drugs in aquatic plants.

4.7 References

Aarti, P. D., Tanaka, R., Tanaka, A. 2006. Effects of oxidative stress on chlorophyll biosynthesis in cucumber (*Cucumis sativus*) cotyledons. Physiologia Plantarum, 128(1), 186–197. doi:10.1111/j.1399-3054.2006.00720.x

Aebi, H (1984) Catalase in vitro. Methods in Enzymology, 6. 105-121

Al-Khateeb, L. A., Hakami, W., Salam, M. A. 2017. Removal of non-steroidal anti-inflammatory drugs from water using high surface area nanographene: Kinetic and thermodynamic studies. Journal of Molecular Liquids, 241, 733–741. doi:10.1016/j.molliq.2017.06.068

Alkimin, G. D., Daniel, D., Dionísio, R., Soares, A. M. V. M., Barata, C., Nunes, B. 2019a. Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is time a factor? Environmental Research, 177, 108609. doi:10.1016/j.envres.2019.108609

Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M., Barata, C., Nunes, B. 2019b. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Sci Total Environ 657, 926–937

Apel, K., Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annual Review of Plant Physiology and Plant Molecular Biology 55:373–399.

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant physiology, 24, 1-15.

Bahar, F. G., Imai, T. 2013. Aspirin Hydrolysis in Human and Experimental Animal Plasma and the Effect of Metal Cations on Hydrolase Activities. Drug Metabolism and Disposition, 41(7), 1450–1456. doi:10.1124/dmd.113.051805

Bailey-Serres, J., Mittler, R. 2006. The Roles of Reactive Oxygen Species in Plant Cells. PLANT PHYSIOLOGY, 141(2), 311–311. doi:10.1104/pp.104.900191

Balbi, T., Montagna, M., Fabbri, R., Carbone, C., Franzellitti, S., Fabbri, E., Canesi, L. 2018. Diclofenac affects early embryo development in the marine bivalve *Mytilus galloprovincialis*. Science of The Total Environment, 642, 601–609. doi:10.1016/j.scitotenv.2018.06.125

Bartha, B. 2012. Uptake and Metabolism of Human Pharmaceuticals in Plants. Identification of Metabolites and Specification of the Defense Enzyme Systems

under Pharmaceutical Exposure (2012) (PhD thesis. Technische Universität München. Lehrstuhl für Ökologischen Landbau und Pflanzenbausysteme)

Bartha, B., Huber, C., Schröder, P. 2014. Uptake and metabolism of diclofenac in Typha latifolia – How plants cope with human pharmaceutical pollution. Plant Science, 227, 12–20. doi:10.1016/j.plantsci.2014.06.001

Borsani, O., Valpuesta, V., Botella, M. A. 2001. Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. Plant Physiology, 126, 1024–1030.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Brooks, P. M., Day, R. O. 1991. Nonsteroidal Antiinflammatory Drugs — Differences and Similarities. New England Journal of Medicine, 324(24), 1716–1725. doi:10.1056/nejm199106133242407

Caverzan, A., Casassola, A., Patussi Brammer, S. 2016. Reactive Oxygen Species and Antioxidant Enzymes Involved in Plant Tolerance to Stress. Abiotic and Biotic Stress in Plants - Recent Advances and Future Perspectives. doi:10.5772/61368

Claessens, M., Vanhaecke, L., Wille, K., Janssen, C.R. 2013. Emerging contaminants in Belgian marine waters: single toxicant and mixture risks of pharmaceuticals Mar. Pollut. Bull., 71 (1–2), 41-50

Cleuvers, M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology Letters, 142(3), 185–194. doi:10.1016/s0378-4274(03)00068-7

Corcoran, J., Winter, M. J., Tyler, C. R. 2010. Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. Critical Reviews in Toxicology, 40(4), 287–304. doi:10.3109/10408440903373590

Csiszár, J., Horváth, E., Váry, Z., Gallé,Á., Bela, K., Brunner, S., et al. 2014. Glutathione transferase supergene family in tomato: salt stress-regulated

expression of representative genes from distinct GST classes in plants primed with salicylic acid. Plant Physiol. Biochem. 78, 15–26. doi: 10.1016/j.plaphy.2014.02.010

Das, K., Roychoudhury, A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Environmental Science, 2. doi:10.3389/fenvs.2014.00053

Davison, C. 1971. Salicylate metabolism in man. Ann N Y Acad Sci, 179:249–68

Dempsey, D. A., Klessig, D. F. 2017. How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans? BMC Biology, 15(1). doi:10.1186/s12915-017-0364-8

Dixon, D. P., Cummins, I., Cole, D. J., Edwards, R. 1998. Glutathione-mediated detoxification systems in plants. Current Opinion in Plant Biology, 1(3), 258–266. doi:10.1016/s1369-5266(98)80114-3

Espineda, C. E., Linford, A. S., Devine, D., Brusslan, J. A. 1999. The AtCAO gene, encoding chlorophyll a oxygenase is required for chlorophyll b synthesis in Arabidopsis thaliana. Proc Nat Acad Sci USA, 96(18):10507–10511

EU - European Union. 2013. Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy Offic. J. Eur. Union L226: 1–17.

Feito, R., Valcárcel, Y., Catalá, M. 2011. Biomarker assessment of toxicity with miniaturised bioassays: diclofenac as a case study. Ecotoxicology, 21(1), 289–296. doi:10.1007/s10646-011-0790-2

Foyer, C. H. 2018. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Environmental and Experimental Botany, 154, 134–142. doi:10.1016/j.envexpbot.2018.05.003

Galati, G., Tafazoli, S., Sabzevari, O., Chan, T. S., O'Brien, P. J. 2002. Idiosyncratic NSAID drug induced oxidative stress. Chemico-Biological Interactions, 142(1-2), 25–41. doi:10.1016/s0009-2797(02)00052-2

Gomes, A., Correia, A. T., Nunes, B. 2019. Worms on drugs: ecotoxicological effects of acetylsalicylic acid on the Polychaeta species *Hediste diversicolor* in terms of biochemical and histological alterations. Environmental Science and Pollution Research. doi:10.1007/s11356-019-04880-1

González-Ortegón, E., Blasco, J., Le Vay, L., Giménez, L. 2013. A multiple stressor approach to study the toxicity and sub-lethal effects of pharmaceutical compounds on the larval development of a marine invertebrate. Journal of Hazardous Materials, 263, 233–238. doi:10.1016/j.jhazmat.2013.09.041

Gonzalez-Rey, M., Bebianno, M. J. 2014. Effects of non-steroidal anti-inflammatory drug (NSAID) diclofenac exposure in mussel *Mytilus galloprovincialis*. Aquatic Toxicology, 148, 221–230. doi:10.1016/j.aquatox.2014.01.011

Grimm, B. 2001. Chlorophyll: Structure and Function. Encyclopedia of Life Sciences. doi:10.1038/npg.els.0001310

Guiloski, I. C., Stein Piancini, L. D., Dagostim, A. C., de Morais Calado, S. L., Fávaro, L. F., Boschen, S. L. et al. 2017. Effects of environmentally relevant concentrations of the anti-inflammatory drug diclofenac in freshwater fish *Rhamdia quelen*. Ecotoxicology and Environmental Safety, 139, 291–300.

Habig, W. H., Pabst, M. J., Jakoby, W. B. 1974. Glutathione S-transferases—the first enzymatic step in mercapturic acid formation. J Biol Chem. 249, 7130–7139.

Hammad, H. M., Zia, F., Bakhat, H. F., Fahad, S., Ashraf, M. R., Wilkerson, C. J., … Shahid, M. 2018. Uptake and toxicological effects of pharmaceutical active compounds on maize. Agriculture, Ecosystems & Environment, 258, 143–148. doi:10.1016/j.agee.2018.02.022

Havaux, M. 2013. Carotenoid oxidation products as stress signals in plants. The Plant Journal, 79(4), 597–606. doi:10.1111/tpj.12386

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. 2010. Effect of exogenous salicylic acid under changing environment: a review. Environ Exp Bot. 68:14–25.

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. 2010. Effect of exogenous salicylic acid under changing environment: A review. Environmental and Experimental Botany, 68(1), 14–25. doi:10.1016/j.envexpbot.2009.08.005

Hayat, Q., Hayat, S., Irfan, M., Ahmad, A. 2010. Effect of exogenous salicylic acid under changing environment: A review. Environmental and Experimental Botany, 68(1), 14–25. doi:10.1016/j.envexpbot.2009.08.005

Hayat, S., Hasan, S.A., Fariduddin, Q., Ahmad, A. 2008. Growth of tomato (*Lycopersicon esculentum*) in response to salicylic acid under water stress J. Plant Int., 3:(4), 297-304

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicology Letters, 131(1-2), 5–17. doi:10.1016/s0378-4274(02)00041-3

Hendry, G. A. F., Price, A. H. 1993. Stress indicators: chlorophylls and carotenoids. In: Hendry, G.A.F., Grime, J.P. (Eds.), Methods in Comparative Plant Ecology. Chapman & Hall, London, pp. 148–152

Hirashima, M., Satoh, S., Tanaka, R., Tanaka, A. 2006. Pigment shuffling in antenna systems achieved by expressing prokaryotic chlorophyllide a oxygenase in Arabidopsis, J. Biol. Chem., 281, 15385–15393.

Hiscox, J. D., Israelstam, G. F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can J. Bot. 57, 1332-1334.

Jaleel, C.A., Riadh, K., Gopi, R. et al. 2009. Antioxidant defense responses: physiological plasticity in higher plants under abiotic constraints. Acta Physiol Plant 31, 427–436. https://doi.org/10.1007/s11738-009-

James, K. C. 1958. The Hydrolysis Of Acetylsalicylic Acid From Aqueous Suspension. Journal of Pharmacy and Pharmacology, 10(1), 363–369. doi:10.1111/j.2042-7158.1958.tb10316.x

Jesus, C., Meijón, M., Monteiro, P., Correia, B., Amaral, J., Escandón, M., … Pinto, G. 2015. Salicylic acid application modulates physiological and hormonal changes in *Eucalyptus globulus* under water deficit. Environmental and Experimental Botany, 118, 56–66. doi:10.1016/j.envexpbot.2015.06.004

Kahn, M. I. R., Fatma, M., Per, T. S., Anjum, N. A., Kahn, N. A. 2015. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. Front Plant Sci. 6:462. doi:10.3389/fpls.2015.00462.

Khan, M. I. R., Fatma, M., Per, T. S., Anjum, N. A., Khan, N. A. 2015. Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants. Frontiers in Plant Science, 6. doi:10.3389/fpls.2015.00462

Koo, Y. M., Heo, A. Y., Choi, H. W. 2020. Salicylic Acid as a Safe Plant Protector and Growth Regulator. The plant pathology journal, 36(1), 1–10. https://doi.org/10.5423/PPJ.RW.12.2019.0295

Kosjek, T., Heath, E., Krbavcic, A. 2005. Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples. Environment International, 31(5), 679–685. doi:10.1016/j.envint.2004.12.001

Koumaki, E., Mamais, D., Noutsopoulos, C. 2017. Environmental fate of non-steroidal anti-inflammatory drugs in river water/sediment systems. Journal of Hazardous Materials, 323, 233–241. doi:10.1016/j.jhazmat.2016.03.026

Kummerová, M., Zezulka, Š., Babula, P., Tříska, J. 2016. Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant Lemna minor. Journal of Hazardous Materials, 302, 351–361. doi:10.1016/j.jhazmat.2015.09.057

Lichtenthaler, H. K. 1987. Chlorophylls and Carotenoids Pigments of Photosynthetic Biomembranes. Methods in Enzymology. 148, 350-382.

Lichtenthaler, H. K. 1987. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Plant Cell Membranes, 350–382. doi:10.1016/0076-6879(87)48036-1

Lindqvist, N., Tuhkanen, T., Kronberg, L. 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Research, 39(11), 2219–2228. doi:10.1016/j.watres.2005.04.003

Loos, R., Marinov, D., Sanseverino, I., Napierska, D., Lettieri, T. 2018. Review of the 1st Watch List under the Water Framework Directive and recommendations for the 2nd Watch List, EUR 29173 EN, Publications Office of the European Union, Luxembourg, ISBN 978-92-79- 81839-4, doi:10.2760/614367, JRC111198

López-Serna, R., Jurado, A., Vázquez-Suñé, E., Carrera, J., Petrović, M., Barceló, D. 2013. Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. Environmental Pollution, 174, 305–315. doi:10.1016/j.envpol.2012.11.022

Ma, R., Wang, B., Lu, S., Zhang, Y., Yin, L., Huang, J., … Yu, G. 2016. Characterization of pharmaceutically active compounds in Dongting Lake, China: Occurrence, chiral profiling and environmental risk. Science of The Total Environment, 557-558, 268–275. doi:10.1016/j.scitotenv.2016.03.053

Mariana Rivas-San Vicente, Javier Plasencia, Salicylic acid beyond defence: its role in plant growth and development, *Journal of Experimental Botany*, Volume 62, Issue 10, June 2011, Pages 3321–3338, https://doi.org/10.1093/jxb/err031

Masuda, T., Fujita, Y. 2008. Regulation and evolution of chlorophyll metabolism, Photochemical and Photobiological Sciences, 7, 1131–1149. https://doi.org/10.1039/b807210h

Mehinto, A. C., Hill, E. M., Tyler, C. R. 2010. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*) Environ. Sci. Technol., 44,2176-2182

Metcalfe, C. D., Miao, X.-S., Koenig, B. G., Struger, J. 2003. Distribution Of Acidic And Neutral Drugs In Surface Waters Near Sewage Treatment Plants In The Lower Great Lakes, Canada. Environmental Toxicology and Chemistry, 22(12), 2881. doi:10.1897/02-627

Métraux, J. P., Raskin. I. 1993. Role of phenolics in plant disease resistance. In: Chet I, editor. Biotechnology in plant disease control. New York: Wiley, p. 191–209.

Mishra, A. 2019. Morphological and Physiological Root Plasticity and Its Relationships with Shoot Growth of Rice with Water Regimes and Microbial Densities, Root Biology - Growth, Physiology, and Functions, Takuji Ohyama, IntechOpen, DOI: 10.5772/intechopen.87099.

Muthulakshmi, S., Lingakumar, K. 2017. Role of salicylic acid (SA) in plants – A review. International Journal of Applied Research. 3(3): 33-37

Näslund, J., Fick, J., Asker, N., Ekman, E., Larsson, D. G. J., Norrgren, L. 2017. Diclofenac affects kidney histology in the three-spined stickleback (*Gasterosteus aculeatus*) at low µg/L concentrations. Aquatic Toxicology, 189, 87–96. doi:10.1016/j.aquatox.2017.05.017

Nkoom, M., Lu, G., Liu, J., Dong, H., Yang, H. 2019. Bioconcentration, behavioral, and biochemical effects of the non-steroidal anti-inflammatory drug diclofenac in *Daphnia magna*. Environmental Science and Pollution Research. doi:10.1007/s11356-018-04072-3

Nunes, B. 2019. Acute ecotoxicological effects of salicylic acid on the Polychaeta species *Hediste diversicolor*: evidences of low to moderate pro-oxidative effects. Environ Sci Pollut Res 26, 7873–7882. https://doi.org/10.1007/s11356-018-04085-y

OECD - Organisation for Economic Co-operation and Development. 2019. Pharmaceutical Market. Available: <https://stats.oecd.org/Index.aspx?DataSetCode=HEALTH_PHMC>.

OECD – Organisation for Economic Co-operation and Development. 2006. Lemna sp. growth inhibition test n. 221. OECD–Guideline for Testing of Chemicals, Paris, 22 p.

Owumi, S. E., Dim, U. J. 2019. Biochemical alterations in diclofenac-treated rats: Effect of selenium on oxidative stress, inflammation, and hematological

changes. Toxicology Research and Application, 3, 239784731987435. doi:10.1177/2397847319874359

Pál, M., Szalai, G., Kovács, V., Gondor, O. K., Janda, T. 2013. Salicylic Acid-Mediated Abiotic Stress Tolerance. Salicylic Acid: Plant Growth and Development, 183–247. doi:10.1007/978-94-007-6428-6_10

Pareek, S., Sagar, N. A., Sharma, S., Kumar, V., Agarwal, T., González-Aguiar, G. A., Yahia, E. M. 2018. Chlorophylls: Chemistry and Biological Functions. Fruit and Vegetable Phytochemicals: Chemistry and Human Health Edition: Second Publisher: Wiley-Blackwell, John Wiley & Sons Ltd. Editors: Elhadi M. Yahia. John Wiley& Sons Ltd.

Pereira, C. D. S., Maranho, L. A., Cortez, F. S., Pusceddu, F. H., Santos, A. R., Ribeiro, D. A., … Guimarães, L. L. 2016. Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. Science of The Total Environment, 548-549, 148–154. doi:10.1016/j.scitotenv.2016.01.051

Pierattini, E. C., Francini, A., Huber, C., Sebastiani, L., Schröder, P. 2018. Poplar and diclofenac pollution: A focus on physiology, oxidative stress and uptake in plant organs. Science of The Total Environment, 636, 944–952. doi:10.1016/j.scitotenv.2018.04.355

Rabiet, M., Togola, A., Brissaud, F., Seidel, J.-L., Budzinski, H., Elbaz-Poulichet, F. 2006. Consequences of Treated Water Recycling as Regards Pharmaceuticals and Drugs in Surface and Ground Waters of a Medium-sized Mediterranean Catchment. Environmental Science & Technology, 40(17), 5282–5288. doi:10.1021/es060528p

Racchi M. L. 2013. Antioxidant Defenses in Plants with Attention to *Prunus* and *Citrus* spp. Antioxidants (Basel, Switzerland), 2(4), 340–369. https://doi.org/10.3390/antiox2040340

Rivas-San Vicente, M., Plasencia, J. 2011. Salicylic acid beyond defence: its role in plant growth and development. Journal of Experimental Botany, 62(10), 3321–3338. doi:10.1093/jxb/err031

Rzymski, P., Drewek, A., Klimaszyk, P. 2017. Pharmaceutical pollution of aquatic environment: an emerging and enormous challenge. Limnological Review, 17(2), 97–107. doi:10.1515/limre-2017-0010

Scheurell, M., Franke, S., Shah, R. M., Hühnerfuss, H. 2009. Occurrence of diclofenac and its metabolites in surface water and effluent samples from Karachi, Pakistan. Chemosphere, 77(6), 870–876. doi:10.1016/j.chemosphere.2009.07.066

Schomburg, D., Salzmann, M. 1991. Enzyme handbook, Part 4. Springer, Berlin, Heidelberg

Senaratna, T., Touchell, S.A., Bunn, O., Dixon, K. 2000. Acetyl salicylic acid (Aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regul., 30: 157–161.

Sharma, P., Jha, A. B., Dubey, R. S., Pessarakli, M. 201). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany, 2012, 1–26. doi:10.1155/2012/217037

Sharma, R., Kaur, R. 2019. Fluoride toxicity triggered oxidative stress and the activation of antioxidative defence responses in *Spirodela polyrhiza* L. Schleiden. Journal of Plant Interactions, 14(1), 440–452. doi:10.1080/17429145.2019.1646826

Simon, P. W. 1997. Plant Pigments for Color and Nutrition. HortScience 32(1):12-13

Srivastava, M. K., Dwivedi, U. N. 1998. Salicylic acid modulates glutathione metabolism in pea seedlings. J. Plant Physiol. 153: 404–409

Stancova, V., Plhalova, L., Blahova, J., Zivna, D., Bartoskova, M., Siroka, Z., Maršálek, P., Svobodova, Z. 2017. Effects of the pharmaceutical contaminants ibuprofen, diclofenac, and carbamazepine alone, and in combination, on oxidative stress parameters in early life stages of tench (*Tinca tinca*). Veterinární medicína. 68:02, 90–97. https://doi.org/10.17221/125/2016-VETMED

Statista. 2019. Revenue of the worldwide pharmaceutical market from 2001 to 2018. Available: <https://www.statista.com/statistics/263102/pharmaceutical-market-worldwide-revenue-since-2001/>.

Suzuki, N., Koussevitzky, S., Mittler, R., Miller, G. 2012. ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ. 35(2):259-70.

Talaat, N. B. 2019. Role of Reactive Oxygen Species Signaling in Plant Growth and Development. Reactive Oxygen, Nitrogen and Sulfur Species in Plants, 225–266. doi:10.1002/9781119468677.ch10

Taub, D. R., Goldberg, D. 1996. Root system topology of plants from habitats differing in soil resource availability. Funct Ecol10:258–64.

Ternes, T. A. 1998. Occurrence of drugs in German sewage treatment plants and rivers Water Res., 32 (11) (1998), pp. 3245-3260

Thomas, K. V., Hilton, M. J. 2004. The occurrence of selected human pharmaceutical compounds in UK estuaries. Marine Pollution Bulletin, 49(5-6), 436–444. doi:10.1016/j.marpolbul.2004.02.028

Trombini, C., Hampel, M., Blasco, J. 2019. Assessing the effect of human pharmaceuticals (carbamazepine, diclofenac and ibuprofen) on the marine clam *Ruditapes philippinarum:* an integrative and multibiomarker approach. Aquatic Toxicology. doi:10.1016/j.aquatox.2019.01.004

Vaughn, C. C. 2010. Biodiversity Losses and Ecosystem Function in Freshwaters: Emerging Conclusions and Research Directions. BioScience, 60(1), 25–35. doi:10.1525/bio.2010.60.1.7

Vlot, A. C., Dempsey, D. A., Klessig, D. F. 2009. Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol. 47:177–206.

Xia, L., Zheng, L., Zhou, J. L. 2017. Effects of ibuprofen, diclofenac and paracetamol on hatch and motor behavior in developing zebrafish (*Danio rerio*). Chemosphere, 182, 416–425. doi:10.1016/j.chemosphere.2017.05.054

Yusuf, M., Hasan, S. A. Ali, B., Hayat, S., Fariduddin, Q., Ahmad, A. 2008. Effect of salicylic acid on salinity induced changes in *Brassica juncea* J. Integrative Plant Biol., 50:(8),1-4

Yusuf, M., Hayat, S., Alyemeni, M. N., Fariduddin, Q., Ahmad, A. 2013. Salicylic Acid: Physiological Roles in Plants. Salicylic Acid: Plant Growth and Development, 15–30. doi:10.1007/978-94-007-6428-6_2

Ziolkowska, A., Piotrowicz-Cieslak, A. I., Rydzynski, D., Adomas, B., Nalecz-Jawecki, G. 2014. Biomarkers of leguminous plant viability in response to soil contamination with diclofenac Pol. J. Environ. Stud., 23,263-269

Chapter 5

Evaluation of ketoprofen toxicity in two freshwater species: effects on biochemical, physiological and population endpoints

Alkimin, G. D., Soares, A. M. V. M., Barata, C., Nunes, B. (2020) Evaluation of ketoprofen toxicity in two freshwater species: effects on biochemical, physiological and population endpoints. Environmental Pollution. 114993. doi.org/10.1016/j.envpol.2020.114993

Evaluation of ketoprofen toxicity in two freshwater species: effects on biochemical, physiological and population endpoints

Alkimin GD[1*], Soares AMVM[1], Barata C[2], Nunes B[1]

[1] Centre for Environmental and Marine Studies (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

5.1 Abstract

Among the most used non-steroidal anti-inflammatory drugs (NSAIDs), ketoprofen (KTF) assumes an important position. Nevertheless, its ecotoxicological effects are in non-target organisms poorly characterized, despite its use and frequency of occurrence in aquatic matrices. Thus, the aim of this study was to evaluate the possible toxicological effects of realistic environmental concentrations of KTF, and worst scenario cases of KTF contamination, in two freshwater species, *Lemna minor* and *Daphnia magna*, by measuring biochemical, physiological and population parameters. To attain this objective, both species were exposed to the same KTF concentrations (0, 0.24, 1.2, 6 and 30 µg/L). *L. minor* plants were exposed during 4d to these levels, and the plants were analyzed in terms of enzymatic activity (catalase (CAT), glutathione S-transferases (GSTs) and carbonic anhydrase (CA)), and pigments content (chlorophyll a, b and total and carotenoids). *D. magna* was acutely and chronically exposed and enzymatic activities (CAT, GSTs and cyclooxygenase (COX)), the feeding rates and reproduction were assessed. In *L. minor*, KTF provoked alterations in all enzyme activities, however, it was not capable of causing any alteration in any pigment levels. On the other hand, KTF also provoked alterations in all enzymatic activities in *D. magna*, but did not affect feeding rates and life-history parameters. In conclusion, exposure to KTF, provoked biochemical alterations in both species, that were not reflected into deleterious effects on physiological and populational traits of *L. minor* and *D. magna*.

Keywords: Non-steroidal anti-inflammatory drugs; *Lemna minor*; *Daphnia magna*; Photosynthetic pigments; Biochemical markers; Reproduction.

5.2 Introduction

Thousands of tons of pharmaceutical substances are used yearly to prevent or treat illnesses (Kosjek et al., 2005), and drugs have been recognized as a large class of chemical contaminants which may originate from human and aquaculture usages, as direct results of excretions of metabolites and residues after metabolism (Eslami et al., 2015), among other sources. The presence and potential effects of these chemicals has been gaining attention due the increase of their discharges, leading to an augmented frequency of their detection in the environment (Kosjek et al., 2005).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs in modern medicine (Meek et al., 2010). It is estimated that the worldwide NSAIDs production is of several kilotons each year (Cleuvers 2004). These drugs are commonly used in the treatment of symptoms such as inflammation, pain, and fever; this usage occurs since their pharmacological activity occurs through cyclooxygenase (COX) inhibition, enzyme responsible for the production of prostaglandins, which are chemical mediators involved in inflammatory processes, leading to swelling and pain (Hernando et al., 2006).

Among the NSAIDs that are now in use for human therapeutics, it is possible to cite ketoprofen (KTF), the third most used NSAID; available data from the bibliography show that, in Croatia and between 2007 and 2013, KTF consumed amounts were just behind values for diclofenac and ibuprofen (Krnic et al., 2015). Beyond its use for the general purposes that NSAIDS are used for, KTF may also be used to treat rheumatoid arthritis and osteoarthritis, and to relieve muscle and joint pain (Prášková et al., 2011). This drug, in humans, is extensively and rapidly metabolized by the liver, mainly via conjugation with glucuronic acid (Gierse et al., 1999) and approximately 80% of the administered dose is excreted in the urine in a 24-hour period after the administration (Prášková et al., 2011); however, there is no information about these parameters for

aquatic organisms, which is now critically required for the assessment of its putative toxicological environmental assessment.

NSAIDs are one of the most frequently detected pharmaceutical compounds in the aquatic environment, and their environmental distribution is widespread (Gentili 2007), including KTF. These drugs, in general, are not efficiently eliminated in sewage treatment plants, being released to surface waters (Martinez-Sena et al., 2016). Thus, KTF is reported in aquatic environment up to 0.3 µg/L and up to 0.87 µg/L in Catalonia (Spain) rivers and effluents respectively (Farré et al., 2001), around 0.2 µg/L in German municipal sewage treatment plant (Ternes, 1998), 0.18 µg/L (Tixier et al., 2003) in Switzerland waste water treatment plant and 5.7 µg/L in effluents of Canadian sewage treatment plants (Metcalfe et al., 2003), besides that, KTF was recorded up to 2 µg/L in Finland influents (Lindqvist et al., 2005).

Considering the large number of NSAIDs, in general, their environmental fates and effects are still poorly understood, despite there are already described persistent and bioaccumulable in aquatic organisms (Geng et al., 2018). This is a pertinent gap in the knowledge of the environmental effects of drugs, since NSAIDs were designed to be biological active in a specific group of organisms (Kosjek et al., 2005). Mammalians, for instance, have both COX isoforms (COX-1 and COX-2, principally), enzymes responsible for the regulation and onset of the inflammatory process (Heckmann et al., 2008a). However, one COX isoform, at least, is generally present in invertebrates and lower vertebrates (Rowley et al., 2005) and, consequently, these organisms may also be affected by NSAIDs too. Consequently, these pharmaceuticals may be responsible for a large number of toxicological effects in non-targets organisms, including macrophyte species (Alkimin et al., 2019a), mollusks (Almeida and Nunes, 2019; Piedade et al., 2020), polychaetes (Gomes et al., 2019) and fish (Nogueira et al., 2019). In relation to this, toxicological effects of KTF has mostly be limited to few fish species such as the *Danio rerio* (Diniz et al., 2015; Rangasamy et al., 2018) and *Cyprinus carpio* (Prášková et al., 2013).

Besides their pharmacological activity, which occurs through COX inhibition, some NSAIDs have the ability to interact with biota, thereby affecting other biological parameters. In humans, NSAIDs are capable of inhibiting the carbonic anhydrase (CA)

activity (Knudsen et al., 2004), an enzyme responsible for carbon dioxide hydration (Lindskog, 1997). NSAIDs also induce the generation of reactive oxygen species (ROS) (Galati et al., 2002; Adachi et al., 2007) that can lead to oxidative stress. The effects of NSAIDs are not limited to the here-described toxicity, since they may also directly compromise the eicosanoids biosynthesis, an important molecule in reproductive traits of crustaceans (Heckmann et al., 2008a), and provoke physiological alterations on macrophyte species (Wrede, 2015). Thus, it is possible to assume that more studies about these effects associated to NSAIDs exposure, namely KTF, are urgently required, in a comprehensive range of organisms. In addition, these studies were performed by testing much higher concentrations than those found in the aquatic environment, considering the already mentioned studies reporting the environmental presence and levels of this drug.

Thus, the aim of this study was to evaluate the toxicological effects of KTF at realistic environmental concentrations and worst scenario case scenarios, in two freshwater species, namely a macrophyte species (*Lemna minor*) and a microcrustacean (*Daphnia magna*), by measuring biochemical, physiological and populational parameters.

5.3 Material and methods

5.3.1 Chemicals

Ketoprofen (CAS number: 22071-15-4) was purchased from Sigma-Aldrich (Belgium) with ≥ 98% of analytical purity; all other chemicals used in this study (buffers, protein determinations) had analytical purity and were purchased from Sigma Aldrich and Bio-rad® laboratories.

5.3.2 Organisms culture and maintenance

All organisms (*Lemna minor* and *Daphnia magna*) were cultured and maintained at the Department of Biology at Aveiro University, as described by Alkimin et al. (2019b) for *L. minor*, and by Daniel et al. (2019) for *D. magna*.

5.3.3 Exposures of test organisms

All experiments performed in this work involved exposing both species to the same range of concentrations, based on already reported environmental levels (up to 5.7 µg/L) (Farré et al., 2001; Metcalfe et al., 2003; Lindqvist et al., 2005). The nominal exposure concentrations were: 0, 0.24, 1.2, 6 and 30 µg/L; the control treatments involved exposing organisms only to the culture medium of each species. The KTF stock solutions were directly prepared in each specific culture medium by dissolving the pure compound.

5.3.4 Macrophytes – *Lemna minor*

Plants of the macrophyte species *L. minor* were exposed in a 6-well plate, filled with 10 mL of medium and/or stock solution per well. The test started with macrophytes covering around 20% of the surface of each well, and had 96 h of duration (Alkimin et al., 2019b), with 10 replicates per condition. Exposures were conducted according to OECD (2006) under controlled conditions (temperature 23 ± 1 °C; continuous light exposure; light intensity, ~84 µmol photons m^{-2} s^{-1}) in a climate chamber (Binder). After the exposure period, *Lemna* fronds from each treatment were collected, and this biomass was divided and placed in Eppendorfs microtubes, and stored at -80 ºC for the analyzes - quantification of pigments (chlorophyll a, b and total and carotenoids) and determination of biochemical endpoints (catalase, CAT; glutathione-S-transferases, GSTs; and carbonic anhydrase, CA, activities).

5.3.5 Microcrustacean – *Daphnia magna*

Three different experiments were performed with *D. magna*. In the first one, juveniles (5 d old) were exposed to KTF during 48 h in 300 mL glass flasks filled with 200 mL of KTF or medium solution. For each concentration, 5 replicates with 12 organisms each were adopted. This experiment was repeated thrice, each one for obtaining biological samples to quantify a specific enzyme (biochemical markers). At the end of the exposure periods, animals were collected with a plastic pipette to Eppendorfs microtubes and stored at -80 ºC for ulterior analyzes (determination of CAT, GSTs, and

COX activities). Feeding rate experiments were conducted in test-chambers consisted of 50 mL glass flasks filled with test solution, where five 5 d old daphnids were positioned (five replicates per treatment). Briefly, this test was used to measure the filtration capacity on algal cells (*Raphidocelis subcapitata*) by test organisms, when exposed to the drug. The animals were exposed for 24 h, in the dark, to all experimental conditions, with $5x10^5$ cells/mL of the algae *R. subcapitata* in the test-chambers. Afterwards, animals were transferred to a new test-chamber (with clean media), also in the dark, for 4 h, with the same algae density (post-exposure period), to evaluate the recovery from exposure to toxicants. Blank controls (media with no daphnids) were included in the experimental design in both cases. At the end of each period, the algae cells densities were spectrophotometrically measured (Thermo Scientific Multiskan (ScanIt Software 2.4.4)) at 440 nm. Finally, the feeding rate was calculated converting the obtained values to proportional algae consumption (%) relative to control. Finally, a chronic reproduction test was conducted, based on the OECD guideline 211 (OECD 2012) with the exposure period modified according to Ribeiro et al. (2011) and Alkimin et al. (2020). The test duration was approximately 16 days and/or the third brood. The experiment was performed in 50 mL glass flasks, and 10 replicates with < 24 h old neonates were used for each concentration. Medium test was totally renewed every other day, and the animals were maintained in the same culture conditions. The parameters day of first brood, number of neonates from first brood, and total number of neonates were the evaluated endpoints.

5.3.6 Quantification of physiological and biochemical parameters

5.3.6.1 Pigments analysis (chlorophylls and carotenoids)

Total, a, and b chlorophylls (TChl; Chl a; Chl b), and carotenoids (Car) amounts were determined spectrophotometrically, according to the method described by Hiscox and Israelstam (1979). Pigments were extracted from the previously exposed fronds of *L. minor* (about 10 mg per replicate - fresh weight, FW) in 1.8 mL of dimethyl sulphoxide (DMSO). The extract was placed in water at 65 ºC during 30 min and allowed to cool in the dark and at room temperature. The obtained supernatants were used to quantify the amounts of chlorophylls (a, b, and total), and carotenoids levels, by spectrophotometry,

by measuring the absorbance of the extracts, at wavelengths of 470, 645, 646, and 663. The calculation of the pigments followed the equations proposed by Arnon (1949) and demonstrated by Hiscox and Israelstam (1979) to be suitable if the extraction was undertaken with DMSO, to calculate the amounts of Chl (a, b, and total), and by Lichtenthaler (1987) to quantify the Car amount. In addition, two ratios were determined: [Chl a] / [Chl b]; and [TChl] / [Car].

5.3.6.2 Tissue homogenization – biochemical markers

For CAT and GSTs determinations, samples were macerated with mortar and pestle (*L. minor*) or sonicated (*D. magna* – Branson sonicator, model 250) in ice-cold phosphate buffer (50 mM, pH 7.0, with 0.1% Triton X-100). Each homogenate sample was composed by ~ eight fronds of *L. minor*, or 12 individuals of *D. magna*, and 1.2 mL phosphate buffer. Homogenized samples were centrifuged at 15,000 g at 4ºC for 10 min. For carbonic anhydrase activity (CA) determination, samples of *L. minor* were macerated in 1 mL ice-cold Tris-sulfate 25 mM (pH 7.5) with 25 mM of sodium sulfate. Homogenized samples were centrifuged at 10,000 g at 4 ºC for 40 min. Cyclooxygenase determination (*D. magna*) was performed using 0.8 mL of 0.1 M Tris-HCl buffer (pH 7.8) with 1 mM EDTA to sonicate animals, followed by a cycle of centrifugation at 10,000 g at 4 ºC for 15 min. After the centrifugation process, supernatants were divided into aliquots, which were used for the different enzymatic determinations, as described below.

5.3.6.3 Catalase activity determination

Catalase activity was determined in a 96-well microplate, by the procedure described by Aebi (1984), based on the degradation rate of the substrate H_2O_2, monitored at 240 nm for 5 min. The results were expressed by considering that one unit of CAT activity equals the number of moles of H_2O_2 degraded per minute, per milligram of protein.

5.3.6.4 Glutathione-S-transferases activity determination

The procedure to determine GSTs activity monitored the increment of absorbance at 340 nm of a thioether resulting from the catalysis of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione by GSTs, as described by Habig et al. (1974), adapted to a 96 well microplate. Results were expressed as millimoles of thioether produced per minute, per milligram of protein.

5.3.6.5 Carbonic anhydrase activity determination

To determine CA activity, the method by Verpoorte et al. (1967) was adapted to 96-well microplates. This methodology is based on the hydrolysis of the substrate p-nitrophenol acetate (pNPAc), catalyzed by CA. This reaction was then monitored for 5 min at a wavelength of 400 nm. One unit of CA is defined as the amount of enzyme needed to hydrolyze one µmol of pNPAc per minute, and the data were expressed in U/milligram of protein.

5.3.6.6 Cyclooxygenase activity determination

This method is based on the ability of cyclooxygenase (COX) to convert arachidonic acid into a hydroperoxy endoperoxide (Prostaglandin G2; PGG2). This is reduced in the presence of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to its alcohol (Prostaglandin H2; PGH2); then, the oxidation of TMPD was monitored during 5 min at 590 nm (Petrovic and Murray, 2010).

5.3.6.7 Protein determination

Protein quantification was performed at 595 nm using the Bradford method (Bradford 1976), adapted to microplate, with bovine γ-globulin as standard (1mg/ml), in order to express enzymatic activities per mg of protein of the analyzed samples. Finally, all spectrophotometric readings were performed in a microplate reader Thermo Scientific Multiskan (ScanIt Software 2.4.4).

5.3.7 Statistical analysis

Data for all parameters were tested for homogeneity and normality, and a one-way analysis of variance (ANOVA) was performed, followed by a post-hoc Dunnett's test (or the equivalent non-parametric test, according to the data). Statistical analysis was performed with SigmaPlot v.14.0. The adopted level of significance was 0.05.

5.4 Results

The lowest concentration of KTF provoked a decrease in GSTs activity in *L. minor*; on the contrary, this enzyme´s activity was increased in plants exposed to all other concentrations ($F_{(4, 28)} = 57.177$, $p < 0.001$; Figure 1A). With the exception of the highest concentration (30 µg/L), KTF increased CAT activity ($F_{(4, 32)} = 23.157$, $p<0.001$; Figure 1B). KTF caused a decrease of CA activity in plants exposed to levels of 0.24 and 1.2 µg/L ($F_{(4, 46)} = 10.055$, $p<0.001$; Figure 1C).

Photosynthetic pigments (Chl a, b and TChl) measured in *L. minor* did not suffer any alteration after KTF exposure ($F_{(4, 47)} = 0.922$, $p = 0.460$; $F_{(4, 47)} = 1.426$, $p = 0.242$ and $F_{(4, 47)} = 1.003$, $p = 0.416$, respectively). No alterations were also detected for Car content ($F_{(4, 47)} = 1.139$, $p = 0.351$,) or for both determined ratios (Chl a/b – $H_{4df} = 4.255$, $p = 0.373$; TChl/Car – $F_{(4, 47)} = 2.022$, $p = 0.108$) (Figure 2A – F).

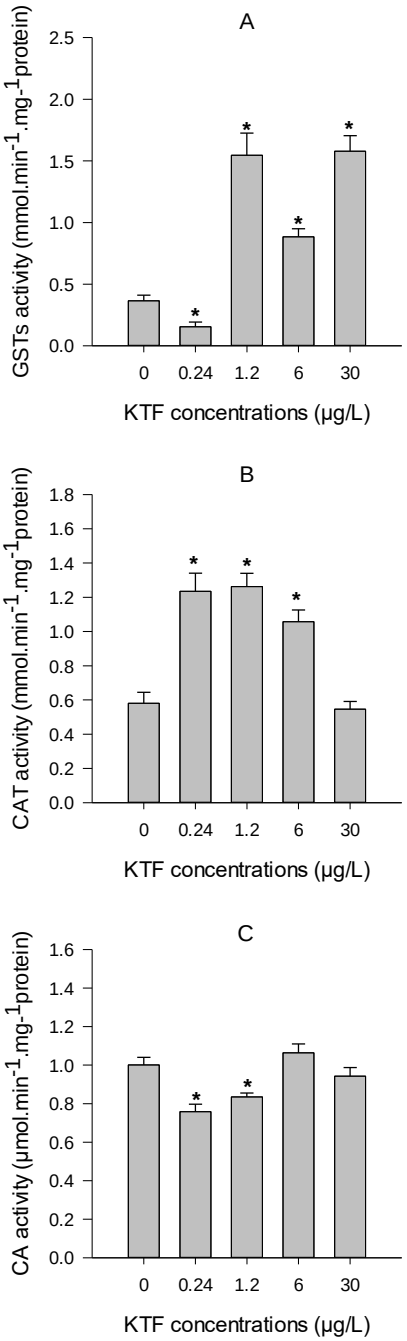

Figure 1 – Effects of ketoprofen exposure in *L. minor* biomarkers upon exposure of 96 hours. A) glutathione S-transferases activity; B) catalase activity; and C) carbonic anhydrase activity. Bars and errors bars are Mean ± SE (N= 10). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

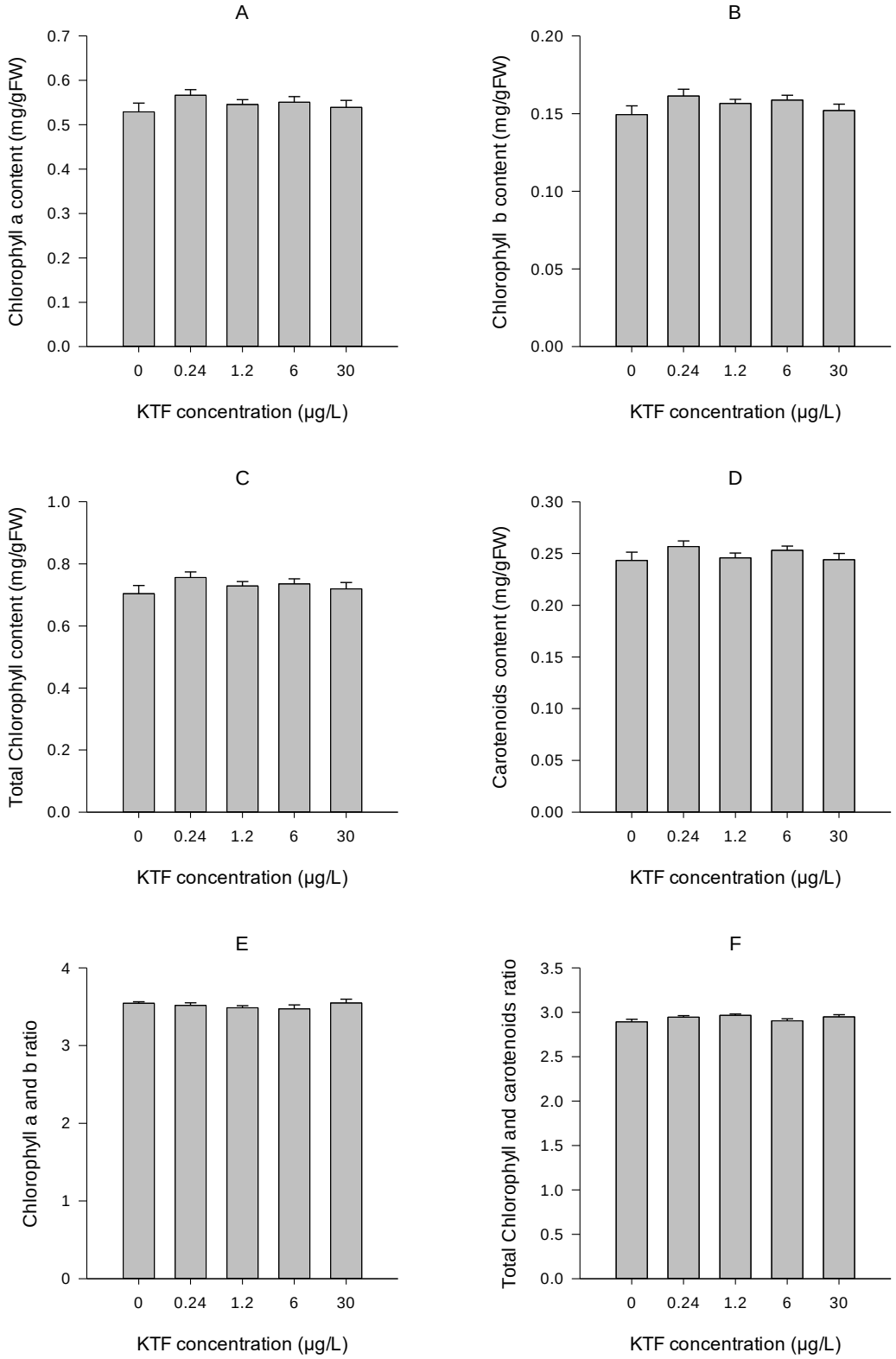

Figure 2 – Effects of ketoprofen exposure in *L. minor* during 96 hours. A) chlorophyll a content, B) chlorophyll b content, C) total chlorophyll content, D) carotenoids content, E) chlorophyll a and b ratio and F) total chlorophyll and carotenoids ratio. Bars and errors bars are Mean ± SE (N= 10). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

In *D. magna*, KTF caused an increase in CAT activity ($F_{(4,20)}$=31.597; p<0.001) in a concentration related manner (figure 3A); GSTs activity also presented an increase, but only statistically different in organisms exposed to concentrations of 1.2 and 6 µg/L ($F_{(4,22)}$=5.048; p=0.007)(figure 3B). On the other hand, COX activity was reduced after KTF exposure ($F_{(4,18)}$=4.695; p=0.013) for organisms exposed to all tested concentrations, in a dose-response manner (figure 3C).

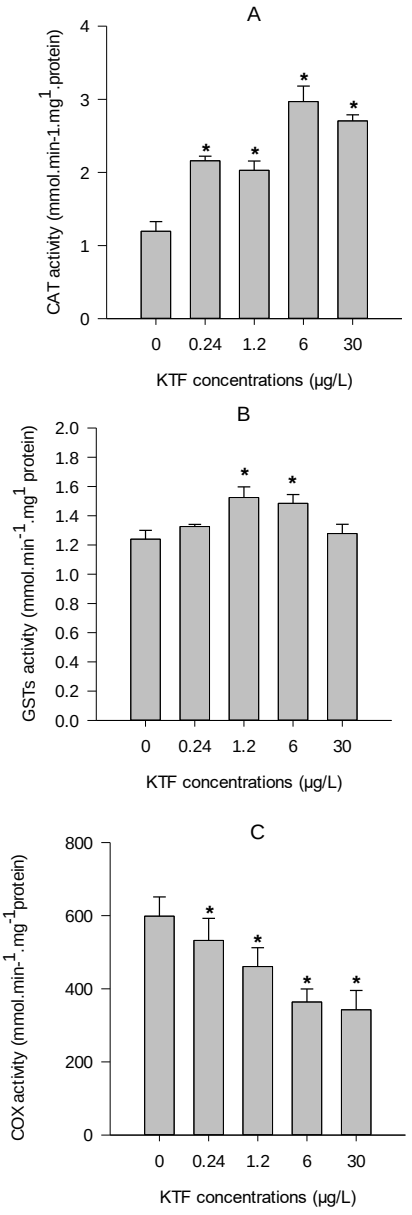

Figure 3 – Effects of ketoprofen in *D. magna* after acute exposure (48h). A) catalase activity B) glutathione S-transferases activity, and C) cyclooxygenase activity. Bars and errors bars are Mean ± SE (N= 5). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

Ketoprofen did not affect *D. magna* feeding rates during exposure ($F_{(4,24)}$= 3.515; p = 0.025) or during recovery ($F_{(4,24)}$= 1.636 p=0.0204; figure 4).

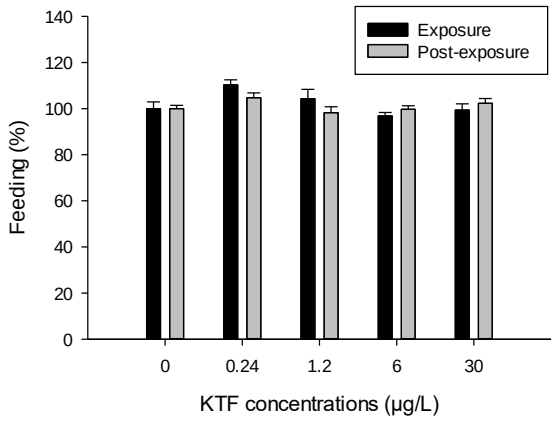

Figure 4 – Effects of ketoprofen in *D. magna* feeding behavior, after exposure and after recovery (post-exposure). Bars and errors bars are Mean ± SE (N= 5). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

Ketoprofen did not affect reproductive endpoints in *D. magna*, which included day of first brood (H_{4df} =4.091; p=0.394; figure 5A), number of neonates from first brood (H_{4df}=2.803; p=0.591; figure 5B), and total number of neonates ($F_{(4, 44)}$=0.938; p=0.452; Figure 5C).

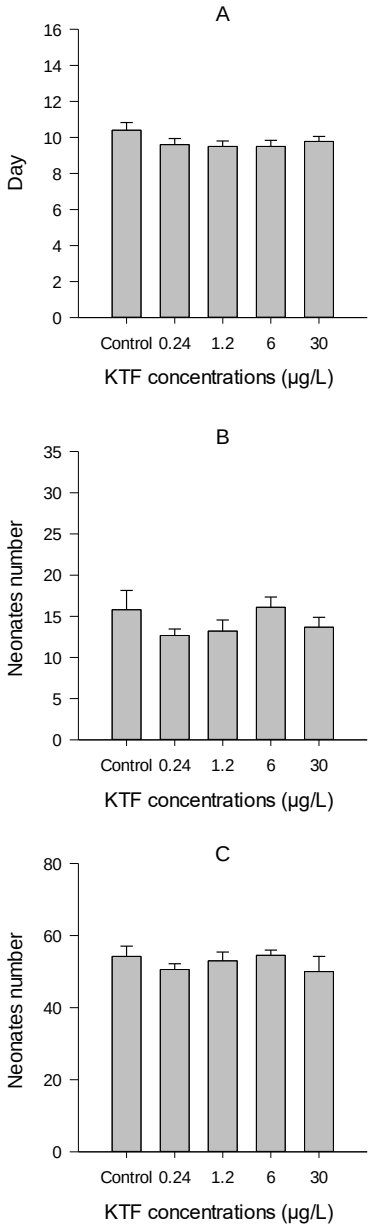

Figure 5 – Ketoprofen effects in reproductive parameters in *Daphnia magna*. A) day of the first brood, B) number of neonates from the first brood and C) total number of neonates (three broods). Bars and errors bars are Mean ± SE (N= 10). * stands for statistical differences in relation to control following ANOVA and Dunnett's or the equivalent non-parametric test.

5.5 Discussion

5.5.1 *Lemna minor*

NSAIDs, including diclofenac and paracetamol, are capable to induce ROS production in *L. minor*, as already demonstrated by Kummerová et al. (2016). This is highly important in our context, considering that reactive oxygen species (ROS), and

particularly hydrogen peroxide (H_2O_2), are generated during the normal plant metabolism and are involved in a diversity of signaling cascades and reactions necessary for the physiological regulation of diverse aspects, such as plant growth (Cheeseman, 2007). Exposure to anthropogenic xenobiotics, such as the referred drugs, may enhance this production, leading to toxic effects in plants. A similar finding was made clear in this study, since CAT activity was increased following KTF exposure. Increased CAT activity must be understood as the activation of enzymatic detoxification mechanisms that to minimize cellular levels of superoxide radicals (O_2^{-}) and H_2O_2. This is the main role of antioxidant enzymes such as CAT (Sairam and Tyagi, 2004). It is thus possible to hypothesize that low levels of KTF were causative of the triggering of an antioxidant response. Conversely, the activity of CAT measured in plants exposed to the highest KTF concentration was closely similar to the control values; this can be assumed as a possible plant resilience mechanism, since macrophytes are known for their ability to recover along time (Wang, 1990), when kept in a contaminated environment by different contaminants, such as metals (Jaramillo et al., 2019) and pharmaceutical drugs (Alkimin et al., 2019a). The normalization of CAT means that plants were able to activate alternative detoxification and antioxidant mechanisms, such as ascorbate peroxidase and superoxide dismutase, another two enzymes very important for the antioxidant defense system of plants (Mittler, 2002). For example, the different affinities of ascorbate peroxidase (APX; μm range) and CAT (mm range) for H_2O_2 indicated that they belong to different classes of ROS-scavenging enzymes, namely, H_2O_2 (Mittler, 2002). This means that the fine modulation of ROS signaling might occurs through APX, whereas CAT might be responsible for the removal of excess ROS during stress (Mittler, 2002).

The alterations of CAT activity may be linked to the observed changes in GSTs activity. Plants exposed to the lowest concentration of KTF had a decrease in GSTs activity. It is possible to suggest that the overproduction of ROS by KTF exposure (signaled by the enhancement of CAT activity) may have caused direct damages in cellular molecules. This a common feature of ROS, and their damaging action may include deleterious alterations of DNA, lipids, and proteins; this often causes a decrease in enzymatic activity by denaturation (Schieber and Chandel, 2014), an effect that may be held accountable for the here-observed decrease of GSTs activity. On the other hand, plants

exposed to the other tested concentrations were capable of increasing their GSTs activity. This pattern is indicative of distinct effects, related to the increase of biotransformation capacity of the organism to cope with the excess of this xenobiotic. Since, this isoenzyme group is part of phase II metabolic mechanisms that is responsible for the detoxification of exogenous compounds; conjugating glutathione (GSH) with compounds containing an electrophilic center to modify the substrate into a more water soluble, less toxic complex (Habig et al., 1974). In addition, GSTs had also a role in resisting pro-oxidative effects, being useful in enhancing plant survival on toxic sites (Cummins et al., 2011). This effect occurs since GSTs contribute for the efficacy of the antioxidant defense system, which does not directly relate to their role in xenobiotic detoxification (Moons, 2005).

Macrophytes (such as *L. minor*) are primary producers in the aquatic environment, consequently being at the basis of the food web of freshwater systems; thus, adverse effects that may occur in these plants can also deleteriously affect the entire aquatic food web. So, if the *L. minor* antioxidant stress system was not able to efficiently reduce the adverse effect of xenobiotics upon exposure, deleterious effects might occur in this species, from cell death to other unpredictable consequences (Demidchik, 2015; Xie et al., 2019). Despite the general absence of data for plant organisms, it is possible to consider the assumptions made by Monaghan et al. (2009), when referring that life-history trade-offs in animals are likely to be associated to oxidative stress and to the antioxidant response, which occur at the most fundamental metabolic activities of living organisms. Such traits are likely to impact not only the individual, but to escalate at higher levels of organization, with unforeseen outcomes. So, changes in plants of *L. minor* may indeed correspond to alterations at the ecosystem level.

Carbonic anhydrase is a zinc-containing enzyme that catalyzes the rapid conversion of CO_2 over water in a proton and bicarbonate ion (HCO_3^- - reversible hydration/dehydration reaction) (Coleman, 2000; Escudero-Almanza et al., 2012). In plants, CA is important in many physiological functions that involve carboxylation or decarboxylation reactions (Moroney et al., 2001) by increasing CO_2 concentrations in the chloroplast to increase the carboxylation rate of the ribulose 1,5-disphosphate carboxylase (RuBisCO) enzyme. This chemical reaction is responsible for the incorporation of CO_2 into carbohydrates during photosynthesis; however, it and can

only use C coming from CO_2, instead of C from carbonic acid or bicarbonate (Escudero-Almanza et al., 2012). In addition, CA also actively participates in the inorganic carbon transportation into actively photosynthesizing cells or away from actively respiring cells (Henry 1996). Few studies reported the effects of pharmaceutical drugs in CA enzymatic activity of macrophytes. Previous data have shown that acetazolamide (diuretic drug) and salicylic acid (NSAID), had the capacity to decrease CA activity, also in a macrophyte species, namely *L. gibba* (unpublished data). However, the here obtained data showed a decrease of CA activity only in plants exposed to the lowest concentrations of KTF (0.24 and 1.2 µg/L), which may indicate hormesis. In general, a hormesis effect represents an adaptive response of organisms to environmental or self-imposed challenges through which they improve its functionality and/or tolerance to more aggressive challenges (Calabrese and Mattson, 2017). Such challenging conditions, in this case, correspond to the higher concentrations of the tested pharmaceutical. The exposed plants showed to be resilient, by decreasing their CA activity. Hormesis effects were also observed in CAT activity, which only increased in plants exposed to the lowest concentrations.

Generally, higher plants have three groups of pigments: carotenoids, chlorophylls, and phycobilins; the most important pigments involved in photosynthesis are chlorophylls, which are prone to be targets for toxic alterations. Changes of the qualitative composition or of quantitative content of pigments, are physiologically important characteristics that indicate the general health condition of plants, informing also on the function of photosynthetic apparatus, including the adaptive responses after being subjected to stressful situations (Belous et al., 2018). Levels of the main photosynthetic pigments here analyzed (Chl a, b and total) did not suffer any alteration after KTF exposure; in addition, the ratio between levels of chlorophylls a and b were not altered. This ratio is a calculation that characterizes the photosynthetic apparatus operation, by indicating the potential photochemical activity of leaves. The absence of effects may lead us to conclude that KTF does not compromise the normal photochemical potential in *L. minor*, despite pervious indications that, in general, NDSAIDs may exert this effect.

Carotenoids are well known free-radical scavengers, also playing an important role in photoprotection of photosynthetic apparatus (Sytar et al., 2013). KTF-exposed plants

did not show changes in Car, in terms of their presence, quantity, and proportion (in comparison with chlorophylls, assessed by the determination of TChl/Car ratio). The ratio of TChl to Car is more informative that the simple measurement of pigment levels, because it indicates the degree of adaptation of plants to light and to adverse conditions. This entire set of results concerning pigments demonstrates that *L. minor* was physiologically adapted to KTF, and no deleterious effects were caused by KTF in terms of the pigments levels of exposed organisms.

Finally, the observed biochemical effects were not evident enough to sustain the occurrence of physiological alterations. Despite the occurrence of biochemical changes caused by KTF exposure, these were not followed by deleterious changes in photosynthetic pigments (Chl a, b, total and Car). Thus, it is possible to assume that the antioxidant and biotransformation systems of *L. minor* were efficient enough to protect these plants from other injuries.

5.5.2 *Daphnia magna*

The genus *Daphnia* is considered as a dominant herbivorous in the zooplankton group, and animals from this genus play an important role in temperate freshwater ecosystems as primary consumers (Smirnov, 2013). Thus, their feeding activity is an important aspect in their ecology, being necessarily assessed in a stressful situation, including exposure to contamination by pharmaceutical drugs. In this work, it was possible to observe that KTF, in the tested concentrations, did not alter feeding, consequently, the recovery period was equal too, and yielded not any substantial effect. However, other NSAIDs, like diclofenac, are capable of reducing *D. magna* feeding rate (Nkoom et al., 2019) in concentrations between 5 and 100 µg/L. However, it is necessary to take into account that Nkoom et al. (2019) experiment was conducted with neonates <24 h old, and our work involved only juveniles (5 d old), which can be more tolerant. Feeding rate can affect a large number of life-history traits that may have context-dependent effects on fitness (Garbutt and Little, 2014), being putatively associated with the reproduction. Indeed, KTF did not affect the studied life-history traits. Data for another crustacean species, namely *Ceriodaphnia dubia*, showed the absence of effects after exposure to KTF at concentrations up to 100 µg/L, with a decrease in the offspring per

female, when animals were exposed to 1 mg/L (Mennillo et al., 2017). This level is nevertheless much higher than the here tested concentrations, and also well above the amounts detected in the environment, as already cited. These data are coherent, since the *D. magna* 48h EC_{50}, is higher than 100 mg/L of KTF (Boström and Berglund, 2015), a value approximately 3300 times higher than the levels tested in this study.

Despite not causing alterations in the feeding behavior and in life-history traits, KTF was able to trigger the *D. magna* antioxidant system, increasing the CAT activity of exposed animals. This occurred most likely as an attempt to cope with the putatively higher levels of generated ROS. Among NSAIDs, KTF has been shown to be more toxic than other drugs from this class to the *D. magna* antioxidant system, generating changes in the antioxidant mechanisms in concentration as low as 0.24 µg/L of KTF ($9.43\ 10^{-10}$ moles/L). Daniel et al. (2019) reported an increase of CAT activity in animals exposed to 2.56 mg/L ($1.69\ 10^{-5}$ moles/L) of paracetamol, and, even a concentration of 50 µg/L ($2.39\ 10^{-7}$ moles/L) of ibuprofen was not able to trigger this system (Wang et al., 2016).

KTF seems also to have activated the biotransformation system in *D. magna*, by increasing the activity of GSTs, a response that occurred for animals exposed to the intermediate concentrations (1.2 and 6 µg/L). Phase II enzymes are crucial in eliminating NSAIDs, but the interaction between GSTs and KTF is poorly understood for aquatic organisms. In other animals, such as mammals, KTF can be eliminated in its free forms: conjugated via glucuronic acid, or as hydroxyl (OH) metabolite, with or without conjugation (Alkatheeri et al., 1999). However, the metabolic pathway of conjugation seems also to occur in aquatic organisms. In fact, KTF triggered the biotransformation system, increasing the GSTs activity, in a fish PLHC-1 cell line (Mennillo et al., 2017). Thus, it is possible to hypothesize that KTF can be excreted following conjugation with glutathione (via GSTs) and/or with glucuronic acid, prior to be delivered to phase II detoxification system. In addition, the lowest (0.24 µg/L) and the highest (30 µg/L) concentrations did not cause any alteration of GST activity. Thus, hermetic effect can be interpreted as follows: at low concentrations hydroxylation processes were effective enough to detoxify the pharmaceutical, turning the phase II biotransformation system of glutathione conjugation redundant (Bartha, 2012). However, in animals exposed to higher concentrations, more complex metabolic

pathways should be activated by the drug (Bartha, 2012) leading to no alteration in the GSTs activity. On the other hand, as already mentioned, ROS have the capacity to cause molecular damage and this effect can be linked to a decrease in enzymatic activity by denaturation (Schieber and Chandel, 2014) and consequently, may be responsible for the decrease of GSTs activity in the higher concentration, attaining values near those of the control treatment.

Eicosanoids are oxygenated metabolites of arachidonic acid (AA) with different functions. Their biosynthesis may occur according to different pathways, such as the cytochrome P450 epoxygenase pathway (epoxyeicosatrienoic acids), lipoxygenage (LOX) pathway (leukotrienes and lipoxins), and cyclooxygenase (COX) pathway (prostanoids: thromboxane and prostaglandins) (Stanley, 2006). As mentioned, COX or PGH_2 synthase is an enzymatic form producing prostaglandins, and it is conserved in crustaceans, such as daphnids (Kyoto Encyclopedia of Genes and Genomes, KEGG). Prostaglandins are signaling molecules with crucial importance in crustacean reproduction and immune system (Smirnov, 2017). NSAIDs can interfere/inhibit prostaglandins biosynthesis, since NSAIDs are competitive inhibitors of some of the involved enzymatic forms, by competing with AA, which is the physiological substrate of COX; NSAIDs thereby affects eicosanoids biosynthesis and their physiological functions (Charlier and Michaux, 2003). Heckmann and co-workers (2008a) demonstrated the existence of a COX pathway in *D. magna*, which appears to be simpler than the COX pathway observed in mammals. In addition, another study indicated that ibuprofen, also a NSAID, is capable of affecting the sequential processes of oogenesis and embryogenesis in daphnids, by interrupting the COX metabolic pathway of eicosanoids (Heckmann et al., 2008b). By acting this way, this drug is responsible for a dose-dependent decrease in *D. magna* reproduction (Han et al., 2010; Heckmann et al., 2007) and also affects reproduction in another crustacean species, namely *Moina macrocopa* (Han et al., 2010). The NSAID effect in COX also was possible to be observed in this study, since KTF exposure clearly decreased COX activity in a dose-response manner, as already discussed here. However, reproductive effects were not observed in *D. magna* after KTF exposure, which means that NSAIDs affect crustacean reproduction at higher concentrations (in the mg/L order) as observed by Han et al. (2010) and Heckmann et al. (2007). These assumptions reinforce the

notion that deleterious NSAIDs effects in the eicosanoids biosynthesis pathway may be important for the reproduction of crustaceans. However, the alterations that were observed in this study constitute a warning for the possible long-term effects of KTF in a worst environmental case scenario.

5.6 Conclusions

In conclusion, both species responded to low, realistic levels of KTF. However, the toxic effects of KTF were only limited to biochemical parameters, without extrapolating to physiological and population modifications. However, changes of the biochemical parameters were clearly different between both species, being *L. minor* more susceptible to low KTF concentrations, hypothesizing the resilience capacity and hormetic effects; while *D. magna*, in general, presented a dose response pattern. This comparison is highly interesting and allows us to assume that KTF toxicity is eminently species-specific. Finally, the results here obtained are a clear indication about the possibility of KTF in causing toxicological effects in the aquatic organisms, even when organisms were exposed to realistic conditions simulating the already described scenarios of contamination in the wild.

5.7 References

Adachi, M., et al. 2007. Nonsteroidal anti-inflammatory drugs and oxidative stress in cancer cells. Histol Histopathol. 22: 437-442. DOI: 10.14670/HH-22.437

Aebi, H. 1984. Catalase in vitro. Methods in Enzymology, 6. 105-121

Alkatheeri, N. A., Wasfi, I. A., Lambert, M. 1999. Pharmacokinetics and metabolism of ketoprofen after intravenous and intramuscular administration in camels. J Vet Pharmacol Ther 22:127–135.

Alkimin, G. D., Daniel, D., Dionísio, R., Soares, A. M. V. M., Barata, C., Nunes, B. 2019a. Effects of diclofenac and salicylic acid exposure on *Lemna minor*: Is time

a factor? Environmental Research, 177, 108609. doi:10.1016/j.envres.2019.108609

Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M., Barata, C., Nunes, B. 2019b. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Science of The Total Environment, 657, 926–937. doi:10.1016/j.scitotenv.2018.12.002

Alkimin, G. D., Paisio, C., Agostini, E., Nunes, B. 2020. Phytoremediation processes of domestic and textile effluents: evaluation of the efficacy and toxicological effects in *Lemna minor* and *Daphnia magna*. Environ Sci Pollut Res 27, 4423–4441. https://doi.org/10.1007/s11356-019-07098-3

Almeida, F., Nunes, B. 2019. Effects of acetaminophen in oxidative stress and neurotoxicity biomarkers of the gastropod *Phorcus lineatus*. Environmental Science and Pollution Research. doi:10.1007/s11356-019-04349-1

Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in *Beta vulgaris*. Plant physiology, 24, 1-15.

Bartha, B. 2012. Uptake and Metabolism of Human Pharmaceuticals in Plants. Identification of Metabolites and Specification of the Defense Enzyme Systems under Pharmaceutical Exposure. (PhD thesis. Technische Universität München. Lehrstuhl für Ökologischen Landbau und Pflanzenbausysteme).

Belous, O., Klemeshova, K., Malyarovskaya, V. 2018. Photosynthetic Pigments of Subtropical Plants, Photosynthesis - From Its Evolution to Future Improvements in Photosynthetic Efficiency Using Nanomaterials, IntechOpen, DOI: 10.5772/intechopen.75193.

Boström, M. L., Berglund, O. 2015. Influence of pH-dependent aquatic toxicity of ionizable pharmaceuticals on risk assessments over environmental pH ranges. Water Research, 72, 154–161. doi:10.1016/j.watres.2014.08.040

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Calabrese, E. J., Mattson, M. P. 2017. How does hormesis impact biology, toxicology, and medicine? Npj Aging and Mechanisms of Disease, 3(1). doi:10.1038/s41514-017-0013-z

Charlier, C., Michaux, C. 2003. Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal antiinflammatory drugs. European Journal of Medicinal Chemistry 38: 645-659.

Cheeseman, J. M. 2007. Hydrogen peroxide and plant stress: a challenging relationship Plant Stress, 1. 4-15

Cleuvers, M. 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. Ecotoxicology and Environmental Safety, 59(3), 309–315.

Coleman, J. R. 2000. Carbonic Anhydrase and Its Role in Photosynthesis. Advances in Photosynthesis and Respiration, 353–367. doi:10.1007/0-306-48137-5_15

Cummins, I., Dixon, D. P., Freitag-Pohl, S., Skipsey, M., Edwards, R. 2011. Multiple roles for plant glutathione transferases in xenobiotic detoxification. Drug Metabolism Reviews, 43(2), 266–280. doi:10.3109/03602532.2011.552910

Daniel, D., Dionísio, R., Alkimin, G. D., Nunes, B. 2018. Acute and chronic effects of paracetamol exposure on *Daphnia magna*: how oxidative effects may modulate responses at distinct levels of organization in a model species. Environmental Science and Pollution Research. doi:10.1007/s11356-018-3788-y

Demidchik, V. 2015. Mechanisms of oxidative stress in plants: From classical chemistry to cell biology. Environmental and Experimental Botany, 109, 212–228. doi:10.1016/j.envexpbot.2014.06.021

Diniz, M. S., Salgado, R., Pereira, V. J., Carvalho, G., Oehmen, A., Reis, M. A. M., Noronha, J. P. 2015. Ecotoxicity of ketoprofen, diclofenac, atenolol and their

photolysis byproducts in zebrafish (*Danio rerio*). Science of The Total Environment, 505, 282–289. doi:10.1016/j.scitotenv.2014.09.103

Escudero-Almanza, D.J. et al. 2012. Carbonic Anhydrase and Zinc In Plant Physiology, Chilean Journal Of Agricultural Research 72(1)

Eslami, A., Amini, M. M., Yazdanbakhsh, A. R., Rastkari, N., Mohseni-Bandpei, A., Nasseri, S., … Asadi, A. 2015. Occurrence of non-steroidal anti-inflammatory drugs in Tehran source water, municipal and hospital wastewaters, and their ecotoxicological risk assessment. Environmental Monitoring and Assessment, 18 7(12). doi:10.1007/s10661-015-4952-1

Farré, M. et al. 2001. Determination of drugs in surface water and wastewater samples by liquid chromatography–mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio fischeri*. Journal of Chromatography A, 938(1-2), 187–197. doi:10.1016/s0021-9673(01)01154-2

Galati, G., Tafazoli, S., Sabzevari, O., Chan, T. S., O'Brien, P. J. 2002. Idiosyncratic NSAID drug induced oxidative stress. Chemico-Biological Interactions, 142(1-2), 25–41. doi:10.1016/s0009-2797(02)00052-2

Gao, X., Geng, J., Du, Y. et al. 2018 Comparative study of the toxicity between three non-steroidal anti-inflammatory drugs and their UV/$Na_2S_2O_8$ degradation products on *Cyprinus carpio*. Sci Rep 8, 13512.

Garbutt, J. S., Little, T. J. 2014. Maternal food quantity affects offspring feeding rate in *Daphnia magna*. Biology Letters, 10(7), 20140356–20140356. doi:10.1098/rsbl.2014.0356

Gentili, A. 2007. Determination of non-steroidal anti-inflammatory drugs in environmental samples by chromatographic and electrophoretic techniques. Analytical and Bioanalytical Chemistry, 387(4), 1185–1202. doi: 10.1007/s00216-006-0821-7.

Gierse, J. K., Koboldt, C. M., Walker, M. C, Seibert, K., Isakson, P. C. 1999. Kinetic basis for selective inhibition of cyclo-oxygenases. Biochem J 339: 607–614.6

Gomes A., Correia A.T., Nunes B. 2019. Worms on drugs: ecotoxicological effects of acetylsalicylic acid on the Polychaeta species *Hediste diversicolor* in terms of biochemical and histological alterations. Environmental Science and Pollution Research, 26, (13), 13619-13629.

Habig W. H., Pabst M. J., Jakoby W. B. 1974. Glutathione S-transferases—the first enzymatic step in mercapturic acid formation. J Biol Chem. 249, 7130–7139.

Han S, Choi K, Kim J, Ji K, Kim S, et al. 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka *Oryzias latipes* and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquatic Toxicology 98: 256-264.

Heckmann, L. H., Callaghan, A., Hooper, H. L., Connon, R., Hutchinson, T. H., et al. 2007. Chronic toxicity of ibuprofen to *Daphnia magna:* Effects on life history traits and population dynamics. Toxicology Letters 172: 137-145.

Heckmann, L. H., Sibly, R. M., Connon, R., Hooper, H. L., Hutchinson, T. H., et al. 2008a. Systems biology meets stress ecology: Linking molecular and organismal stress responses in *Daphnia magna.* Genome Biology 9: R40 1-15

Heckmann, L.-H., Sibly, R. M., Timmermans, M. J., Callaghan, A. 2008b. Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. Frontiers in Zoology, 5(1), 11. doi:10.1186/1742-9994-5-11

Henry, R. P. 1996. Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annual Review of Physiology 58, 523–538.

Hernando, M. D., Heath, E., Petrovic, M., Barceló, D. 2006. Trace-level determination of pharmaceutical residues by LC-MS/MS in natural and treated waters. A pilot-survey study. Analytical and Bioanalytical Chemistry, 385(6), 985–991.

Hiscox, J. D., Israelstam, G. F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can J. Bot. 57, 1332-1334.

Jaramillo, E., Duarte, C., Labra, F.A. et al. 2019. Resilience of an aquatic macrophyte to an anthropogenically induced environmental stressor in a Ramsar wetland of southern Chile. Ambio 48, 304–312 https://doi.org/10.1007/s13280-018-1071-6

KEGG – Kyoto Encycloedia of Genes and Genomes <https://www.genome.jp/kegg/>.

Knudsen, J. F., Carlsson, U., Hammarström, P. et al. 2004. The Cyclooxygenase-2 Inhibitor Celecoxib Is a Potent Inhibitor of Human Carbonic Anhydrase II. Inflammation 28, 285–290. https://doi.org/10.1007/s10753-004-6052-1

Kosjek, T., Heath, E., Krbavcic, A. 2005. Determination of non-steroidal anti-inflammatory drug (NSAIDs) residues in water samples. Environment International, 31(5), 679–685. doi:10.1016/j.envint.2004.12.001

Krnic D, Anic-Matic A, Dosenovic S, Draganic P, Zezelic S, Puljak L. 2015. National consumption of opioid and nonopioid analgesics in Croatia: 2007–2013. Ther Clin Risk Manag. 11:1305-1314.bhttps://doi.org/10.2147/TCRM.S86226

Kummerová, M., Zezulka, Š., Babula, P., Tříska, J. 2016. Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant *Lemna minor*. Journal of Hazardous Materials, 302, 351–361. doi:10.1016/j.jhazmat.2015.09.057

Sairam R, Tyagi A. 2004. Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci-Bangalore. 86:407–421.

Lichtenthaler, H. K. 1987. Chlorophylls and Carotenoids Pigments of Photosynthetic Biomembranes. Methods in Enzymology. 148, 350-382.

Lindskog, S. 1997. Structure and mechanism of carbonic anhydrase. Pharmacology & Therapeutics, 74(1), 1–20. doi:10.1016/s0163-7258(96)00198-2

Lindqvist, N., Tuhkanen, T., Kronberg, L. 2005. Occurrence of acidic pharmaceuticals in raw and treated sewages and in receiving waters. Water Research, 39(11), 2219–2228. doi:10.1016/j.watres.2005.04.003

Martinez-Sena, T., Armenta, S., Guardia, M., Esteve-Turrillas, F. A. 2016. Determination of non-steroidal anti-inflammatory drugs in water and urine using selective molecular imprinted polymer extraction and liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis, 131, 48–53. doi:10.1016/j.jpba.2016.08.006

Meek, I. L., Van de Laar, M. A. F. J., Vonkeman, E. H. 2010. Non-Steroidal Anti-Inflammatory Drugs: An Overview of Cardiovascular Risks. Pharmaceuticals, 3(7), 2146–2162. doi:10.3390/ph3072146

Mennillo, E., Arukwe, A., Monni, G., Meucci, V., Intorre, L., Pretti, C. 2017. Ecotoxicological properties of ketoprofen and the S(+)-enantiomer (dexketoprofen): Bioassays in freshwater model species and biomarkers in fish PLHC-1 cell line. Environmental Toxicology and Chemistry, 37(1), 201–212. doi:10.1002/etc.3943

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., Hirsch, R. 2003. Occurrence of Neutral and Acidic Drugs In The Effluents Of Canadian Sewage Treatment Plants. Environmental Toxicology and Chemistry, 22(12), 2872. doi:10.1897/02-469 doi:10.1016/j.watres.2005.04.003

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance Trends Plant Sci., 7, 405-410 PMID: 12234732 52

Monaghan, P., Metcalfe, N. B. and Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters, 12: 75-92. doi:10.1111/j.1461-0248.2008.01258.x

Moons, A. 2005. Regulatory and Functional Interactions of Plant Growth Regulators and Plant Glutathione S-Transferases (GSTs). Vitamins & Hormones, 155–202. doi:10.1016/s0083-6729(05)72005-7

Moroney, J. V., Bartlett, S. G., Samuelsson, G. 2001. Carbonic anhydrases in plants and algae. Plant, Cell and Environment, 24(2), 141–153. doi:10.1111/j.1365-3040.2001.00669.x

Nkoom, M., Lu, G., Liu, J., Dong, H., Yang, H. 2019. Bioconcentration, behavioral, and biochemical effects of the non-steroidal anti-inflammatory drug diclofenac in *Daphnia magna.* Environmental Science and Pollution Research. doi:10.1007/s11356-018-04072-3

Nogueira, A. F., Pinto, G., Correia, B., Nunes, B. 2019. Embryonic development, locomotor behavior, biochemical, and epigenetic effects of the pharmaceutical drugs paracetamol and ciprofloxacin in larvae and embryos of *Danio rerio* when exposed to environmental realistic levels of both drugs. Environmental Toxicology. doi:10.1002/tox.22819

OECD. 2006. *Lemna sp*. growth inhibition test n. 221. OECD–Guideline for Testing of Chemicals, Paris, 22 p.

OECD. 2012. Test No. 211: *Daphnia magna* reproduction test, OECD guidelines for the testing of chemicals, section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264185203-en

Petrovic N., Murray M. 2010. Using N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to Assay Cyclooxygenase Activity In Vitro. In: Armstrong D. (eds) Advanced Protocols in Oxidative Stress II. Methods in Molecular Biology (Methods and Protocols), vol 594. Humana Press, Totowa, NJ

Piedade, F., Bio, S., Nunes, B. 2019. Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel *Mytilus* spp. Environmental Toxicology and Pharmacology, 103276. doi:10.1016/j.etap.2019.103276

Praskova E, Voslarova E, Siroka Z, Macova S, Plhalova L, Bedanova I, Marsalek P, Pistekova V, Svobodova Z. 2011. Comparison of acute toxicity of ketoprofen to juvenile and embryonic stages of *Danio rerio*. Neuro Endocrinol Lett. 2011;32 Suppl 1:117-20.

Prášková, E., Štěpánová, S., Chromcová, L., Plhalová, L., Voslářová, E., Pištěková, V., … Svobodová, Z. 2013. The effects of subchronic exposure to ketoprofen on

early developmental stages of common carp. Acta Veterinaria Brno, 82(3), 343–347. doi:10.2754/avb201382030343

Rangasamy, B., Hemalatha, D., Shobana, C., Nataraj, B., Ramesh, M. 2018. Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to anti-inflammatory drug ketoprofen. Chemosphere. doi:10.1016/j.chemosphere.2018.09.013

Ribeiro F., Ferreira, N. G. C., Ferreira, A., Soares, A. M. V. M., Loureiro, S. 2011. Is ultraviolet radiation a synergistic stressor in combined exposures? The case study of *Daphnia magna* exposure to UV and carbendazim. Aquat Toxicol 102(1–2):114–122. https://doi.org/10.1016/j.aquatox.2011.01.007

Rowley, A. F., Voga, C. L., Taylor, G. W., Clare, A. S. 2005. Prostaglandins in non-insectan invertebrates: recent insights and unsolved problems. Journal of Experimental Biology, 208(1), 3–14. doi:10.1242/jeb.01275

Schieber, M., Chandel, N. S. 2014. ROS Function in Redox Signaling and Oxidative Stress. Current Biology, 24(10), R453–R462. doi:10.1016/j.cub.2014.03.034

Smirnov, N. N. 2013. Physiology of the Cladocera. Academic Press, San Diego, CA, USA.

Smirnov, N. N. 2017. Physiology of the Cladocera. 2nd Edition. Elsevier. ISBN 978-0-12-805194-8

Stanley, D. 2006. Prostaglandins and Other Eicosanoids In Insects: Biological Significance. Annual Review of Entomology, 51(1), 25–44. doi:10.1146/annurev.ento.51.110104.151021

Sytar, O., Kumar, A., Latowski, D. et al. 2013. Heavy metal-induced oxidative damage, defense reactions, and detoxification mechanisms in plants. Acta Physiol Plant 35, 985–999 https://doi.org/10.1007/s11738-012-1169-6

Ternes, T. A. 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Research, 32(11), 3245–3260. doi:10.1016/s0043-1354(98)00099-2

Tixier, C., Singer, H. P., Oellers, S., Müller, S. R. 2003. Occurrence and Fate of Carbamazepine, Clofibric Acid, Diclofenac, Ibuprofen, Ketoprofen, and Naproxen in Surface Waters. Environmental Science & Technology, 37(6), 1061–1068. doi:10.1021/es025834r

Verpoorte, J. A., Suchinta, M., Edsall, J. T. 1967. Esterase activities of human carbonic anhydrase B and C. J. Biol. Chem. 242, 4221–4229.

Xie, X., He, Z., Chen, N., Tang, Z., Wang, Q., Cai, Y. 2019. The Roles of Environmental Factors in Regulation of Oxidative Stress in Plant. BioMed Research International, 2019, 1–11. doi:10.1155/2019/9732325

Wang, L., Peng, Y., Nie, X., Pan, B., Ku, P., Bao, S. 2016. Gene response of CYP360A, CYP314, and GST and whole-organism changes in *Daphnia magna* exposed to ibuprofen. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 179, 49–56. doi:10.1016/j.cbpc.2015.08.010

Wang, W. 1990. Literature review on duckweed toxicity testing. Environmental Research, 52(1), 7–22. doi:10.1016/s0013-9351(05)80147-1

Wrede, J. D. 2015. Pharmaceutically active compounds might bioaccumulate and cause effects on the free – floating macrophyte *Ceratophyllum demersum*. PhD thesis. Lebenswissenschaftliche Fakultät der Humboldt-Universität zu Berlin. 103p.

Chapter 6

Daphnia magna responses to fish kairomone and chlorpromazine exposures

Alkimin, G. D., Nunes, B., Soares, A. M. V. M., Bellot, M., Gómez-Canela, C., Barata, C. (2020). *Daphnia magna* responses to fish kairomone and chlorpromazine exposures. Chemico-biological Interaction. 109123. doi.org/10.1016/j.cbi.2020.109123

Daphnia magna responses to fish kairomone and chlorpromazine exposures

Alkimin, GD[1*], Nunes, .[1], Soares, AMVM [1], Bellot, M[2], Gómez-Canela, C[2], Barata, C[3]

[1] Centre for Environmental and Marine Studies (CESAM), Campus de Santiago, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] Department of Analytical Chemistry and Applied (Chromatography section), School of Engineering, Institut Químic de Sarrià-Universitat Ramon Llull, Via Agusta 390, Barcelona, 08017, Spain

[3] Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18, 08034 Barcelona, Spain

6.1 Abstract

To avoid being preyed, organisms must be able to identify predatory threats by sensing molecules released by predators (kairomones), and to employ effective strategies to avoid detection by predators. Furthermore, in the wild, organisms are also exposed to chemicals that may alter their behavioral traits, such as neuroactive pharmaceuticals. Considering the co-occurrence of both types of chemicals, their possible interaction needs to be studied. To address this topic, the aim of this study was to verify the effects of fish kairomone (FK – a chemical associated to putative predation by fish) and chlorpromazine (CPZ - neuroactive pharmaceutical drug, environmental contaminant), isolated and in combination, in different functional endpoints of *Daphnia magna*, such as oxygen consumption, feeding rate, behavior and reproduction. Among these endpoints, oxygen consumption was only affected by the combination of compounds (FK + CPZ). On the other hand, feeding rate was affected by all treatments, being smaller than control. For life history traits and phototactic behavior, the effects of FK predominated over the ones caused by CPZ exposure, incrementing the reproductive output of females t, leading to greater population growth rates and decreasing swimming behavior.

Keywords: Predation; pharmaceutical effects; oxygen consumption; feeding rate; reproduction; behavior swimming.

6.2 Introduction

Among aquatic animals, chemical signals can mediate many intra- and inter-specific interactions (DeMile et al., 2016) and even visual species respond strongly to such cues (Hay, 2009). In aquatic systems, chemical cues are recognized as being highly important for the detection of predators, thereby determining prey survival (Mirza and Chivers, 2000). Alarm cues and predatory cues (i.e. kairomones) are the first two primary stimuli extracted from the sensory landscape as information about the potential predation (Schoeppner and Reylea, 2009). In addition, many predator–prey relationships are mediated by the release and detection of kairomones. The importance of these agents as chemical mediators is extreme, since they are responsible for the induction of different responses, such as modifications in behavior, feeding, and reproduction (Lima and Dill, 1990).

A biological interaction during which one organism kills and feeds on another organism is one of predation. This relationship not only shapes natural ecosystems but is also likely to occur in anthropogenically impacted environments (Birkhofer et al., 2017). To avoid becoming someone's meal, an organism must be able to identify predatory threats and employ effective strategies to avoid being detected by predators. In the event that such avoidance fails, animals can trigger behaviors to prevent predators from attacking, and also deploy tactics that will increase their chances of surviving the attack, in the case if it does occur (Rosier and Langkilde, 2011). This can be achieved by avoiding the same habitat that is occupied by predators, or by being active at different times of the day or altering the behavioral activity in response to changes in predatory risk (Rosier and Langkilde, 2011), among others. Besides these behavioral alterations, predator-induced responses are important aspects of the population and even community ecology of freshwater zooplankton (Dodson, 1989). This happens since a good perception of danger and a correct anti-predator strategy should increase the chances of survival at the population level, regardless the species-specific reaction (Šmejkal et al., 2018).

Furthermore, in the wild individuals interact with other individuals of the same (or of other species) and are exposed to chemicals. Among the array of environmental pollutants, one may find pharmaceutical compounds, which can alter population dynamics, animal behavior, reproduction, health and other features (Daniel et al., 2018; Falcão et al., 2019). In terms of behavioral alterations, especially those drugs with neuroactive properties are particularly troublesome. Pharmaceutical drugs are a global concern in aquatics environments, since the number of these chemicals that are found in the wild is ever increasing (aus der Beek et al., 2016). One of these pharmaceuticals is chlorpromazine (CPZ), a low-cost aliphatic phenothiazine, widely available and used in treatments of psychiatric disorders worldwide, including schizophrenia (Adams et al., 2014). Its primary antipsychotic activity is due to the blockade of dopamine (D2) receptors in the mesolimbic pathway of the brain (Bryan, 2011). This pharmaceutical has been shown to cause toxicological effects in non-target organisms, such as macroinvertebrates (Oliveira et al., 2015a), macrophytes (Alkimin et al., 2019), and fish (Li et al., 2008), and it has been found in different aquatic compartments in concentrations ranging from 1 to 364 ng/L (Roberts and Bersuder, 2006; Yuan et al., 2013).

The toxic effects of environmental chemicals needs a better and more realistic comprehension about their consequences and their impact; to attain this purpose, the organisms must be analyzed in more complex interaction scenarios, mimicking the actual and more realistic conditions of the ecosystem in which organisms live in (Beklioglu et al., 2010). Ecotoxicological studies focusing on the crosstalk between predation avoidance (and the effects of chemicals that act as chemical signals during this intercourse) and pharmaceuticals are adequate to study the joint contribution of natural and anthropogenic stressors. Despite the increasing number of studies dealing with the effects on living organisms caused by a large set of both natural and anthropogenic stressors, the characterization of these two combined factors is still scarce, despite being recognized as very important to understand how environmental contamination can affect the natural responses of aquatic animals to predation. *Daphnia* species are sensitive to a large number of substances and can be easily cultured under laboratory conditions, being therefore very useful in ecotoxicology as a model organisms (Bownik, 2017). In addition, *Daphnia* species seem particularly sensitive to

chemicals that are released by their predators, and somehow involved in their predation. The adaptive responses induced by predator kairomones are a key element in the modulation of individual fitness, which is the result of the contribution of both survival and reproduction success. Therefore, the success of a population is the consequence of individual survival and fitness, consequently, for the maintenance of *Daphnia* populations in aquatic ecosystems, the protection conferred by kairomone-mediated antipredator defenses is vital (Barry, 2002).

Daphnia species are ecologically important due to their role in many aquatic food webs. *Daphnia* act as primary consumer, and thereby control populations of primary producers; in addition, they also provide a food source for many secondary consumers (DeMille et al., 2016). Consequently, the responses of *Daphnia* towards aquatic contamination and/or kairomones may affect the entire ecosystem. To address this topic, the aim of this paper was to verify the effects of fish kairomone (indicative of predation) and chlorpromazine (environmental contaminant), alone and in combination, in different functional endpoints in *Daphnia magna*.

6.3 Material and methods

6.3.1 Chemicals

Chlorpromazine hydrochloride (CAS: 69-09-0) was purchased from Sigma-Aldrich (USA) with 98% of purity. All other used reagents had analytical purity.

6.3.2 *Daphnia* culture and maintenance

Clone $P_1$32,85 was obtained from two generations of intraclonal mixes within clone P_1, isolated from a small pond which contained fish (located at Driehoeksvijver, Heusden, The Netherlands; animals were isolated in August 1986). According to De Meester (1993) organisms from this clone, in the presence of fish kairomones, became negatively phototactic. Cultures of 10 animals/L were maintained in ASTM hard water (ASTM, 1994) and fed every other day with 5×10^5 cells/mL of the freshwater algae of the species *Raphidocelis subcapitata*. Three times a week the culture medium was

renewed and a set up to of 14h:10h light:dark cycle and 20 ± 2 °C was used as photoperiod and temperature, respectively.

6.3.2 Chemical stability experiment

The stability of the studied chemicals in ASTM water was assessed measuring the concentration of 1000 µg/L of the tested chemicals in water in freshly prepared and old (24, 48 h) test solutions by liquid chromatography connected to a triple quadrupole detector (Xevo TQS, Acquity Waters, Mildford, USA) (LC-MS/MS). The analysis was performed in selected reaction monitoring (SRM) mode using two transitions from de precursor ion to the product ion to identify each compound. The system and data management were processed using MassLynx v4.1 software package.

6.3.3 Obtaining Fish kairomone

To obtain fish kairomone (FK), two 8 cm juvenile fish (*Leuciscus idus*) were allowed to swim in a12 L aquarium, filled with ASTM hard water medium for 24 h, after which the medium was filtered (0.045 µm). This procedure was repeated for every experiment, to use a fresh FK solution for every experiment. Only for the reproduction tests, a single FK preparation was used in three media changes. This method is routinely used to simulate fish predation risk (De Meester and Cousyn, 1997; Jansen et al. 2013). The kairomone dilution was roughly to 1 fish in 50 L.

6.3.4 Experimental conditions

The experimental conditions for *D. magna* exposures were defined as control, FK (fish-kairomone conditioned water); CPZ (10 µg/L of CPZ); and the combination of both treatments (FK + CPZ). These conditions are designated along this study as Control, FK, CPZ, and FK + CPZ respectively. The dilution of 1:8 of FK was chosen in order to avoid interference of ammonia on the toxic effects elicited on *D. magna*, as demonstrated by previous experiments performed by our research group; and the concentration of 10 µg/L of CPZ was chosen after the analysis of the results obtained for the behavior preliminary tests (data not shown).

6.3.5 Respiration assay – oxygen consumption

Respiration rate was assessed by measuring the oxygen consumption through the difference between the initial and final oxygen concentrations and converted to proportional oxygen (%) relative to control. To attain this purpose, five replicates with four 15 d old synchronized daphnids (Clone $P_1$32,85) per experimental treatment were used. The animals were maintained during 24 h in the dark at 20 ± 2 ºC in 50 mL gastight syringes (Hamilton, USA – final volume of 30 mL of test-solution). Oxygen interface (model 928, Strathkelvin Instruments, Glasgow, UK) was used to measure initial and final oxygen concentration. Media with no daphnids (blank controls) for each treatment were used to correct natural oxygen depletion and to avoid the interference by algae oxygen consumption no food was added.

6.3.6 Feeding rate (F)

The test-chambers (five replicates per treatment) consisted of 100 mL glass flasks filled with test-solution, each replicate was composed by five 15 d old daphnids (Clone $P_1$32,85). Briefly, the animals were submitted during 24 h, in the dark, to all experimental conditions, with $5x10^5$ cells/mL of *R. subcapitata* in the test-chambers. Afterwards, the animals were transferred to a new test-chamber (clean media), also in the dark, for 4 h with the same algal concentration (post-exposure period) to evaluate the recovery from exposure to toxicants. Blank controls (media with no daphnids) were included in both cases. At the end of each period, the algal concentrations were spectrophotometrically measured (Du® 730) at 440 nm (Allen et al., 1995). Finally, the feeding rate was calculated converting the obtained values to proportional algae consumption (%) relative to control.

6.3.7 Swimming behaviour assay

The swimming tracks of the 15 d old females from clone $P_1$32,85 pre-exposed for 24 h to the four studied treatments were assessed using the arena video tracking system described by Simão et al. (2019). The pre-exposure period was chosen taking into account that after 3 h of FK exposure, it is possible to detect changes in behaviour of

daphnids, according to De Meester and Cousyn (1997). Groups of five adults were pre-exposed to the tested treatments in 100 mL media containing food *(R. subcapitata*, $5x10^5$ cells/mL) for 24 h prior to behavioral tests, each with five replications. Five behavioral trials were performed per treatment. In each trial, groups of five organisms from two different randomized treatments were distributed among the two arenas filled with 50 mL of test solution. Animals were then acclimated in the dark (only infrared light on) for 5 min before video recording. For the behavioral analysis, animals were initially subjected to a dark period (5 min), and then to a moderate light intensity period (10 lux, 15 min,). The software EthoVision XT12 video tracking (video-recording at 20 frames per s) was used as a tool to analyze the position changes of each animal. The first step of this procedure was of dividing the arenas in three virtual zones: bottom, middle and top, to simulate a water column. Then, the social interaction module was used to analyze, individually, tracks of 5 organisms and to determine time spent in the top of water column (virtual zone determined in the arena; %), total distance moved (cm) and the distance inter-organisms (aggregation) measured by the average distance (cm) among individual animals in the arena. For statistical analysis three periods were considered: the last two minutes of the dark period, the first two and the last ten minutes of light period, hereafter referred as dark, first and late light, respectively. Values were determined per min. In addition, the used software measures the complete tracking in the two dimensions. So, it also includes the different types of organisms' behavior, with the exception of the rotational behavior of animals (turning around, without displacement in the x-y axis). Besides, the use of 15 d old organisms was necessary to ensure that test organisms had a minimum size to be reliably detected by the hardware + software. This age was also chosen, since it corresponds to the complete development of phototaxis. Finally, animals with the same age were used in the feeding and oxygen consumption tests, to allow the possibility of comparing data from all these endpoints, and, in our laboratory conditions, 15 d old organisms have an average size of 2.76 ± 0.2 cm (N = 56, Mean ± SD).

6.3.8 Reproduction assay

Reproduction test was conducted according to OECD recommendations (OECD, 2012). Organisms less than 24-h old were exposed, individually, in 100 mL glass flasks, to

each experimental condition and a total of 10 replicates per condition was used. The organisms were maintained and fed as previously described in *Daphnia* culture and maintenance section and every other day the medium was renewed. Throughout the 21-d assay period the individuals were monitored daily and the endpoints evaluated were: total number of neonates, brood number, time to first brood, neonates in the first brood and Intrinsic rate of population increase (*r*), this last one calculated using the Euler's ($1=\sum^{n}_{x=0} e^{-rx} l_x m_x$) where *r* is the population increase rate (per day), x is the age in days (0 … n), lx is the survival probability in the age x, and mx is the fecundity in the age x. Pseudo-values from replicates were generated using the jackknife procedure (Meyer et al., 1986).

6.3.9 Data analysis

Treatment effects on life-history and behavioral traits, proportional oxygen consumption and feeding rates were compared against the control by one-way ANOVA followed by post-hoc Dunett's test. Prior to analyses, data were checked for ANOVA assumptions of normality and variance homoscedasticity. After transformation, not normally distributed data were compared using a Kruskal-Wallis test followed by Wilcoxon and Wilcox test (Zar, 1996). These analyzes were performed using the statistical software SigmaPlot 14.0 and the adopted significance was $p < 0.05$.

6.4 Results

Obtained results indicated that the tested compound was stable in ASTM water since concentrations in freshly prepared solutions (N = 4, Mean ± SE, 1092.8 ± 10.4 µg/L) were similar to those aged for 24 (1073.5 ± 5.6 µg/L) and 48 h (1063.2 ± 11.5 µg/L).

The oxygen consumption was smaller ($F_{3;12} = 4.091$, $p<0.05$) than control in the combination (FK + CPZ) (fig. 1). On the other hand, feeding rates (fig. 2) were significantly smaller ($p<0.05$) in all treatments, during the exposure ($F_{3;16} = 4.825$) and post-exposure ($F_{3;15} = 5.444$) periods, relative to controls.

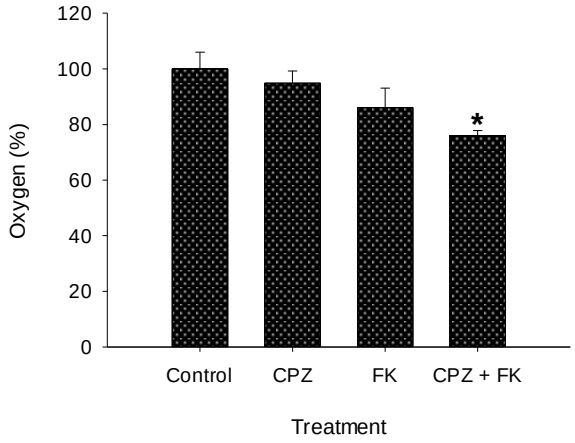

Figure 1. Effects of chlorpromazine (CPZ), of fish kairomone (FK) and of the mixture (CPZ+FK) on *D. magna* oxygen consumption. For each parameter, mean and standard error are shown. * stands for statistical differences in relation to respective control following ANOVA and post-hoc test (or the equivalent non-parametric test).

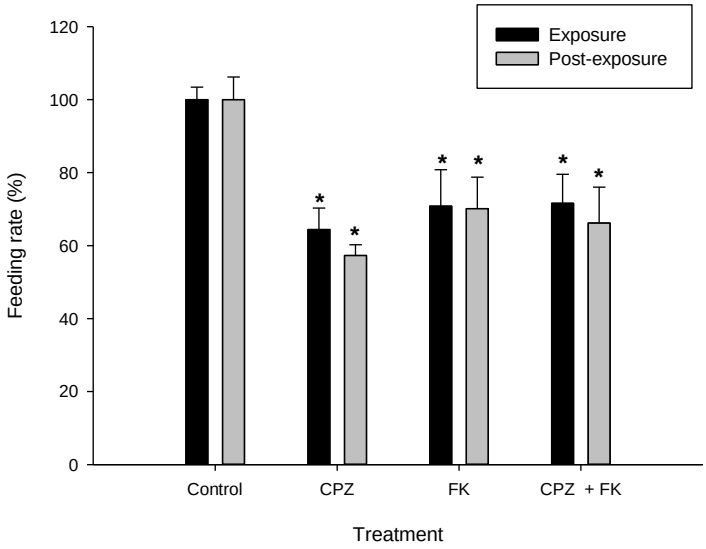

Figure 2. Feeding rate (cells/h/org) of *D. magna* exposed to chlorpromazine (CPZ), to fish kairomone (FK) and to the mixture (CPZ+FK). For each parameter, mean and standard error are shown. * stands for statistical differences in relation to respective control following ANOVA and post-hoc test (or the equivalent non-parametric test).

Figure 3 includes the behavioral responses per min in the left panel graphs (fig. 3A, C, E) and grouped by the three analyzed periods (right panel graphs, Fig 3B, D, F). Females exposed to fish waterborne kairomone of fish (FK) alone or with CPZ were situated closer to the bottom (fig. 3A) when light was present, and those exposed to FK

and CPZ moved less and aggregated less when light was on (fig 3C, E). Consequently, there were significant differences in the % of time that animals swam close to the surface, between FK and FK+CPZ treatments and controls ($F_{3;87}$ = 17.660, p<0.001, fig. 3B). The combination of CPZ+FK was able to decrease significantly the moved distance in all analyzed periods (dark – $F_{3;81}$ = 8.945, p<0.001; fist light – H_{3df} = 18.359, p<0.001; late light – $F_{3;79}$ = 12.934, p<0.001) (fig. 3D). In addition, FK exposure caused a significant decrease (H_{3df} = 13.051, p = 0.005) in the inter-organismic distance in the dark, while CPZ+FK caused an increase (H_{3df} = 22.015, p<0.001) in a late light period (fig. 3F).

The cumulative number of neonates per female (fig. 4A) was significantly higher (H_{3df} = 16.037, p<0.001) in females exposed to FK treatments (FK, CPZ+FK), whereas the clutch size of the first brood was larger in all exposed females ($F_{3;34}$ = 4.592, p<0.01, fig. 4B) in comparison to control. CPZ, alone, was capable of causing a delay (H_{3df} = 25.497, p<0.001; fig. 4C), approximately of 4 days, in the age of the first brood. This pharmaceutical was also responsible for a decrease (H_{3df} = 18.145, p<0.001) in the total number of broods (fig. 4D). Finally, the population growth rate (fig. 4E) was significantly higher (H_{3df} = 36.018, p<0.001) in organisms exposed to FK (FK, FK+CPZ).

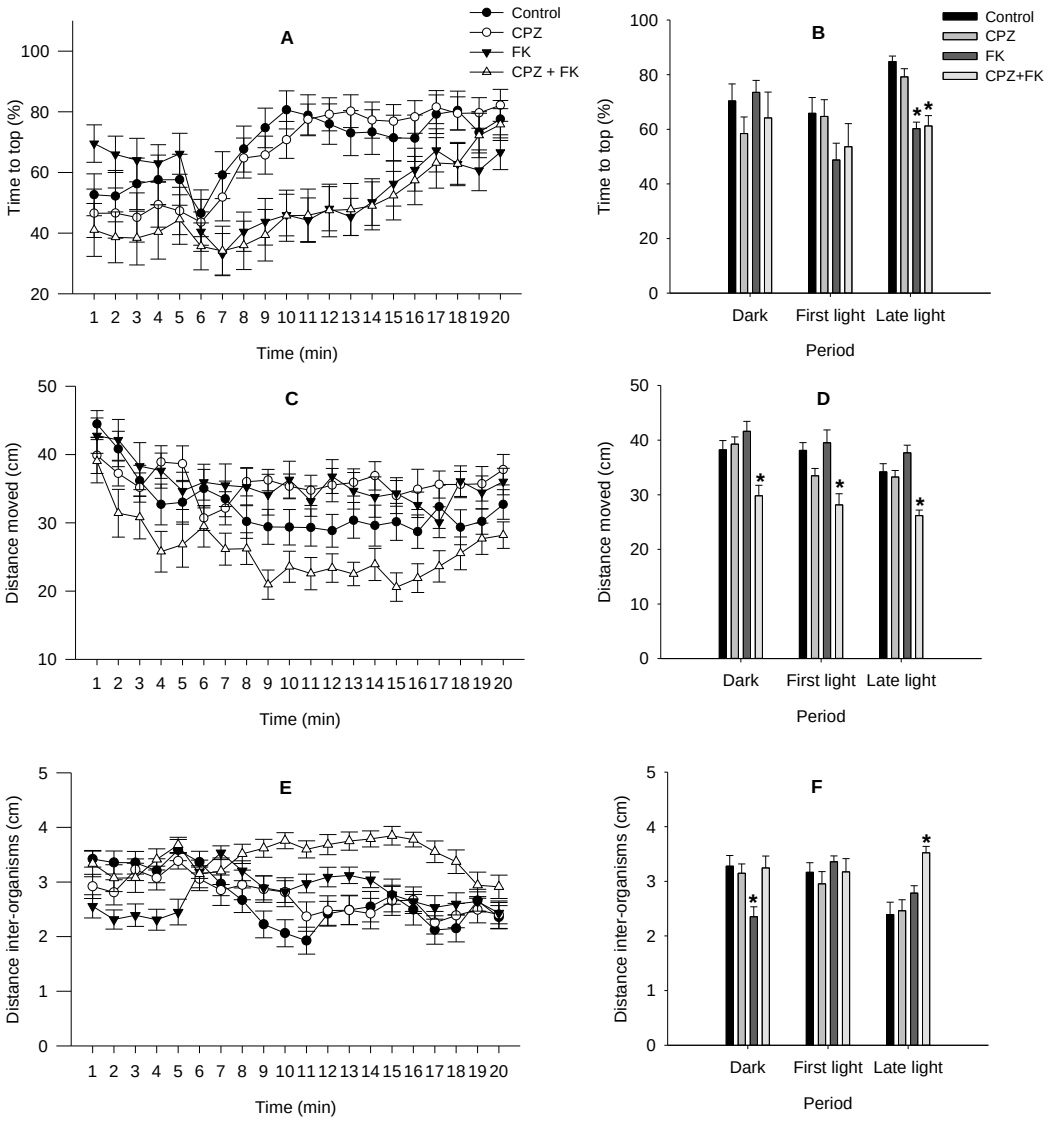

Figure 3. Effects of chlorpromazine (CPZ), of fish kairomone (FK) and of the mixture (CPZ+FK) on *D. magna* behavior, plus control. (A) and (B) correspond to time to top (%), (C) and (D) to distance moved (cm) and (E) and (F) to inter-organisms distance. (A), (C) and (E) correspond to the results on the integra and (B), (D) and (F) to the results divided in categories (dark, first light, and late light). For each parameter, mean and standard error are shown. * stands for statistical differences in relation to control following ANOVA and post-hoc test (or the equivalent non-parametric test).

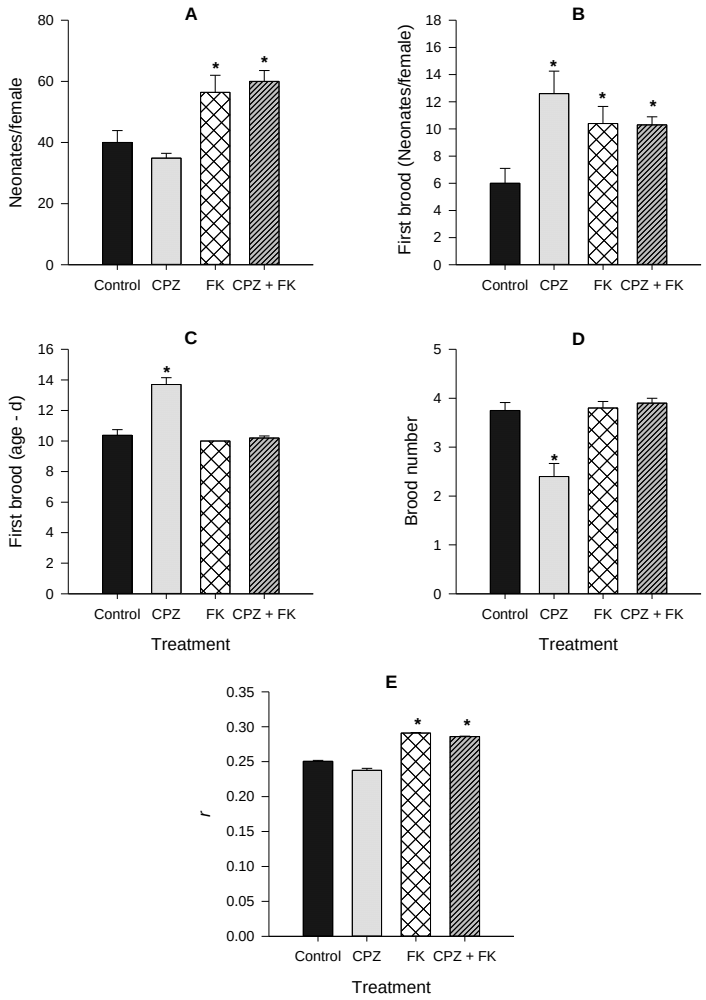

Figure 4. Effects of chlorpromazine (CPZ), fish kairomone (FK) and the mixture (CPZ+FK) in *D. magna* reproductive parameters. (A) Total number of neonates per female; (B) Total number of neonates in the first brood; (C) Age of the first brood (d); (D) Total number of broods and (E) Population increase rate per day (*r*). For each parameter, mean and standard error are shown. * stands for statistical differences in relation to control following ANOVA and post-hoc test (or the equivalent non-parametric test).

6.5 Discussion

Zooplanktonic organisms, and in particular, *Daphnia* species, have been shown to exhibit responses to predator-specific chemicals (i.e. kairomones) in different parameters, as life history and behavioral traits (Larsson and Dodson, 1993). However, the association of this natural stressor with anthropogenic ones (i.e. pharmaceuticals) is less studied, and this study brings some perspectives about this potential association. The here-adopted strategy is a comprehensive framework that includes a series of multiple endpoints with high physiological importance, whose impairment may result in strong population effects.

As a sensitive metabolic biomarker and a high potential for toxicity screening, monitoring oxygen consumption can be an advantageous tool when compared to mortality (Zitova et al., 2009). In *Daphnia* spp. the oxygen consumption, in the absence of food, is an important tool to provide estimates about costs of physiological maintenance, which include swimming/ventilation and basal metabolism, activities that are essential to life (Campos et al., 2012). In this study, the oxygen consumption was smaller when the organisms were exposed to the mixture, that is, less oxygen was consumed. This can be an indication that anthropogenic and natural stressors can interact and cause metabolic alterations in exposed animals. Although not significantly, oxygen consumption in CPZ and FK alone were smaller than control, thus it is possible to hypothesize that the joint effect of both stressors was additive. Concomitant with the reported oxygen effects, females exposed to the combination of FK and CPZ also moved less, thus, requiring lower oxygen consumption. In addition, another explanation for the here observed effects is the relationship between oxygen consumption and distance moved; both analyzed endpoints suffered a decrease after exposure to the combination FK+CPZ. Thus, these two effects possibly indicate that impaired oxygen consumption is associated with shorter distance moved, i.e. reduced swimming activity.

In metabolic or respiration rate, food ingestion is an energetically expensive action and can represent up to 40% of this expense of energy consumption (Philippova and Postnov, 1988; Barber et al., 1994). The maintenance costs may increase in the presence of pollutants by increasing the rate of detoxification, a demand side effect. However, more often, these compounds decrease metabolic rates inhibiting food ingestion (supply side effects) when food is present (Barber et al., 1994). Beside that, a functional and fitness link between food impairment and feeding behavior can be a crucial endpoint measurable through the feeding performance (Loureiro et al., 2012) which is related to the reproductive outputs (Pieters et al., 2005; Barata and Baird, 2000). Filter-feeding zooplanktonic organisms demand movement of appendages coordinated by the nervous system to assure food filtration (Villarroel et al., 1999). During this process, *Daphnia*'s feeding apparatus repeats constantly these movements, but not in the same intervals, making it a dynamic system (Peñalva-Arana et al., 2007). Therefore, loss of coordination can be caused by toxicants affecting the nervous system and consequently reducing rates of filtration, as observed in this study. This hypothesis may be

corroborated by Rocha et al. (2014) after exposing *D. magna* to neuroactive compounds such as neostigmine and pyridostigmine, which compromised neuronal transmission by cholinesterasic impairment, altering behavioral features. Additionally, fish predation can represent a risk to prey animals, and this stimulus can trigger an adaptation of prey defense; this should represent a balance between avoiding being preyed (benefit), and reductions in fitness (cost) caused by the deployment of the defensive measures (Ramcharan et al., 1992) and consequently a reduction in the feeding rate. Related to post-exposure period (4 h of recovery), daphnids presented similar results, with a reduction in feeding rate in all experimental conditions in comparison with the control. This result shows that in the time frame here adopted, the organisms were not capable to recover from the exposure in any of the tested conditions. This result is in line with data obtained by Villarroel et al. (1999) and Mc William and Baird (2002) after exposing *D. magna* to several contaminants. Overall, the here reported results for oxygen consumption, feeding and swimming speed indicated that, upon the presence of fish kairomone in water, females moved to a lesser extent, hence reducing their oxygen consumption and feeding rates. These parameters have been investigated in other researches, e.g., Pestana et al. (2013) also found a decrease in filtration after a 24 h exposure to FK and to alarm cues (crushed *Daphnia*), however, the authors reported an increase in oxygen consumption. These differences can be assumed by the age of exposed animals and the use of different clones. Contrary to the present study Pestana et al. (2013) used 4 d old juveniles, while our study was conducted with 15 d old adults, and behavior differences can be related with the daphnids age and genotype (De Meester, 1991, 1992). These effects, in general, result from reversible behavioral responses, which can include changes in feeding behavior, increased vigilance, decrease activity, alertness and change in the swimming speed (Pijanowska, 1997). All the above-mentioned behaviors can alter respiration and feeding rates.

A phototactic response is an orientated reaction to light stimuli (Ringelberg, 1964). In *D. magna* this characteristic is heritable (varying from clone to clone), and can be distinguished between positively, intermediately and negatively phototactic genotypes (De Meester, 1991). Phototactic-positive genotypes spend much time in the upper water level, whereas phototactic-negative genotypes spend most of their time close to the bottom sediments (Ebert, 2005). The behavior of juveniles is less clone-specific than

that of adults (De Meester, 1992). The here studied clone is a phototactic-positive genotype, thereby explaining why organisms spent most of the time in the top of water column. Thus, this behavior was affected by FK in late light exposure, since the animals remained less time near the top of the water column. This happened because, in general, the phototactic behavior may be modulated by environmental factors, such as the presence of predators, temperature and food availability, which are likely to fluctuate continuously in the wild (Michels et al., 1999), and the fish presence can increase the phototactic negative response of organism (Eber, 2005). On the other hand, and according to Barrozo et al. (2014) there are some evidences that specific dopaminergic signaling pathways are involved in the decrease of *Daphnia* swimming behavior. This trend confirmed after exposing these organisms to neurotoxins that specifically act on such dopaminergic neurotransmission route. Nevertheless, CPZ, a dopamine antagonist, was not capable of changing the phototactic behavior of daphnids, either alone or in combination with the natural stressor (FK), since animals exposed to the combination (CPZ+FK) presented the same behaviour was when exposed only to FK. This similar trend possibly indicates that no interaction between stressors occurred, concerning this specific endpoint.

In general, various mechanisms of action can be affected by neurotoxic substances, which may result in depression or excitation of the nervous system, which in turn may induce opposite effects on the cumulative moved distance (Bownik, 2017). Chlorpromazine is a dopamine antagonist, mostly blocking dopamine receptors, thus, blocking these receptors causes diminished neurotransmitter binding in the forebrain and consequently cause a depression in the nervous system. Considering that daphnids also have dopaminergic neurotransmission (Hartline and Christie, 2010), it is possible to suggest a similar pathway. However, these effects seem not to have happened in this study when the organisms were exposed to the pharmaceutical alone. Interestingly, only the combination of CPZ with waterborne fish kairomone (and not both stressors alone) decreased swimming speed; light also decreased aggregation rates. Both effects may diminish the ability of *D. magna* females to escape from fish predation since most fish prefer to prey on isolated prey that swim slower (Allen, 1920). It is possible that an additional stress, FK in this case, can consequently increase the *D. magna* susceptibility to CPZ toxicity. This type of interaction was also reported by other studies: the presence

of predator increased the mortality of tadpoles when exposed to carbaryl (Relyea and Mills, 2001) and predation was responsible for an increase of the toxic effects provoked by esfenvalerate in *Artemia* sp., resulting in a population decrease (Beketov and Liess, 2006). However, evaluations regarding the putative effects of xenobiotics (namely chemicals whose environmental presence derives directly from anthropogenic contaminant) considering simultaneously the occurrence natural compounds (e.g. FKs), are still scarce within the scope of plankton ecotoxicology (Guitierrez et al., 2012). This is aggravated if one considers only studies focusing on the interaction of such FKs with pharmaceutical drugs, since most of the studies conducted so far were carried out with metals and pesticides (Gutierrez et al., 2012; Hanzato, 1999; Scherer et al., 2013; Pestana et al., 2010). On the other hand, the prediction of potential effects of these combinations, and the characterization of such interactions, can be a complicated issue since the nature and origin of FKs was not fully elucidated yet, despite the efforts of several work teams. Ringelberg and Van Gool (1998) suggested that FKs may in fact be a bacteria present in the fish skin. In addition, Boriss et al. (1999) proposed that trimethylamine, a tertiary amine produced by fish, is the compound responsible for triggering the defensive behavior of *Daphnia* against predatory fish. In addition, other chemicals have been identified, being extracted from fish water; these chemicals are 5-hydroxidy-4-decanolide (Tjossem, 1990), 6-hydroxy-5-tetradec-2-enolide (Lass et al., 2001) and phenylalanine-proline diketopepirazine (Dawidowicz et al., 1990). However, as previously cited, these are assumptions, and the complete nature, origin, and composition of FKs are still unclear. Furthermore, the precise fundamental pathways by which kairomone perception occurs in daphnids remain to be determined, which is a critical step for the understanding of mechanisms, which are activated and induce *Daphnia* defenses (Weiss et al., 2015a). However, these same authors showed that, in *D. longicephala*, the first antennae are a location of kairomone-detecting chemoreceptors, a finding in agreement with data for other cladocerans (Ekerholm and Hallberg, 2002; Hallberg et al., 1992). We are thus able to hypothesize that this mechanism, being conserved, may also occur in *D. magna* as one of the pathways for the detection of kairomones. Considering the absence of information about the putative mechanisms of action of these kairomones in freshwater crustaceans, it is impossible to predict the type and extent of the interaction of kairomones with environmental toxicants. However, kairomones are able to trigger neuronal responses, which are

responsive without any pre-existent learning process. In rodents, this chemosensing system exists in specialized structures, such as the vomeronasal organ and the main olfactory epithelium. These receptors are specialized in sensing kairomones from the external media, sending information about their presence for central integration (Stowers and Kuo. 2016). The underlying mechanisms of chemosensing of kairomones, and the subsequent biological response, are still not elucidated in rodents, and are not likely to have been also described in crustaceans. But it is possible to suggest that, being based on neuronal networks, these processes are likely to be affected and compromised by the exposure to neuroactive chemicals, including pharmaceutical drugs such as CPZ.

Many chemicals are able to decrease reproduction and delay first reproduction (brood) (Heckmann et al., 2007; Oliveira et al., 2015b), often by impairing food acquisition (Barata and Baird, 2000). There are, however, trade-offs between producing more offspring and delayed reproduction (Barata and Baird, 1998). In this study, CPZ delayed reproduction and increased the offspring production in the first brood, but not in subsequent ones. As a result, females exposed to CPZ had equivalent levels of population growth rates (*r*) as control ones. Additionally, predators' exudates can exert a selective pressure in planktonic animals and, in response, many morphological aspects, life-history and behavioural traits can suffer changes (Pijanowska and Stolpe, 1996). In aquatic environments size-selective predation can greatly influence both community and population dynamics by altering the onset of reproduction, the size of the first clutch and hence the age structure of population (Weider and Pijanowska, 1992). For example, fish prefer to predate on larger prey, thus *Daphnia* individuals should reproduce more efficiently and earlier upon fish predation (Barata et al., 2001; Riessen, 1999). Our results are in agreement with previous studies since females exposed to FK alone, or to FK and CPZ, produced more offspring in the first clutch. Again, CPZ did not affect life-history responses when combined with FK. Following detection, predator-specific signals are transferred via independent neurosignalling pathways (Mitchell et al., 2017). Responses of *D. pulex* e. g. to fish predators are mediated via the suppression of the inhibitory GABAergic pathways, resulting in the release of ecdysteroids (Dennis et al., 2014; Weiss et al., 2012, 2015b). Ecdysteroids promote the reallocation of energy from somatic growth to reproduction, which results in the life-history changes described above (Mitchell et al., 2017). The here obtained

results show this trend. This outcome occurred even when the exposed organism had the same number of broods than control animals, confirming the reallocation of energy provoked by ecdysteroids to reproduction. This trend also supports the hypothesis formulated by Stibor (2002): the predator can induce life history shifts, which are not the result of changes in metabolism but rather the result of changes in energy allocation induced early in the development, as previously cited. In addition, the interaction between CPZ and FK resulted in the alteration of some life history parameters; this effect may be associated to the known CPZ mode of action, which occurs by blocking dopamine (D2) receptors (Bryan, 2011). This is of critical importance, since the dopaminergic systems in daphnids are involved in the recognition of predator (by sensing FKs), and in triggering the defensive system. The dopaminergic system of daphnids seems to be interconnected with endocrine pathways, namely in the control of juvenile hormone and ecdysone levels, which are important in the physiological regulation of crustacean growth, thereby causing significant alterations in the life history traits of these organisms (Weiss et al., 2015b). Furthermore, Tams and co-workers (2019) suggested that the life history alterations in crustaceans are not necessarily related to changes in gene expression caused by exposures to predator. This relationship can occur just marginally, and may also occur by additional posttranslational processes, such as miRNA-mediated gene regulation or increased degradation (Schwarzenberger et al., 2009). However, we must assume that all mechanisms of prey-predator interactions have not been fully elucidated. Finally, the non-consistent patterns of response in terms of several endpoints here analyzed may be attributed to the specificity of the mechanisms of action, both of the drug and of the FK itself. In addition, the mechanisms by which CPZ and FK interact remain little elucidated in the literature. Thus, more studies of this nature are required to understand the mode of action of isolated chemicals, and underlying mechanisms of drug+FK interaction, that may serve as the basis for the here-observed modifications.

6.6 Conclusions

In summary, the tested natural and anthropogenic stressors (FK and CPZ, respectively) acted jointly decreasing oxygen consumption rates, swimming speed and aggregation, but operated independently on feeding. Concerning life history traits and phototactic

behavior, the effects of FK predominated over those of CPZ, making females reproduce to a greater extent and hence having greater population growth rates and also decreasing positive phototactic behavior. Finally, according to the analyzed endpoints, it is also possible to assume that these two stressors are capable to interact, since some of the analyzed parameters were only altered in organisms exposed to the combination of both stressors. This assumption indicates the ecological importance of the here obtained data, and of this type of study. Consequently, more studies with this type of combination are necessary to understand how aquatic organisms can act/react when challenged by two different risk sources in the environment. However, it is necessary to take into account that in the environment, organisms are challenged by more risks of predation (other predator organisms are present) and by more drugs. Consequently, it is difficult to predict the response resulting from interactions in the natural environment.

6.7 References

Adams, C. E., Awad, G. A., Rathbone, J., Thornley, B., Soares-Weiser, K. 2014. Chlorpromazine versus placebo for schizophrenia. Cochrane Database of Systematic Reviews 1. DOI: 10.1002/14651858.CD000284.pub3

Alkimin, G. D., Daniel, D., Frankenbach, S., Serôdio, J., Soares, A. M. V. M., Barata, C., Nunes, B. 2019. Evaluation of pharmaceutical toxic effects of non-standard endpoints on the macrophyte species *Lemna minor* and *Lemna gibba*. Sci Total Environ 657, 926–937

Allen, W.E. 1920. Behaviour of loon and sardines. Ecology, 1, 309-310.

Allen, Y., Calow, P., Baird, D. 1995. A mechanistic model of contaminant-induced feeding inhibition in *Daphnia magna* Environ. Toxicol. Chem., 14 1625-1630

ASTM. 1994. Standard guide for conducting renewal life - cycle toxicity tests with *Daphnia magna*. ASTM (Eds.), Annual Book of ASTM Standards, E 1193–94, American Society of Testing and Materials, Philadelphia, PA, pp. 512-518

Aus der Beek, T., Weber, F.-A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A. 2016. Pharmaceuticals in the environment-Global occurrences and

perspectives. Environmental Toxicology and Chemistry, 35(4), 823–835. doi:10.1002/etc.3339

Barata, C., Baird, D. J. 2000. Determining the ecotoxicological mode of action of toxicants from measurements on individuals: Results from short duration chronic tests with *Daphnia magna* Straus. Aquatic Toxicology, 48, 195– 209.

Barata, C., Baird, D. J., 1998. Phenotypic plasticity and constancy of life-history traits in laboratory clones of *Daphnia magna* straus: Effects of neonatal length. Functional Ecology 12, 442-452.

Barata, C., Baird, D., Soares, A., 2001. Phenotypic plasticity in *Daphnia magna* straus: Variable maturation instar as an adaptive response to predation pressure. Oecologia 129, 220-227.

Barber, I., Baird, D. J., Calow, P., 1994. Effect of cadmium and ration level on oxygen consuption, RNA concentration and RNA-DNA ratio in two clones of *Daphnia magna* Dtraus. Aquatic toxicology 30, 249-258.

Barrozo, E., Fowler, D., Ingebretson, J., Beckman, M. 2014. The role of dopaminergic signaling in *Daphnia magna* swimming. FASEB 28, 839– 845.

Barry, M. J. 2002. Progress toward understanding the neurophysiological basis of predator-induced morphology in *Daphnia pulex*. Physiol. Biochem. Zool. 75, 179-186. doi:10.1086/339389

Beketov, M. A., Liess, M. 2006. The influence of predation on the chronic response of *Artemia* sp. populations to a toxicant. Journal of Applied Ecology, 43(6), 1069–1074. doi:10.1111/j.1365-2664.2006.01226.x

Beklioglu, M., Banu Akkas, S., Elif Ozcan, H., Bezirci, G., Togan, I. 2010. Effects of 4-nonylphenol, fish predation and food availability on survival and life history traits of *Daphnia magna* straus. Ecotoxicology, 19(5), 901–910. doi:10.1007/s10646-010-0470-7

Birkhofer, K., Bylund, H., Dalin, P., Ferlian, O., Gagic, V., Hambäck, P. A., et al. 2017. Methods to identify the prey of invertebrate predators in terrestrial field studies. Ecology and evolution, 7(6), 1942–1953. doi:10.1002/ece3.2791

Boriss, H., Boersma, M., & Wiltshire, K. H. 1999. Trimethylamine induces migration of waterfleas. Nature, 398(6726), 382–382. doi:10.1038/18796

Bownik, A. 2017. *Daphnia* swimming behaviour as a biomarker in toxicity assessment: A review. Science of The Total Environment, 601-602, 194–205. doi:10.1016/j.scitotenv.2017.05.199

Bryan, J. 2011. How chlorpromazine improved the treatment of schizophrenic patients. The Pharmaceutical Journal, 286, 249 | URI: 11069402

Campos, B., Piña, B., Barata, C. 2012. Mechanisms of Action of Selective Serotonin Reuptake Inhibitors in *Daphnia magna*. Environmental Science & Technology, 46(5), 2943–2950. doi:10.1021/es203157f

Daniel D., Dionisio R., Alkimin G. D., Nunes B. 2019. Acute and chronic effects of paracetamol exposure on *Daphnia magna*: how oxidative effects may modulate responses at distinct levels of organization in a model species. Environmental Science and Pollution Research, 26(4), 3320-3329

Dawidowicz, P., Pijanowska, J., Ciechomski, K. 1990. Vertical migration of *Chaoborus* larvae is induced by the presence of fish. Limnology and Oceanography, 35(7), 1631–1637. doi:10.4319/lo.1990.35.7.1631

De Meester, L. 1991. An analysis of the phototactic behaviour of *Daphnia magna* clones and their sexual descendants. Hydrobiologia 225:217. https://doi.org/10.1007/BF00028400

De Meester, L. 1992. The phototactic behaviour of male and female *Daphnia magna* Anim. Behav., 43, 696-698

De Meester, L. 1993. Genotype, fish-mediated chemicals, and phototactic behaviour in *Daphnia magna* Ecology, 74:1467-1474.

De Meester, L., Cousyn, C. 1997. The change in phototactic behaviour of a *Daphnia magna* clone in the presence of fish kairomones: the effect of exposure time. Hydrobiologia, 360:169. https://doi.org/10.1023/A:1003119827390

DeMille, C. M., Arnott, S. E., Pyle, G. G. 2016. Variation in copper effects on kairomone-mediated responses in *Daphnia pulicaria*. Ecotoxicology and Environmental Safety, 126, 264–272.doi:10.1016/j.ecoenv.2015.12.038

Dennis, S. R., LeBlanc, G. A. and Beckerman, A. P. 2014. Endocrine regulation of predator-induced phenotypic plasticity. Oecologia 176, 625-635.

Dodson, S. I. 1989. The ecological role of chemical stimuli for the zooplankton: predator-induced morphology in *Daphnia*. Oecologia, 78(3), 361–367. doi:10.1007/bf00379110

Ebert, D. Ecology, Epidemiology, and Evolution of Parasitism in Daphnia [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2005. Chapter 2, Introduction to Daphnia Biology. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2042/

Ekerholm, M. and Hallberg, E. 2002. Development and growth patterns of olfactory sensilla in malacostracan crustaceans. In The Crustacean Nervous System (ed. K. W. Wiese), pp. 376-385. Berlin, Heidelberg: Springer

Falcão, B., Marques, M., Nunes, B. 2019. Behavioural and biochemical effects of the antifouler and antidandruff zinc pyrithione on the freshwater fish *Gambusia holbrooki*. Fish Physiology and Biochemistry. 45(4):1495-1512

Gutierrez, M. F., Paggi, J. C., Gagneten, A. M. 2012. Infodisruptions in predator–prey interactions: Xenobiotics alter microcrustaceans responses to fish infochemicals. Ecotoxicology and Environmental Safety, 81, 11–16. doi:10.1016/j.ecoenv.2012.04.001

Hallberg, E., Johansson, K. U. I., Elofsson, R. 1992. The aesthetasc concept: structural variations of putative olfactory receptor cell complexes in Crustacea. Microsc. Res. Tech. 22, 325-335. doi:10.1002/jemt.1070220403

Hanazato, T. 1999. Anthropogenic chemicals (insecticides) disturb natural organic chemical communication in the plankton community. Environmental Pollution, 105(1), 137–142. doi:10.1016/s0269-7491(98)00203-6

Hartline, D. K., Christie, A. E. 2010. Immunohistochemical mapping of histamine, dopamine, and serotonin in the central nervous system of the copepod *Calanus finmarchicus* (Crustacea; Maxillopoda; Copepoda). Cell Tissue Res 341, 49–71 doi:10.1007/s00441-010-0974-8

Hay, M. E. 2009. Marine Chemical Ecology: Chemical Signals and Cues Structure Marine Populations, Communities, and Ecosystems. Annual Review of Marine Science, 1(1), 193–212. doi:10.1146/annurev.marine.010908.163

Heckmann, L., Callaghan, A., Hooper, H., Connon, R., Hutchinson, T., Maund, S., Sibly, R. 2007. Chronic toxicity of ibuprofen to *Daphnia magna*: Effects on life history traits and population dynamics. Toxicology Letters, 172(3), 137–145. doi:10.1016/j.toxlet.2007.06.001

Ioannou, C. C., Tosh, C. R., Neville, L., Krause, J. 2007. The confusion effect—from neural networks to reduced predation risk. Behavioral Ecology, 19(1), 126–130. doi:10.1093/beheco/arm109

Jansen, M., Vergauwen, L., Vandenbrouck, T., Knapen, D., Dom, N., Spanier, K. I., Cielen, A., De Meester, L. 2013. Gene expression profiling of three different stressors in the water flea *Daphnia magna*. Ecotoxicology, 22(5), 900–914. doi:10.1007/s1064013-1072-y

Larsson, P., Dodson, S. 1993. Chemical communication in planktonic animals. Arch. Hydrobiol. 129: 129–155

Lass, S., Boersma, M., Wiltshire, K. H., Spaak, P., Boriss, H. 2001. Does trimethylamine induce life-history reactions in *Daphnia*?. Hydrobiologia, 442(1/3), 199–206. doi:10.1023/a:1017538524539

Li, Q., Zhou, N., Zhang, Y. 2008. Luo Toxic effects of chlorpromazine on *Carassius auratus* and its oxidative stress J. Environ. Sci. Health B, 43, 638-643

Lima, S. L., Dill, L. M. 1990. Behavioral decisions made under the risk of predation: a review and prospectus. Canadian Journal of Zoology, 68(4), 619–640. doi:10.1139/z90-092

Loureiro, S., Meyer, T. L., Ferreira, A. L. G., Amorim, M. J. B., Soares, A. M. V. M. 2012. Single and joint effects of perchlorates to *Daphnia magna*: additivity and interaction patterns Fresenius Environ. Bull., 21 844-852

Mc William, R. A., Baird D. J. 2002. Post-exposure feeding depression: a new toxicity endpoint for use in laboratory studies with *Daphnia magna*. Environ. Toxicol. Chem., 21, 1198-1205

Meyer, J. S., Ingersoll, C. G., Mcdonald, L.L., Boyce, M. S. 1986. Estimating uncertainty in population growth rates: jackknife vs. bootstrap techniques. Ecology, 67, 1156-1166

Michels, E., Leynen, M., Cousyn, C., De Meester, L., Ollevier, F. 1999. Phototactic behavior of *Daphnia* as a tool in the continuous monitoring of water quality: Experiments with a positively phototactic *Daphnia magna* clone. Water Research, 33(2), 401–408. doi:10.1016/s0043-1354(98)00213-9

Mirza R. S., Chivers D. P. 2000. Predator-recognition training enhances survival of brook trout: Evidence from laboratory and field-enclosure studies. Canadian Journal of Zoology, 78, 2198–2208. https://doi.org/10.1139/z00-164

Mitchell, M. D., Bairos-Novak, K. R., Ferrari, M. C. O. 2017. Mechanisms underlying the control of responses to predator odours in aquatic prey. The Journal of Experimental Biology, 220(11), 1937–1946. doi:10.1242/jeb.135137

Mitchell, M. D., Bairos-Novak, K. R., Ferrari, M. C. O. 2017. Mechanisms underlying the control of responses to predator odours in aquatic prey. The Journal of Experimental Biology, 220(11), 1937–1946. doi:10.1242/jeb.135137

OECD (2012) Test No. 211: *Daphnia magna* reproduction test, OECD guidelines for the testing of chemicals, section 2. OECD Publishing, Paris. https://doi.org/10.1787/9789264185203-en

Oliveira L.L.D., Antunes S.C., Goncalves F., Rocha O., Nunes B. 2015a. Evaluation of ecotoxicological effects of drugs on *Daphnia magna* using different enzymatic biomarkers. Ecotoxicology and Environmental Safety. 119, 123-131.

Oliveira, L. L. D., Antunes, S. C., Gonçalves, F., Rocha, O., Nunes, B. 2015b. Acute and chronic ecotoxicological effects of four pharmaceuticals drugs on cladoceran *Daphnia magna*. Drug and Chemical Toxicology, 39(1), 13–21. doi:10.3109/01480545.2015.1029048

Peñalva-Arana, D. C., Moore, P. A., Feinberg, B. A., DeWall, J., Strickler, J. R. 2007. Studying Daphnia feeding behavior as a black box: a novel electrochemical approach. Hydrobiologia, 594(1), 153–163. doi:10.1007/s10750-007-9080-7

Pestana, J. L. T., Baird, D. J., Soares, A. M. V. M. 2013. Predator threat assessment in *Daphnia magna*: the role of kairomones versus conspecific alarm cues. Marine and Freshwater Research, 64(8), 679. doi:10.1071/mf13043

Pestana, J. L. T., Loureiro, S., Baird, D. J., Soares, A. M. V. M. 2010. Pesticide exposure and inducible antipredator responses in the zooplankton grazer, *Daphnia magna* Straus. Chemosphere, 78(3), 241–248. doi:10.1016/j.chemosphere.2009.10.066

Philippova, T. G., Postnov, A. L., 1988. The effect of food quantity on feeding and metabolic expenditure in Cladocera. Internationale Revue der gesamten Hydrobiologie und Hydrographie 73, 601-615.

Pieters, B. J., Paschke, A., Reynaldi, S., Kraak, M. H. S., Admiraal, W., Liess, M. 2005. Influence of Food Limitation on the Effects of Fenvalerate Pulse Exposure on Life History and Population Growth Rate of *Daphnia Magna*. Environmental Toxicology and Chemistry, 24(9), 2254. doi:10.1897/04-563r.1

Pijanowska, J. 1997. Alarm signals in Daphnia? Oecologia 112(1), 12–16. doi:10.1007/S004420050277

Pijanowska, J., Stolpe, G. 1996. Summer diapause in *Daphnia* as a reaction to the presence of fish. Journal of Plankton Research, 18(8), 1407–1412. doi:10.1093/plankt/18.8.1407

Ramcharan, C. W., Bodson, S. I., Lee, J. 1992. Predation risk, prey behavior, and feeding rate in *Daphnia pulex*. Can. 1. Fish. Aquat. Sci. 49: 159-1 65.

Relyea, R. A., Mills, N. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings of the National Academy of Sciences, 98(5), 2491–2496. doi:10.1073/pnas.031076198

Riessen, H. P., 1999. Predator-induced life history shifts in *Daphnia*: A synthesis of studies using meta-analysis. Canadian Journal of Fisheries and Aquatic Sciences 56, 2487-2494.

Ringelberg, J. 1964. The positively phototactic reaction of *Daphnia magna* Straus. – A contribution to the understanding of diurnal migration Neth. J. Sea Res., 2, 319-406

Ringelberg, J., Van Gool, E. 1998. Do bacteria, not fish, produce 'fish kairomone'?. Journal of Plankton Research, 20, 1847-1852

Roberts, P.H.; Bersuder, P. 2006. Analysis of OSPAR priority pharmaceuticals using high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry J. Chromatogr. A, 1134, 143-150

Rocha, R., Gonçalves, F., Marques, C., Nunes, B. 2014. Environmental effects of anticholinesterasic therapeutic drugs on a crustacean species, *Daphnia magna*. Environ Sci Pollut Res Int. 21(6):4418-29. doi: 10.1007/s11356-013-2339-9

Rosier, R. L., Langkilde, T. 2011. Behavior under risk: how animals avoid becoming dinner. Nat Educ Knowl 2(11), 8

Scherer, C., Seeland, A., Oehlmann, J., Müller, R. 2013. Interactive effects of xenobiotic, abiotic and biotic stressors on *Daphnia pulex*—Results from a multiple stressor experiment with a fractional multifactorial design. Aquatic Toxicology, 138-139, 105–115. doi:10.1016/j.aquatox.2013.04.014

Schoeppner, N. M., Relyea, R. A. 2009. Interpreting the smells of predation: how alarm cues and kairomones induce different prey defences. — Funct. Ecol. 23, 1114-1121

Schwarzenberger, A., Court,s C., Von Elert, E. 2009. Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free Microcystis aeruginosa. BMC Genomics 10, 527

Simão, F. C. P., Martínez-Jerónimo, F., Blasco, V., Moreno, F., Porta, J. M., Pestana, J. L. T., Soares, A. M. V. M., Demétrio, R., Barata, C. 2019. Using a new high-throughput video-tracking platform to assess behavioural changes in *Daphnia magna* exposed to neuro-active drugs. Science of The Total Environment. 662, 160–167. doi:10.1016/j.scitotenv.2019.01.187

Šmejkal, M., Ricard, D., Sajdlová, Z., Čech, M., Vejřík, L., Blabolil, P., … Peterka, J. 2018. Can species-specific prey responses to chemical cues explain prey susceptibility to predation? Ecology and Evolution, 8(9), 4544–4551. doi:10.1002/ece3.4000

Stibor, H. 2002. The role of yolk protein dynamics and predator kairomones for the life history of *Daphnia magna*. Ecology, 83, 362–369.

Stowers, L., Kuo, T.-H. (2016). Chapter 1 - Specialized Chemosignaling that Generates Social and Survival Behavior in Mammals. In Chemosensory Transduction. The Detection of Odors, Tastes, and Other Chemostimuli, pp 3-27. Academic Press. DOI: 10.1016/B978-0-12-801694-7.00001-9

Tams, V., Nickel, L. H., Ehring, A., Cordellier, M. 2019. Insights into the genetic basis of predator-induced response in Daphnia - a comparative transcriptomic approach. BioRxiv. doi: https://doi.org/10.1101/503904

Tjossem, S. F. 1990. Effects of fish chemical cues on vertical migration behavior of *Chaoborus.* Limnology and Oceanography, 35, 1456–1468. doi:10.4319/lo.1990.35.7.1456

Tjossem, S. F. 1990. Effects of fish chemical cues on vertical migration behavior of Chaoborus. Limnology and Oceanography, 35(7), 1456–1468. doi:10.4319/lo.1990.35.7.1456

Villarroel, M. J., Ferrando, M. D., Sancho, E., Andreu, E. 1999. *Daphnia magna* Feeding Behavior after Exposure to Tetradifon and Recovery from Intoxication. Ecotoxicology and Environmental Safety, 44(1), 40–46. doi:10.1006/eesa.1999.1817

Weider, L. J., Pijanowska, J. 1993. Plasticity of Daphnia Life Histories in Response to Chemical Cues from Predators. Oikos, 67(3), 385. doi:10.2307/3545351

Weiss, L. C., F., Leese, C., Laforsch, R., Tollrian. 2015b. Dopamine is a key regulator in the signalling pathway underlying predator-induced defences in Daphnia. Proc. R. Soc. B Biol. Sci. 282, 20151440

Weiss, L. C., Kruppert, S., Laforsch, C. and Tollrian, R. 2012. Chaoborus and Gasterosteus anti-predator responses in *Daphnia pulex* are mediated by independent Cholinergic and Gabaergic neuronal signals. PLoS ONE 7, e36879.

Weiss, L. C., Leimann, J., Tollrian, R. 2015a. Predator-induced defences in *Daphnia longicephala*: location of kairomone receptors and timeline of sensitive phases to trait formation. Journal of Experimental Biology, 218(18), 2918–2926. doi:10.1242/jeb.124552

Yuan, S., Jiang, X., Xia, X., Zhang, H., Zheng, S., 2013. Detection, occurrence and fate of 22 psychiatric pharmaceuticals in psychiatric hospital and municipal wastewater treatment plants in Beijing, China. Chemosphere 90, 2520–2525.

Zar, J. H. 1996. Biostatistical Analysis. 3rd ed. Prentice Hall.

Zitova, A., Cross, M., Hernan, R., Davenport, J., Papkovsky, D. B. 2009. Respirometric acute toxicity screening assay using *Daphnia magna*. Chemistry and Ecology, 25(3), 217–227. doi:10.1080/02757540902936851

Chapter 7

Final remarks

According to the here-obtained data, pharmaceutical drugs have been shown to be toxic to aquatic organisms, at several distinct levels, including to macrophyte species and macroinvertebrates. This thesis brought along 5 research chapters, condensing new insights and perspectives about this subject, in an ecotoxicologically integrated view, using different approaches to try understanding the complexity of this theme in different scenarios. Chapters 2, 3, and 4 were instrumental to overlook ecotoxicological assays involving macrophyte species. When compared with other groups of well-established invertebrates such as *Daphnia* species, the comparison of the raw number of publications is highly unfavorable to macrophytes. The still little use of macrophytes as test organisms in ecotoxicology may be realized through a simple search in common search engines for scientific information, searching for keywords with an association between the term "ecotoxicology" with "macrophytes" or "*Daphnia*". These searches bring values of 205 and 1569 published documents, respectively. If one replaces the term "ecotoxicology" by "pharmaceutical", the results of the searches fall to 66 and 350 studies on macrophytes and *Daphnia,* respectively. As we can observe, the number of published documents with *Daphnia* is almost 450% higher than those found for aquatic plants. This can be interpreted as a sign for the necessity of studies using plants as test organisms, since, environmentally, macrophytes are as important as all other taxa of aquatic organisms.

Macrophytes are of undisputable importance in freshwater ecology, and their presence conditions the survival of a diversity of other organisms; overall, the good function of an ecosystem strongly depends on macrophytes, as mentioned along this thesis, since these organisms have several important functions, including being primary producers, that is, they serve as an important source of food for many types of fish and some species of birds and aquatic mammals. Macrophytes also participate in the cycling of nutrients and may function as shelters for some species of animals, and provide shade for those sensitive organisms at high intensities of solar radiation. Consequently, the impairment of macrophyte populations may impact the environmental balance in unpredicted ways. In ecological terms, the here presented results are extremely important. The effects that were observed in the different experiments described in this thesis showed that environmentally realistic concentrations of pharmaceutical drugs can jeopardize physiological and biochemical parameters of macrophyte species. These

effects, if occuring in natural environments as a consequence of the prolonged environmental presence of pharmaceutical drugs, may cause effects in populations of macrophyte species, thereby disturbing the aquatic environment, affecting all the above mentioned specific functions of macrophytes.

On the other hand, Chapter 2 showed that the use of non-standard endpoints in ecotoxicological evaluations based on macrophyte species as test species are likely to be used under varied circumstances, serving the purpose of attaining distinc objectives, and may therefore be considered promising tool. This chapter also reported that different *Lemna* species may show different response patterns when exposed to the same contaminants. However, this topic needs to be addressed by the scientific community in a broader manner, in order to complement standard testing guidelines with macrophytes with new testing methods, with improved reliability, reduced duration, and high ecological significance. This is necessary, since the most commonly adopted international guidelines for ecotoxicity testing using macrophytes as test organisms are limited to the determination of endpoints such as growth, yield, and biomass. These parameters reflect only a very narrow suite of endpoints and physiological parameters, and their underlying mechanisms are far from being the only processes that can be affected by contaminants. In fact, effects on such pathways do not represent at all the complexity of biological systems (including in plants), and of environmental interactions. By adopting standardized guidelines, we will be certainly limited in the extension of our findings, since the collected biological and ecological information about pharmaceutical toxicity using these standard bioassays is restricted.

The necessity of new approaches to determine putative ecotoxicological effects of pharmaceutical drugs (and contaminants in general), was brought to light in Chapter 3. In this Chapter, it was clear that time is a factor that can influence not just the intensity, but the types of effects of contaminants. Considering the obtained data, different durations of exposure need to be taken into account in experimental designs to be adopted when aquatic plants are used as test organisms. This is a critical assumption when selecting biomarkers as toxicological endpoints, since without a judicious selection of putative pathways that are likely to be activated or altered only after prolonged exposures to toxicants, it will be possible to comprehensively ascertain their toxicity. Furthermore, in this chapter it was reported that different pharmaceutical

compounds belonging to the same pharmacological and therapeutic group (NSAIDs) affected differently the tested *Lemna* species. Beyond the fact that similarly acting pharmaceuticals may have different toxicological mechanisms in aquatic plants, aquatic biota are exposed in the wild to compounds of distinct origin, chemical classes, and proveniences. Chapter 4 focused on the combined presence of two chemicals: one was a drug, while the second was a drug that is also a natural plant hormone. The results showed that, when plants were co-exposed to both substances, the natural hormone was able to strongly modulate the plant response. Again, these data reinforced the concept that exposure of plants to environmentally realistic conditions may generate unpredictable, even paradoxical effects, which deserve further attention.

Chapter 5 presented a comparative toxicological study of two distinc species (*Lemna* and *Daphnia*) exposed to similar concentrations of a NSAID compound, ketoprofen. To do that, equivalent biochemical and physiological responses were assessed. Results reported that ketoprofen enhanced antioxidant responses in both species and did not cause physiological effects (it did not alter pigments in *Lemna*, and feeding rates in *Daphnia*). These results indicated that chemicals may act similarly across distinc trophic and phylogenetically distant species. More specificaly, the results of this chapter indicated that oxidative stress effects may be similar across distant species, thus suporting the species read-across theory. However, the effects of drugs on the biochemical parameters were clearly different between both species, being *L. minor* more susceptible to low pharmaceutical concentrations, allowing to hypothesize about the resilience of this particular species, and the possibility of establishment of hormetic effects; while *D. magna*, in general, responding according to a clear dose response pattern. In environmental terms, these details are important, since they may imply that some species may be highly sensitive or resistant, granting different organisms competitive advantages (and disadvantages) that may be of ecological concern. In this thesis, this comparison was highly interesting and allowed us to assume that pharmaceutical toxicity can be species-specific, indicating the necessity of more studies from this nature. The here presented work evidenced that these differences did in fact occur. Besides the observed results in this chapter being limited to the alterations at the biochemical level, they were clearly different between both species, as above mentioned. Thus, this set of results addresses the already mentioned possibility, of

establishing an ecological advantage from one organism to another, suggesting that different organisms may have different abilities to adapt and thrive under unfavorable environmental conditions, consequently having different ecological success.

Finally, Chapter 6 evidenced that drugs may not only interact with each other (or with alternative anthropogenic chemicals), but their effects may be altered also by naturally occurring substances, such as fish kairomones. This chapter addressed interactive effects of anthropogenic and natural stressors, on oxygen consumption, feeding rate, behavior, and life history traits in *D. magna*. This chapter deserves to be highlighted, since some of the observed alterations were only observed when animals were co-exposed to the mixture (natural + anthropogenic stressor). That means some ecotoxicological effects may only be observed under scenarios of more complexity and realism, which require the adoption of fully dedicated approaches, encompassing the normal complexity of aquatic ecosystems, and novel systems of observation, including of subtle behavioral changes. Chapter 6 was eminently related to the findings described in Chapter 4, which brought the effects on macrophytes, focusing on the interactions between two compounds, being one an antropogenic stressor (pharmaceutical drug) and the other, a natural stressor to aquatic plants (phytohormone), a compound that is also a pharmaceutical drug. This last Chapter studied the single and joint effects of two compounds, with evident similarities to those analysed in Chapter 4; in the case of Chapter 6, the anthropogenic stressor was also a pharmaceutical, while the natural stressor was the fish kairomone. However, Chapter 6 analysed the effects of these substances on an invertebrate species. By simultaneously analyzing and comparing the major findings described in both chapters, it was possible to assume that complex interactions are occurring in the environment all the time; however, and despite the evident differences, in general, the results that were obtained while testing the combinations of the several stressors presented commom results, and alterations caused by exposures to single compounds were largely different from those obtained for combined chemicals. The data and patterns from these two chapters drew our attention to the necessity of more investigations with this experimental design and this level of complexity.

After exploring the main subjects of this thesis, it was possible to conclude that the ecotoxicological research on pharmaceutical drugs still has a long way to go, exactly to

study never before addressed issues that now require more attention from researchers. In general, we may conclude that integrating a further step in the complexity of ecotoxicological test systems is now mandatory. By answering to the initial proposed questions, we are able to suggest that non-standard endpoints are suitable to study ecotoxicological effects in macrophyte species, but definitely durations of exposures are a highly important feature. In fact, time modulated the response, namely its type and nature, and not only its extent. In addition, the co-occurrence of anthropogenic and natural chemicals being released from plants, namely endogenous compounds, can modulate the responses of challenged macrophyte species. The thesis also demonstrate that the same pharmaceutical can have different effects in different species, even at the same trophic level (*L. minor – L. gibba*), or in different trophic levels (*L. minor – D. magna*). In case of same genus species, and considering that both species share the same habitat, this difference in the toxic response, in ecological aspects, can be assumed as a competitive advantage, and may grant a stronger dissemination and growth capacity of one species over the others, when in contaminated environments. On the other hand, considering only species from different trophic levels, this advantage can cause a long-term food chain disturbance and may consequently affect the ecosystem balance. We also conclude that some responses may only be observed in a realistic scenario when different interactions (both chemical and biological, e.g. pharmaceutical and natural stressor) occur. This is of extreme ecological relevance, since it grantedus a highly valuable insight into toxic responses, and further consequences at a larger, ecosystem level, scale of organization.

In conclusion, pharmaceutical drugs have been shown to be toxic in some extent to aquatic organisms; more attention is however required to analyse the effects of this vast group of chemicals on aquatic plants. New experimental designs and approaches need to be developed, implemented, and validated to understand the toxicological effects of a complex environment, as aquatic ecosystems, especially when considering the use of aquatic plant species, and freshwater crustaceans as test organisms.