Universidade de Aveiro Departamento de Biologia
Ano 2020

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Assessment of single and combined effects of two pharmaceuticals in non-target species – evaluation of possible interactions

Avaliação dos efeitos individuais e em mistura de dois fármacos em espécies não alvo – avaliação de possíveis interações

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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, Equiparado a Investigador Auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

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“The important thing is not to stop questioning. Curiosity has its own reason for existence.”

-Albert Einstein

o júri

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palavras-chave

Acetazolamida; ácido salicílico, biomarcadores; *Lemna gibba*; *Mytilus* spp.; *Phorcus lineatus*.

resumo

O aumento da qualidade e da esperança média de vida têm sido em parte relacionados com o uso de fármacos. Estes são usados nas mais variadas áreas de atividade humana, e representam um grande avanço no tratamento de doenças e no bem-estar das populações. Contudo, este aumento do uso de fármacos tem levado a um aumento da frequência de deteção dos mesmos em ecossistemas aquáticos. Isto deve-se, entre outras razões, à incapacidade das estações de tratamento de águas residuais de removerem na totalidade estes compostos. De entre as classes mais usadas e encontradas no ambiente, podem identificar-se os anti-inflamatórios não esteroides (AINEs), que correspondem a medicamentos largamente usados a nível global como analgésicos, anti-inflamatórios e antipiréticos. Um exemplo desta classe é o ácido salicílico. À semelhança dos restantes compostos da classe onde se insere, o ácido salicílico é um inibidor da enzima cicloxigenase (COX), que é responsável pela síntese de mediadores de inflamação e pirogénicos, nomeadamente prostaglandinas e tromboxanos. Do ponto de vista ambiental, existe uma outra classe de fármacos que é igualmente importante, mas para a qual não existe um manancial de dados muito extenso. Esta classe, a dos diuréticos, caracteriza-se não pela sua abundância, mas pela sua capacidade de afetar pontos críticos em vias metabólicas de organismos não alvo, ou pela possibilidade de modelar a toxicidade de outros fármacos. De entre os diuréticos mais importantes, destacam-se particularmente os do subgrupo dos inibidores da anidrase carbónica, enzima que é responsável pelo equilibro ácido-base nos organismos bem como outros processos chave, entre eles a fixação de carbono pelas plantas, e a disponibilidade de cálcio em artrópodes. O objetivo deste trabalho foi avaliar os efeitos de concentrações ambientalmente relevantes de acetazolamida (ACZ) e do ácido salicílico (SA) (individualmente e em mistura binárias) em organismos não-alvo (nomeadamente *Lemna gibba*, *Mytilus* spp. e *Phorcus lineatus*) usando marcadores bioquímicos (enzimas como a CA, COX), dados biométricos e pigmentos fotossintéticos. Dados de pigmentos fotossintéticos, nomeadamente clorofilas, determinados em *L. gibba* mostraram a capacidade da ACZ em inibir a sua síntese. No entanto em mistura com o SA, estes efeitos foram revertidos, mostrando o potencial de fito-proteção deste fármaco. Em indivíduos do género *Mytilus*, exposição a ACZ demonstrou a capacidade deste fármaco em inibir a CA principalmente nas brânquias. Por outro lado, a exposição à mistura de ambos os fármacos levaram a um aumento da inibição da COX no manto, sugerindo uma modulação pela ACZ na permanência e excreção do SA em organismos deste género. Finalmente, indivíduos da espécie *Phorcus lineatus*

não registaram alterações nos padrões de biomarcadores avaliados. Em conclusão, os fármacos (nomeadamente SA e ACZ) podem ter efeitos muito diferentes em organismos distintos, sendo que a mistura dos fármacos aqui testados parece ser benéfica na planta (invertendo a redução dos conteúdos de pigmentos fotossintéticos causados pela exposição a ACZ) e prejudicial no mexilhão (a co-exposição aos dois compostos acentuou a inibição da COX). Assim podemos concluir que os efeitos causados por fármacos podem variar de organismo para organismo bem como na extensão dos danos causados pela interação/modulação de vários contaminantes. O que pode levar a alterações nos indivíduos o que pode provocar alterações na função ecológica das espécies.

keywords

Acetazolamide; salicylic acid, biomarkers; *Lemna gibba*; *Mytilus* spp.; *Phorcus lineatus*

abstract

Increase in life quality is partially linked to the use of pharmaceuticals. These are used in several areas and represent a great advancement in disease treatment and population welfare. Nevertheless, the increase in pharmaceutical usage lead to an increase in the detection of these compounds in the water courses. This happens because wastewater treatment plants are unable to fully remove these contaminants. From the most detected classes in the environment are non-steroid anti-inflammatory drugs (NSAIDs). NSAIDs are used as analgesics, anti-inflammatory and antipyretic. An example of this class is salicylic acid (SA) which similar to this class acts by inhibiting the activity of cyclooxygenase (COX). Which, in its turn is responsible for the synthesis of inflammatory mediators. From an environmental point of view, another class of interest, is that of the diuretics, particularly the subgroup of carbonic anhydrase inhibitors. Pharmacologically, this class acts by inhibiting the activity of enzyme carbonic anhydrase (CA). This enzyme is responsible for acid-base balance in organisms as well as key processes such as carbon obtention in plants, calcium mobilization in arthropods among others. The goal of this work was to evaluate the effects of environmental realistic concentrations of ACZ and SA (individually and in mixture) in non-target species (namely *Lemna gibba*, *Mytilus* spp.,and *Phorcus lineatus*) using biomarkers (enzymes such as CA, COX), photosynthetic pigments and biometrics. Photosynthetic pigments from *L. gibba* showed ACZ capacity to inhibit their synthesis. Nevertheless, in mixture with SA, these effects were reversed, showing the phyto-protection capacity of SA. In organisms from the genus *Mytilus* spp. exposure to ACZ display the capacity of this pharmaceutical to inhibit CA, especially in gills. When in mixture, the decrease in COX activity suggest ACZ modulates SA excretion. Finally, in organisms from *P. lineatus* the here tested pharmaceuticals did not caused any straightforward pattern in evaluated enzymes. In conclusion, pharmaceuticals can have different effects in different organisms. Being pharmaceutical mixture here tested capable of being beneficial to plants (by reversed photosynthetic pigment reduction) or be harmful in mussels (increased inhibition of COX activity).

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List of Acronyms

µg – microgram
ACZ – Acetazolamide
ASA – Acetyl salicylic acid
CA – Carbonic anhydrase
Ca^{2+} - Calcium
CAI – Carbonic anhydrase inhibitor
Car – Carotenoids
CESAM – Centro de Estudos do Ambiente e do Mar
Chl – Chlorophyll
Chl a – Chlorophyll a
Chl b – Chlorophyll b
CO_2 – Carbon dioxide
COX – Cyclooxygenase
EDTA - Ethylenediamine tetraacetic acid
FR – Filtration rate
FW – fresh weight
H_2O – Water
HCO_3^- - Bicarbonate ion
LHC – Light Harvesting Complex
M – Molar
mg – milligram
mL – milliliter
mM – millimolar
ng – nanogram
nm – namometer
NSAIDs – Non steroid anti-inflammatory drugs
PG – Prostaglandins
pH - power of hydrogen
pNPAc - p-nitrophenyl acetate
PS I – Photosystem I

PS II – Photosystem II

PSs – Photosystems

SA – Salicylic acid

SH – Shell hardness

TChl – Total chlorophyll

TMPD - N,N,N’,N’-tetrametil-p-phenylenediamine

WHO – World Health Organization

WWTP – Wastewater treatment plant

Chapter 1

General introduction

1. Pharmaceuticals in the environment

Pharmaceuticals are substances which are used in several activities, namely human and veterinary medicines (Kaczala & Blum, 2016), in agriculture, aquaculture (He et al., 2016; Singh & Singh, 2018) and cattle breeding (Lalouckova & Skřivanová, 2019), to fight or treat diseases, or to increase well-being in individuals. Thus, the contribution of pharmaceuticals to modern lifestyle cannot be neglected. Due to these roles, the usage and consumption of pharmaceutical drugs has been increasing worldwide (IQVIA, 2019). The increasing usage of pharmaceutical drugs resulted consequently in their release into the environment. In fact, pharmaceuticals are one of the classes of environmental contaminants which are being systematically detected in the aquatic compartment (Sui et al., 2015; Chander et al., 2016; Fan et al., 2020). When it comes to agricultural and aquaculture usages, pharmaceuticals reach the environment by leaching, or as a results of direct application and dispersion (in the case of aquaculture; Singh & Singh, 2018). However, in human and animal medicine practices, these substances are administered, and excreted by urine and/or faeces, mostly in their unaltered form (from 50 to 80%; Ueda et al., 2009), or as metabolites (Fent et al., 2006). This excreted substances are released in sewages which afterwards are redirected to systems of wastewater treatment. There, in order to remove these contaminants from wastewater before re-entering the environment, a series of specific systems of wastewater treatment were implemented, by adopting industrial removal methodologies, namely wastewater treatment plants (WWTP). WWTPs are urban infrastructures that reproduce intensively the biodegradation processes that naturally occur in rivers (Martin & Vanrolleghem, 2014). Therefore, they remove all sort of contaminants, which pharmaceutical drugs are included (Patel et al., 2019). To attain this purpose, several processes, of biological, chemical, and physical nature, are used (Li & Yang, 2018), and their joint effect results in varying rates of removal of pharmaceutical drugs from wastewater, depending on chemical nature of the pharmaceutical, as well as the type of processes which are applied. This role by WWTPs represents a significant reduction in the concentration of pharmaceutical drugs which varies from 30 to 95%, and in some cases, it reaches 100% (Gracia-

Lor et al., 2012; Kermia et al., 2016). Nevertheless, some pharmaceuticals can resist even to the most advanced processes of wastewater treatment, and persist in the environment (Fan et al., 2020). In fact, Angeles et al., (2020) reviewed the different efficiency of these new methods of pharmaceutical removal, concluding that some processes are better for specific pharmaceuticals classes, but none of the methods are completely efficient at completely removing the pharmaceuticals. This lack of efficiency in the removal of drugs from wastewater lead to their ultimate release into receiving waters. In general terms, all pharmaceutical classes have been reported in the aquatic compartment, being some of the most representative, such as the groups of the antibiotics, lipid regulators, β-blockers, steroids and related hormones, antidepressant, tranquillizers and non-steroid anti-inflammatory drugs (NSAIDs) (Nikolaou, 2007; Chander et al., 2016; Fan et al., 2020) and have the potential to cause toxic effects (Fent et al., 2006). Nevertheless, other classes which are not as frequently detected also deserve attention, since they could also cause deleterious effects on non-target organisms, or can modulate or interact with other chemicals also present in these complex aquatic matrices, increasing or decreasing their toxicity for example the class of diuretics.

Pharmaceuticals present characteristics that contribute to some environmental risks, namely moderate lipophilicity (which can reflect in a capacity to bioaccumulate), resistance to degradation and, potential biological effects, even in non-target organisms (Fent et al., 2006). Despite the relative low concentrations in which pharmaceuticals reach the environment (usually they are detected in the range of ng/L to µg/L; Sui et al., 2015; Chander et al., 2016 ; Fan et al., 2020), several studies have shown that, even at these concentrations, pharmaceutical drugs can exert deleterious effects on a vast array of model organisms, including microcrustaceans (*Daphnia* magna, Daniel et al., 2019; Dionísio et al., 2020a); fish (*Danio rerio* zebrafish; Nowakowska et al., 2020) and non-model organisms such as marine polychaetes (*Hediste diversicolor*; Gomes et al., 2019), and marine mussels (*Mytilus spp.*; Piedade et al., 2019) among others. These alterations can occur in several levels of biological organization such as enzyme activity alterations (Daniel et al., 2019; Nowakowska et al., 2020), histological alterations

(Gomes et al., 2019), reproductive and behavioural alterations (Sudin et al., 2019; Dionísio et al., 2020a). These alterations can be caused by sub-lethal concentrations of pharmaceutical drugs, significantly lower that the concentrations that may cause death of exposed organisms. Consequently, these low concentrations in which drugs may be found are environmentally relevant, considering that may result in deleterious effects in non-target exposed biota. Organisms can even bioaccumulate those contaminants, increasing their concentrations in their tissues, favouring deleterious effects, or enhancing the transfer of these chemicals to other levels of the food web (Streit, 1998; Schäfer et al., 2015).

In addition, in the environment, chemicals are present in complex matrices (Lopes et al., 2016). Therefore, in terms of environmental effects posed by drugs, this complexity is important to consider, since interactions among these chemicals are possible to occur, which can lead to uncharacterized effects or increase/reduction of already known effects through antagonism/synergism/additive/potentiation, or even effects that were not observed when evaluating the effects of isolated compound (EC COM, 2012). A clear example of such modulatory effects were shown to occur by Varano et al., (2017), who described the antagonistic effects of fluoxetine and propranolol as determined in *Daphnia magna*, or the increase in the toxicity of several anti-inflammatory drugs in *D. magna* (Cleuvers, 2004).

Another important factor that can modulate the absorption of contaminants, their toxicity and excretion, is the pH of external media, especially of aquatic organisms. In fact, the absorption of xenobiotics by organisms is well related to medium pH. This principle is well documented and in water, weak acid molecules are better absorbed in basic media (Crouthamel et al., 1971; Martinez & Amidon 2002; Mitra & Kesisoglou 2013). Despite being a generic principle, that is especially valid for simple processes (cellular and organ levels), this seems also to be valid for environmental processes of absorption of toxicants from the external media. Nevertheless, the acid-base regulation in living organisms is an intricate and complex group of processes which uses both bicarbonate and non-bicarbonate buffers in both the intracellular and the extracellular media. These mechanisms are effective for the immediate defense against volatile (mainly CO_2) and non-

volatile (organic and inorganic) acids before excretion by the lungs and kidneys, respectively (Pham et al., 2015). An alteration of this homeostatic sate could result in acidification or alkalization. When these alterations are caused through specific phenomena, such as impairments in the excretion of CO_2 in lungs (respiratory acidosis) or increase of cellular respiratory/metabolism activity (metabolic acidosis). both scenarios result in an increase of CO_2 which reacts with water producing HCO_3^-, which acidifies the media. Moreover, there are several chemicals who can interfere with this homeostasis. A few examples are the ingestion of strong acid precursors (e.g. ethanol, propofol, paracetamol), loss of alkali in gastrointestinal track (e.g. cholestyramine, magnesium sulphate) or alteration in renal excretion of endogenous acid-base regulators caused for example by, lithium, acetazolamide and heparin, among others (Pham et al., 2015).

1.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

Among the most used pharmaceuticals worldwide, one may find non-steroid anti-inflammatory drugs (Sharma & Kaushik, 2017). The drugs of this class are used as antipyretic, analgesic, and anti-inflammatories (Zarghi & Arfaei, 2011; Wongrakpanich et al., 2018). This class is well absorbed in the gastrointestinal track and have high bioavailability. Pharmacologically, NSAIDs are competitive inhibitors of both isoforms of the enzyme cyclooxygenase (COX-1 and COX- 2), compromising its activity (Fokunang et al., 2018). This enzyme mediates the bioconversion of arachidonic acid into prostaglandins (PGs) which act as inflammatory mediators, in fever and pain status, as well as acting as vasodilators, and contributing for the protection of the gastric mucosa (by lowering the acid secretions, enhancing the mucosal blood flow and stimulating the mucus formation and bicarbonate secretion). Thromboxanes, which are also synthetized from PGs, are also involved in platelet aggregation and are vasoconstrictors (Zarghi & Arfaei, 2011).

Pharmaceuticals from this class represent low toxicity when used at therapeutic dosages (Wongrakpanich, 2018), are inexpensive, accessible (sometimes sold as

over the counter formulations), and some of these drugs do not have effective therapeutic alternatives. These characteristics make them one of the most used drugs worldwide, up to 10 % from all prescribed medication worldwide (Onder et al., 2004) and in addition to not being totally removed from WWTPs (Garcia-Lor, 2012), makes them one of the most detected in the aquatic environment (Sui et al., 2015; Chander et al., 2016; Fan et al., 2020).

There are several subgroups of NSAIDs, such as derivatives of indoleacetic acid and iodoacetic acid (e.g. indometacin), derivates of phenylacetic acid (e.g. diclofenac), aryl propionic acid derivatives (e.g. ibuprofen), and the salicylates [e.g. salicylic acid (SA)], among others (Carvalho et a., 2004). In fact, salicylates are the largest group, being used since the XVIII century (Hedner & Everts, 1998). Additionally, acetylsalicylic acid (sold under the name of Aspirin®; ASA) has its annual consumption above one hundred billion tablets, with a yearly production close to 40,000 tons (Freches, 2017). SA is the active metabolite resultant from the de-acetylation of ASA. Concerning SA metabolism in humans, in therapeutic dosages it is mostly (80%) metabolized in the liver, through the conjugation with amino acids (glycine) and with glucuronic acid, leading to the formation of salicyluric acid and acyl and phenolic glucuronides, respectively (Bojić et al., 2015). After conjugation, these compounds, are mainly excreted by the kidneys in which urinary pH is directly related to SA excretion rate. Alkalinisation of the urine pH greatly enhances the excretion of SA and of its metabolites (Proudfoot et al., 2003), due to the acidic nature of SA (pKa= 2.98; Sergeant & Dempsey, 1979).

Additionally, SA (and NSAIDS in general) are the group of pharmaceuticals most prone to interact with others and provoke adverse conditions (Wongrakpanich, 2018). NSAIDs have the potential to interact with diuretics such as thiazide and cause an increase in systolic blood pressure (Moore et al., 2015). They can also interact with antithrombotic such as aspirin by causing competition for access to the acetylation site of platelet-expressed COX-1 resulting in an increased risk of clot formation (Wongrakpanich, 2018). Additionally, these drugs can interact with selective serotonin reuptake inhibitors and tricyclic antidepressants causing gastrointestinal bleeding by altering NSAIDs metabolism, resulting in an increase of their effects (Moore et al., 2015).

Despite being mostly used as a human pharmaceutical, SA is also a phytohormone of natural occurrence (Maruri-López et al., 2019). In fact, this substance, is used by plants in key physiological processes, such as photosynthesis (Janda et al., 2014), growing (Rivas-San Vicente & Plasencia, 2011) and response to biotic and abiotic stress (Husen et al., 2018; Li et al., 2020). As a hormone, SA is also involved in plant processes such as seed germination, vegetative growth, thermogenesis, flower formation, seed production and senescence, among others (Rivas-San Vicente & Plasencia, 2011). SA can also help maintaining cellular redox homeostasis through the regulation of antioxidant enzymes activity in plants (Slaymaker et al., 2002). Additionally, SA can attenuate effects caused by contaminant exposure by acting in the above-mentioned processes (Wang & Zhang, 2017). Nevertheless, the most important mechanisms behind SA protection against xenobiotics are the activation of antioxidant mechanisms in response to contaminant/microorganism exposure (Hayat et al., 2012). Other important role by SA is related to an increase in photosynthetic pigments, among them chlorophyll, to increase photosynthesis to grant the plant a higher energetic reserve to fight these adverse conditions (Morales et al., 2020)

When it comes to environmental presence, this pharmaceutical is found in surface waters, and in effluents of WWTPs, as well as in salt-water environments. Despite this wide dispersion, SA has an environmental half-life of up to 13 days (Blaug & Wesolowski, 1959; Onah, 2004), being degraded by microorganisms and by natural processes such as sunlight (Ayyash et al., 2015), being mostly removed in WWTPs (Heberer, 2002). Nevertheless, due to his high usage, SA is found in concentrations from 0.05 – 330 µg/L (Metcalfe et al., 2003; Carballa et al., 2004; Martin et al., 2012) in effluents of WWTPs, and even in coastal zones of Belgium, SA was reported at concentrations up to 0.855 µg/L (Claessens et al., 2013).

1.2 Diuretics

Other class of interest is the diuretics, which are pharmaceuticals that promote diuresis, which means that they increase the excretion of water from an organism (Oh & Han, 2015). The ecological interest of analysing the ecotoxicological effects

of diuretics is due to their capacity, not only to cause deleterious effects on non-target organisms, but also by being involved in the modulation of the toxicity of other contaminants. There are several types of diuretics, namely potassium-sparing diuretics, thiazide diuretics, loop diuretics, and carbonic anhydrase inhibitors. This classification is proposed take into account the specific location within the nephron. Potassium sparing diuretics act on the cortical collecting duct, by inhibiting sodium reabsorption. Thiazide diuretics are responsible for an impairment in sodium and chlorine reabsorption in the distal convoluted tubule. Loop diuretics prevent the reabsorption of sodium chloride in the ascending limb of the loop of Henle. Finally, and most importantly, carbonic anhydrase inhibitors act in the distal collecting duct, preventing this way the reabsorption of sodium, water and bicarbonate ion, and increasing potassium loss (Roush et al., 2013)

From a pharmacological point of view, carbonic anhydrase inhibitors act by inhibiting the activity of the enzyme carbonic anhydrase (CA; Carta & Supuran, 2013). This enzyme is responsible for the interconversion of bicarbonate ion (HCO_3^-) into carbon dioxide (CO_2) and water (H_2O). This reaction is important in several key biological processes, namely, to maintain the homeostatic state of an organism, and gas exchanges (Shuttleworth et al., 2006). In addition, CA is one of the enzymes responsible for the calcium (Ca^{2+}) available for calcification processes (Marin et al., 2012), as well as in processes of bone resorption (Lehenkari et al., 1998), and tumorigenicity (Benej et al., 2014). In plants, CA is involved in processes such as carboxylation or decarboxylation reactions, in both photosynthesis and respiration process (Moroney et al., 2001).

An example of this class is acetazolamide (ACZ). This pharmaceutical is part of the list of essential medicines published by the World Health Organisation (WHO) (WHO, 2019), being used in human therapeutics for the treatment of glaucoma, altitude sickness, epilepsy and as a diuretic (Low et al., 2012; Liu et al., 2016). In terms of pharmacological effects, this pharmaceutical inhibits the enzyme CA. Nevertheless, its pharmacokinetics is not fully described. In fact, in humans, ACZ does not suffer any kind of metabolism, being excreted mostly in urine in its unaltered form (Ritschel et al., 1998). However, no information is available for most species, and consequently, data on its potential environmental risk is very

scarce. In fact, the lack of studies evaluating the environmental concentrations of this pharmaceutical makes difficult to assess the effects of these pharmaceutical under realistic conditions of exposure, since it is impossible to adopt realistic levels of exposure. To the present date, this pharmaceutical has been found in WWTP effluents in concentrations of ng/L (Singer et al., 2016).

2. Test-Organisms

From an ecotoxicological point of view, it would be beneficial to test a broad range of organisms against every potential contaminant in order to assess its fully ecotoxicological profile. However, this would not constitute a logistically sound approach. Moreover, contaminants can have different profiles and deleterious effects on different taxa. Nevertheless, due to ethical, logistic, and economical constrains (Yip et al., 2016), the assessment of toxic effects posed by contaminants should be prioritized in key species. One of the groups that should be prioritized are primary producers, namely plants (Sandermann, 2004). Plants are at the base of most food webs and are in direct contact with contaminants. Taking this in consideration, the use of plant species as test organisms in the ecotoxicological assessment of the effects of contaminants is a priority. Nevertheless, due to the possible effects of different contaminants on different taxa, other key species should be considered, namely those playing key physiological roles in the ecosystem, such as primary feeders and filter-feeder organisms. These characteristics make such organisms especially vulnerable to contaminant exposure.

2.1 *Lemna gibba*

A group of plants which are often used in ecotoxicological studies are from the genus *Lemna sp.* namely *Lemna gibba* (Mkandawire et al., 2014). This aquatic macrophyte (commonly known as duckweed), has two parts: a floating (or partial submerged) frond and a thin root originating from the centre of each frond (Wang, 1990). These vascular plants usually have two or more fronds (Wang, 1990). The

reproduction occurs asexually by vegetative propagation where the rapid production of new fronds occurs (Wang, 1990). Due to their wide distribution and ecological role (providing food for small aquatic animals, shelter to small invertebrates, oxygen production, and participation in the nutrient cycle), *L. gibba* is a key organism in the ecosystem (Wang et al., 1990). In addition, studies have shown the potential bioremediation effect of these plants (Gupta & Prakash, 2014) highlighting even more their ecological potential role.

In terms of ecotoxicological assays, due to its characteristics, namely rapid production of large quantity of biomass, ecological representativity, asexual reproduction, and the possibility to quantify parameters that are not promptly quantifiable in vertebrates due to ethical issues (such as growth and mortality; Cedergreen et al., 2009), this species is frequently used. Moreover, previous data from the literature have shown that this species is sensitive towards some pharmaceutical among them NSAIDs (Alkimin et al., 2019a; 2019b).

2.2 *Mytillus* spp.

Another group of organisms that should be used to determine potential deleterious effects of environmental contaminant are organisms who are both in direct contact with the contaminants and are first order consumers in food webs. These organisms are especially important due to their link with other levels in the food web (Bergström & Lindgarth, 2016).

Among the organisms following this criteria, one may refer filter feeders as organisms from the genus *Mytillus sp*. This genus of mussels has a wide distribution being found in rocky beaches worldwide (Brooks & Farmen, 2013). Additionally, they are resistant to a variety of contaminants and have a long-life cycle which makes possible to assess bioaccumulation of several contaminants (Teixiera et al., 2017). All above mentioned characteristics makes this genus an ecological representative of filter feeders and addition they have been used in biomonitoring marine environments (Li et al., 2016).

In addition to being used as sentinel organisms, this genus has also been used in ecotoxicological studies due to their sedentary behaviour, feeding behavior (filtering), easy capture, and maintenance in laboratory (Piedade et al., 2019).

2.3 *Phorcus lineatus*

In some geographical locations, organisms of the genus *Mytillus* are accompanied by animals from other species. Among these, one may find organisms from the class Gastropoda. Similarly, to mussels, gastropods are suitable to be used as test organisms in ecotoxicological tests. One of the most significant species from this class, is *Phorcus lineatus* (Archeogastropoda: Trochidae; toothed top shell), which is an herbivorous species of marine snails, that exhibit a set of interesting characteristics, such as small dimensions, wide distribution along the European Western coast, natural abundance, simple maintenance in laboratory conditions, easy capture (due to their reduced mobility), continuous availability through the year (Crothers, 2001; Cunha et al., 2007).

3. Use of biomarkers to evaluate the toxicity of pharmaceutical drugs

The use of tools to assess contaminant exposure and effects is the main objective of ecotoxicology. This objective may only be attained by evaluating the contaminant effects on non-target organisms. To do so, several strategies have been developed, including the use of biomarkers, which are biological alterations resulting from the exposure to a xenobiotic (Forbes et al., 2006). There are several types of biomarkers, enzymatic, non-enzymatic, behavioural, reproductive, histological, among others. The selection of biomarkers in a study should reflect the effects of the specific contaminant, to better extrapolate and interpret the obtained results, in order to conclude about the entire set of effects and consequences that a given xenobiotic may cause in exposed biota, ultimately allowing to conclude about putative environmental effects. Moreover, biomarkers should also reflect complementary biological processes, be sensitive and responsive to the exposure to contaminants (Forbes et al., 2006). Additionally,

biomarkers should reflect key biological processes or metabolic pathways, that regulate processes that condition the organism's well-fare and/or surviving (Forbes et al., 2006), which may have consequences at higher levels of organization, *i.e.* at the ecosystem level. Nevertheless, the ecological relevance of some of these biomarkers can sometimes be argued, especially those that give insights of the subcellular (enzymatic and non-enzymatic) functioning. To address this problem, complementary biomarkers of several organisation levels should be used.

3.1 Specific biomarkers of plants

Plants have an exclusive and distinctive physiological feature, which is related to the process of photosynthesis. This process uses energy of the sun, atmospheric CO_2 and water, to synthesize sugars, which in turn fee the plant. This process is the generic base of most food webs, and most organisms depend on them. Photosynthetic pigments, such as chlorophylls (Chl) and carotenoids (Car) are the main constituents of the photosystems (PSs) both I and II; McElroy & Kopsell, 2009). PSs are complexes of photosynthetic pigments arranged in the form of a cluster, for an efficient absorption and utilization of sunlight energy. PS are divided in two main complexes: the antenna complex, and the reaction centre. The antenna complex is where the photons arrive to the plant, specifically to the light harvesting complex (LHC). LHC which is composed by molecules of both chlorophylls (a and b) and carotenoids who are responsible for collecting the photon energy and passing it to second structure, the reaction centre. There, a molecule of chlorophyll a and a final receptor (NADP+) receives the energy from excited electrons and converts them into chemical energy (Vinyard et al., 2013; Caffarri et al., 2014). Both Chl and Car are also involved in in essential processes besides light harvesting, since are also instrumental in energy transfer and photoprotection, by dissipating excessive energy and neutralizing reactive molecules which could damage the PSs, thereby causing adverse effects on photosynthesis (McElroy & Kopsell, 2009; Caffarri et al., 2014). PS II uses light energy to split water molecules, resulting in the release of free protons which contribute for the membrane electrochemical potential (Vinyard et al., 2013). This

mechanisms is the main driving force of photosensitizes and consequently, alterations in Chl and/or Car content could result in alterations in photosynthetic rates, by compromising the efficacy of the harvesting of photon energy, or by not preventing the reaction of reactive molecules with LHC. Ulterior alterations could eventually lead to the death of the organism, thereby affecting the food web (Simkin et al., 2019).

3.2 Biomarkers determined in filter feeders

Another physiological process which is essential to several organisms is filtration. Filtration is the process by which some aquatic organisms feed; consequently, it can also be responsible for the removal of several particles and contaminants from the environment, which are sometimes accumulated in the organism's tissues, being transferred to other species when predated (Hartmann et al., 2016). Marine species of bivalves are known to alter their feeding behaviour when in the presence of organic and inorganic contaminants, such as metals (arsenic), detergents (sodium dodecyl sulphate) and anaesthetics (trichloroethylene), among others (Giari et al., 2017), restoring their normal feeding rates when exposure cesses, a phenomenon known as a defensive behavior (Riisgård et al., 2011; Hartmann et al., 2016). Nevertheless, there are contaminants with the capacity of altering this capacity at molecular levels. Such compounds usually act by inhibiting neuro-muscular transmission and could affect organisms at a longer term after exposure (Ayad et al., 2011). Alterations in filtration rates could jeopardize organism's welfare interfering with food ingestion, leaving the organisms debilitated and potentially more sensitive to other stress factors (Hartmann et al., 2016). These effects could, ultimately compromise the natural ability of filter organisms to perform filtration of the surrounding watery media (Riisgåd et al., 2011).

4. Previous studies

Several studies have been conducted to assess the effects of environmental contaminants in non-target organisms. In fact, pharmaceuticals are among the contaminants who are raising more concerns in terms of environmental risk assessment (Godoy & Kummeow, 2017). Among the most studied classes of drugs that are found in the environment are the NSAIDs. Due to their wide usage and different mechanisms of metabolism, their ecotoxicological effects are diverse. In fact, a representative of this class, SA was tested under several scenarios in several non-target species showing its potential oxidative effects with alterations in antioxidants enzymes, genotoxicity, in several aquatic organisms, namely zebrafish (*Danio rerio*, Zivna et al., 2016), marine polychaetes (*Hediste diversicolor*: Gomes et al., 2019; Nunes, 2019), *Salmo truta fario* (Nunes et al., 2015), and *Daphnia magna* (Goméz-Oliván et al., 2014).

Moreover, the potential of seawater acidification to modulate the absorption of acidic substances, namely SA, was also evaluated in *Gibulla umbilicalis*, allowing to conclude that environmental pH could alter the toxic effects of this pharmaceutical (Dionísio et al., 2020b). Additionally, Cleuvers (2004) tested the effects of mixtures of ASA with other NSAIDS, namely diclofenac, ibuprofen and naproxen. This study assessed standard endpoints, namely immobilization, on the microcrustacean *Daphnia magna*, and growth of the green algae *D. subspicatus*, showing increased toxicity when organisms were exposed to mixtures of these pharmaceuticals. When it comes to SA effects in plants, there are several studies evaluating its potential phyto protector effect, especially when plants were exposed to chemical contaminants. The observed effects of SA included the activation of antioxidant mechanisms in response to contaminant/microorganism exposure (Rivas San-Vicent et al., 2011; Hayat et al., 2012; Radwan et al., 2019) and/or increase in photosynthetic pigments content. This was particularly evidenced by Alkimin et al. (2020) when environmental realistic concentration of SA were able to revert the extensive oxidative damages caused by co-exposure to diclofenac in the macrophyte *Lemna minor*.

When it comes to ACZ effects, its ecotoxicological effects are not well characterized, and most studies analysed only its effects on plants, namely

spinach (*Spinacia oleracea* L.; Swader & Jacobson, 1972), maize (*Zea mays* L.; Stembler & Jursinic, 1983), and corals (Bertucci et al., 2013).

Moreover, the effects of SA may be assessed by studying alterations of its pharmacological target, COX activity. Finally, the modulatory effect of both pharmaceuticals should be assessed once ACZ could modulate SA excretion, through pH modulation, and SA could revert ACZ deleterious effects in plants.

5. Objectives

This work intended to evaluate the ecotoxicological effect of two pharmaceuticals and their potential interaction non-target organisms.

- to characterize the effects of the diuretic drug acetazolamide in three non-target organisms, in terms of enzymatic activities, namely CA activity.
- to evaluate the effects of acetazolamide and salicylic acid on the aquatic macrophyte *Lemna gibba* photosynthetic (chlorophyll a, b, total and carotenoids) and the potential of SA to protect this species against contaminant exposure.
- to evaluate the potential interactions between ACZ and SA, namely the excretion profiles of these drugs, and the extent of toxic effects in the species *L. gibba, Mytilus spp. and P. lineatus*.

6. Thesis structure

Present chapter (**Chapter 1**) is essentially a literature review describing the problem of environmental pharmaceutical contamination, and particularly the here studied pharmaceuticals (salicylic acid and acetazolamide). In addition, this chapter also described the test organisms (*Lemna gibba*, *Mytillus spp.*, and *Phorcus lineatus*) and potential evaluation endpoints that may be used to address the toxicity of xenobiotics in these species as well as previous works donne with these species and pharmaceuticals.

The next 3 chapters organize the experimental work sequentially and were built following the specific layout commonly used in journal articles. In fact, these 3 chapters constitute three distinct manuscripts, summarizing the main findings obtained in the experimental component of this thesis. **Chapter 2** focus on the

effects of environmental realistic concentrations of salicylic acid and acetazolamide (both in single exposures, and in mixtures) on the aquatic macrophyte *Lemna gibba*. This research focused on the effects of both drugs on key enzymatic features, namely CA activity. It also analysed potential effects of the drugs on photosynthetic apparatuses, namely by measuring the contents of chlorophylls a, b, and total, and the levels carotenoids. Additionally, the potential protective effects of SA on contaminant exposure were also discussed in this chapter.

On **Chapter 3,** the effects of the same pharmaceuticals (salicylic acid and acetazolamide) in the marine mussel *Mytillus spp.* were discussed. This chapter focus on biomolecular parameters (namely CA, COX) as well as behavioural (feeding rate) and morphological (shell biometrics and shell hardness). The effects on acetazolamide on changes of DSA excretion were discussed, based on its capacity to cause metabolic acidification.

Chapter 4 evaluated the effects that were attained after exposing the species *Phorcus lineatus* to salicylic acid and acetazolamide (both in single and combined exposures) for different exposure periods. The effects were observed on carbonic anhydrase and cyclooxygenase activities, on the marine gastropod *Phorcus lineatus*. Similar to the previous chapter, potential interactions between ACZ in SA excretion were also discussed.

Finally, **Chapter 5** presents the final remarks and conclusions of all obtained results, as well as some future prospects.

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Chapter 2

Single and combined effects of the drugs salicylic acid and acetazolamide: adverse changes in physiological parameters of the freshwater macrophyte, *Lemna gibba*

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1. Abstract

Pharmaceutical drugs are among the most used chemicals, for human and veterinary medicines, aquaculture and agriculture. Pharmaceuticals are biologically active molecules, having also environmental persistence, thereby exerting biological effects on non-target species. Among the most used pharmaceuticals, one may find salicylic acid (SA), a non-steroid anti-inflammatory drugs (NSAIDs), and acetazolamide (ACZ), a diuretic drug that acts by inhibiting the activity of carbonic anhydrase (CA). In this work, single and combined effects of SA and ACZ were assessed in the aquatic macrophyte *Lemna gibba* L., focusing on physiological parameters, namely photosynthetic pigments, (chlorophyll a, b and total (Chl a, b and TChl) as well as carotenoids (Car)). In addition CA activity. The highest concentrations of ACZ, caused a decrease in the contents of all chlorophylls; this effect was however reverted by SA exposure. Both ACZ and SA levels caused a decrease in CA activity. In conclusion, both pharmaceuticals have the capacity to cause alterations in *L. gibba* enzymatic activity and photosynthetic pigments content. Additionally, SA seems to exert a protective effect on this species against deleterious effects caused by ACZ.

2. Keywords: drugs; enzymatic biomarkers; photosynthetic pigments; phyto-protection

3. Introduction

In the past decades, the presence of pharmaceuticals in the environment has been recognized as an emerging issue, thereby deserving more attention, both from the scientific community and from the general public. From the literature, it is possible to conclude that the aquatic compartment is especially affected by a comprehensive profile of contamination, which includes, among many others, pharmaceutical drugs (Fent et al., 2006; Ebele et al., 2016). This occurs since the aquatic compartment is the final destination of such chemicals, resulting from

agricultural lixiviates, sewage discharges, direct disposal, or use in aquaculture (Fent et al., 2006; Chowdhury et al., 2015; Sui et al., 2015). Once in the aquatic compartment, pharmaceuticals have the capacity to exert deleterious effects on non-target species (Fentet al., 2006). Due to the high number of potential interactions between pharmaceuticals and non-target organisms, it is difficult to fully evaluate the ecotoxicological effects of this class of contaminants, especially when in complexes environmental matrices (Brain et al., 2004; Farré et al., 2008).

One of the most frequently detected class of pharmaceutical contaminants are non-steroidal anti-inflammatory drugs (NSAIDs). This class is one of the most prescribed drugs in the world and has become a growing concern in ecotoxicological terms (Bonnefille et al., 2018). A good representative of NSAIDs is salicylic acid (SA), which is the main (and most pharmacologically important) metabolite of acetylsalicylic acid. SA is responsible for the inhibition of the main therapeutic target of NSAIDs, the cyclooxygenase enzymes, at inflammation sites, preventing the production of prostaglandins, considered as important inflammatory mediators (Mitchell et al., 1997). Furthermore, in plants, SA is considered a phytohormone, involved in processes of growth, development and interaction between plants and pathogens, as well as being implicated in responses to environmental stress (Rivas-San Vicente & Plasencia, 2011). Its beneficial effects in plants are widely recognized, being SA exogenous administration sometimes used as a method to increase crop production (Joseph et al., 2010). However, and due to his high usage, SA is found almost ubiquitously in effluents of wastewater treatment plants (WWTPs) at concentrations of the ng/L to µg/L (Metcalfe et al., 2003; Claessens et al., 2013). Despite this wide dispersion, SA has a short environmental half-life time, being mostly removed at WWTPs (Heberer, 2002). Considering these two roles, as a beneficial phytohormone in plants, and its role as an environmental pollutant, the putative effects of SA in plants are difficult to ascertain and quantify, especially if one considers that plant exposure may also occur simultaneously to other chemicals; most probably, the combination of chemicals, doses, durations of exposure, and the intrinsic sensitivity of the plant species, are major factors in this crosstalk between toxicity and protection.

Diuretics are another class of therapeutic drugs widely used in human therapeutics. These substances promote diuresis, *i.e.* increase the excretion of water, being this effects fully documented in vertebrate organisms (Roush et al., 2014; Oh & Han, 2015). Among the most used diuretics, one may find carbonic anhydrase inhibitors (CAI; Carta & Supuran, 2013), which act by supressing the activity of carbonic anhydrase (CA), the enzyme responsible for the interconversion of bicarbonate ion (HCO_3^-) into carbon dioxide (CO_2) and water, and for the *in vivo* formation of carbonic acid (Badger & Price, 1994). This activity results in the control of acid-base balance and the transport of CO_2 in vertebrate organisms (Oh & Han, 2015). These drugs are used in the treatment of glaucoma, altitude sickness and epilepsy, and an example of a drug of this class is acetazolamide (ACZ; Low et al., 2012; Liu et al., 2017). ACZ was first introduced in 1952 and it was included in the World Health Organization's List of Essential Medicines, which summarizes the safest and most effective medicines needed in a health system (WHO, 2019). Due to his high usage, it is has been found in wastewater effluents in concentrations of ng/L range (Singer et al., 2016).

Living organisms in the wild are not exposed to a single contaminant, being sometimes exposed to complex matrices that simultaneously include hundreds or thousands of contaminants (Thrupp et al., 2018). Those contaminants may interact and generate new effects or increase/attenuate effects caused by exposures to single chemicals (EC COM, 2012). Since most contaminants end up in aquatic systems, it is generally accepted that aquatic organisms are specially affected by these contaminants. To address this issue, the selection of adequate test organisms to study the impacts of contaminants on non-target organisms, namely those at the basis of aquatic food webs, often relies on plant species. Therefore, the freshwater macrophyte species *Lemna* spp. is considered a good model species in ecotoxicological analysis, due to its characteristics, namely rapid growth, easy to produce large quantity of biomass, ecological representativity, asexual reproduction, and the possibility to quantify parameters that are not promptly quantifiable in vertebrates due to ethical issues (Cedergreen et al., 2009). In addition, previous data from the literature have shown that this species is sensitive to pharmaceuticals contamination (Alkimin et al., 2019a; 2019b).

The aim of this work was to evaluate the effects of the pharmaceuticals ACZ and SA (exposure to single compounds, and also to mixtures of both chemicals) on the macrophyte species *Lemna gibba* L., in terms of pigments content, namely chlorophyll a, b, and total (Chl a, b TChl) as well as carotenoids content (Car) and carbonic anhydrase (CA), after a 5 day exposure.

4. Material and Methods

4.1 Chemicals

All pharmaceutical drugs were purchased from Sigma Aldrich, with purities>98%: acetazolamide (CAS: 59-66-5) and salicylic acid (sodium salt form; CAS 54-21-7) and all other chemicals used in this study have analytical purity.

4.2 *Lemna gibba* assay

Tests were performed by exposing plants to concentrations of ACZ and SA based on already reported environmental concentrations, and also on worst-case scenarios of contamination. SA concentrations ranging from 0.855 up to 330 µg/L were already documented in WWTP effluents (Metcalfe et al., 2003; Claessens et al., 2013). ACZ was detected in WWTP effluents in concentrations up to 180 ng/L (Singer et al., 2016). A stock solution for each pharmaceutical was prepared in modified Steinberg media (OECD, 2006). From here, each tested concentration was prepared by diluting the stock solution into final nominal concentrations of 10, 100, and 1.000 µg/L of ACZ (C1; C2; C3, respectively), and of 100, 1.000, and 10.000 µg/L of SA (C4; C5; C6, respectively). In addition to concentrations of each drug, we also tested combinations of the two drugs, 10 µg/L ACZ + 100 µg/L SA (C7), and 1.000 µg/L ACZ + 10.000 µg/L SA (C8), based on the lowest and highest concentrations of each pharmaceutical previously selected. Additionally, one negative control (C0) was used.

Lemna gibba plant cultures were obtained from laboratory kept axenic cultures, established at the Centre for Environmental and Marine Studies (CESAM) at University of Aveiro, and reared in 1 L glass flasks, with approximately 200 mL of modified Steinberg medium, according to the general recommendations from OECD 221 (OECD, 2006), under controlled conditions (temperature 20 ± 2 °C; photoperiod 16 h:8 h; light intensity, ~84 µmol photons m^{-2} s^{-1}). The exposure was performed in 6-well microplates, where each well represented an individual replicate, in a total of 10 replicates per treatment. In each plate, a well was used for control, and each of the remaining 5 wells had test solutions, one well for each of the tested pharmaceuticals. At the beginning of the test, similar plants (3-5 plants, with 7-9 fronds in total) were placed in each well (individually exposed replicate), with 10 mL of modified Steinberg media (OECD, 2006). Exposures were conducted under controlled conditions (continuous light; temperature 23 ± 0.2 ºC; light intensity ~84 µmol photons m^{-2} s^{-1}; OECD, 2006) in a climatic chamber (Binder KBW 720). Media was totally renewed every other day to maintain the concentrations of both drugs. The adopted testing guideline was modified to accommodate a duration of exposure of 5 days, according to Alkimin et al. (2019b). The exposure time was adapted to a shorter version of the guideline since Alkimin et al., (2019a) showed that another aquatic macrophyte (*Lemna minor*) was responsive to contaminants after a 4-day.

4.3 Pigments analyses (chlorophylls and carotenoids) and enzymatic activities (CA)

The quantification of pigments, namely of chlorophyll a (Chl a), chlorophyll b (Chl B), total chlorophyll (TChl) and carotenoids (Car) after each replicate (8 in each treatment) was transferred to a microtube and fresh weight (FW) was determined. To each replicate, a volume of 1.8 mL of dimethyl sulfoxide (DMSO) was added. These tubes (sample and DMSO) were placed in a water bath, at a temperature of 65ºC for 30 min, and allowed to cool overnight in the dark. The next day, samples were vortexed (10s), and centrifuged for 5 minutes at 15.000g at 4ºC (Eppendorf 5810R centrifuge). The absorbance of supernatants was spectrophotometrically

read at 470, 645, 646, and 663 nm in a spectrophotometer Thermo Scientific Multiskan (SkanIt Software 2.4.4 RE for Multiskan Spectrum). The levels of pigments were calculated according to the equations (1; 2; 3) proposed by Arnon (1949) - shown by Hiscox & Israelstam (1979) to be virtually identical to Chl extracted in DMSO. For the calculation of CAR content, equation 4, proposed by (Lichtenthaler, 1987), was used. Results were expressed in mg pigment/g FW.

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

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

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

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

For the determination of carbonic anhydrase (CA) activity, tissues were homogenized in 1 ml of Tris-sulphate 25 mM (pH 7.5) buffer and then centrifuged at 10,000g for 40 minutes (Eppendorf 5810R centrifuge). CA activity was assayed according to Verpoorte et al. (1967), where degradation of p-nitrophenyl acetate (pNPAc) by CA was followed at 400 nm; results were expressed as µmol of oxidized pNPAc per minute per milligram of protein (U). The quantification of total soluble protein of samples was performed at 595 nm using the Bradford method (Bradford, 1976), adapted to microplate, with 1 mg/mL bovine γ-globulin as standard. All parameters were spectrophotometrically measured, and the readings were performed in a microplate reader Thermo Scientific Multiskan (SkanIt Software 2.4.4 RE for Multiskan Spectrum).

4.4 Statistical analysis

Both one-way analysis of variance (ANOVA) assumptions (homogeneity of variance and normal distribution) were tested prior to the statistical analysis. Afterwards an ANOVA was performed using software SigmaPlot v.14. A post-hoc test (Dunnett) was performed with a significance level of $\alpha = 0.05$.

5. Results

Acetazolamide was responsible for a decrease in the contents of pigments, namely Chl a ($F_{(8;83)}$= 6.137, p<0.05); Chl b ($F_{(8;91)}$= 5.929, p<0.05); TChl ($F_{(8;87)}$,= 4.452 p<0.05) and CAR ($F_{(8;91)}$= 5.23, p<0.05), which was significant in plants exposed to the highest concentration of ACZ (C3; Fig. 1). Despite the absence of significant alterations in pigment levels caused by SA, it was possible to observe a pattern of increased amounts of pigments in plants according to a dose-response relationship (Fig 1). The here tested mixture only caused an increase in Chl a levels (Fig. 1A).

In terms of enzymatic activities, ACZ caused a decrease in CA activity, in plants exposed to all tested concentrations (C1-C3; Fig. 2C; $F_{(8;70)}$= 20.648, p<0.05). Mixtures of ACZ and SA, caused no alteration in any of the tested parameters (Fig. 1 and 2), with the exception of the inhibition of CA activity in plants exposed to the highest concentrations of both drugs combined (C8; Fig. 2).

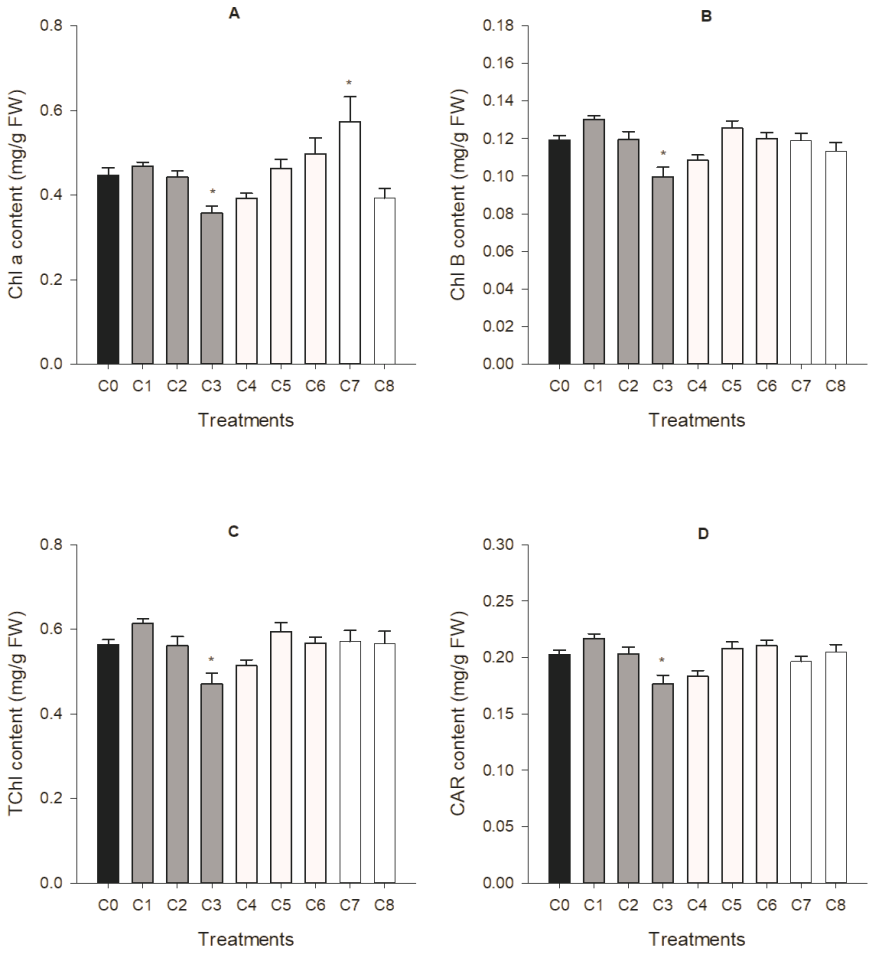

Figure 1. Effects of ACZ and SA on *L. gibba* on pigment content, Chl a (A), Chl b (B), TChl (C) and CAR (D) content. x-axis represents treatments (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L acetazolamide (ACZ); C4, C5, C6 – 100, 1,000, 10,000 µg/L salicylic acid (SA); C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). Each bar represent means ± se. n=10. * stands for statistical differences (Dunnett's test: $p < 0.05$) between treatments and the control group (C0).

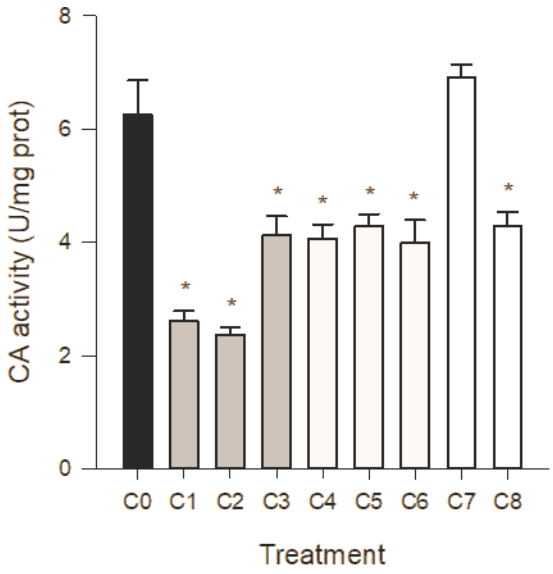

Figure 2. Effects of ACZ and SA on *L. gibba* enzymatic activities CA. x-axis (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L acetazolamide (ACZ); C4, C5, C6 – 100, 1,000, 10,000 µg/L salicylic acid (SA)ACZ; C4, C5, C6 – 100, 1,000, 10,000 µg/L SA; C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). Each bar represent means ± se. n=10. * stands for statistical differences (Dunnett's test: $p < 0.05$) between treatments and the control group (C0).

6. Discussion

Photosynthetic pigments, namely chlorophylls and carotenoids, are involved in essential photosynthetic processes (e.g. light harvesting, excitation, energy transfer and photoprotection; Caffarri et al., 2014). PS II uses light energy to split water molecules, resulting in the release of free protons which contribute for the membrane electrochemical potential (Vinyard et al., 2013). The hereby obtained results show that exposure to ACZ caused a decrease in the amounts of all pigments, in plants exposed to the highest concentration of this drug. This phenomenon may be a consequence of the capacity shown by ACZ to inhibit PS II, as already evidenced in isolated chloroplasts, from both spinach (*Spinacia oleracea L.*; Swader and Jacobson, 1972) and maize (*Zea mays L.*; Stemler and Jursinic, 1983). This inhibition of PS II can cause photobleaching, an effect that corresponds to a loss of photosynthetic pigments due to structural alterations in the molecules of pigments caused by light exposure, thereby compromising photosynthesis (Mooney et al., 1974; Dominy & Williams, 1986; Thomas & Matile, 1988; Andreeva et al., 2007). However, when plants of *L. gibba* were exposed to mixtures of ACZ and SA, the pigment losses that were observed after ACZ single exposure did not occur. So, it is possible to suggest that such deleterious effects for all pigments caused by ACZ alone were somehow reverted, and even restored to control levels, by the joint presence of ACZ+SA. This reversion of toxic effects may be due to the capacity of SA to prevent pigment loss (Li et al., 1992; Çag et al., 2009; Utami et al., 2018), leading to an increase in the levels of photosynthetic pigments, by reducing their degradation caused by oxidative stress (Radwan et al., 2019). This effect may be a direct consequence of the physiological effects of SA, which acts on plants as a phytohormone, and can protect them from unfavourable scenarios. Among these functions, SA may contribute for the activation of antioxidant mechanisms, such as catalase, superoxide dismutase, ascorbate peroxidase, and increased levels of ascorbate (El-Esawi et al., 2017).

Carbonic anhydrase is a metalloenzyme responsible for the interconversion of CO_2 into HCO_3^- (Khalifah, 1971). In plants, CA is of extreme importance due to its involvement in the regulation of physiological processes such as carboxylation or

decarboxylation reactions, in both photosynthesis and respiration (Moroney et al., 2001). Furthermore, CA is also involved in inorganic carbon transport into photosynthetic active cells, or away from respiring cells (Henry, 1996). The here obtained results showed that single exposure to ACZ was, in general, causative of CA inhibition. This result was expected since ACZ is an inhibitor of CA, and other studies have reported a similar inhibition of this enzyme in plants, caused by this pharmaceutical drug (Moroney et al., 2001; Shitov et al., 2018). In addition, SA exposure also yielded a somewhat similar result. The here reported inhibition of CA caused by SA may be explained considering the capacity of some salicylates to bind to the active site of this enzyme (Bayram et al., 2008; Medina-Puche et al., 2017), rendering it hydrolytically inactive. Furthermore, Medina-Puche et al. (2017) suggested that SA may be responsible for a downregulation of CA activity, resulting in deleterious effects in CO_2 fixation and signalling in which CA is involved in plants, namely to assure stomatal movements, controlling this way the available CO_2 inside a plant (Hu et al., 2015). Additionally, CA is also involved in such processes as lipid production, an effect that was acknowledged during the germination phase of cotton seedlings (Hoang & Chapman, 2002). Our evaluation of the effects caused by mixtures of ACZ and SA, yielded two different responses. Exposures to mixtures of low levels of both drugs, caused no significant alterations in enzymatic biomarkers, namely CA; this absence of effects was also verified in pigment levels, apart from Chl a, which was significantly increased. This can suggest the establishment of protective effects by SA when plants were under stress, in this case exposure to ACZ. However, an opposite pattern was observed in plants exposed to mixtures of high levels of both drugs, where an inhibition of CA was observed. It is thus possible to hypothesize that the extension of the protective response reported for low levels of the two drugs is limited by the amount of available SA; when plants were exposed to a stronger aggression (higher amounts of ACZ), this beneficial effect by SA was abolished, since SA concentrations were not able to counteract ACZ deleterious effects.

As already referred, salicylic acid is a well-known phytohormone, being involved in the regulation of several key processes in plants. Besides being a regulator of the antioxidant responses (please see above), it is also involved in various

phytochemical processes, including photosynthesis, and in the regulation of pigment content and of enzymes levels, such as RuBisCo and CA (Rivas-San Vicente & Plasencia, 2011). However, exogenous SA can exert other physiological effects, mostly based on the dose and analysed plant species (Rivas-San Vicente & Plasencia, 2011), as well as the duration of exposure (Alkimin et al., 2019b). While in higher doses it has been reported to decrease chlorophyll content in cowpea, wheat, and *Arabidopsis* (Rao et al., 1997; Chandra & Bhatt, 1998; Moharekar et al., 2003), at lower doses an increase of CO_2 assimilation, chlorophyll content, as well as augmented CA activity, were observed in *Brassica juncea* (Fariduddin et al., 2003). All these effects seem to be beneficial to plants. Nevertheless, the here tested single doses of SA seemed not to affect *L. gibba* photosynthetic pigments. Similar results were obtained by Alkimin and colleagues (2019b) after exposing another macrophyte species, namely *Lemna minor*, to SA. Considering these results, it is difficult to infer about the biological meaning of the present findings, but it is possible to suggest that effects of exogenous SA are species-specific, as well as dose specific.

In conclusion, ACZ and SA, alone or in combination, have the potential to cause significant physiological alterations that may encompass deleterious effects on the macrophyte species *Lemna gibba*. Some of such deleterious effects can be reverted to control condition by SA exposure, given the already documented protective capacity of this chemical, namely in terms of photosynthetic pigments and CA activity. ACZ had the ability to inhibit CA, plant capacity to perform gas exchanges which can ulterior cause a photosynthesis impairment. Further studies are needed to better understand mechanisms of SA protection and interaction with other pharmaceuticals.

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Chapter 3

Toxicity of two drugs towards the marine filter feeder *Mytilus* spp, using a biomarker approach based on biochemical and shell integrity parameters

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1. Abstract

The increasing presence of anthropogenic contaminants in the environment may constitute a challenge to non-target biota considering that most contaminants can exert deleterious effects. Salicylic acid is a non-steroid anti-inflammatory drug (NSAIDs) which exert its activity by inhibiting the enzyme cyclooxygenase (COX). Another class of drugs is those of diuretics, in which acetazolamide (ACZ) is included. This pharmaceutical class acts by inhibiting carbonic anhydrase (CA), a key enzyme in acid-base homeostasis (by regulation of pH) and responsible for bio-availability of Ca^{2+} for shell formation processes. In this work, we evaluated the chronic (28-day) ecotoxicological effects of SA and ACZ (alone and in combination) on individuals of the marine mussel species *Mytillus spp.*, using enzymatic namely COX and CA, and morphological and physiological (shell hardness, shell index and feeding behaviour) biomarkers. In terms of CA activity, exposure to ACZ and SA, this enzyme was inhibited by the highest concentration of ACZ in gills, but no effects occurred in the mantle tissue. In terms of COX activity, this enzyme was not altered after exposure to the single chemicals. However, animals exposed to the mixture of ACZ and SA evidenced a significant inhibition of COX activity. Morphological and physiological processes (namely, feeding, shell index and shell hardness) were not affected by the here tested pharmaceuticals. Nevertheless, mixtures of contaminant can cause an additional stress factor to organisms in the wild.

2. Keywords: carbonic anhydrase; cyclooxygenase; acidosis; shell hardness; mixture

3. Introduction

Environmental contamination by micropollutants of organic nature is raising increasing concern. Recently, more reports and studies have detected the presence of several classes of contaminants, among which pharmaceutical drugs raise special attention. Pharmaceuticals are chemicals used in both human and veterinary medicines, but also in agriculture and aquaculture practices

(Chowdhury et al., 2015; Sui et al., 2015). Due to their high usage, environmental persistence and capacity to exert biological effects, pharmaceuticals are prone to cause deleterious effects in non-target organisms (Fent et al., 2006).

Among the already reported pharmaceuticals classes that occur in the environment, non-steroid anti-inflammatory drugs (NSAIDs) are one of the most frequently found, and their levels are considerable. The pharmacological target of the drugs of this pharmacotherapeutic class are cyclooxygenases (COX, 1 and 2), which are enzymatic forms responsible for the biosynthesis of prostaglandins (PGs; Wongrakpanich et al., 2018). The biological role of these biomolecules is high, since these substances are important in the regulation of key physiological processes, such as inflammation, fever and pain (Wongrakpanich et al., 2018). A major representative of this class is salicylic acid (SA), a chemical known for being a strong inhibitor of COX enzymes, used as anti-inflammatory, analgesic and antipyretic drug (Peesa et al., 2016). SA is the most important bioactive metabolite of acetylsalicylic acid (ASA; Mitchell, et al., 1997). Consequently, its environmental presence is common, being however detected in relatively low concentrations in the environment (Patel et al., 2013; Martá et al., 2018). Despite being a common drug (used in the form of SA, and by being metabolized *in vivo* from ASA), it is naturally degraded in the wild, being also easily photodegraded (Gangwang et al., 2012). Considering its extensive degradation when in the wild, but its simultaneous high input rates into the environment, this drug may be considered a pseudo pollutant. This explains why it is still continuously found in the aquatic ecosystem, in concentrations of the range of the ng/L to µg/L, namely in surface waters (Martá et al., 2018).

Another class with a great human usage is that of the diuretics. These substances promote diuresis, which means that they increase water excretion from organisms (Oh & Han, 2015). There are several types of diuretics which include carbonic anhydrase inhibitors (CAI). An example of this class is acetazolamide (ACZ), a drug which acts by inhibiting carbonic anhydrase (CA) activity (Low et al., 2012; Liu et al., 2016). This enzyme is responsible for the maintenance of acid-base balance, and for the transport of carbon dioxide in animals' blood (Oh & Han, 2015). This enzyme also plays an important role in the homeostasis of calcium

(Ca^{2+}) ions in molluscs (Wang et al., 2017; Cardoso et al., 2019). ACZ is used in human therapeutics for the treatment of several illnesses, including altitude sickness, and glaucoma. Despite being used for several decades (WHO, 2019), studies to evaluate the ecotoxicological potential and the environmental presence and fate of this pharmaceutical are still scarce. In fact, there is almost a total absence of studies measuring ACZ concentrations in the aquatic compartment. As far as we know, only one study has detected ACZ in water, and its measured concentrations in the aquatic compartment are under the ng/L (Singer et al., 2016).

Regarding the evaluation of ecotoxicological effects posed by drugs, it is necessary to bear in mind the number of different chemicals simultaneously present in the environment (Thrupp et al., 2018). Thus, it is possible that these chemicals may interact with each other, leading to new, uncharacterized pharmacological and toxicological effects, thereby altering magnitude and type of effects that occur after exposures to single drugs (EC COM, 2012).

To study the effects of environmental contaminants, it is necessary to use organisms which are likely to be affected by the chemicals under study. A suitable group to be used in the ecotoxicological assessment of chemicals is that of marine mussels, particularly the genus *Mytillus* spp., due to the fact that they are sessile filter-feeders (Riisgård et al., 2011), and thereby tend to accumulate waterborne contaminants of multiple types (Beyer et al., 2017) for long periods, without seeking new areas of refuge. This set of features makes mussels a good candidate to characterize contamination profiles in aquatic systems (Riisgård et al., 2011), namely in marine and estuarine areas. Mussels were also reported to be suitable test organisms to be used in ecotoxicological assays due to their wide distribution, resistance to many contaminants, being also easy to capture and maintain under laboratory (Piedade et al., 2020).

The assessment of effects of both ACZ and SA (single and combined exposures), were undertaken by using individuals of the marine bivalve mussel, *Mytillus spp*, by measuring the activities of enzymatic biomarkers, namely cyclooxygenase (COX) and carbonic anhydrase (CA), which were used to address the activation of the known specific pharmacological pathways for both drugs. Shell biometrics and

shell hardness were assessed to study possible effects of this pharmaceuticals in the processes of mineralization, shell absorption and shell integrity. Animals feeding rate was assessed in order to evaluate if the here tested pharmaceuticals could impair the filtration rates of these organisms compromising their health and nutritional status, and consequently their ecological role.

4. Material and Methods

4.1 Animal collection and quarantine

Animals were collected at Barra, Ria de Aveiro, Portugal (40°38'34.5"N 8°44'07.7"W) during low tide period. This location is mainly subjected to naval traffic (Oliveira et al., 2009), and no major discharge points of untreated domestic sewage, the main source of human pharmaceuticals, are present at this location. Due to the co-existence of two *Mytilus* species (*M. edulis* and *M. galloprovincialis)* the occurrence of hybridization is possible. Therefore, at this site, a mixture of pure and hybrid individuals may naturally occur (Coghlan & Gosling, 2007). Since no morphological character can be reliably used to separate these populations, we were not able to identify which exact species was used in this study, and organisms were designated as *Mytilus spp*.

After collection and screening, where apparently healthy animals with 5 ± 1 cm of shell length were brought to the laboratory and submitted to a quarantine period of 15 days. During the quarantine period, animals were kept in 60 L aquaria under controlled conditions (salinity 30 made with Tropic Marin Pro-Reef® sea salt, temperature 20 ± 1ºC, pH 8 ± 0.5, photoperiod 16 L: 8D, and continuous aeration with a density of 150 animals per 60L). During this period, animals were fed twice a week with a commercial *Tetraselmis sp.* formulation (Phyto bloom prof ©) with 150.000 cells per animal. Medium was renewed once a week and dead animals were immediately discarded.

4.2 Exposure

Animals were exposed in 1 L plastic containers to concentrations of both ACZ and SA based on levels already reported to occur in the environment (Singer et al., 2016), and on worst case scenarios. These concentrations, for exposure to single drugs, were 10, 100, and 1.000 µg/L of ACZ (C1; C2; C3 respectively); 100, 1.000 and 10.000 µg/L of SA (C4; C5; C6). Exposures with combinations of both drugs were performed using 10µ g/L of ACZ + 100 µg/L SA (C7) and 1.000 µg/L ACZ + 10.000 µg/L SA (C8), in addition to a negative control group (C0, without pharmaceutical). Stock solutions of the pharmaceuticals were prepared in distilled water. 10 animals per treatment (replicates), for a total of 90 animals, were individually exposed in 1 L plastic bottles, for a period of 28 days (chronic exposure). Media were totally renewed trice a week, and animals were fed with *Tetraselmis sp.* (Phyto bloom prof ©) at a density of 150.000 cells per animal, everyday 2 days (after media were renewed). Experimental conditions were the same as those adopted for the quarantine period. Tests were performed following an adapted version of the Ecological Effects Test Guidelines: OPPTS 850.1710. Oyster BCF (Environmental Protection Agency EPA, 1996) After 28 days of exposure, animals were sacrificed and dissected in ice-cold sea water (4ºC) and tissues of interest (gills and mantle) were separated and stored at -80ºC until enzymatic assays.

4.3 Feeding test

After 27 days of exposures, 7 randomly chosen animals were transferred to clean (drug free) media containing $3.5*10^8$ cells of *Tetraselmis sp.* (Phyto bloom prof ©) per litre and were kept for one hour, to allow the filtration and uptake of algal cells. Media samples had their absorbance immediately measured at 440 nm and compared with the control treatment (Thermo Scientific Multiskan (SkanIt Software 2.4.4 RE for Multiskan Spectrum)). Following this period, animals were returned to test media.

4.4 Biometrical parameters

In terms of biometrical parameters (namely shell length, width and animal weight) these were assessed before the onset of the chronic test. At the 28th day, animals were removed from test media and were again weighted (scales - GH-252), and had their shells measured using calipers. Furthermore, after the removal of their soft tissues, shells were dried for later assessment of their resistance. This tests were made with as described in Dionísio et al. (2020). Briefly, random shells from the tested organisms were submitted to vertical pressure until rupture. The maximum force (N) needed to do so was measured using a Texture Analyser (Stable Micro Systems; Godalming, UK) equipped with a 30 kg loading cell. The data was collected by the apparatus software Texture Exponent 32 (version 6.1.12.0, Stable Micro Systems; Surrey, UK). A 4 mm diameter metal cylinder probe (TA-56) was used to apply a growing force at the shell's apex point; the probe was lowered at a speed of 1 mm/s. Since the dimensions of the shells will directly influence their resistance, the SH was normalized by dividing the maximum force with their ratio of diameter/height.

4.5 Biomarker quantifications

Tissue samples for the quantification of CA activity were homogenized in Tris-sulphate 25 mM (pH 7.5); for COX activity determination, samples were homogenized in 0.1M Tris-HCl (pH 7.8) with 1mM EDTA. All homogenizations were performed in a Brandson Sonifier 250 (constant cycle ultrasounds for approximately 30 seconds), followed by a centrifugation at 10,000g for 40 minutes at 4ºC for CA; and 10,000g for 15 minutes at 4ºC for COX. All samples were centrifuged in (Eppendorf 5810R centrifuge).

CA activity was measured according to Verpoorte et al. (1967) where the increment in absorbance resulting from the degradation of p-nitrophenyl acetate (pNPAc) by CA was followed at 400 nm. Results were expressed as µmol of oxidized pNPAc per minute per milligram of protein (U).

COX activity was measured by using the method described by Petrovic & Murray (2009), by monitoring the oxidation, at 590 nm, on N,N,N',N'-tetrametil-p-phenylenediamine (TMPD) by PGG_2 resulting from conversion of arachidonic acid

by COX. Results were expressed as nmol of TMPD oxidized per minute per mg of protein (U).

Total soluble protein quantification of samples was performed at 595 nm using the Bradford method (Bradford, 1976), adapted to microplate, with 1mg/mL bovine γ-globulin as standard.

All spectrophotometrical readings were performed in a microplate reader Thermo Scientific Multiskan (SkanIt Software 2.4.4 RE for Multiskan Spectrum). Results were expressed in U/mg protein.

4.6 Statistical analysis

Both one-way analysis of variance (ANOVA) assumptions (homogeneity of variance and normal distribution) were tested prior to the statistical analysis. Afterwards an ANOVA was performed using software SigmaPlot v.14. A post-hoc test (Dunnett) was performed with a significance level of $\alpha = 0.05$.

5. Results

In terms of CA, this enzyme seemed to present in higher levels in *Mytillus* spp gills. Moreover, it was in this tissue that ACZ caused an inhibition of this enzyme, in animals exposed to the highest concentration, and also in mussels exposed to the least concentrated mixture (C7; Fig. 3 B; $F_{(8;80)} = 2.859$, $p<0.05$). No pattern was observed in terms of CA activity in the mantle (Fig. 3 A; $F_{(8;60)} = 3.087$, $p<0.05$). COX activity was again higher in gills than in the mantle. Additionally, this enzyme activity decreased, despite not always statistically significantly, in both tissues of animals exposed to both mixtures (Fig. 3 C; D; Gills: $F_{(8;53)} = 4.246$, $p<0.05$; Mantle: $F_{(8;54)} = 2.277$, $p<0.05$).

In other parameters namely feeding, shell index and shell hardness, the here tested pharmaceuticals caused no alterations, for any of the tested concentrations (Fig. 4; Feeding: $F_{(8;56)} = 1.764$, $p=0.108$; Shell hardness: $F_{(8;47)} = 0.934$, $p=0.500$; Shell index: $F_{(8;48)} = 0.775$, $p=0.627$)

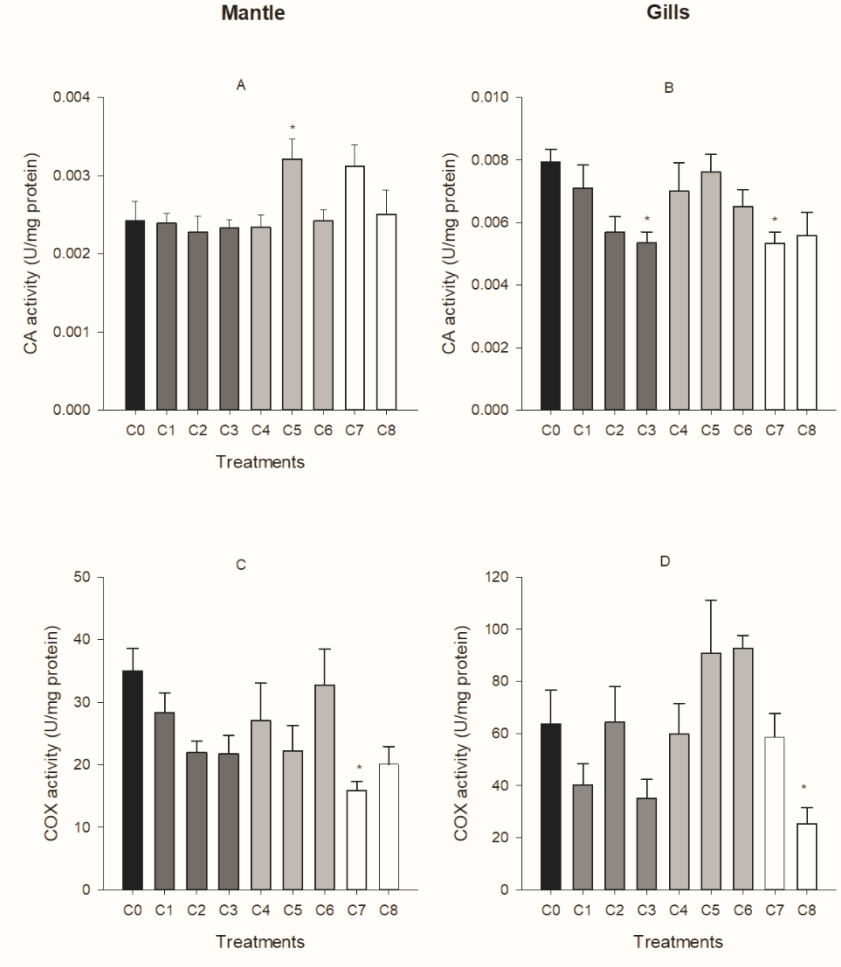

Figure 3. Effects of ACZ and SA on *Mytillus spp.* CA (A) and COX (C) in mantle and CA (B) and COX (D) in gills. x-axis represents treatments (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L ACZ; C4, C5, C6 – 100, 1,000, 10,000 µg/L SA; C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). n=10. * stands for statistical differences (Dunnett's test: $p < 0.05$) among treatments and the control group (C0).

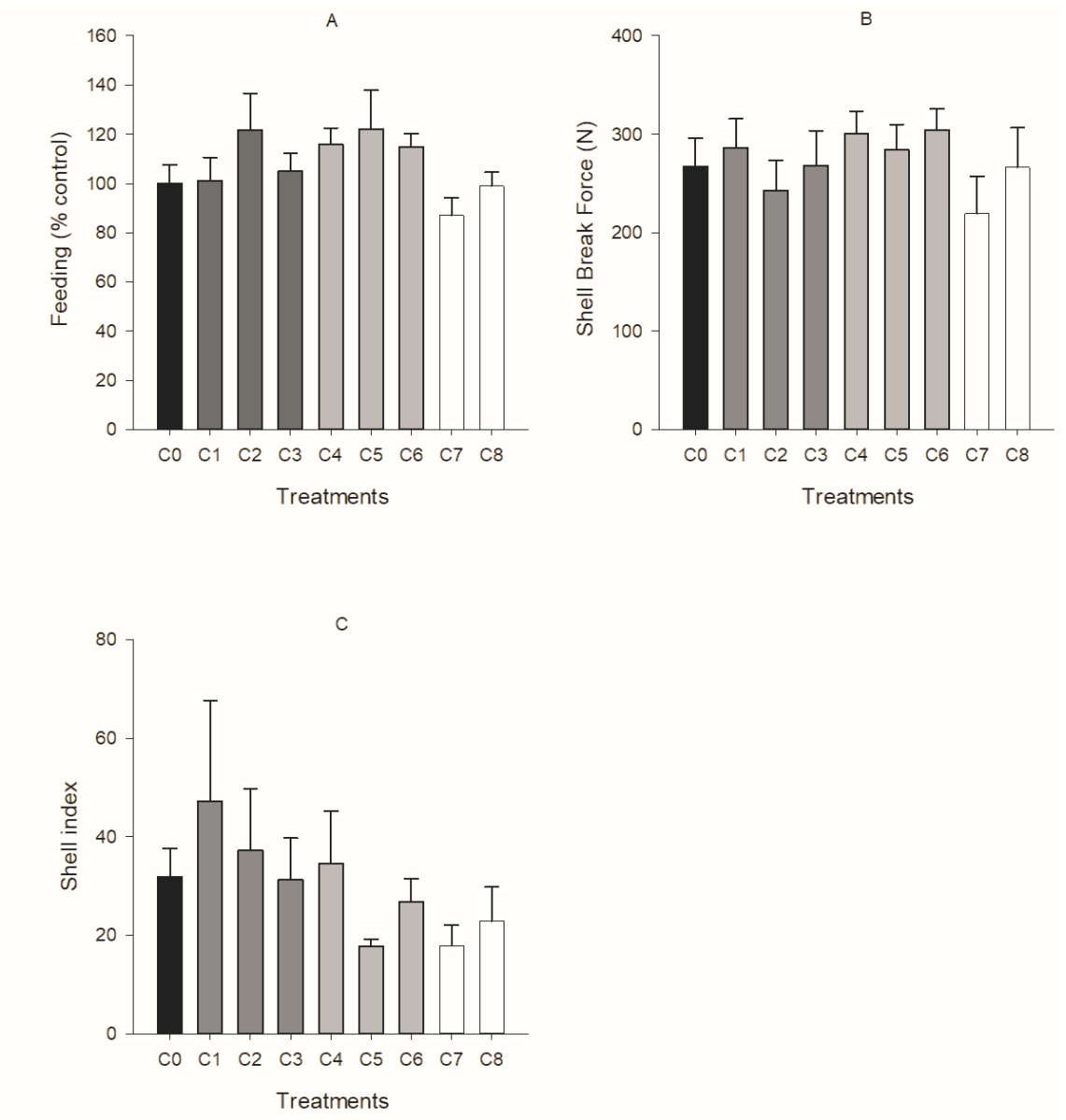

Figure 4. Effects of ACZ and SA on *Mytillus spp.* Feeding (A), shell break force (B) and shell index (C). x-axis represents treatments (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L ACZ; C4, C5, C6 – 100, 1,000, 10,000 µg/L SA; C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). n=10.

6. Discussion

The effects of ACZ as a CA inhibitor have been demonstrated in several marine species (Bertucci et al., 2013). This particular effect was thought to be responsible for the acidosis observed in the haemolymph, of *Cancer productus* as demonstrated by McMahon et al. (1984). This acidosis caused by ACZ is extremely important for the discussion of the excretion of acidic drugs, such as SA. The excretion of SA is highly pH dependent, being more easily absorbed in lower pH values due to its acidic nature. Under such conditions of higher pH, SA occur in its water-soluble form, being promptly excreted from vertebrates (Vree et al., 1994). In a condition of acidosis, salicylates are more likely to stay in the organism, and are prone to exert possible toxic effects. Exposure to NSAIDs may cause an inhibition of COX activity (Hinz et al., 2000); SA, being a NSAID, is expected to cause a similar inhibition of these enzymatic forms. The hypothesis that ACZ could cause acidosis, thereby increasing the accumulation of SA in the organism, was somehow validated by the hereby obtained data. The absence of significant decreases in COX activity in mussels exposed to separate treatments of each drug was not expected. Nevertheless, when animals were exposed to combined treatments of SA and ACZ, a significant decrease of COX activity in both tissues (gills and mantle) was registered. This can be explained by considering the above-mentioned mechanism of SA absorption, for which drugs that inhibit carbonic anhydrase can strongly contribute. By inhibiting CA activity, ACZ could lead to an increment of HCO_3^- which can cause an acidosis scenario, compromising the excretion of SA. This way, SA stays in higher concentrations and for longer periods inside the previously exposed animal.

Another important role of CA in molluscs is related to the regulation of intracellular pH, which in turn modulates calcium availability in shell mineralization processes (Wang et al., 2017; Caricato et al., 2018; Rodriguez-Navarro et al., 2019). By being involved in the pH regulation, CA contributes directly for the availability of Ca^{2+} from most calcium forms with which molluscs are included. Consequently, CA activity is one of the mechanisms responsible for the availability of Ca^{2+}, a cation of fundamental importance in the mantle in order to occur shell synthesis (Marin et

al., 2012). This role was already reported to occur in other marine organisms (namely corals) where CA inhibition by ACZ can lead to significant decreases (up to 73 %) in the biomineralization process (Bertucci et al., 2013) which can consequently affect shell hardness and thickness leading to an increase vulnerability against erosion and predation. Nevertheless, ACZ caused no significant alteration in CA activities measured in the mantle of exposed mussels. This can suggest that, despite the considerable duration of the test, ACZ exposure may have not being long enough to allow attaining concentrations in mantle tissues to deleteriously interfere with CA activity, in order to cause significant adverse or otherwise inhibitory effects, namely in processes related to the growth of shells. In addition, the animals that were used for this bioassay were adults, and their shells may be already stable. The impact of these drugs may occur with more adverse consequences in early life stages, where shell development is still occurring.

The hereby data showed an inhibition of CA activity, caused by the highest concentration of ACZ, in gills but not in mantle tissue. This difference may be due to the nature of this animals; being filter feeders, gills are the first organs to be affected by waterborne xenobiotics (Aldoghachi et al., 2016). Since CA is involved in the control of CO_2 levels in fish and in some invertebrates' gills, the inhibition of this enzyme in this tissue may have repercussions in the capacity of gas exchange, and consequently on the environmental fitness of exposed animals. McMahon et al. (1984) showed that, when individuals of the crustacean *Cancer productus* were injected with ACZ, they had an increase in CO_2 levels in their haemolymph.

According to Hartmann et al., (2015), alterations in filtration rates in mussels may be caused by closure of valves, which is a mechanism of defence against contaminants. In fact, valve closure, is described as a response mechanism when *Mytillus galloprovincialis* were exposed to cypermethrin (Ait Ayad et al., 2011). In other marine species, namely *Anodonta woodiana*, valve opening was significantly affected by several different contaminants, such as metals (arsenic), detergents (sodium dodecyl sulphate) and anesthetic (trichloroethylene), among others (Giari

et al., 2017). These authors suggest using this behaviour, among others, to assess the extent of exposure of these organisms to contaminants.

In terms of ecological traits, and in direct relation with valve closure, feeding rate is extremely important since xenobiotics that compromise this endpoint may directly jeopardize the animals' fitness (Hartman et al., 2016). The here tested pharmaceuticals did not cause any alteration in the feeding rate of individuals of *Mytillus spp.* Nevertheless, other authors have shown that NSAIDs have the potential to modify the feeding behaviour of mussels (Solé et al., 2010). However, this is not a straightforward pattern, since this response seems to be highly species-specific. In terms of ASA, the SA precursor, Piedade et al. (2020) similarly to this study, showed no alterations in the feeding rate of *Mytillus spp.* exposed to ASA, suggesting SA does not cause alterations in this genus filtration rate neither by decreasing filtration or increasing food uptake.

In bivalves, shells (valves) offer protection against abiotic and biotic factors, such as predation (Sherker et al., 2017). Structural alterations in valves can jeopardize animal survival, by reducing shell thickness or creating abnormalities which can ultimate be weak point. The here obtained results showed that shell biometrics and resistance were not significantly affected by both pharmaceuticals. Considering that ACZ is a CA inhibitor, and that this enzyme plays a key role in Ca^{2+} availability for shell formation (Marin et al., 2012), effects on shell features could be expected. An inhibition of this enzyme is likely to cause a decrease in shell formation and shell resistance (Bertucci et al., 2013). Despite the absence of effects in the here used animal models, a similar effect of skeleton weakening was already reported to occur in corals (Bertucci et al., 2013). Exposure to conditions that compromise the activity of CA (namely exposure to ACZ and ethoxzolamide, two CA inhibitors) are causative of a significant impairment in calcium fixation and incorporation into the calcium-based shell matrices, thereby leading to the weakening of their integrity, and affecting growth and survival. Nevertheless, those alterations were not observed in our experimental organisms. Another possible explanation for the absence of significant results in terms of shell features may be due to the average life span of the here used animals. Some individuals of the genus *Mytillus* can live up to 20 years, and a 28-day exposure during their adult

stage may not be long enough to significantly cause alterations in shell hardness. Nevertheless, it is necessary to keep in mind that other life stages (namely larval stages) as well as already damaged animals (by wave action or predators) may have different sensitivity in terms of this endpoint, since their shells are not yet formed and consolidated, and disruptions in this process may lead to adverse effects. Considering the fact that global climate changes are a reality, and that ocean acidification can also cause a significant decrease in shell biomineralization (Marin et al., 2012), the effects caused by pharmaceuticals as environmental contaminants can contribute as an additional factor for shell thickening. This can lead to an additional vulnerability to predators and to wave erosion, since shells are calciferous structures which are not only used for structural and integrity purposes but also for predator defence (Sherker et al., 2017).

In conclusion, data from specific biomarkers, namely COX and CA that were selected as direct indicators of the known pharmacological effects of both drugs, seem to support the assumption that metabolic acidosis might have occurred, since COX activity was only inhibited when SA was in mixture with ACZ. This may highlight the potential threat to organisms posed by mixtures of contaminants in the environment, which can potentiate the effects of single chemicals, and alter the response patterns of organisms. Nevertheless, shell index, shell hardness and feeding behaviour were not affected by the here tested concentrations of both drugs. This may mean that these processes are relatively stable in adult organisms and are not prone to be compromised by these chemicals.

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Chapter 4

Evaluation of single and combined effects of two pharmaceuticals on *Phorcus lineatus* enzymatic activity under two different exposure periods

Submitted to Environmental Science and Pollution Research

1. Abstract

Pharmaceutical drugs are among the most used chemicals, for human and veterinary medicines, aquaculture and agriculture. Pharmaceuticals are environmentally persistent, biologically active molecules, thereby having the potential to exert biological effects on non-target species. Among the most used pharmaceuticals, one may find salicylic acid (SA), a non-steroid anti-inflammatory drug (NSAID), and acetazolamide (ACZ), a diuretic that acts by inhibiting the activity of carbonic anhydrase (CA). In this work, the effects of both single and combined effects of these drugs were assessed on the marine gastropod *Phorcus lineatus*, by measuring key enzymatic activities, namely carbonic anhydrase (CA) and cyclooxygenase (COX), under two different exposure periods (14 and 28 days). We observed no straightforward pattern of enzymatic response in all treatments of both pharmaceutical, on both tissues analysed, and for both exposure regimes. We assume that this species is nor responsive to the hereby tested pharmaceuticals, a finding that may be due to general mechanisms of response to adverse conditions, such as reduction of metabolism, of heart rate, of filtration rates, and to the increase production of mucus. All these functional adaptations can mitigate the deleterious caused by adverse conditions, without triggering biochemical responses. In conclusion, the species *P. lineatus* seems not to be sensitive in terms of these specific enzymatic pathways to these contaminants, under the adopted conditions.

2. Keywords: acetazolamide; salicylic acid; carbonic anhydrase; cyclooxygenase; gastropod

3. Introduction

Pharmaceuticals are widely used in several areas such as human and veterinary medicines (Kaczala & Blum, 2016), but are also used in cattle raising (Laloučková & Skřivanová, 2019) and aquaculture (He et al., 2016; Singh & Singh, 2018). This wide usage and consequently metabolism and excretion, in both the form of conjugates or unaltered parental drug, leads their discharge into sewages. To treat the excreta from pharmaceutical drugs, municipal systems were established, namely wastewater treatment plants (WWTP) in which contaminants are removed through a variety of physical-chemical processes, before the water re-enters the environment (Martin & Vanrolleghem, 2014; Patel et al., 2019). Nevertheless, some contaminants, in which pharmaceuticals are included, are in some part resistant to these processes and may persist in the effluents from WWTPs and in the wild (Fan et al., 2020). Consequently, this process leads to an ever-increasing presence in the environment, with a solid tendency for an augmented frequency of detection of these substances in the environment, by a large number of studies, in concentrations ranging from ng/L to µg/L (Chander et al., 2016; Fan et al., 2020).
Among those drugs of particular interest, one may find a class such as non-steroid anti-inflammatory drugs (NSAIDs). This class is one of the most used worldwide and consequently released in the environment (Sharma & Kaushik, 2017). Pharmacologically, this class acts by inhibiting both isoforms of the enzyme cyclooxygenase (COX-1 and COX-2), preventing the synthesis of prostaglandins and tromboxanes (Fokunang et al., 2018). These molecules are involved in the inflammatory process, mediating pain and fever (Zarghi & Arfaei, 2011). NSAIDs act by pharmacologically inhibiting the biosynthesis of this intermediates, thereby acting as antipyretic, analgesic and anti-inflammatory substances. One of the main drugs of this class is salicylic acid (SA), which is used as an analgesic and antipyretic. SA results from the de-acetylation of acetyl-salicylic acid (ASA), being ASA principal active principle. From there, it is uptaken by the liver, where it is conjugated with glycine and glucuronic acid, leading to the formation of salicyluric acid, and acyl and phenolic glucuronide, respectively the most important forms of conjugation. Nevertheless, a small portion of SA is hydroxylated into gentisic acid

(Bojić et al., 2015). From the environmental contamination point of view, due to its relatively short environmental half-life, SA is found low in concentrations ranging up to 4 µg/L in WWTP effluents (Metcalfe et al., 2003; Carballa et al., 2004; Martín et al., 2012) and almost up to 1 µg/L in Belgium coastal zones (Claessens et al., 2013). Moreover, SA has been shown to cause deleterious effects on non-target organisms, including in antioxidant enzymatic activities of zebrafish (Zivna et al., 2016), on marine polychaete *Hediste diversicolor* (Gomes et al., 2019; Nunes, 2019), on the freshwater *Salmo truta fario* (Nunes et al., 2015), and on the freshwater microcrustacean *Daphnia magna* (Goméz-Oliván et al., 2014)

Another class of ecotoxicological interest are diuretics, which are drugs which promote diuresis (Oh & Han, 2015). There are several types of diuretics, considering the mechanism and location within the nephron where they exert their activity, to promote the diuresis. Among commercially available diuretics, one may find potassium-sparing diuretics, thiazide diuretics, and carbonic anhydrase inhibitors (CAI; Roush et al., 2013). This last group exerts its pharmacological effects by inhibiting the activity of the enzyme carbonic anhydrase (CA; Carta & Supuran, 2013), which is a key enzyme in a variety of processes such as interconversion of bicarbonate ion (HCO_3^-) into carbon dioxide (CO_2) and water (H_2O). This reaction is important in several key biological processes, namely for maintaining the acid base homeostatic state of an organism, gas exchanges (Shuttleworth et al., 2006), being also one of the enzymes responsible for the calcium (Ca^{2+}) for the calcification processes (Marin et al., 2012) as well as in processes of bone resorption (Lehenkari et al., 1998), and tumorigenicity (Benej et al., 2014). An example of a pharmaceutical from this group is acetazolamide (ACZ). ACZ is used for the treatment of glaucoma, altitude sickness and epilepsy (Low et al., 2012; Liu et al., 2016). Despite not being metabolized, in humans, ACZ has the capacity of causing metabolic acidosis, which can affect the absorption, metabolism and excretion of other pharmaceuticals (Pham et al., 2015). This particular pharmaceutical has been used since the 1950's and is included in the list of essential medicines by the World Health Organization However, very few studies were ever published assessing its effects on non-target organisms (WHO, 2019). In fact, the environmental concentrations of ACZ have been only

determined in one study, and its levels were found to be in the range of ng/L (Singer et al., 2016).

The presence of pharmaceuticals in the environment can deleteriously alter the physiology of non-target marine species; among these, organisms from the class Gastropod are included. This class is used as a test species in ecotoxicological studies, since these animals are known to be them adaptable to a variety of different environments, contributing to their wide distribution. From this class, the herbivorous marine snail *Phorcus lineatus* (Archeogastropoda: Trochidae) may be highlighted, due to its reduced mobility, easy sampling, continuous availability throughout the year (Crothers, 2001; Cunha et al., 2007), and sensitivity to contaminants, specifically pharmaceuticals (Almeida & Nunes, 2019). These characteristics make the use of this species an adequate candidate in ecotoxicological studies.

In addition to the type of contaminant, and the judicious selection of test organisms, the performance of ecotoxicological bioassays is strongly conditioned by the duration of the exposure period. Guidelines usually suggest strict exposure periods, divided in chronic and acute exposures based on the organism's life cycle duration, and its life spam. Nevertheless, intermediate exposure periods also should be assessed in order to characterise the effects of contaminants along time, and therefore trace a pattern of response to infer if the duration of exposure is a factor which affects the toxicity of a given contaminant. The study of the time course of the intoxication is also important logistically, in order to propose shorter exposure periods than those recommended by testing guidelines.

In this study, we evaluated the effects of SA and ACZ, after single exposures and in combination, on *P. lineatus* enzymes, namely COX and CA activities, to indirectly assess the effects of the pharmaceuticals, Moreover, two different exposure times (14 and 28 days) were evaluated in order to assess *P. lineatus* response thought time to these contaminants.

4. Material and Methods

4.1 Animal sampling and quarantine

These top shells usually occupy the upper half of the intertidal zone (Desai, 1966). Organisms from the species *P. lineatus* were manually collected during the low tide period, at a rocky beach of Vila Nova de Gaia, North of Portugal (41° 07′17″N, 8° 40′00″W), which is mostly free from anthropogenic sources of chemical contamination. The selected animals were 16 ± 1 mm in height and weight 1.9 ± 0.4 g.

After collection, animals were brought to laboratory facilities in plastic boxes with seawater from the sampling site. After arriving at the laboratory facilities, animals were transferred to 60 L plastic boxes with artificial seawater, with a salinity 30 ± 1 (Tropic Marin Pro-Reef® sea salt), mechanical filtration, continuous aeration, temperature of 19 °C ± 1 °C, photoperiod of 16 h L:8 h D, in a final density of 20 organisms/L. Where Animals were kept in these conditions, for a 14- day period, for quarantine, depuration and acclimation. Animals were fed weekly with vegetable protein concentrate (Sparos®). Animals were inspected daily, and dead animals were immediately removed.

4.2 Exposure

Animals were individually exposed in 200 mL glass flasks with artificial seawater (salinity 30± 1; Tropic Marin Pro-Reef® sea salt) in the same conditions as those described for the quarantine period, to concentrations of ACZ and SA based on levels already found in the environment and worst case scenarios. SA concentrations ranging from 0.855 up to 330 µg/L were already documented in WWTP effluents (Metcalfe et al., 2003; Claessens et al., 2013). ACZ was detected in WWTP effluents in concentrations up to 180 ng/L (Singer et al., 2016). A stock solution of each pharmaceutical was prepared in distilled water. From here, each tested concentration was prepared by diluting the stock solution into final nominal concentrations of 10, 100, 1.000 µg/L of ACZ (C1; C2; C3, respectively), and of 100, 1.000, 10.000 µg/L of SA (C4; C5; C6, respectively). In addition to concentrations of each drug, we also tested combinations of the two drugs, 10 µg/L ACZ + 100 µg/L SA (C7), and 1.000 µg/L ACZ + 10.000 µg/L SA (C8), based on the lowest and highest concentrations of each pharmaceutical previously

selected. In addition, a negative control (C0) was also used. 7 replicas per treatment were adopted, in a total of 63 animals. To test if the duration of the exposure could affect *P. lineatus* capacity to cope with contaminants, a sub-chronic and a chronic exposure (14 and 28 days respectively) were adopted. Following these periods, animals were sacrificed by immersion in ice cold seawater. The gut and the muscular foot were mechanically removed, dissected, and isolated with scalpels on Petri dishes; immediately after removal, tissues were inserted in Eppendorf microtubes, and stored at -80º C for until enzymatic determination.

4.3 Sample preparation and enzyme quantification

Tissue samples, previous collected and stored at -80ºC, were then processed for the determination of enzymatic activities. For the determination of carbonic anhydrase (CA) activity, tissues were homogenized (Brandson Sonifier 250 in constant cycle ultrasounds for approximately 30 seconds) in 1 mL of Tris-sulphate 25 mM (pH 7.5) buffer and then centrifuged at 10,000g for 40 minutes (Eppendorf 5810R centrifuge). CA activity was assayed according to Verpoorte et al. (1967) where degradation of p-nitrophenyl acetate (pNPAc) by CA was followed at 400 nm, and results were expressed as µmol of oxidized pNPAc per minute per milligram of protein (U).

For the determination of COX activity determination, samples were homogenized (Brandson Sonifier 250 in constant cycle ultrasounds for approximately 30 seconds) in 0.1 M Tris-HCl (pH 7.8) with 1 mM EDTA. Afterwards, a centrifugation of 10,000g for 15 minutes at 4ºC (Eppendorf 5810R centrifuge). COX activity was measured by using the method described by Petrovic & Murray (2009), by monitoring the oxidation, at 590 nm, on N,N,N',N'-tetrametil-p-phenylenediamine (TMPD) by PGG2 resulting from the conversion of arachidonic acid by COX. Results were expressed as nmol of TMPD oxidized per minute per mg of protein (U)

The quantification of total soluble protein of samples was performed at 595 nm using the Bradford method (Bradford, 1976), adapted to microplate, with 1mg/mL

bovine γ-globulin as standard. All parameters were spectrophotometrically measured, and the readings were performed in a microplate reader Thermo Scientific Multiskan (SkanIt Software 2.4.4 RE for Multiskan Spectrum).

4.4 Statistical analysis

Both ANOVA assumptions (homogeneity of variance and normal distribution) were tested prior to the statistical analysis. Afterwards a one-way analysis of variance (ANOVA) was performed using software SigmaPlot v.14. A post-hoc test (Dunnett) was performed with a significance level of $\alpha = 0.05$.

5. Results

ACZ and SA exposure did not cause any alteration in terms of CA activity in both gut (Fig. 5 A and C; respectively: $F_{(8;51)} = 2.848$, $p=0.012$; $F_{(8;53)} = 5.855$, $p<0.05$) and muscle (Fig. 5 B and D; respectively: $F_{(8;53)} = 2.024$, $p<0.065$; $F_{(8;53)} = 2.253$, $p=0.292$). Moreover, results obtained after exposing animals to the different exposure periods (14 and 28 days) also did not show any straightforward alterations of CA activity when compared to control animals. In addition, both pharmaceuticals did not cause any alteration of COX activity in both tissues (gut in muscles), following both exposure regimes (14 and 28 days; Fig. 6; gut 14 days: $F_{(8;46)} = 1.509$, $p=0.187$; muscle 14 days: $F_{(8;43)} = 0.921$, $p=0.511$; gut 28 days: $F_{(8;43)} = 1.107$, $p=0.382$; muscle 28 days: $F_{(8;57)} = 3.105$, $p<0.05$).

In terms of mixture of contaminants, none of the here tested conditions caused alterations of CA or COX activity, in both tested tissues (gut and muscle) at both exposure times (14 and 28 days; Fig 5 and 6).

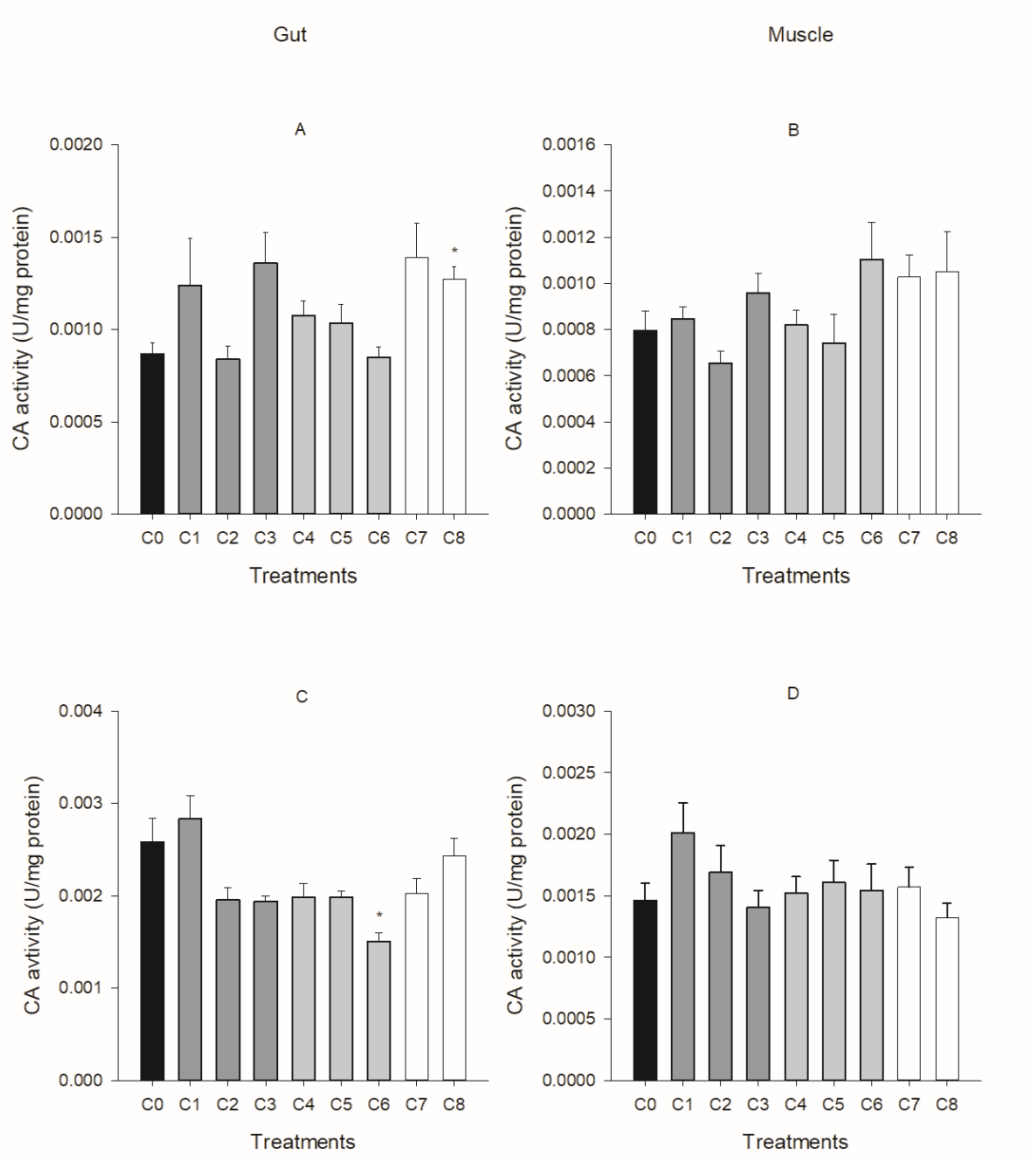

Figure 5. Effects of ACZ and SA on *P. lineatus* carbonic anhydrase (CA) activity. Data for gut (A and C) and muscle (B and D) after a 14-day exposure (A and B) and 28-day exposure (C and D). x-axis represents treatments (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L acetazolamide (ACZ); C4, C5, C6 – 100, 1,000, 10,000 µg/L salicylic acid (SA); C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). Each bar represent means ± se. n=7. * stands for statistical differences (Dunnett's test: $p < 0.05$) between treatments and the control group (C0).

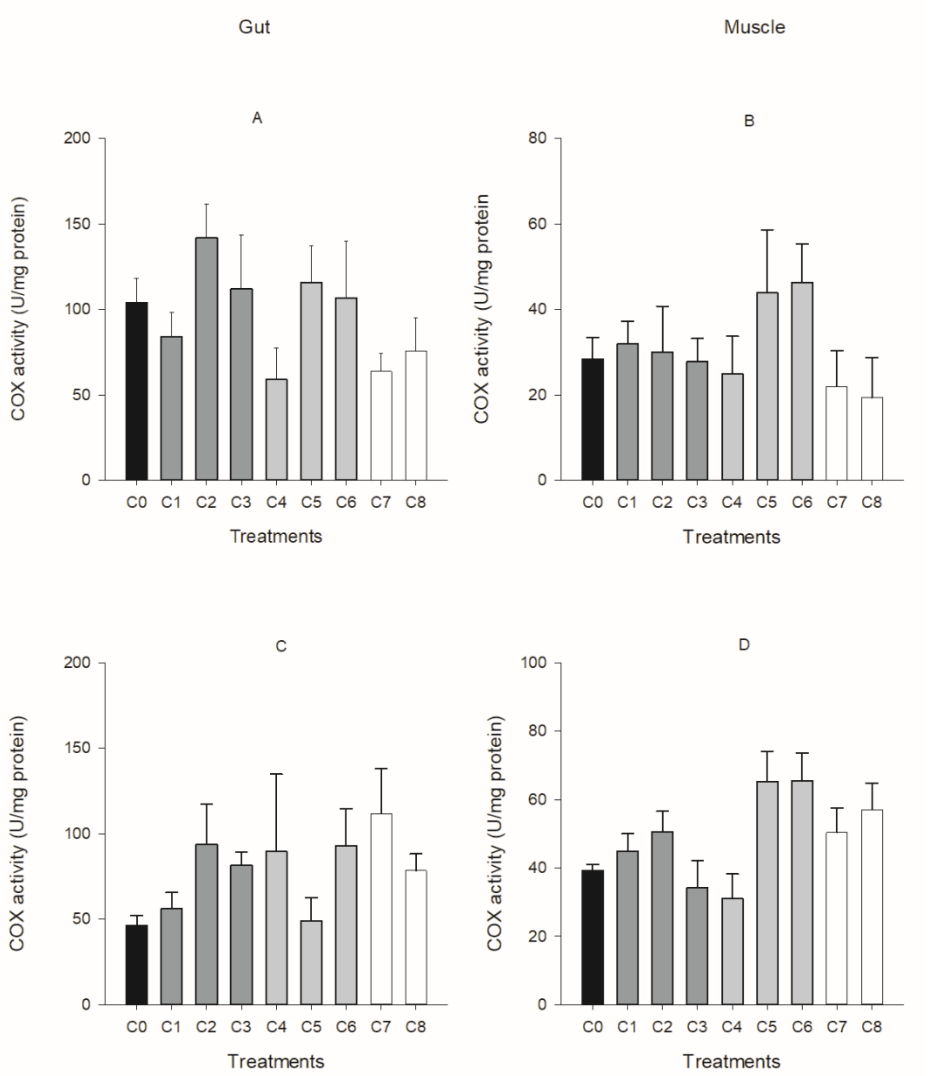

Figure 6. Effects of ACZ and SA on *P. lineatus* cyclooxygenase (COX) activity. Data for gut (A and C) and muscle (B and D) after a 14-day exposure (A and B) and 28-day exposure (C and D). x-axis represents treatments (C0 – 0 µg/L; C1, C2, C3 – 10, 100, 1,000 µg/L acetazolamide (ACZ); C4, C5, C6 – 100, 1,000, 10,000 µg/L salicylic acid (SA); C7 – 10 µg/L ACZ + 100 µg/L SA; C8 – 1,000 µg/L ACZ + 10,000 µg/L SA). Each bar represent means ± se. n=7.

6. Discussion

Carbonic anhydrase is an enzyme responsible for the regulation of a variety of physiological processes. Among them, one may find processes of acid base homeostatic maintenance, gas exchanges (Shuttleworth et al., 2006), and the regulation of the available calcium (Ca^{2+}), which is used for calcification processes (Marin et al., 2012). The involvement in these processes makes CA of extreme importance in living organisms. The here evaluated concentrations of ACZ did not cause any alterations in CA activity in both tested tissues, and for both tested exposure periods. These data were contradictory to the common patters of pharmacological activity, especially of ACZ, which is a CA inhibitor. No pattern was observed in CA activity was observed when exposing individuals of *P. lineatus* to SA concentrations. This absence of effects can be due to the high variety of CA isoforms that exist in biota; some of these forms, are low activity enzymes, sulfonamide-resistant, usually well represented in muscles of mammals (Jeffery et al., 1986; Frémont et al., 1989). Due to the wide distribution of this enzyme through several taxa, it is expectable that this isoform may be also present in molluscs, particularly in *P. lineatus*. In fact, the activity of CA was assessed in several marine invertebrates, and it was concluded that each species has a value of basal CA activity which depends on the evaluated tissue, being their sensibility towards ACZ also variable (Nielsen & Frieden, 1972)

SA is a NSAID, and the main pharmacological effects caused by this class of drugs is the inhibition of both isoforms of COX activity (Fokunang et al., 2018). Consequently, it was expectable that this enzyme activity would be inhibited in our experimental animals after being exposed to SA. Nevertheless, this inhibition was not observed in both tissues of the here exposed *P. lineatus*, under both tested exposure times. A possible explanation for this is that the used concentrations of SA are not enough to cause observable effects on COX activity. Another possible explanation are the inhibitory effects of SA are not particularly evident in some species, making SA a weak COX inhibitor (Hinz et al., 2000). In fact, Dionisio et al. (2020) also reported the absence of COX activity inhibition when exposing another marine gastropod, *Gibulla umbilicalis*, to SA. This can suggest that marine

gastropods are not sensitive to this pharmaceutical in terms of COX activity. In fact, the comparison between COX enzymes that occur in corals and in mammals demonstrate that these enzymes have a slight difference in their active sites (an amino acid substitution), causing the invertebrates enzyme to be nonresponsive to COX – 2 inhibitor (Koljak et al., 2001). This can explain the lack of responsiveness of COX activity towards NSAIDs in invertebrates.

Moreover, the general absence of patterns of alterations can be due the presence and production of mucus. Mucus is a secretion produced by several aquatic and terrestrial organisms which can help animals in a variety of ways such as mating, energy saving, pathogen prevention, contaminant prevention and excretion (Ng et al., 2013). In fact, this secretion has been found to be increased in adverse conditions (increased temperature, metal exposure) in the marine gastropod *Nucella lapillus* (Leung et al., 2000). This can suggest the involvement of mucous in the protection against other types of contaminants. In fact, Gomes et al. (2019) registered an increase in volume of mucous glands in the marine polychaete *Hediste diversicolor* after SA exposure. This can suggest a mechanism of protection against SA deleterious effects could be trough mucus.

Additionally, another physiological trait of this species should be considered when assessing the effects of contaminants, which is related to the presence of an operculum. Similarly to other groups, such as molluscs (mussels), this operculum allows a specific behaviour which makes possible to isolate animals from the external media, thereby preventing their exposure to adverse conditions, such as exposure to contaminants, and to environmental changes such as salinity drops (Hoyaux et al., 1976; Berger & Kharazova, 1997). However, this behaviours is only possible for a few hours, not being able to protect animals for a long time (HRS-BRENKO, 2006; Chapperon & Seuront, 2011; Marshall & McQuaid, 2011). Nevertheless, this behaviour can significantly modulate the effects of contaminants in exposed animals. Another mechanism that is activated in animals to deal with adverse condition consists in reducing the metabolic rate, reducing the water exchange in the mantle cavity, and/or decreasing the cardiac activity. This set of effects leads to a decrease of gill perfusion, consequently reducing the contact between internal tissues and external water (Liu et al., 1990).

In conclusion, marine gastropod species *P. lineatus* does not seem sensitive to ACZ and SA in terms of CA and COX activity. This absence of deleterious changes may could be due to several reasons such as mucus production or concentrations not high enough to cause detectable alterations in these parameters. Moreover, the two exposure periods may not be long enough to cause observable effects on the here evaluated endpoints, namely CA and COX activity.

7. References

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Chapter 5

Final remarks and future perspectives

The present work assessed the effects of two widely used pharmaceuticals, acetazolamide (ACZ) and salicylic acid (SA), on the activities of key enzymes, namely carbonic anhydrase (CA) and cyclooxygenase (COX), and other key physiological processes, including photosynthetic pigments such as chlorophyll a (Chl a), b (Chl b), total (TChl), carotenoids (Car), shell hardness (SH), filtration rate (FR) and shell index, determined on several non-target organisms, namely *Lemna gibba* (Chapter 2), *Mytillus spp.* (Chapter 3), and *Phorcus linatus* (Chapter 4).

This work made it possible to understand the very different effects of each pharmaceutical in organisms of each taxa. The profile and extension of deleterious effects, in organisms exposed to both single and mixtures of the mentioned drugs, was also very distinct. In fact, in *L. gibba*, the effects caused by single exposure to ACZ resulted in the reduction of content of photosynthetic pigment, namely of Chl a, Chl b, TChl and Car. However, this effect was reverted, close to normal control values, when plants of this species were simultaneously exposed to ACZ and SA. This set of results highlights the capacity of ACZ to jeopardize the photosynthetic efficiency of plants by altering photosynthetic pigment content. On the other hand, plants co-exposed to ACZ and SA had their pigments reverted to normal levels, suggesting that SA has a capacity to exert phyto protective effects.

Nevertheless, the scenario was different when animals of the genus *Mytilus spp.* were exposed to the same conditions. In this case, co-exposure to SA and ACZ, showed that SA did not act as a protector, but as an additional stress factor. In fact, the toxic effects that were observed after single exposures to ACZ resulted only in the inhibition of CA activity, and for the highest concentrations. When animals were simultaneously exposed to SA, this same effect was observed at lower concentrations of both pharmaceuticals. When it comes to COX activity, no straightforward pattern was observed after exposures to the isolated pharmaceuticals. However, when animals were exposed to mixtures of the two drugs, an inhibition of COX was observed in both tissues (gills and mantle), suggesting that the modulation of pH by ACZ caused a possible metabolic acidification scenario, thereby altering SA absorption and excretion, and causing more deleterious effect, namely by favoring the inhibition of COX activity. When it comes to other physiological parameters, namely FR, SH and shell index, no

alterations were observed in animals exposed to all treatments. This can be due to the phase of growth/development of the animals that were used. Fully grown and developed animals seem to be substantially less prone to suffer adverse effects on their shells, and alterations in CA activity may not end up in shell alterations. Additionally, filtering rate was also not affected. Nevertheless, the evaluation of potential effects of the here evaluated pharmaceuticals may be interesting if performed on not fully developed animals, since their shell development, which is key to their survival, is more likely to be affected.

The final species used in this study was *Phorcus lineatus* which after being submitted to the same pharmaceuticals, and in the same conditions as those described for the above-mentioned species, did not seem to respond in a similar manner. In fact, *P. lineatus* did not trigger a straightforward pattern of response, when it comes to CA and COX activity. This can be due to several mechanisms of defense present in these organisms; among them, we may suggest the closure of the operculum, isolating animals from the external media and from adverse conditions; in addition, it is also possible that the production of mucus, which acts as barrier from chemicals, was also activated. All these mechanisms can contribute to the higher resistance of this species to tested pharmaceuticals.

More studies need to be undertaken to assess the potential effects of ACZ and SA under a climate change scenario, to increase the body of knowledge about how an additional stress factor (namely, water pH) to and can modulate the responses of aquatic organisms to contamination scenarios. Moreover, the assessment of the adverse effects posed by other mixtures of pharmaceuticals has the potential to unravel potential interactions, that are likely to cause new, undescribed effects but that reflect environmental realistic conditions, especially when considering that a large array of contaminants are simultaneously present in complex aquatic matrices, and these chemicals often interact in the environment.

In conclusion, the potential effects of ACZ and SA are very different among non-target species. Therefore, the selection of test organisms should reflect the possibilities of different effects and, if possible, different taxa should be used to comprehensively evaluate the ecotoxicological effects of environmental contaminants, including pharmaceutical drugs. Additionally, the assessment of

mixtures of contaminants should also be assessed since the here observed toxic effects caused by individual or combined chemicals were totally different.