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A multi-biomarker assessment of single and combined effects of oxytetracycline and ciprofloxacin on *Mytilus spp.*

Avaliação baseada em biomarcadores dos efeitos isolados e combinados da oxitetraciclina e da ciprofloxacina em *Mytilus spp.*

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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 do Doutor Bruno André Fernandes de Jesus da Silva Nunes do Departamento de Biologia da Universidade de Aveiro

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“I had my ups and downs but I always find the inner strength to pull myself up”
- Queen B

o júri

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Após um longo e exigente percurso, que culminará na obtenção do tão desejado grau de mestre, é preciso glorificar aqueles que apesar de não serem aqui citados merecem toda a gratificação da minha pessoa. Primeiramente, e, como não podia deixar de ser, o meu orientador, o Doutor Bruno Nunes que me aceitou como sua aluna para o desenvolvimento desta tese de mestrado e que apesar do ano invulgar que vivemos e todos os contratempos que tiveram que ser enfrentados sempre me ajudou, guiou e incentivou a trabalhar com ética, responsabilidade e espírito de equipa e que foi sem dúvida uma peça chave para o desenvolvimento deste trabalho.

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palavras-chave

Mexilhões; antibióticos; ecotoxicologia biomarcadores enzimáticos; genotoxicidade.

resumo

Os antibióticos são uma classe de fármacos amplamente utilizada pelo mundo inteiro para tratar infeções, quer em humanos como em animais. Devido ao seu amplo uso, estes fármacos acabam por ser libertados no ambiente, uma vez que as estações de tratamento de águas residuais não são eficientes na sua eliminação. Isto faz com que estes compostos se encontrem amplamente distribuídos em ambientes aquáticos, justificando a crescente preocupação quanto ao impacto que os mesmos podem ter nas espécies lá presentes. Dessa forma, este trabalho visa caracterizar a toxicidade decorrente da exposição aguda (96h) e crónica (28 dias) a dois antibióticos, oxitetraciclina (0, 0.3125, 0.625, 1.25, 2.5 e 5 μgL^{-1}) e ciprofloxacina (0, 0.005, 0.013, 0.031, 0.078 e 0.195 mgL^{-1}) e a sua mistura (0, 0.005 mgL^{-1} CIP + 0.3125 μgL^{-1} OTC, 0.031 mgL^{-1} CIP + 1.25 μgL^{-1} OTC, e 0.195 mgL^{-1} CIP + 5 μgL^{-1} OTC) em mexilhões, *Mytilus spp*. As concentrações selecionadas implementadas nos dois tipos de exposição foram definidas tendo por base valores encontrados em ambiente aquático, sendo, portanto, ecologicamente relevantes. A análise dos efeitos toxicológicos foi efetuada pela avaliação de diversos parâmetros de stress oxidativos (actividades das enzimas superóxido dismutase e catalase), de biotransformação (atividade das isoenzimas glutationa S-transferases) e peroxidação lipídica (níveis de substâncias reativas ao ácido tiobarbitúrico). Estes parâmetros foram avaliados em três tecidos, nas brânquias, na glândula digestiva e no músculo. Foi também avaliado um parâmetro de genotoxicidade através do teste do cometa na hemolinfa e por último um parâmetro para avaliar a influência dos fármacos na dureza da concha dos organismos expostos. No caso da oxitetraciclina, este antibiótico causou dano oxidativo nas brânquias dos organismos expostos durante 28 dias às quatro concentrações mais altas (0.625, 1.25, 2.5, and 5 μgL^{-1}). No que diz respeito aos biomarcadores de genotoxicidade, foi possível observar um aumento do dano genotóxico com o aumento das concentrações, quer para a exposição aguda como para a crónica. Contudo, para o biomarcador da dureza das conchas, nenhuns efeitos foram observados após exposição a este antibiótico, mas é de salientar uma tendência de diminuição da dureza das conchas para a exposição crónica a oxitetraciclina. A exposição a ciprofloxacina resultou em dano oxidativo para as três concentrações mais altas (0.031, 0.078 e 0.195 mgL^{-1}), na glândula digestiva dos mexilhões expostos durante 28 dias a este antibiótico. A exposição aguda e crónica a este antibiótico foi capaz de induzir nos organismos expostos genotoxicidade, com um aumento caracterizado por um padrão dose-resposta crescente para ambas as exposições. Para o parâmetro da dureza das conchas não foram registadas alterações nos organismos expostos, independentemente do tempo de exposição. A mistura dos dois antibióticos foi feita tendo em consideração concentrações causadoras de efeitos nas

exposições isoladas, contudo para nenhuma das exposições foi possível observar dano oxidativo apesar de alterações nas defesas antioxidantes. Contudo, a mistura destes dois antibióticos mostrou ser capaz de causar danos genotóxicos, quer para a exposição aguda como para a crónica, sendo possível observar um aumento do dano com o aumento da concentração da mistura. Mais uma vez, para o parâmetro da dureza das conchas nenhuma alteração foi observada, após ambas as exposições. Este conjunto de dados permitem-nos inferir e reforçar a necessidade de avaliar os efeitos que estes compostos podem ter em organismos não-alvo. Ambos os antibióticos mostraram-se potenciais indutores de efeitos deletérios aos organismos expostos bem como a sua mistura. A exposição capaz de causar mais efeitos deletérios foi a oxytetraciclina. Contudo com os resultados de todas as exposições podemos concluir que estes fármacos induzem alterações nos organismos expostos podendo resultar em consequências para as populações, sendo de extrema importância ecológica a avaliação do impacto deste tipo de compostos.

keywords Mussels; antibiotics; ecotoxicology; enzyme biomarkers; genotoxicity.

abstract

Antibiotics are a class of drugs widely used throughout the world to treat infections in both humans and animals. Due to their widespread use, these drugs are eventually released into the environment, as wastewater treatment plants are not efficient in eliminating them. This causes these compounds to be widely distributed in aquatic environments, justifying concerns about the impact they may have on the species present there. Thus, this work aims to characterize the toxicity arising from acute (96h) and chronic (28 days) exposure to two antibiotics, oxytetracycline (0, 0.3125, 0.625, 1.25, 2.5 and 5 μgL^{-1}) and ciprofloxacin (0, 0.005, 0.013, 0.031, 0.078 and 0.195 mgL^{-1}) and their mixture (0, 0.005 mgL^{-1} CIP + 0. 3125 μgL^{-1} OTC, 0.031 mgL^{-1} CIP + 1.25 μgL^{-1} OTC, and 0.195 mgL^{-1} + 5 μgL^{-1}) in mussels, *Mytilus spp.* The selected concentrations implemented in both exposure types were defined based on values found in the aquatic environment and are therefore ecologically relevant. The analysis of toxicological effects was carried out by evaluating several parameters of oxidative stress (activities of the enzymes superoxide dismutase and catalase), biotransformation (activity of the isoenzymes glutathione S-transferases) and lipid peroxidation (levels of thiobarbituric acid reactive substances). These parameters were evaluated in three tissues, the gills, the digestive gland, and the muscle. A genotoxicity parameter was also evaluated by the comet assay in the haemolymph and finally a parameter to evaluate the influence of the drugs on the shell hardness of the exposed organisms. In the case of oxytetracycline, this antibiotic caused oxidative damage in the gills of organisms exposed for 28 days at the four highest concentrations (0.625, 1.25, 2.5, and 5 μgL^{-1}). With regard to the genotoxicity biomarkers, an increase in genotoxic damage could be observed with increasing concentrations for both acute and chronic exposure. However, for the biomarker of shell hardness, no effects were observed after exposure to this antibiotic, but a decreasing trend in shell hardness is noteworthy for chronic exposure to oxytetracycline. Exposure to ciprofloxacin resulted in oxidative damage for the three highest concentrations (0.031, 0.078 and 0.195 mgL^{-1}), in the digestive gland of mussels exposed for 28 days to this antibiotic. Acute and chronic exposure to this antibiotic was able to induce genotoxicity in the exposed organisms, with an increase characterized by an increasing dose-response pattern for both exposures. For the parameter of shell hardness, no changes were recorded in the exposed organisms regardless of the exposure time. The mixture of the two antibiotics was made taking into consideration concentrations causing effects in the isolated exposures, however for none of the exposures was it possible to observe oxidative damage despite changes in antioxidant defences. However, the mixture of these two antibiotics was shown to be capable of causing genotoxic damage for both the acute and chronic exposures, and an increase in damage could be observed with increasing concentration of the mixture. Again, for the shell hardness parameter no change was observed after both exposures. This set of data allows us to infer and reinforce the need to evaluate the effects that these compounds may have on non-target organisms. Both antibiotics were shown to be capable of causing deleterious effects to exposed organisms as well

as their mixture. The exposure capable of causing the most deleterious effects was oxytetracycline. However, with the results of all the exposures we can conclude that these drugs induce changes in exposed organisms that can result in consequences for the populations, and that it is of extreme ecological importance to evaluate the impact of these types of substances.

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List of Acronyms

CAT – Catalase

CDNB - 1-chloro-2,4-dinitrobenzene

CIP - Ciprofloxacin

CYP - Cytochrome P450

DNA - Deoxyribonucleic acid

EC – Comet Assay

EMA – European Medicines Agency

ERA – Environmental Risk Assessment

EU - European Union

FQs - Fluoroquinolones

GABA - gamma-Aminobutyric acid

GPx – Glutathione peroxidase

GSH – Reduced glutathione

GST - Glutathione-S-transferase

LOEC - Lowest Observed Effect Concentration

LMPA - Low Melting Point Agarose

LPO - Lipid peroxidation

MDA - Malondialdehyde

MN – Micronucleus

mRNA - Messenger RNA

NADPH - Nicotinamide adenine dinucleotide phosphate

NAPQI - N-acetyl-p-aminobenzoquinoneimine

NMPA – Normal Melting Point Agarose

NOECs - No Observed Effect Concentration

OECD - Organisation for Economic Co-operation and Development

OTC - Oxytetracycline

PBS - Phosphate-buffered saline

PBT – Persistence, Bioaccumulation and Toxicity

PEC – Predicted environmental concentration

RNA - Ribonucleic acid

RNS – Reactive Nitrogen species

ROS - Reactive Oxygen species

RQ- Risk Quotient

SOD - Superoxide dismutase

TBA - Thiobarbituric acid

TBARS - Thiobarbituric acid reactive substances

WWTPs - Wastewater treatment plants

I. General Introduction

As the largest representative of the aquatic environment, the ocean has been considered a reservoir of contaminated garbage and waste, at least until the end of the 1950s, mainly due to its own vastness, which would theoretically exceed the human capacity to change it and would allow eliminating all human waste. However, for some time, human activities have demonstrated that this conception was naive, if not erroneous (Carson, 2010). Nevertheless, the presence of pollutants in aquatic ecosystems has greatly increased in recent years. Aquatic ecosystems are now considered the final destination of many pollutants, which can come from natural sources, or from sources of anthropogenic contamination (Altindang et al., 2008). One of the main consequences of the models and patterns of production and consumption adopted by today's society is the generation of waste (Baudrillard, 2007; Thompson et al., 2009), a major environmental problem that demands more and more attention for a search for solutions, mainly with regard to marine pollution (Thompson et al., 2009; Derraik, 2002). In the past, contamination by organic matter, metals (such as copper, cadmium, chromium, lead, iron; Siddiqui and Pandey, 2019; Islam et al., 2015; Shanbehzadeh et al., 2014; Nandini et al., 2007), and pesticides (such as dimethoate, glyphosate, propanil, diuron, terbutryn, carbendazim, chlorfenvinphos and many others; Nunes et al., 2015 Abrantes et al., 2010; Engenheiro et al., 2005), were the most known forms of pollution (Sharma, 1994; Jain and Singh, 2003), however, since the 1970s, the presence of drugs in aquatic ecosystems, especially freshwater systems, was detected (Fick et al., 2009).

Drugs can reach aquatic ecosystems in various ways, including human excretion, inappropriate disposal, toilets and landfills, applications in veterinary medicine, hospital wastewater, among others (Breton and Boxall, 2003; Daughton and Ternes, 1999). Wastewater treatment plants (WWTPs) are however, the main source of pharmaceutical drugs in the wild. This occurs since WWTPs are not effective in completely removing these drugs, so these chemicals are introduced continuously into aquatic ecosystems (Jones et al., 2007; Kuch & Ballschmiter, 2001; Sedlak et al., 2000). Studies have shown that specific factors (such as the treatment temperature, redox conditions, chemical conditions as well as the hydraulic retention time at the waste treatment plant) influence the rate of removal of pharmaceutical products (Kosma et al., 2014). Drugs such as acetaminophen and ibuprofen have a high rate of degradation and can be degraded almost entirely (Gracia-Lor et al., 2012), however some compounds are only partially degraded, such as diclofenac and carbamazepine (Ziylan and Ince, 2011). Removal efficiency rates of pharmaceutical drugs are around 80%, except for

carbamazepine, diclofenac and ketoprofen (among others) due to their more recalcitrant characteristics (Matamoros et al., 2009). According to Kimura et al. (2005) for the case of ketoprofen, recalcitrance can be attributed to the two extended aromatic rings that this compound presents making them more difficult to be degraded; for diclofenac, its resistance to degradation may be due to the presence of chlorine in its structure. However, there are still few studies that focus on the bioactive metabolites associated with these drugs, and that can also cause harmful effects on ecosystems (Li et al., 2019).

As a consequence of their elimination at WWTPs, pharmaceutical drugs are frequently found in the aquatic environment. More than 559 active substances of pharmaceutical compounds have been detected in wastewater effluents and surface waters (Milmo, 2014) including antibiotics, anticonvulsants, anti-inflammatories, antidyslipidemic (Fent et al., 2006), antipyretics, cytostatics, and hormones (Ganiyat, 2008). This scenario results mostly from the ineffective disposal of these substances, after their use. Despite the overall efficacy in the removal of drugs at WWTPs, and the dilution effect when drugs reach the aquatic environment, the environmental contamination by drugs can occur in concentrations of parts per billion (ppb) or parts per trillion (ppt) (Jones et al., 2007). However, some studies report values of 1 mgL^{-1} (oxytetracycline, ciprofloxacin, losartan and cetirizine) in treated industrial effluents and recipient waters (Li et al., 2008; Larsson et al., 2007;). The dilution concept is extremely important for the management of waste discharges, but dilution is not a self-purification mechanism, as it does not lead to a reduction of the polluting constituent in the environment. However, the dilution mechanisms are important as they promote the reduction of toxicity due to the decrease in the concentration of toxic residues in the biota during the initial dilution and exposure period. In the study conducted by Minguez et al. (2014) it is possible to verify that the concentrations found in freshwater ecosystems vary greatly when compared to those found in saltwater ecosystems due to the dilution effect. These measurements were made in the Basse-Normandie region (France) and correspond to the maximum values measured for fresh and saltwater. For example, the value found for carbamazepine concentration in freshwater was of 21.1 $ngL^{-1,}$ whereas for saltwater this value was of 1.9 ng/L; another good example of this dilution effect is paracetamol, where the measured values for freshwater were of 305.5 ngL^{-1}, whereas for saltwater the value was much lower, of 3.3 ngL^{-1}.

The majority of the environmental studies published so far have identified and quantified these pollutants in low levels, which are not high enough to attain acute effects in organisms environmentally exposed. However, there is evidence that these pharmaceutical

compounds are present in concentrations sufficiently high to cause chronic effects on biota (Crane et al., 2006). Many studies have showed this type of low dose, chronic effect, for a large variety of compounds. For instance, Marques et al. (2004) have shown that salicylic acid was able to adversely affect the reproduction of *Daphnia magna* and *Daphnia longispina*. Studies conducted with *Pseudokirchneriella subcapitata*, exposed to the drugs carbamazepine, diclofenac and clofibric acid have demonstrated that these substances could impair growth of individuals of these species. Exposure of the organisms *Brachionus calyciflorus* and *Ceriodaphnia dubia*, also yielded a reduction in reproduction. (Ferrari et al., 2004, 2003).

Pharmaceutical drugs are particularly significant in environmental terms, since they have a series of characteristics that justify their relevance. Among these features, drugs have a high capacity for bioaccumulation or environmental persistence. Bioaccumulation is the process of assimilation and retention of chemical substances used by the environment by organisms. Absorption can occur directly, when substances are incorporated into the organism from the environment (water, soil, sediment), or indirectly, through the ingestion of food containing these substances. Bioaccumulated substances are not biodegradable, and due to various reasons, are not metabolized or excreted by animals (or both processes occur extremely slowly), so that throughout the entire life span of an organism, these substances become increasingly concentrated in its tissues (Borgå, 2013; Arnot & Gobas, 2006). Bioaccumulation can lead to a process called biomagnification (or trophic magnification), which consists of the transfer of bioaccumulated chemical substances from one trophic level to another (Wang, 2016). Some examples of pharmaceutical drugs whose presence in the wild may result in bioaccumulation are diclofenac, oxazepam, diphenhydramine, hydroxyzine and trimethoprim (Lagesson et al., 2016). The concept of environmental persistence is an important characteristic that can alter the potential that a given substance must promote harmful effects, and to be transported to remote locations (Mackay & Webster, 2005). The persistence of organic compounds is governed by ratios related to their rate of removal through biological or chemical processes, such as biodegradation, hydrolysis, atmospheric oxidation and photolysis (Calamari, 2002; Klecka et al., 2000). Some examples of persistent pharmaceutical compounds are carbamazepine, diclofenac, and ibuprofen, among others (Bu et al., 2016).

In addition, drugs are usually excreted not in their original form (parental compound), but as metabolites (Garza et al., 2019). Drugs in the wild may be further transformed, being able to acquire new forms (degradation products) (Kummerer, 2009c; Fent et al., 2006;). Drugs can be biotransformed by oxidation, reduction, hydrolysis, hydration, conjugation, condensation or

isomerization; but, whatever the process, the objective is to facilitate their excretion. The metabolites formed in metabolic reactions are more polar and therefore more readily excreted (Gerba, 2019). Degradation products are the result of a series of transformations that occur to a certain pharmaceutical substance and/or excipient. Some are biotic transformations as is the case of biotransformation and others are abiotic transformations, such as hydrolysis and photodegradation (Trautwein & Kummer, 2012; Escher & Fenner, 2011). This type of transformation is in general the main removal pathway in the organisms, but also in WWTPs, Degradation of pharmaceutical drugs usually occurs in a series of steps, from which the resultant degradation products are formed, which may in turn have similar properties as the original substances, such as persistence, bioaccumulation potential and even toxicity (Melo et al., 2014; Schenker et al., 2007). Both metabolites and degradation products may have new modes of action and new target organs (Jones et al., 2007; Fent et al., 2006; Alavijeh, 2005). This intrinsic property increases, for some compounds, their potential for bioaccumulation and persistence in the environment (Bottoni et al., 2010; Christensen, 1998).

1. Pollution in marine ecosystems

The oceans constitute the largest volume of life on Planet Earth (1.37 billion cubic kilometres) covering about 72% of its surface (Harris et al., 2014). The average depth of the ocean is approximately 3700 meters and connectivity is one of the main features that oceans have. The oceans play an extremely important role for the life of the planet, they are responsible for the generation of oxygen, they absorb carbon dioxide, they recycle nutrients, and they are also responsible for regulating global climate changes and temperature (Deutsch et al., 2015).

Nowadays, one of the major concerns that humanity faces is related to the ecological status of oceans and a considerable number of studies have strongly suggested that ocean's health is in jeopardy and needs urgent attention (Halpern et al., 2015, Rojas-Rocha, 2014; Halpern et al., 2012). As a result, marine environments are deteriorating consistently, and the loss of marine biodiversity increasingly undermines the ocean's ability to provide ecosystem services, impairing its ability to recover from challenges and damages (Worm et al., 2016).

1.1. Pharmaceuticals in the environment

At the end of the 19th century, the pharmaceutical industry was already well established and with great growth prospects (Pushpakom et al., 2018; Achilladelis and Antonakis, 2001;). Large-scale production and the accessibility to large amounts of pharmaceutical drugs gave rise to their indiscriminate use, and also of hygiene and personal care products. Such activity has become a widespread habit in urban society. Due to supply, use and disposal of these products, there has also been an increase in the amount of effluents containing this type of substances over the years, as well as a significant increase in these substances in water bodies (Mittal & Garg, 1994).

In the past 50 years, population growth has occurred at an unprecedented pace, with the world population currently totalling 7.5 billion people (Anson et al., 2019). Such growth has had a major impact on public health, which makes the implications for the future also worrying. The increase in concerns in the area of health occurs simultaneously with the constant growth of industrial production of personal care products, and also of pharmaceutical products, designed to reduce risks and combat the various diseases that plague humanity. Annually, thousands of tons of pharmacologically active compounds are used worldwide (Christen et al., 2010; Jones et al., 2003). Being fundamental for the prevention and treatment of several pathologies, drugs can, however, cause undesired effects on non-target organisms (terrestrial and aquatic), consequently altering the activity and balance of ecosystems (Ziylan et al., 2011; Celiz et al., 2009).

Drugs are an extensive and diversified group of organic compounds used in high quantities all over the world (Caliman et al., 2009). At the European level alone, more than 3,000 active ingredients are available on the market (Christen et al., 2010; Redshaw et al., 2008) and similarly to other organic pollutants, the source of contamination of ecosystems by therapeutic agents is primarily anthropogenic (Mompelat et al., 2009). However, the use of veterinary drugs in aquaculture and in the treatment of intensive animal breeding (cattle, pigs and birds) represents the main route of entry of drugs (namely antibiotics) into the environment, which can cause contamination of both aquatic and terrestrial environments (Boxall et al., 2004). Drugs used as food additives in fish farming are discharged directly into the aquatic environment, and a large part of these medications are not consumed by fish (Jacobsen & Berglind, 1988). High use of antibiotics (often indiscriminately) associated with their consequent release into the environment is a serious concern.

1.1.1. Transport and environmental dissemination of pharmaceuticals

Pharmaceutical compounds can be used for the purpose of providing deficient elements to the body, such as vitamins, minerals and hormones; prevention of diseases or infections; temporary blocking of a normal function; correction of a deregulated function; detoxification of the organism; and auxiliary diagnostic agents (Wenzel et al., 2001). After use, the molecules of pharmaceutical drugs are absorbed, distributed, and partially metabolized in the human body, being finally excreted to the sewage system, which are sent to WWTPs. Pharmaceutical drugs may reach WWTPs as unchanged compounds (or slightly transformed) and mainly as conjugates with polar molecules, which are the result from the metabolism process. However, not all countries have WWTPs, according to the report of the UNESCO (2017). On average, high-income countries treat about 70% of the municipal and industrial wastewater they generate. That ratio drops to 38% in upper middle-income countries, and only to 28% in lower middle-income countries. In low-income countries, only 8% of wastewater undergoes treatment of any kind. These estimates support the often-cited estimate that, globally, over 80% of all wastewaters is discharged without treatment.

The excretion of drugs can be carried out by the lungs, gastrointestinal tract, kidneys and also by body secretions, such as sweat, breast milk and tears. However, among all these routes, the major contribution for the excretion of drugs is by the kidneys (Kenakin, 2017; Aldred, 2009; Maddison et al., 2008). For the case of antibiotics, a large amount is excreted unaltered or as active metabolites via urine and faeces, being this two the most important pathways for this class of compounds (ECDC/EFSA/EMA, 2015; Kemper, 2008). These conjugates can, sometimes, be cleaved during wastewater treatment, and the original drugs are then released into the aquatic environment, mainly in the effluents of WWTPs (Kasrzyk-Hordern et al., 2008).

Among the main routes of entry of drugs into the environment, two stand out: production of drugs, and their elimination/excretion. In addition, it should be noted that the organism to which a given drug is administered also contributes to the way in which that drug is introduced into the environment. Of the two main routes of entry of drugs into the environment, special attention must be paid to excretion after use, since it is the main source of drugs in the environment after its administration to humans and animals (Madureira et al., 2010; Nikolaou et al., 2007; Jjemba, 2002; Kummerer, 2001).

In addition to this situation, drugs whose expiration date has expired or are in unnecessary quantities are often eliminated by the majority of the population through the sanitation network, via direct rejection in the plumbing of houses, or through the network of collection of urban waste. In this way, drugs find a new way of entering the environment capable of contaminating both soil and water sources (Nikolaou et al., 2007; Fent et al., 2006; Jones et al., 2003). On the other hand, the drugs used in veterinary medicine have even more ways of entering the environment. Contamination through intensive livestock farming represents one of the main routes for transporting veterinary drugs (Caliman et al., 2009). The contamination of water sources by direct application of drugs, namely antibacterials, is added, as is the case in aquacultures (Mompelat et al., 2009; Carlsson et al., 2006; Boxall, 2004) and it is possible to conclude that the sources of environmental contamination by veterinary drugs is very wide.

Finally, it is also necessary to mention that during the process of production of therapeutic agents, the release of residues that ultimately reach surface water sources can occur (Ziylan et al., 2011; Mompelat et al., 2009; Boxall, 2004). However, this seems to be a less important route compared to indiscriminate and continuous use, excretion and improper disposal of drugs, regardless of whether they are for human or animal use (Kummerer, 2009c; Jones et al., 2003).

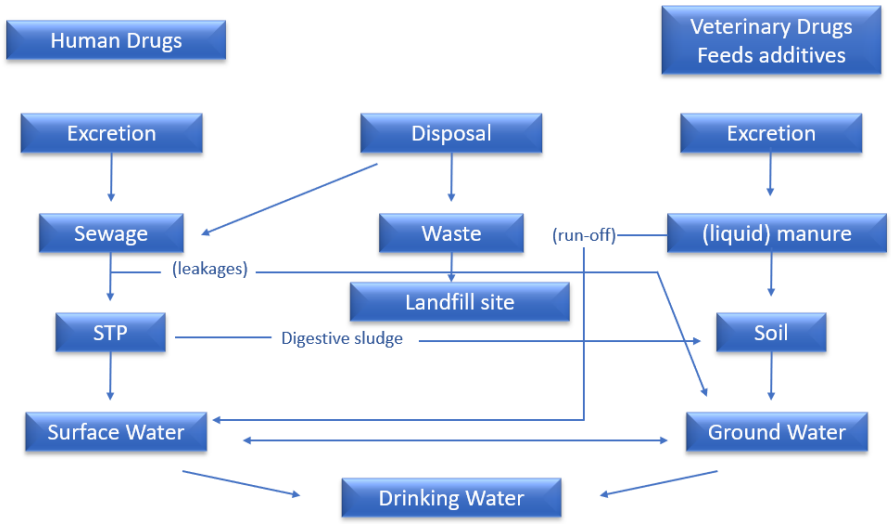

Figure 1 - Pharmaceutical Compounds in Water and Wastewater: Sources, Impact and Elimination (Source: Ternes, 1998).

The excretion of drugs and their metabolites ends the pharmacological activity in the body, as they only stop influencing an individual's biochemical properties when they are definitively eliminated by excretory pathways (Namiesnik et al., 2014). The rate of excretion of the parent substance depends on the drug, the dosage, as well as the organism to which it is administered (Melo et al., 2009).

However, the processes commonly used for wastewater treatment are not efficient enough to allow the total and complete removal of these compounds due to their chemical structure (Christen et al., 2010; Caliman et al, 2009; Nunes, 2006; Fent et al., 2006; Jones et al., 2003). Indeed, there are studies (Nikolaou et al., 2007; Bila et al., 2003) that demonstrate that many drugs are persistent in the environment and are therefore not completely eliminated in the treatment of WWTP.

After its excretion and introduction into the environment, structural changes can occur through a variety of biotic and abiotic processes to both the original compounds and their metabolites. Drugs can be transformed incompletely by microorganisms, such as bacteria and fungi, in the environment, as well as by sunlight and other abiotic chemical processes (Trautwein et al., 2008; Gröning et al., 2007).

Structural transformations of molecules (including pharmaceuticals) can also be the result of technological processes, such as the treatment of effluents by oxidation, hydrolysis and photolysis (Méndez-Arriaga et al., 2008; Pérez-Estrada et al., 2007) that occur in WWTPs. The resulting molecules with new properties (including pharmacological and toxicological) are called transformation products (Längin et al., 2008). The most commonly operated WWTPs can integrate four treatment phases: preliminary treatment, primary treatment, secondary treatment and tertiary treatment. However, the need to use these steps depends on the type and production process of the waters to be treated (Pei et al., 2019). In the preliminary treatment, consisting only of physical-chemical processes, the removal of the floats is done through the use of grids and coarse sieves, and the separation of residual water from the sands using sand channels (Gray, 2005). Among the pharmaceutical compounds that are eliminated at WWTPs, the ones that have higher removal rate are naproxen, sulfamethoxazole, roxithromycin and some oestrogens (Carballa et al., 2007). The primary treatment is also made up solely of physical-chemical processes. In this step, pre-aeration, flow equalization, neutralization of the effluent load from an equalization tank are carried out, and then liquid or solid particles are separated through flocculation, flocculation and sedimentation processes, using a sedimentation tank. The sludge resulting from this treatment is subjected to an anaerobic

digestion process in an anaerobic digestor or septic tank, and reduces the load of organic chemicals and pathogens (Gray, 2005). Among these organic chemicals, one may find a considerable number of pharmaceutical drugs, which are removed in this specific phase of wastewater treatment.

Secondary treatment consists of biological processes followed by physical-chemical processes. In the biological process, two different types of treatment can be used: 1) aerobic, where depending on the effluent characteristic, activated sludge tank (the air is inflated with surface aerator), aerated lagoons with macrophytes, percolating beds or biodiscs (Samer, 2015; Samer et al., 2014; Lin, 2007); 2) anaerobic, lagoons or anaerobic digesters can be used. The physical-chemical process consists of one or more secondary sedimenters. In this stage, the biological flakes are sedimented, leaving the liquid, after this treatment, free of biological solids or flakes (Ersahin et al., 2011; Samer, 2012). The sludge resulting from this treatment is dried in drying beds, filter bags or press filters. This process has been shown to reduce the load of a considerable number of pharmaceutical drugs, including ibuprofen (Fortunato et al., 2016), fluoroquinolones (Zhang et al., 2019), verapamil (Trautwein et al., 2008), salicylic acid (Carballa et al., 2004; Metcalfe et al., 2003a) and paracetamol (Roberts and Thomas, 2006), among many others. However, the efficacy of removal of drugs is not constant. The biological decomposition of micropollutants such as dissolved drugs increases with the increase in the hydraulic retention time (time that the effluent is retained in the tanks) and with the age of the sludge present in the treatment with activated sludge (Fent et al., 2006). The rates of elimination of these in WWTPs are quite variable, depending on the drugs, which include compounds with different chemical properties, the equipment and processes involved, and other factors such as weather conditions (Fent et al., 2006).

Tertiary treatment is also made up solely of physical-chemical processes. At this stage, pathogenic microorganisms are removed through the use of maturation and nitrification ponds. The most advanced methodologies for the removal of drugs from wastewater are chemical oxidation (including ozonation, and catalytic oxidation), ultraviolet (UV) radiation, membrane filtration, membrane reactors, and activated carbon adsorption, among others (Verlicchi et al., 2012). Finally, the resulting water is subject to disinfection through adsorption (using activated carbon) and, if necessary, treatment with chlorine and ozone (Russel, 2006). Some of the compounds that are eliminated in these processes (namely of oxidation) are non-steroidal anti-inflammatory drugs (e.g. diclofenac), B-blockers such as metoprolol, and antivirals (such as oseltamivir) (Ghosh et al., 2010; Wert et al., 2009; Ternes, 1998).

1.1.2. Environmental impacts of drugs

The growing number of studies on the environmental impact of drugs and their derivatives (Kumar et al., 2010; Madureira et al., 2010; Caliman et al., 2009 Nikolaou et al., 2007; Carlsson et al., 2006; Crane et al., 2006; Ferrari et al., 2004;) have shown a growing concern with the environmental fate of numerous organic compounds used in the pharmaceutical industry, due to their potential as environmental pollutants.

Currently, in the European Union (EU), the approval of new pharmacological agents requires an estimation of the potential environmental risks (by means of an environmental risk assessment (ERA) procedure) associated to their use, as well as an evaluation of the aspects related to quality, safety and effectiveness (Laenge et al., 2006). The procedure described by the European Medicines Agency (EMA) follows a tiered approach.

The ERA for pharmaceutical drugs is carried out with a methodology consisting of 3 phases. The ERA only proceeds to the next tier when risk cannot be excluded based on defined criteria. In phase I, the $LogP_{ow}$ is determined (accessed by OECD 107 or 123) and the predicted environmental concentration (PEC) value is calculated. The $LogP_{ow}$ value consists of the octanol/water partition coefficient and can be used to predict how fast a chemical will (or will not) accumulate in living organisms. The more fat-soluble the chemical, the more it will accumulate in living organisms (fatty tissue) and therefore in the environment. Water-soluble substances are more readily eliminated and generally have a lower bioaccumulation potential. If the value of $LogP_{ow} > 4.5$, it is necessary to perform a persistence, bioaccumulation and toxicity (PBT) assessment that consists of a test to estimate the environmental behaviour of a compound. If the PEC < 0.01 ug/L (and the drug does not present any specific concern due to compound-specific characteristics, such as endocrine disruptors), it is not necessary to carry out further tests. But if this value is equal or higher (or presents a specific concern) then further tests are necessary.

The phase II of this process, more specifically tier A, involves several tests that are carried out to identify the fate of medicinal products in the environment and their potential effects on representative organisms. Tests that may be used at this stage include the determination of adsorption/desorption, which is an important route for the elimination of highly toxic organic compounds (OECD 106), and biodegradation (OECD 301), and toxicity assessment. This is performed using measures of algal growth inhibition test (OECD 201),

daphnia reproduction test (OECD 211), fish early life stage test (OECD 210) among others. If the tests show that a risk exists, then it is necessary to go to phase III (tier B) which consists of studies follow from tier A risk assessment. If these new results do not show risk, further testing is not necessary (ERA, 2015). ERA's existing regulatory concepts are established considering a set of short-term ecotoxicological studies on three to four different species, environmental behaviour and also the application of assessment factors to correct the uncertainty inherent in ERA. Based on theoretical considerations and experience with a very limited but well-investigated number of examples, considering that drugs are highly biologically active compounds, the suitability of this risk assessment procedure for all substances can be questioned since effects in the long-term can occur in much lower concentrations and follow toxicodynamic mechanisms different from those extrapolated in short-term studies (Länge and Dietrich, 2002). In these cases, the application of assessment factors to derive the chronic no observed effect concentration (NOECs) appears to be problematic. Although long-term tests with a variety of organisms provide a complete database for the assessment of environmental risks, this is unattainable for all drugs due to time, money and animal welfare restrictions (Epel and Smital, 2001; Pfluger and Dietrich, 2001). However, there is a large number of drugs that have been marketed for a long time ("legacy drugs"), and for which no environmental risk assessment studies have been carried out, since regulatory guidelines requiring the environmental risk assessment of drugs did not exist at the time. Some of these substances have already been withdrawn from the market due to their toxic potential mostly, but not only, to humans. However, others continue to be widely used, some with clear evidence of their potential toxic effects to non-target organisms, and some without any consubstantiated knowledge about their effects on non-target organisms.

1.2. Acute and chronic effects of pharmaceutical drugs

Ecotoxicological tests assess the adverse effects of exposure to different concentrations of a chemical substance in individuals of a given species. Ecotoxicological tests can be carried out using aquatic or terrestrial organisms, according to the type of study, and of its objectives. These studies can be carried out at the level of the individual, the population, the community and even the ecosystem, and in some cases may last for several years. This chronic approach becomes very important to understand at what levels xenobiotics may induce long term deleterious effects on organisms, and are only possible with long periods of exposure, thereby increase the realism of laboratory controlled conditions, and increasing the overall relevance of obtained data.

The quantification of subindividual parameters in organisms exposed to toxic agents is currently a modality of toxicity assessment that focuses on the influence that compounds can have on specific biochemical and enzymatic pathways (Ankley and Johnson, 2004; Hutchinson 2004). More than classic individual parameters, such as mortality, the evaluation of subindividual parameters allows establishing causal relationships between toxic aggression and the compromise of multiple mechanisms of physiological regulation, which can cause observable toxicological phenomena at the individual level, or even compromise the population development of the analysed species (Martins, 2013). Subindividual parameters can function as early assessment responses, which indicate subtle changes in the organization and functionality of exposed organisms, long before the occurrence and establishment of damage (Walker et al., 2004). Considering these arguments, subindividual effects may be the best strategy to evaluate the effects of pharmaceutical drugs since this type of compounds are not supposed to cause environmental effects at the individual.

Acute toxicity tests aim to estimate the dose or concentration of a toxic agent that would be capable of producing a specific measurable response in a test organism or population, in a relatively short period of time, usually 24 to 96 hours. However, the experimental designs of acute tests can suffer adaptations, taking into account the characteristics of the organisms, namely their life-span. (Gherardi-Goldstein et al., 1990). Despite being important for comparative terms, of benchmark toxicity values, or to define thresholds of toxicity, acute tests are not realistic, especially in environmental terms. Acute tests do not reflect realistic exposure scenarios to which organisms are exposed in their habitats, as they focus on short-term periods, and often at concentrations much higher than those found in environmental matrices. It is much more likely that an environmentally exposed organism is subjected to pollutants for a rather prolonged period, instead of being exposed for hours or days. To study these prolonged effects, it is important to establish chronic durations of exposure. By increasing the exposure duration, sometimes for the entire duration of the life span, of a given organism, or during one or more generations of a given species, it is possible to ascertain long term (more realistic) effects. This chronic exposure may allow to observe the more subtle effects of the toxics, such as reduced growth and alterations of reproduction. To analyse such long-term effects, only chronic exposures are likely to measure realistic effects, similar to those that may occur in the wild (EPA, 2002). Chronic toxicity tests are, therefore, long-term tests aimed at studying the non-lethal effects on organisms, from their prolonged exposure to sub-lethal concentrations. These effects are generally assessed through specific biomarkers (histological, haematological, behavioural,

etc.), used to detect chronic changes, such as physiological disorders, somatic and/or gametic tissue deformities, changes in the organism's growth and reproduction, among others (Crane et al., 2006). In addition, they allow the calculation of NOEC, concentration of toxic agent considered safe, and LOEC (Lowest Observed Effect Concentration) (Magalhães & Filho, 2008). Studies on possible chronic effects of drugs individually and/or in mixtures, can provide important information about the risk of these compounds in the aquatic ecosystem (Fent et al., 2006; Cleuvers, 2003).

The disposal and arrival of these compounds, pharmaceutical drugs, to environmental matrices causes several types of problems, since the vast majority of these chemicals are persistent in the environment, as well as their degradation products. Even those with short half-lives, are likely to cause chronic effects due to their continuous introduction into the environment, being considered pseudo persistent compounds; in addition, they may have pronounced effects due to the mechanism of synergistic action (Reis-Filho et al., 2007).

Most drug risk assessment studies are based on acute exposures (Cunningham et al., 2004, Webb, 2004a; Ayscough et al., 2002; Halling-Sørensen et al., 1998). However, in recent years, concern have arisen about the possibility of environmental effects being caused by prolonged exposure to low concentrations of pharmaceutical products. Although the most recent studies indicate that the concentrations of drugs detected in environmental samples are low (Celiz et al., 2009; Gibs et al., 2007; Kim & Aga, 2007; Bila et al., 2003), usually in the order μgL^{-1} or ngL^{-1} (Christen et al., 2010; Melo et al., 2009; Mompelat et al., 2009; Carlsson et al., 2006; Fent et al., 2006; Voulvoulis et al., 2006; Boxall, 2004), there are several studies from the literature that demonstrate that a wide range of these compounds are persistent and ubiquitous in the environment (Madureira et al., 2010; Caliman et al., 2009; Crane et al., 2006; Ferrari et al., 2004; Stackelberg et al., 2004; Bila et al., 2003; Jones et al., 2003).

1.3. Mixtures

Drugs are generally not released into the environment as isolated, single compounds, but rather as complex mixtures, being associated to other pollutants, especially in aquatic ecosystems. As a result, in the environment, the organisms are seldom exposed to isolated drugs, but rather to these complex chemical mixtures. There is limited information about the interactions that may occur when such chemicals are simultaneously present in an aquatic matrix, being possible to accept that their toxic effects may increase in this condition

(Schwarzenbach et al., 2006). Drugs, being excreted from patient´s organisms in the form of metabolites, may keep some of their bioactivity, being as bioactive (or even more bioactive) than the parent drug itself (Martins et al., 2013).

The occurrence of pharmaceutical mixtures implies that general frameworks for determining the environmental risk of individual pharmaceuticals may not adequately reflect their actual environmental risk when they are present in mixtures. For example, using the risk quotient (RQ) as a risk assessment measure estimates the environmental risk from the predicted concentration of an individual pharmaceutical product and available ecotoxicological data (Altenburger et al., 2003). They do not consider the environmental risk of metabolites or transformation products in the same assessment, being one of their most important limitations. In addition, hazard categorization schemes developed to assess the inherent environmental harms of pharmaceuticals (such as the Stockholm PBT classification) and many ecotoxicological studies involving test organisms often compile risk data from individual pharmaceuticals, rather than of complex mixtures, that indeed occur in realistic scenarios (Luo et al., 2014).

2. Antibiotics

Antibiotics are natural or synthetic compounds, capable of inhibiting growth or causing the death of bacteria (Rahman and Sarker, 2020; Grenni et al., 2018;). They are considered as bactericidal, when the bacterium dies, or bacteriostatic, when they promote the inhibition of microbial growth (Walsh, 2003). Antibiotics of natural or semi-synthetic origin are mainly for medicinal use and are classified into β-lactams, tetracyclines, aminoglycosides, macrolides, cyclic peptides, streptogramins, among others. Antibiotics of synthetic origin are classified into sulfonamides, fluoroquinolones and oxazolidines (Pupo et al., 2006; Abraham, 2003); for each class the mechanism of antibiotic action is different. The presence of antibiotics in the environment has long been reported in several scientific studies. Compounds such as sulfamethoxazole, sulfamethoxine, different sulfonamides, dehydro-erythromycin, were detected in WWTP, wastewater and groundwater. Particularly for antibiotics, their environmental concerns have been traditionally associated mainly to the alteration in the genetic makeup of microorganisms with direct and indirect effects by contact in low concentration, allowing the selection of multi-resistant bacteria (Schawartz et al., 2003; Kolár et al., 2001; Davison, 1999;). The high use of antibiotics (often indiscriminately), associated with their consequent release into the environment, is the main cause of the increase in bacterial

resistance, exacerbated by the fact that this resistance is maintained even in the absence of antibiotics, that is, this phenomenon is irreversible. Therefore, the natural occurrence of antibiotic resistance in bacterial populations can cause serious public and environmental health problems, and this fact can also be considered an indicator of the potential presence of other drug residues in the environment (Iwu et al., 2020; Kraemer et al., 2019;). However, chemical toxicity caused by antibiotics has been suggested also as a major adverse effect, requiring further research.

1.1. Oxytetracycline

Oxytetracycline (OTC) was the second tetracycline to be discovered in 1950, from cultures of the bacteria species *Streptomyces rimosus*, being marketed with the designation of terramycin (Finlay et al., 1950). Tetracyclines are light sensitive compounds, and when they decompose, they turn brown, thus contributing to the poor quality of water in ecosystems and thus becoming dangerous to the organisms present in these ecosystems (Yanong, 2003b). The OTC degradation in seawater is dependent on light and temperature and on the aqueous solution this degradation depends on pH (Choo, 1994). As OTC has been used for a long time in aquaculture, it may be considered an environmental contaminant which, consequently, raises concerns about the impacts of this drug on public health (Tonguthai, 1996).

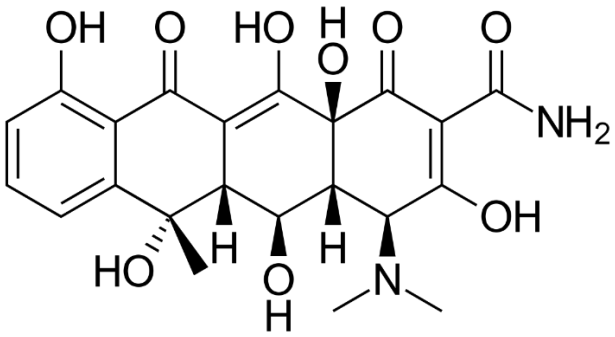

Figure 2 - Chemical structure of oxytetracycline (Adapted from Kim et al., (2005)).

Figure 2 shows the chemical structure of OTC with molecular weight equal to 460.4 g mol^{-1} and solubility in water> 100 mg mL^{-1} (21°C). It is one of the most significant contaminants in aquatic fauna and flora since, its excessive use has contributed for the emergence of bacterial resistance with serious consequences for human health.

OTC is effective against several microorganisms, and is almost always used indiscriminately (Sarmah et al., 2006; Kim et al., 2005; Zhu et al., 2001;). Its mechanism of action consists in the inhibition of bacterial protein synthesis. OTC is indicated to treat diseases such as sinusitis, bronchitis, cholera, acne, urinary tract infections, recurrent fever, leptospirosis, among others (Guerra et al. 2016; Jayanthi and Subash, 2010). OTC is still one of the most used antibiotics in aquaculture and veterinary medicine (Rodrigues et al. 2017a).

OTC is used orally, and its absorption varies from 60-80% with the remainder eliminated by faeces and urine for domestic sewage, with the final destination of rivers and lakes (Halling-Sorensen et al., 2002). Release of its residues, without proper treatment of effluents from the pharmaceutical industries, is a considerable environmental source of this drug.

In surface waters, concentrations of OTC ranging from 0.0003 to 340 ngL^{-1} (Pailler et al. 2009; Calamari et al. 2003; Kolpin et al. 2002) were detected, and in Chinese rivers its presence was reported in concentrations ranging from 0.235 to 0.712 mgL^{-1} (Li et al. 2008).

2.1. Ciprofloxacin

Ciprofloxacin (CIP) is a synthetic antibiotic belonging to the group of fluoroquinolones, firstly marketed in 1987, being one of the most prescribed antibiotics today (De Witte et al., 2009b). CIP was developed by Bayer researchers in Germany, and belongs to the third generation of quinolones, being active against gram-negative bacteria and widely used in the treatment of urinary, respiratory and gastrointestinal infections, in addition to infections in the skin, bones and joints. Thus, like all drugs, this antibiotic is not fully metabolized by the body and is partially excreted into the environment (Frade et al., 2014). Figure 3 shows the chemical structure of CIP with molecular weight equal to 331.34 g mol^{-1} and solubility in water 30 mg mL^{-1} (20°C).

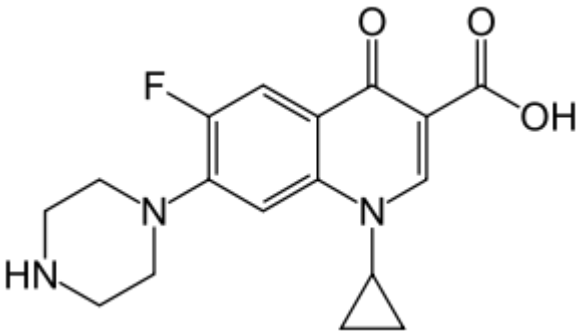

Figure 3 - Chemical structure of ciprofloxacin (Adapted from Halling-Sorensen, 2000).

Fluoroquinolones act by inhibiting type II bacterial topoisomerases (topoisomerase II and topoisomerase IV - they can inhibit only one or both). Topoisomerase II or DNA gyrase is an enzyme that allows the transcription and replication of DNA by producing its negative super helicoidization. Thus, when inhibited, it causes the relaxation of the super-spiral bacterial DNA and consequent break of the DNA strand, making its replication impossible. Topoisomerase II is the main target of quinolones in gram-negative bacteria. Inhibition of topoisomerase IV promotes, in turn, a loss of chromosome stabilization during cell division, being the main target in the fight against gram-positive bacteria (Hooper and Jacoby, 2016).

The main route of excretion of CIP is via the renal and extra-renal routes, being mostly excreted in its unchanged form, with only small amounts of its metabolites being excreted through the urine and faeces (Sörgel et al., 1991; Bergan et al., 1988). The rate of excretion of this compound in its original form varies from 50% to 70% after metabolism (Mompelat et al., 2009; Hirsch et al., 1999). This antibiotic is found in tributaries of wastewater stations in concentrations of 667.1 ngL^{-1}, and in effluents in concentrations reaching 309.2 ngL^{-1} (Seifrtová et al., 2008).

3. Biomarkers

The increasing influx of pollutants into aquatic ecosystems has led to the need for a better understanding and evaluation of the biological effects of these contaminants on aquatic biota. Several studies have used biomarkers as functional tools to assess the toxicity of a wide variety of compounds on natural populations (Rodrigues et al., 2011). Biomarkers can be defined, in ecotoxicological terms, as a change in a biological response (from molecular changes

through cellular and physiological responses, to behavioural changes) that reflects the toxic effects of chemicals dispersed in the environment, and to which organisms are exposed to (Islas-Flores et al., 2013). There are molecular, cellular and individual-level biomarkers. The two most important characteristics of biomarkers are to the possibility of identifying the interactions that occur between contaminants and living organisms, and to measure sublethal effects. Hence the importance and current interest in incorporating biomarker analysis into environmental contamination assessment programs (Jesus & Carvalho, 2008).

Biomarkers are biological responses reflecting the individuals' exposure to chemical agents and/or environmental stressors (Van der Oost et al., 2003), evidenced by cellular, biochemical or molecular changes measured in biological media such as tissues, cells or fluids (Hulka et al. 1990). As the common aspects among different organisms are accentuated mainly at the molecular level, many molecular biomarkers have the advantage of being applied to a wide variety of living organisms (Lam & Gray, 2003). Still, they comprise the determination of substances, their biotransformation products and interaction with biomolecules in organisms (Yusa et al., 2012). Various biological parameters can be changed as a result of the interaction between a chemical agent and an organism. However, to be used as a biomarker, it is necessary that this parameter can be quantitatively determined and that there is a correlation with the intensity of exposure and / or the biological effect of the substance.

In ecotoxicological studies, the use of a battery of biomarkers is recommended, since only the assessment of a single biological response may not reflect broadly and comprehensively the alterations to the physiology of living organisms that a given environmental contaminant can cause (Zorita et al., 2008). In addition, the concomitant use of several biomarkers is essential to minimize misinterpretations in cases of complex pollution situations (Pacheco & Santos, 2002). In these studies, it is essential that the most appropriate choice will include species indicating the quality of the environment under study. Thus, and within the use of biomarkers, great emphasis has been given to the development of assays aimed at quantifying the activity of enzymes that play a key role in common physiological processes, such as neurotransmission, detoxification, respiration or the antioxidant response (Alkimin et al., 2020; Nunes et al., 2020; Rebelo et al., 2020; Antunes et al., 2016).

For all that has already been mentioned, the use of biomarkers in Ecotoxicology has several advantages, as they allow to observe in advance the existence of contamination by toxic biologically significant substances. Biomarkers are also useful to identify species or populations at risk of contamination, by analysing the magnitude of the contamination, predicting the degree

of severity of the effects caused by the pollutants, and establishing causal relationships between chemical species and effects on specific metabolic pathways that may justify more complex biological responses, such as behavioural changes and reproductive alterations (Brandão et al., 2011).

3.1. Types of biomarkers

The use of biomarkers has been increasingly proposed as a suitable approach for monitoring and management of the marine and freshwater environments, proving to be extremely effective. Over the years, many of the biomarkers that have been developed are considered efficient in providing an early warning of deleterious effects on biological systems, and for estimating biological effects due to certain contaminants. Notwithstanding it is important to realize that the possibilities for applying a biomarker depend on the objective chosen for environmental monitoring and the actual contamination scenario (Adedeji et al. 2012a).

3.1.1. Biomarkers of peroxidative damage and biotransformation

Oxidative stress parameters constitute an important group of biomarkers, due to the fact that several toxic compounds existing in the environment (or their metabolites) can exert toxic effects in this domain (Winston & Di Giulio, 1991). Reactive oxygen species (ROS) are unstable and extremely reactive molecules capable of transforming other molecules with which they interact. ROS are produced as by-products during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation. They have the potential to cause a number of deleterious events. It was originally thought that only phagocytic cells were responsible for ROS production as their part in host cell defense mechanisms. Recent work has demonstrated that ROS has a role in cell signalling, including apoptosis; gene expression; and the activation of cell signalling cascades. It should be noted that ROS can serve both intra- and intercellular messengers. In addition, ROS are formed as necessary intermediates of metal catalysed oxidation reactions (Hancock et al., 2001). The amount of ROS produced by the organisms is virtually equal to the amount that is eliminated. ROS are now known to be biologically important in various physiological systems, including adaptation to hypoxia; regulation of autophagy, immunity, and differentiation; cognitive function; regulation of fertility and longevity (Bardaweel et al., 2018; Sena and Chandel, 2012). According to Livingstone (2003), organisms are able to adapt to the increased production of ROS by also increasing regulation by

antioxidant defences. However, the amount of ROS can be altered, which leads to the disturbance of the redox state; this phenomenon is known as oxidative stress, and occurs due to an imbalance between the high levels of produced ROS, and the ineffective scavenging of ROS by the antioxidant defence. Oxidative stress is a situation in which the concentration of ROS in steady state is transiently or chronically increased, surpassing the capability of antioxidant defensive mechanisms, and disrupting cell metabolism and its regulation, damaging cell constituents (Lushchak, 2011). Oxidative stress results from an imbalance between reactive oxygen species (ROS) and the organism's antioxidant systems (Barata et al., 2005). The failure of the antioxidant defence to counteract the excess production of ROS can cause oxidative damage, such as enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Kovacic, 1986). Defence systems that inhibit the formation or limit the action of oxyradicals, are only a part of antioxidant defences, which may also include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and also phase II metabolic enzymes such as glutathione S-transferases (GSTs). GSTs are important mediators in oxidative stress responses. The formation of ROS leads to the conversion of polyunsaturated fatty acids into lipid hydroperoxides. These in return can give rise to highly reactive a,b-unsaturated aldehydes, such as, 4-HNE. 4-HNE is toxic at high concentrations, but at low concentrations, it has important signalling functions, affecting cell proliferation, apoptosis, and differentiation (Sawicki et al., 2003). GSTs have a protective effect at several different stages in this pathway. GSTs can detoxify 4-HNE by conjugation with glutathione (Sawicki et al., 2003; Singh et al., 2001).

SOD is indispensable key enzyme in the first line of antioxidant defences, that acts by catalysing the reduction of the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) (Liu et al., 2014). Catalase (CAT) has a double role, being able to function as a catalyst in the dismutation of H_2O_2 molecules, and also in the peroxidation by behaving only as an electron acceptor, in which electron donors are oxidized via one-electron transfers releasing radicals (Ahmad, 1995). A deficiency in catalase activity results in excessive accumulation of hydrogen peroxide due to insufficient decomposition. If hydrogen peroxide is not broken down by catalase, additional reactions convert it into ROS that can damage DNA, proteins, and cell membranes (Nandi et al., 2019). Glutathione peroxidase (GPx) is a tetrameric cytosolic enzyme, dependent or not on selenium, the selenium-dependent form being the one with the greatest expression. This antioxidant enzyme belongs to a family of enzymes capable of removing hydrogen peroxide and

other peroxides by coupling water reduction to oxidation of reduced glutathione (GSH) (Nunes et al., 2006).

GSTs represent a family of widely distributed enzymes that catalyse the conjugation of various xenobiotics with reduced glutathione (GSH). In its role of detoxification, it is responsible for conjugation of a large number of xenobiotics with GSH, which translates into increased hydrophilicity of lipophilic toxics, thereby reducing the likelihood of these compounds to bind to other macromolecules, such as DNA (Schelin et al., 1983).

The levels of thiobarbituric acid reactive substances (TBARS) are considered to be a valuable indication of oxidative damage of cellular components, and expresses the levels of lipid peroxidation of membrane lipids. TBARS are formed as a by-products of lipid peroxidation, after the oxidative attack of membrane lipids by reactive oxygen species. Malondialdehyde (MDA) is the most important end product of this process, and its levels represent the levels of several end products formed through the decomposition of lipid peroxidation products. MDA (and other substances resulting from this oxidative attack of membrane lipids) reacts with thiobarbituric acid (TBA) forming a pink chromogen. The intensity of the peak resulting from this chromogen is proportional to the levels of all thiobarbituric reactive substances (TBARS), conventionally expressed in terms of MDA concentrations (Skakun and Vysotski, 1982).

3.1.2. Biomarkers of genotoxicity

In order to analyse the action of pollutants on the genetic material of organisms, toxicological genetics emerges. This line of research integrates concepts from genetics (mechanisms of inheritance) and classical toxicology (toxicokinetics and toxidynamics) (Aardema and MacGregor, 2002). Many of the pollutants that enter different ecosystems are capable of interacting directly or indirectly with the organisms' genetic material, affecting its structure or function. These compounds are designated genotoxic, and their effects can be detected even when in sublethal concentrations (Grisolia, 2002). Genotoxic compounds and/or their metabolites (free radicals, for example) can generate the disruption of both a single and double strand of DNA; they can oxidize the nitrogenous bases in the DNA molecule, or establish covalent bonds to the genetic material, forming DNA adducts (Newman and Clemens, 2008)

Several genotoxic evaluation techniques have been developed in order to quantify and understand the mode of action of compounds that are dangerous to organisms. These

techniques are within the group of effect biomarkers and are capable of recognizing many classes of damage, in a wide variety of cells and in different organisms, thus providing information at the level of individual cells of the organisms (Rojas et al., 1999).

One of the mostly used tools for genotoxicity assessment is the comet assay (EC) that corresponds to an electrophoresis of individual cells on an agarose gel. This technique is based on the migration of fragments of DNA generated by the breaking of the strands in an alkaline medium, due to the action of genotoxic agents that generate alkaline-sensitive sites. The greater the amount of DNA damage, the greater the migration of fragments from the degraded nucleus towards the positive electrode during electrophoresis, giving the appearance of a comet, being this is an indication of the susceptibility of the tested organism to genotoxic xenobiotics. Ostling and Johanson (1984) were the first to develop the gel electrophoresis technique to detect genetic damage. Later, Singh et al. (1988) modified the technique in alkaline conditions (pH> 13), allowing the exposure of alkaline-sensitive sites, thus increasing the sensitivity of the assay. Due to its simplicity and high sensitivity, this determination was suggested for environmental monitoring studies, being able to evaluate the genotoxic effect of a wide variety of compounds such as pesticides and industrial and domestic sewage, among others (Do Amaral et al., 2015; Knight et al., 2004).

Another technique used for the measurement of genetic damage induced by chemicals is the micronucleus (MN) test. This procedure corresponds to a simple, fast and sensitive technique that allows obtaining an index of cytogenetic damage (Sánchez-Galán et al., 1998). The MN test was developed by Schmid (1975) to analyse the formation of MNs in rodents, and later Hooftman and Raat (1982) adapted the test to work with aquatic organisms under laboratory conditions. The mechanism of formation of MNs occurs during the blood cell proliferation phase, where these cells are in continuous division. During cell division, the genotoxic agent acts, which can cause a chromosome break, releasing a fragment (clastogenesis) or altering the disruption of sister chromatids, caused by spindle dysfunction, leading to the loss of one of them (aneugenesis). These abnormalities remain until the end of cell division, not being reintegrated into the nucleus, starting to constitute an MN, which is visualized in the cytoplasm (Krishna and Hayashi, 2000). In the same way as for the comet assay, the micronucleus test has been widely used to assess the impact of pollutants on aquatic organisms, after direct or indirect exposure in vivo to chemicals (Izquierdo et al., 2003). The test was widely used in bivalves, both in biomonitoring studies (Kolarević et al., 2016; Klobučar et al., 2003; Rank and Jensen, 2003),

and in the evaluation of the effects of different pollutants on different organisms (Štambuk et al., 2008; Ching et al., 2001).

4. Mussels

Currently, there is a large industry for the cultivation of bivalves, and among the most cultured species, one may find mussels. The genus *Mytilus* belongs to the family Mytilidae and to the phylum MOLLUSCA (Mollusks). This genus is one of the most diverse marine genera, occurring in estuaries and ocean habitats, both in intertidal and subtidal zones, occupying a diversity of substrates, being distributed in all major oceans and rivers in the world. It is found, in Europe, in three taxonomic forms (*Mytilus edulis*, *Mytilus galloprovincialis*, and *Mytilus trossulus*) being the first two predominant species. These forms can be recognized by the analysis of morphological characteristics and by genetic markers (López et al., 2002; Gardner and Thompson, 2001;). *M. edulis* and *M. galloprovincialis*, co-exist in some areas, such as the north, south and west coasts of Ireland and the north and west coasts of France. These two species are known to hybridize (Schneider et al., 2004; López et al., 2002), and such hybrids have been found in Western Europe, between Portugal and the Boreal province.

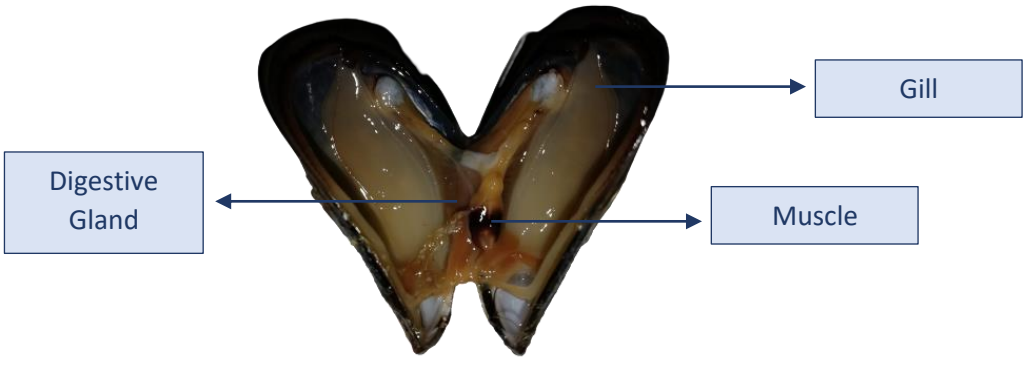

Figure 4 - Mussel (Photo acquired by the author).

The mussel, as a bivalve mollusc, is generally described as an animal that does not have an internal skeleton and its body is enclosed in a shell, of two equal parts, the valves, joined by a ligament (Camacho, 1991). The body is formed by two symmetrical lobes and the mantle from which the foot protrudes. Between the mantle and the visceral mass, one may find the gills, responsible for gas exchanges and capturing food (Cannuel et al., 2009). According to Giufrida et al. (2003), the main vital functions of mussels (such as nutrition, breathing, and excretion), are based on filtration; mussels are filter feeders, and feed on suspended matter and phytoplankton (Wong and Levinton, 2004). The shell closes under the action of the adductor muscles, the main constituents of the muscular system. The shell is mainly composed of calcium carbonate crystals embedded in a protein matrix, called conquioline, and is covered on the outer surface by a thin film resembling a skin also of a protein nature, the periostracum. The valves join through the ligament, of conquioline, which due to its elasticity, is responsible for opening the shell (Camacho, 1991). The foot is a muscular organ, reddish orange, in the shape of a tongue. At the posterior end of the foot, one may find the bison secretory gland, which is formed by a series of filaments, of a protein nature, terminated in small adhesive discs, with which the mussel is fixed (Camacho, 1991).

Mussels have no sexual dimorphism, although cases of hermaphroditism are known, and the proportion of males and females in the population is similar, with females being slightly more numerous than males (Mills and Côté, 2003).

Mytilus sp. have been shown to be one of the most successful model organisms for time-integrated responses to complex mixtures of pollutants (UNEP/RAMOGE,1999). Mussels have been widely used in the past as biological indicators of pollution in monitoring programs. Mussels are considered good organisms for biomonitoring mainly because they are sessile organisms, which feed through filtration, being therefore able to accumulate in its tissues many of the contaminants present in sea water (Besada et al., 2011; Naimo, 1995; Widdows & Donkin, 1992; Bayne, 1989; Fossato et al., 1989;). In addition, they have a rapid response at the molecular, cytological, and physiological levels in the presence of certain contaminants (Conceição et al., 2008). In addition, mussels have a wide geographical distribution, allowing the survey of extensive coastal areas. They are present in very wide geographical areas (Thain et al., 2008), being particularly abundant in coastal and estuarine waters and, are found in large quantities (Chandurvelan et al., 2015; Bolognesi et al., 2006; Farrington et al., 1983). Nowadays mussels are organisms widely used in laboratory studies under controlled conditions (Gagné and Burgeot, 2013), for a multiplicity of purposes.

II. Material and methods

1. Test organisms

The mussels used in this work were manually collected near the mouth of the Ria de Aveiro (40 ° 38 ′ 34.5 ″ N, 8 ° 44 ′ 07.7 ″ W) during the low tide period. The Ria de Aveiro is an estuarine environment located to the northwest of the Portuguese coast, being mainly affected by naval traffic (Oliveira et al., 2009), not inculcating the risk of contamination by untreated domestic and industrial sewage. It is a place protected from wave action but is subjected to a tidal cycle. Salinity was measured on site at the time of sampling and had a value of 30. Two species of mussel coexist at this site (*M. edulis* and *M. galloprovincialis*) and their interspecific hybrids coexist. Since the characteristics of individual from hybrid zones are morphologically and ecologically indistinguishable (Lourenço et al., 2015; Coghlan and Gosling, 2007; Daguin et al., 2001), the organisms will be referred to as *Mytilus spp*.

400 organisms of similarly sized shell length (4.5 to 5.5 cm) were collected, and were later transported to the laboratory in plastic boxes with water from the sampling site. After arrival, the organisms were cleaned, to remove all materials adhered to the shells, such as small invertebrates and algae. The organisms were kept in 15L aquariums with artificial sea water at 30 ± 1 salinity (Tropic Marin® Sea Salt). The organisms were continuously aerated and were kept at a temperature of 20 ± 1 ° C, a pH of 7.8 ± 0.10, and a photoperiod of 16 h L: 8 h D. The organisms were fed every 2 days with a suspension of *Chlorella vulgaris* seaweed (1×10^5 cells/ml), optimized to provide basic nutritional needs and keep animals healthy for several weeks. The water in the systems was renewed weekly, and the dead organisms were removed and discarded immediately. Organisms whose shells were open and did not close after stimulation were considered dead. The organisms were considered suitable for experimental procedures since the mortality after the collection did not exceed 10%. The organisms were submitted to a quarantine period lasting 15 days before being used for any test.

2. Pharmaceuticals and stock solutions

The compounds ciprofloxacin and oxytetracycline (with purity ≥ 99%), were purchased from Sigma-Aldrich Chemical. The stock solutions were prepared before the tests, for ciprofloxacin a

100 mgL^{-1} stock solution was prepared and for the 10 mgL^{-1} oxytetracycline; both solutions were prepared in artificial sea water (similar to acclimatization).

3. Acute exposure

The acute exposures were carried out under controlled conditions similar to those adopted during the period of acclimatization of the organisms. The same regimes of photoperiod, temperature, salinity and pH were used. For the exposure to single chemicals, the final nominal concentrations tested were: 0, 0.3125, 0.625, 1.25, 2.5, and 5 μgL^{-1} of OTC and 0, 0.005, 0.013, 0.031, 0.078, 0.195 mgL^{-1} of CIP. These concentrations were established based on values found in surface waters; for CIP, the concentrations detected in wastewaters were of 309.2 ngL^{-1} (Seifrtová et al., 2008); for OTC the concentrations were established according to Rodrigues et al. (2017), that chose the concentrations based on environmental values (0.0003 ngL^{-1} – 0.712 mgL^{-1}; Wei et al. 2011; Li et al. 2008; Calamari et al. 2003; Kolpin et al. 2002). For the test with the mixture of drugs, the concentrations were: 0, 0.005 mgL^{-1} CIP + 0.3125 μgL^{-1} OTC, 0.031 mgL^{-1} CIP + 1.25 μgL^{-1} OTC and 0.195 mgL^{-1} CIP + 5 μgL^{-1} OTC; these concentrations were established taking in consideration the concentrations of the isolated exposures (previous step) that caused significant effects. Each test had a total of 10 replicates for each condition tested, and all replicates were exposed individually, in plastic bottles, one individual mussel per bottle, in a volume of 0.5L of seawater. The tests lasted 96h, and at 48h the medium was renewed. The exposed organisms were not fed during the tests. At the end of each test, the organisms were removed by hand from the exposure apparatuses, and haemolymph was collected from all organisms. After collection, all organisms were sacrificed and dissected; gills, muscle and digestive gland were removed for biochemical analysis. The sacrifice was performed in cold sea water (4 °C), and the samples were stored in Eppendorf microtubes and subsequently frozen at -80 °C until being processed for biomarker assays.

4. Chronic exposure

The chronic exposures were also carried out under controlled conditions similar to those adopted during the period of acclimatization of the organisms (and for the acute tests). The same regimes of photoperiod, temperature, salinity and pH were used. For the exposures to single chemicals, their final nominal concentrations tested were: 0, 0.3125, 0.625, 1.25, 2.5 and

5 μgL^{-1} of OTC and 0, 0.005, 0.013, 0.031, 0.078, 0.195 mgL^{-1} of CIP. The concentrations of the mixtures to which the animals were chronically exposed were 0, 0.005 mgL^{-1} CIP + 0.3125 μgL^{-1} OTC, 0.031 mgL^{-1} CIP + 1.25 μgL^{-1} OTC, and 0.195 mgL^{-1} CIP + 5 μgL^{-1} OTC. These concentrations were established based on values found in surface waters (already mentioned). Each test had a total of 10 replicates for each condition tested and all replicates were exposed individually, in plastic bottles, one individual mussel per bottle in a volume of 0.5L of seawater. The tests lasted 28 days, with renewal of medium and addition of food every two days (*C. vulgaris* suspension, 1×10^5 cells/mL). At the end of each test, animals were removed by hand from the exposure apparatuses, and haemolymph was collected from all organisms. After collection, all organisms were sacrificed and dissected; gills, muscle and digestive gland were removed for biochemical analysis. The sacrifice was performed in cold salted water (4°C), and the samples were stored in Eppendorf microtubes and subsequently frozen at -80°C until they are processed for biomarker assays.

5. Biochemical determinations

The determination of the activity of the enzyme SOD (Total, copper-Zinc (Cu-Zn) and manganese (Mn)) was based on the method described by Flohé & Otting (1984). The total activity of SOD is represented by the reaction of cytochrome C with peroxide radicals, these radicals are produced by the system of xanthine-xanthine oxidase. The inhibition of the activity of Cu-Zn SOD by the potassium cyanide allows the quantification of que isolated activity of Mn SOD. For all the forms, the enzymatic activity was spectrophotometrically followed at λ = 550 nm and the activity was expressed in mmol/min/mg of protein.

Catalase activity (CAT) was determined by spectrophotometry, measuring the decrease in absorbance at 240 nm, as described by Aebi (1984), due to the decomposition of hydrogen peroxide (H_2O_2) in water (H_2O) and O_2. Changes in the absorbance of the samples were monitored spectrophotometrically at 240 nm for 30 seconds, and the activities were expressed as nmoles of H_2O_2 consumed per minute, per milligram of protein.

The activity of glutathione-S-transferases (GSTs) was also determined by spectrophotometry, according to Habig et al. (1974). The GSTs catalyse the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione (GSH), forming a thioether whose formation can be followed by increasing the absorbance at a wavelength of 340 nm. The results were expressed as nmol of thioether produced per minute, per milligram of protein.

The lipid peroxidation level (LPO) was measured by quantifying thiobarbituric acid reactive substances (TBARS) (Buege & Aust, 1978). In the presence of thiobarbituric acid (TBA) and heat (water bath at approximately 100 °C), MDA-like compounds reacted with TBA, which led to the formation of a coloured end product. This formation was spectrophotometrically quantified at λ = 535 nm. TBARS concentrations were expressed as MDA equivalents and then calculated in M/mg of protein.

The determination of the protein concentration of the samples was carried out according to the spectrophotometric method of Bradford (1976) (wavelength 595 nm), adapted to microplate (96 wells) using bovine γ-globulin as a standard (1mg/mL), in order to express enzymatic activities as a function of protein content of samples.

6. Comet Assay

DNA damage was estimated using a slightly modified version of the comet assay (Singh et al. (1988) as described by Almeida et al. (2011). All procedures related to the comet assay technique were conducted using yellow light to avoid UV-induced DNA damage. Haemolymph of seven mussels from each condition was collected, in a volume of approximately 200uL. Microscopic slides were coated with 1% normal melting point agarose (NMPA) in H_2O (1g to 100ml). After collection, the haemolymph cells for each mussel were centrifuged at 3000 rpm for 3 min (4°C). The pellets with isolated cells were resuspended in 0.8% low melting point agarose (LMPA), the slide was filled with mini-gels. Each mini-gel was composed of 10µl of cell suspension with LMPA. Subsequently, the slides were placed at 4°C for 15 minutes to solidify the agarose. Then, the slides with the incorporated cells were immersed in a lysis buffer (0.2M NaOH, 100mM Na_2.EDTA·$2H_2O$, 10mM TRIS and 1.5M NaCl, 1% Triton X-100, 10% DMSO, pH 10, 4°C) for 1 h, for the diffusion of cellular components and immobilization of DNA in agarose. After the lysis step, the slides were gently placed in an electrophoresis chamber containing electrophoresis buffer (0.3M NaOH, 1mM EDTA, pH 13, 4°C). The slides were gently submerged and left in this solution for 15 min to allow the DNA to unfold. Electrophoresis was performed for 15 min at 25 V and 300 mA. After electrophoresis was completed, the slides were removed and washed with PBS and then with cold water. After this washing, the slides were dehydrated with 70% alcohol for 10 minutes, and then 100% alcohol for another 10 minutes. The slides were kept in the dark until they were stained. To stain the slides, a solution of 0.01 mg/ml of ethidium bromide (EtBr) was used with a Pasteur pipette in order to fully cover the mini-gels. After these

20 minutes, the slides were washed with cold water, allowed to dry at room temperature, but protected from light. To observe the slides, a drop of distilled water was placed on each mini-gel, followed by the slide to cover all the mini-gels. The slides were analysed under a fluorescence microscope, equipped with a 510-560 nm excitation filter, and a 590 nm barrier filter. For each sample, 100 comets (50 per mini-gel) were analysed, based on a visual evaluation system based on 5 recognizable classes of the comet, from class 0 (no damage, no visible tail) to class 4 (almost all DNA in the tail, insignificant in the head) illustrated in the figure below (Figure 5). Each of the comets was given a value according to the class in which it was placed.

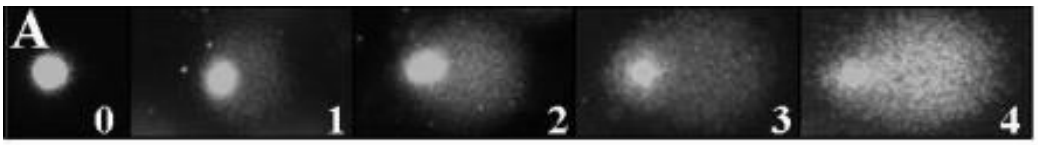

Figure 5 - Comet classes: 0, no or minimal damage; 1, low damage; 2, mid damage; 3, high damage; 4, extreme damage (Adapted from Rodrigues et al., 2016).

7. Shell hardness

The hardness of the shells from randomly chosen mussels of each treatment was tested using the equipment TA-X2i Texture Analyser (Stable Micro Systems; Godalming, UK). With the shells firmly held in a metal surface, a 4 mm diameter metal cylinder probe (TA-56) was lowered at a constant speed of 1 mm/s until the rupture of the shell occurred; the force was applied vertically along the axis at bivalve shell apex. Using a 30 kg load cell, the maximum force (N) applied to the shell was registered by the apparatus, using the software Texture Exponent 32 (version 6.1.12.0, Stable Micro Systems; Surrey, UK). The results were expressed as a function of the force (N) necessary to cause the rupture of the organisms' shells.

8. Statistical analysis

The analyses were statistically assessed using a One-way ANOVA followed by the Dunnett's test to determine the occurrence of significant differences between the responses observed in treated groups compared with the control treatment. Data were initially analysed for homogeneity of variance with the Levene test. A significance level of 0.05 was always used to infer statistically significant results.

III. Results

1. Biomarkers determined in animals exposed to oxytetracycline

a. Gills

In the acute exposure, the SOD activity decreased significantly in the animals exposed, for Total SOD ($F_{(5,46)}$ = 18.071; p<0.001) in all concentrations, and for Mn SOD ($F_{(5,46)}$ = 7.296; p<0.001) for all concentrations except the lowest (0.31 μgL^{-1}) (Figure 6a). Animals exposed to all the concentrations of OTC for 28 days showed a statistically significant decrease in both Total SOD ($F_{(5,44)}$ = 48.418; p <0.001) and Mn SOD ($F_{(5,44)}$ = 40.445; p<0.001) activity in their gill tissues for all the concentrations (Figure 6b).

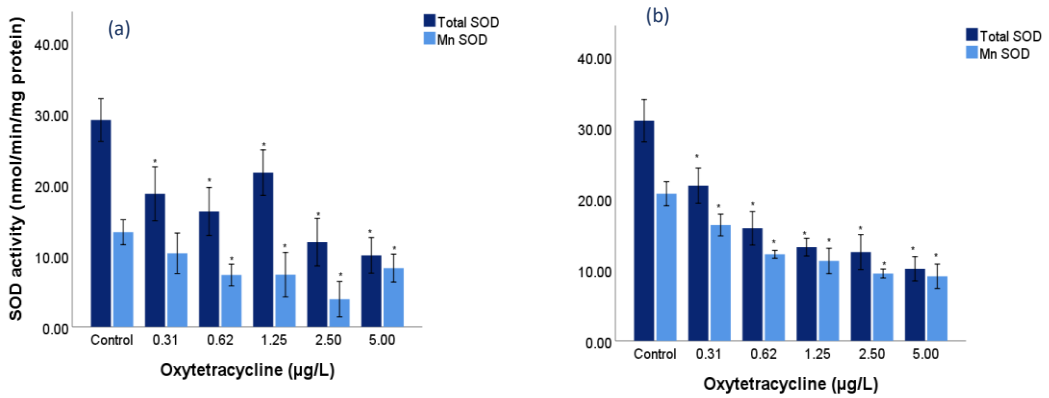

*Figure 6 - Results for SOD activity (determined in gills), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The results obtained for catalase (Figure 7) showed a statistically significant increase in the activity at the highest concentration of OTC tested, for the acute exposure ($F_{(5,47)}$ =6.015; p=<0.001) and for the chronic exposure ($F_{(5,43)}$ =4.454; p=0.002).

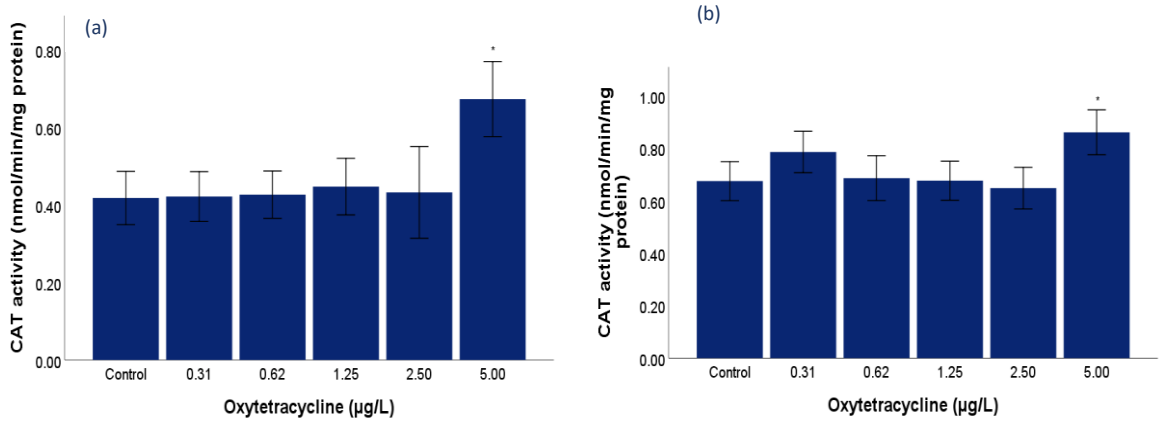

*Figure 7 - Results for CAT activity (determined in gills), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

For the acute exposure (Figure 8a) the results of GSTs activity showed a decrease for animals exposed to the highest concentration (5µgL^{-1}) of OTC tested ($F_{(5,46)}$ =4.339; p=0.003). GSTs activity after chronic exposure to OTC showed an increase for the lowest concentration, followed by a decrease of the enzyme activity for the remaining concentrations tested ($F_{(5,43)}$ =16.652; p<0.001) (Figure 8b).

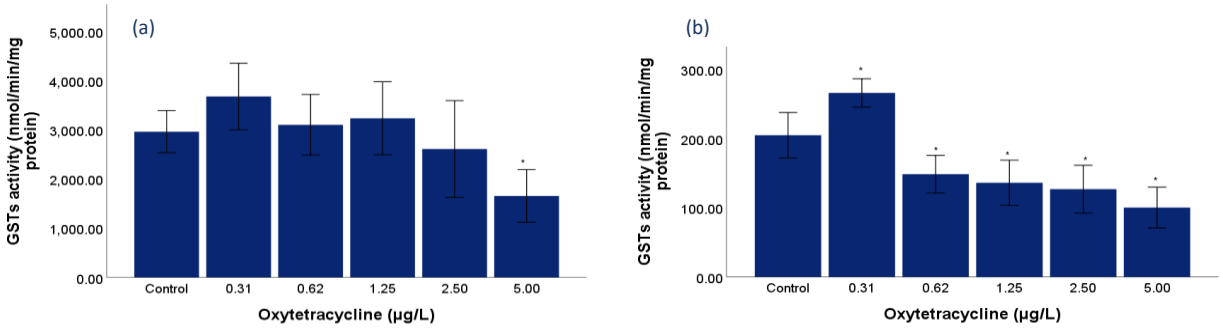

*Figure 8 - Results for GSTs activity (determined in gills), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

In the case of the TBARS levels, for the acute exposure (Figure 9a), the results showed no differences after exposure of the organisms to OTC ($F_{(5,47)}$ =0.413; p=0.838). However, the exposure for a period of 28 days (Figure 9b) was causative of an increase in the lipid peroxidation levels ($F_{(5,44)}$ =6.215); p<0.001).

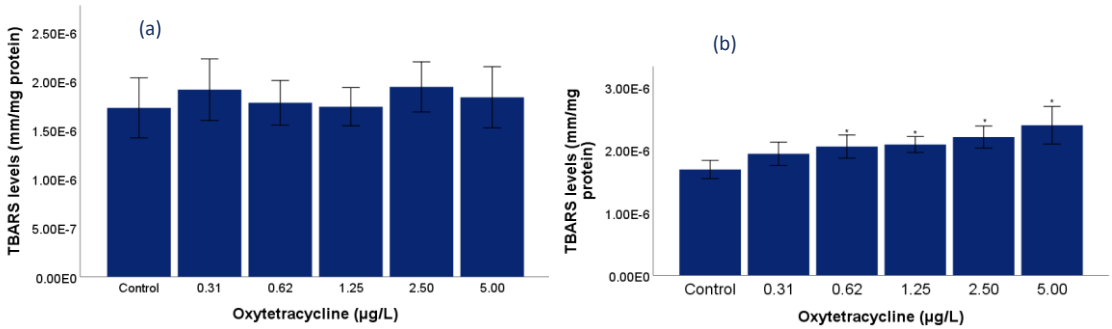

*Figure 9 - Results for TBARS levels (determined in gills), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

b. Digestive gland

The acute exposure of mussels to OTC caused a decrease on the SOD activity. For Total SOD ($F_{(5,46)}$ = 14.902; p<0.001) is possible to observe a decrease for all the concentrations tested, for Mn SOD ($F_{(5,46)}$ = 4.039; p=0.004) this decrease was also noted, except for the highest concentration (5µgL^{-1}). The digestive gland of mussels, after chronic exposure to OTC, showed no significant differences for Total SOD ($F_{(5,43)}$ = 2.171; p=0.075), however for the Mn SOD ($F_{(5,43)}$ = 4.863; p=0.001), a decreased activity was possible to observe, except for the last concentration (Figure 10).

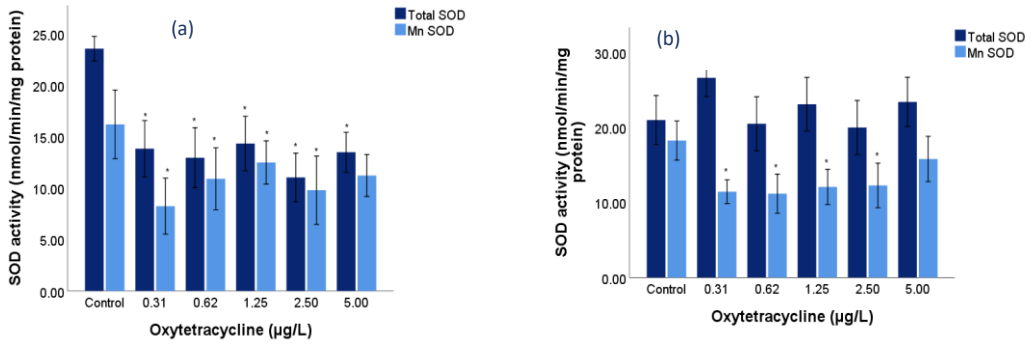

*Figure 10 - Results for SOD activity (determined in digestive gland), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The catalase activity, measured in the digestive gland after acute exposure show an increase for the two highest concentrations of OTC ($F_{(5,47)}$ =6.852; p<0.001) (Figure 11a). For the chronic exposure (Figure 11b), the catalase activity also increased ($F_{(5,44)}$ =25.689; p<0.001).

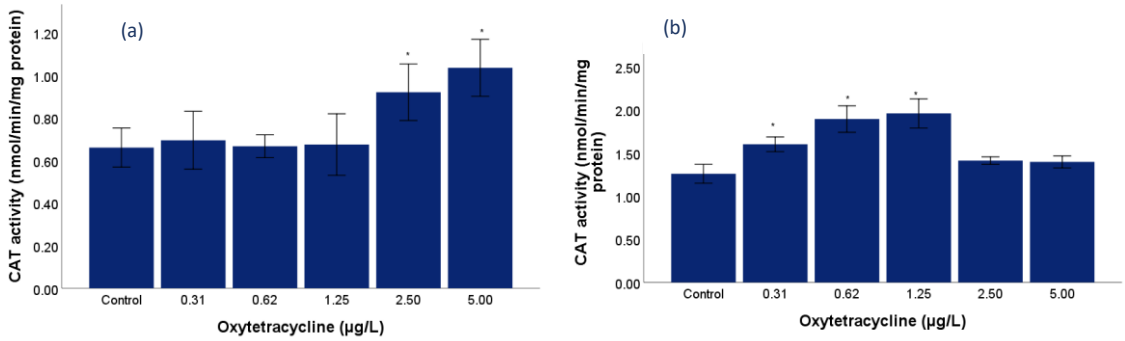

*Figure 11 - Results for CAT activity (determined in digestive gland), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

GTSs results obtained for animals acutely exposed to OTC (Figure 12a) showed a decrease in the activity for the three highest concentrations ($F_{(5,47)}$ =13.214; p<0.001). In organisms exposed to OTC for 28 days, no significant alterations were observed in terms of GSTs activity ($F_{(5,43)}$ =3.275; p=0.014) (Figure 12b).

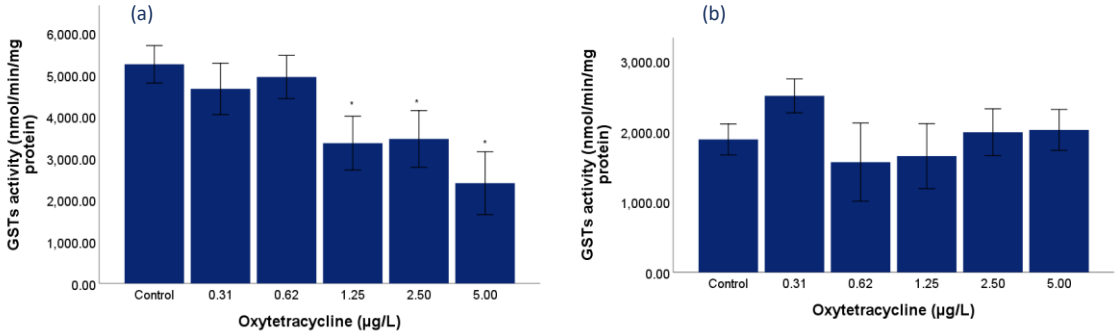

*Figure 12 - Results for GSTs activity (determined in digestive gland), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The acute exposure of organisms to OTC (Figure 13a) did not cause any effects in the levels of TBARS ($F_{(5,47)}$ =0.869; p=0.509). For the TBARS levels in the digestive gland of the organisms exposed for 28 days to OTC, it was possible to observe a decrease in the lipid peroxidation levels, for animals exposed to the concentrations 1.25 μgL^{-1} and 2.50 μgL^{-1} ($F_{(5,44)}$ =5.000; p=0.001) (Figure 13b).

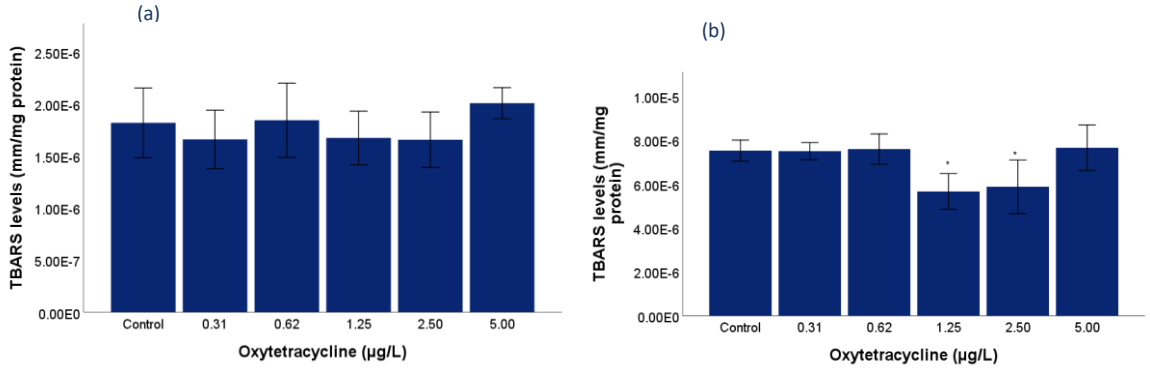

*Figure 13 - Results for TBARS levels (determined in digestive gland), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

c. Muscle

The muscle tissue of the animals acutely ($F_{(5,47)}$ =31.426; p<0.001) and chronically ($F_{(5,42)}$ = 3.207; p<0.001) exposed to OTC showed an increase of the activity of catalase (Figure 14). In the acutely exposed animals, the increase was significant for the two highest concentrations, while the chronic exposure resulted in no alterations for almost all concentrations tested.

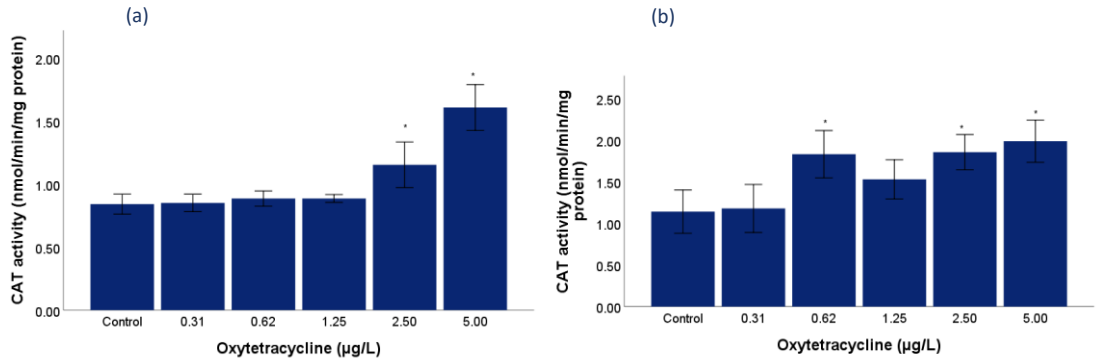

*Figure 14 - Results for CAT activity (determined in muscle), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

In terms of TBARS levels measured in the muscle tissue (Figure 15), no alterations were observed for animals acutely ($F_{(5,47)}$ =1.921; p=0.109) or chronically exposed to OTC ($F_{(5,42)}$ = 3.207; p=0.015).

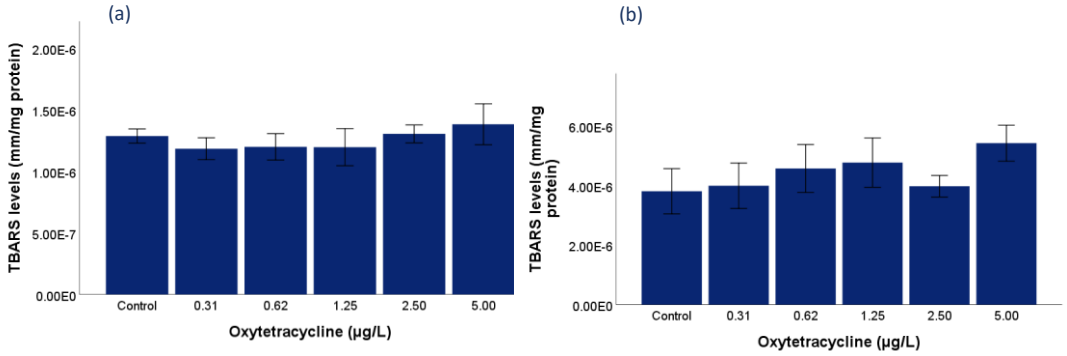

Figure 15 - Results for TBARS levels (determined in muscle), after exposure to OTC: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars.

2. Biomarkers determined after exposure to ciprofloxacin

a. Gills

The exposure of organisms to CIP caused a decrease in the activity of SOD (Figure 16). The acute exposure of organisms caused a decrease of Total SOD ($F_{(5,52)}$ =21.614; p<0.001) only for the two highest concentrations of CIP tested (0.078 and 0.195 mgL^{-1}). Mn SOD ($F_{(5,52)}$ =13.327; p<0.001) results showed an increase only for animals exposed to the concentration 0.031 mgL^{-1}. For Total SOD, and for all the concentrations tested in the chronic exposure, it was possible to see a decrease of activity ($F_{(5,51)}$ =13.859; p<0.001). For Mn SOD, a decrease of its activity ($F_{(5,51)}$ =4.264; p=0.003) was also observed, with statistical differences for the highest concentrations (0.078 mgL^{-1}, and 0.195 mgL^{-1}).

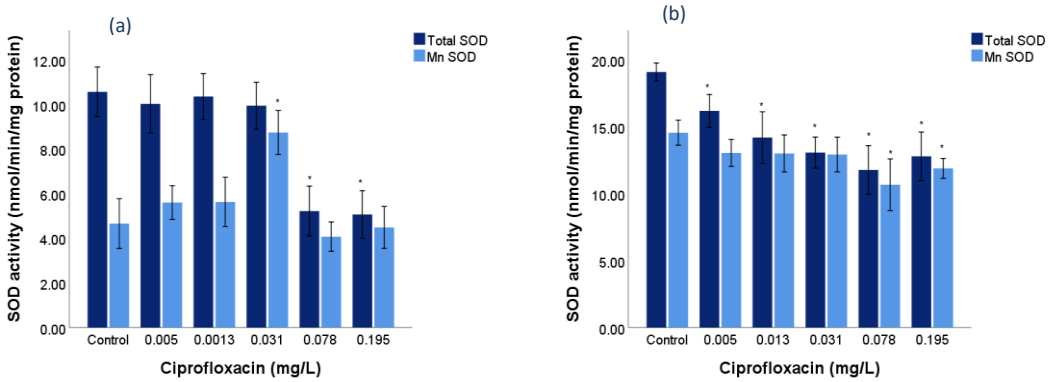

*Figure 16 - Results for SOD activity (determined in gills), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

Following the acute exposure (Figure 17a), catalase activity s was increased ($F_{(5,52)}$ =5.731; p<0.001). The catalase activity measured in gills of animals after chronic exposure demonstrated a dose-dependent increase ($F_{(5,51)}$ =22.651; p<0.001) (Figure 17b).

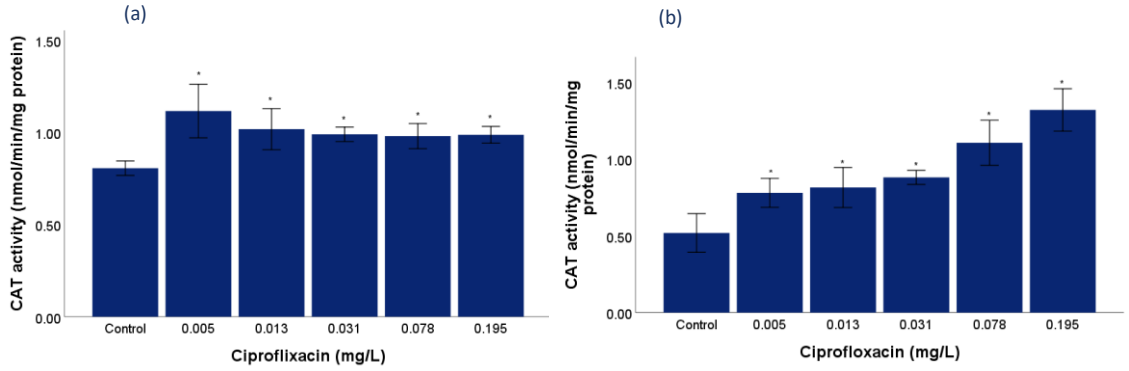

*Figure 17 - Results for CAT activity (determined in gills), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The GSTs activity in gills showed opposite results, for acute and chronic exposures. For the acute exposure, the GSTs activity (Figure 18a) decreased with statistical differences for the concentrations 0.013, 0.078, and 0.195 mg /L ($F_{(5,50)}$ =3.765; p=0.006). For the chronic exposure (Figure 18b), GSTs activity increased in animals exposed to all concentrations of CIP ($F_{(5,51)}$ =6.001; p<0.001).

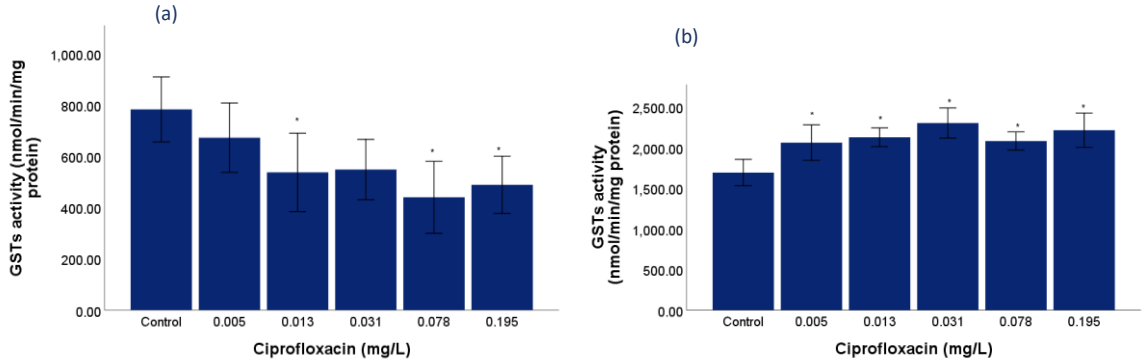

*Figure 18 - Results for GSTs activity (determined in gills), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The TBARS levels after acute ($F_{(5,52)}$ =1.893; p=0.112) and chronic exposures ($F_{(5,51)}$ =6.900; p<0.001) measured in the gills tissues (Figure 19) were significantly decreased for the highest concentrations of CIP.

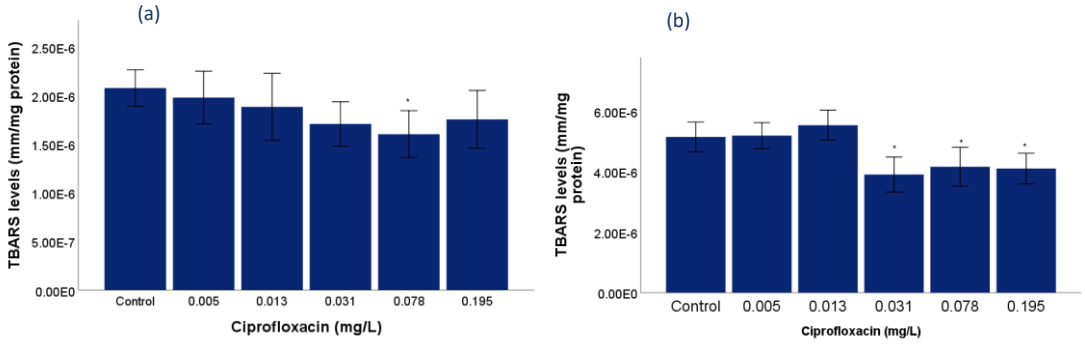

*Figure 19 - Results for TBARS levels (determined in gills), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

b. Digestive Gland

SOD activity quantified in the digestive gland after exposure to CIP was decreased. In the acutely exposed animals, the decrease was only observed for Total SOD ($F_{(5,52)}$ =17.380; p<0.001) in all concentrations. For Mn SOD ($F_{(5,52)}$ =3.948; p=0.004) no alterations were observed. For Total SOD in chronic exposure (Figure 20), it was possible to observe a significant statistical decrease in animals exposed to all concentrations tested, except for 0.031 mgL^{-1} ($F_{(5,51)}$ =6.866; p<0.001); and for Mn SOD, the same pattern was observed, with statistical differences been only attained for animals subjected to the highest concentrations ($F_{(5,51)}$ =2.752; p=0.028).

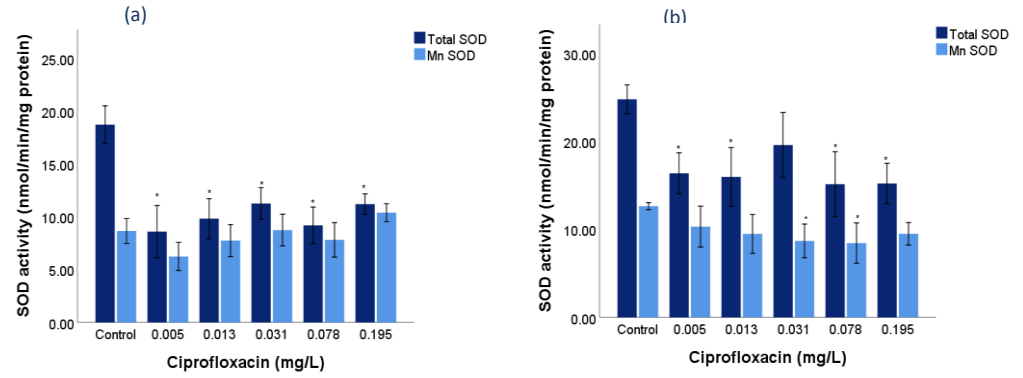

*Figure 20 - Results for SOD activity (determined in digestive gland), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

CAT activity measured in digestive gland of organisms exposed during 96h to CIP (Figure 21a) showed an increase ($F_{(5,52)}$ =9.310; p<0.001) for all concentrations tested. The results obtained for the activity of catalase in mussels after 28 days of exposure of organisms to CIP,

measured in the digestive gland of organisms (Figure 21b) showed an increase ($F_{(5,51)}$ =15.172; p<0.001) for all concentrations.

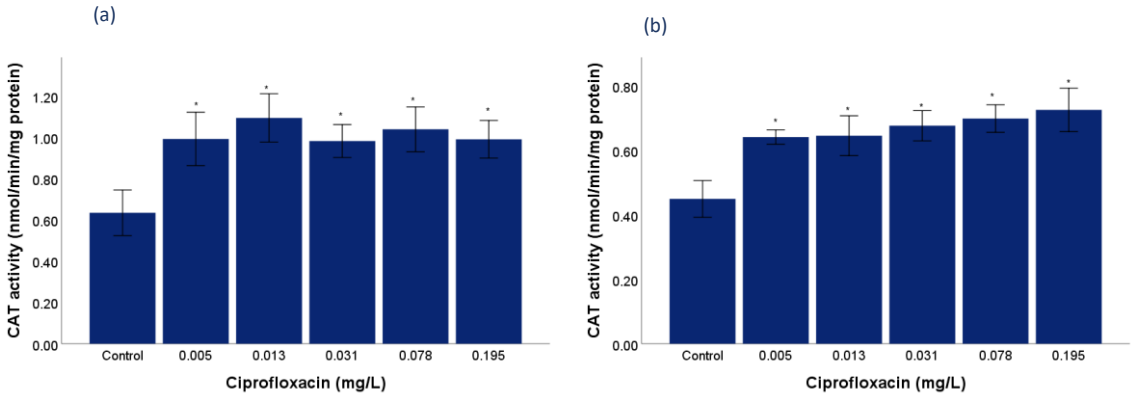

*Figure 21 - Results for CAT activity (determined in digestive gland), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

Acute exposure of organisms to CIP (Figure 18a) caused a decrease of GSTs activity ($F_{(5,52)}$ =4.941; p=0.001) for the concentration 0.013 mgL^{-1}. Regarding the GSTs activity (Figure 22b) measured after the chronic exposure, in the digestive gland tissue, it was possible to observe that the enzymatic activity increased, with statistical differences for the two highest concentrations ($F_{(5,51)}$ = 3.927; p=0.004).

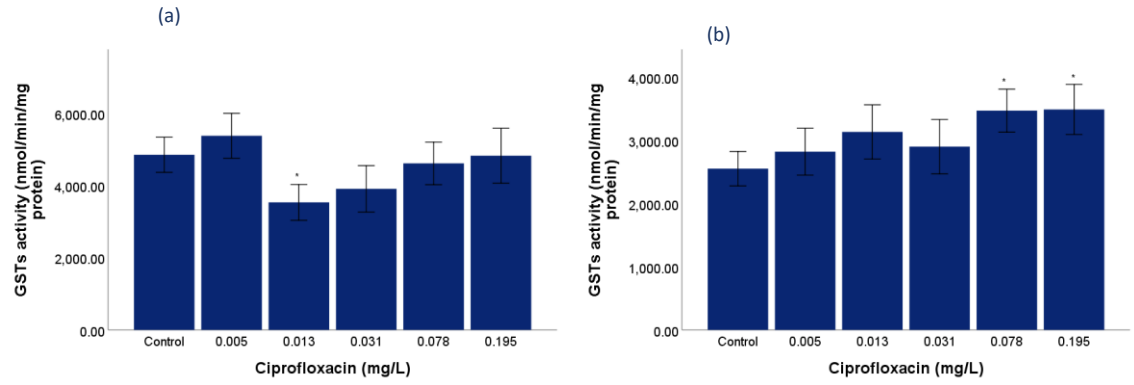

*Figure 22 - Results for GSTs activity (determined in digestive gland), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The TBARS levels for the acute exposure (Figure 23a) to CIP yielded a decrease of its levels ($F_{(5,52)}$ =2.748; p=0.028) with statistical differences only for animals exposed to the concentration of 0.013 mgL^{-1}. Concerning the TBARS levels of chronically exposed animals

(Figure 23b) the results showed an increase of the levels of this biomarker, in the digestive gland of the exposed organisms, which was dose-dependent ($F_{(5,50)}$ = 6.558; p=0.000).

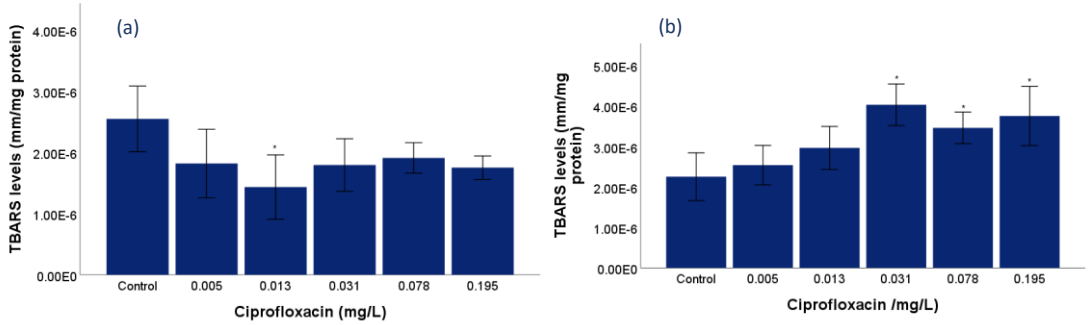

*Figure 23 - Results for TBARS levels (determined in digestive gland), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

c. Muscle

Acute exposure of organisms to CIP (Figure 24a) resulted in no alterations on the catalase activity measured in muscle tissue ($F_{(5,52)}$ =1.610; p=0.174). For the muscle tissue (Figure 24b) of the animals exposed to CIP for a period of 28 days, the catalase activity showed an increase for the three lowest concentrations ($F_{(5,51)}$ =31.293; p<0.001).

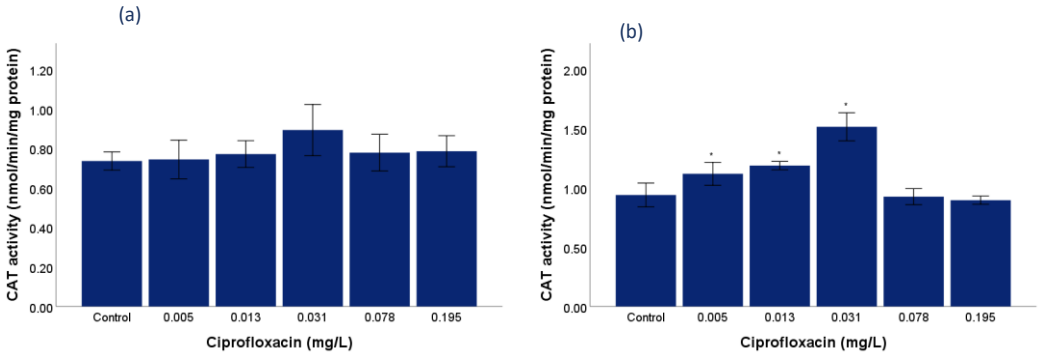

*Figure 24 - Results for CAT activity (determined in muscle), after exposure to CIP: (a) acute exposure; (b) chronic exposure. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

No effects were observed for TBARS levels after acute expose of organism to CIP (Figure 25a) in the muscle tissue ($F_{(5,52)}$ =0.286; p=0.919). The TBARS levels, in muscle tissue of chronically exposed organisms (Figure 25b) showed a decrease, but only with statistical differences for animals exposed to the concentration of 0.078 mgL^{-1}, except for the last concentration, for which a non-significant increase was observed ($F_{(5,51)}$ =4.281; p<0.001).

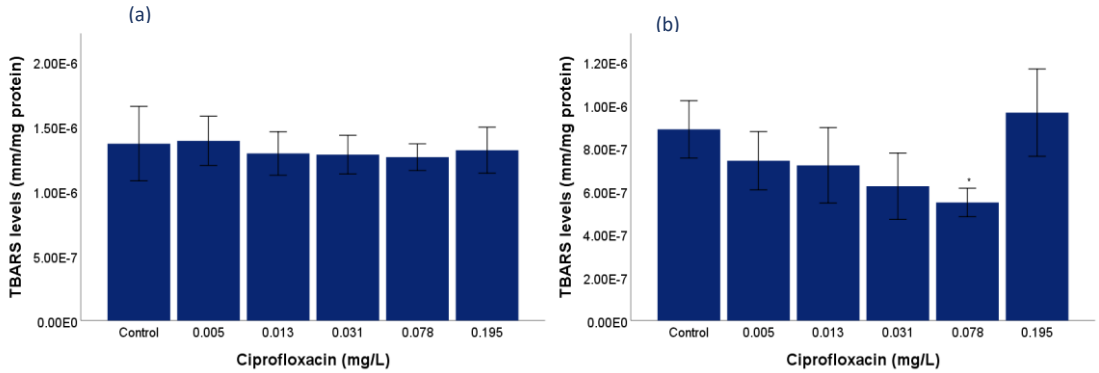

*Figure 25 - Results for TBARS levels (determined in muscle), after exposure to CIP: (a) acute exposure; (b) chronic exposure. results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

3. Biomarkers determined after exposure to a mixture of oxytetracycline and ciprofloxacin

    a. Gills

Animals acutely exposed to the mixture of antibiotics showed decreasing alterations in the SOD activity, Total ($F_{(3,33)}$ =5.132; p=0.005) and Mn ($F_{(3,33)}$ =4.119; p=0.014), but only for the second concentration (0.031 mgL^{-1} CIP + 1.25 μgL^{-1} OTC). After the chronic exposure, a decrease was observed in organisms for all treatments, for both Total ($F_{(3,34)}$ =9.899; p<0.001) and Mn ($F_{(3,34)}$ =04,940; p=0.006) SOD (Figure 26).

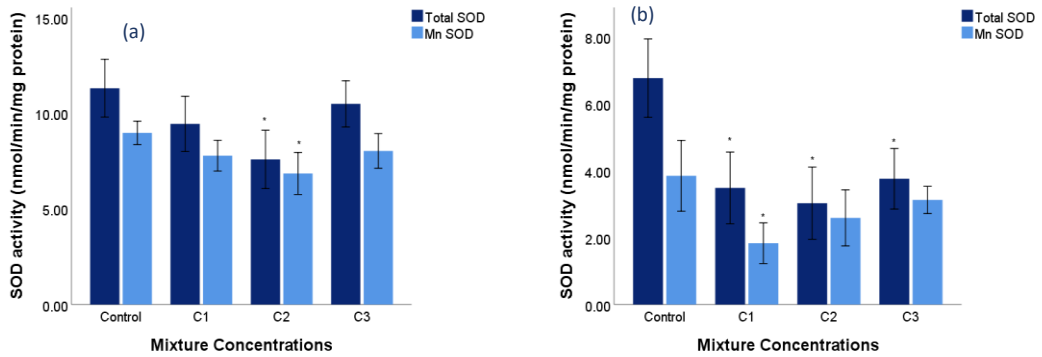

*Figure 26 - Results for SOD activity (determined in gills), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The catalase activity in the gills of acutely exposed organisms ($F_{(3,33)}$ =13.238; p<0.001) was decreased, for two of the concentrations tested (C1- 0, 0.005 mgL^{-1} CIP + 0.3125 μgL^{-1} OTC; C2- 0.031 mgL^{-1} CIP + 1.25 μgL^{-1}) (figure 27a). For the chronic exposure ($F_{(3,34)}$ =1.752; p=0.175) no alterations were observed for this biomarker (figure 27b).

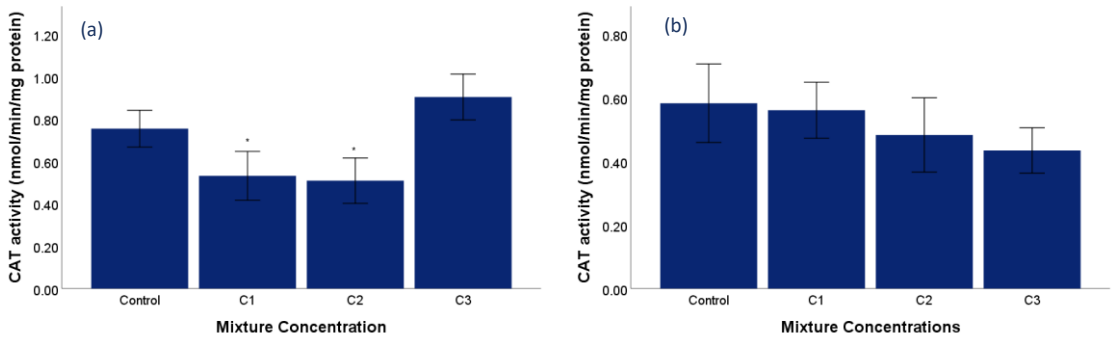

*Figure 27 - Results for CAT activity (determined in gills), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The results obtained for GSTs after acute exposure ($F_{(3,33)}$ =11.996; p<0.001) to the mixture of antibiotics showed an increase for the lowest concentration tested, and a decrease for the highest concentration (Figure 28a). The chronic exposure ($F_{(3,32)}$ =5.199; p=0.005) yielded a decrease of GSTs activity in the animals exposed to the second concentration (Figure 28b).

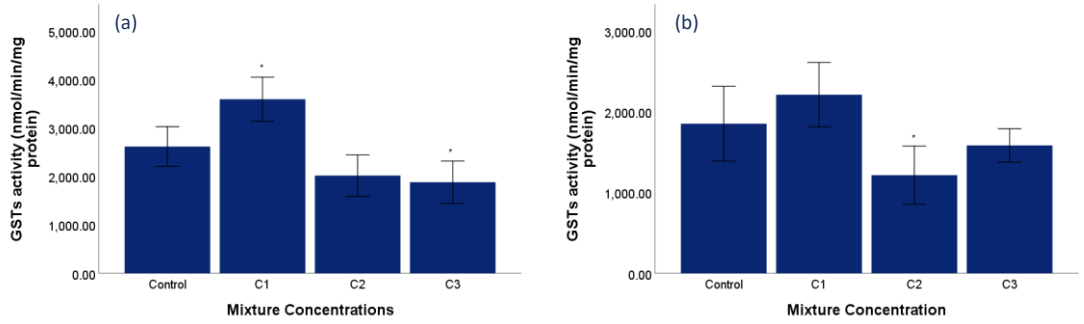

*Figure 28 - Results for GSTs activity (determined in gills), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The results for TBARS levels did not present any alterations, after both acute ($F_{(3,33)}$ =1.245; p=0.309) and chronic ($F_{(3,33)}$ =1.376; p=0.267) exposures to the mixture of antibiotics, in the gills of mussels (Figure 29).

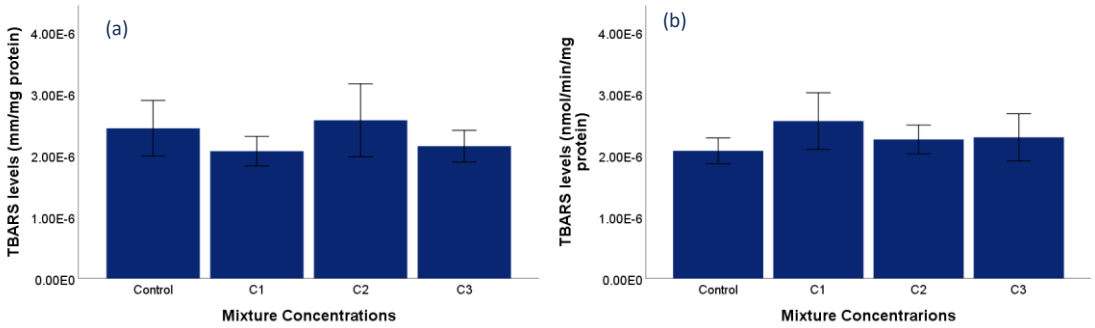

Figure 29 - Results for TBARS levels (determined in gills), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Results correspond to the average of 10 replicates, with the corresponding standard error bars.

b. Digestive gland

SOD results for the digestive gland of exposed organisms showed a decrease in the activity of this enzyme. In acutely exposed organisms, this decrease occurred for both Total ($F_{(3,33)}$ =25.891; p<0.001) and Mn SOD ($F_{(3,33)}$ =14.965; p<0.001), and for all the concentrations of the mixture tested (Figure 30a). The chronic exposure also yielded a decrease of the SOD activity for both Total ($F_{(3,34)}$ =46.471; p<0.001) and Mn ($F_{(3,34)}$ =8.930; p<0.001), but with statistical differences only for the two highest concentrations (Figure 30b).

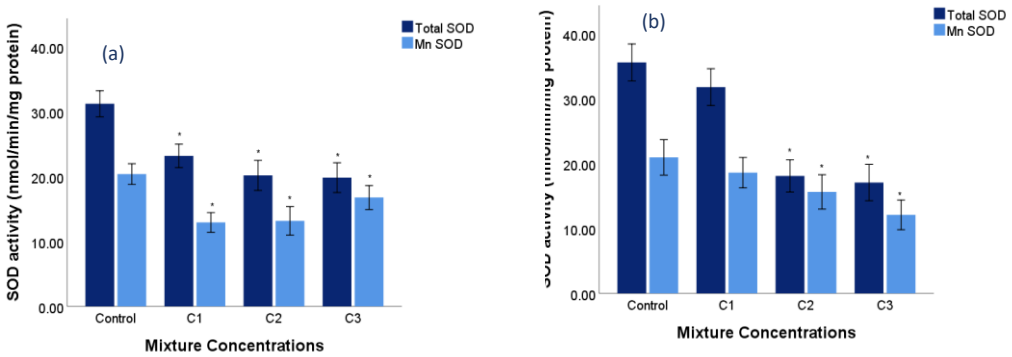

*Figure 30 - Results for SOD activity (determined in digestive gland), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The results obtained for catalase activity in animals acutely exposed to the mixture of the two antibiotics showed a decrease ($F_{(3,33)}$ =5.664; p=0.003) (Figure 31a). For the chronic exposure, the catalase activity increased in a dose-dependent manner ($F_{(3,34)}$ =10.418; p<0.001) (Figure 31b).

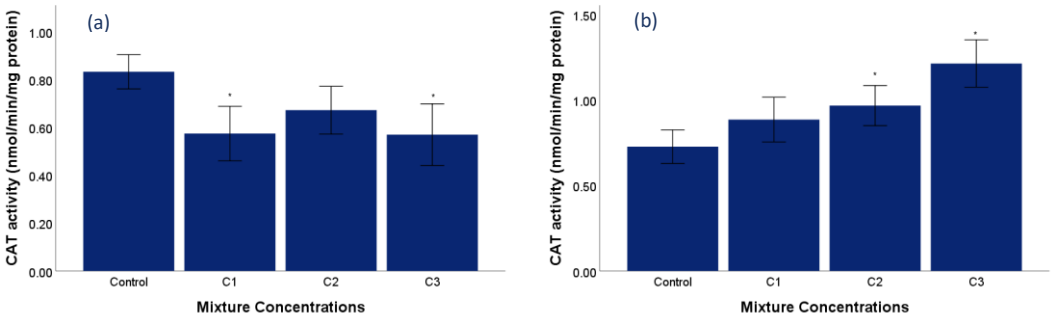

*Figure 31 - Results for CAT activity (determined in digestive gland), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L + 5 µg/L. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

GSTs activity in the digestive gland of mussels acutely exposed to the mixture of antibiotics ($F_{(3,33)}$ =6.565; p=0.001) increased for all the concentrations tested (Figure 32a). However, for the chronic exposure ($F_{(3,34)}$ =9.303; p<0.001), an increase of GSTs activity was reported, for the lowest concentration. On the contrary, for the other two concentrations, the values of GSTs activity decreased (Figure 32b).

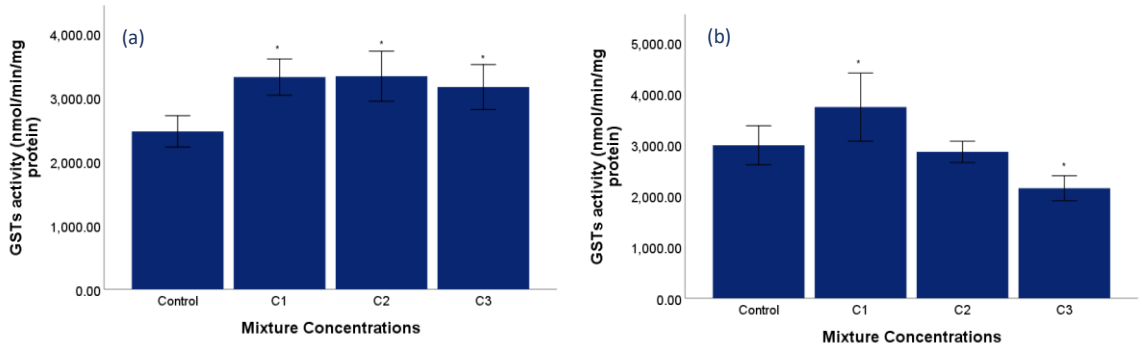

*Figure 32 - Results for GSTs activity (determined in digestive gland), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L + 5 µg/L. Results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

No significant differences in terms of TBARS levels were found in the digestive gland of mussels acutely ($F_{(3,33)}$ =2.476; p=0.079) and chronically ($F_{(3,33)}$ =1.523; p=0.226) exposed to the mixture of antibiotics (Figure 33).

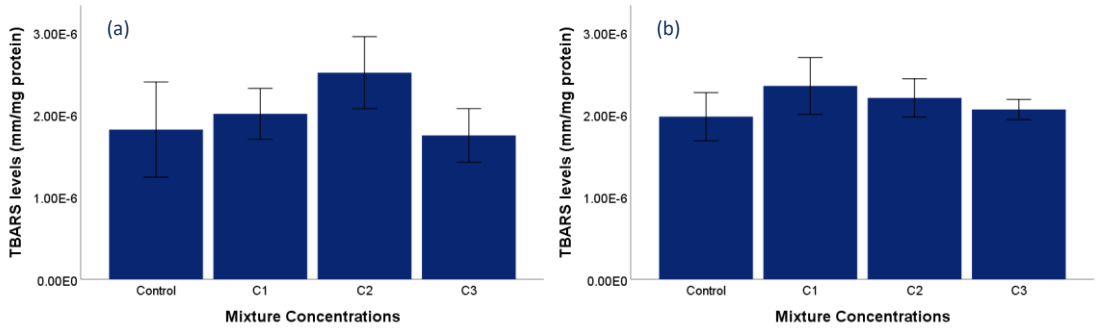

Figure 33 - Results for TBARS levels (determined in digestive gland), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L + 5 µg/L. Results correspond to the average of 10 replicates, with the corresponding standard error bars.

c. Muscle

Catalase activity in the muscle of animals acutely ($F_{(3,33)}$ =12.908; p<0.001) exposed to the mixture of the antibiotics increased for all the concentrations tested (Figure 34a). Contrarily, for the chronic exposure ($F_{(3,34)}$ =15.828; p<0.001) the catalase activity decreased for all the concentrations tested (Figure 34b).

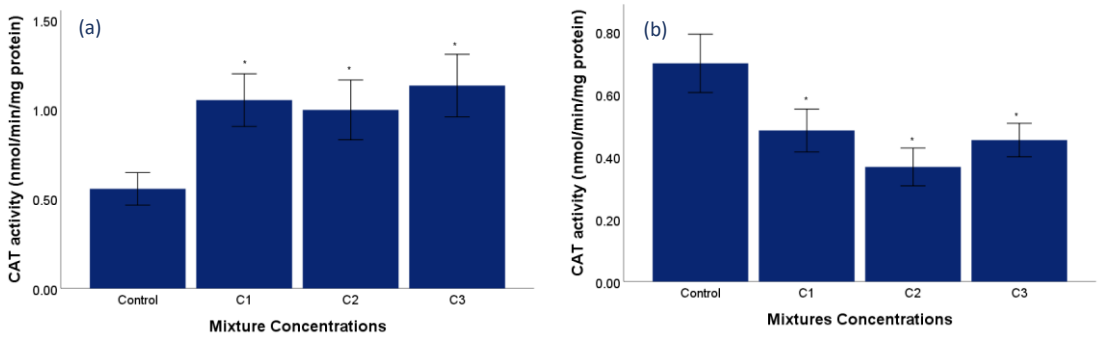

*Figure 34 - Results for CAT activity (determined in muscle), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. results correspond to the average of 10 replicates, with the corresponding standard error bars. * - Stands for significant differences when compared to control (p < 0.05).*

The results concerning the TBARS levels did not show any alterations in the muscle tissue of organisms, for both acute ($F_{(3,33)}$ =0.353; p=0.787) and chronic ($F_{(3,34)}$ =2.355; p=0.089) exposures to the mixture of oxytetracycline and ciprofloxacin (Figure 35).

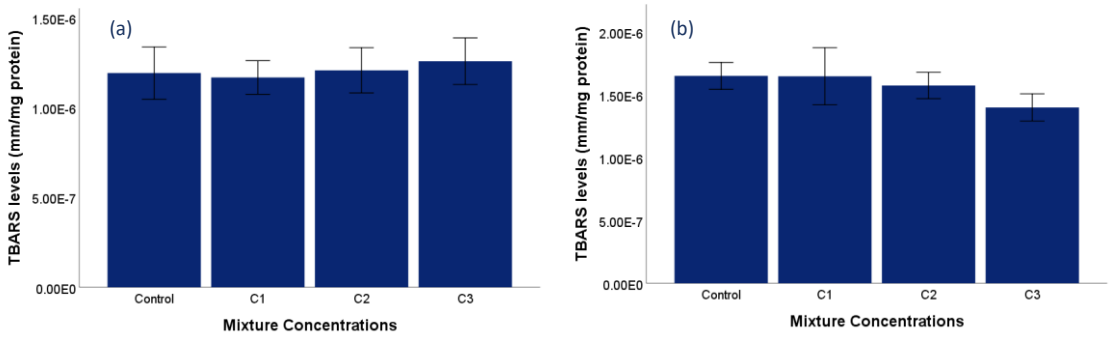

Figure 35 - Results for TBARS levels (determined in muscle), after exposure to a mixture of oxytetracycline and ciprofloxacin: (a) acute exposure; (b) chronic exposure. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Results correspond to the average of 10 replicates, with the corresponding standard error bars.

4. Genotoxicity

a. Exposure to oxytetracycline

Mussels exposed to OTC revealed a significantly higher DNA damage, measured as GDI, after both acute ($F_{(5,36)}$ =545.805; p<0.001) and chronic ($F_{(5,36)}$ =1465.079; p<0.001) exposures (Figure 36). Individuals that were exposed to concentrations of OTC showed higher percentage of damages from classes 1 and 2 for both exposures, and lower percentage of classes 0 and 1, for both exposures (Table 1; Figure 36). The results showed a concentration-dependent pattern for both exposures.

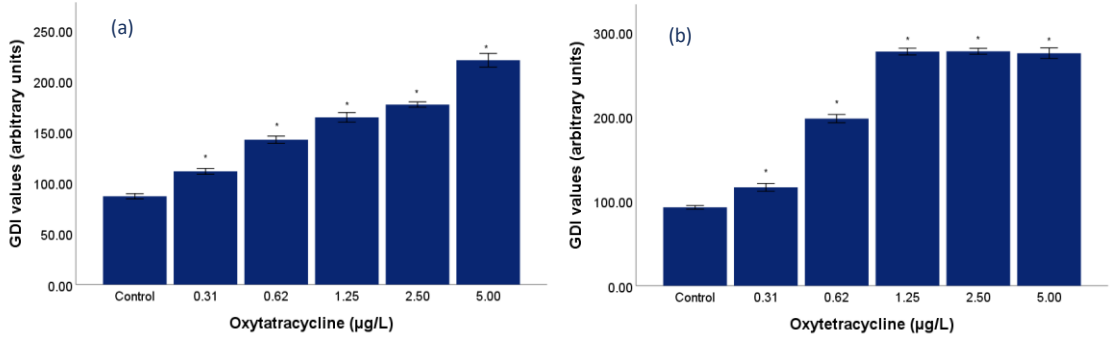

*Figure 36 - Mean GDI (expressed as arbitrary units), measured by the comet assay, in hemolymph cells of mussels after acute (a) and chronic (b) exposures to oxytetracycline. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

For the acute assay, significant differences in several damage classes were detected, specifically: for class 0 ($F_{(5,36)}$ =557.435; p<0.001) the frequency decreased for all concentrations; class 1 ($F_{(5,36)}$ =56.114; p<0.001) also presented a decrease for all concentrations; damages of class 2 were increased ($F_{(5,36)}$ =277.321; p<0.001) for all concentrations; for damages of class 3, it was possible to observe an increase of their frequency ($F_{(5,36)}$ =168.311; p<0.001) for the

concentrations of 0.625; 1.25; 2.50, and 5.00 μgL^{-1}; and for class 4 damages ($F_{(5,36)}$ =346.800; p<0.001), an increase only for the concentration 5.00 μgL^{-1} was observed. For the chronic assay, significant differences were detected for several damage classes, specifically: class 0 changes ($F_{(5,36)}$ =835.025; p<0.001) were decreased for all concentrations; damages of the class 1 ($F_{(5,36)}$ =171.698; p<0.001) were increased, in animals exposed to the concentrations of 0.3125 and a decrease for 1.25; 2.50 and 5.00 μgL^{-1}; class 2 damages ($F_{(5,36)}$ =69.978; p<0.001) were increased in animals subjected to the concentrations of 0.3125 and 0.625, with a decrease of their frequency for organisms exposed to2.50 and 5.00 μgL^{-1}; damages from class 3 ($F_{(5,36)}$ =484.286; p<0.001) were increased for the concentrations 0.625; 1.25; 2.50 and 5.00 μgL^{-1}; and changes of class 4 ($F_{(5,36)}$ =971.593; p<0.001) were also increased for the organisms exposed to concentrations of 1.25; 2.50 and 5.00 μgL^{-1}.

*Table 1- Mean frequency (%) of each DNA damage class (± standard error), measured by the comet assay, in hemolymph cells of mussels after acute (a) and chronic (b) exposures to oxytetracycline. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

OTC Concentrations		**0**	**1**	**2**	**3**	**4**
Acute exposure	CTRL	33.1 ± 1.1	46.8 ± 2.5	20.0 ± 2.8	0.0 ± 0.0	0.0 ± 0.0
	0.3125	**26.0 ± 1.6*↓**	**37.7 ± 1.4*↓**	**35.1 ± 2.5*↑**	**1.1 ± 1.6**	0.0 ± 0.0
	0.625	**15.7 ± 1.4*↓**	**29.7 ± 3.1*↓**	**50.8 ± 1.6*↑**	**3.7 ± 1.8*↑**	0.0 ± 0.0
	1.25	**9.4 ± 1.5*↓**	**24.8 ± 1.6*↓**	**57.4 ± 0.9*↑**	**8.2 ± 2.7*↑**	0.0 ± 0.0
	2.5	**2.6 ± 2.2*↓**	**31.7 ± 3.5*↓**	**51.7 ± 1.4*↑**	**14.0 ± 1.6*↑**	0.0 ± 0.0
	5	**0.0 ± 0.0*↓**	**22.6 ± 5.1*↓**	**43.7 ± 2.9*↑**	**24.0 ± 2.3*↑**	**9.7 ± 1.4*↑**
OTC Concentrations		**0**	**1**	**2**	**3**	**4**
Chronic exposure	CTRL	38.0 ± 1.6	31.1 ± 2.2	30.8 ± 1.9	0.0 ± 0.0	0.0 ± 0.0
	0.3125	**18.8 ± 1.9*↓**	**45.7 ± 4.5*↑**	**35.4 ± 5.0*↑**	0.0 ± 0.0	0.0 ± 0.0
	0.625	**0.0 ± 0.0*↓**	29.4 ± 3.0	**43.1 ± 2.8*↑**	**27.4 ± 3.9*↑**	0.0 ± 0.0
	1.25	**0.0 ± 0.0*↓**	**4.8 ± 1.9*↓**	28.3 ± 1.4	**51.4 ± 2.2*↑**	**15.4 ± 2.5*↑**
	2.5	**0.0 ± 0.0*↓**	**18.0 ± 2.6*↓**	**19.1 ± 3.0*↓**	**30.0 ± 3.0*↑**	**32.9 ± 1.6*↑**
	5	**1.7 ± 2.4*↓**	**17.1 ± 1.9*↓**	**18.0 ± 2.8*↓**	**30.3 ± 2.1*↑**	**32.8 ± 1.6*↑**

b. Exposure to ciprofloxacin

The organisms exposed to CIP evidenced a significantly higher DNA damage, measured as GDI, following both acute ($F_{(5,36)}$ =1039.963; p<0.001) and chronic ($F_{(5,36)}$ =1182.615; p<0.001)

exposures (Figure 37). Individuals that were exposed to concentrations of CIP showed higher percentage of damages from classes 2 and 3, for both exposures, and lower percentage of classes 3 and 4, for both exposures also (Table 2; Figure 37). The results showed a concentration-dependent pattern for both exposures.

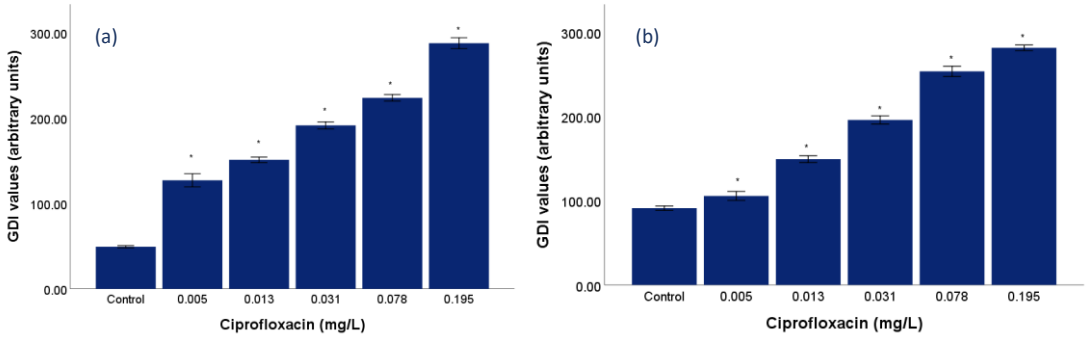

*Figure 37 - Mean GDI (expressed as arbitrary units), measured by the comet assay, in hemolymph cells of mussels after acute (a) and chronic (b) exposures to ciprofloxacin. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

For the acute assay, significant differences were detected in several damage classes, specifically: class 0 changes ($F_{(5,36)}$ =1134.076; p<0.001) showed a decrease of frequency for all concentrations; the frequency of class 1 damages ($F_{(5,36)}$ =235.930; p<0.001) was increased for the lowest concentrations (0.013 mgL^{-1}), and decreased for the remaining concentrations; class 2 alterations ($F_{(5,36)}$ =176.110; p<0.001) had their frequency increased for all concentrations; the frequency of class 3 damages were also increased ($F_{(5,36)}$ =689.242; p<0.001) for 0.013; 0.031; 0.078 and 0.195 mgL^{-1}; and for class 4 damages ($F_{(5,36)}$ =346.058; p<0.001) the frequency of detection was increased for 0.031; 0.078, and 0.195 mgL^{-1} of CIP. For the chronic assay, significant differences were detected in several damage classes, specifically: class 0 had a decrease of frequency ($F_{(5,36)}$ =426.676; p<0.001) for all concentrations; class 1 ($F_{(5,36)}$ =285.137; p<0.001) also presented a decrease on its frequency for all concentrations; changes of the class 2 ($F_{(5,36)}$ =29.534; p<0.001) evidenced an increase of their frequency for all concentrations; for class 3 changes ($F_{(5,36)}$ =254.807; p<0.001) the frequency also increased for the concentrations 0.013; 0.031; 0.078 and 0.195 mgL^{-1}; and class 4 damages were characterized also by an increase of their frequency ($F_{(5,36)}$ =735.024; p<0.001) for 0.013; 0.031; 0.078 and 0.195 mgL^{-1} of CIP.

*Table 2- Mean frequency (%) of each DNA damage class (± standard error), measured by the comet assay, in haemolymph cells of mussels after acute (a) and chronic (b) exposures to ciprofloxacin. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

CIP Concentrations		0	1	2	3	4
Acute exposure	CTRL	55.4 ± 2.2	40.6 ± 3.0	4.0 ± 1.6	0.0 ± 0.0	0.0 ± 0.0
	0.005	**15.7 ± 2.1*↓**	**46.0 ± 1.6*↑**	**38.0 ± 5.8*↑**	1.7 ± 2.1	0.0 ± 0.0
	0.0013	**13.4 ± 1.5*↓**	**34.3 ± 1.8 *↓**	**40.0 ± 3.2*↑**	**12.3 ± 2.1*↑**	0.0 ± 0.0
	0.0031	**1.4 ± 1.9*↓**	**32.3 ± 1.8 *↓**	**44.6 ± 2.5*↑**	**16.8 ± 1.6*↑**	**4.8 ± 1.9*↑**
	0.0078	**1.1 ± 1.0*↓**	**23.4 ± 1.9*↓**	**35.1 ± 1.9*↑**	**31.1 ± 1.6*↑**	**9.1 ± 1.6*↑**
	0.195	**0.0 ± 0.0*↓**	**10.8 ± 2.5 *↓**	**15.1 ± 2.2*↑**	**49.4 ± 2.8* ↑**	**24.6 ± 2.2*↑**
CIP Concentrations		**0**	**1**	**2**	**3**	**4**
Chronic exposure	CTRL	31.1 ± 1.1	46.6 ± 2.2	22.3 ± 2.6	0.0 ± 0.0	0.0 ± 0.0
	0.005	**26.3 ± 2.7*↓**	**43.1 ± 3.2*↓**	**29.1 ± 5.0*↑**	1.4 ± 0.9	0.0 ± 0.0
	0.013	**21.1 ±1.6*↓**	**28.6 ± 1.5*↓**	**32.3 ± 1.4*↑**	**14.6 ± 2.7*↑**	**3.1 ± 1.5*↑**
	0.031	**11.1 ± 1.6*↓**	**22.0 ± 1.6*↓**	**38.0 ± 1.6*↑**	**17.4 ± 3.6 *↑**	**11.4 ± 1.5*↑**
	0.078	**1.7 ± 1.7*↓**	**13.4 ± 3.4*↓**	**34.3 ± 1.4*↑**	**30.6 ± 1.5*↑**	**20.0 ± 1.6*↑**
	0.195	**0.0 ± 0.0*↓**	**9.4 ± 1.5*↓**	**31.1 ± 1.1*↑**	**26.6 ± 1.5*↑**	**32.6 ± 1.5*↑**

a. Exposure to mixtures of oxytetracycline and ciprofloxacin

The mussels exposed to mixtures of the two antibiotics revealed a significantly higher DNA damage, measured as GDI, in both acute ($F_{(5,36)}$ =403.737; p<0.001) and chronic ($F_{(5,36)}$ =1219.249; p<0.001) exposures (Figure 38). Individuals that were exposed to concentrations of the mixture showed higher percentage of damages from classes 1 and 2 for acute exposure, and from classes 2 and 3 for chronic exposure; in addition, these same animals evidenced lower percentage of classes 3 and 4 for acute exposure, and classes 0 and 4 for the

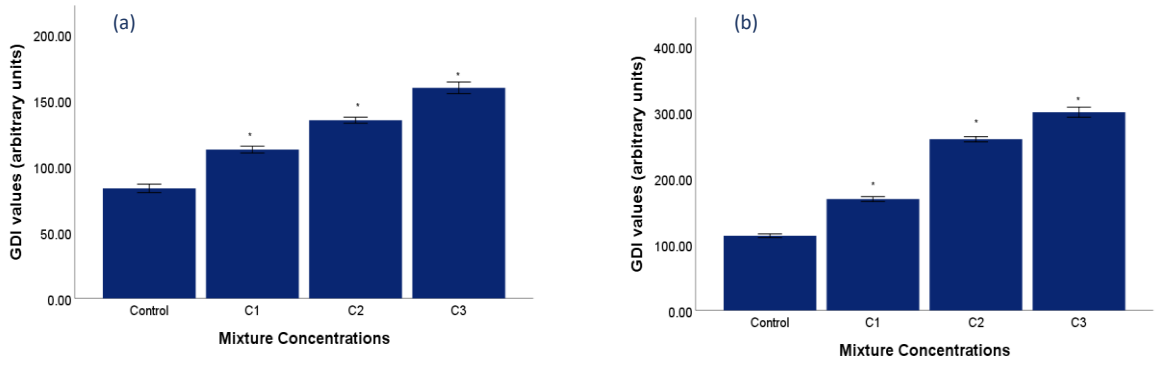

*Figure 38- Figure 38 - Mean GDI (expressed as arbitrary units), measured by the comet assay, in hemolymph cells of mussels after acute (a) and chronic (b) exposures to a mixture of oxytetracycline and ciprofloxacin. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

chronic exposure (Table 3; Figure 38). The results showed a concentration-dependent pattern for both exposures.

For the acute assay, significant differences were detected in several damage classes, specifically: for class 0 ($F_{(5,36)}$ =222.605; p<0.001) it was possible to see a decrease of their frequency for all concentrations; class 1 ($F_{(5,36)}$ =502.275; p<0.001) showed the same trend for all concentrations; however for class 2 damages ($F_{(5,36)}$ =375.584; p<0.001) an increase of their frequency was observed for all concentrations; class 3 damages also presented an increase of detection frequency ($F_{(5,36)}$ =64.233; p<0.001) for all concentrations. For the chronic assay, significant differences were detected in several damage classes, specifically: the class 0 ($F_{(5,36)}$ =587.871; p<0.001) showed a decrease of detection frequency for all concentrations; class 1 ($F_{(5,36)}$ =284.347; p<0.001) also showed the same trend for all concentrations; class 2 ($F_{(5,36)}$ =221.610; p<0.001) showed an increase of detection, for the lowest concentration, and a decrease for the highest concentration; damages from both class 3 ($F_{(5,36)}$ =775.512; p<0.001) and class 4 ($F_{(5,36)}$ =629.795; p<0.001) showed an increase in detection frequency; class 3 for all the concentrations, and class 4 for the two highest concentrations.

*Table 3- Table 4- Mean frequency (%) of each DNA damage class (± standard error), measured by the comet assay, in hemolymph cells of mussels after acute (a) and chronic (b) exposures to a mixture of oxytetracycline and ciprofloxacin. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Data are expressed as mean ± standard error (SE), 10 mussels per condition. * - Stands for significant differences when compared to control (p < 0.05).*

MIX Concentrations		**0**	**1**	**2**	**3**	**4**
Acute exposure	CTRL	36.8 ± 1.1	44.8 ± 1.1	16.3 ± 0.8	2.0 ± 1.6	0.0 ± 0.0
	C1	**30.6 ± 1.5*↓**	**34.0 ± 1.6 *↓**	**27.4 ± 2.8*↑**	**8.0 ± 1.6*↑**	0.0 ± 0.0
	C2	**26.0 ± 1.6*↓**	**28.0 ± 1.6*↓**	**30.8 ± 1.9*↑**	**15.1 ± 1.1* ↑**	0.0 ± 0.0
	C3	**18.8 ± 1.1* ↓**	**16.8 ± 1.1*↓**	**50.0 ± 1.6*↑**	**14.3 ± 3.1*↑**	0.0 ± 0.0
MIX Concentrations		**0**	**1**	**2**	**3**	**4**
Chronic exposure	CTRL	21.7 ± 1.8	44.6 ± 2.2	32.6 ± 2.7	1.1 ± 1.5	0.0 ± 0.0
	C1	**16.0 ± 1.6 *↓**	**19.7 ± 3.1*↓**	**46.0 ± 1.6*↑**	**16.0 ± 1.6*↑**	2.3 ± 1.4
	C2	**0.0 ± 0.0 *↓**	**15.1 ± 1.9*↓**	30.8 ± 1.1	**33.1 ± 1.1*↑**	**20.8 ± 1.6*↑**
	C3	**0.0 ± 0.0 *↓**	**9.42 ± 2.2*↓**	**19.7 ± 1.8*↓**	**31.7 ± 1.4 *↑**	**39.1 ± 3.2 *↑**

5. Shell Hardness

a. Oxytetracycline

Concerning the results of the shell hardness, both acute ($F_{(5,36)}$ =1.268; p=0.293) and chronic ($F_{(5,36)}$ =1.538; p=0.198) exposures to OTC did not result in any significant alterations (Figure 39a). However, it is possible to see a dose dependent decrease of this parameter in chronically exposed animals (Figure 39b), despite the absence of statistically significant differences among treatments.

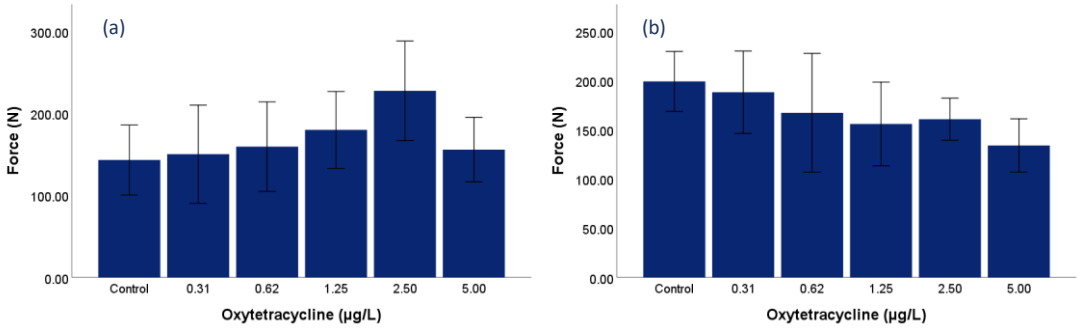

Figure 39 - Mean Force (N), measured in mussels after acute (a) and chronic (b) exposures to a mixture of oxytetracycline. Data are expressed as mean ± standard error (SE).

b. Ciprofloxacin

Results concerning shell hardness, for both acute ($F_{(5,36)}$ =0.725; p=0.608) and chronic ($F_{(5,36)}$ =0.631; p=0.677) exposures to CIP, did not show any significant alterations among the concentrations tested (Figure 40).

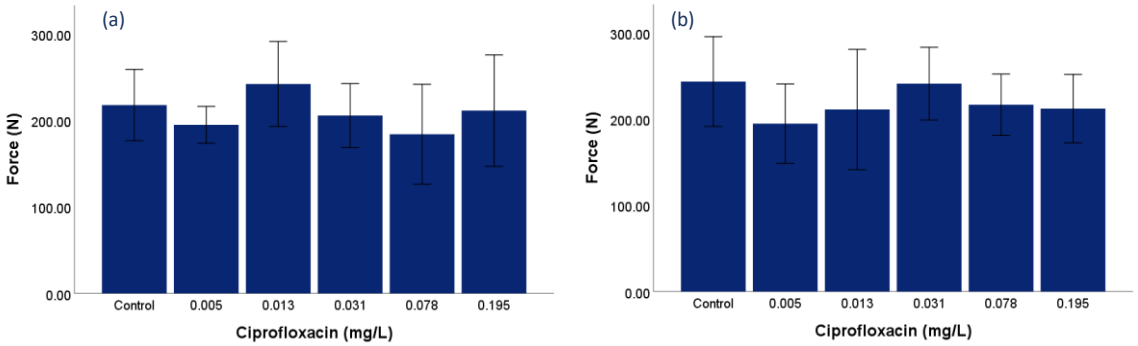

Figure 40 - Mean Force (N), measured in mussels after acute (a) and chronic (b) exposures to a mixture of ciprofloxacin. Data are expressed as mean ± standard error (SE).

c. Mixture to oxytetracycline and ciprofloxacin

The exposure to the mixtures of the two antibiotics for both acute ($F_{(3,36)}$ =0.214; p=0.886) and chronic ($F_{(3,36)}$ =0.723; p=0.545) exposures did not yield any significant alteration in the force needed to break the shell of the organisms (Figure 41).

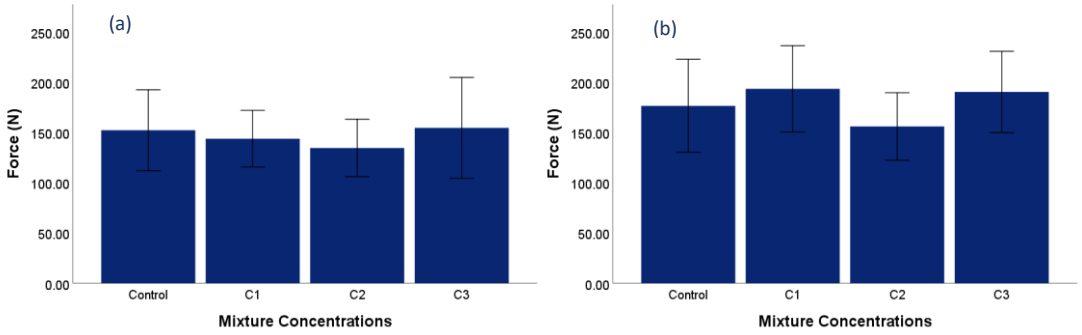

Figure 41 - Mean Force (N), measured in mussels after acute (a) and chronic (b) exposures to a mixture of oxytetracycline and ciprofloxacin. C1- 0, 0.005 mg/L CIP + 0.3125 µg/L OTC; C2- 0.031 mg/L CIP + 1.25 µg/L OTC; C3- 0.195 mg/L CIP + 5 µg/L OTC. Data are expressed as mean ± standard error (SE), 10 mussels per condition.

IV. Discussion

1. Oxytetracycline exposure

OTC is a biologically active antibiotic, and due to its widespread use, ineffective elimination in WWTPs, and its potential for contamination of aquatic environments, its toxicity has been assessed for different groups of aquatic organisms, through the use of different endpoints. Previous ecotoxicological data have shown that OTC is able to cause adverse effects to non-target organisms. The metabolism of OTC frequently results in the production of toxic intermediates, particularly reactive oxygen and nitrogen species (ROS and RNS), such as hydroxyl and peroxynitrite radicals. OTC also inhibits mitochondrial β-oxidation, causing a disruption in the respiratory chain and producing superoxide anion, which is decisive to produce more ROS and RNS (Rodrigues et al. 2017a; Pari and Gnanasoundari 2006). Peroxynitrite is a potent oxidant that can disturb cellular energy status by inactivating fundamental mitochondrial enzymes, possibly leading to protein changes and, consequently, dysfunction (Barzilai and Yamamoto 2004). A study by Kołodziejska et al. (2013) investigated the aquatic toxicity of some antibiotics, including OTC, and the results showed an effect of OTC in *Lemna minor* (EC50 = 2.96 mg / L), *Scenedesmus vacuolatus* (EC_{50} = 18.0 mg / L) and *D. magna* (EC_{50} = 114 mg / L). Feng-Jiao et al. (2014) also reported that the cycle of cell growth, nutrition, antioxidant capacity, lipid peroxidation, photosynthesis and respiration of the diatom *Conticribra weissflogii* were affected by 24h exposure to OTC (1.0 - 10.0 mgL^{-1}). However, such toxic effects were only attained at concentrations that are considerably high, when compared to the values that are found in the wild. Studies on toxic doses of antibacterial drugs (such as OTC) for aquatic wildlife, are available only for a few species, and very limited to short-term test data, providing no information on the toxicity resulting from the long-term exposure, and the possibility of translating these adverse effects into more realistic scenarios of exposure of organisms to these xenobiotics. The scarce information available about sub-lethal and sub-individual parameters, measured in realistic toxicity scenarios, becomes increasingly important as it allows us to have a more accurate perception of the deleterious effects that the organisms are facing.

There are several studies in the literature indicating that tetracyclines (including OTC) are metabolized by several organisms, triggering antioxidant responses and/or oxidative stress, namely in *Oncorhynchus mykiss* (Rodrigues et al., 2017a and 2017b; Yonar 2012; Yonar et al., 2011), *Danio rerio* (Oliveira et al., 2013), *Gambusia holbrooki* (Nunes et al., 2015b), *Portunus tribuberculatus* (Ren et al., 2017), and *Eisenia fetida* (Dong et al., 2012). So, the triggering of antioxidant defence mechanisms seems to be a generic response to exposure to tretracyclines.

In agreement with this trend, our data showed a similar response. Exposure of mussels to OTC caused a decrease in SOD activity in both tissues. Gills exhibited a dose-dependent decrease in SOD activity for all the concentrations tested, following both acute and chronic exposures. For the digestive gland, the SOD results also showed a decrease of activity, after acute and chronic exposure to OTC. These results are not entirely surprising considering that some studies already reported similar responses after exposure to OTC. The exposure of rainbow trout (*O. mykiss*) to OTC (100 mg OTC per kg fish weight) by Yonar (2012) showed a statistically significant decreased in SOD activity. A study conducted by Elia et al. (2014) aimed to simulate pharmacological treatment of carp, *Cyprinus carpio*, with therapeutic levels (75 mg/kg) of oxytetracycline (OTC), and at higher doses (150, 300 mg/kg) for 10 days. After 10 days of exposure, it was possible to observe a decrease in SOD activity for the higher dose of OTC (300 mg/kg). This trend is not limited to fish, and crustaceans seem also to respond similarly, also showing a decrease in the SOD activity. Ren et al. (2017) obtained similar results in SOD activity after exposed larvae of the crab *P. tribuberculatus* to OTC (0.3; 3, and 30 μgL^{-1}) in all conditions tested. Wei et al. (2008) demonstrated that there is an interaction between tetracyclines and CuZnSOD activity. With increasing volume of tetracycline analogues (i.e, increasing concentration of tetracycline analogues), the auto-oxidation of pyrogallol becomes faster, which indicates a decrease in CuZnSOD activity. OTC causes the greatest decrease in CuZnSOD activity, from 64.1–66.7%. Experimental results demonstrate that OTC (and other tetracyclines) can quench CuZnSOD fluorescence and that this quenching is static. The ability of CuZnSOD to scavenge the radical superoxide (O_2^-) would be reduced when its activity is decreased by tetracycline analogues.

The establishment of oxidative conditions is also reinforced by data obtained for CAT activity. The acute exposure of mussels to OTC resulted in an increase of CAT activity for all the tissues tested. The enzymatic activity for CAT in chronic tests was not similar for both tissues tested, but in general it was possible to also observe an increase of its activity. In gills, an increase was only observed for mussels exposed to the highest concentration of OTC in both acute and chronic exposure (5 μgL^{-1}). In the digestive gland of the acutely exposed animals, the CAT activity significantly increased for the two highest concentrations (2.50, and 5 μgL^{-1}). On the other hand, data for the chronic exposure showed that, in the digestive gland, an increase of CAT activity was also noticeable, following a dose-dependent pattern for the three lowest concentrations (0.31; 0.62 and 1.25 μgL^{-1}). For the last tissue tested, the muscle, CAT activity increased in animals acutely and chronically exposed to this substance. Following the acute exposure, only the two highest concentrations were able to cause an increase in CAT activity. In the case of the

chronic exposure, this increase was noticed for all concentrations, with statistically significant differences being reported for the concentrations of 0.62, 2.50, and 5 μgL^{-1}. Nunes et al. (2015) also obtained an increase on CAT activity after acute exposure of *G. holbrooki* to tetracycline, which is an antibiotic from the same therapeutic class, and shares structural similarities with OTC. A study of the effect of tetracycline and chlortetracycline in earthworms, *E. fetida,* also showed that these compounds are involved in the establishment of oxidative stress conditions, evidenced by the increase of CAT activity (Dong et al., 2012). Elia et al. (2014) studied the pharmacological treatment of carp, *C. carpio* with oxytetracycline (OTC), by contaminating feed at therapeutic levels (75 mg/kg), and at higher doses. (150, 300 mg/kg) for 10 days. After 10 days of exposure, it was possible to observe an increase in catalase activity for all tested doses of OTC.

Although OTC can cause alterations in the antioxidant defence, which are probably due to an increase in H_2O_2, it can also cause opposite effects, but through different mechanisms. Chi et al. (2010) showed that CAT activity was depressed by increasing concentrations of this drug, due to molecular interactions or directly binding of OTC to CAT, thereby altering the enzymatic activity. This effect may be, at least partially, responsible for the results obtained in this work, following the chronic exposure, for which the two highest concentrations of OTC were capable of slightly decreasing the activity of CAT in digestive gland, however with no significant differences when compared to control. The exposure to oxytetracycline enhanced the antioxidant response in the catalase activity of the organisms suggesting the occurrence of oxidative effects, since it was possible to observe a general increase of this enzymatic activity for exposed animals.

GSTs is a group of isoenzymes that detoxifies endogenous (intracellular metabolites) and exogenous substances (drugs, pesticides, and other pollutants), by conjugating them with GSH, which converts a reactive lipophilic molecule into a non-reactive, water-soluble conjugate that can be excreted (Kim et al., 2010; Hayes et al., 1995). Thus, GSTs play an important role in protecting tissues from toxicity of xenobiotics, including oxidative stress. An increase in GSTs activity indicates a protective measure against the toxicity of lipid peroxidation induced by xenobiotics (Leaver and George, 1989). The results for GSTs activity in both tissues showed different results for the acute and chronic exposures, following OTC exposure. GSTs activity after acute exposure to OTC showed an inhibition of this enzymatic activity in the gill tissue, following both acute and chronic exposures. The trend was the same after acute exposure for the digestive gland, being possible to observe an inhibition of the enzymatic activity. However, in the chronic

exposure, no significant alterations were observed for all the concentrations tested. Ren et al. (2017) also observed a decrease in the GSTs activity of the larvae of the crab *P. tribuberculatus* after being exposed to OTC (0.3; 3 and 30 μgL^{-1}). Data obtained by Yonar (2012) for GSTs showed a decrease of its activity in the kidneys of rainbow trout after expose to OTC. The involvement of OTC in GSTs activity has already been established in some studies. Some works from the literature indicate a decrease in GSTs activity, and a downregulation of GSTs mRNA expression, after exposure to OTC (Ren et al., 2017; Wang et al., 2016; Yonar, 2012). This trend was already described for aquatic organisms, and might indicate high levels of stress which may lead to the collapse of the GSTs enzymatic pathway due to enzyme denaturation or cofactor exhaustion (Oliveira et al., 2013; Ren et al., 2017). However, the inhibition of GSTs activity can also be associated to the reduction of levels of intracellular GSH, which are susceptible of being conjugated, indicating a toxic consequence of pollutants (i.e., OTC), resulting in a decreased capacity to detoxify with the involvement of GSTs.

The determination of the TBARS levels is one of the most used methods to monitor the occurrence of lipid peroxidation (Hsiao et al., 2011). The results obtained after acute exposure to OTC in all tissues tested showed no alterations of the TBARS levels, suggesting an absence of lipid peroxidation consequent to OTC exposure. However, after chronic exposure, the TBARS levels in gills showed a dose-dependent increase in animals exposed to all the concentrations of OTC. This increase of TBARS levels in gills after chronic exposure to OTC is in agreement with the insufficiency of antioxidant and detoxification mechanism to cope with the pro-oxidative effects of OTC. For the digestive gland, the alterations observed only occurred following chronic exposures, for the mussels exposed to the levels of 1.25 μgL^{-1} and 2.50 μgL^{-1}, which had levels of lipid peroxidation below control values. For muscle tissue, no differences were observed following the acute exposure. In the chronic exposure, it was possible to observe an increase of TBARS levels in the muscle of the mussels for the highest concentration (5.00 μgL^{-1}). In our study, both antioxidant and detoxification mechanisms were insufficient to cope with the pro-oxidative effect by OTC, and damage did occur, reflected by an increase in TBARS levels in gills, after chronic exposure up to OTC. It has already been shown that high doses of OTC can cause the peroxidation of membrane lipids, a detrimental process attributed to free radicals (Pari and Gnanasoundari 2006). Rodrigues et al. (2017a) obtained somewhat similar results for the TBARS levels after exposing the fish *O. mykiss* to 0.3125 μgL^{-1} of OTC. Ren et al. (2017) also demonstrated an increase on the TBARS levels after exposing larvae of the crab *P. tribuberculatusa* to OTC. These data suggest that OTC may cause deleterious changes, of

oxidative nature, in tissues of exposed fish. OTC metabolites, such as peroxynitrite and hydroxyl radicals, have the ability to react with membrane components, such as lipids (Barzilai and Yamamoto 2004), resulting in LPO, the initial step of cellular membrane damage, culminating in membrane destabilization and disintegration (Ren et al. 2017; Yonar, 2012; Gnanasoundari and Pari, 2006;), alterations in membrane fluidity and permeability, and affecting cellular structure and function (Chi et al. 2014). It is possible to have a global perspective of the damage caused by OTC on cell membranes. Chi et al. (2014) demonstrated that OTC can inhibit ATPase activity, affecting structurally and functionally the cell membrane of human red blood cells. ATPase function is to maintain the ionic and osmotic balances inside and outside the cell, maintaining transmembrane electrochemical gradients, and in cellular energy metabolism. If the integrity of the membranes is compromised, the entire cellular processes can be affected. Lipid peroxidation refers to the oxidative degradation of lipids, by which free radicals capture electrons from lipids in cell membranes, altering the structural integrity of cell membranes. Lipid peroxidation is an auto-oxidation process and consists of initiation, propagation, and termination (Gahalain et al., 2011). Furthermore, tetracyclines possess potential metal binding sites, forming stable complexes with di and trivalent cations, being susceptible to chelation (Chopra and Roberts, 2001; Wang et al. 2016).

Rodrigues et al. (2017b) reported that the stable complexes formed between tetracyclines (as OTC) and metals can compromise the normal functioning of many other cellular processes, such as ATPases, which require metal cofactors for their activities, in gills, thereby threatening the integrity of the cell membrane. The here observed increment of lipid peroxidation may be due to radicals and reactive intermediates, since they are able to attack the lipids of the cell membrane and lead to the destabilisation and disintegration of the membrane, leading to the alteration of critical membrane properties, such as increase of the area per lipid, a decrease of the membrane thickness, and a disorder of lipid tails (Boonnoy et al., 2015). These compounds interact with each other and affect membrane proteins causing function impairment, increased nonspecific permeability to ions, fluidity changes and inactivation of membrane-bound receptors and enzymes.

In conclusion, it is possible to state that exposure to OTC was able to induce changes in antioxidant defences of exposed organisms. The responses observed for the different tissues had the same trend, with the exception of the gills for TBARS levels following chronic exposure. The most affected tissue in exposed organisms were the gills, where for chronic exposure it is possible to evidence the occurrence of oxidative damage. On the contrary, for the other tissues

there was no oxidative damage, despite the observed changes of antioxidant mechanisms. However, this type of alterations in antioxidant defences and the occurrence of oxidative damage in some tissues of the exposed animals may have deleterious implications for the organisms.

a. Genotoxicity

OTC metabolism generates ROS that can cause single and double strand breaks in DNA (Khan et al., 2003). It is possible to infer that genotoxicity is an indirect result of oxidative stress (oxidative DNA damage). Regardless of its origin, disruption of ROS cell balance leading to constant oxidative stress is a common factor in genomic instability syndromes - disorders that reflect a defect in the DNA damage response, which in turn leads to modification and potentially consequences to the cell (Cooke et al. 2003; Barzilai and Yamamoto, 2004). In addition to damaging DNA, ROS also disrupts the function of DNA repair proteins (Guilherme et al. 2010). Scientific studies carried out established that OTC has a genotoxic potential (Liu et al. 2012; Botelho et al. 2015), leading to chromosomal breaks/alterations. The results of the comet assay for both acute and chronic exposures showed that OTC caused DNA damages. The here obtained results, for both durations of exposure, allowed observing an increase of GDI values for all the concentrations tested. A study conducted with *O. mykiss* exposed to OTC, also evidence that acute and chronic exposures to this antibiotic could cause damage to the DNA. The author, Rodrigues et al. (2017), concluded that exposure to OTC resulted in the establishment of genotoxic alterations with the induction of DNA strand breaks in blood cells (increase of GDI values). Despite these results, it is not possible to state that the observed genotoxic effects are a direct consequence of oxidative stress, since this condition was not clearly established after exposure to OTC, as discussed in the previous section. The observed genotoxicity can be explained by the binding of OTC to DNA. In fact, from a biochemical standpoint, OTC and tetracyclines in general have a strong affinity for DNA, which results in the formation of TC-DNA binary complexes, which in turn may cause changes in the secondary structure of the native DNA duplex (Khan et al. 2003; Khan and Musarrat, 2003). Khan and Musarrat (2003) suggest that the binding of tetracyclines to DNA causes disturbances in the secondary structure of DNA, which can be harmful if damaged DNA is not effectively repaired. Thus, these conformational changes in DNA structure may possibly be reflected in the observed genotoxic damage.

2. Ciprofloxacin exposure

CIP is an antibiotic from the group of fluoroquinolones, which is classified by the World Health Organization (WHO) as a critically important class of antibiotics for human use (Eggen et al. 2011; Who 2007). CIP belongs to the third-generation of fluoroquinolones, which is often detected in the different environmental media due to its long-term persistence (Pan et al. 2018; Picó and Andreu 2007). In addition, CIP inhibits enzymatic activities that play a key role in the DNA replication process (Pan et al. 2018). Ciprofloxacin is partially excreted unchanged, but may also be partially metabolized by CYP1A2, and is an inhibitor of CYP1A2 (Panja et al. 2019; Best, 2013). In addition, an increase of reactive oxygen species in bacterial cells in response to ciprofloxacin has already been shown (Goswami et al., 2006). Considering the toxicological profile of CIP, detection of fluoroquinolone antibiotics such as CIP in the aquatic environments is quite important due to their potential adverse health effects. The previously calculated EC_{50} for the algae *P. subcapitata* is 2.97 mgL^{-1} (Lützhøft et al., 1999), and for the cyanobacteria *Microcystis aeruginosa* it is of 0.017 mgL^{-1} (Robinson et al., 2005). The EC_{50} for duckweed (*L. minor*) was above the CIP environmental concentrations (3.75 mgL^{-1}), as shown by the study of Martins et al. (2012). This means that environmental concentrations of CIP can challenge aquatic organisms. In a chronic assay, low concentrations of CIP induced impairments to life-history parameters of *D. magna* and so CIP was considered a real risk for aquatic organisms (Martins et al., 2012).

The exposure of mussels to the antibiotic CIP resulted in alterations in some of the enzymatic biomarkers. For SOD activity, the results of the acute and chronic exposures showed a significant decrease for the two tissues tested, except for Mn SOD in acutely exposed animals, that showed a slight increase for gills, and no alterations in the digestive gland tissue. A study conducted by Pan et al. (2016) to unravel the molecular mechanism of fluoroquinolones-induced oxidative stress, with the involvement of SOD, reported that Fluoroquinolones (FQs) can alter the activities of antioxidant enzymes, by decreasing their activity. This decrease of antioxidant activity following exposure to FQs was observed by the authors, and was also reported in the present work. This effect may indicate that CIP could affect the Cu/Zn-SOD activity, intensifying the potential risk of oxidative stress. The here described decreased of SOD activity may be due to the excessive production of superoxide radicals, which, after their transformation into H_2O_2, cause an oxidation of the cysteine in the enzyme and thereby deactivate SOD activity (Li et al., 2007). The substantial decline in SOD activity at the highest concentration of CIP may be attributed to the high accumulation of the superoxide radical, derived from an imbalance

between the rates of detoxification and production of $O_2^{\cdot-}$ and exceeding the capacity of SOD to respond to the superoxide radical levels. Aderemi et al. (2018) obtained similar results for SOD activity measured in the microalgae *Raphidocelis subcapitata* after exposure to 19.1 µM of CIP. This type of response to CIP exposure, with an impairment of SOD activity, is well described in humans. Talla and Veerareddy (2011) conducted a study to evaluate oxidative stress induced by fluoroquinolones used for the treatment for complicated urinary tract infections. The authors suggested that the efforts of SOD to remove the continuously generated free radicals initially increased due to an induction of SOD activity, but later in the toxic process, a significant decrease of SOD activity occurred, resulting in oxidative cell damage. Hence, when the generation of reactive free radicals overwhelms the antioxidant defence, lipid peroxidation of the cell membrane occurs. This causes disturbances in cell integrity leading to cell damage/death. Talla and Veerareddy (2011) studied the repeated administration of CIP in humans and observed an increase of free radical adduct generation by CYP450 mediated metabolism, which accumulated in the cell, resulting in increased ROS and substantial reduction in antioxidant defence.

The results obtained for CAT activity suggest that CIP metabolism may result in oxidative alterations. In the acutely exposed organisms, their gill and digestive gland tissues showed an increase of CAT activity when exposed to this compound, but for the muscle tissue no changes were observed. This result is not at all surprising, since this tissue does not contain as much cytochrome p450 (which is responsible for the bioactivation and metabolism of most xenobiotic compounds, including of CIP, as discussed previously), as other tissues (King, 2017; Shankar & Mehendale, 2014). This antibiotic caused effects in the activity of CAT for all the tissues after the chronic exposure. For gills and digestive gland, the activity increased in a dose-dependent manner, and animals exposed to all concentrations showed an increase of activity. For the muscle tissue the effect was the same for the lowest three concentrations (0.005; 0.013 and 0.031 mgL^{-1}), with a significant dose-dependent increase in CAT activity. For the two highest concentrations (0.078 mgL^{-1} and 0.195 mgL^{-1}) the activity decreased below the levels of control. These results are consistent with those also reported in some studies from the literature. CIP is known to cause increases on the activity of CAT. In a study conducted by Nie et al. (2013), the algae *P. subcapitata*, after being exposed to CIP, showed an increase of this enzyme´s activity. Ng et al. (2008) found that the freshwater algae species *C. vulgaris* after exposure to CIP showed a dual profile concerning the CAT activity. An induction of CAT activity was observed at low levels of exposure, and a decrease of this antioxidant enzyme activity at higher concentrations of this drug, suggesting oxidative effects. Nogueira and Nunes (2020) conducted a study to access the

effects of low levels of ciprofloxacin in the polychaete *Hediste diversicolor* and after 96 hours of exposure the authors observed an increase of the catalase activity. Bartoskova et al., (2014) evaluated the effect of a fluoroquinolone (norfloxacin) in *D. rerio* and for all the concentrations tested (0.0001; 0.1; 1; 10 and 30 mgL^{-1}), CAT activity was significantly increased. Qin and Liu (2013) characterized the oxidative stress response of two fluoroquinolones (Ciprofloxacin and Enrofloxacin) in humans, measured in terms of catalase activity in erythrocytes. The authors suggested that the structural and functional changes in the CAT activity were closely associated with increased risk of oxidative stress induced by both fluoroquinolones. Our results suggest a clear indication of an excessive H_2O_2 generation. These results show that adverse changes occurred in the beginning of the intoxication process, with a short-term activation of the antioxidant defence. However, longer periods of exposure suggest that an oxidative condition is transient, which does not require the activation of the antioxidant defence mechanisms for prolonged periods, suggesting that exposed organisms are able to adapt to the presence of the triggering chemical.

GSTs activity was significantly reduced for the gill and digestive gland tissues of acutely exposed organisms. FQs, such as CIP, can inhibit the activities of GSTs in tissues of experimental animals, in vitro and in vivo (Xiao et a., 2020). An identical trend was observed by Dionísio et al. (2020) for crustaceans of the species *D. magna* after being acutely exposed to this same drug; however, longer durations of exposure resulted in a contrary effect, reflected by an increase of this enzymes activity. Results obtained following the chronic exposure showed that CIP was able to cause alterations in the activity of GSTs, in the gills and digestive gland, with an increase of its activity along the concentrations tested. In gills, significant alterations were visible in animals exposed to all concentrations. In the digestive gland, these differences were only reported in animals exposed to the two highest concentrations (0.078 mgL^{-1} and 0.195 mgL^{-1}). These results indicate that low concentrations of CIP may lead to the activation of the phase II conjugation pathway (namely GSTs), and may also indicate the occurrence of oxidative stress since we have an increase of this activity. According to Neuman et al. (2015), FQs conjugate with GSH to promote detoxification. This response is usually expected because of the important role that GSTs plays in catalysing the conjugation of the tripeptide glutathione with the xenobiotics in the phase II of the biotransformation process that will promote its elimination from the organisms (Li et al., 2007; Richardson et al., 2008). The microalga *P. subcapitata* after being exposed to CIP, also evidenced an increase in GSTs activity (Nie et al., 2013). Bartoskova et al. (2014) also found an increase in the activity of GSTs of *D. rerio* when exposed to norfloxacin, for 96h, even at the

lowest concentration (0.0001 mgL^{-1}). Lui et al., (2014) found similar results, with an increase of GSTs activity of male goldfish (*Carassius auratus*) when exposed to norfloxacin (0.016 mgL^{-1}) for different periods of time (1, 2, 4 and 7 days). In addition, a study was made to evaluate the efficacy of GSTs as a biomarker to serve as a diagnostic tool to the exposure of fish to the quinolones norfloxacin and ofloxacin, in northern Taihu lake in China, the hepatic GSTs activity measured in exposed fish increased significantly (Lu et al., 2012).

The results obtained after the acute exposure showed a decrease in TBARS levels for gill and digestive gland tissue, after exposure to CIP, with may be the result of a successful activation of the antioxidant defence (Nunes et al., 2018). For the muscle tissue, the acute exposure of organisms to CIP yielded no changes. The here obtained TBARS data for chronically exposed organisms showed different results depending on the tissue. For gills, the levels of TBARS for the lowest concentrations (0.005 mgL^{-1} and 0.013 mgL^{-1}) were not different from those of the control group, but for the three highest concentrations, the levels of TBARS significantly decreased below control values. For the digestive gland, the observed pattern of response was totally different, since TBARS levels increased along with the concentrations, with statistically differences for the last three concentrations (0.031; 0.078 and 0.195 mgL^{-1}). For the muscle tissue, the levels of TBARS decrease with the increase of the concentration of CIP, except for the highest concentration (0.195 mgL^{-1}), for which the TBARS levels increased above the values of the control. Taking into account the literature and numerous studies carried out with this type of compound in other organisms, it would be expected that CIP could have caused oxidative damage. However, this effect was only observed for the digestive gland, and following the chronic exposure. For the remaining conditions, it is possible to observe a decrease in TBARS levels which, although rare, has also been shown by other studies. It is already known that some fluoroquinolones have the ability to decrease the levels of TBARS. Nunes et al. (2018) also obtained a decreased of TBARS levels when exposed *D. magna* to ciprofloxacin (0.005 mgL^{-1} and 0.013 mgL^{-1}). Zivna et al. (2015) obtained similar results when exposing the common carp, *C. carpio*, to ciprofloxacin. Hsiao et al., (2011) suggests that these results occur as a consequence of the ability of fluoroquinolones to produce reactive nitrogen species, which are likely to react with the superoxide radical to form peroxynitrite. This last metabolite can bind to an atom of molybdenum from xanthine oxidase. This binding inhibits the activity of xanthine oxidase, which generates superoxide. Inhibition of xanthine oxidase and decreased concentrations of superoxide may lead to a reduction in lipid peroxidation (Zivna et al., 2016). Nunes et al. (2018) suggested that one possible explanation for this pattern is the successful activation of the

antioxidant defence system at low dosages, and its failure at higher levels of exposure. In chronically exposed animals, it was possible to observe oxidative damage in the digestive gland tissue, with an increase of its TBARS levels.

The results obtained for this exposure at low, ecological relevant levels of CIP, allows to suggest that CIP was able to induce oxidative damage in the digestive gland of organisms exposed for 28 days to this compound. For the remaining tissues, although the deleterious effects were notorious, there was no oxidative damage. The digestive gland showed to be the most sensitive tissue, however the results for all the tissues tested were quite similar. Exposure to low concentrations of CIP for long periods of time were able to induce effects that may be deleterious to the tested species. This effect can have implications at an individual level by damaging cells, proteins, and even DNA, suggesting that chronic effects must not be underestimated and should be used as an important and mandatory approach in pharmaceutical drugs risk assessment policies.

a. Genotoxicity

Quinolones are drugs widely used in human and veterinary medicines. Quinolones act by inhibiting DNA gyrase (bacterial topoisomerase II) or topoisomerase IV, and are known to have mild inhibitory effects on eukaryotic topoisomerase II (Akasaka et al., 1998), which is responsible for the double-strand DNA breakage/reassembly reaction (Liu and Wang, 1999). Inhibitors of topoisomerase II have been shown to interfere with the topoisomerase II breakage/assembly reaction, resulting in single-stranded breaks (SSBs) and double-stranded breaks (DSBs) in humans (Fukuda et al., 1996). Given these data on DNA breakdown by topoisomerase II inhibitors, some quinolones may have genotoxic and carcinogenic potential (Heisig, 2009). In this work we can confirm the occurrence of genotoxicity after both acute and chronic exposures to CIP, since the results obtained for the comet assay corroborate this finding. In both exposures, it was possible to observe a dose-dependent increase of the genotoxic damage. The data obtained with this study for the comet assay showed that CIP has the potential to induce DNA strand breaks, since the exposures of mussels, acute and chronic, resulted in an increase of GDI values. Data concerning the potential genotoxicity of CIP, are yet insufficient to predict the outcomes of its exposure. The study performed by Isidori et al. (2005) showed that an antibiotic from the same class of CIP, ofloxacin, could cause genotoxic effects on non-target organisms. Gurbay et al. (2006) performed a study exposing rodent astrocytes to 150 and 300 mgL^{-1} of CIP,

and observed a significant increase in DNA damage. It is also known that CIP can cause genotoxicity in humans, a study performed by Gorla et al. (1999) showed the occurrence of genotoxicity in humans lymphocytes exposed to concentrations from 0-50 μgL^{-1} of CIP. Failure of antioxidant defence mechanisms can lead to oxidative stress, which in turn can lead to molecular damage, especially in cellular macromolecules such as DNA (Bartoskova et al., 2013). Therefore, genotoxicity may be a consequence of the establishment of oxidative stress, as the increase in intracellular oxygen levels is related to the accumulation of chromosomal aberrations (Conger & Fairchild, 1962). However, with the results obtained following this exposure, it is not possible to state that the observed genotoxicity is being caused by ROS, since we did not have a clear scenario of oxidative stress after exposure to CIP, despite evidence indicating the activation of antioxidant defence mechanisms. However, CIP was able to induce genotoxic effects, which allows us to infer that despite the absence of an oxidative stress scenario, this antibiotic was able to cause adverse effects at a sub-individual level in the exposed organisms. It is known that CIP inhibits the bacterial gyrase; however, when in high concentrations, it may also inhibit also the functionally related eukaryotic topoisomerase-II, which resulted in genotoxic effects in several in vitro tests (Herbold et al., 2001). The results here obtained that indicate the occurrence of genotoxicity may have occurred due to this mechanism.

3. Exposure to mixtures of the two drugs

The enzymatic activity of SOD after exposure of the organisms to a mixture of the two antibiotics showed similar results to those obtained for the individual exposures. In general terms, it was possible to observe a decrease in this enzyme´s activity for both the gills and for the digestive gland, following the acute and chronic exposures. Despite the generic absence of data, there are already some studies that assess the effects of drug mixtures. However, the trend that resulted from such studies, especially in terms of oxidative stress effects, do not always coincide with the patterns of toxic response that we obtained in our assays. Zhang et al. (2021) evaluated the effects of a mixture of sulfamethoxazole and erythromycin on the micro algae *R. subcapitata*. The enzymatic activity of SOD increased after exposure to the mixture of these two compounds. SOD activity also increased for a mussel species, *M. galloprovincialis*, after exposure to a mixture of ibuprofen, diclofenac and fluoxetine (Gonzalez-Rey et al., 2014). Gonzalez-Rey et al. (2014) exposed the organisms to two different combinations of the mixture of the three drugs and evaluated the enzymatic activity of SOD after 3, 7 and 15 days in two different tissues, gills and digestive gland. For both tissues, it was possible to observe an increase in this enzymatic

activity over the exposure time. A study by Yang et al. (2019) exposed a species of fish, *C. auratus*, to a mixture of ofloxacin, sulfamethoxazole and ibuprofen in order to assess the deleterious effects resulting from this exposure. It was possible to observe an increase in the enzymatic activity of SOD for all tested concentrations. Another study carried out with the fish *D. rerio* also showed an increase in SOD activity after exposure to a mixture of two antibiotics (norfloxacin and sulfamethoxazole) (Yan et al., 2016). So, from these results it is possible to assume that mixtures of pharmaceutical compounds similar to those here-tested generally induce an increase in SOD activity, contrarily to our data. The results for the mixture of the two antibiotics reflect the same trend as isolated exposures; in fact, this trend was a priori expected, since the mixture also caused a decrease in the SOD activity. However, we have to take in consideration that the two antibiotics may interact with each other according to mechanisms that are not well known.

The here observed effects in terms of the CAT enzyme activity varied according to the tissue, and to the duration of exposure. Following the acute exposure, it was possible to evidence a decrease in CAT activity for the gills and digestive gland; for the muscle tissue, it was possible to devise an increase of this enzyme´s activity. With regard to the chronic exposure, we had a decrease in the enzymatic activity for the gills and muscle and an increase for the digestive gland. Beghin et al. (2021) carried out a study to evaluate the effects of a mixture of 5 drugs (paracetamol, carbamazepine, diclofenac, naproxen and irbesartan) on a fish species, *O. mykiss*. The enzyme activity was measured in the liver of these animals, and after 1, 7, 21 and 42 days of exposure. The enzymatic activity of CAT was inhibited for the entire period tested. Another study performed with fish, *D. rerio*, showed an increase in CAT activity after exposure to the mixture of norfloxacin and sulfamethoxazole (Yan et al., 2016). This response observed by Yan et al. (2016) is in agreement with the response obtained after the exposures to each of the single compounds, for which it was also possible to observe an increase in catalase activity. A study by Gonzalez-Rey et al. (2014) evaluated the impact of three drugs, ibuprofen, diclofenac and fluoxetine, on *M. galloprovencialis*. The authors reported an increase of CAT activity in the gills after 15 days of exposure to this mixture. Individuals of the crayfish species, *Procambarus clarkii*, after being exposed to a mixture of ibuprofen, ciprofloxacin and flumequine, showed an increase of CAT, significant only for those animals exposed to the highest concentration of the mixture (100 μgL^{-1}) and after 7 days of exposure (Trombini et al., 2021).

The results obtained in this work for the enzymatic activity of GSTs were quite similar for animals both acutely and chronically exposed to the mixture of drugs. For the gills, it was

possible to observe a decrease in this isoenzyme´s activity for both exposures (acute and chronic). For the digestive gland, it was possible to observe an increase in the enzymatic activity. Zhang et al. (2021) observed a decrease in GSTs activity after exposure of the microalgae *R. subcapitata* to a mixture of sulfamethoxazole and erythromycin, which was observed only for the highest concentration of the tested mixture. In turn, Gonzalez-Rey et al. (2014) obtained an increase in the activity of GSTs, after exposing *M. galloprovincialis* to a mixture of 3 drugs, ibuprofen, diclofenac and fluoxetine and obtained an increase in enzymatic activity for the gills and digestive gland of the mussels. A study by Li et al. (2011) with *C. auratus*, assessed the toxicity of a mixture of sulfamethoxazole and caffeine, and obtained a decrease in the enzymatic activity of GSTs. The authors suggested that the results demonstrated that the tested pharmaceuticals resulted in a significant GSTs induction in goldfish, and they all showed full bell-shaped concentration-response curves. Another study carried out with the fish *C. auratus* by Liu et al. (2014) showed an increase in the enzymatic activity of GSTs after exposure to a mixture of norfloxacin and sulfamethoxazole in the liver of the species. Mixtures of pharmaceutical drugs may involve interactions among the substances themselves, or among degradation products formed during metabolic processes (Cedergreen, 2014). When two drugs are used together, they are likely to interact in toxicological terns, resulting in the increment of their joint toxicity (additive, synergistic, or potentiation effects), or a reduction in the toxicological outcome (antagonism) (Greco et al., 1995). Direct interactions between drugs are also possible and may occur when two drugs are mixed prior to the absorption (Khan et al., 2011). These factors can contribute to the variations that may exist in terms of enzymatic activities. Depending on the organisms and the interactions of the compounds present in the mixtures, we can obtain different responses in the enzymatic patterns, however more studies are needed to understand the possible interactions between the two compounds.

The results obtained for TBARS levels did not show changes for any of the tissues, following both acute and chronic exposures. We can infer that, despite the enzymatic alterations, there was no cellular damage in the exposed organisms. However, in animals exposed to both compounds alone, there was peroxidative damage. In animals exposed only to OTC there was damage to the gills, while in those animals exposed only to CIP there was damage to the digestive gland. Considering this pattern, it was expected that there would be damage to organisms exposed to the mixture of these two compounds. Studies in the literature demonstrate that oxidative stress after exposure to mixtures of drug is not always observed. Gonzalez-Rey et al. (2014) carried out a study evaluating the toxicity of ibuprofen, diclofenac

and fluoxetine in *M. galloprovincialis* and observed oxidative stress in these organisms in both tissues tested, gills and digestive gland, for the entire period of exposure. Nonetheless, a study carried out by Trombini et al. (2021) evaluated the toxicity of the mixture of ibuprofen, ciprofloxacin and flumequine in *P. clarkia*, and concluded for the absence of oxidative stress in organisms exposed to this mixture of drugs. The evaluation of the effects that mixtures have on organisms is extremely important, since they simulate in a more realistic way, the environments in which the organisms are found. In nature, organisms are exposed to a cocktail of compounds.

a. Genotoxicity

For the mixture of the two antibiotics, the results were in line with those obtained for the isolated exposures. For all the concentrations tested, in both acute and chronic durations of exposure, the GDI values increased in a dose-dependent way. Some studies have already highlighted the deleterious effects that antibiotics can cause in terms of genotoxicity. A study performed by Rocco et al. (2011) determined that two antibiotics, erythromycin and lincomycin, could cause genomic damage in zebrafish (*D. rerio*). Another study performed in embryos of the same species showed the genotoxic effects of the antibiotic amoxicillin (Chowdhury et al., 2020). Rodrigues et al. (2016) performed a study evaluating the genotoxicity of the antibiotic erythromycin in in peripheral blood cells of the species *O. mykiss*, and the author concluded that erythromycin has the potential to induce DNA strand breaks in blood cells. Liu at al. (2012) performed a study to evaluate the genotoxicity of six pharmaceuticals (erythromycin, sulfamethazine, sulfathiazole, chlortetracycline, oxytetracycline, and diclofenac), and concluded that these pharmaceuticals induced the DNA damages that stall DNA replication, leading to chromosomal breaks. Taking into account the results obtained for the exposures to single antibiotics, it was expected that the mixture would cause genotoxicity to the organisms. However, this genotoxicity cannot be associated with an oxidative stress scenario since we did not obtain oxidative damage in any of the analysed tissues. In comparison with the results obtained for exposures to single compounds, it cannot be inferred that there are significant changes, since exposures to both drugs resulted in similar percentages of genotoxic damage. From an ecological point of view, the protection of genetic diversity in natural populations is important for the survival of the population (Wurgler and Kramers, 1992; Anderson and Wild, 1994). Genotoxic compounds have the ability to cause mutations in organisms that can have heredity consequences. If these DNA damages are not repaired, they can cause biological consequences at the cellular and morphological level for the animal and, ultimately, at the

community and population level (Lee and Steinert, 2003). The detection of carcinogenic effects on aquatic organisms is necessary to assess the health of aquatic organisms, as well as to prevent carcinogens from entering the food chain for humans (De Flora et al., 1991). As many chemicals with genotoxic potential are emitted to surface water, genotoxicity tests are gaining importance.

4. Shell hardness

The results of this parameter did not show significant changes, neither for the single exposures to the two antibiotics, OTC and CIP, nor to their mixture. However, following the chronic exposure to OTC, it was possible to observe a dose-dependent decrease in the required force to crush the animals´ shells, although without significant changes when compared to the control. This decrease may be due to the fact that OTC interacts with calcium, a relationship that has been documented by some studies in the literature (Agwuh, 2006; Simmons et al., 1983; Likins and Pakis, 1964 Finerman and Milch, 1963). Tetracyclines have a high affinity to form chelates with polyvalent metallic cations such as Fe^{3+}, Fe^{2+}, Al^{3+}, Mg^{2+} and Ca^{2+}. Chelation is a type of bonding of ions and molecules to metal ions (Katlam et al., 2017). This ability to chelate to metals, such as calcium, results in tooth discoloration when tetracycline is administered to children (Hlavka et al., 2000). The mechanism by which TCs interfere with the mineralization process is poorly understood. Several in vitro studies (Kaitila 1971; West & Storey 1972) have indicated that tetracyclines exert their major effect on bone by inhibiting calcification. Bevelander et al. (1960) concluded that the inhibition of mineralization after tetracycline administration is likely due to the reduction in the number of free cations that subsequently results in the formation of mineral-deficient bone. It has also been suggested that TCs induce calcification defects by inhibiting calcium accumulation in the mitochondria of cells in preparation for mineralization (Shapiro et al. 1977). This intramitochondrial accumulation of calcium is postulated as a first essential step in the calcification process (Duvvuri and Lood, 2021; 2010; Lehninger, 1970). Mussel shells are composed by several layers of calcium carbonate precipitated in an organic matrix. Since this antibiotic has the ability to bind calcium ions there is a possibility that this interaction decreases the availability of calcium to the shell matrix, similarly to what happens for the bone tissue of vertebrates, as previously described. However, more studies would be needed in order to understand the impacts of this interaction on organisms, since it is the first time that this biomarker has been used to assess the impact resulting from the exposure to this type of compounds on the resistance and integrity of the shells of organisms.

V. Conclusion

Pharmaceutical compounds can reach the aquatic environment through different routes. These substances can exert subtle acute and chronic effects and can exert adverse effects at the biochemical level, which may affect populations and communities. Despite not being able to trigger visible acute lethal effects most of the times, pharmaceutical drugs may cause almost imperceptible effects, sometimes of unknown mechanisms of toxicity. The work developed within the scope of this thesis tried to address the different levels susceptible of being altered by oxytetracycline and ciprofloxacin, and by the mixture of both, through the evaluation of biochemical parameters at the level of oxidative stress, genotoxicity, and shell integrity in mussels. The here obtained results allowed us to state that these organisms can be used for biomonitoring of the marine environment, and to assess the toxicity of several compounds, including pharmaceutical drugs. The adopted strategy based on different biomarker assessments, of enzymatic activity as well as genotoxicity and shall integrity analysis, proved to be very useful tools to assess ecotoxicity in laboratory conditions. After the exposure to oxytetracycline, the most affected tissue was the gills, this being the only tissue for which the occurred oxidative damage was evidenced by the increase in lipid peroxidation. The genotoxicity data allow us to conclude that this compound is capable of causing genotoxic effects in organisms exposed to this antibiotic. Regarding the shell hardness of the exposed organisms, although we did not obtain significant differences, it was possible to observe a tendency for the reduction of shell hardness after exposure to this compound. This set of data allowed us to conclude that the tested organisms showed some sensitivity, however more studies are needed to describe the interaction of oxytetracycline and calcium under realistic conditions of exposure. Following the exposure to Ciprofloxacin, the most affected tissue was the digestive gland, since this was the only tissue where an increase in the level of TBARS was evidenced, allowing us to infer about the occurrence of oxidative damage. The genotoxicity data allowed us to conclude that this compound has genotoxic effects, since it caused DNA damage. The shell hardness was not affected since no trend or change could be observed. Exposure to the mixture of the two antibiotics did not induce oxidative damage to the exposed organisms, however with regard to genotoxicity it is possible to conclude that the mixture of compounds was able to result in very significant effects. The shell hardness parameter showed no changes resulting from exposure to the mixture of the two antibiotics. Of all the exposures made, the organisms showed greater sensitivity to oxytetracycline with regard to enzymatic parameters, the most affected tissue being the digestive gland, where it was possible to report an increase in lipid peroxidation. With

regard to genotoxicity, oxytetracycline induced greater damage since it caused a higher percentage of damages of class 3 and 4, compared to the exposure to ciprofloxacin and the mixture of the two antibiotics. It was also the only exposure for which it was possible to observe a slight effect, suggesting a reduction of the shell hardness of the exposed organisms. This set of results was obtained for concentrations of high ecological relevance, that are similar to those already reported to occur in the wild. This allows us to suggest that the presence of these compounds in the environment can result in deleterious effects on non-target aquatic organisms which, in turn, can affect their populations. In conclusion, the work carried out in this thesis, as well as the set of scientific data obtained here, contribute to a better understanding of the effects that drugs, namely antibiotics, which are widely used and often found in aquatic matrices, may have on non-target organisms such as mussels.

VI. Bibliography

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