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COMO É QUE OS AGENTES ANTINEOPLÁSICOS AFETAM A SOBREVIVÊNCIA E A HOMEOSTASIA CELULAR EM *DAPHNIA MAGNA*?

HOW DO ANTINEOPLASTIC AGENTS AFFECT THE SURVIVAL AND CELLULAR HOMEOSTASIS OF *DAPHNIA MAGNA*?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-Toxicologia e Análise de Risco, realizada sob a orientação científica da Doutora Maria Pavlaki, Investigadora do Departamento de Biologia da Universidade de Aveiro e da Professora Doutora Susana Patrícia Mendes Loureiro, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Doxorubicina; Oxaliplatina; Agentes Antineoplásicos; Toxicidade Aguda; Misturas; Stress oxidativo; Neurotoxicidade; *Daphnia magna*

resumo

Antineoplásicos são componentes vitais da quimioterapia contra o cancro e são desenhados para inibir a proliferação de células tumorais por meio de interações com o DNA e modificações nos fatores de crescimento celular. A sua libertação nos efluentes municipais e hospitalares suscita preocupações quanto aos potenciais riscos para organismos aquáticos não alvo, especialmente quando coexistem em misturas complexas. É investigada a imobilização, resposta antioxidante e bioquímica, bem como a neurotransmissão em *Daphnia magna* quando expostas a dois diferentes agentes antineoplásicos, doxorubicina (DOX) e oxaliplatina (OXA), com o objetivo de avaliar o impacto dos seus efeitos tóxicos individuais e em misturas. As toxicidades combinadas foram avaliadas usando dois modelos distintos, Adição de Concentração e Ação Independente, bem como seus possíveis desvios para sinergismo (causando efeito mais severo) ou a antagonismo (efeito menos severo), dependentes da dose aplicada, ou dependência do rácio entre as doses aplicadas para cada item da mistura e/ou combinação. Os efeitos agudos da exposição individual após 48 horas revelaram que cinquenta por cento de imobilização de *D. magna* ocorre em EC_{50}=0,79 mg/L de DOX e EC_{50}=59,61 mg/L de OXA. A exposição de misturas de DOX e OXA apresentou um melhor ajuste para os padrões de desvio de dependência do rácio em ambos os modelos, mostrando sinergismo em concentrações mais altas de OXA e antagonismo em concentrações mais baixas de DOX. A avaliação de biomarcadores para neurotoxicidade, stresse oxidativo e danos na membrana celular foi realizada por meio da exposição de *D. magna* a concentrações subletais de DOX e OXA. Os biomarcadores incluíram atividades da Acetilcolinesterase, Catalase, Glutationa peroxidase, Glutationa Redutase, Glutationa S-transferase, Sistema de transporte de Eletrões, Citocromo c Redutase e Peroxidação Lipídica. Análise de biomarcadores mostra que diferentes mecanismos foram utilizados para atenuar o impacto a nível celular do stress oxidativo induzido por cada antineoplásico individualmente em *Daphnia*. Quando em misturas, foi observado diminuição da acetilcolinesterase, especialmente em concentrações mais elevadas de OXA. Os dados ecotoxicológicos apresentados neste estudo, não apenas destacam o potencial risco ambiental associado a compostos antineoplásicos, particularmente no que diz respeito aos seus efeitos sinérgicos, mas também enfatizam a importância da integração de modelos estatísticos para avaliar o impacto ambiental desses compostos, uma vez que o seu comportamento será diferente do impacto causado por stressores individuais.

keywords Doxorubicin; Oxaliplatin; Antineoplastic Agents; Acute toxicity; Mixtures; Oxidative stress; Neurotoxicity; *Daphnia magna*

abstract

Antineoplastic agents, vital components of cancer chemotherapy, are designed to inhibit tumor cell proliferation through interactions with DNA and modifications of cellular growth factors. Their release into municipal and hospital effluents raises concerns about potential risks to non-target aquatic organisms, especially when they coexist in complex mixtures. This study investigates the immobilisation, antioxidant and biochemical response and neurotransmission of *Daphnia magna* when exposed to two distinct antineoplastic agents, doxorubicin (DOX) and oxaliplatin (OXA), with the objective to evaluate the impact of their single and mixture toxic effects. The combined toxicities were assessed using two distinct models, Concentration Addition and Independent Action, alongside with their possible deviations, like synergism, (causing a more severe effect) or antagonism (less severe effect), effects dependent on "dose level" (different deviations at high and low concentration levels) or those dependent on "dose ratio" (deviations depend on the mixture's composition). Acute effects of single exposure after 48 hours found a fifty percent of immobilisation of *D. magna* occuring at EC_{50}=0.79 mg/L of DOX and at EC_{50}=59.61 mg/L of OXA. Mixture exposure of DOX and OXA displayed a better fit for dose ratio deviation patterns for both models, showing synergism at higher OXA concentrations while antagonism was mainly caused by increasing DOX concentrations. The evaluation of biomarkers for neurotoxicity, oxidative stress, and cell membrane damage was conducted through *D. magna*'s exposure to sub-lethal concentrations of DOX and OXA. Biomarkers included Acetylcholinesterase, Catalase, Glutathione peroxidase, Glutathione Reductase, Glutathione S-transferase, Electron Transport System, Cytochrome c Reductase activities, and Lipid Peroxidation. Biomarker analysis elucidates that different mechanisms have been employed to attenuate the cellular-level impact of oxidative stress induced by each isolated antineoplastic on *Daphnia*. When exposed to mixtures, it is observed a decrease on acetylcholinesterase, especially at higher concentrations of OXA. The ecotoxicological data presented in this study not only highlight the potential environmental risk associated with anticancer drugs, particularly concerning their synergistic effects, but also emphasize the importance of integrating statistical models to assess the environmental impact of these compounds, as their behaviour will be different to the impact the single stressors induce.

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Abbreviations

AChE	Acetylcholinesterase
ANOVA	One-way analysis of variance
ASTM	American Society for Testing and Materials
BHT	2,6-Di-tert-butyl-4- 256 methylphenol
CA	Concentration Addition
CAT	Catalase
CI	Confidence intervals
CDNB	1-chloro-2,4- dinitrobenzene
CYP c Reductase	Cytochrome c Reductase
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DR	Dose ratio
DL	Dose level
DTNB	5,5′-dithiobis (2- nitrobenzoic acid)
EC_{50}	Concentration estimated to cause 50 per cent of effect
ETS	Electron transport system
EU	European Union
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-*S*-transferase
IA	Independent Action
INT	p-iodonitrotetrazolium
LOEC	Lowest observed effect concentration
LPO	Lipid peroxidation
NADPH	Nicotinamide adenine dinucleotide phosphate
NOEC	No observed effect concentration
OECD	Organization for Economic Cooperation and Development

OXA	Oxaliplatin
PMS	Post-mitochondrial supernatant
PROT	Protein
ROS	Reactive oxygen species
S/A	Synergism/antagonism
SE	Standard error
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TOP II	Topoisomerase II
WWTP	Wastewater treatment plant

Chapter 1

General Introduction

1.1 Introduction

1.1.1 Overview of pharmaceuticals in the environment

Pharmaceuticals are widely employed compounds designed to prevent, diagnose, and treat diseases, improving and revolutionizing the healthcare system, alongside with quality of life and longevity for the living beings in human and veterinary medicine (Fent *et al*., 2006; González Peña *et al*., 2021). Over the last decades, the global consumption of pharmaceuticals has been increasing due to advances in research and development, increased accessibility to healthcare and driven by a growing demand for drugs to treat a wide spectrum of medical conditions (Van Boeckel *et al*., 2014; Adeleye *et al*., 2022). However, as the pharmaceutical market grows and consumption rises, the discharge of pharmaceuticals and their metabolic byproducts ends up finding their fate into the environment (González Peña *et al*., 2021). Together with the limited discharge regulation and appropriate management and treatment methods, this class of compounds commonly classified as emerging contaminants, has prompted an overall growing awareness by different entities that try to create a framework to control their release based on assessment of environmental fate and effects (Ferrando-Climent *et al*., 2014).

Recently, the global pharmaceutical market has experienced substantial growth. The revenue generated by the pharmaceutical industry production in Europe reached approximately €340,000 million in 2022, more than double compared to the figures from 2000; additionally, in 2022, it was invested an estimated €44,500 million in research and development exclusively in Europe (EFPIA, 2023). While market sales in Europe for prescription pharmaceuticals in 2022 constituted 22.4% of the global pharmaceutical retail, North America held the largest share, at 52.3% (EFPIA, 2023). Brazil, China and India market and research economies are currently experiencing a rapid growth that is expected to result in a gradual shift of economic activities from Europe to these fast-growing markets (EFPIA, 2023).

In the European Union (EU) about 5000 different substances are used in human medicine and 10000 in the US, such as analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators, neuroactive compounds, and many others (Fent *et al*., 2006; Dong *et al*., 2013; S. R. Hughes *et al*., 2013). According to González Peña *et al*. (2021), in 2017, musculoskeletal drugs were the largest pharmaceutical market globally, followed by cardiovascular and oncological drugs. Furthermore, Health at a Glance report from OECD countries, noticed a raise in anti-hypertensives drugs consumption by approximately 65% between 2000 to 2019, doubled the consumption of anti-depressants and anti-diabetic medicines, and have an even higher increased tendency for lipid-modifying agents, for the same period (OECD, 2021). IQVIA Institute for Human

Data Science, predicts that alongside with lipid-modifying agents and anti-diabetics drugs, over the next four years, immunology and oncology therapy areas will be the most significant contributors to the growth of the global medicine market (Arias *et al*., 2021). Oncology is projected to be the area where is expected more spending to increase over 63% as new drugs continue to be introduced for the treatment of cancer and its access will be better (Arias *et al*., 2021).

1.1.2 Pharmaceuticals' metabolism and biochemical cellular alterations

Once in the body, a pharmaceutical drug undergoes a series of processes known as ADME: absorption, distribution, metabolism, and elimination. Pharmacologically active organic molecules typically exhibit lipophilic characteristics, often remaining uncharged or only partially ionized under physiological pH conditions, which would significantly prolong the duration of their action if the drug's effects were solely reliant on renal excretion (Correia, 2012). An alternative mechanism involves the metabolization of this drugs substances', where they undergo chemical alteration to create compounds that are more easily excreted from the body, generally, lipophilic xenobiotics converted into more polar substances (Correia, 2012; Susa *et al*., 2023). This process referred as biotransformation occur primarily in the liver. Pharmaceuticals biotransformation in the body, is catalysed by specific cellular enzymes, primarily located in subcellular compartments such as the endoplasmic reticulum, mitochondria, cytosol, lysosomes, or the nuclear envelope and plasma membrane (Correia, 2012).

These reactions can generally be categorized into two major groups, known as phase I and phase II reactions. Phase I reactions (e.g., oxidation, reduction, hydrolysis) typically convert the parent drug into a more polar metabolite by introducing or revealing a functional group (e.g., -OH, -NH2, -SH) (Correia, 2012). If phase I metabolites are sufficiently polar, they can be readily excreted. However, numerous phase I products are not rapidly eliminated and subsequently undergo conjugation mechanisms such as methylation, acetylation, sulfation, glucuronidation, and glycine or glutathione conjugation, where an endogenous substance combines with the newly introduced functional group to create a highly polar conjugate (Correia, 2012; Susa *et al*., 2023). These conjugation reactions are characteristic of phase II metabolism. Various drugs undergo these sequential biotransformation reactions, although in some cases, the parent drug may already possess a functional group that can form a conjugate directly, and in these cases, phase II reactions may actually precede phase I reactions in some instances (Correia, 2012).

Metabolic byproducts are often less pharmacodynamically active than the parent drug, and in some cases, they may even be entirely inactive. However, certain biotransformation products can exhibit enhanced activity or toxic properties (Correia, 2012; Susa *et al*., 2023).

The cytochrome P450 system is one of the most important enzyme systems of phase I, a microsomal superfamily of isoenzymes that catalyses the oxidation of many pharmaceuticals drugs (Correia, 2012). The electrons are supplied by NADPH to CYP450 reductase, a flavoprotein that transfers electrons from NADPH to CYP450 and reducing the oxidized P450-drug complex (Correia, 2012; Susa *et al*., 2023). Various P450 isoforms have been identified in the human liver, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP4A11, and CYP7. CYP3A4, in particular, plays a crucial role being responsible for metabolizing over 50% of prescription drugs by the liver (Correia, 2012).

Reactive oxygen species (ROS) are produced during various cellular metabolic processes characterized by incomplete reduction of oxygen. The primary ROS generated are singlet oxygen ($^1O_2$), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}HO$); these molecules differ in cellular reactivity and potential to cause damage to lipids, proteins, and DNA (Regoli & Giuliani, 2014). An organism that shows an imbalance of ROS and cannot handle its detoxification in an effective way, is described as being under oxidative stress (Figure 1.1) (D'Autréaux & Toledano, 2007).

Under normal conditions, the antioxidant system, comprising of low molecular weight scavengers and antioxidant enzymes, prevents the adverse effects of oxyradicals (Regoli *et al*., 2011; Regoli & Giuliani, 2014). Scavengers neutralize ROS through direct reactions, becoming temporarily oxidized before being reconverted by specific reductases (Regoli & Giuliani, 2014). They can also halt the propagation of lipid peroxidation reactions on membranes. Reduced glutathione (GSH), a tripeptide, is the most abundant cytosolic scavenger, neutralizing various reactive species and acting as a cofactor for antioxidant glutathione-dependent enzymes (Regoli & Giuliani, 2014).

Enzymatic antioxidants, in contrast to scavengers that interact with multiple types of ROS, perform highly specific reactions with particular substrates. One such enzyme is superoxide dismutase, which catalyses the conversion of superoxide into hydrogen peroxide, a reactive oxygen species that requires subsequent enzyme reduction assistance with detoxification, like catalase (CAT) or glutathione peroxidases (GPx) (Regoli *et al*., 2011; Regoli & Giuliani, 2014). Hydrogen peroxide can be efficiently converted to hydroxyl radicals via the Fenton reaction, a powerful initiator of the membrane lipid peroxidation (Regoli *et al*., 2011).

Glutathione peroxidases uses reduced GSH as an electron donor to catalyse the reduction of hydrogen peroxide to water (Regoli & Giuliani, 2014). GPx and specific isoforms of glutathione S-transferases (GST) reduce lipid hydroperoxides to alcohols, simultaneously oxidizing GSH to oxidized glutathione (GSSG) (Regoli *et al*., 2011; Regoli & Giuliani, 2014). GSTs can also facilitate the conjugation of GSH adducts to both foreign and endogenous lipophilic compounds (D'Autréaux & Toledano, 2007; Regoli *et al*., 2011). Reconversion from oxidized glutathione to GSH by glutathione reductase (GR), although not a genuine antioxidant enzyme, is crucial for maintaining the correct GSH/GSSG ratio and the intracellular redox state in organisms (Regoli & Giuliani, 2014).

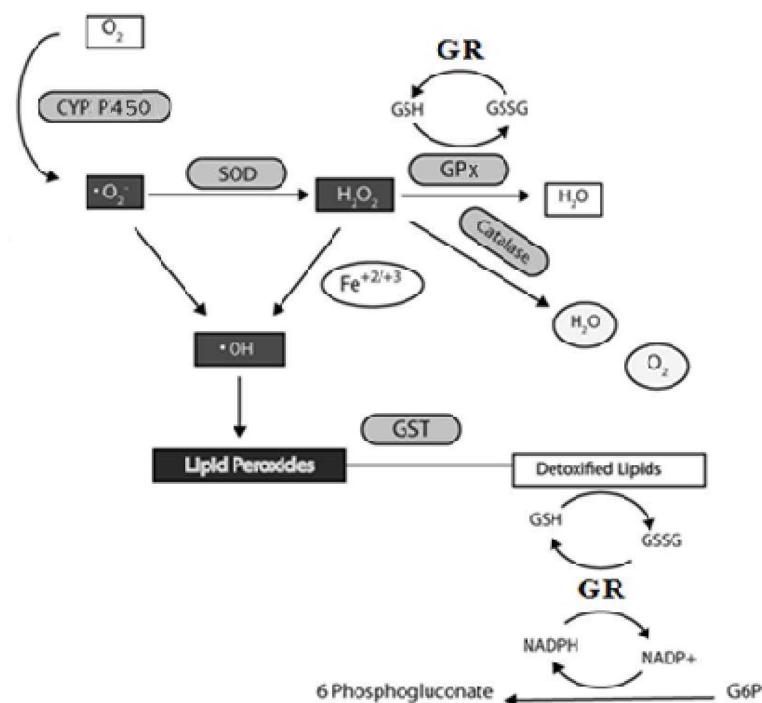

Figure 1.1 | Scheme for oxidative stress pathways; SOD - superoxide dismutase, CAT - catalase, GPx, - glutathione peroxidase, GR - glutathione reductase, GST - glutathione S transferase, CYP P450 - cytochrome P450 and G6P - glucose 6 phosphate (from Shaban *et al*., 2014).

Mitochondria, highly dynamic and versatile organelles, play a pivotal role in cellular metabolism, stress responses, and the maintenance of homeostasis (Chen *et al*., 2023). They serve as hubs for essential biochemical processes, including adenosine triphosphate production, fatty acid synthesis, generation of ROS, oxidative phosphorylation, and calcium homeostasis (Nolfi-Donegan *et al*., 2020; Chen *et al*., 2023). Despite variability across cell types, mitochondrial adenosine triphosphate generation and ROS production are intricately linked through the electron transport

system (ETS). This linkage underscores the significance of efficient ETS function in providing insights into both physiological mechanisms and disease pathogenesis (Nolfi-Donegan *et al*., 2020; Chen *et al*., 2023).

The inner mitochondrial membrane integrates ETS complexes I-IV, facilitating the transfer of electrons from reduced substrates to molecular oxygen. ROS generation in the inner mitochondrial membrane are regulated by antioxidant enzymes, such as superoxide dismutase and GPX (Nolfi-Donegan *et al*., 2020). The proximal dismutated ROS generated by the ETS are H2O2 species, that exit the mitochondria, mediating cytosolic cell signalling and participating in various cellular processes (Nolfi-Donegan *et al*., 2020), demonstrating ETS essential role of mitochondrial ROS signalling and as a biomarker for understanding the impact of environmental stressors.

Acetylcholinesterase, an essential enzyme within cholinergic nerve synapses, is synthesized in nerve terminals through the incorporation of choline (Ritter *et al*., 2020). Choline is transported into the nerve terminal via specific transporters, analogous to those responsible for various neurotransmitters. However, this transporter's primary role is to facilitate the transport of choline, making it a precursor in the synthesis process rather than directly involved in terminating neurotransmitter actions (Ritter *et al*., 2020).

The principal function of acetylcholinesterase in cholinergic synapses is to regulate nerve impulse transmission by catalysing the hydrolysis of the neurotransmitter acetylcholine (Lockridge *et al*., 2018; Ritter *et al*., 2020). In cases where acetylcholinesterase is inhibited, there is an accumulation of acetylcholine, leading to excessive overstimulation of acetylcholine receptors that results in the paralysis of muscles essential for locomotion and breathing, and disrupting the rhythm-generating centre in the brain, and ultimately causing respiratory failure and neuropathological conditions (Lockridge *et al*., 2018). These effects underscore the critical role of acetylcholinesterase in maintaining proper neuromuscular function and the significant consequences of its inhibition (Lockridge *et al*., 2018).

Acetylcholinesterase (AChE) activity has been used as a biomarker for environmental pollution in aquatic environments, particularly pesticides and heavy metals, but more recently studies have started to display pharmaceuticals can affect AChE activity in organisms such as polychaetes, mussels and fishes (Solé *et al*., 2010; Li *et al*., 2012; Fonseca *et al*., 2017).

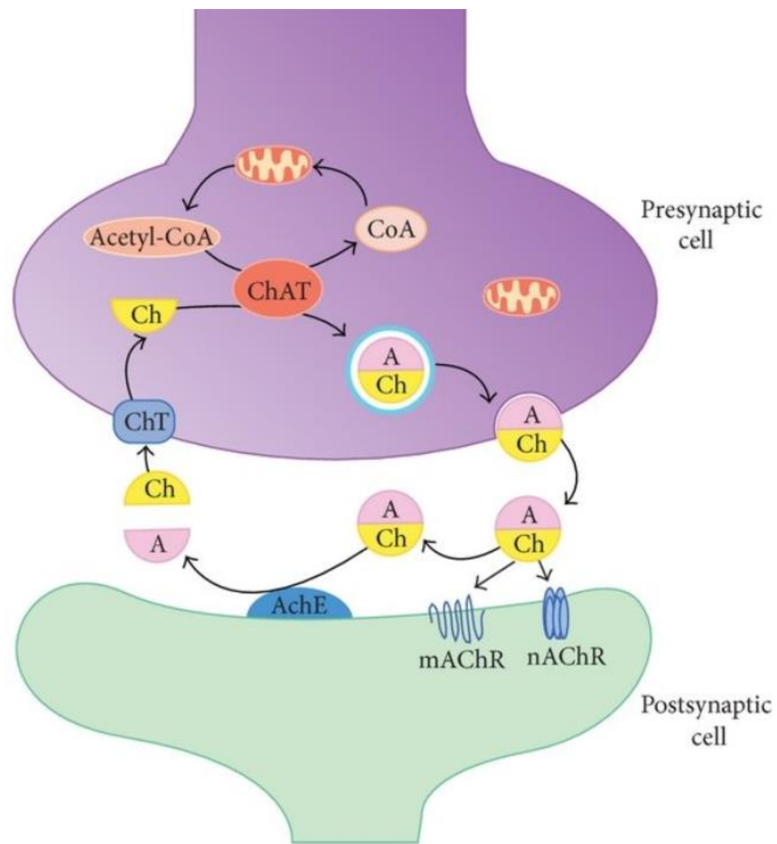

Figure 1.2 | Synthesis of acetylcholine in synapse; A – acetyl, AChE – acetylcholinesterase, Ch – choline, ChAT – acetylcholine transferase, ChT – choline transporter, CoA – coenzyme A, mAChR – muscarinic receptor and nAChR – nicotinic receptor (from Toledo Ibarra *et al*., 2013).

1.1.3 Sources and pathways of pharmaceuticals in the environment

Pharmaceuticals were first detected in the aquatic environment in the 1970s (Hignite & Azarnoff, 1977; Besse *et al*., 2012). Since then, this topic has been extensively discussed and documented in the literature over the past decades, reporting their sources, occurrence, fate, degradation, elimination, effects as well as recognizing their worldwide status as an environmental concern and a potential human health risk (González Peña *et al*., 2021; Ibáñez *et al*., 2021).

During their manufacture, use, and disposal, pharmaceuticals enter aquatic ecosystems through different pathways, either in their original form or as metabolites, following their usage, physiological excretion, and disposal. They can be released continuously to the environment from point but also nonpoint sources such as unregulated domestic effluents, agricultural outputs to fields and aquatic systems (Patel *et al*., 2019). The ability of each compound to be sorbed onto soils and sediments, their biotransformation properties, and the micro-organisms present in treatment facilities contribute to the environmental contaminant load (Patel *et al*., 2019).

Municipal wastewater is the principal point source of these substances to the urban sewage system, which transports remnants of human pharmaceuticals after normal use and disposal of unwanted medicines, hospital and pharmaceuticals industries' wastewater (Fent *et al.*, 2006; Bavumiragira *et al.*, 2022). In urbanized regions, these pharmaceuticals can subsequently find their way to wastewater treatment plants (WWTPs) (Figure 1.3). However, some pharmaceuticals, due to their physico-chemical properties aren't readily degraded and persist in the conventional treatment process since WWTPs weren't specifically designed with the purpose to remove these drugs (Patel *et al.*, 2019; Hawash *et al.*, 2023). As a result, WWTPs are often unable to fully eliminate pharmaceuticals from the influent streams. The effectiveness of pharmaceutical removal at WWTPs varies widely, with removal rates ranging from 2% to 100% for different drugs and across different WWTPs (Bavumiragira *et al.*, 2022). Examples of resistant cases of pharmaceuticals with less than 10% of efficiency are carbamazepine, atenolol, acetylsalicylic acid, diclofenac, mefenamic acid, propranolol, atenolol, clofibric acid, and lincomycin, among others (Patel *et al.*, 2019). Consequently, treated effluents from WWTPs contain residual pharmaceuticals, eventually leading to contamination in rivers, lakes, estuaries, and on rare occasions, groundwater and drinking water sources (Fent *et al.*, 2006; Hawash *et al.*, 2023). In addition to these sources, veterinary pharmaceuticals can also enter aquatic systems via manure application to fields and subsequent runoff, but also via direct application in aquaculture (Fent *et al.*, 2006; Bavumiragira *et al.*, 2022).

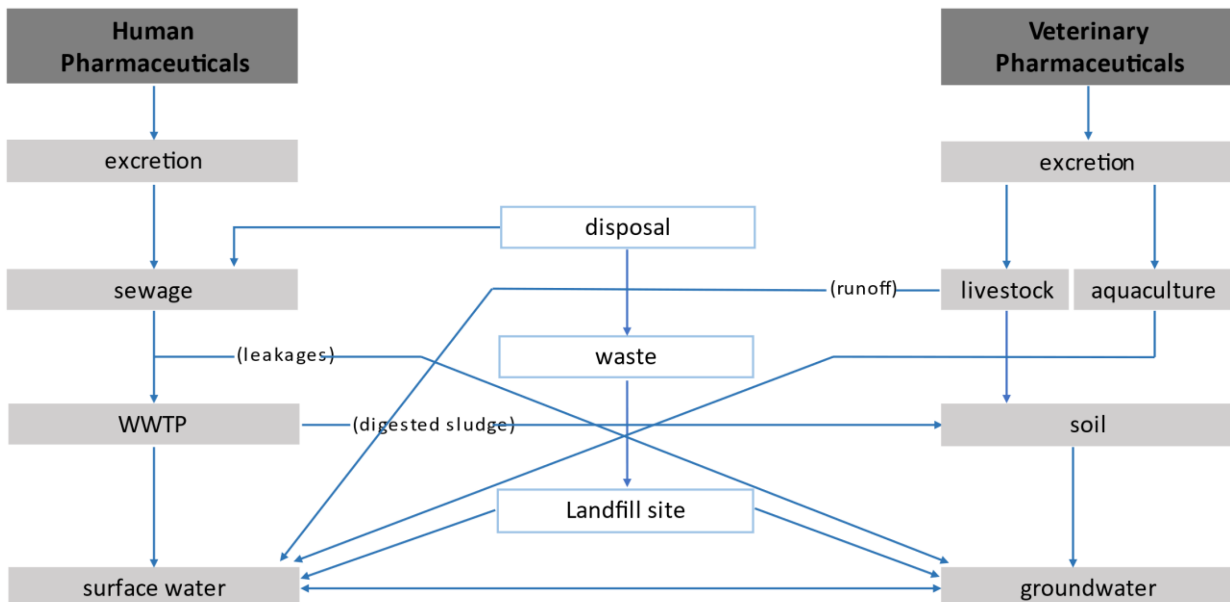

Figure 1.3 | Sources, pathways, and fate of pharmaceuticals in the environment (World Health Organization, 2012).

1.1.4 Occurrence and detection of pharmaceuticals in the environment

Understanding the behaviour and fate of pharmaceuticals and their metabolites in aquatic ecosystems remains a complex area of study, especially when considering the multitude of substances available for consumption. Characterized by their low volatility, these compounds are primarily dispersed in the environment through aqueous transport and can also propagate through the food chain (Fent *et al*., 2006).

These compounds along with their metabolites, exhibit different features designed to have a specific mode of action and site target activity, and often hold a degree of persistence in the body, making them an interesting subject for ecotoxicological studies concerning effects on biota, and human health (Fent *et al*., 2006; Patel *et al*., 2019). In contrast to traditional chemical contaminants, pharmaceuticals can be, in most cases, bioactive and lipophilic substances intentionally formulated to be effective at very low concentrations, and, at some cases, resistant to environmental degradation, accumulative in life forms, and remain biologically active (Patel *et al*., 2019; Chavoshani *et al*., 2020; Bavumiragira *et al*., 2022). Most of these compounds exhibit polarity, having more than one ionizable group, with their ionization behaviour influenced by the pH of the surrounding medium (Patel *et al*., 2019). They contain a chemically complex molecules with a large variety of structures, shapes, molecular masses, and functionalities, and were originally developed for a specific activity in a target organism (Patel *et al*., 2019).

Pharmaceuticals, despite being designed for chemical stability, undergo various transformation and degradation processes once released into the environment that can shape the fate of these compounds in the water and their byproducts that are either more or less toxic compared to the parental compound (Bavumiragira *et al*., 2022). These processes, including photodegradation, hydrolysis, oxidation, reduction, microbial degradation, and biodegradation, play a crucial role in attenuating pharmaceutical contaminants, however, they can also lead to further transformations, reducing their environmental stability (Patel *et al*., 2019; Bavumiragira *et al*., 2022).

The spatial distribution of pharmaceuticals in aquatic systems varies globally due to differences in consumption rates and the mobility properties of these compounds (Hawash *et al*., 2023). Studies conducted thus far have generally reported relatively low concentrations of pharmaceuticals in groundwater, surface waters, and treated water, typically below 100 ng/L (Hawash *et al*., 2023).

Hawash *et al*. (2023)'s review comprehensively assessed recently published articles, quantifying around 94 pharmaceuticals' residues in surface waters. Notable findings include the

detection of acetaminophen in Spain (up to 440 ng/L) (Mijangos *et al*., 2018) and Mexico (up to 4460 ng/L) (Rivera-Jaimes *et al*., 2018), carbamazepine in South Korea (up to 899.9 ng/L)(Na *et al*., 2019) and the USA (up to 249.3 ng/L)(Batt *et al*., 2016), diclofenac in Spain (up to 650 ng/L) (Mijangos *et al*., 2018), 17β-estradiol in Brazil (up to 6806 ng/L)(Montagner *et al*., 2019), ibuprofen in Portugal (up to 1.32 μg/L) (Paíga *et al*., 2016), and tramadol in the UK (670 ng/L)(White *et al*., 2019).

Despite typically low environmental concentrations, pharmaceuticals remain a significant ecotoxicological concern for aquatic organisms, emphasizing the need to monitor and measure their presence in the environment to comprehensively assess their ecological impact. Advances made in instrumentation and analytical techniques have enabled the detection and quantification of low concentrations (ng/L) of several pharmaceuticals across various environmental matrices. Gas chromatography with mass spectrometry (GC-MS) and liquid chromatography with mass spectrometry (LC-MS) are advanced methods commonly employed for detecting pharmaceutical compounds in aqueous media, being its selection dependent on the physical and chemical properties of the target compound (World Health Organization, 2012; Ibáñez *et al*., 2021). In the case of many polar pharmaceuticals, liquid chromatography-mass spectrometry is the preferred method due to their low volatility because when gas chromatography is employed, high boiling points are necessary to elevated column temperatures, which can lead to decomposition of these compounds (Patel *et al*., 2019). These techniques allowed the determination and quantification of almost 3000 biologically active compounds in the environment (Patel *et al*., 2019). Nonetheless, the reliable identification and quantification of pharmaceuticals, particularly in the case of metabolites in complex samples such as wastewater still poses significant challenges (Ibáñez *et al*., 2021).

1.1.5 Policy measures and strategies towards environmental regulation of pharmaceuticals

Despite rigorous safety regulations enforced by pharmaceutical organizations and regulatory bodies like the European Medicines Agency, significant environmental challenges persist (Chavoshani *et al*., 2020). The EU, in 1990, provided one of the first legal limits for antibiotics in milk (4-1500 μg/kg) and other food products of animal origin (25-6000 μg/kg) (Chavoshani *et al*., 2020).

In 2000, the EU introduced the Water Framework Directive and marked a pioneering approach to protect water matrices (Hawash *et al*., 2023). The directive established a Watch List, aimed at identifying potential water pollutant substances that must be monitored by EU Member States at least every four years due to their significant risk to aquatic systems. Over the years, this

list and directive has featured various pharmaceutical products that are updated based on the availability of data to assess whether these substances pose a potential threat to aquatic systems or not (Hawash *et al*., 2023). Hospital liquid waste, including pharmaceuticals, require to be treated as waste, properly collected, and disposed of (Carraro *et al*., 2018; Adeleye *et al*., 2022;). However, the EU does not provide specific guidelines for hospital wastewater management, leaving member states to establish their own practices and upgrading wastewater treatment with advanced treatment steps is not always feasible nor sustainable in view of energy- and material use of these additional steps (Gildemeister *et al*., 2023). Some countries have regulation on hospital wastewater pretreatment before being introduced into municipal streams (Carraro *et al*., 2018; Adeleye *et al*., 2022).

During 2006, the European Medicines Agency released a guideline describing how to evaluate the potential risks of pharmaceutical products entering the environment, only focused on the environmental risks associated with the usage and not with storage, disposal, synthesis, or the manufacture of these substances (Aguirre-Martínez *et al*., 2016).

To address the issue of harmful effects of pharmaceuticals in the environment, the concept of ecopharmacovigilance has gained global attention, promoting detection, evaluation, understanding and prevention, as a plan strategy to identify potential risks and hazards imposed by these substances (Jose *et al*., 2020). As pharmaceutical concentrations in the environment increase, conducting Environmental Risk Assessments (ERA) for both existing and new drugs becomes crucial (Jose *et al*., 2020). Many countries and organization like the Organization for Economic Cooperation and Development (OECD) have adopted these ERA procedures to protect the nature (Jose *et al*., 2020).

The guidelines on the ERA of the medicinal products for human in the EU is described in Article 8(3) of Directive 2001/83/EC. This directive states that pharmaceuticals causing potential risk to the environment must be reported and analysed but lacks specific details regarding the environmental protection goals and mandatory risk mitigation measures for dangerous substances (Jose *et al*., 2020; Gildemeister *et al*., 2023). In 2019, new guidelines were adopted by the European Medicines Agency and the European Commission, that included the hazard assessment in addition to current risk evaluation increasing data transparency, closing data gaps, and controlling the production of pharmaceuticals (Jose *et al*., 2020; Gildemeister *et al*., 2023). An ERA involves a series of steps for the assessment of pharmaceuticals that, in specific cases, will require a range of studies compliant with Good Laboratory Practice-compliant on ecotoxicity according to Organisation for

Economic Co-operation and Development (OECD) test guidelines and on physico-chemical behaviour in environmental compartments including sewage treatment (Gildemeister *et al*., 2023).

More recently, in April 2023, the European Commission adopted a proposal for a new Directive and Regulation, aimed at revising and replacing the current overarching pharmaceutical legislation (European Commission, 2023). This novel legislation, for the first time, empowers authorities to reject, suspend, or amend drug authorizations in the case of environmental harm, should sufficient risk mitigation measures are not in place. The proposed regulation also introduces post-authorization requirements, necessitating the conduct of environmental risk assessments for products authorized prior to the existing regulation (European Commission, 2023).

Expanding the environmental monitoring of pharmaceuticals stands out as a critical action in the strategic approach, achieving awareness of actual drug concentrations across various environmental compartments, enhancing environmental risk assessment studies and implementing more targeted measures when necessary (Cristóvão *et al*., 2020). Additionally, exploring innovative treatment technologies for pharmaceutical removal is of great significance.

1.1.6 Cancer, antineoplastic drugs and environmental impact

Cancer is a generic term to designate a large group of diseases, in which the control of growth is lost in one or more cells, leading to hematological malignancies or a solid mass of cells known as a tumor that can invade adjoining parts of the body and spread to other organs (Thurston & Pysz, 2021; WHO, 2022). If diagnosed sufficiently early, a primary tumor can be removed surgically or treated by other means, such as radiotherapy, chemotherapy, targeted therapy, or antibody-based agents, yet, most tumors are not diagnosed early enough, and death ultimately occurs (Thurston & Pysz, 2021).

Cancer is a leading global cause of death, with nearly 10 million deaths worldwide in 2020 (Figure 1.4), accounting for approximately 26% (1.2 million) of all deaths EU countries in 2019, secondly only to cardiovascular diseases (*Health at a Glance: Europe 2022*, 2022; WHO, 2022). The most common cancers in the world are breast, lung, colon and rectum and prostate cancers as represented in Figure 1.3 (Ferlay *et al*., 2020; WHO, 2022). In Portugal, 60,467 new cancer cases were confirmed in 2020, with colorectal cancer being the one with the highest incidence, followed by breast and prostate cancers (Gouveia *et al*., 2019). The Instituto Nacional da Farmácia e do Medicamento (Infarmed, Portugal) provided the consumption data of 171 different antineoplastic drugs over 9 years, from 2007 to 2015 (Santos *et al*., 2017). Between 30-50% of cancer cases are

preventable, and mortality rates can also be reduced through earlier diagnosis and the provision of more timely and effective treatments (*Health at a Glance: Europe 2022*, 2022). Effective treatment of cancer relies on early detection, accurate diagnosis and access to surgery, chemotherapy and/or radiotherapy (WHO, 2022).

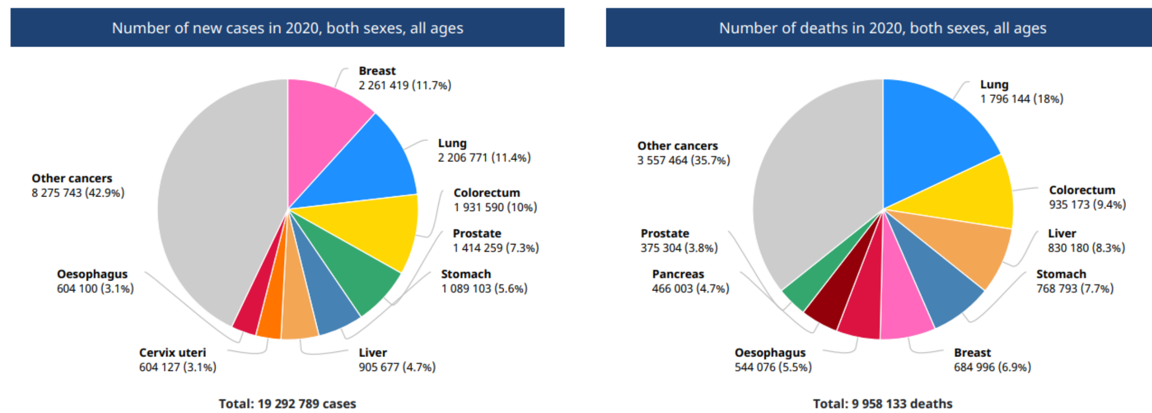

Figure 1.4 | Number of new cases and number of deaths related to worldwide cancer in 2020 (from Ferlay *et al*., 2020).

The rising incidence of cancer in modern societies has been and will continue to lead to an increase in the use of antineoplastic agents (also known as cytostatic or anticancer drugs), and consequently, the consumption of these drugs has surged in recent years, resulting in their inevitable discharge into the environment in the coming years (Ferrando-Climent *et al*., 2014; Gouveia *et al*., 2019). Chemotherapy treatments are primarily administered within hospital facilities and after the end of each treatment the patients are often discharged to go home; there are also the cases for specific chemotherapy treatments that can be delivered at home. Antineoplastic agents and their metabolites, though less studied for their occurrence and risk assessment compared to other pharmaceuticals, have potent mechanisms of action, not exclusively targeting cancer cells and thus, pose a potential risk to various organisms as they are excreted and enter sewage systems through hospital, home, and pharmacy industry effluents (Gouveia *et al*., 2019; Cristóvão *et al*., 2020). Martín *et al* (2014) reported measured environmental values of up to 190 ng/L in effluent wastewater for several cytotoxic compounds, namely cytarabine, doxorubicin, iphosphamide and paclitaxel, among others. Tamoxifen, an antiestrogen used in endocrine therapy, has been found in concentrations of up to 200 ng/l in surface waters (Roberts & Thomas, 2006), while predicted environmental concentrations for capecitabine, an antimetabolite, may reach up to 117 ng/L (Besse *et al*., 2012).

Antineoplastic agents are known for their genotoxic, mutagenic, carcinogenic, teratogenic, and embryotoxic properties. These effects primarily arise from their ability to enable the proliferation of malignant cells by interfering with DNA structure and function, acting quickly against cells division, and also by disrupting nucleic function and protein production throughout the entire cell division cycle (Figure 1.6) (Zounková *et al*., 2007; Kwok *et al*., 2017; Mukherjee *et al*., 2021). According to the mechanism of therapeutical action, antineoplastic agents are primarily classified as alkylating agents, antimetabolites, plant alkaloids, antitumor antibiotics, kinase inhibitors, hormones, platinum compounds and monoclonal antibodies (Brunton *et al*., 2018; Damasceno *et al*., 2023). Antineoplastic drugs are effective against both proliferating and resting cells, preventing abnormal cell division, and ultimately disrupting mitosis (Kwok *et al*., 2017; Mukherjee *et al*., 2021). Some of these antineoplastic compounds can act by blocking the synthesis of DNA, interfere with DNA function by becoming incorporated into it, interact directly with DNA by cross-linking mechanisms, (intercalation between base pairs, alkylation or methylation in the major groove) interfere with the structural proteins important for the processing of DNA (Brunton *et al*., 2018; Kwok *et al*., 2017). There are, however, in addition to the modes of action previously discussed, a wide range of antineoplastic agents that use distinct mechanisms and approaches to fight against cancer cells.

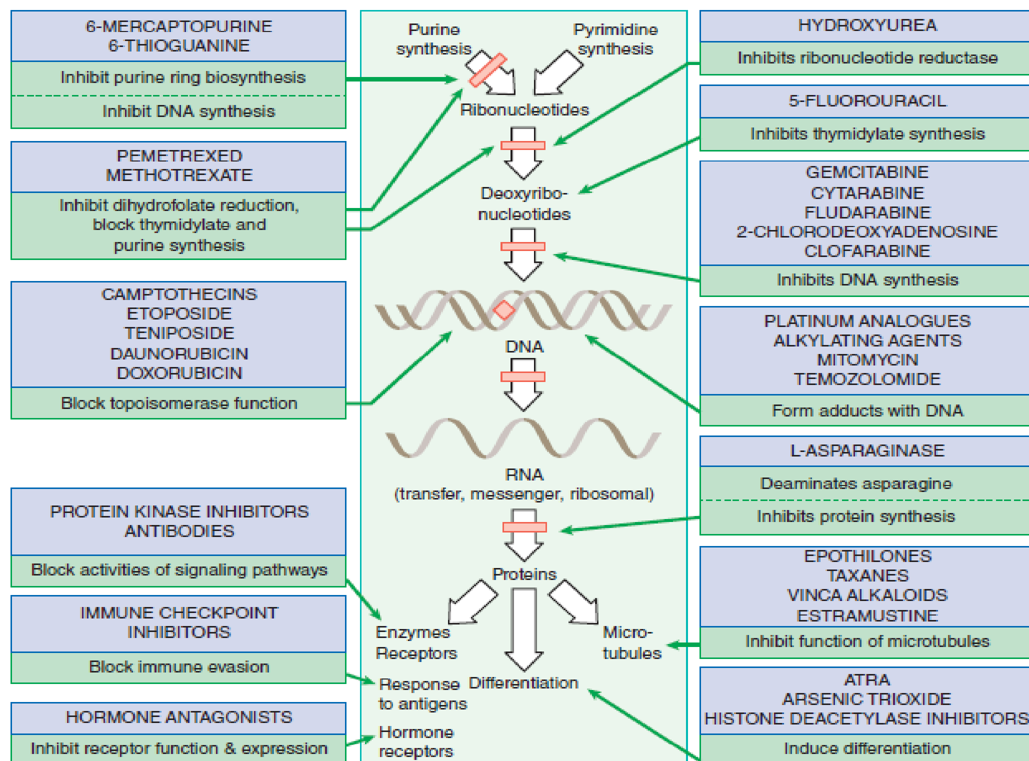

Figure 1.5 | Mechanisms and sites of action of some of the antineoplastic drugs used in the treatment of cancer (from Brunton *et al*., 2018).

The administration of combinations of antineoplastic drugs takes advantage of the different mechanisms of action (Kwok *et al*., 2017). By using agents that act at different phases of the cell cycle, synergistic effects and an increase in the collective antitumor effect may be obtained without an increase in undesirable side effects (Fig. 4) (Kwok *et al*., 2017). Classic chemotherapy agents are not tumor cell–specific and kill all cells actively undergoing cell division, resulting in the unintended destruction of normal host cells in the gastrointestinal tract, bone marrow, hair follicles, and other tissues (Kwok *et al*., 2017). Some antineoplastic drugs were already identified as carcinogenic to humans, such as etoposide, cyclophosphamide, tamoxifen, azathioprine, busulfan and chlorambucil (Gouveia *et al*., 2023). Others, as doxorubicin, cisplatin, dacarbazine and mitoxantrone have been classified as probably or possibly carcinogenic to humans (Gouveia *et al*., 2023). Still, most of antineoplastic agents are unclassified since there is a lack of toxicological studies as they still can pose potential for environmental risks (Gouveia *et al*., 2023).

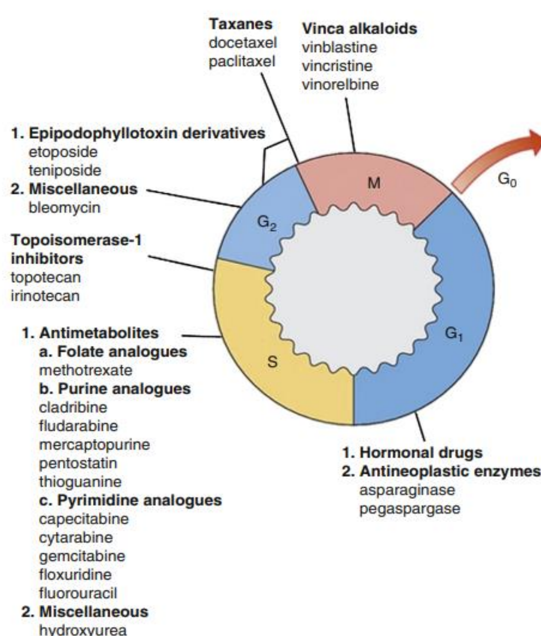

Figure 1.6 | Cell cycle sites of antineoplastic activity: G0, Resting phase; G1, period before DNA synthesis, during which the enzymes necessary for DNA synthesis are synthesized; G2, period of specialized protein and RNA synthesis and the manufacture of mitotic spindle apparatus; M, mitosis; S, DNA synthesis, during which DNA is replicated (from Kwok *et al*., 2017).

1.1.7 Ecotoxicity of antineoplastic agents

The environmental impact of pharmaceuticals, specifically antineoplastic drugs, has become a subject of growing concern. As a wide range of pharmaceuticals has been reported in

different compartments of the environment, also anticancer substances have been primarily reported in hospital effluents and wastewater treatment plants, especially in the last two decades, and together with household wastewater these sources become significant contributors to the presence of these drugs in aquatic environments, such as surface and groundwater (Roberts & Thomas, 2006; Besse *et al*., 2012; Martín *et al*., 2014).

Aherne *et al*. (1985) and Richardson & Bowron (1985) were among the first to publish the presence of antineoplastic drugs in the environment in 1980s while they were addressing the distribution of several pharmaceuticals in the sewage of hospital wastewater and other aquatic compartments. Since then, different authors have published environmental data of some of the most commonly used compounds in anticancer therapy like Steger-Hartmann *et al*. (1996), Ternes (1998), Mahnik *et al*. (2006, 2007), Catastini *et al*. (2008), Isidori *et al*. (2009), Liu *et al*. (2010), Yin *et al*. (2010), Martín *et al*. (2011), Perrodin *et al*. (2013), Negreira *et al*. (2014), Gómez-Canela *et al*. (2014), Česen *et al*. (2015), Azuma *et al*. (2016) Ferrando-Climent *et al*. (2014b) and Gouveia *et al*. (2023), were most of the antineoplastic agents are detected in low concentrations, ranging from µg/L to mostly ng/L.

Most of the data in the literature, work mostly with matrices like hospital effluents, WWTPs influents and effluents and in scarcer cases with surface water, to detect potential sources of these contaminants. Data regarding the levels of these compounds in surface water, groundwater, drinking water and soil is almost inexistent, and there is an even greater deficiency on the understanding of the presence of metabolites of these antineoplastic drugs in the environment.

Ecotoxicological assays conducted to date have primarily focused on some of the most used antineoplastic drugs in patient treatments, such as 5-fluorouracil, cyclophosphamide, ifosfamide, imatinib, tamoxifen, etoposide, cisplatin, doxorubicin, and a few others (Damasceno *et al*., 2023). Antineoplastics can be considered hazardous as they can cause eco-, geno- and cytotoxicity to several aquatic organisms (Zounkova *et al*., 2010; Damasceno *et al*., 2022). Commonly prescribed chemotherapeutic drugs, such as cyclophosphamide and ifosfamide, cause toxicity and teratogenic effects to *Danio rerio* embryos (Weigt *et al*., 2011). Parrella *et al*. (2014) showed that all the six antineoplastic agents (5-fluorouracil, capecitabine, cisplatin, doxorubicin, etoposide, and imatinib) tested in two *Daphnia* species promoted DNA damage at environmentally relevant concentrations. When looking at parental compounds and their metabolites, Zounkova *et al*. (2010) provided insight on the toxicity of 3 antineoplastic drugs by testing different trophic levels and evidencing the parental compounds being more toxic compared to their metabolites, which they exerted low to no toxicity.

In their review, Damasceno *et al*. (2023) conducted a comprehensive assessment of the impact of antineoplastic drugs. Their research revealed that, for compounds like the ones mentioned before, the most commonly used test organisms are *Pseudomonas putida*, *Raphidocelis subcapitata*, and *Synechococcus leopoliensis* for microorganisms; *Daphnia magna*, *Brachionus calyciflorus*, and *CerioDaphnia dubia* for invertebrates; *Lemna minor* for aquatic plants; and *Danio rerio* for vertebrate organisms. These tests encompass a range of endpoints, including mortality, immobilization, growth inhibition, not often reproduction, and, in the case of vertebrates, malformations. The EC_{50} values determined by each researcher conducting the experimental assay exhibited wide variations, influenced by the specific compound, the endpoint under evaluation, and the selected test organism. One of the conclusions one can draw from the Damasceno *et al.* (2013) review is the fact that most antineoplastic agents were more toxic to lower trophic levels, such as invertebrates.

The typology of tests commonly employed to assess toxic effects predominantly consists of short-term acute tests, with limited employment of chronic tests featuring longer exposure periods in accordance with standardized guidelines, a practice driven by animal welfare considerations and screening purposes (Fent *et al*., 2006). The compounds in question often target site-specific receptors and exhibit distinct modes of action that can operate un-specifically in organisms. The typical conducted assays are not explicitly designed to detect secondary effects on test organisms, such as impacts on organs, tissues, cells, alterations in metabolism, or the presence of compound mixtures that may induce synergistic or antagonistic effects (Fent *et al*., 2006).

1.1.8 Combined effects of antineoplastic agents

The residues of pharmaceuticals drugs are not released into the environment as single compounds, but rather as complex combinations of parent compounds and their metabolites. Neglecting the consequences of combined toxicity poses a significant challenge because it is the most realistic scenario in natural environments (Pavlaki *et al*., 2011; Silva *et al*., 2022). Different contaminants may induce adverse effects to non-target organisms through different and often unknown mechanisms of action, making it difficult to predict their joint toxicity and understand potential interactions (Silva *et al*., 2022). Low concentrations of antineoplastic agents in the environment, when combined, may potentially affect organisms compared to exposure to a single substance (Besse *et al*., 2012).

Research has investigated the combined toxic effects of various antineoplastic drugs on aquatic organisms, although it's not extensive. For instance, Brezovšek *et al*. (2014), Kundi *et al*. (2016) and Parrella *et al*. (2014) have all contributed valuable insights to this field. They investigated binary mixtures of antineoplastic agents, including 5-fluorouracil, cisplatin, etoposide, and imatinib mesylate, and observed intriguing interactions within these combinations. In their research, Parrella *et al*. (2014) found a synergistic tendency when exposing *CerioDaphnia dubia* to mixtures containing imatinib mesylate. On the other hand, Brezovšek *et al*. (2014) noted synergism between 5-fluorouracil and cisplatin in relation to *Raphidocelis subcapitata* and *Synechococcus leopoliensis*, and synergistic effects between 5-fluorouracil and imatinib mesylate on *Raphidocelis subcapitata*. In their study, Fonseca *et al*. (2019) conducted research on a ternary mixture of cisplatin, cyclophosphamide and tamoxifen. The conducted experiment employed a concentration design mirroring these antineoplastic agents' presence in the environment and assessed their toxic effects on *Nereis* diversicolor. Different endpoints, including behaviour, neurotoxicity, antioxidant enzymes, biotransformation metabolism, and genotoxicity, were examined to evaluate the impact of this mixture.

These studies collectively highlight the complex nature of interactions within these drug mixtures, indicating that the outcomes can be both compound-specific and species-specific and relying solely on single compound toxicity data is inadequate for accurately predicting the potential environmental risks associated with antineoplastic drugs.

To evaluate and predict the effects of known mixtures, two base reference models are applied, the concentration addition model (CA) (Loewe & Muischenek, 1926) and the independent action model (IA) (Bliss, 1939). The CA is a model applied when it's assumed that substances in a mixture act by the same mode of action, whereas the IA model is applied when that substances in a mixture do not act by the same mode of action (Bliss, 1939; Loewe & Muischenek, 1926). However, deviations from these two concepts can also occur in less complex mixtures of chemicals, deviations such as synergism or antagonism, dose level and dose ratio dependency exist (Figure 1.7) (Jonker *et al*., 2005; Pavlaki *et al*., 2011). Deviations from the reference models, such as synergism or antagonism, should be addressed when assessing the effects of chemicals in a mixture, which may result from various factors, including external exposures (e.g., binding and transport), toxicokinetics (from absorption, distribution, metabolism to excretion), or toxicodynamic processes (interaction at the target site) (Silva *et al*., 2022).

The MixTox model allows to detect more complex deviations that diverges from the reference models of CA and IA (Jonker *et al*., 2005). This model can describe how these well-

established mixture toxicity principles are incorporated in a coherent data analysis procedure enabling detection and quantification of dose level (deviation is dependent of the dose of each component in the mixture) and dose ratio (deviation is dependent of the ratio of the two components of the mixture) specific synergism or antagonism from both the concentration addition and the independent action models (Jonker *et al*., 2005).

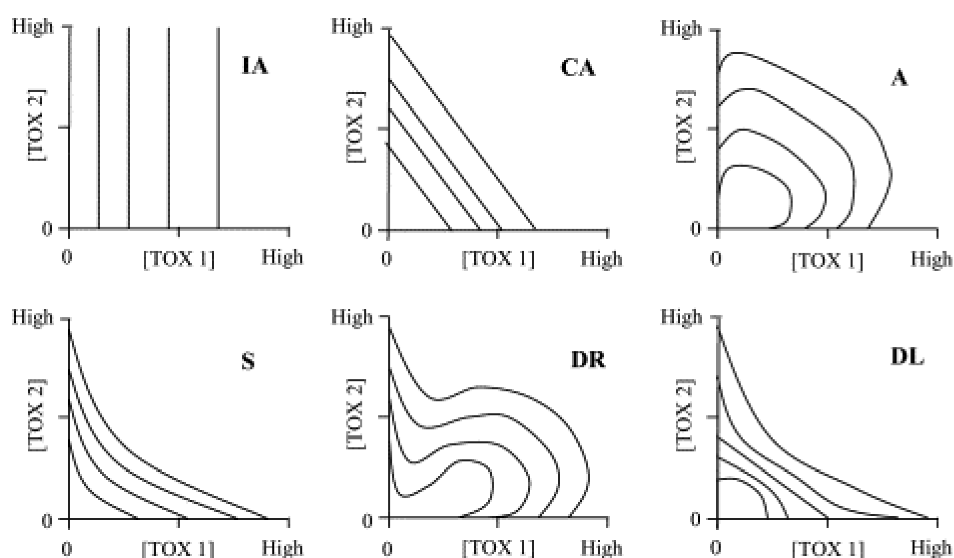

Figure 1.7 | Binary mixture concentration–response relationships illustrating independent action (IA) and concentration addition (CA) and all the four deviation patterns from these reference models: antagonistic deviation (A), synergistic deviation (S), dose ratio dependent deviation (DR) and dose level dependent deviation (DL) (from Ferreira *et al*., 2008). Representation of 2D isobolic curves.

1.1.9 *Daphnia magna* as a test organism

Daphnia, a water flea of the Cladocera order, has been a model organism for hundreds of years and among the best studied research ecological models to date (Ebert, 2022). With over 100 species of freshwater planktonic organisms, *Daphnia* exhibits a rather consistent body architecture and a body length ranging from a size of 0.5 mm to 6 mm or more (Ebert, 2022; Tkaczyk *et al*., 2021). These organisms can be found in standing freshwater environments, ranging from small pools to vast lakes, although they do not typically inhabit seawater like some of their related genera that colonize saltwater lakes and estuaries (Ebert, 2022). Daphnids are often undertaken as the pivotal role of keystone species in ponds and lakes, functioning as primary consumers. Their diet comprises bacteria, algae, cyanobacteria, protozoans, and other suspended particles, further solidifying their

significance in aquatic food webs, where they serve as both prey for fish and diverse invertebrate predators (Ebert, 2022; Tkaczyk *et al*., 2021).

Daphnia are well known for their ability to reproduce asexually through cyclic amictic parthenogenesis, and under favourable conditions, they can propagate asexually for many years (Ebert, 2022). However, when environmental conditions become unstable, they can switch to sexual reproduction, in which case, they will initially produce males asexually, followed by haploid eggs that need fertilization for reproduction (Ebert, 2022). As neonates are released into the external environment, they undergo four to six molting events to reach maturity, after which they continue to grow and moult in regular intervals, after each brood release, throughout their life (Ebert, 2022).

D. magna as a model organism, offers a multitude of practical advantages and remarkable attributes. These include straightforward laboratory conditions, short life cycle, parthenogenetic reproduction, high fecundity, easy handling, and low cost of maintenance, adhering to the 3Rs principles (replacement, reduction, and refinement (Bownik, 2020; Tkaczyk *et al*., 2021). *Daphnia* has a notable cloning capability, enabling the generation of numerous clonal offspring and the preservation of genetic lines for multiple generations under controlled laboratory conditions (Ebert, 2022). The relatively small size of *Daphnia*, compared to other model organisms, permits the simultaneous use of several individuals in a single experimental setup. *Daphnia*'s mobility is readily observable, making it a common choice for tests related to immobilization, lethality, and reproduction (Bownik, 2020; Tkaczyk *et al*., 2021). Another advantageous characteristic of D. *magna* is its transparent body, facilitating the concurrent measurement of various physiological endpoints, including heart activity, feeding parameters, and swimming behaviour parameters (Tkaczyk *et al*., 2021).

The use of *D. magna* in toxicological studies dates back to 1944 to evaluate industrial wastewater substances' toxicity (Anderson, 1944). Over the years, *Daphnia* has been extensively applied in pharmaceutical testing, exploring the toxicological effects from various therapeutic classes, including analgesics, antibiotics, antineoplastic drugs, antidepressants, antidiabetics, antiepileptic drugs, anti-inflammatory drugs, antipsychotics, beta-blockers, and lipid-regulating agents, using OECD tests as a basis (Tkaczyk *et al*., 2021). Up to now, toxicity tests are based on common OECD-standardized tests, assessing immobilization and reproduction effects (Tkaczyk *et al*., 2021).

1.2 Objective

The present study aimed to assess the single and combined ecotoxicological effects of two stressors to the non-target organism model *Daphnia magna* at a molecular and individual level. The substances tested were two antineoplastic agents, doxorubicin and oxaliplatin.

To reach the objective set in the present work, ecotoxicological standardized tests were used to evaluate the acute toxicity of each stressor as well as their combinations on *D. magna*. To assess the biochemical alterations of single and binary combinations of doxorubicin and oxaliplatin to *D. magna*, different biomarkers were measured, namely lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferases (GST), acetylcholinesterase (AChE), electron transport system (ETS) and cytochrome c reductase.

1.3 Dissertation structure

The current dissertation is organized in three chapters as it follows:

Chapter 1: General Introduction.

A general introduction to the subject studied is given along with the aim of the present dissertation. Chapter 1 is focused on the metabolism, occurrence, and detection of pharmaceuticals in the environment providing examples of environmental studies as well as the regulations all pharmaceuticals are being reigned along with the 2023 proposal to Reform of the EU pharmaceuticals legislation. It is also referring to the several ecotoxicity studies with antineoplastic agents using aquatic organisms providing insight on the eventual effects of those agents when act at an individual level or in a mixture exposure and stress the importance for a robust environmental hazard and risk assessment.

Chapter 2: Impact of Antineoplastic Agents in the Survival and Cellular Homeostasis of *Daphnia magna*;

In chapter 2, is given an introductory exploration into the theme of antineoplastic drugs within the environment. It delves into their significance as contaminants and underscores the importance of comprehending their effects in aquatic ecosystems, often in conjunction with a combination of other substances, and the potential ramifications of these mixtures on various organisms. It is presented the procedures undertaken and elucidates the outcomes assembled from subjecting *D. magna* to acute exposures of doxorubicin and oxaliplatin, both individually and in combination, and additionally, the response to oxidative stress on sub-lethal concentrations with both compounds.

Chapter 3, Remarks and Future Perspectives.

In Chapter 3, final remarks are being presented along with future perspectives and directives for a more robust hazard assessment of antineoplastic agents in aquatic organisms.

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Chapter 2

Impact of Antineoplastic Agents in the Survival and Cellular Homeostasis of *Daphnia Magna*

2.1 Introduction

In recent years, the presence of pharmaceuticals in aquatic ecosystems has raised concerns due to their potential fate, effects and overall impact in the environment, thus highlighting their classification as emerging contaminants. Among these pharmaceuticals, antineoplastic compounds have drawn particular attention, given their potent cytotoxic properties and widespread clinical use.

Cancer, a prevailing global health challenge, accounted for nearly 10 million deaths worldwide in 2020, underscoring the urgency for sustained research and comprehensive strategies to confront and mitigate its far-reaching consequences (Health at a Glance: Europe 2022, 2022; WHO, 2022). The growing incidence of cancer in modern societies has been leading to increasing use of antineoplastic agents, and consequently, this rise in drug consumption has inevitably translated into their release into the environment throughout the years (Ferrando-Climent *et al.*, 2014; Gouveia *et al.*, 2019).

Antineoplastic agents, characterized by their genotoxic, mutagenic, carcinogenic, teratogenic, and embryotoxic properties, were specifically designed to directly or indirectly disrupt DNA structure and function, and rapidly arresting the cell division cycle or potentially triggering cell death through apoptosis (Kwok *et al.*, 2017; Mukherjee *et al.*, 2021; Zounková *et al.*, 2007). After antineoplastics' administration in patients, the discharge of chemotherapeutic drugs into the aquatic environment, whether in their original form or as metabolites generated within patients, has been primarily attributed to the effluents from municipal and hospital wastewater systems (Liu *et al.*, 2010; Booker *et al.*, 2014; Fonseca *et al.*, 2017). There is considerable variability in the pharmacokinetics' antineoplastics agents when administrated and show strong interpatient variation, nonetheless, the average percentage of unchanged parent compound excreted in urine can range from negligible to above 75% (Booker *et al.*, 2014). The mechanisms of action of antineoplastic agents do not exclusively target cancer cells, and together with their metabolites, these compounds raise concerns about their potential risk to various non-target organisms as they are excreted and subsequently enter sewage systems. It's worth noting that wastewater treatment plants (WWTPs) have limited efficacy in removing them (Cristóvão *et al.*, 2020; Gouveia *et al.*, 2019).

As result of a limited efficacy of wastewater treatment plants for both domestic and hospital residual waters, antineoplastic agents have been released into the environment over the years without adequate restrictions, mainly because WWTPs were not explicitly designed to eliminate such substances (Olalla *et al.*, 2018). As a result, there is a pressing need to enhance the capabilities of such treatment plants by incorporating advanced treatment technologies such as ozonisation, photocatalysis, ultraviolet systems or membrane bioreactors (Olalla *et al.*, 2018; Mukherjee *et al.*,

2021). The presence of these antineoplastic agents has been detected in effluents at concentrations usually ranging from ng to µg/L (Cristóvão *et al.*, 2020; Liu *et al.*, 2010; Mahnik *et al.*, 2006, 2007; Martín *et al.*, 2011, 2014; Mukherjee *et al.*, 2021; Negreira *et al.*, 2014; Yin *et al.*, 2010).

While present in the environment at relatively low concentrations, these compounds have the potential to affect various organisms due to their unique mode of action and effectiveness at very low doses, yet research on their ecotoxicological effects and the associated human health risks in aquatic environments remains limited (Parrella *et al.*, 2014).

Doxorubicin, an anthracycline class of chemotherapeutic drugs, is an antibiotic initially isolated from *Streptomyces peacetius*, is essential in treating breast and esophageal carcinomas, solid tumors, osteosarcomas, Kaposi's sarcoma, soft tissue sarcomas, and Hodgkin and non-Hodgkin lymphomas (Varela-López *et al.*, 2019; Vincent *et al.*, 2013). The exact mechanism of action of doxorubicin is multifaceted and not entirely understood but dominant mechanism of action involves impairing the activity of topoisomerase II (TOP II), which results in DNA intercalation (Coldwell *et al.*, 2008; Varela-López *et al.*, 2019). Doxorubicin acts as a TOP II trap at cleavage sites, stabilizing the cleavage complex and preventing DNA resealing, leading to an increased number of double-strand DNA breaks (Coldwell *et al.*, 2008; Varela-López *et al.*, 2019). Several alternative mechanisms have been proposed for doxorubicin's broad-spectrum activity, as the formation of Doxorubicin-DNA adducts, which activate DNA damage responses (Coldwell *et al.*, 2008; Varela-López *et al.*, 2019), and doxorubicin’s ability to generate free radicals that produce hydrogen peroxide and hydroxyl radicals that cause damage to DNA and cell membranes (Rivankar, 2014).

Oxaliplatin, a platinum compound, is employed in the treatment against colorectal cancer, one of the most common human cancers, and together with other antineoplastic compounds to treat different gastroesophageal and pancreatic cancers (Alcindor & Beauger, 2011). Alcindor & Beauger (2011) observes that relatively few pharmacodynamic studies were performed, possibly because of an assumption that oxaliplatin and cisplatin shared the same mechanism of action. Oxaliplatin exerts its cytotoxic effect primarily by inducing DNA damage through the formation of intrastrand, interstrand, and protein cross-links that disrupt DNA replication and transcription processes, ultimately leading to cell death (Alcindor & Beauger, 2011; O’Dowd *et al.*, 2023). Once inside the cell nucleus, it exhibits a particular affinity for guanine-rich sequence regions, forming DNA monoadducts (Alcindor & Beauger, 2011). The formation of DNA adducts by oxaliplatin initiates a cascade of events, including inhibition of DNA synthesis, transcriptional blockage, and ultimately cell apoptosis (O’Dowd *et al.*, 2023).

The residues of pharmaceuticals are not released into the environment as single compounds, but rather as complex combinations of parent compounds and their metabolites. In order to understand the interactions between the antineoplastics compounds, binary mixtures were tested to predict their interactions and gain more insights into the complex responses of aquatic species to antineoplastic agents. Two reference models are primarily used for predicting the joint effects of mixtures: the Concentration Addition (CA) and the Independent Action (IA) models. Compounds with the same mode of action are expected to act in accordance with the CA model (Loewe & Muischenek, 1926), while those with different mechanisms of action are considered to follow the IA model (Bliss, 1939). However, deviations from these two concepts can also occur such as synergism or antagonism and should be addressed when assessing the effects of chemicals in a mixture. Such deviations may result from various factors, including external exposures (e.g., binding and transport), toxicokinetics (from absorption, distribution, metabolism to excretion), or toxicodynamic processes (interaction at the target site) (Silva *et al.*, 2022).

The present study aimed to assess the ecotoxicological effects of two antineoplastic agents, doxorubicin and oxaliplatin, when acting on the model organism *Daphnia magna*, from a molecular to an individual level. Acute standardized tests were conducted to evaluate the individual and combined toxicity on daphnid organisms at 24 and 48h of exposure. Additionally, and taking into consideration the antineoplastic agents' mode of action, any biochemical alterations at sub-lethal concentrations, including lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferases (GST), acetylcholinesterase (AChE), electron transport system (ETS), and cytochrome c reductase, will be assessed for a more robust approach.

2.2 Materials and Methods

2.2.1 Chemicals

The chemical compounds used in this study were Oxaliplatin (OXA), with an analytical purity of ≤100% (CAS No. 61825-94-3, Sigma-Aldrich, USA), and Doxorubicin hydrochloride (DOX) with an analytical purity of ≥ 99.13% (CAS No. 25316-40-9, TargetMol, USA). Test solutions for all exposures were performed with a stock solution prepared with the respective test compounds dissolved in culture artificial medium, while submerged for 5 minutes in an ultrasound bath, based on the American Society for Testing and Materials moderated-hard-water medium (ASTM, 2002).

2.2.2 Test organism and laboratory culture

The present work was conducted using the microcrustacean *Daphnia magna* Straus clone K6, under culture in our laboratory for more than 10 years and maintained in glass beakers in laboratory cultures. *D. magna* cultures were maintained in artificial medium based on the American Society for Testing and Materials moderated-hard-water medium (ASTM, 2002), under controlled temperature conditions ranging from 20 ± 2 °C, with a photoperiod of 16h:8h light/dark in non-aerated containers (OECD, 2004). Daphnids were housed in 1 L glass containers, each containing 800 mL of culture medium and a group of 20 individuals (only female). Cultures were fed, and medium was renewed three times a week. The food regime consisted of a suspension of unicellular algae, *Raphidocelis subcapitata*, at a concentration of $3×10^5$ cells/mL, and supplemented with organic extract (Marinure seaweed extract supplied by Glenside Organics, Ltd.) (Baird *et al.*, 1989). New cultures and all the subsequent experiments were performed using neonates less than 24 hours old from third to fifth brood from the parental cultures. In accordance with the OECD procedure (OECD, 2004), a reference substance, potassium dichromate ($K_2Cr_2O_7$), was employed to evaluate the sensitivity of the daphnids.

2.2.3 Toxicity Tests

2.2.3.1 Acute single exposure

Acute immobilisation tests with *D. magna* were carried out according to OECD Guideline 202 (OECD, 2004). Neonates (<24 hours old) were exposed to the respective test solutions and negative control (ASTM cultures medium) for a period of 48 hours. Exposure conditions were maintained at a controlled room temperature (20 ± 2 °C) and a photoperiod 16:8 h light/dark, with no food added. Immobilization was assessed at 24 hours and 48 hours after exposure started. Single compound toxicity tests were performed with five replicates for all concentrations of both test substances, with each replicate containing five organisms. Measurements for pH, conductivity, and dissolved oxygen were taken for each concentration and control. In the case of the OXA exposure, the test solutions remained unchanged throughout the 48 hours exposure period, while for the DOX exposure, test solutions were renewed at the 24 hours mark.

Preliminary tests with DOX found that this compound exhibited characteristics of chemical instability in the test solution within the time frame of the acute *D. magna* exposure, and therefore, the OECD guidelines were followed, and the solution was replaced at 24 hours (OECD, 2019).

The nominal concentrations for DOX tested were 0.20, 0.55, 1.53, 4.29 and 12 mg/L, and for OXA were 15.24, 27.43, 49.38, 88.89 and 160.00 mg/L. Concentrations chosen for the single exposure were selected after information considered in literature (Parrella *et al*., 2014) and (Zounková *et al*., 2007) and preliminary range finding tests.

2.2.3.2 Acute mixture exposure

The mixture compounds acute immobilisation test set up was also based on OECD Guideline 202 (OECD, 2004), but decreasing the number of replicates per treatment to one, allowing the use of a greater number of treatments within the test, to achieve a more comprehensive understanding of the chemical's response when combined. Control had six replicates and single compounds had two replicates. As in the single compound tests, each individual replicate of the treatments had five neonates (<24 hours old). Measurements were taken for pH, conductivity, and dissolved oxygen in the control group, as well as in the single and mixture compounds treatments where the concentration of each compound tested was the highest. During the mixture exposure, test solutions were renewed at 24h.

To evaluate binary mixture toxicity effects of DOX and OXA, concentrations were chosen based on the results of the single exposure tests, presenting a full factorial experimental design (Figure 2.1). The toxic unit (TU) approach was used, with one TU being defined as the exposure concentration that promotes 50% of the effect measured of each substance (EC_{50}) (Gestel & Hensbergen, 1997; Jonker *et al.*, 2005). The experimental design consisted of the single compound exposures of *D. magna* of six concentrations each and of 36 treatments with combinations of both compounds (Figure 2.1).

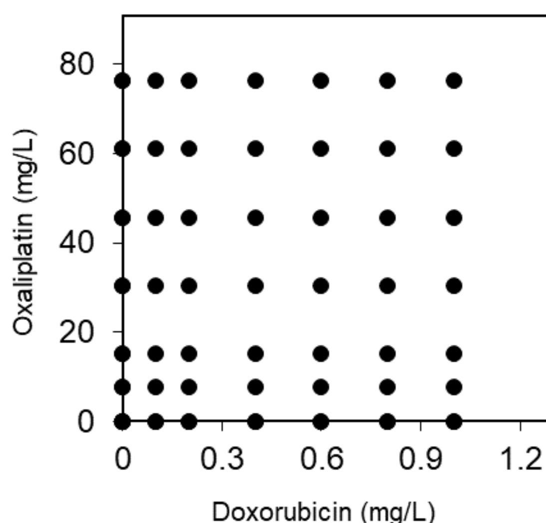

Figure 2.1 | Full factorial design of the combinations used for the doxorubicin and oxaliplatin mixture toxicity test.

2.2.4 Sub-lethal exposure for biomarkers determination

D. magna's exposure to obtain biological samples to quantify biochemical markers was divided in three parts, single exposure to DOX, single exposure to OXA and mixture exposure of DOX and OXA. Neonates (< 24h hours old) were exposed to each treatment for 48h. For all treatments, including controls, five replicates were prepared, having fifty organisms each in 100 ml test solutions for each replicate. Concentrations tested (Table 2.1) were chosen based on the NOECs (no observed effect concentration) obtained from the acute toxicity tests performed previously.

Table 2.1 | Sub-lethal concentrations of doxorubicin and oxaliplatin antineoplastics for biomarkers determination.

Test	Doxorubicin concentration (mg/L)	Oxaliplatin concentration (mg/L)
	0.01	-
	0.03	-
Single Doxorubicin exposure	0.05	-
	0.10	-
	0.20	-
	-	0.95
	-	1.91
Single Oxaliplatin exposure	-	3.81
	-	7.63
	-	15.25

Mixture Doxorubicin and Oxaliplatin exposure	***Mix 1***	0.01	0.95
	Mix 2	0.05	3.81
	Mix3	0.20	15.25
	Mix 4	0.01	15.25
	Mix 5	0.20	0.95

After 48h of exposure, 50 organisms from each replicate were transferred to an Eppendorf microtube, snap frozen in liquid nitrogen, and stored at -80°C until analysis was performed.

Previously frozen samples were thawed on ice and homogenized in 1.8 mL of phosphate buffer (0.2M, pH 7.4) using a sonicator. After homogenization, an aliquot of 150 µL of homogenate sample from each replicate was separated for lipid peroxidation (LPO) analysis. The remaining homogenate was divided into aliquots of 250 µL for acetylcholinesterase (AChE), 250 µL for electron transport system (ETS), 550 µL for catalase (CAT), glutathione-S-transferases (GSTs), glutathione reductase (GR), and glutathione peroxidase (GPx) and 450 µL for Cytochrome c Reductase (CYP c Reductase). Aliquot for CAT, GST, GR and GPx was centrifuged at 10,000 g for 20 minutes at 4 °C to isolate the post-mitochondrial supernatant (PMS), which was transferred to new Eppendorf tubes. Except for the homogenate aliquot separated for LPO levels determination, which was performed immediately, all other aliquots were treated with the Halt™ Protease and Phosphatase Inhibitor at a concentration of 10 µL/mL, as recommended by the manufacturer and afterwards stored at -80°C until the respective enzyme activity analysis of each biochemical parameter. All biomarker determinations were performed spectrophotometrically in micro-assays set up in 96 well plates.

Protein content (PROT) of the samples was determined through the spectrophotometric method of Bradford adapted to microplates (Bradford, 1976), using bovine γ-globulin as the standard and the absorbance read at 600nm.

For LPO determination, 4% 2,6-Di-tert-butyl-4- 256 methylphenol (BHT) in methanol was added to the homogenate aliquots (if sample had to be preserved be freezing again, this step would be done first). LPO levels were based on the quantification of thiobarbituric acid reactive substances (TBARS), according to the protocols described by Bird and Draper (1984) and Ohkawa *et al.* (1979), adapted for microplates. Absorbance was measured at 535 nm using a molar extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$. Lipidic peroxidation was determined measuring the absorbance of the substances reactive to thiobarbituric acid and expressed as nmol of TBARS per mg of protein.

Catalase activity was determined based on the method described by (Claiborne, 1985), using PMS and a reaction solution with hydrogen peroxide to measure the substrate decomposition at 240 nm for 2 min. Results were expressed as µmol of H_2O_2 consumed per minute per mg of protein, using a molar extinction coefficient of 40 M^{-1} cm^{-1}.

GPx activity was calculated by the method of Mohandas *et al* (1984), adapted for microplates. GSH and hydrogen peroxide were added lastly to start the reaction in the microplate The enzymatic activity was determined by measuring the absorbance extinction of NADPH at 340 nm for 3 min and was expressed as nmoles of NADPH oxidized per min per mg of protein.

GR activity was determined according to the methodology described by Mohandas *et al* (1984). The methodology was adapted for microplates, using of PMS and a solution with GSSG to start the reaction. Enzyme activity was determined by measuring the absorbance extinction of NADPH at 340 nm for 3 min and the results were expressed as nmol of NADPH oxidized per mg of protein.

Glutathione S-Transferase activity was determined based on the method described by Habig *et al.* (1974). GST activity was determined with PMS and by assessing GSH conjugation with 1-chloro-2,4- dinitrobenzene (CDNB), at 340 nm, every 20 seconds for 5 min using extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}. The enzyme activity was expressed as nmol of substrate hydrolysed per minute per mg of protein.

Electron transport system activity was measured using the p-iodonitrotetrazolium (INT) reduction assay, protocol based on the protocol from De Coen and Janssen (1997) and adapted by Rodrigues *et al.* (2015). INT solution was added after homogenised aliquots were centrifuged 1000g for 10 minutes at 4°C, and the rate of INT reduction in the presence of the non-ionic detergent Triton X-100 was measured with an absorbance of 490 nm for 3 min. The oxygen consumption rate was calculated based on the stoichiometric relationship in which 2 µmoles of formazan formed corresponds to the consumption of 1 µmole of oxygen and expressed in mJ per hour per mg of protein. The amount of formazan formed was calculated using a molar extinction coefficient of $1.59x10^4M^{-1}$ cm^{-1}.

Previously separated aliquots containing homogenate were centrifuged at 2000g for 3 min at 4ºC. The obtained supernatant was immediately assayed for AChE activity according to the Ellman method (Ellman *et al.*, 1961) adapted to the microplate (Guilhermino *et al.*, 1996). Enzymatic activity was initiated by adding the reaction solution, a mixture of potassium-phosphate buffer (0.1

M; pH 7.2), 0.075 M acetylthiocholine iodide and 10 mM 5,5′-dithiobis (2- nitrobenzoic acid) (DTNB), after an incubation period of 10 minutes, three readings were performed 5 min apart from each other with an absorbance of 414 nm. AChE was expressed in nmol of substrate hydrolysed per minute per mg of protein, using a molar extinction coefficient of $1.36 \times 10^{-3}\ M^{-1}\ cm^{-1}$.

Cytochrome c Reductase activity was measured using the Assay Kit Cytochrome c Reductase (NADPH) (Catalog Number CY0100; Sigma-aldrich). CYP c Reductase transfers electrons from NADPH to several oxygenases, the most important of which is the cytochrome P450 family of enzymes, responsible for xenobiotic metabolism. This assay measures the reduction of cytochrome c by NADPH-cytochrome c reductase in the presence of NADPH. Positive control solution was prepared using the Cytochrome c Reductase (NADPH) (Catalog Number C9363) diluted 10 times with Enzyme Dilution Buffer (Catalog Number E0155) 300 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA and 0.5 mg/ml bovine serum albumin.

The working solution was made by adding 9 mg of Cytochrome c to 20 ml of the Assay Buffer (Catalog Number A8477) 300 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA. After, it was added 384 µl of Cytochrome c Oxidase Inhibitor Solution (Catalog Number C9238) 50 mM potassium cyanide in water. Prior to use, working solution was warmed up to 25°C. NADPH Solution was made by adding 3.4 mg NADPH (Catalog Number N6505) to 4 ml of ultra-pure water.

Previously separated aliquots containing homogenate were centrifuged at 1000g for 10 min at 4 °C and the supernatant was extracted for posterior analysis, were 20 µL for each technical replica was used. Positive controls were performed by using 20 µL of positive control solution. After 200 µL of the working solution was added and to start the reaction 40 µL of NADPH solution was added in each well plate. Absorbance was read at 550 nm for a period of 3 min and the CYP c Reductase was expressed in nmol of substrate hydrolysed per minute per mg of protein, using a molar extinction coefficient of $21.1 \times 10^{-3}\ M^{-1}\ cm^{-1}$.

2.2.5 Data analysis

The EC_{50} values obtained for DOX and OXA single exposure test to *D. magna* were calculated by fitting the data to the non-linear regression model, $Y(X_i)=100/[1+(EC_{50}/X_i)\text{^}HillSlope]$, with $Y(x_i)$ as an normalized response, between 100% down to 0%, decreasing as X_i, exposure concentration, increases and HillSlope describes the steepness of the curve. The dose response regression curve was calculated using GraphPad Prism 8.0.1 software. The normality and homoscedasticity of the

data for all exposures were assessed through the Shapiro–Wilk ($p < 0.05$) and Levene ($p > 0.05$) tests, respectively. To obtain the NOEC and LOEC (lowest observed effect concentration) values for each of the compounds after the single toxicity tests, a parametric analysis of variance (ANOVA) and the multiple comparisons Dunnett's post hoc test (GraphPad Prism 8.0.1 software) was performed.

For the prediction of the joint toxicity of the combined antineoplastic agents the models used were Concentration Addition and Independent Action. The two models and their deviations (Synergism/Antagonism, Dose Level Dependency, and Dose Ratio Dependency) were compared using the method of maximum likelihood and the best fit was chosen using the MixTox tool and their plots made with the software SigmaPlot 14.0. The interpretation of each reference model parameters (*a* and *b*) value that define the functional form of the deviation pattern was made based on the information provided by Jonker *et al.* (2005).

2.3 Results

2.3.1 Acute single toxicity

The antineoplastic agents tested showed an increasing immobilisation of *Daphnia magna* as the concentration applied increased (Figure 2.2). After 48h of exposure the concentration that caused 50% of effect for doxorubicin (DOX) was set at 0.79 mg/L, NOEC was 0.20 mg/L and LOEC was 0.55 mg/L. For oxaliplatin (OXA), the EC_{50} was 59.61 mg/L, the NOEC was 15.24 mg/L and the LOEC was 27.43 mg/L. Results of both single antineoplastic agents' exposure at 24 and 48h are summarized in Table 2.2.

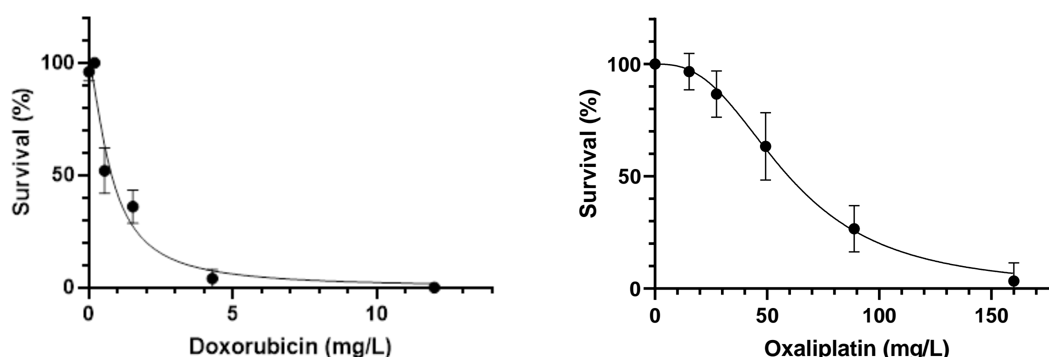

Figure 2.2 | Concentration response curve of *Daphnia magna* acute immobilisation test (OECD 202) after 48h of exposure to doxorubicin (left) and oxaliplatin (right) (mg/L) presented as mobile organisms in percentage of survival (mean±SE bars).

Table 2.2 | Concentration that caused 50% of effect (EC_{50}) in *Daphnia magna* after 24 h and 48h of exposure to doxorubicin and oxaliplatin (mg/L). No observed effects concentration (NOEC) and lowest observed effect concentration (LOEC) values in *Daphnia magna* after 48h exposure to doxorubicin and oxaliplatin (mg/L). EC_{50} fit parameters: confidence interval (CI) 95% and standard error (SE). Fitness of non-linear regression analysis expressed as R^2 is the coefficient of determination.

		Antineoplastic Agents	
Test Duration		**Doxorubicin (mg/L)**	**Oxaliplatin (mg/L)**
24h	**EC_{50}**	9.91	211.30
	CI 95 %	[5.13; 36.28]	[180.90; 285.90]
	SE	3.88	21.76
	R^2	0.56	0.69
48h	**EC_{50}**	0.79	59.61
	CI 95 %	[0.61; 1.04]	[54.46; 65.20]
	SE	0.10	2.63
	R^2	0.94	0.89
	NOEC	0.20	15.24
	LOEC	0.55	27.43

2.3.2 Acute mixture toxicity

Conceptual models, such as Concentration Addition and Independent Action, were employed to predict the combined toxicity of chemical mixtures. The summarized results of the MIXTOX model after the exposure of *D. magna* to a mixture of DOX and OXA after 48h can be found in Table 2.3.

Initially, the mixture effects were modelled using both the Concentration Addition (CA) and the Independent Action (IA) models as well as their deviations. After adding parameter *a* to the Concentration Addition model in order to describe synergism or antagonism, and parameter *b* that indicates the doses where synergism changes to antagonism, the tested deviation pattern for dose ratio-dependency (DR) was the one that more significantly improved the fitting of the data, decreasing the SS to the value of 49.39 significantly ($p(\chi^2) < 0.05$). Parameter *a* had a value of -2.39, which indicates synergism except for those mixture ratios where *b* is positive (*b* value of 5.34), and values indicate antagonism was mainly caused by DOX.

For the IA model, the DR deviation had the most significant reduction of the SS value, down to 51.24, and provided a significantly better fit than the reference model ($p(\chi^2) < 0.05$), and as well the fitting of S/A ($p(\chi^2) = 0.04, < 0.05$). Parameter *a* had a value of -7.83, which indicates synergism except for those mixture ratios where *b* is positive (*b* value of 8.31), and values indicate antagonism was mainly caused by DOX.

As observed, the DR deviation pattern appears to be the utmost best adjust to the data from the acute test immobilisation exposure of *D. magna* to the compounds combined.

Table 2.3 | Summary of the mixture analysis of *Daphnia magna*'s 48h immobilisation test effects exposed to doxorubicin and oxaliplatin.

	Concentration Action				**Independent Action**			
	Reference	S/A	**DR**	DL	Reference	S/A	**DR**	DL
max	0.98	0.98	0.98	0.98	0.98	0.98	0.98	0.98
$\beta_{doxorubicin}$	4.00	5.71	7.42	5.10	6.40	8.16	6.74	5.19
$\beta_{oxaliplatin}$	4.06	3.93	3.21	4.17	3.12	3.63	3.66	2.55
$EC_{50doxorubicin}$	1.20	1.05	0.94	1.10	0.96	1.04	1.00	1.14
$EC_{50oxaliplatin}$	72.83	67.27	81.36	70.40	55.96	73.00	75.82	74.36
a	-	0.71	-2.39	-0.02	-	-3.99	-7.83	-0.03
b	-	-	5.34	19.22	-	-	8.31	307.51
R^2	0.72	0.72	0.78	0.72	0.69	0.75	0.77	0.75
SS	62.79	60.93	49.39	62.42	68.06	55.51	51.24	54.36
$p(\chi 2)$	-	0.172	0.001	0.83	-	0.0004	0.0002	0.001
df	-	1	2	2	-	1	2	2

Max is the control response; β is the slope of the individual dose response curve; EC_{50} is the mean effect concentration; *a* and *b* are parameters of the function; SS is the sum of squared residuals; $p(\chi^2)$ indicates the outcome of the likelihood test, being achieved significant differences at < 0.05; df the degrees of freedom. S/A is synergism or antagonism, DR is dose ratio dependent deviation from the reference and DL is dose level deviation from the reference.

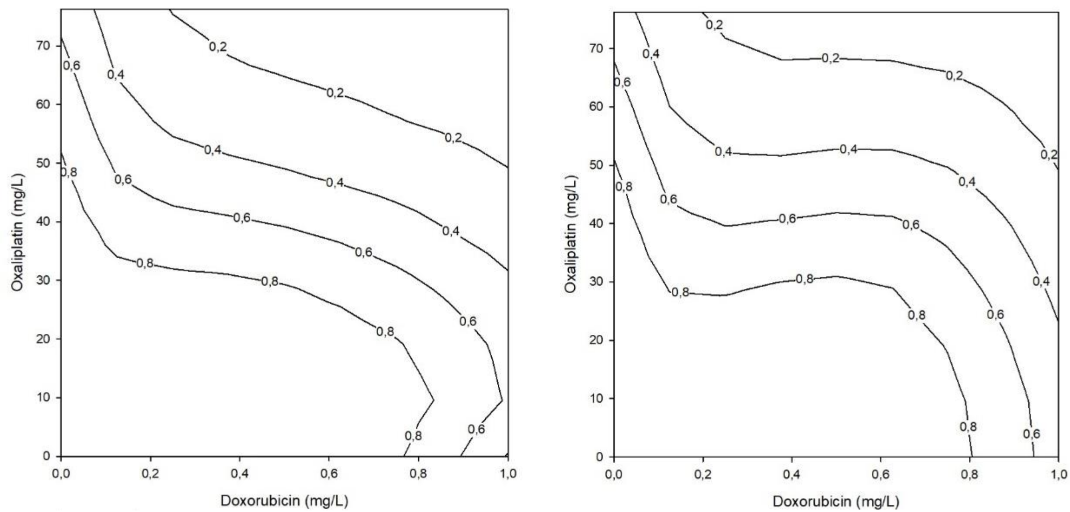

Figure 2.4 | Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the 48h immobilisation test data of *Daphnia magna* exposed to doxorubicin (mg/L) and oxaliplatin (mg/L), showing a dose ratio deviation to the CA model (left) and to the IA model (right).

2.3.3 Biochemical biomarkers

Results in Figure 2.4 show that, except for LPO and CYP c Reductase, no significant differences were found between the control and all treatments of DOX exposure in *D. magna*. Oxidative damage by LPO was significantly increased in organisms exposed to 0.1 and 0.2 mg/L of DOX (Figure 2.4.a). CYP c Reductase also increased significantly in the last concentration, 0.2 mg/L, of DOX (Figure 2.4.g).

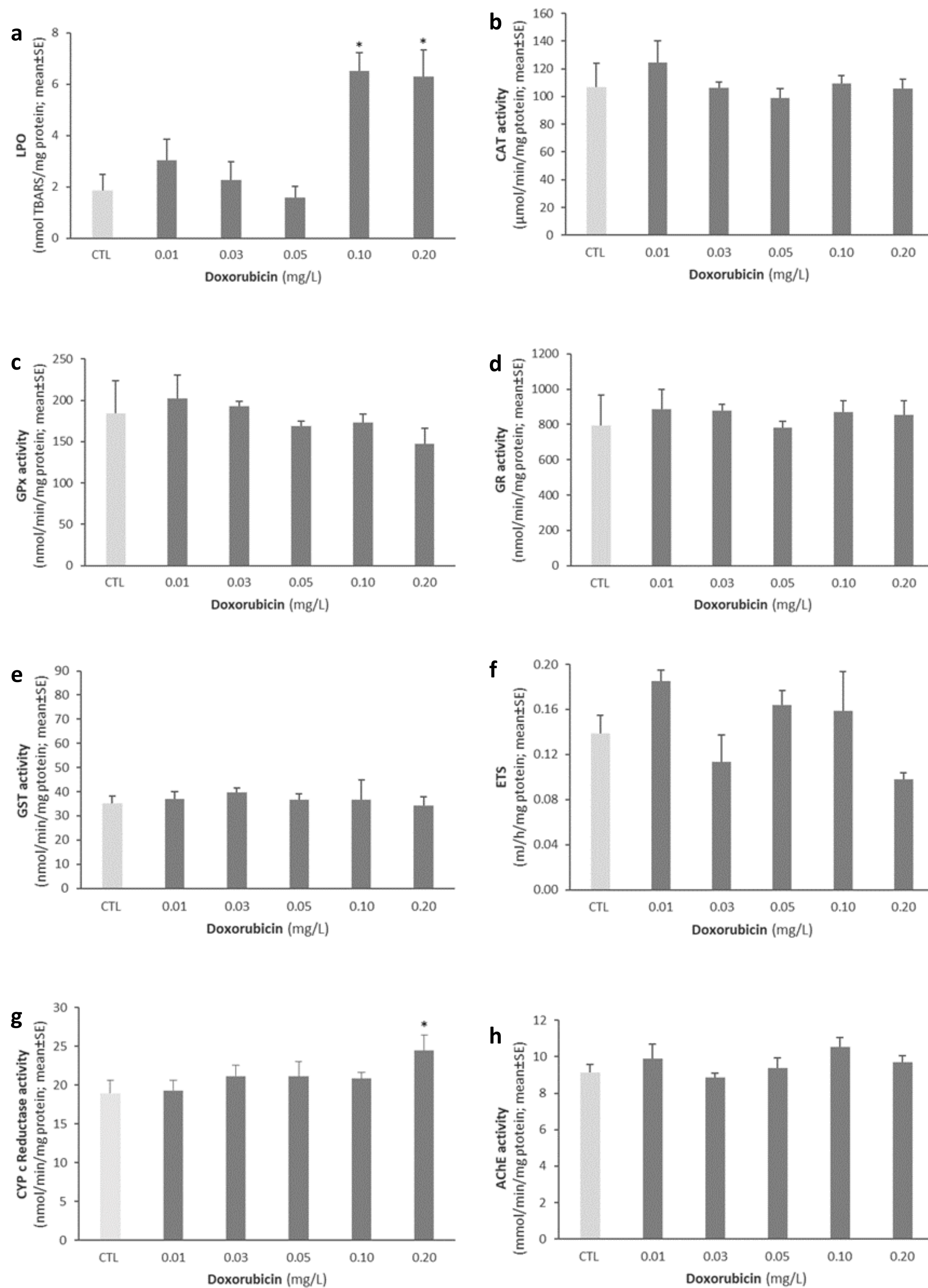

Figure 2.5 | Effects on biochemical endpoints measured in *Daphnia magna* after 48h of exposure to sub-lethal concentrations of doxorubicin: **a)** LPO – lipid peroxidation (nmol TBARS/mg protein; mean±SE); **b)** CAT activity – catalase activity (µmol/min/mg protein; mean±SE); **c)** GPx activity – glutathione peroxidase activity (nmol/min/mg protein; mean±SE); **d)** GR activity – glutathione

reductase activity (nmol/min/mg protein; mean±SE); **e)** GST activity – glutathione-S-transferase activity (nmol/min/mg protein; mean±SE); **f)** ETS – electron transport system (mJ/h/mg protein; mean±SE); **g)** CYP c Reductase activity – cytochrome c reductase activity (nmol/min/mg protein; mean±SE); and **h)** AChE activity – enzymatic activity of acetylcholinesterase (nmol/min/mg protein; mean±SE). CTL is the negative control. Statistically significant differences represented by * (ANOVA, Dunnett´s test, $p < 0.05$).

Figure 2.5 shows that antioxidant enzymes were affected, and oxidative damage was promoted when *D. magna* was exposed to OXA. LPO activity was significantly reduced in the last concentration of OXA, 15.25 mg/L, when compared with the control (Figure 2.5.a). Significant differences were found in concentration 7.63 mg/L of OXA, for CAT, GPx and GR (Figure 2.5.b-d). GST activity decreased at the three highest concentrations of OXA showing significant differences when compared to the control (Figure 2.5.e). ETS was reduced in all tested concentrations of OXA (Figure 2.5.f), while the electron transfer from CYP c Reductase's activity was increased when *D. magna* was exposed to 3.81 mg/L of OXA (Figure 2.5.g). No statistical differences were found in AChE activity when organisms were exposed to OXA (Figure 2.5.h).

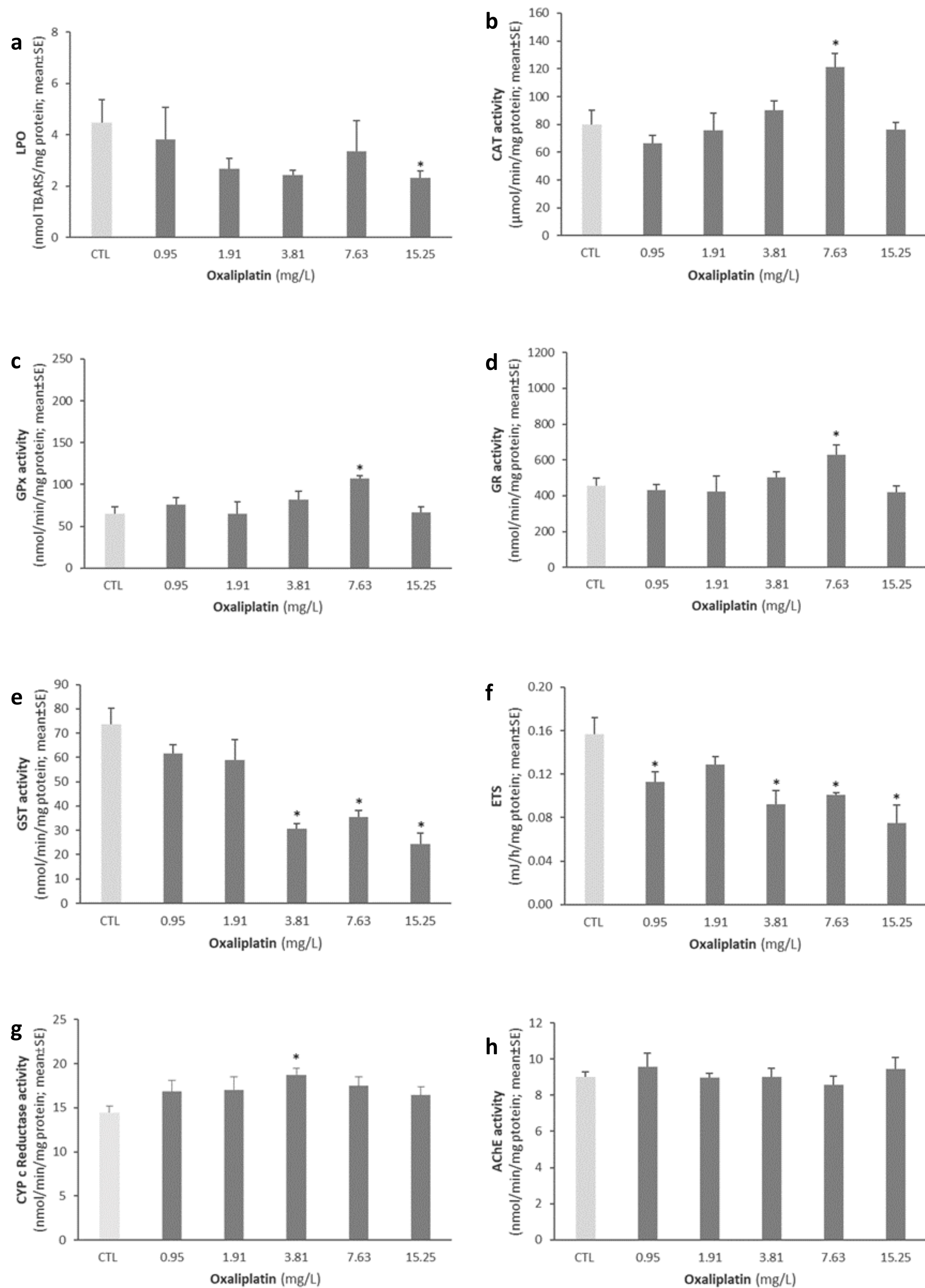

Figure 2.6 | Effects on biochemical endpoints measured in *Daphnia magna* after 48h of exposure to sub-lethal concentrations of oxaliplatin: **a)** LPO – lipid peroxidation (nmol TBARS/mg protein; mean±SE); **b)** CAT activity – catalase activity (µmol/min/mg protein; mean±SE); **c)** GPx activity – glutathione peroxidase activity (nmol/min/mg protein; mean±SE); **d)** GR activity – glutathione

reductase activity (nmol/min/mg protein; mean±SE); **e)** GST activity – glutathione-S-transferase activity (nmol/min/mg protein; mean±SE); **f)** ETS – electron transport system (mJ/h/mg protein; mean±SE); **g)** CYP c Reductase activity – cytochrome c reductase activity (nmol/min/mg protein; mean±SE); and **h)** AChE activity – enzymatic activity of acetylcholinesterase (nmol/min/mg protein; mean±SE). CTL is the negative control. Statistically significant differences represented by * (ANOVA, Dunnett's test, $p < 0.05$).

Results from the biochemical analysis of the exposure combinations of DOX and OXA in *D. magna* are presented in Figure 2.6. No statistical differences were found in LPO, GPx, GR and GST activity when organisms were exposed to the five different mixtures (Figure 2.6.a, c-e). Catalase activity was significantly inhibited in Mix 4 (0.01 mg/L of DOX and 15.25 mg/L of OXA) (Figure 2.5.b). Exposure to Mix 2 (0.05 mg/L of DOX and 3.81 mg/L of OXA), Mix 3 (0.20 mg/L of DOX and 12.25 mg/L of OXA), Mix 4 (0.01 mg/L of DOX and 15.25 mg/L of OXA) and Mix 5 (0.20 mg/L of DOX and 0.95 mg/L of OXA) caused a significant decrease in the electron transport system, ETS (Figure 2.5.f). CYP c Reductase activity was decreased significantly in Mix 3 (Figure 2.5.g). Both, Mix 3 and Mix 4, presented statistical differences for AChE, were its activity is reduced (Figure 2.5.h).

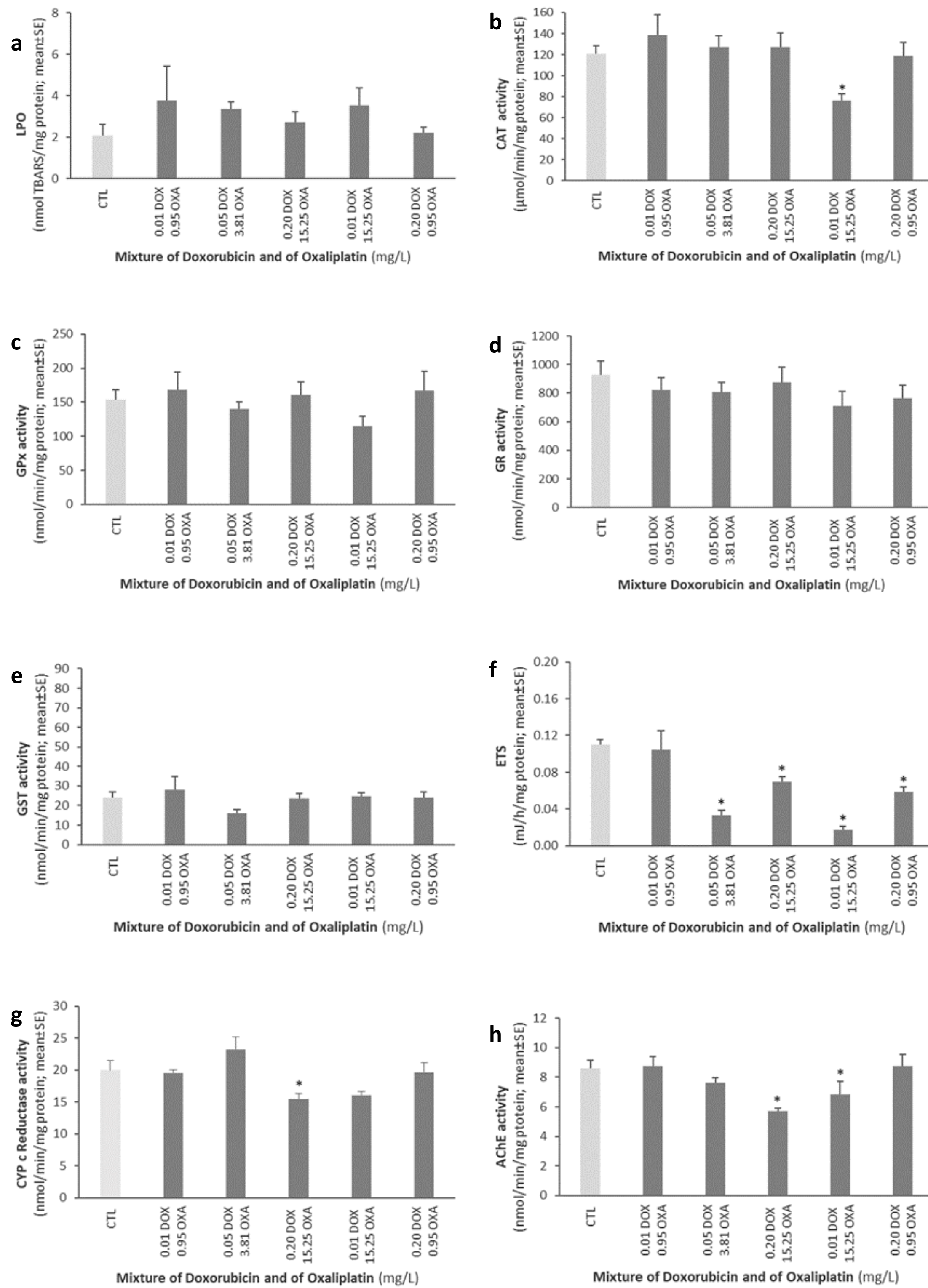

Figure 2.7 | Effects on biochemical endpoints measured in *Daphnia magna* after 48h of exposure to sub-lethal concentrations of doxorubicin and oxaliplatin mixtures: **a)** LPO - lipid peroxidation (nmol TBARS/mg protein; mean±SE); **b)** CAT activity – catalase activity (µmol/min/mg protein; mean±SE); **c)** GPx activity – glutathione peroxidase activity (nmol/min/mg protein; mean±SE); **d)** GR activity –

glutathione reductase activity (nmol/min/mg protein; mean±SE); **e)** GSTs activity – glutathione-S-transferases activity (nmol/min/mg protein; mean±SE); **f)** ETS – electron transport system (mJ/h/mg protein; mean±SE); **g)** CYP c Reductase activity – cytochrome c reductase activity (nmol/min/mg protein; mean±SE); and **h)** AChE activity – enzymatic activity of acetylcholinesterase (nmol/min/mg protein; mean±SE). CTL is the negative control. Statistically significant differences represented by * (ANOVA, Dunnett's test, $p < 0.05$).

2.4 Discussion

2.4.1 Single toxicity effects

Chemical instability of DOX was also reported in a sturdy by Parrella *et al.* (2014), while Kajander *et al.* (2021) did not observe a decline in the concentration of OXA over time in different test solutions in their reports.

The EC_{50} and LOEC values obtained in this study are considerably higher, approximately 10^{-6} times above than the concentrations of DOX found in the few available literature reports on water from hospitals and wastewater treatment influent and effluent, where concentrations are characterized in the order of nanograms per litre or lower (Mahnik *et al.*, 2006, 2007; Yin *et al.*, 2010; Martín *et al.*, 2011, 2014; Negreira *et al.*, 2014; Gouveia *et al.*, 2023), except for Souza *et al.* (2018) that determined levels ranging from 2.43 to 4.64 µg/L. However, no EC_{50} values for prolonged chronic studies involving this compound were found, which makes it challenging to assess the effects of prolonged exposure.

Regarding the acute toxicity of the single compound DOX in *D. magna*, the EC_{50} at 48 hours of exposure observed in this experimental set (0.79 mg/L) was slightly lower than values reported in the literature for the same organism and test, with values of 2.0 mg/L (Zounková *et al.*, 2007) and 2.14 mg/L (Parrella *et al.*, 2014). It's worth noting that the 95% confidence intervals reported by Zounková *et al.* (2007) ranged from 0.52 to 4.8 mg/L, while in the present study ranged from 0.61 to 1.04 mg/L showing an overlapping of values, thus indicating that the EC_{50}s may not be statistically different.

A literature assessment of DOX toxicity in aquatic organisms revealed that the antineoplastic agent had a 24 h EC_{50} of 5.18 mg/L for mortality in the aquatic species, *Ceriodaphnia dubia* (Parrella *et al.*, 2014), which is more comparable to the observations in *D. magna* in this experiment at 24 hours of exposure. In *Brachionus calyciflorus*, a growth inhibition EC_{50} at 48 hours was recorded at 7.7 mg/L (Parrella *et al.*, 2014). However, for the crustacean *Thamnocephalus platyurus*, DOX exhibited a higher toxic effect, with an EC_{50} of 0.31 mg/L at 24 hours of exposure (Parrella *et al.*,

2014). Han *et al.* (2015) reported a lesser toxic effect of DOX on the fish *Danio rerio*'s embryonic stage, with an EC_{50} of 16.96 mg/L at 72 h. Comparing the model species *D. magna*'s sensitivity to DOX with other aquatic organisms enriches our understanding of the compound's ecological impact, providing a more comprehensive interpretation of the study's findings.

Damasceno *et al.* (2023) conducted a comprehensive study analysis of various ecotoxicological parameters, applying a species sensitivity distribution (SSD) approach to assess the impact of antineoplastic compounds, imatinib, cisplatin, and 5-fluorouracil. Their SSD curves reveal that, overall, *Danio rerio* exhibits lower sensitivity compared to *Daphnia magna*, *Ceriodaphnia dubia*, *Brachionus calyciflorus*, and *Thamnocephalus platyurus* when exposed to these substances.

D. rerio is a vertebrate organism with a more complex body structure and detoxification system compared to the invertebrate *D. magna*. Furthermore, the mode of exposure differs, as daphnids are in direct contact with the surrounding environment during short-term tests, whereas zebrafish embryos are enclosed within the yolk sac for a portion of the test duration, providing some protection from the external environment. Kovács *et al.* (2016) investigated the acute and sub-chronic effects of four antineoplastic agents on embryos, early-life stages, and adult *D. rerio*, were it found cisplatin and imatinib to be more toxic to adults than to embryos at 96 hours. One plausible explanation for this observation could be attributed to the limited metabolic capacity of the embryo or its slower drug uptake (Damasceno *et al.*, 2023; Knöbel *et al.*, 2012).

SSD curves from Damasceno *et al.* (2023) also indicate that the sensitivity position of *Thamnocephalus platyurus* varies among all evaluated species depending on the antineoplastic agent to which they were exposed. In contrast, *Ceriodaphnia dubia* and *Brachionus calyciflorus* appear to exhibit higher sensitivity to the assessed compounds, contrasting with our own observation for *D. magna* when compared to the existing literature. *Daphnia* may exhibit greater sensitivity to specific antineoplastic agents compared to other species, due to differences in their physiological and biochemical responses. This heightened sensitivity in *D. magna* could be attributed to variations in their molecular or cellular mechanisms, which make them more susceptible to the toxic effects of certain antineoplastic agents. Further research is needed to explore the underlying factors that contribute to these differences in sensitivity among aquatic species.

D. magna exposure to OXA resulted in a 48 h EC_{50} value (59.61 mg/L) approximately 75.46 times higher than the one observed with DOX. This suggests that DOX exerts a higher level of toxicity on the tested organism. The difference in toxicity between DOX and OXA could be attributed to differences in their mechanisms of action. DOX, due to its ability to TOP II (Coldwell *et al.*, 2008;

Varela-López *et al.*, 2019) and to generate free radicals (Tacar *et al.*, 2013), can cause more extensive damage and have a greater impact on the survival of organisms. OXA, although it causes DNA damage through disruption and adduct formation (Alcindor & Beauger, 2011; O'Dowd *et al.*, 2023), may do so in a way that is generally less harmful compared to DOX, resulting in lower toxicity, as observed in the acute immobilisation test exposure in *D. magna*.

It's important to note that there is limited available literature on the ecotoxicity of OXA and no studies with *D. magna* or similar species. Ren *et al.* (2022) conducted tests with cisplatin, a platinum-based antineoplastic agent similar to OXA, on *D. magna* where it states a 48 h EC_{50} value of 1.77 mg/L in terms of immobilization, indicating a considerably higher level of toxicity compared to OXA. A similar pattern was observed when a freshwater alga, *Chlorella vulgaris*, was exposed to three different platinum-based antineoplastic agents, OXA, carboplatin and cisplatin. Cisplatin demonstrated higher toxicity, in terms of growth inhibition of *C. vulgaris*, compared to carboplatin and OXA (Dehghanpour *et al.*, 2020).

The concentration of OXA in a hospital effluent in Iran was determined by Ghafuria *et al.* (2018) to range from 3.18 to 850 µg/L. These concentrations, while lower than the 24- and 48h EC_{50} values found for *D. magna*, may be considered relevant for assessing potential long-term exposure effects, such as effects in reproduction and feeding outputs or even long-term survival and eventually assist in the environmental risk assessment of OXA.

Oxidative stress can trigger a series of adaptive responses within protective systems, leading to modifications in macromolecules and, ultimately, cellular and tissue damage (Regoli & Giuliani, 2014). To counter the excessive production of reactive oxygen species (ROS), the organism can enhance antioxidant activity by enhancing the performance of natural ROS-neutralizing enzymes like CAT, glutathione peroxidase GPx, and superoxide dismutase (SOD) (Regoli & Giuliani, 2014).

DOX, as an antibiotic antineoplastic agent, can directly affect the cell membrane by binding to plasma proteins, leading to enzymatic electron reduction of DOX to a semiquinone radical form under aerobic conditions and then reoxidised (cycle), donating an electron to oxygen to form superoxide (Tacar *et al.*, 2013; Asensio-López *et al.*, 2017). This process can result in the formation of highly reactive oxyradicals and hydroxyl free radicals, which can induce oxidative damage to cell proteins, phospholipids, DNA, and lipid peroxidation (Matczak *et al.*, 2009; Tacar *et al.*, 2013). In the case of *D. magna*, it was observed that oxidative damage due to lipid peroxidation increased at the two highest concentrations of DOX, 0.1 and 0.2 mg/L. This increase could be attributed to the free radicals generated by the redox cycle of the compound, potentially impacting the integrity of the cell membrane. However, none of the tested antioxidant enzymes were increased, compared to the

organisms from the control, to prevent further oxidative damage, nor in phase I or phase II glutathione conjugation reactions. The limited duration of exposure may not allow the organism enough time to upregulate additional enzymes at higher concentrations. In such scenarios with increased production of ROS, these compounds could evade the organism's defense mechanisms and interact with lipids, ultimately causing damage.

DOX activation is initiated within the oxidoreductase family, transforming the drug into a semiquinone free radical through electron reduction, via NADPH and cytochrome P450 reductase, into a semiquinone radical (Bartoszek, 2002; Finn *et al.*, 2011). This process has been identified as a significant contributor to DOX's cytotoxic effects, however, the role of NADPH-cytochrome P450 reductase in modulating DOX toxicity is complex (Bartoszek, 2002; Finn *et al.*, 2011). When *D. magna* is exposed to a highest concentration of DOX, an increase in the activity of CYP c Reductase can be observed, potentially promoting a reaction within the cytochrome family to metabolize this antineoplastic agent. Furthermore, no alterations in the activity of AChE were observed at any of the tested concentrations for this antineoplastic agent, indicating no neurotoxicity was promoted to *D. magna*.

The single exposure to OXA in *D. magna* resulted in alterations to the antioxidant enzymes involved in metabolic biotransformation via two different pathways, CAT and GPx, competing for the same substrate H_2O_2, a ROS species. Furthermore, GR activity also increased, highlighting the redox cycle function with GR playing a crucial role in supporting the replenishing of GPx. These alterations are visible across the concentration of 7.63 mg/L and exhibit a bell-shaped behaviour for these biomarkers. In bell-shaped curves, an increasing biomarker response is observed at low doses, reaching a maximum, followed by a decrease at higher concentrations of the exposure substance. These patterns represent a biphasic response, while monotonic responses are characterized by a continuous increase or decrease without reaching a maximum or minimum (Colas & Faucheur, 2023). Dose-response behaviours in the form of hormetic curves, both bell-shaped and U-shaped, have previously been observed in the context of antineoplastics and anti-angiogenic agents within tumor biology (Reynolds, 2010). Among various compounds reported to exhibit these behaviors, cisplatin, a platinum compound of the same family as OXA, stands out with biphasic responses at low concentrations in rats (Albertsson *et al.*, 2009; Reynolds, 2010). Fonseca *et al.* (2017) also found similar patterns in some of the biomarkers tested, when *Nereis diversicolor* was exposed to cisplatin, suggesting a complex interaction among the compounds and the signalling pathways that

contribute to dynamic monotonicity in a response that doesn't increases or decreases proportionally to the xenobiotic exposure.

Defense biomarkers tend to follow a bell-shaped dose-response curve, while damage biomarkers typically exhibit a linear trend, either increasing or decreasing, depending on the response patterns linked to the inherent mechanisms of these biomarkers (Colas & Faucheur, 2023). Molecular, sub-cellular, and cellular processes that play a role in an organism's defense against stress are induced at low concentrations and so, the concentrations of defense biomarkers can display a bell-shaped curve, increasing as the contaminant concentration rises as an effort to preserve cellular integrity (Colas & Faucheur, 2023). However, when the contaminant concentrations become excessively high, these defense mechanisms become overwhelmed, resulting in a gradual reduction in their response at the highest concentrations (Colas & Faucheur, 2023).

According to literature, GST catalyzes the conjugation of glutathione (GSH) to electrophilic xenobiotics, to inactivate them and facilitate their excretion from the body, participating in the role of detoxification of platinum derivatives, including OXA (Fronik *et al.*, 2022; Lecomte *et al.*, 2006; O'Dowd *et al.*, 2023). A possible interaction between OXA and GST may have occurred during a single exposure, that could have made the substrate unavailable for analysis during the determination of GST enzymatic activity, leading to an inhibition pattern on the enzyme's activity. Fonseca *et al.* (2017) tested cisplatin concentrations of 0.1, 10 and 100 ng/L on *N. diversicolor*. Significant differences were observed only at the highest concentration, 100 ng/L, when compared to the control group where a depletion on the GST enzymatic activity is observed. The authors also discussed the interaction between GSH, GST and cisplatin to form conjugation, that can generate thiyl radicals capable of producing ROS, and consequent depletion on both GST and GSH activity within the cells.

The activity of CYP c Reductase in OXA exposure also exhibits an inverted bell-shaped or U-shaped pattern. Its activity differs from the control at an intermediate concentration of 3.81 mg/L, probably indicating a supply chain of electrons for a CYP P450-related family involved in defense mechanisms against the compound inside the cell and a correlated need of the organism for detoxification of OXA. On the other hand, the electron transport chain in the mitochondrial membrane undergoes several changes across the tested concentrations of OXA, with ETS activity decreasing overall and promoting damage that can affect the aerobic energy metabolism of cells. Van Loenhout *et al.* (2020) reports that during chemotherapy, when there is a high production of reactive oxygen species (ROS), two mechanisms may be involved, one related to mitochondrial

processes and the other to the antioxidant system. Cisplatin has been reported to induce a loss of mitochondrial membrane potential and inhibit respiratory complexes, leading to the disruption of the electron transport system (ETS) in the mitochondrial membrane and electron leakage (Marullo *et al.*, 2013; Van Loenhout *et al.*, 2020).

2.4.2 Mixture toxicity effects

In order to gain a better understanding and predict the combined toxicity of the antineoplastics DOX and OXA, the Concentration Addition and Independent Action models were employed. The choice between these models usually depends on the similarity between the mode of action of the stressors, but it can also be based on target biological site. DOX´s primarily mode of action is hypothesized to be inhibition of TOP II that leads to more DNA breaks, which is different from OXA, an antineoplastic platinum-based agent that forms cross-links with DNA. However, some have reported the possible formation of doxorubicin-DNA adducts (Coldwell *et al.*, 2008; Vincent *et al.*, 2013; Varela-López *et al.*, 2019), comparable to the mode of action of OXA. Ultimately, both compounds impact the cell's DNA, resulting in the end in disruptions to DNA function and biosynthesis, implying that they target the same biological site. Given their different main mode of action, the IA model would be the primary choice for predicting their combined toxicity. However, due to their shared overall target of cellular DNA, the CA model was also considered.

The results from the mixture of DOX and OXA exposure in *D. magna* show a dose ratio dependency pattern for both CA and IA models. Within the range of concentrations studied for immobilisation, a synergistic response is observed for higher concentrations of OXA combined with lower concentrations of DOX but the response changes to mainly antagonism if the opposite is observed, lower concentrations of OXA combined with higher concentrations of DOX. These results suggest that the antineoplastic OXA primarily drives the toxic effects within this binary exposure scenario in the *D. magna* organism, while when DOX is present in higher ratios in the mixture, an antagonistic pattern is observed.

The CAT biomarker was inhibited in the 0.01 DOX + 15.25 OXA mg/L mixture, and the rest of the tested mixtures did not differ from the control, likely indicating that this is not the primary pathway chosen for H_2O_2 detoxification. However, GPx, which competes for the same substrate, showed no differences in this mixture when compared to the control, suggesting that neither of these pathways is the main process for ROS detoxification, and neither is detoxified through conjugation with GST phase II metabolism, which also showed no differences. This contrasts to

single OXA exposures, where based on GST activity it was presumed to be the main metabolic pathway for detoxifying higher OXA concentrations. Mixture 0.01 DOX + 15.25 OXA mg/L, as denoted by the deviation from the isobolic surface pattern derived from acute combined exposure results, appears to be closer to a possible synergism. Therefore, the elevated OXA concentrations in this mixture could be responsible for increased damage compared to the other mixtures tested.

The ETS activity was affected in four of the five combination tests, specifically in the mixtures of 0.05 DOX + 3.81 OXA mg/L, 0.2 DOX + 15.25 OXA mg/L, 0.01 DOX + 15.25 OXA mg/L, and 0.2 DOX + 0.95 OXA mg/L. In comparison to the results from single exposures, where DOX did not show differences in the activity of this biomarker, but OXA did, a pattern emerges indicating that the combination of these compounds may be acting synergistically, increasing the potentiation of the mixture. In these mixtures, the high concentrations of OXA and DOX appear to interfere with the electron transport system in the mitochondrial membrane and the respiratory system of the cells in the *Daphnia* organisms.

As CYP c Reductase show effects in the single exposure to DOX for the highest concentrations and OXA for intermediate concentration, the cumulative results of the compounds were not expected to cause inhibition in two of combined mixtures where the concentrations do not correspond to the ones in individual exposure. However, CYP c Reductase showed significant activity inhibition only in the 0.2 DOX + 15.25 OXA mg/L mixture, although the 0.01 DOX + 15.25 OXA mg/L mixture exhibited a similar pattern, but it wasn't significantly different. In mixtures, CYP c Reductase inhibition appears to be related to higher concentrations of OXA, whereas in singles, the higher concentrations did not show differences when compared to the control, with only a slight increase in the intermediate concentration suggesting that CYP c Reductase, may not be one of the cellular detoxification pathways for these compounds.

Acetylcholinesterase inhibition was observed in the mixtures of 0.2 DOX + 15.25 OXA mg/L and 0.01 DOX + 15.25 OXA mg/L, potentially leading to the accumulation of acetylcholine in nervous receptors that may cause neurotoxic effects on the test organism. Based on the results of the individual compound exposures, inhibition of AChE activity in the mixtures exposure was not expected. However, AChE inhibition is observed at concentrations where OXA is present in higher concentrations, combined with the highest concentration and with the lowest concentration of DOX mixtures, and consistent with the mixtures acute immobilisation test results, where OXA is hypothesized to be the compound that generates more toxic effects when combined. To date, there is no literature available regarding this AChE biomarker in *Daphnia magna* exposed to OXA or DOX that can explain this behaviour.

However, Penta *et al*. (1983) reported that cisplatin, a platinum-based antineoplastic agent, is capable of inhibiting acetylcholinesterase (AChE) in humans within a range of 27%. This inhibition often occurs during chemotherapy treatment (Lazarevic-Pasti *et al*., 2017). In a study by Fonseca *et al*. (2017), a hormetic response to cisplatin exposure was observed in *Nereis* diversicolor. At a concentration of 10 ng/L, this compound increased AChE activity, but at 100 ng/L, it significantly inhibited AChE activity. At the lower concentration, the organism appeared to mitigate the effects of the compound, but at the higher concentration, probable neurotoxicity became evident (Fonseca *et al*., 2017). According to Bhagavan (2002), AChE substrates are susceptible to nucleophilic attacks by the oxygen atom of the serine hydroxyl group present in the enzyme. The electrophilic reactive products of hydrolysis, containing platinum (Pt), may play a pivotal role in the inhibition of AChE activity (Fonseca *et al*., 2017). Interestingly, while single exposure to OXA did not demonstrate notable effects, when combined with DOX in mixtures, the interaction between these two antineoplastic agents may induce reactions that led to neurotoxic damage, particularly at higher concentrations of OXA within these mixtures.

2.4 Conclusions

The present study showed that DOX appears to be more toxic, in terms of survival, than OXA based on the EC_{50} values observed in the present study. However, OXA showed to promote more evident molecular effects at sub-lethal concentrations compared to DOX. Such differences can be attributed to the antineoplastic agents' different mechanisms of action. Even though OXA induces lower acute effects, at sub-lethal concentrations it elicits significant molecular alterations that may not promote direct cell death, eventually translated to *Daphnia* survival, but an attempt of the cells to repair the damage. On the other hand, DOX appears to trigger an immediate response, as it is known to be a highly cytotoxic agent, which results to cell death and thus lower *Daphnia* survival, reducing the chances of the organism's cells to respond to the hazard.

Even though, the concentrations tested here are not environmentally relevant, when it comes to an Environmental Risk Assessment (ERA) approach these concentrations, depending on the safety factor applied, can correspond to measured concentrations encountered in effluents and/or surface waters, thus providing significant input in environmental impact of DOX and OXA to aquatic organisms and the environment.

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Chapter 3

Remarks and Future Perspectives

3.1 Remarks and Future Perspectives

The aquatic environment serves as the ultimate repository for effluents from WWTP and can contain a mixture of xenobiotic compounds, including pharmaceuticals, due to the incomplete efficiency of WWTP treatment processes (Damasceno *et al.*, 2023). Cytostatic drugs, which are administered to cancer patients undergoing chemotherapy, have been detected in surface waters worldwide, raising concerns for both environmental and human health and like many pharmaceuticals, cytostatic drugs are only partially metabolized by the human body, with a portion of the administered dose excreted in urine and faeces (Ferrando-Climent *et al.*, 2014; Gouveia *et al.*, 2022). The limited degradation of most cytostatic drugs, as well as their metabolites, within conventional wastewater treatment plants allows them to enter surface and groundwater systems, presenting potential risks to aquatic ecosystems and the broader environment (Ferrando-Climent *et al.*, 2014; Gouveia *et al.*, 2022). This situation underscores the importance of addressing the impact of pharmaceutical contaminants on our water resources and ecosystems.

The present study has yielded valuable insights into the acute toxicity of both Doxorubicin (DOX) and Oxaliplatin (OXA) when exposed to *Daphnia magna*. Notably, DOX emerged as the more potent toxicant when tested individually. For both compounds, the calculated EC_{50} values, as well as the LOEC and NOEC values, exceeded those reported in the literature for detecting these antineoplastic agents in the environment. This may suggest a reduced risk of exposure to aquatic environments. However, in Environmental Risk Assessment (ERA) studies a safety factor, e.g., 1000-fold, is often used to mitigate uncertainties and ensure a conservative approach in the analysis of chemical hazards. This approach is used to safeguard the protection of the environment, and subsequently human health, by taking into consideration potential variations, such as individual susceptibility, limitations in the data and experimental approach as well as complex interactions in the ecosystem. Looking at the most sensitive parameter for DOX in *D. magna*, we can conclude that LPO was affected at a concentration of 0.1 mg/L. Taking into consideration the safety factor of 1000, this value drops to 0.1 µg/L, a concentration often encountered in environmental samples (wastewater effluents), as Martín *et al.* (2014) consistently reported. These results show the urgent need to assess antineoplastics agents in order to obtain ecotoxicological data for environmental protection.

As mentioned above, exposure to concentrations below the NOEC for both compounds resulted in alterations in the activity of the antioxidant defense system, lipid peroxidation, the mitochondrial electron transport system affecting cellular respiratory potential, and changes in

cytochrome reductase activity—all of which manifested after 48 hours of exposure. These findings suggest that even minor changes occurring within a short timeframe may, when considered in an environmental context of prolonged exposure, amplify into effects of greater relevance and consequence for aquatic organisms. Therefore, it is of interest to explore the effects of these compounds in a long-term chronic test involving *Daphnia magna* and mixtures scenarios, at lower concentrations similar to those found in the environment. Additionally, chronic tests could be paired with the use of molecular techniques, e.g., biomarkers for oxidative stress (as we presented in Chapter 2), but also include transcriptomics and metabolomics approaches, to better understand how these types of compounds may promote alterations to organisms at a cellular and molecular level (transcriptome and metabolome) all throughout a longer exposure period. Martins *et al.* (2021) used a multigenerational approach to assess toxicity effects on *B. calyciflorus* of 5-fluorouracil and DOX exposure and found that the population became non-viable after a second generation exposure to DOX, emphasizing the lack of ability to recover from the compound.

Exposure to the combined compounds did not follow a linear pattern but rather exhibited a complex interplay of toxic dose dependent responses in *D. magna*. Synergistic behaviours were observed at higher OXA concentrations combined with lower DOX concentrations, while higher DOX concentrations were associated with antagonistic patterns. This underscores the importance of studying contaminant mixtures, as their combined effects may differ from individual exposures, thus posing a greater impact on organisms coming in contact with antineoplastics agents. These patterns extended to the exposure of combined DOX and OXA concentrations, where it was observed that higher OXA concentrations seemed to result in more disparate effects compared to the control group. The mixtures 0.01 DOX + 15.25 OXA mg/L and 0.2 DOX + 15.25 OXA mg/L appeared to be the most affected after 48 hours of exposure, suggesting that OXA might be the primary influencer of toxic effects, though the influence of DOX should not be disregarded when present in sufficiently concentrated amounts.

In contrast to single exposures, neurotoxicity was observed in the mixtures. This not only reinforces the effect of a possible synergism at higher OXA concentrations but also raises questions about the possible interaction pathway of these compounds, which together inhibit acetylcholinesterase and may cause nerve damage to *D. magna*. Additionally, opposing effects of cytochrome reductase between single and mixed exposures were noted. This suggests the potential involvement of CYP P450 in the detoxification process at certain concentrations of single DOX and OXA exposures but not in the case of mixtures. To supplement this information, analysing the

activity of specific isoforms of CYP P450, such as CYP 3A4, a key player in drug detoxification (Baldwin *et al.*, 2009), would be of great interest.

This study significantly advances our comprehension of the acute toxicity of DOX and OXA in *Daphnia magna*, while also highlighting the broader implications for assessing and managing pharmaceutical contaminants in aquatic environments. It underscores the pressing need for ongoing research and the formulation of strategies to protect our freshwater ecosystems amidst evolving environmental challenges.

3.2 References

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