Universidade de Aveiro
2022

Beatriz Casal de Sousa

Avaliação de desinfectantes para combate da SARS-CoV-2 em *Danio rerio* - Uma análise embrionária, comportamental e bioquímica

Assessment of SARS-CoV-2 disinfectants in *Danio rerio* – *An e*mbryonic, behavioral, and biochemical analysis

Universidade de Aveiro
2022

Beatriz Casal de Sousa

Avaliação de desinfectantes para combate da SARS-CoV-2 em *Danio rerio* - Uma análise embrionária, comportamental e bioquímica

Assessment of SARS-CoV-2 disinfectants in *Danio rerio* – *A e*mbryonic, behavioral, and biochemical analysis

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Bruno André Fernandes de Jesus da Silva Nunes, Equiparado a Investigador Principal do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e da Doutora Paula Inês Borralho Domingues, Equiparada a Investigadora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro do POCTI no âmbito do III Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio.

“If you aim to be something you are not, you will always fail. Aim to be you. Aim to look and act and think like you. Aim to be the truest version of you. Embrace that you-ness. Endorse it. Love it. Work hard at it.”

In “The Midnight Library” – Matt Haig

o júri

presidente	Doutora Isabel Maria Cunha Antunes Lopes Investigadora Principal em Regime Laboral, CESAM & Departamento de Biologia, Universidade de Aveiro
arguente	Doutora Sara Raquel Boaventura Rodrigues Investigadora, CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental
orientador	Doutor Bruno André Fernandes de Jesus da Silva Nunes Equiparado a Investigador Principal, CESAM & Departamento de Biologia, Universidade de Aveiro

agradecimentos

Agradeço em primeiro lugar ao meu orientador, Doutor Bruno Nunes, pela já longa orientação, por todas as oportunidades que me deu para crescer como Bióloga, mas também como pessoa. Agradeço por nunca me ter dito o que fazer, mas ajudar-me a percorrer o caminho que escolhi trilhar. Obrigada pelas discussões científicas, filosóficas e teológicas. Obrigada! Agradeço de igual modo à Doutora Inês Domingues, pois sem ela esta dissertação também não seria possível. Obrigada pela sua paciência no laboratório e boa disposição.

Em segundo lugar agradeço aos meus pais, irmã e cunhado, sem os quais esta dissertação não seria possível. Obrigada por me ouvirem e darem força!

À minha tia, pela força e coragem que me ensinou a ter. Pelo seu exemplo de vida e fé. Obrigada! Estarás sempre nos nossos corações.

Ao Loureiro por me ter apoiado e levantado quando o cansaço era grande.

Obrigada aos meus amigos de longa data, que mesmo longe me apoiam e são exemplos de força de vontade. Obrigada por serem almas velhas comigo.

À Filipa e ao David por toda a paciência que tiveram comigo ao longo deste percurso. Agradeço por me terem mostrado este lado belo da ciência - a entreajuda. Obrigada pelos telefonemas tardios, pelos brainstormings e pelos desabafos. Sem vocês este percurso também não teria sido possível.

Ao David e à Sara, que por percorrer este mesmo percurso, me ajudou. Obrigada pela partilha de dificuldades e pela ajuda no laboratório.

Ao Gilberto, que apesar de não me ter acompanhado na dissertação, me abriu os horizontes para a ciência e me ajudou a desenvolver autonomia e espírito crítico. Obrigada!

A todas as pessoas nas instalações do peixe zebra, em especial à Carla e à Cátia, pelos debates científicos e acima de tudo pelas gargalhadas. Sem vocês a minha passagem por este laboratório não teria sido cheia de alegria. Obrigada!

Ao pessoal do 37 e das salas ao lado no Departamento de Biologia, pela ajuda incansável que tiveram comigo.

A todos os meus colegas que conheci ao longo destes 5 anos no departamento. A todos um grande obrigado! Hoje sou quem sou devido a vós.

palavras-chave

Zebrafish, disinfetantes, COVID-19, THY, BAC, SDBS, embriotoxicidade, comportamento, biomarcadores.

resumo

O aumento de incidência da SARS-CoV-2 à escala global decretou no início de 2020 uma situação de pandemia. Dentro das várias medidas de mitigação propostas por todos os governos e entidades responsáveis da área da Saúde Humana, o uso de desinfetantes foi uma das mais consensuais. Os desinfetantes passaram a ser aplicados nas residências, no exterior, em contexto hospitalar e para uso pessoal. Dado o aumento da procura, é expectado que os mesmos possam chegar em maiores quantidades aos ambientes aquáticos. Avaliar os possíveis efeitos destes compostos torna-se ainda mais imperativo, uma vez que se prevê um crescimento de 10% do uso de agentes desinfetantes até 2027. Entre as classes de desinfetantes mais usadas e mais frequentemente encontradas no ambiente, identificamos os tensioativos catiónicos e aniónicos, e aqueles cujas substâncias ativas são compostos naturais. Para o âmbito deste trabalho, foram assim selecionados o cloreto de benzalcónio (BAC), um agente tensioativo catiónico pertencente à família dos compostos de amónio quaternário, o sal do ácido dodecilbenzeno sulfónico de sódio (SDBS), agente tensioativo aniónico, e o timol (THY), um composto derivado de plantas com múltiplas aplicações. Apesar de haver alguns estudos sobre as propriedades e efeitos destes ingredientes ativos em organismos aquáticos, os seus efeitos adversos em espécies aquáticas continuam em grande parte desconhecidos. Nesse sentido, este estudo teve como objetivo avaliar, em larvas com cinco dias, da espécie *Danio rerio,* através de exposições agudas, biomarcadores embrionários, comportamentais (atividade total, comportamento tigmotático e movimentos erráticos) e metabólicos, como a atividade das enzimas de fase I (CYP 1A1 e CYP 1A2), enzimas da defesa antioxidante (catalase, CAT; glutationa peroxidase, GPx) e, por fim, as isoenzimas de metabolismo de fase II, glutationa-S-transferases (GSTs). As concentrações dos 3 agentes às quais os animais foram expostos partiram de níveis destes compostos já reportados em diversas matrizes aquáticas (efluentes, águas municipais e rios), para obter uma maior relevância dos dados.

A exposição de animais a BAC resultou num aumento da mortalidade às 48h após uma exposição a uma concentração de 2,5 mg/L, sendo igualmente responsável pelo aumento do número de malformações, como edemas pericárdicos e malformações na cauda. Em termos comportamentais, a atividade total, o comportamento tigmotático e o número de movimentos erráticos (classe I) foram também aumentados em animais expostos a uma concentração de 2,5 mg/L. Este aumento geral de atividade pode relacionar-se com alterações no balanço iónico e com alterações no equilíbrio das vias colinérgicas. Os biomarcadores metabólicos mostraram um aumento da sua atividade, no caso da CYP 1A1 e CAT (em animais expostos a 0.5 mg/L), e uma inibição da CYP 1A2, GPx e GSTs em animais expostos às restantes concentrações (0,1; 0,5 e 2,5 mg/L). Estes resultados sugerem que, numa primeira fase, o BAC é metabolizado pelo CYP 1A1, levando à produção de espécies reativas de oxigénio (ROS), aumentando a atividade da CAT. A possível continua produção de ROS levou à inibição da GPx e das GSTs. A exposição ao SDBS foi responsável por um aumento da mortalidade às 96h (animais expostos a 5 mg/L) mas nenhuma malformação predominante foi observada. Em termos comportamentais, verificou-se um aumento da atividade total e do número de movimentos erráticos (classe I) que presumivelmente se deverá às alterações nos níveis de acetilcolinesterase (AChE) verificadas em estudos posteriores. Nos biomarcadores enzimáticos observou-se um aumento da atividade da CAT o que traduz uma possível ativação da defesa antioxidante. Em contraste, foi observada uma inibição geral das enzimas GPx e GSTs, o que poderá advir do aumento de ROS. O aumento geral dos níveis de ROS, de uma forma direta ou indireta, podem promover a desnaturação enzimática, dado a sua interação com proteínas, lípidos, ácidos nucleicos, levando ao dano oxidativo. A inibição da CYP 1A1 e da CYP 1A2 reportada no nosso estudo deve-se possivelmente à especificidade do composto poder ser metabolizado por outras isoenzimas. Finalmente, a exposição a THY levou também a um aumento da mortalidade às 96h, e a uma elevada incidência de edemas pericárdicos. No que toca à atividade natatória e comportamento tigmotático (parâmetro da distância percorrida na zona periférica) verificou-se uma diminuição. Quanto aos movimentos erráticos, observou-se um aumento significativo para animais expostos a todas as concentrações. Alterações no balanço das correntes iónicas dos iões potássio (K^+) e sódio (Na^+) e nos níveis de AChE verificadas em estudos anteriores, podem traduzir os mecanismos subjacentes a estas alterações. Foram também verificadas inibições da atividade enzimática da CYP 1A1, CYP 1A2, CAT e GSTs. O THY é um composto antioxidante, que promove a sua ação ou pela destruição do peróxido de hidrogénio (através das enzimas antioxidantes), ou pela neutralização de radicais peróxidos (transferindo o H para o ROO*). Porém, o THY, em grandes quantidades pode também agir como pro-oxidante. Dadas estas propriedades os dados reportados no presente estudo sugerem que esta diminuição da atividade enzimática geral se deva as propriedades antioxidantes deste princípio ativo.

Concluímos assim que os desinfetantes testados em concentrações ambientalmente relevantes causam efeitos adversos no organismo modelo aqui estudado. Os efeitos observados levantam ainda mais preocupações dado que a produção e consumo destes compostos irão continuar a aumentar.

keywords

Zebrafish, disinfectants, COVID-19, THY, BAC, SDBS, embryotoxicity, bahaviour, biomarkers.

abstract

Due to the increasing incidence of SARS-CoV-2 on a global scale a pandemic was declared at the beginning of 2020. Among the various mitigation measures proposed by all governments and entities responsible for human health, the use of disinfectants was one of the most consensual. Disinfectants are now applied in homes, outdoors, in hospitals, and for personal use. Given the increased demand, it is expected that they may reach aquatic environments in greater quantities. Assessing the possible effects of these compounds becomes even more imperative, as the use of disinfecting agents is expected to grow by 10% by 2027. Among the most commonly used and most frequently encountered classes of disinfectants in the environment, we identify cationic and anionic surfactants, and those whose active substances are natural compounds. For the scope of this work, benzalkonium chloride (BAC), a cationic surfactant belonging to the family of quaternary ammonium compounds, the salt of sodium dodecylbenzene sulfonic acid (SDBS), an anionic surfactant, and thymol (THY), a plant-derived compound with multiple applications, were thus selected. Although there are some studies on the properties and effects of these active ingredients on aquatic organisms, their adverse effects on aquatic species remain largely unknown. In this sense, this study aimed to evaluate, in five-day-old larvae of the species *Danio rerio*, through acute exposures, embryonic, behavioral (total activity, thigmotactic behavior and erratic movements) and metabolic biomarkers, such as the activity of phase I enzymes (CYP 1A1 and CYP 1A2), the phase II metabolism isoenzymes, glutathione-S-transferases (GSTs) and, finally, antioxidant defense enzymes (catalase, CAT; glutathione peroxidase, GPx). The concentrations of the 3 agents to which the animals were exposed were taken from levels of these compounds already reported in various aquatic matrices (effluents, municipal waters, and rivers) to obtain greater relevance of the data.

Exposure of animals to BAC resulted in increased mortality at 48h after exposure to a concentration of 2.5 mg/L, and was also responsible for an increased number of malformations, such as pericardial edema and tail malformations. Behaviorally, total activity, thigmotactic behavior and the number of erratic movements (class I) were also increased in animals exposed to a concentration of 2.5 mg/L. This overall increase in activity may be related to changes in the ionic balance and alterations in the balance of cholinergic pathways. Metabolic biomarkers reported an increase in their activity, in the case of CYP 1A1 and CAT (in animals exposed to 0.5 mg/L), and an inhibition of CYP 1A2, GPx and GSTs in animals exposed to the remaining concentrations (0.1; 0.5 and 2.5 mg/L). These results suggest that, in a first step, BAC is metabolized by CYP 1A1, leading to the production of reactive oxygen species (ROS), increasing CAT activity. The possible continued production of ROS led to the inhibition of GPx and GSTs. SDBS exposure was responsible for increased mortality at 96h (animals exposed to 5 mg/L) but no predominant malformation was observed. In behavioral terms, there was an increase in total activity and in the number of erratic movements (class I) which is presumably due to the changes in acetylcholinesterase (AChE) levels observed in other studies. In the enzymatic biomarkers an increase in CAT activity was observed which translates to a possible activation of antioxidant defense. In contrast, a general inhibition of the enzymes GPx and GSTs was observed, which may stem from increased ROS. The general increase in ROS levels, in a direct or indirect way, may promote enzyme denaturation, given their interaction with proteins, lipids, nucleic acids, leading to oxidative damage. The inhibition of CYP 1A1 and CYP 1A2 reported in our study is possibly due to the specificity of the compound being metabolized by other isoenzymes. Finally, THY exposure also led to increased mortality at 96h, and a high incidence of pericardial edema. Regarding swimming activity and thigmotactic behavior (parameter of distance traveled in the peripheral zone) a decrease was observed. As for erratic movements, a significant increase was observed for animals exposed to all concentrations. Changes in the balance of ionic currents of potassium (K^+) and sodium (Na^+) ions and in AChE levels reported in previous studies, may translate the mechanisms underlying these changes. Inhibition of the enzymatic activity of CYP 1A1, CYP 1A2, CAT and GSTs were also verified. THY is an antioxidant compound, which promotes its action either by destroying hydrogen peroxide (via antioxidant enzymes), or by neutralizing peroxide radicals (by transferring H to ROO*). However, THY, in large amounts can also act as a pro-oxidant. Given these properties the data reported in the present study suggest that this decrease in overall enzymatic activity is due to the antioxidant properties of this active ingredient.

We thus conclude that the disinfectants tested at environmentally relevant concentrations cause adverse effects in the model organism studied here. The observed effects raise further concerns given that the production and consumption of these compounds will continue to increase.

List of Contents

List of figures

List of Acronyms

(%TDM) – Total percentage of distance moved

(%TTM) – Total percentage of time traveled

ACh – Acetylcholine

AChE – Acetylcholinesterase

AEO-7 – Fatty alcohol polyoxyethylene ether-7

BAC – Benzalkonium chloride

CAT – Catalase

CDNB - 1-chloro-2,4-dinitrobenzene

CYP 450 – Cytochrome P450

DNA – Deoxyribonucleic acid

Dpf - Days post-hatching

EROD – 7-ethoxy-resorufin O-demethylase

FET – Fish embryo toxicity test

GGS -Oxidized glutathione

GPx – Glutathione peroxidase

GSG – Reduced glutathione

GSTs – Glutathione S-transferases

H_2O_2 – Hydrogen peroxide

LAS - Linear alkylbenzene sulfonate

LC_{50} – Lethal concentration 50%

MROD – 7-methoxyresorufin O-demethylase

NADPH - Nicotinamide adenine dinucleotide phosphate

OH* - Hydroxyl radical

PPE – Personal protective equipment

QACs – Quaternary ammonium compounds

REACH – European chemicals policy animal research

ROS – Reactive oxygen species

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus

SDBS – Sodium dodecylbenzene sulfonic acid salt

SOD - Superoxide dismutase

SULTs – Sulfotransferases

THY – Thymol

UDP - uridine 5'-diphospho-glucuronosyltransferase

US EPA – United States Environmental Protection Agency

USFWS – U.S. Fish and Wildlife Service

WHO – World Health Organization

WWTP’s – Wastewater treatment plants

Chapter I - General Introduction

1. Footprint of SARS-CoV-2 in the Environment

The outbreak of severe acute respiratory syndrome coronavirus (SARS-CoV-2) led to a global crisis (World Health Organization [WHO], 2020). This virus has a high transmission rate, and is transmitted directly from suspended particles in the air, or from indirect contact via surfaces (Beninde et al., 2015). The health crisis, initiated in 2019, is responsible at the moment, for the death of millions of people (Aronson et al., 2017; Barghi et al., 2018). Although the toll of this pandemic in our daily life is already known, no footprint of SARS-CoV-2 in the environment was taken into consideration up to now. However, some very recent studies have been conducted reflecting the adverse effects of some actions adopted to tackle this outbreak. Measures decreed to prevent the spread of the virus, such as the use of personal protective equipment (PPE), hand sanitizers, and disinfection products, was of extreme importance at the time. Since all PPE must, and should, be disposable to cause no more harm, an increasing spread of PPE, such as personal masks, became a frequent environmental scenario (Panseri et al., 2019; Nabi et al., 2020).

Disinfection methods and subsequent products, which that could put a stop to transmission vectors, were one of the most important measures to control the pandemic, and to face future viral outbreaks (McDonnell and Burke, 2011; Roy et al., 2020). Despite the consensual importance of vaccination protocols, disinfection methods will continue to play a crucial role. Only in China, the disinfection market rose 14% from 2019 to 2020. The massive growth on disinfectant products was encompassed by regulatory agencies, and in the United States of America the Environmental Protection Agency (US EPA) released the List N Tool: COVID-19 Disinfectants, which in only two years grew by 43% (Hora et al., 2020; US EPA, 2022). The N-List Tool: COVID-19 Disinfectants, described these products according to their active ingredient, application in institutional, healthcare or in residential sites, surface type (hard nonporous and food contact), and contact time required to produce effect (US EPA, 2022). Furthermore, the boom in the spread of disinfectants in urban environments (e.g., streets) and closed spaces (e.g., offices) led to an increasing amount of these compounds to enter different environmental compartments (BBC, 2020). Considering the deleterious effects already known for some disinfectants, it is of extreme importance to identify the potential hazardous to animals. One study conducted in the region of Chongqing, China, established that 17 different species, such as birds, weasels and wild boars, died from

exposure to disinfectants spread in the streets (You, 2020). Food and water resources were also found to be contaminated by different disinfectant products (Sepp et al., 2019; Zhang et al., 2020). Since there is no indication that the production of these compounds will decelerate in a near future, scientific evidence is of extreme importance, so that society, politics, and decision makers can used such products without affecting or creating hazards to other forms of life (WHO, 2020).

1.1 Disinfectants

This work focused on active ingredients, selected from the N-List Tool, based on criteria such as frequent use, and 'ecological friend' indication (US EPA, 2022). A disinfectant is defined as a chemical agent that is able to destroy, inactive or reduce the number of pathogens, such as bacteria, viruses, and fungi (Merriam-Webster Dictionary, 2022). These compounds are especially applied on hard surfaces or in water. The most common chemical disinfectants used worldwide are chlorine, calcium, various forms of hypochlorite, phenol, ethanol, and quaternary ammonium compounds (US EPA, 2022).

Quaternary ammonium compounds (QACs) are one of the classes whose market value grew more for the past years. Since 2015, this class grew to a market value of 1104.6 million US dollars in 2022, and the tendency is not to reduce its production (Hora et al., 2020; Precision reports, 2022). Benzalkonium chloride (BAC) is the second most frequently found quaternary ammonium in the environment (Clara et al., 2007; Martínez-Carballo et al., 2007; Pati and Arnold, 2020). The high concentrations found in different water matrices (216 ng/L to 1386 ng/L), when compared to other QACs, is due to its wide application in household products, medical drugs, industrial and agriculture products (Tezel and Pavlostathis, 2011; European Medicines Agency, 2017; Choi et al., 2018). Most QACs are effectively removed from the environment (~90%), however only ~75% of the total volume of QACs reach wastewater treatment plants (WWTPs) (Tezel and Pavlostathis, 2011; Hora et al., 2020). So, it is possible that large amounts of BAC could be found in the environment, as reported in Taiwanese rivers, with concentrations between 2.5 to 65 µg/L, and in effluents from WWTPs in a range of 0.5 to 4.1 µg/L, which could threaten the organisms environmentally exposed to these chemicals (Zhang et al., 2015; Huang et al., 2017;). The toxicity of BACs occurs at the level of the cell membrane, as reported in different organism

(Waller et al., 1996; Rosen et al., 2001; Eleftheriadis et al., 2002; Okahara and Kawazu, 2013). Also, this compound is associated with the induction of oxidative stress, through the overproducton of superoxide anion and hydroperoxide.

The second compound analyzed in this work belongs to the class of anionic surfactants, which is the most commonly used among surfactants. The market value of surfactants, due to their vast qualities as disinfectants and detergents, is predicted to reach to a 58.5 billion dollars gross value by 2027 (Research, 2020). Sodium Dodecylbenzene Sulfonic Acid salt (SDBS) is the most abundant anionic surfactant from this class in the environment (Steber, 2007; Niraula et al., 2012). As a consequence, concentrations up to 416 µg/L have been found in surface waters (Fox et al., 2000). SDBS penetrates the phospholipidic membrane leading to the disruption of the cellular membrane (Zhang et al., 2005). This could led to the disruption of the normal function of the cell, and ultimately to the death of the zebrafish embryo (Zhang et al., 2005). Behavioral changes were also reported in previous studies connected to alterations in the cholinergic pathways (Feng et al., 2008; Wang et al., 2014; Zhang et al., 2015). In addition, SDBS promotes the generation of reactive oxygen species (ROS), so possible oxidative damage in different organisms may be promoted (Wibbertmann et al., 2011). The toxicity of SDBS can thus be observed at the embryonic, behavioral, and metabolic level.

Finally, the last compound explored in this thesis is Thymol (THY), which is considered to be less toxic, being a more ‘ecofriendly’ disinfectant (Corbett, 2020). Similarly, to the compounds presented above, THY has been widely applied in different contexts. The first use of this natural monoterpene phenol was documented in the Greek, Roman, and Egyptian cultures, as a food preservative, in medicine, and in mumification processes (Didry et al., 1994; Mahmoud, 1994; Yanishlieva et al., 1999). More recently, due to its antimicrobial, antifungal, antioxidant, antiseptic and antiviral properties, this compound has been applied in personal care products, treatment solutions for aquaculture purposes, pesticides, and disinfectants (Shapiro et al., 1994; Yilmaz et al., 2011; Carayon et al., 2014). Thymol has been detected in the Tamagawa river in concentrations between 0.5 to 3.0 ng/L, and in fish fillets in 1989 (Heil and Lindsay, 1989; Nakada et al., 2008). Although THY can protect against the action of free radicals, this compound has also been shown to increase the level of ROS (Bullangpoti et al., 2018). In addition to these effects,

exposure to THY can lead to alterations in the ionic balance of calcium and potassium ions (Ebashi, 1965; Szentandrássy et al., 2003).

These three disinfectants raise environmental concerns, due to their wide application, vast commercialization, rising market, and due to the high environmental concentrations already found in the aquatic compartments before SARS-CoV-2.

2. Test organism – *Danio rerio*

Zebrafish (*Danio rerio*) is a small freshwater fish considered as an emerging model organism (Dooley and Zon, 2000; Shin and Fishman, 2002). This native fish from India and South Asia rivers belongs to the Cyprinid family (Scholz et al., 2008). With at least one ortholog for ~70% of human genes, this animal opened new paths for understanding human diseases (Postlethwait et al., 1998; Howe et al., 2013). In addition to its genetic similarities, zebrafish ha important advantages over rodent models. The high number of offspring, transparency of the embryos, short-life span, small body frame, and easy maintained under laboratory conditions are some of the extensive list of desirable traits (Kimmel et al., 1995; Nagoor-Meeran et al., 2017). Given the vast advantages of this model organism, this fish has become an important model for ecotoxicological studies (Nagoor-Meeran et al., 2017).

Embryos hatch in a short period of time (48h to 72h), and zebrafish larvae reach maturity after three months (Nagel, 2002). When they reach adulthood, they can measure 3 to 4 cm in length. Their body is latterly flattened, with 5-7 dark blue stripes and easy to distinguish when they reach sexual maturity (Spence et al., 2008). Females have characteristically swollen bellies, and males have a slenderer body with an anal fin (Kimmel et al., 1995; Spence et al., 2008). During the external fertilization, each female can produce hundreds of eggs which are then fertilized by resealed male sperm. However, the number of eggs produced can vary according to water temperature, fish age and other abiotic factors (Kimmel et al., 1995). As soon as the fertilization occurs, cytoplasm accumulates protecting the nucleus of the zygote, and after a short period of time, cleavage stage begins (Kimmel et al., 1995). Only after 24h the entire body is formed. Before the hatching stages, the embryo feeds on their reserves and from some nutrients found in the medium (Kimmel et al., 1995). According to the European Union Legislation, embryos of *D. rerio* can be used in laboratory tests due to the inexistence of regulations (Scholz et al., 2008).

Alongside with the optimal conditions in the early life stages, the small size of larvae and number of embryos that reach this stage brings new pathways to behavioral trials (Kimmel et al., 1995). Also, the maintenance of this species is less expensive when compared to rodent models. Therefore, zebrafish gathers important features for genetic, behavioral, and ecotoxicology research.

3. General biomarkers

The use of biomarkers allows us to assess the contamination of xenobiotics and their possible effects on a variety of organisms. A biologic biomarker is a measurable and evaluated substance, structure or process, used as an indicator of normal biological function or to predict the outcome of the exposure to a determined xenobiotic (WHO, 2001). WHO has stated that any measurement that can reflect an interaction between a biological system and a potential hazard, of physical, chemical or biological origin, can be considered as a biomarker (WHO, 2001). Biomarkers can be of enzymatic, non-enzymatic, reproductive, development, and behavioral nature, among others. In every study, a careful selection of biomarkers must be made. Every chosen biomarker should translate the possible effects of the specific contaminant, so the possible impairments could be entirely understood, which would then allow us to estimate the possible environmental effects (Forbes et al., 2006). Despite the value of the information given by biomarkers, the selection process and their transmission to more relevant organization levels should be kept in mind. Biomarkers that give us insights of enzymatic and non-enzymatic alterations must be complemented by other biomarkers so that possible impairments can be translated into higher levels of organization (Forbes et al., 2006).

3.1 Embryonic biomarker

The fish embryo acute toxicity test (FET) was proposed to assess the possible impairments, through a variety of embryonic biomarkers, on the early life stages of zebrafish caused by exposure to a determined xenobiotic (OCDE, 2013). The FET test requires a small amount of individuals, and small amounts of the test substance. The transparency of zebrafish embryos provides optimal conditions to assess a variety of parameters. During the

short period of the test (96h), tail detachment, coagulation, existence of cardiac rhythm, pigmentation, presence of somite's and edema, and finally, hatching delay are registered every 24 hours (OCDE, 2013). According to the OCDE 236 guideline, any organism with a positive outcome in any of these parameters during the exposure time, is considered dead (OCDE, 2013). Although these criteria are defined in the OCDE 236 guideline, more criteria can be added according to our necessities. The FET test is an optimal assay to understand scenarios of chronic responses, at the onset of early life stages.

In addition, and according to the framework of the European Regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the use of animals in research should respect the 3 Rs policy (reduction, replacement, and refinement). FET uses animals in their early stages as replacement of adult fish. In these stages, the organism is still developing a sensory nervous apparatus, so the use of these embryos is a way to minimize suffering inherent to our research (refinement). Finally, the small number of embryos used per evaluation, makes FET assays compatible with European chemicals policy (Russell and Burch, 1959).

3.2 Behavioral biomarkers

Behavioral research with fish is also a key biomarker to assess contamination of xenobiotics. Fish display a variety of complex behavior, which are somewhat similar to other model organisms, such as socially mediated learning, ritualized aggression, mating behavior, communication, spatial navigation, thigmotaxis behavior, avoidance, and Pavlovian conditioning (Behrend and Bitterman, 1963; Suboski, 1988; Reebs, 1996; Miklósi et al., 1997; Payne, 1998; Miklósi and Andrew, 1999; Talton et al., 1999; Colwill et al., 2005; Drew et al., 2005; Schnörr et al., 2012). Several tests, such as T-maze, novel diving tank test, thigmotaxis evaluation, and others, have been developed to assess the different behaviors mentioned before (Levin et al., 2007; Levin and Cerutti, 2009; Schnörr et al., 2012). Alterations in behavioral performance after the organism has been exposed to pharmaceuticals, insecticides, disinfectants or other xenobiotics, have been reported in the literature (Phillips et al., 2002; Correia et al., 2017; Almeida et al., 2019; Dionísio et al., 2020; Nogueira and Nunes, 2020; Sousa and Nunes, 2020; Vieira et al., 2021; Santos et al., 2021).

It is then of extreme importance to determine alterations in the motor behavior in young zebrafish to visualize the adverse effects of early toxicant exposure. Since swimming behavior is of critical importance for survival (feeding, reproduction, and avoidance), even subtle behavioral alterations can be a sign of potential hazard to the animal (Little and Brewer, 2001). Behavioral biomarkers such total distance, standardized thigmotaxis behavior and assessment of erratic movements are some of the indicators are some of the indicators to measure changes in behavior explored in our work. Thigmotaxis is a standard assay to evaluate anxiety-like behavior (Schnörr et al., 2012). In novel environments this organism tends to stay closer to the walls, thus remaining longer in the defined peripheral zone. For this purpose, two areas were defined; an inner area with 12.25 mm ray, and an outer area with 4 mm from the first area. Finally, abrupt changes in direction (erratic movements) could also be a good indicator of alterations in behavior (Zhang et al., 2017). The different classes of angles were defined according to the study of Zhang et al. (2017). Angles with higher amplitude (90° to 180°) constitutes class 1. Class 2 and 3 consists in angles between 30° to 90°, and 10° to 30°, respectively. Finally, class 4 comprises angles with amplitudes between 0° to 10° (Figure 1). Organisms with a higher number of class 1 angles indicate the presence of erratic movements by the exposed organism (Zhang et al., 2017).

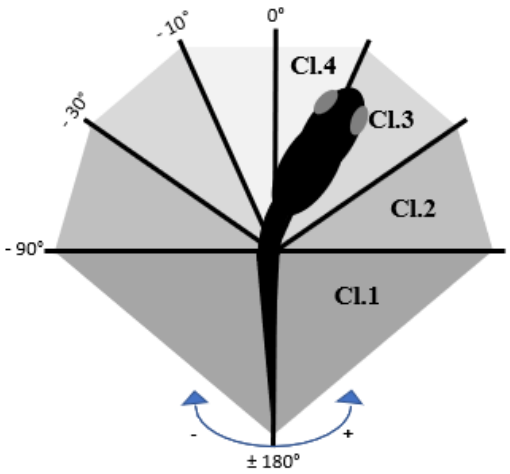

Figure 1 - Schematic representation of angles performed by larvae of D. rerio, grouped in classes (Cl.1, Cl.2, Cl.3, and Cl.4), obtain in behavior assay. The schematic is not at scale.

3.3 Metabolic biomarkers

Finally, metabolic pathways are of extreme importance to the homeostasis of several organisms. The interaction of xenobiotics with biological systems usually involves the activation of metabolic pathways, that in some cases, can led to distinct outcomes, including oxidative stress, inhibition or induction of detoxification enzymes, and adverse changes in

neuro-muscular junctions. Xenobiotics are first metabolized in animals through phase I, and II systems. The enzymes involved in this metabolic processes are of extreme importance to increase the hydrophilicity, through phase I reactions, such as hydrolysis, reduction and oxidation, and phase II conjugations (Holth et al., 2008). The regulation or abnormal behavior of these enzymes are key points to assess the effects in aquatic species (Holth et al., 2008). BAC, SDBS, and THY have the ability to alter normal metabolic pathways of the cell (Thalhamer et al., 2011; Wibbertmann et al., 2011; Yu et al., 2021). More specifically, the metabolism of BAC, SDBS, and THY have been associated to an increase of ROS; THY itself is a powerful antioxidant agent (Wojdyło et al., 2007; Wibbertmann et al., 2011; Meeran and Prince, 2012; Seguin et al., 2019) . So, metabolism by enzymes such as those from the cytochrome P450 (CYP 450) complex, and enzymes of the antioxidant defense system, such as catalase (CAT), and glutathione peroxidase (GPx), can be used as biomarkers to assess the generation of ROS. Also, assessing the activity of glutathione S-transferases (GSTs) can be useful to investigate if this enzyme can be involved in the detoxification mechanism of the compounds selected. For example, in humans reported in a previous study, THY can be detoxified by uridine 5'-diphospho-glucuronosyltransferase (UDP) and sulfotransferases (SULTs) (Thalhamer et al., 2011). An activation of GSTs can be in place, as reported in the study of Vieira et al., (2021), in animals exposed to 1 mg/L of THY or inhibited if the primary pathways by which the compound is detoxified are other.

4. Previous studies

Although there is a considerable amount of work already produced to evaluate the possible effects of disinfectants, there is a consensus that more information concerning the possible metabolic pathways, and impairments of disinfectants is needed. As mentioned before BAC, SDBS, and THY are some of the most used disinfectants worldwide. However, their action and metabolic pathways are not fully understood, especially in aquatic organisms.

BAC and SDBS (a cationic, and an anionic surfactant, respectively) are known to be ROS generators. Antunes et al. (2016), after an exposure to BAC, in concentrations up to 1.050 mg/L, reported an enzymatic inhibition of CAT, and an increase in the activity of acetylcholinesterase (AChE), which are key endpoints of oxidative damage and

neurotoxicity, suggesting possible behavior, and enzymatic impairments. CYP 450 could also be involved in the metabolism of BAC, although the only study mentioning this possible relationship was conducted in humans (Seguin et al., 2019). BAC also promoted embryos delayed hatching, mortality, and morphological malformations in zebrafish (Sreevidya et al., 2018). SDBS induced an increment in the enzymatic activity of CAT, GPx, and GSTs, in *Lateolabrax japonicus* fish exposed to 1 mg/L (Jifa et al., 2005). Also, no significant differences were observed in the development of zebrafish embryos exposed to SDBS in the µg/L range (Wang et al., 2015). These two compounds increased the amount of ROS in *Oncorhynchus mykiss* and in *L. japonicus* (Jifa et al., 2005; Antunes et al., 2016). The antioxidant defense of the fish species mentioned above, can lead to the increase of the enzymatic activity of CAT, GPx, GSTs, or inhibit these same enzymes when the rate of production of ROS is higher than the amount of ROS that the organism can tackle (Cserháti et al., 2002; Jifa et al., 2005; Wu et al, 2010).

THY, being a powerful antioxidant, due to the phenolic hydroxyl group, is known to protect against the possible adverse effects of free radicals (Nagoor-Meeran and Prince, 2012). This protection is conferred by increasing antioxidant enzymes such as superoxide dismutase (SOD), CAT, GPx, and GSTs, as reported in previous studies, in rats and fish models (Wojdyło et al., 2007; Nagoor-Meeran and Prince, 2012; Vieira et al., 2021). Although there is a general consensus on the antioxidant properties of thymol, phenolic compounds can also act as prooxidants, promoting the generation of ROS (Shalaby and Horwitz, 2015; Bayliak et al., 2016; Krishnan et al., 2019). Krishnan et al. (2019), have shown the potential of this phenol to increase the levels of ROS, and to diminish CAT activity in the µM range.

5. Objectives

The aim of this our was to assess the possible adverse effects of disinfectants, proven to be effective against SARS-CoV-2, on zebrafish embryos, and larvae. For that purpose, 3 different disinfectants were selected, and we exposed zebrafish embryos and larvae to a range of concentrations. To evaluate the possible deleterious effects of these compounds, a battery of assays was used to assess alterations at the embryonic level (FET), behavioral alterations (activity, thigmotaxis, and erratic movements), and biochemical markers

(activities of phase I isoenzymes CYP 1A1, CYP 1A2; activity of the phase II detoxification pathway, GSTs); and activities of enzymes from the antioxidant defense system, CAT, GPx.

6. Thesis structure

Chapter I is a literature review describing the potential issue of environmental contamination due to the increase in the use of disinfectants, favored by the SARS-CoV-2 outbreak. In this general introduction, common points of the three disinfectants selected are described (test organism and biomarkers). At the end, results from previous works are presented to establish a baseline of knowledge.

The following three chapters constitute three distinct works for further publication, one per each tested chemical. More specifically, ***Chapter 2*** focuses on the effects of environmental concentrations of BAC. In ***Chapter 3***, we discuss the possible toxic effects of SDBS, and in ***Chapter 4***, we evaluate the possible impairments resulting from the exposure of fish to THY.

Finally, ***Chapter 5*** presents the final discussion integrating all data resulting from exposure to the selected compounds, and some final perspectives.

7. References

Almeida, A.R., Salimian, M., Ferro, M., Marques, P.A., Goncalves, G., Titus, E., and Domingues, I. (2019). Biochemical and behavioral responses of zebrafish embryos to magnetic graphene/nickel nanocomposites. *Ecotoxicology and Environmental Safety*, *186*, 109760. https://doi.org/10.1016/j.ecoenv.2019.109760

Antunes, S.C., Nunes, B., Rodrigues, S., Nunes, R., Fernandes, J., and Correia, A.T. (2016). Effects of chronic exposure to benzalkonium chloride in *Oncorhynchus mykiss*: Cholinergic neurotoxicity, oxidative stress, peroxidative damage and genotoxicity. *Environmental Toxicology and Pharmacology*, *45*, 115–122. https://doi.org/10.1016/j.etap.2016.04.016

Aronson, M.F.J., Lepczyk, C.A., Evans, K.L., Goddard, M.A., Lerman, S.B., MacIvor, J.S., and Vargo, T. (2017). Biodiversity in the city: key challenges for urban green space management. *Frontiers in Ecology and the Environment*, *15*(4), 189–196. https://doi.org/10.1002/fee.1480

Bayliak, M.M., Burdyliuk, N.I. and Lushchak, V.I. (2016). Effects of pH on antioxidant and prooxidant properties of common medicinal herbs. *Open life Sciences, 11*(1), 298-307. https://doi.org/10.1515/biol-2016-0040

Barghi, M., Jin, X., Lee, S., Jeong, Y., Yu, J.P., Paek, W.K., and Moon, H.B. (2018). Accumulation and exposure assessment of persistent chlorinated and fluorinated contaminants in Korean birds. *Science of the Total Environment*, *645*, 220–228. https://doi.org/10.1016/j.scitotenv.2018.07.040

BBC. (2020). Coronavirus: Wild animals enjoy freedom of a quieter world. Retrieved from https://www.bbc.com/news/world-52459487

Behrend, E.R., and Bitterman, M.E. (1963). Sidman Avoidance in the Fish 1 . *Journal of the Experimental Analysis of Behavior*, *6*(1), 47–52. https://doi.org/10.1901/jeab.1963.6-47

Beninde, J., Veith, M., and Hochkirch, A. (2015). Biodiversity in cities needs space: A meta-analysis of factors determining intra-urban biodiversity variation. *Ecology Letters*, *18*(6), 581–592. https://doi.org/10.1111/ele.12427

Bullangpoti, V., Mujchariyakul, W., Laksanavilat, N., and Junhirun, P. (2018). Acute toxicity of essential oil compounds (thymol and 1,8-cineole) to insectivorous guppy,

Poecilia reticulata Peters, 1859. *Agriculture and Natural Resources*, *52*(2), 190–194. https://doi.org/10.1016/j.anres.2018.06.011

Carayon, J.L., Téné, N., Bonnafé, E., Alayrangues, J., Hotier, L., Armengaud, C., and Treilhou, M. (2014). Thymol as an alternative to pesticides: Persistence and effects of Apilife Var on the phototactic behavior of the honeybee *Apis mellifera*. *Environmental Science and Pollution Research*, *21*(7), 4934–4939. https://doi.org/10.1007/s11356-013-2143-6

Choi, S.M., Roh, T.H., Lim, D.S., Kacew, S., Kim, H.S., and Lee, B.M. (2018). Risk assessment of benzalkonium chloride in cosmetic products. *Journal of Toxicology and Environmental Health – Part B: Critical Reviews*, *21*(1), 8–23. https://doi.org/10.1080/10937404.2017.1408552

Clara, M., Scharf, S., Scheffknecht, C., and Gans, O. (2007). Occurrence of selected surfactants in untreated and treated sewage. *Water Research*, *41*(19), 4339–4348. https://doi.org/10.1016/j.watres.2007.06.027

Colwill, R.M., Raymond, M.P., Ferreira, L., and Escudero, H. (2005). Visual discrimination learning in zebrafish (*Danio rerio*). *Behavioural Processes*, *70*(1), 19–31. https://doi.org/10.1016/j.beproc.2005.03.001

Corbett, K. (2020). EPA Approves Thymol Cleaners to Kill Coronavirus - So What Is Thymol? Retrieved from: https://www.housebeautiful.com/lifestyle/cleaning-tips/a32291832/epa-approves-thymol

Correia, D., Almeida, A.R., Santos, J., Soares, A.M.V.M., and Domingues, I. (2017). Behavioral effects in adult zebrafish after developmental exposure to carbaryl. *Toxicology Letters, 280(2017),* S206-S213. http://dx.doi.org/10.1016/j.toxlet.2017.07.565

Cserháti, T., Forgács, E., and Oros, G. (2002). Biological activity and environmental impact of anionic surfactants. *Environment International*, *28*, 337–348. https://doi.org/10.1016/j.memsci.2004.04.007

Didry, N., Dubreuil, L., and Pinkas, M. (1994). Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharmaceutica Acta Helvetiae*, *69*(1), 25–28. https://doi.org/https://doi.org/10.1016/0031-6865(94)90027-2

Dionísio, R., Daniel, D., Alkimin, G.D., and Nunes, B. (2020). Multi-parametric analysis of ciprofloxacin toxicity at ecologically relevant levels: Short- and long-term effects

on *Daphnia magna*. *Environmental Toxicology and Pharmacology, 74.* https://doi.org/10.1016/j.etap.2019.103295

Dooley, K., and Zon, L.I. (2000). Zebrafish: A model system for the study of human disease. *Current Opinion in Genetics and Development*, *10*(3), 252–256. https://doi.org/10.1016/S0959-437X(00)00074-5

Drew, M., Zupan, B., Cooke, A., Couvillon, P.A., and Balsam, P. (2005). Temporal Control of Conditioned Respondig in Goldfish. *Journal of Experimental Psychology: Animal Behavior Procesesses, 31*(1), 31–39. https://doi.org/https://doi.org/10.1037/0097-7403.31.1.31

Ebashi, E. (1965). The sarcoplasmic reticulum and excitation-concentration coupling. *Molecular Biology of Muscular Contraction*, 197–206.

Eleftheriadis, H., Cheong, M., Sandeman, S., Syam, P.P., Brittain, P., Klintworth, G.K., and Tyson, S.L. (2002). Corneal toxicity secondary to inadvertent use of benzalkonium chloride preserved viscoelastic material in cataract surgery. *Evidence-Based Eye Care, 3*(4), 184–185. https://doi.org/10.1097/00132578-200210000-00008

European Medicines Agency. (2017). Questions and answers on benzalkonium chloride used as an excipient in medicinal products for human use. *Ema/Chmp/704195/2013*, *44*(828), 1–10.

Feng, T., Li, Z.B., Guo, X., and Guo, J. (2008). Effects of trichlorfon and sodium dodecyl sulphate on antioxidant defense system an acetylcholinesterase of *Tilapia nilotica* in vitro. *Pesticide Biochemistry and Physiology*, *92*, 107–113.

Forbes, V.E., Palmqvist, A., and Bach, L. (2006). The use and misuse of biomarkers in ecotoxicology. *Environmental Toxicology and Chemistry*, *25*(1), 272–280. https://doi.org/10.1897/05-257R.1

Fox, K., Holt, M., Daniel, M., Buckland, H., and Guymer, I. (2000). Removal of linear alkylbenzene sulfonate from a small Yorkshire stream: Contribution to GREAT-ER project 7. *Science of the Total Environment*, *251–252*, 265–275. https://doi.org/10.1016/S0048-9697(00)00389-2

Heil, T.P., and Lindsay, R.C. (1989). Environmental and industrial factors relating to flavor tainting of fish in the upper Wiscosin river. *Journal of Environmental Science and Health*, 527–552.

Holth, T.F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G.I., and Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (*Danio rerio*) following exposure to produced water components. *Aquatic Toxicology*, *90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Hora, P.I., Pati, S.G., McNamara, P.J., and Arnold, W.A. (2020). Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications. *Environmental Science and Technology Letters*, *7*(9), 622–631. https://doi.org/10.1021/acs.estlett.0c00437

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., and Stemple, D.L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*(7446), 498–503. https://doi.org/10.1038/nature12111

Huang, N., Wang, T., Wang, W. L., Wu, Q.Y., Li, A., and Hu, H.Y. (2017). UV/chlorine as an advanced oxidation process for the degradation of benzalkonium chloride: Synergistic effect, transformation products and toxicity evaluation. *Water Research*, *114*, 246–253. https://doi.org/10.1016/j.watres.2017.02.015

Jifa, W., Zhiming, Y., Xiuxian, S., You, W., and Xihua, C. (2005). Comparative researches on effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate upon *Lateolabrax japonicus* biomarker system. *Environmental Toxicology and Pharmacology*, *20*(3), 465–470. https://doi.org/10.1016/j.etap.2005.05.006

Kimmel, C.B., Ballard, W.W., Kimmer, S.R., Ullmann, B., and Schilling, T.F. (1995). Zebrafish as an alternative vertebrate model for investigating developmental toxicity - the triadimefon example. *International Journal of Molecular Sciences*, *203*, 253–310. https://doi.org/10.3390/ijms18040817

Krishnan, M., Kim, D.K., Gie Kim, S., and Kang, S.C. (2019). Thymol exposure mediates pro-oxidant shift by regulating Nrf2 and apoptotic events in zebrafish (*Danio rerio*) embryos. *Environmental Toxicology and Pharmacology*, *65*, 1–8. https://doi.org/10.1016/j.etap.2018.11.001

Levin, E.D., Bencan, Z., and Cerutti, D.T. (2007). Anxiolytic effects of nicotine in zebrafish. *Physiology and Behavior*, *90*(1), 54–58. https://doi.org/10.1016/j.physbeh.2006.08.026

Levin, E.D., and Cerutti, D.T. (2009). Behavioral Neuroscience of Zebrafish. In *Methods of Behavior Analysis in Neuroscience* (2 nd edition).

Little, E.E., and Brewer, S.K. (2001). Neurobehavioral toxicity in fish. *Toxicol. Enviroment*, *2*, 139–174.

Mahmoud, A.L. (1994). Antifungal action and antiaflatoxigenic properties of some essential oil constituents. *Letters in Applied Microbiology*. https://doi.org/https://doi.org/10.1111/j.1472-765X.1994.tb00918.x

Martínez-Carballo, E., Sitka, A., González-Barreiro, C., Kreuzinger, N., Fürhacker, M., Scharf, S., and Gans, O. (2007). Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. Application to surface, waste and indirect discharge water samples in Austria. *Environmental Pollution*, *145*(2), 489–496. https://doi.org/10.1016/j.envpol.2006.04.033

McDonnell, G., and Burke, P. (2011). Disinfection: Is it time to reconsider Spaulding? *Journal of Hospital Infection*, *78*(3), 163–170. https://doi.org/10.1016/j.jhin.2011.05.002

Meeran, M.F.N., and Prince, P.S.M. (2012). Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol-induced myocardial infarcted rats. *Journal of Biochemical and Molecular Toxicology*, *26*(9), 368–373. https://doi.org/10.1002/jbt.21431

Merriam-Webster dictionary. (2022). Definition of disinfectant. Retrieved from https://www.merriam-webster.com/dictionary/disinfectant

Miklósi, Á., and Andrew, R.J. (1999). Right eye use associated with decision to bite in zebrafish. *Behavioural Brain Research*, *105*(2), 199–205. https://doi.org/10.1016/S0166-4328(99)00071-6

Miklósi, A., Andrew, R.J., and Savage, H. (1997). Behavioural lateralisation of the tetrapod type in the zebrafish (*Brachydanio rerio*). *Physiology and Behavior*, *63*(1), 127–135. https://doi.org/10.1016/S0031-9384(97)00418-6

Nabi, G., Wang, Y., Hao, Y., Khan, S., Wu, Y., and Li, D. (2020). Massive use of disinfectants against COVID-19 poses potential risks to urban wildlife. *Environmental Research*, *188*, 109916. https://doi.org/10.1016/j.envres.2020.109916

Nagel, R. (2002). DarT: The embryo test with the Zebrafish *Danio rerio* – a general model in ecotoxicology and toxicology. *Alternatives to Animal Experimentation*, *19*, 38–48.

Nagoor-Meeran, M.F. and Prince, P. (2012). Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol-induced myocardial

infarcted rats. *Journal of Biochemical and Molecular Toxicology, 26*(9), 368-373. https://doi.org/10.1002/jbt.21431

Nagoor-Meeran, M.F., Javed, H., Taee, H. Al, Azimullah, S., and Ojha, S.K. (2017). Pharmacological properties and molecular mechanisms of thymol: Prospects for its therapeutic potential and pharmaceutical development. *Frontiers in Pharmacology*, *8*, 1–34. https://doi.org/10.3389/fphar.2017.00380

Nakada, N., Kiri, K., Shinohara, H., Harada, A., Kuroda, K., Takizawa, S., and Takada, H. (2008). Evaluation of pharmaceuticals and personal care products as water-soluble molecular markers of sewage. *Environmental Science and Technology*, *42*(17), 6347–6353. https://doi.org/10.1021/es7030856

Niraula, T.P., Shah, S.K., Bhattarai, A., and Chatterjee, S. (2012). Cationic Surfactants and their uses in different fields. *TUTA Journal*, 33–37.

Nogueira, A. and Nunes, B. (2021). Acute and chronic effects of diazepam on the polychaete *Hediste diversicolor*: Antioxidant, metabolic, pharmacologic, neurotoxic and behavioral mechanistic traits. *Environmental Toxicology and Pharmacology, 82*, 103538. https://doi.org/10.1016/j.etap.2020.103538

OCDE. (2013). OECD guidelines for testing of chemicals, 509–511. https://doi.org/10.3109/9781841848570-66

Okahara, A., and Kawazu, K. (2013). Local toxicity of benzalkonium chloride in ophthalmic solutions following repeated applications. *Journal of Toxicological Sciences*, *38*(4), 531–537. https://doi.org/10.2131/jts.38.531

Panseri, S., Chiesa, L., Ghisleni, G., Marano, G., Boracchi, P., Ranghieri, V., and Tecilla, M. (2019). Persistent organic pollutants in fish: biomonitoring and cocktail effect with implications for food safety. *Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, *36*(4), 601–611. https://doi.org/10.1080/19440049.2019.1579926

Pati, S.G., and Arnold, W.A. (2020). Comprehensive screening of quaternary ammonium surfactants and ionic liquids in wastewater effluents and lake sediments. *Environmental Science: Processes and Impacts*, *22*(2), 430–441. https://doi.org/10.1039/c9em00554d

Phillips, T.A., Summerfelt, R.C., and Atchison, G.J. (2002). Environmental, biological, and methodological factors affecting cholinesterase activity in walleye

(*Stizostedion vitreum*). *Archives of Environmental Contamination and Toxicology*, *43*(1), 75–80. https://doi.org/10.1007/s00244-002-1134-2

Postlethwait, J.H., Yan, Y.L., Gates, M. A., Horne, S., Amores, A., Brownlie, A., and Talbot, W.S. (1998). Vertebrate genome evolution and the zebrafish gene map. *Nature Genetics*, *18*(4), 345–349. https://doi.org/10.1038/ng0498-345

Precision reports. (2022). Global Quaternary Ammonium Compounds Market Growth 2022-2028. Retrieved from https://www.marketwatch.com/press-release/quaternary-ammonium-compounds-market-size-2022-global-top-manufacturers-analysis-covid-19-impact-business-strategies-industry-share-demand-trend-opportunities-and-challenge-forecast-until-2028-2022-02-16

Reebs, S.G. (1996). Time-place learning in golden shiners (Pisces: Cyprinidae). *Behavioural Processes*, *36*(3), 253–262. https://doi.org/10.1016/0376-6357(96)88023-5

Research, A.M. (2020). Surfactants Market by Type (Anionic, Non-ionic, Cationic, Amphoteric, and Others) and Application (Household Detergents, Personal Care, Industrial and Institutional Cleaners, Food Processing, Oilfield Chemicals, Agricultural Chemicals, Textiles, Emulsion Polymerization (Plastics), Paints and Coatings, Construction, and Others): Global Opportunity Analysis and Industry Forecast, 2020–2027. Retrieved March 28, 2022, from https://www.alliedmarketresearch.com/surfactant-market

Rosen, M.J., Li, F., Morrall, S.W., and Versteeg, D.J. (2001). The relationship between the interfacial properties of surfactants and their toxicity to aquatic organisms. *Environmental Science and Technology*, *35*(5), 954–959. https://doi.org/10.1021/es0015141

Roy, A., Parida, S.P., and Bhatia, V. (2020). Role of disinfection and hand hygiene: a COVID-19 perspective. *International Journal Of Community Medicine And Public Health*, *7*(7), 2845. https://doi.org/10.18203/2394-6040.ijcmph20203025

Russell, W.M., and Burch, R. (1959). The Principles of Humane Experimental Techniques. *Methuen, London, UK*.

Santos, J., Barreto, Â., Almeida, C., Azevedo, C., Domingues, I., Amorim, M.J.B. and Maria, V.L. (2021). *Aquatic Toxicology, 238,* 105930. https://doi.org/10.1016/j.aquatox.2021.105930

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., and Champagne, D.L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*, *228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., and Voelker, D. (2008). The zebrafish embryo model in environmental risk assessment – Applications beyond acute toxicity testing. *Environmental Science and Pollution Research*, *15*(5), 394–404. https://doi.org/10.1007/s11356-008-0018-z

Seguin, R.P., Herron, J.M., Lopez, V., Dempsey, J.L., and Xu, L. (2019). Metabolism of Benzalkonium Chlorides by Human Hepatic Cytochromes P450. *Chemical Research in Toxicology*, *32*(12), 2466–2478. https://doi.org/10.1021/acs.chemrestox.9b00293.Metabolism

Sepp, T., Ujvari, B., Ewald, P. W., Thomas, F., and Giraudeau, M. (2019). Urban environment and cancer in wildlife: Available evidence and future research avenues. *Proceedings of the Royal Society B: Biological Sciences*, *286*(1894). https://doi.org/10.1098/rspb.2018.2434

Shalaby, S., and Horwitz, B. A. (2015). Plant phenolic compounds and oxidative stress: integrated signals in fungal–plant interactions. *Current Genetics*, *61*(3), 347–357. https://doi.org/10.1007/s00294-014-0458-6

Shapiro, S., Meier, A., and Guggenheim, B. (1994). The antimicrobial activity of essential oils and essential oil components towards oral bacteria. *Molecular Oral Microbiology*. https://doi.org/https://doi.org/10.1111/j.1399-302X.1994.tb00059.x

Shin, J.T., and Fishman, M. C. (2002). From zebrafish to human: Modular medical models. *Annual Review of Genomics and Human Genetics*, *3*(24), 311–340. https://doi.org/10.1146/annurev.genom.3.031402.131506

Sousa, B., and Nunes, B. (2020). Reliability of behavioral test with fish: How neurotransmitters may exert neuromodulatory effects and alter the biological responses to neuroactive agents. *Science of the Total Environment*, *734*, 139372. https://doi.org/10.1016/j.scitotenv.2020.139372

Spence, R., Gerlach, G., Lawrence, C., and Smith, C. (2008). The behaviour and ecology of the zebrafish, *Danio rerio*. *Biological Reviews*, *83*(1), 13–34. https://doi.org/10.1111/j.1469-185X.2007.00030.x

Sreevidya, V.S., Lenz, K.A., Svoboda, K.R., and Ma, H. (2018). Benzalkonium chloride, benzethonium chloride, and chloroxylenol – Three replacement antimicrobials are more toxic than triclosan and triclocarban in two model organisms. *Environmental Pollution*, *235*, 814–824. https://doi.org/10.1016/j.envpol.2017.12.108

Steber, J. (2007). The Ecotoxicity of Cleaning Product Ingredients. In *Handbook for Cleaning / Decontamination of Surface*, 721–746. https://doi.org/https://doi.org/10.1016/B978-044451664-0/50022-X

Suboski, M. (1988). Acquisition and social communication of stimulus recognition by Fish. *Behavioural Processes*, *16*(3), 213–244. https://doi.org/https://doi.org/10.1016/0376-6357(88)90041-1

Szentandrássy, N., Szentesi, P., Magyar, J., Nánási, P., and Csernoch, L. (2003). Effect of thymol on kinetic properties of Ca^{2+} and K^{+} currents in rat skeletal muscle. *BMC Pharmacology*, *3*, 1–8. https://doi.org/10.1186/1471-2210-3-9

Talton, L.E., Higa, J.J., and Staddon, J.E.R. (1999). Interval schedule performance in the goldfish *Carassius auratus*. *Behavioural Processes*, *45*(1–3), 193–206. https://doi.org/10.1016/S0376-6357(99)00018-2

Tezel, U., and Pavlostathis, S.G. (2011). Role of Quaternary Ammonium Compounds on Antimicrobial Resistance in the Environment. In Patricia L. Keen and M.H.M. . Montforts (Eds.), *Antimicrobial Resistance in the Environment* (Vol. 20). https://doi.org/https://doi.org/10.1002/9781118156247.ch20

Thalhamer, B., Buchberger, W., and Waser, M. (2011). Identification of thymol phase I metabolites in human urine by headspace sorptive extraction combined with thermal desorption and gas chromatography mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, *56*(1), 64–69. https://doi.org/10.1016/j.jpba.2011.04.014

United Sates Environmental Protection Agency [US EPA]. (2022). List N: Disinfectants for Use Against SARS-CoV-2. Retrieved January 20, 2022, from https://www.epa.gov/%0Apesticide-registration/list-n-disinfectants-use-against-sars-cov-2

Vieira, R., Venâncio, C., and Félix, L. (2021). Teratogenic, oxidative stress and behavioural outcomes of three fungicides of natural origin (*Equisetum arvense, mimosa tenuiflora*, thymol) on zebrafish (*Danio rerio*). *Toxics*, *9*(1), 1–15. https://doi.org/10.3390/toxics9010008

Waller, D.L., Fisher, S.W., and Dabrowska, H. (1996). *FISH-CULTURIST*. *58*, 77–84.

Wang, Q., Liu, N., Wang, J., Wu, Y., and Wang, L. (2014). Physiological changes and acetylcholinesterase activity in th cladoceran *Moina macrocopa* (Straus, 1820) exposed to mercury and sodium dodecylsulphate. *Crustaceana*, *87*(14), 1678–1690.

Wang, Y., Zhang, Y., Li, X., Sun, M., Wei, Z., Wang, Y., and Gao, A. (2015). Exploring the Effects of Different Types of Surfactants on Zebrafish Embryos and Larvae. *Nature Publishing Group*, 1–10. https://doi.org/10.1038/srep10107

Word Health Organization [WHO]. (2001). WHO International Programme on Chemical Safety Biomarkers in Risk Assessment: Validity and Validation. Retrieved from http://www.inchem.org/documents/ehc/ehc/ehc222.htm

Word Health Organization [WHO]. (2020). WHO Characterizes COVID-19 as a Pandemic? Retrieved from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/events-as-they-happen

Wibbertmann, A., Mangelsdorf, I., Gamon, K., and Sedlak, R. (2011). Toxicological properties and risk assessment of the anionic surfactants category: Alkyl sulfates, primary alkane sulfonates, and α-olefin sulfonates. *Ecotoxicology and Environmental Safety*, *74*(5), 1089–1106. https://doi.org/10.1016/j.ecoenv.2011.02.007

Wojdyło, A., Oszmiański, J., and Czemerys, R. (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry*, *105*(3), 940–949. https://doi.org/10.1016/j.foodchem.2007.04.038

Wu, Z., Yu, D., Li, J., Wu, G., and Niu, X. (2010). Growth and antioxidant response in *Hydrocharis dubis* (Bl.) Backer exposed to linear alkylbenzene sulfonate. *Ecotoxicology*, *19*(4), 761–769. https://doi.org/10.1007/s10646-009-0453-8

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., and Raneva, V.G. (1999). Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. *Food Chemistry*, *64*(1), 59–66. https://doi.org/10.1016/S0308-8146(98)00086-7

Yilmaz, E., Çoban, D., Kirim, B., Guller, M., and Ozturk, S. (2011). The effects of the Carvacrol and Thymol addition to *Pangasius hypophthalmus* feeds in different amount on the growth performance.

You, T. (2020). More than 100 wild animals drop dead near coronavirus epicentre in China after workers 'sprayed too much disinfectant'to prevent coronavirus. *Daily Mail*, Retrived from: https://www.dailymail.co.uk/news/article-8029271/100-wild-animals-drop-dead-near-coronavirus-epicentre.html

Yu, X., Kamali, M., Van Aken, P., Appels, L., Van der Bruggen, B., and Dewil, R. (2021). Advanced oxidation of benzalkonium chloride in aqueous media under ozone and

ozone/UV systems – Degradation kinetics and toxicity evaluation. *Chemical Engineering Journal*, *413*(July 2020), 127431. https://doi.org/10.1016/j.cej.2020.127431

Zhang, B., Chen, X., Pan, R., Xu, T., Zhao, J., Huang, W., and Yin, D. (2017). Effects of three different embryonic exposure modes of 2, 2', 4, 4'-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. *Chemosphere*, *169*, 542–549. https://doi.org/10.1016/j.chemosphere.2016.11.098

Zhang, C., Cui, F., Zeng, G., Jiang, M., Yang, Z., Yu, Z., and Shen, L.. (2015). Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. *Science of the Total Environment*, *518–519*, 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007

Zhang, Y., Tan, Y., Stormer, H.L. and Kim, P. (2005). Experimental observation of the quantum Hall effect and Berry's phase in graphene. *Nature*, *438*, 201–204.

Zhang, H., Tang, W., Chen, Y., and Yin, W. (2020). Disinfection threatens aquatic ecosystems. *Science*, *368*(6487), 145–146.

Chapter II | Effects of SARS-CoV-2 through the eyes of the freshwater fish *Danio rerio*: an embryonic, behavioral, and metabolic perspective after acute exposure to benzalkonium chloride

1. Abstract

The outbreak, in 2019, of the severe acute respiratory syndrome coronavirus (SARS-CoV-2) lead to a tremendous pressure on the disinfectant market. Although the pandemic is now on a downward state, the perspective for the disinfectant market is to increase. A higher demand for disinfectants creates a potential environmental problem since residues of these substances are likely to end up in the aquatic medium. Benzalkonium chloride (BAC), a popular disinfectant product, was already found at concentrations between 2.5 to 65 µg/L, and between 0.5 to 5 mg/L in Taiwanese rivers, and effluents from European hospitals, respectively, before the pandemic stroke. So now it is presumable that these levels could increase (Zhang et al., 2015). Although some information is available about the possible impairments of BAC exposure to aquatic organism, the studies available are still limited. So, our aim was to provide more evidence to elucidate possible impairments, and metabolic pathways involved after an acute exposure of individuals of the freshwater fish species *Danio rerio*, to different concentrations 0.1, 0.5, and 2.5 mg/L. After the Fish Embryo Toxicity (FET) assay, we reported a higher mortality at 48h in animals exposed to a concentration of 2.5 mg/L. Also, a higher number of malformations such as pericardial edema and tail malformations, were observed. An increase in the overall swimming activity, thigmotaxis behavior, and on erratic movements were observed in animals exposed to the highest concentration (2.5 mg/L). This increment in activity is probably due to the disruption in the ionic balance of the membrane, and a possible inhibition of AChE, that led to a hyperstimulation of the postsynaptic nerve. At the enzymatic level, an increase in the detoxification enzyme CYP 1A1, and in the antioxidant defense (increased catalase activity) was observed at 1 mg/L exposure. These changes in the enzymatic activities reported in our study suggest which BAC is metabolized by CYP 1A1 producing hydrogen peroxide (H_2O_2), that in turn is used by catalase (CAT). Finally, our results reported an inhibition of the phase I enzyme CYP 1A2, the phase II enzyme GSTs, and the antioxidant enzyme GPx. Given the activation of CYP 1A1, and CAT, we propose that the inhibition of GPx and GSTs reported in our study could be due to the increase in reactive oxygen species (ROS), resulting from the metabolism of BAC. Since the concentrations tested in this study were based on levels already reported in effluents before the pandemic, the adverse embryonic, behavioral, and

metabolic effects here reported may be even greater, given the tendency for this compound to thrive in the market.

2. Keywords

BAC; Covid-19; FET; Thigmotaxis; Antioxidant defense.

3. Introduction

The pandemic of the severe acute respiratory syndrome coronavirus (SARS-CoV-2) led to a great pressure on the market to create and increase the production of already existing and new disinfectants. There are two major classes of disinfectants, oxidizing and non-oxidizing. Oxidizing disinfectants, such as hypochlorites and hydrogen peroxide, destroy all cellular matter, while non-oxidizing disinfectants, including alcohols (ethanol, and propanol), and quaternary ammonia compounds (benzalkonium chloride, and tetrabutylammonium chloride, among others), disrupt the natural metabolic pathways of the cell (Gomes, 2019). In low concentrations, although with some exceptions, disinfectants can pose risks to aquatic organisms. Fatty alcohol polyoxyethylene ether-7 (AEO-7), for example, a non-oxidizing disinfectant, has been considered as 'super toxic' (LC_{50} of 15.35 μg/L for zebrafish) by the U.S. Fish and Wildlife Service (USFWS) (Al-Asmakh et al., 2020). Also, the oxidant disinfectant sodium hypochlorite, with an LC_{50} of 48 mg/L calculated for zebrafish, is considered by the United States Environmental Protection Agency (US EPA) to be 'highly toxic' to aquatic organisms (United Sates Environmental Protection Agency [US EPA], 1991; Magalhães et al., 2007).

Among the various types of disinfectants described in List N Tool: COVID-19 Disinfectants by EPA, discriminated according to the active ingredient, application site (institutional, healthcare, residential), surface type, and contact time (the amount of time required for the product be effective), the most recommended products were surface disinfectants (586 products) (US EPA, 2022; Nowak et al. 2021). As active ingredients, the US EPA list provides 35 different products. Among the most applied products, we may find ethanol, isopropanol, hydrogen peroxide, various forms of hypochlorites, and quaternary ammonium compounds (QACs) (US EPA, 2022).

The production of quaternary ammonium compounds constituted 22,68 million kg in 2015 (Hora et al., 2020). Since the outbreak of the pandemic of SARS-CoV-2, the QACs market has reached 1104.6 million US dollars, in 2022 (Precision reports, 2022). In 2020, there were 216 products described in List N Tool, and nowadays this number has increased to 277 chemicals that may be used as disinfectants (US EPA, 2022). However, this growing trend is not expected to decrease, but rather to increase up to 10% by 2027, which translates into a market of 1.63 billion US dollars (Hora et al., 2020; Precision reports, 2022).

As a result of the increased growth in production, QACs reach the environment faster than before the SARS-CoV-2 outbreak. It is predicted that most of the QACs partially reach sewers and wastewater treatment plants (WWTPs) (~75%) (Hora et al., 2020; Tezel and Pavlostathis, 2011). WWTPs remove almost 90% of the total volume of QACs that reaches these facilities, through their absorption to biosolids and their degradation. Despite the high percentage of removal from WWTPs, they only partially remove such substances, due to the high production volume of these chemicals (Tezel and Pavlostathis, 2011). So, a considerable amount of QACs is still found in the aquatic environments in concentrations of 1 to 60 ug/L (Hora et al., 2020).

Quantitative and ionic liquid analysis from 13 different water samples have indicated benzalkonium chloride as a substance occurring in high concentrations, ranging from 216 ng/L to 1386 ng/L, compared to other quaternary ammonium compounds (Tezel and Pavlostathis, 2011). This is probably due to the widespread use of BAC as anti-microbial household cleaners and soaps (Hora et al., 2020). The first use and commercialization of these BACs dates back to 1947 (Pereira and Tagkopoulos, 2019). Since then, their application has been vast, starting with household products (fabric softeners, cosmetics, hygiene products) to medical (e.g., nasally administered drugs), agricultural, and industrial products (Tezel and Pavlostathis, 2011; European Medicines Agency, 2017; Choi et al., 2018).

BACs reach the aquatic environment via WWTPs, where BACs present in treated effluents are discharged in aquatic environments (Zhang et al. 2015); via the increased usage in outdoors spaces, where rainwater carries this compound to sewage waters to WWTPs, or directly to aquatic compartments (Van De Voorde et al. 2012); industrial discharges have been also involved in this problematic, as already reported to occur in a pharmaceutical manufacturing complex, in South Korea (Kim et al., 2020). Consequently, BACs are found

in riverine systems, and Zhang et al. (2015) reported concentrations between 2.5 and 65 µg/L in Taiwanese rivers. Also, effluents from Australian WWTPs had concentrations of BAC between 0.5 and 4.1 µg/L. Additionally, Kummerer et al. (1997) reported 0.5 to 5 mg/L of BAC in the effluents of European hospitals. All these records were published before the SARS-CoV-2 outbreak, so higher concentrations are to be expected now, due to the increase in the production and use of such chemicals (Hora et al., 2020).

Because of its chemical and physical characteristics, especially the length of their alkaline chain (C-12), BAC activity is exerted directly on the cell membrane, as reported in the membranes of zebra mussels, green algae (*Selenastrum capricornutum*), and fish (*Pimephales promelas,* and *Ictalurus punctatus*) (Waller et al., 1996; Rosen et al., 2001; Eleftheriadis et al., 2002; Okahara and Kawazu, 2013). Biotransformation, and biodegradation of BAC, by *Aeromonas hydrophila*, takes place by first splitting the alkyl chain from its quaternary nitrogen (Zhang et al., 2011). Then, produced benzyldimethylamine is converted to ammonia through demethylation, debenzylation, and demethylation processes. BAC can also be degraded in water through the Fenton reaction (H_2O_2/Fe^{2+}) process (Zhang et al., 2016). In this process, Fe^{2+} reduces hydrogen peroxide (H_2O_2), producing hydroxyl radicals (HO*). This reaction induces a considerable increase in HO* radicals. In turn, these free radicals can enhance oxidative stress (Lu et al., 2020).

As a consequence of the biodegradation processes, an induction of oxidative stress accompanied by the overproduction of hydroperoxide and superoxide anion, demonstrated on epithelial human cells, leads to an increase in mitochondrial mass, degradation of deoxyribonucleic acid (DNA), and lipid membranes. Finally, the factors mentioned above may trigger apoptosis (Karbowski et al., 1999; Matsuhashi et al., 1997; Pauloin et al., 2008; Yi et al., 2022). Given the properties of this chemical, it is expected that organisms exposed to this compound will exhibit alterations in biomarkers of oxidative stress (Pauloin et al., 2008; Clouzeau et al., 2012). Some studies have already demonstrated an increase in oxidative stress resulting from an exposure to sodium dodecylbenzene sulfonic acid salt (SDBS) and alkylbenzene sulfonate, to *Lateolabax japonicus* and *Oncorhynchus mykiss*, respectively (Hofer et al., 1995; Jifa et al., 2005). Given the general consensus of an increment in oxidative stress, Antunes et al. (2016) reported an enzymatic inhibition of catalase (CAT). Glutathione S-transferases (GSTs) enzyme detoxifies xenobiotics by increasing their hydrophilicity, raising its excretion rate. However, this enzyme can also act

as an antioxidant enzyme to protect from oxidative damage, when the generation of reactive oxygen species (ROS) through the metabolism of the compound occurs (Liu et al., 2010). Mediated by GSTs enzyme, reduced glutathione actively scavenges ROS, preventing oxidative toxicity (Liu et al., 2010; Das and Roychoudhury, 2014). A downward trend was reported, when individuals of the fish species *O. mykiss* were chronically exposed to concentrations up to 1.050 mg/L of BAC (Antunes et al., 2016). An increase in GSTs activity translates into an activation of this detoxification pathways. However, when an inhibition is verified, we can assume, for example, that the ROS produced due to the exposure to the toxicant can decrease the GSTs activity. Inhibition of this phase II, by xenobiotics, in fish species (*Brycon amazonicus* and *O. mykiss*) and in rats, due to the excessive ROS, has already been described (Letelier et al., 2006; Monteiro et al., 2010; Antunes et al., 2016). Despite not being demonstrated in fish, BAC is metabolized in humans, by the cytochrome P450 (CYP 450), namely by the CYP4 family of isoforms, and by CYP 2D6 (Seguin et al., 2019).

Toxicity of this cationic surfactant was also detected in the embryonic development of zebrafish, with delayed hatching, increased embryonic mortality, and morphological malformations (Sreevidya et al., 2018). Since membrane disruption, oxidative stress, and cholinergic neurotoxicity can occur, the process required for neurotransmission can also be impaired (Shephard and Whiting, 1992; Guilhermino et al., 2000; Nunes et al., 2005). Cholinergic toxicity occurs when acetylcholine (ACh) is not degraded, and accumulates in the synaptic cleft (Lott and Jone, 2022). Some controversy on the cholinesterase inhibition has been debated over the years. On one hand, some studies report that detergents could be toxic to several organisms *in vivo*, and *in vitro*, such us *Daphnia magna*, *Moina macropa*, *Mytilus galloprovincialis,* and *Tilapia nilotica*, (Martínez-Tabche et al., 1997; Guilhermino et al. 1998; Guilhermino et al., 2000; Feng et al., 2008; Wang et al., 2014). On the other hand, the neurotoxicity through cholinesterase inhibition by detergent agents, is unlikely to occur. Nunes et al. (2011) proposed that acetylcholinesterase (AChE) inhibition reported in other studies may be an experimental artifact. In this study, the author observed that after homogenizing the tissue, the SDS detergent used tended to form micelles. These in turn can dissolve portions of the cell membrane forming micelles containing AChE, inhibiting AChE activity. However, Shephard and Whiting (1992) showed BACs ability to precipitate, which could reduce the total amount of protein and increase the relative amount of AChE in

samples, considering that the activity of AChEs is expressed as a function of total soluble protein of samples. Given this evidence, it is vital to understand the potential toxic effects on fish by measuring the motor function (hyper- or hypoactivity) of aquatic organisms. Swimming activities are key point for survival of aquatic organisms, so impairments in locomotor system should be assessed to ascertain about the toxicity of detergents.

Zebrafish (*Danio rerio*) have been increasingly used as a standard model for ecotoxicological studies (Scholz et al., 2008; Hollert and Keiter, 2015; Bambino and Chu, 2017), considering their possible direct contact with polluted water, position in the trophic web, wide distribution, and ecological role. In addition, their easy maintenance in laboratory conditions, high reproduction rates, rapid development, and transparency of the embryos, favors the use of zebrafish as an optimal species to perform ecotoxicological studies (Marple et al., 2004; Selderslaghs et al., 2012; Bambino and Chu, 2017).

Since BAC is a non-oxidant disinfectant capable of altering the normal metabolic pathways of the cell, and given the evidence already presented above, the aim of this study was to assess the toxicological changes in *D. rerio*, after being acutely exposed to BAC (Yu et al., 2021). We propose a multi- faceted approach, which includes (1) alterations in embryonic development, through a FET assay; (2) behavior analysis, through an evaluation of the swimming activity, thigmotaxis behavior, and erratic movements and (3) a multi-biomarker approach by measuring the activity of phase I, and II enzymes and antioxidant defense mechanisms. Phase I reactions were evaluated by measuring the activities of cytochromes P450, CYP 1A1 and CYP 1A2 were analyzed. Finally, phase II reactions were also assessed by the quantification of GSTs activity. This enzyme can also, participate in the defense against oxidative stress (Van der Oost et al., 2003). Since BACs have been demonstrated to be a potential generator of ROS, the activities of enzymes that are capable of degrading these products were assessed, such as CAT and Glutathione peroxidase (GPx). Endogenous enzymatic and non-enzymatic antioxidants are important to convert ROS to nontoxic metabolites (Bebe and Panemangalore, 2003). The key enzymes for that process are CAT and GPx.

4. Materials and methods

4.1 Chemicals

Benzalkonium Chloride (CAS 63449-41-2, purity ≥ 95%) was purchased from Sigma Aldrich®. Bradford reagent was obtained from Biorad®, UK. All other chemicals used for media or buffers to enzymatic determinations were obtained from Sigma-Aldrich® or Merck-Millipore®.

4.2 Test Organisms

For this study *D. rerio*, provided by the Biology Department of the University of Aveiro (Portugal), were used. The organisms were maintained under a photoperiod cycle of 12h:12h light/dark, at 27 ± 1°C. The water in the circulation system was held at 800 ± 50 µs, with Spectrum Brands salt, oxygen saturation above 95%, and pH at 7.5 ± 0.5. Zebrafish embryos were harvested after 1h30 of natural spawning and maintained in system water, at 27 ± 1°C, for 5 days. Larvae with 5 days post-hatching (dph) were exposed to the selected compound, and behavioral and enzymatic assays were performed. However, to perform the FET, embryos were collected and exposed immediately for 96h (OCDE, 2013).

In all trials, the organisms were exposed to 3 environmental concentrations: 0.1, 0.5, and 2.5 mg/L of BAC. These concentrations were established by multiplying the minimal concentration for which effects were already reported by a factor of 5 (0.1 mg/L) (Antunes et al., 2016).

All assays were performed according to the optimal conditions mentioned previously (photoperiod, temperature, dissolved oxygen, and pH).

4.3 FET

To perform a FET, the embryos were thoroughly cleaned with system water, and checked under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon). The FET was initiated as soon as possible after the selection of eggs, and 20 eggs per treatment were randomly distributed in 24-well plates. Every 24h, the medium was renewed (2 ml of medium per well), and the following parameters were analyzed: coagulation, hatching,

presence of cardiac rhythm, presence of somites, tail detachment, hatching delay, edema, and pigmentation. The organisms were considered dead whenever there was coagulation, absence of cardiac rhythm, somite, or no detachment of the tail, according to the OCDE 236 guideline (OCDE, 2013).

4.4 Behavioral Tests

Organisms with 5-dph, one per each well, were placed in 24-well microplates. Larvae presenting deformities or exhibiting physical abnormalities that could impair locomotor behavior were excluded. For each concentration, 96 larvae were randomly selected, and distributed. Each larvae was placed in 2 ml solution per well (Oliveira et al., 2021). After 24h of exposure, the movement was tracked using the Zebrabox (Viewpoint, Lyon, France), using a 25 frame per second infrared camera. First overall activity was assessed through the total distance traveled. The readings included a habituation period of 6 minutes. Lights were kept on during this period, thereafter the lights were turned off abruptly, for 4 min and the swimming behavior was registered by an infrared camera (Schnörr et al., 2012; Andrade et al., 2016). Parameters such as total distance, total time in movement, were recorder every minute. Thigmotaxis was studied by discriminating movement performed by larvae in an inner area of the well (12.25 mm ray), and an outer area (4 mm from the outline of the first area) to assess preference for the edges of the well. Abrupt changes of direction (erratic swimming) were measured defining 4 classes of angles according to Zhang et al. (2017). Class 1 includes angles with higher amplitude (90°- 180°), class 2, and 3 contain angles between 30°- 90°, and 10°-30°, respectively. Class 4 comprises angles between 0° to 10°. The data collected were pre-treated to obtain the percentage of distance moved (%TDM) in the peripheral zone. Thus, the thigmotaxis was calculated as the ratio between the distanced traveled in the outer zone, and the total distance traveled. The percentage of this parameter was obtained by multiplying by a factor of 100, to minimize individual differences (Bouwknecht and Paylor, 2008). Also, the percentage of time spent in the outer area (%TTM) was obtained as the ratio between the time spent in the peripheral zone and the total duration of the test. Also, to minimize the individual differences, the value was multiplied by a factor of 100 (Bouwknecht and Paylor, 2008).

4.5 Enzymatic determinations

For the determination of the enzymatic activity of catalase, glutathione-S-transferases, glutathione peroxidase, CYP 1A1, and CYP 1A2, 96 larvae with 5-dpf per treatment were exposed, for a period of 24h, to a range of concentrations of BAC. The 96 animals per-treated, were grouped in 12 samples with 8 larvae per sample. However, in the case of GPx, 10 larvae per set was used. These assays were carried out under the conditions mentioned above. After a 24h exposure, the samples were frozen in microtubules with 1mL of phosphate buffer 50mM, pH= 7.0, with Triton X-100 0,1%, to determine the enzymatic activity of GPx, CAT and GSTs. To assess CYP 1A1, and CYP 1A2 activities, a solution of Tris HCL 50 nM, pH=8.0, 0.15M KCL, 1mM dithiothreitol was added instead. Samples were then stored at -80°C. Enzymatic activity was determined for GPx, GST, and CAT by homogenizing and centrifuging (Megafuge 8R, Thermo Scientific) samples at 15000 *g*, or 10000 *g* for CYP 1A1, and CYP 1A2 for 10 min, at 4°C.

In order to determine the enzymatic activity of CYP 1A1 and CYP 1A2, the method proposed by Cheah et al. (1995) was followed. The supernatant resulting from the first centrifugation was removed to a new set of Eppendorfs microtubes and a solution of 12.5 mM sucrose with 8mM CaC_{12}, at pH=7.4, was added to obtain microsomes from aggregation with calcium. After centrifuging samples at 1696 *g* for 10 min, at 4°C, the supernatant was discarded and the pellet resuspended with 0.1M of phosphate buffer pH=7.4, with 1mM EDTA, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate and 0.4% (w/v), and Triton X-100. Subsequently on a microplate reader, the respective reaction solution (7-ethoxyresorufin for CYP 1A1, and methoxyresorufin for CYP 1A2), and NADPH were added to the samples. The activity was measured spectrofluorometrically (Hitachi, F-7000, with the FL Solutions 2.1 for F-7000 software), between λex=540nm, and λem=590nm, and expressed as pmol/min/mg of protein (Ferreira et al. 2010).

To access GSTs activity, a phase II biomarker, the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione was catalyzed by glutathione S-transferases, according to the method described by Habig et al. (1974). The generation of a thioether from this conjugation leads to an increase in absorbance at 340nm. Glutathione-S-transferases activity was expressed as unit of nanomoles of thioether produced per min/mg of protein.

CAT activity was measured as described by Aebi (1984), following a decrease of absorbance at a wavelength of 240 nm, in the microplate reader (Multiskan Spetrum, Thermo Scientific) with SkanIt ^{TM} software, due to the decomposition of H_2O_2. Enzymatic activities were expressed considering one unit of activity equals the number of consumed H_2O_2 moles per minute, per milligram of protein.

GPx activity was measured indirectly from the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), at 340 nm, by following the protocol proposed by (Flohé and Gunzler, 1984). Oxidation of NADPH occurs when oxidized glutathione (GGS) is reduced to glutathione in its reduced form (GSH). In turn, the enzyme GPx oxidizes GSH and H_2O_2. The total GPx, determined with cumene hydroperoxide, was expressed in units of nmol of oxidized NADPH per minute and per milligram of protein.

For all determinations, total protein concentration was assessed following the methodology described by Bradford (1976). To express all enzymatic activities per mg of protein, a standard of 1mg/ml of y-globulin was used.

4.6 Statistical analysis

A one-way analysis of variance (ANOVA) was used to assess the differences effects obtain among treatments. Results that passed the requirements of the Kolmogorov-Smirnov normality test, or when they failed to meet these criteria, a Kruskal-Wallis test, was performed. A Dunnett test was applied to infer significant differences among the concentrations, and the control. To infer statistically significant results the GraphPad software was used, and a significance level of 0.05 was used.

5. Results

5.1 FET

The FET showed that BAC did not induce significant mortality in zebrafish embryos. Figure 2 presents the cumulative lethality, where higher mortality was observed for animals exposed to higher concentrations, starting after 48h.

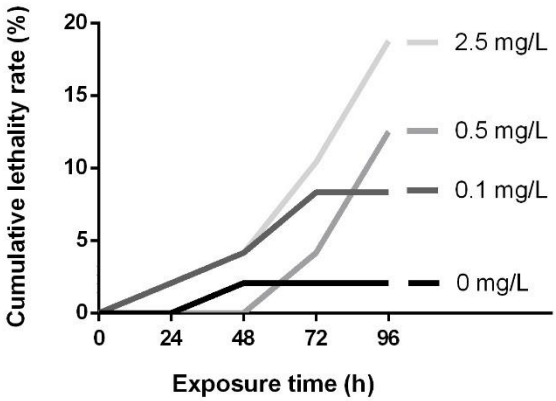

Figure 2 - Cumulative lethality rate (%), after a 96h exposure to BAC. N=96.

In addition, we were also able to observe other alterations, such as delayed hatching in 7% of the embryos exposed to 0.1 mg/L, and by 2.3% of the embryos in concentration of 0.5 mg/L (Figure 3 B1,2, and 4, C2, and 3). Pericardial edema was also registered in 2.3 %, and by 10% of the embryos after an exposure to 0.1 mg/L, and 0.5 mg/L, respectively (Figure 3 B3, C4, and 5, and D1). Finally, tail malformations were also registered in 5% of the embryos in 0.1 and 0.5 mg/L, and by 10% in 2.5 mg/L (Figure 3 D2, and 3), after an acute exposure of *D. rerio* to BAC.

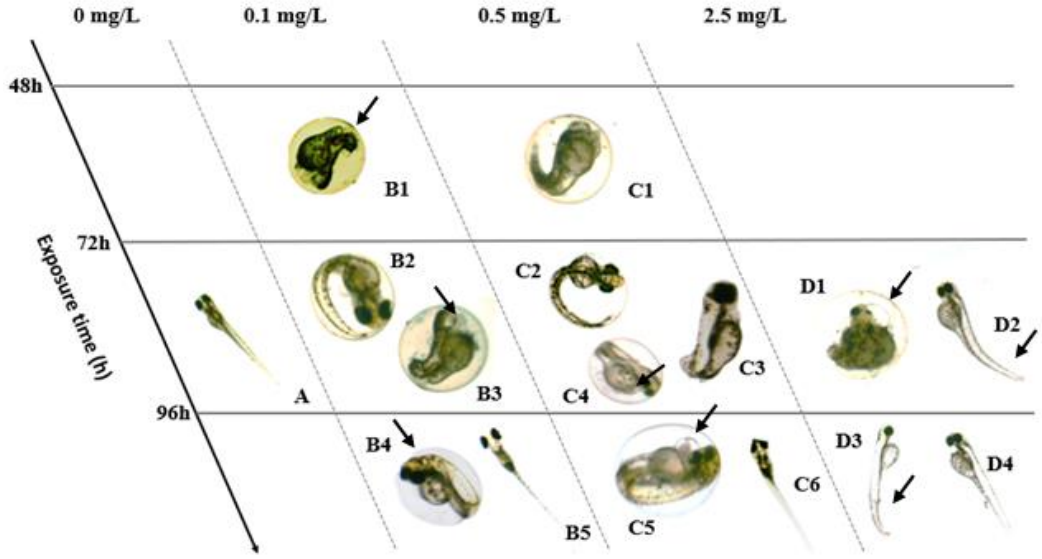

Figure 3 - Observed effects along the 96h exposure to BAC. A), B5), C6), and D4) Normal development; B1,2, and 4), and C2), and 3) Hatching delay; C1) Dead embryo; D2), and D3) Tail malformation; B3), C4), and 5), and D1) Pericardial edema.

5.2 Behavioral Tests

The first behavioral test measured the total distance moved during the assay. A significant increase was observed in animals exposed to 2.5 mg/L, when compared to the control ($F_{[3, 356]} = 9.146$, $p < 0.0001$) (Figure 4).

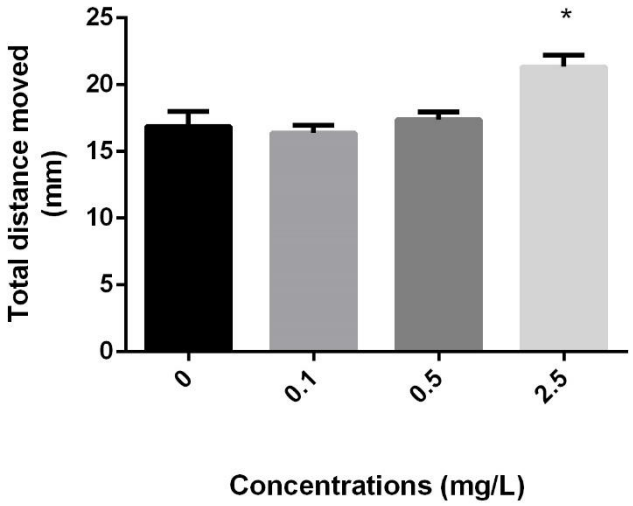

Figure 4 - Total distance moved (mm) after an acute exposure of D. rerio to different concentrations of BAC. Results are represented as mean standard error. n= 96. Significant differences from the control, p< 0.05.

Benzalkonium chloride significantly increased the %TDM ($F_{[3,452]} = 8.700$, $p<0.0001$) in animals exposed to the highest concentration tested (Figure 5A). Also, a significant increase was reported in the %TTM ($F_{[3,447]} = 11.66$, $p<0.0001$) in animals exposed to the highest concentration, when compared to the control group (Figure 5B).

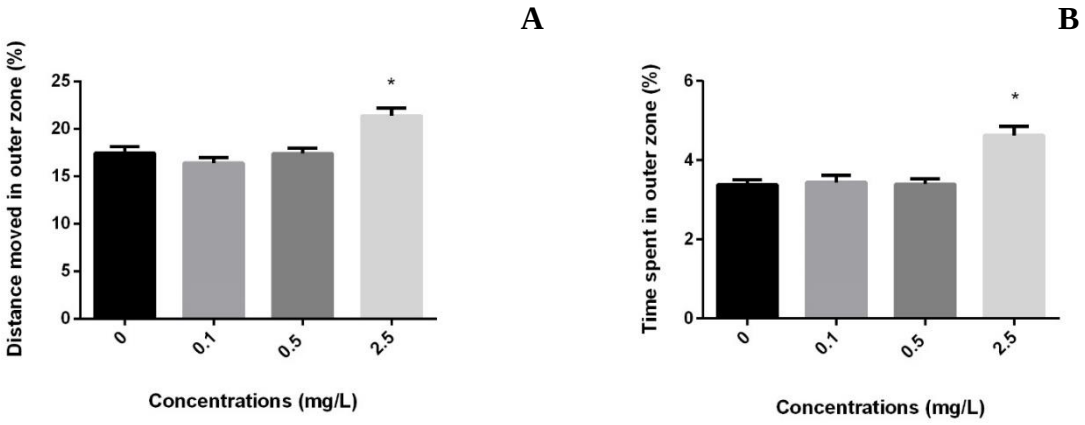

Figure 5 - (A) Effects on distanced travel in the outer zone, and (B) time spent in the outer zone, made after an acute exposure of *D. rerio* to BAC. Data are represented as mean ± standard error. n= 96. Significant differences from the control, p<0.05.

The frequency of the angles performed was also measured, and a significant increase was observed in organisms exposed to 2.5 mg/L, class 1, when compared to the control group ($F_{[9,48]} = 1.508$, $p<0.05$) (Figure 6).

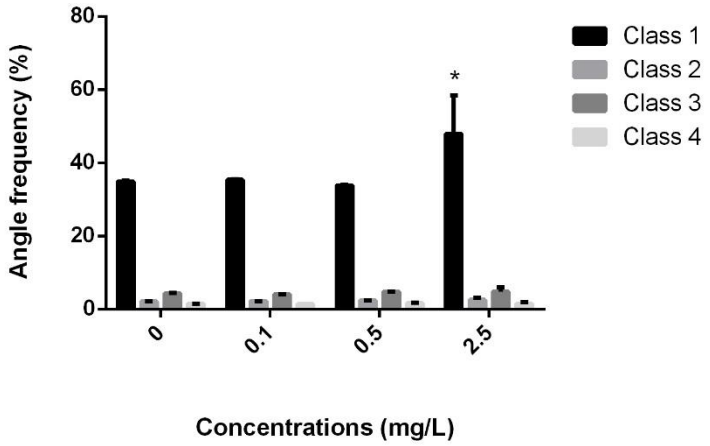

Figure 6 - Results of the angle frequency determined in *D. rerio*, after an acute exposure to BAC. Data are represented as mean ± standard error. n=96. Significant differences form control, $p< 0.05$.

5.3 Enzymatic determinations

5.3.1 Phase I metabolic biomarkers

Significant differences were found in the animals exposed to 0.5 mg/L with an increment in CYP 1A1 activity ($F_{[3,36]} = 11.52$, $p<0.0001$), when compared to the control (Figure 7A). Also, a significant decrease in CYP 1A2 was observed in animals exposed to the concentrations of 0.5, and 2.5 mg/L, ($F_{[3,32]} = 4.342$, $p=0.0112$) (Figure 7B).

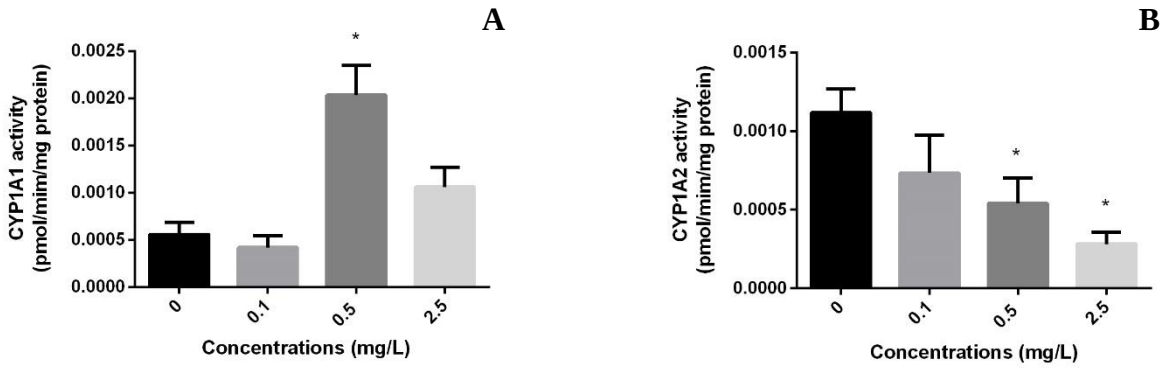

Figure 7 - Results of CYP 450 enzymes determined in *D. rerio*, after an acute exposure to BAC. A) CYP 1A1 activity; B) CYP 1A2 activity; Data are represented as mean ± standard error. n=12 Significant differences from the control, $p< 0.05$.

5.3.2 Phase II metabolic biomarkers

GSTs activity was significantly decreased, in all concentrations, when compared to the control ($F_{[3,44]} = 18.77$, $p<0.0001$), after an acute exposure (Figure 8).

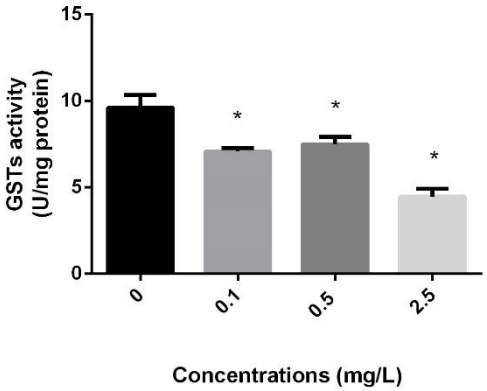

Figure 8 - GSTs activity determined in individuals of *D. rerio*, after an acute exposure to BAC; Data are represented as mean ± standard error. n=12. Significant differences from the control, $p<0.05$.

5.3.3 Oxidative stress biomarkers

In terms of CAT activity, significant differences were only observed in animals exposed to the concentration of 0.5 mg/L, with an increment in this endpoint ($F_{[3,37]} = 2.968$, $p= 0.0443$) (Figure 9A). In terms of GPx activity, there was a significant decrease of this parameter in animals exposed to the highest concentrations ($F_{[3,35]} = 9.093$, $p= 0.0001$) (Figure 9B).

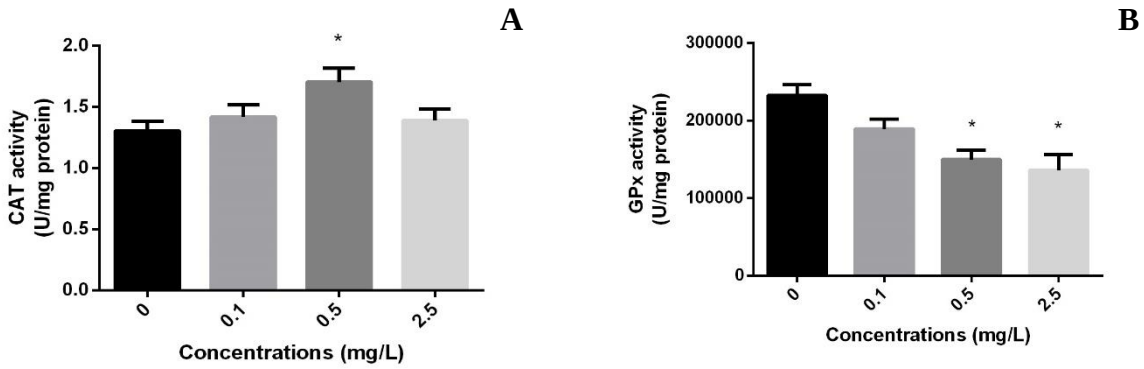

Figure 9 - Results of oxidative stress after an acute exposure of *D. rerio* to BAC. (A) CAT activity; (B) GPx activity; Data are represented as mean ± standard error. n=12. Significant differences from the control, $p<0.05$.

6. Discussion

BAC has already been implicated in the development of genotoxicity, cholinergic toxicity mediated by membrane disruption, and oxidative stress (Lei et al., 2015; Antunes et al., 2016). Metabolically, BAC can be decomposed into carbon dioxide, water, ammonia, and chlorines ions, through demethylation, debenzylation, and demethylation pathways, via intermediates such as 4-hydroxybenzoic acid, decanoyl acid, benzyl dimethyl amine, and dodecanal. It can also be decomposed through the Fenton reaction, producing hydroxyl radicals, as reported in bacteria in aqueous medium (Raymond, 2009; Zhang et al., 2016; Seguin et al., 2019). BAC has the particularity of being able to bind to acetylcholine muscarinic receptors, which in turn are linked to G-Couple proteins.

The large positively charged molecules of BAC are poorly absorbed by gastrointestinal tract (Arugonda, 1999). Despite the different possible routes of entry of BAC, Xue et al. (2004) have provided evidence that the major route of exposure is by inhalation, and is discharged mostly by feces in rats. Thus, we can expect that changes in zebrafish are most prone to be observed at the gill level, as reported to occur in *O. mykiss* by Kierkegaard et al. (2020). Activation of metabolic pathways, such as CYP 450, have been also reported occur in the human liver (Seguin et al., 2019).

6.1 FET

Previous studies conducted about the toxicity of BAC demonstrated the deleterious effects at the embryonic level, with increasing mortality and malformations (Cao et al., 2009; Sreevidya et al., 2018). Although our results did not showed significant mortality or malformations, our mortality spiked after the first 48h in embryos exposed to the highest concentration (2.5 mg/L), as well as some malformations (tail malformations and pericardial edema), similar to what was reported in other studies (Cao et al., 2009; Sreevidya et al., 2018). Sreevidya et al. (2018) reported a higher mortality rate in all concentrations tested (0.1, 0.25, and 0.5 mg/L), from 48h onward, when compared to the embryos exposed for 24h.

Also, in the study of Sreevidya et al. (2018), malformations in terms of the spine curvature and edema were observed in embryos exposed to the highest concentration, as in our study. Body curvature and pericardial edema suggest kidney problems and cyst formation (Cao et al., 2009). The development of pericardial edema in larvae is linked to a decline in renal function due to the organisms inability to maintain the water balance (Cosentino et al., 2010; Swanhart et al., 2011). Although no studies reflect the potential nephrotoxicity in zebrafish, microinjections of BAC in rats resulted in higher concentrations of this substance in kidney tissue (Xue et al., 2004). Also, in the study of Kierkegaard et al. (2020), small amounts of BAC were found in *O. mykiss* kidney. Since BAC can often be directly discarded in human urine, we suggest that this compound may, in an active way, have interfered with the normal function of the kidney (Antunes et al., 2016).

The main effect observed on the embryos, on previous studies, was delayed hatching, which usually occurs within the first 48h (Sreevidya et al., 2018). However, in our study the

majority of the larvae hatched in the first 48h, and no significant delay in hatching was observed. Nonetheless, we observed an increased delay in hatching (7%) in larvae exposed to a concentration of 0.1 mg/L, when compared to the 2.3% delay in hatching in animals exposed to a concentration of 0.5 mg/L. Major delays in hatching were also observed in animals exposed to the lowest concentration (0.1 mg/L) in the study by Sreevidya et al. (2018). The number of embryos presenting delay in hatching decreased in a dose-depended manner. As the number of delayed embryos per hatch diminished, the mortalities increased. This dose dependent response reported in our study is corroborated by Sreevidya et al. (2018), where an increase in mortality was followed by a decrease in hatching delay, due to increasing concentrations. Surfactants penetrate the phospholipidic membrane, leading to the weakening of its integrity, and structures of the cellular membrane. This disruption leads to extravasation of the cell contents, thereby disrupting the normal function of the cell, that in turn can lead to the death of the zebrafish embryo, since the embryonic membrane protecting the embryo has a similar constitution and structure as that of a cell membrane (Kimmel et al., 1995; Mustapha and Bawa-Allah, 2020).

Due to the previously mentioned interaction with the cellular membrane, sorption, and destabilization, we can assume that the observed delay in embryo hatching is due to the membrane destabilization carried out by the presence of the benzyl group, and the affinity of BAC for phospholipids (Timmer and Droge, 2017; Sreevidya et al., 2018; Kierkegaard et al., 2020). BAC belongs to the surfactant category and is likely to penetrate the phospholipidic membrane that constitutes the yolk sac, leading to a destabilization of the cellular membrane. The embryonic membrane of the zebrafish embryo has several functions, including protecting the embryo from chemical and physical damage, and maintaining exchanges with the external environment (Hwang and Chou, 2013; Gordon et al., 2019). These exchanges are carried out in a similar manner as to other cell membranes, controlled by ionic exchanges (Chang and Hwang, 2011; Hwang and Chou, 2013). BAC, due to its chemical characteristics, inserts itself in the cell membrane, promoting its disruption, and changing the ionic balance. It is likely that these alterations could lead to an increase in the concentration of the compound reaching the organism, disrupting its osmotic balance, therefore causing stress to the organism (Hwang et al., 2011; Hwang and Chou, 2013; Guh et al, 2015). Thus, we suggest that this delay in hatching may be due to the changes in ion

channels promoted by the insertion of the xenobiotic molecule in the membrane (Xia and Onyuksel, 2000; Timmer and Droge, 2017).

6.2 Behavioral Tests

The main mode of action of QACs, including BAC, is through the disruption of all lipid bilayers by interactions with the alkyl chain of the compound, and by changing the electric charge on the membrane surface, through nitrogen ions (Wessels and Ingmer, 2013). In studies conducted in bacteria, changes in porins and in efflux pumps were found (Wessels and Ingmer, 2013). So, it is expected that BAC could promote alterations in the ionic balance of the cellular membrane and disrupting the normal transport between the extracellular space and the intracellular space. Alterations on the ionic potential of the membrane can lead to a disruption of the membrane, which can ultimately lead to impairments in locomotor activity (Lei et al., 2015). In addition, alterations in the spontaneous tail movements towards the yolk sack, due to impairments in fish motor system, carried out by unbalance of ion content in the membrane, can be a possible reason for the delayed hatch (Ogungbemi et al., 2019).

Our results showed a significant increment in the total swimming activity in animals exposed to the last concentration of 2.5 mg/L. Also, in the thigmotaxis behaviour significant differences in animals exposed to the higher concentration (2.5 mg/L), in both parameters of the total distance and time spent, were observed. Thigmotaxis is a valid tool to evaluate anxiety-like behaviour in 5-dph larvae (Schnörr et al., 2012). Typically, during light periods, zebrafish display a low level of activity. For that reason, we focused our results only in the dark period. There was a gradual, though slight, increase in the distance moved and time spent in the outer zone. This increase indicates that the organism exposed to 2.5 mg/L of BAC displayed a more anxious behaviour. Larvae exposed to 2.5 mg/L of BAC also presented a higher number of class 1 angles, suggesting erratic swimming behaviour (Almeida et al., 2019).

Although no significant changes were observed in animals exposed to the lowest concentrations (0.5, and 1 mg/L), a general tendency towards an increment in all the behavioral parameters was observed, which we can suggest may be related to impairments in the cholinergic pathways. According to the literature, alteration at the membrane level produced by BAC could indeed cause impairments in locomotor activity, through an

unbalance of ions. However, we suggest that the main effects reported in our study could be a result of impairments at the cholinergic pathways. In the work by Antunes et al. (2016), an increase in the AChE activity was observed. It has been reported that a variety of compounds could lead to impairments in the cholinesterase pathway. Pesticides, such as organophosphorus, carbamates, pharmaceuticals and disinfectants, can inhibit or activate AChE (Ostergaard et al., 1989; Saglio et al., 1996; Phillips et al., 2002; Antunes et al., 2016). During the neurotransmission event in cholinergic neurons, ACh is released, and binds to nicotinic and muscarinic ACh receptors on the post-synaptic membrane. AChE terminates the signal by hydrolyzing ACh (Colovic et al., 2013). An inhibition of the AChE activity creates an overload of ACh in the synaptic clef, leading to a hyper-stimulation of the post-synaptic membrane, and can ultimately lead to paralysis and dead. Therefore, a relationship between the activity of AChE and swimming behavior can be established (Tilton et al., 2012; Bonansea et al., 2016; Pullaguri et al., 2020). However, this inhibition and increase in the AChE activity is not straightforward since an increase in activity in acetylcholinesterase can also lead to an increased swimming activity (Bonansea et al., 2016). Notwithstanding the above mentioned, we believe that the increased activity of the organisms reported in our study was due to the observed inhibitions reported in the literature, for many different organisms (Martinez-Tabche et al., 1997; Guilhermino et al. 1998; Guilhermino et al., 2000; Feng et al., 2008; Wang et al., 2014). When an inhibition of AChE occurs, the ACh released into the synaptic cleft is not hydrolyzed, and in turn occurs a hyperstimulation of the postsynaptic nerve due to the increased amount of ACh (Colovic et al., 2013; Lott and Jone, 2022;). This hyperstimulation can lead to an increase in activity as reported by other studies (Nunes et al., 2011; Tilton et al., 2012; Bonansea et al., 2016; Pullaguri et al., 2020; Lott and Jone, 2022).

6.3 Enzymatic determinations

6.3.1. Phase I metabolism

Previous studies have shown that quaternary ammonium salts are metabolized by cytochrome P450 in humans (Seguin et al., 2019). Their chemical structure permits the direct conjugation or oxidation of the nitrogen heteroatom but could also be susceptible to N-

dealkylation and C-H oxidations, catalyzed by the CYP enzymes (Seguin et al., 2019). Nevertheless, our results showed an increase in the enzymatic activity of CYP 1A1 in organisms exposed to 0.5 mg/L, while an inhibition of CYP 1A2 was observed in organisms exposed to the highest concentrations. To our knowledge, the increase in the enzymatic activity of CYP 1A1 reported in our study is the first reporting that CYP 450 may be involved in the metabolism of BAC in fish. Metabolism via CYP 450 may lead to an increase in free radicals (Winston and Giulio, 1991; Turrens, 2003). However, a marked inhibition of CYP 1A2 was also reported in our study, which was also observed in CYP4 enzymes and CYP2D6 in humans, after being exposed to this chemical (Seguin et al., 2019). We propose that the differences reported in our work result from the different affinities of the xenobiotic towards the isoenzymes, and the possible rise of ROS. On one side, the different locations of these two enzymes in the membrane, their affinities, and their three-dimensional structures can explain the increase observed in CYP 1A1, and an inhibition of CYP 1A2 (Park et al., 2015; Kapelyukh et al., 2019). CYP 1A2 is located in ordered sites of the reticulum endoplasmic (RE) membrane and is expressed in a higher amount in fish (Park et al., 2015). On the other hand, CYP 1A1 is expressed in less quantity, and is located in more disordered sites of the membrane (Park et al, 2015). Ordain domains are considered detergent-resistant, whereas less organized domains are more soluble (Park et al., 2015). Another possible reason for the differences in enzyme activity reported in our study results from the ability of the enzyme complex itself to produce ROS (Ekstrom and Ingelman-Sundberg, 1989; Winston and Giulio, 1991; Turrens, 2003). The increase in superoxide and hydroxyl radicals, produced by CYP 450 or other metabolic pathway of this compound, could inhibit its activity. The possible increment in ROS reported by several studies in different model organisms (*O. mykiss*, *L. japonicus*, *Carassius gibelio*, and *B. amazonicus*) exposed to quaternary ammonium salts may lead to an increase in O_2^-, and H_2O_2. The rising amount of ROS could lead to a denaturation and inhibition of CYP 1A2 (Ekstrom and Ingelman-Sundberg, 1989; Hofer et al., 1995; Debbasch et al., 2001; Jifa et al., 2005; Monteiro et al., 2010).

6.3.2. Phase II metabolism biomarkers

Concerning the activity of GSTs, a key group of enzymes involved in phase II detoxification, it was possible to observe a significant inhibition, in fish exposed to all tested concentrations (0.1, 0.5, and 2.5 mg/L). This phase II metabolic enzyme detoxifies endogenous and exogenous chemicals, such as peroxides, lipids, and xenobiotics (Van der Oost and Vermeulen, 2003; Čolak and Žorić, 2019). Since their main function is to detoxify, this enzyme plays a key function by increasing the excretion rate of a vast number of xenobiotics (Atli and Canli, 2010). Gheorge et al. (2020) demonstrated a 45% inhibition of GSTs activity, on *C. carpio* after an exposure to 1 mg/L of benzalthonium chloride. In this study, the low levels of GSH in liver samples led to the possible decrease in GSTs activity accompanied by the inhibition through ROS (Gheorghe et al., 2020). So, other mechanisms may be in place to promote GSTs inactivation. Antunes et al. (2016) also demonstrated a downward trend in GSTs activity, suggesting that this pathway was not induced following exposure to BAC. However, in rats exposed to BAC, an increase in the activity of this biomarker was observed (Swiercz et al., 2008). However, we must bear in mind that the study conducted by Swiercz et al. (2008) used other animal models, an in *in vitro* conditions. Also, it has been demonstrated by some studies that enzymes such as CAT and GSTs can be inactivated by superoxide anion and hydrogen peroxide (Atli and Canli, 2010). Since BAC is known to produce superoxide anions and hydrogen peroxide, we can assume that the inhibition of the enzymatic activity of GSTs reported in our work can be due to the possible increase in hydrogen peroxide (Hofer et al., 1995; Debbasch et al., 2001; Jifa et al., 2005; Raymond, 2009; Monteiro et al., 2010; Zhang et al., 2016).

6.3.3. Antioxidant defence biomarkers

Although there is no evidence regarding the metabolic pathways of BAC, exposures to this compound, and its subsequent metabolism, can lead to a possible increase of ROS as suggested by other studies, and our results (Hofer et al., 1995; Debbasch et al., 2001; Jifa et al., 2005; Monteiro et al., 2010). After exposure to quaternary ammonium compounds, organisms such as *O. mykiss*, *L. japonicus*, *C. gibelio*, and *B. amazonicus*, showed a significant increase in O_2^-, and H_2O_2 (Hofer et al., 1995; Debbasch et al., 2001; Jifa et al., 2005; Monteiro et al., 2010). We assume that given the information present in the literature

concerning the alteration of activity of several antioxidant enzymes, and the presence of ROS, that oxidative damaged may occur (Ekstrom and Ingelman-Sundberg, 1989; Hofer et al., 1995; Jifa et al., 2005; Monteiro et al., 2010; Antunes et al., 2016; Gheorghe et al., 2020;). Also, there is evidence that BAC, when dissolved in water, can be degraded by the Fenton process (Raymond, 2009; Zhang et al., 2016). The Fenton pathway converts H_2O_2 into HO*. Knowing that this pathway is active in the metabolism of BAC, we suggest that there will be implications on the normal enzyme activity, due to the increment in free radicals (Raymond, 2009; Seguin et al., 2019). Our results showed an increase in CAT activity in animals exposed to 0.5 mg/L of BAC, suggesting an activation of the antioxidant defense, derived from the production of hydroperoxide and superoxide anions, possibly due to the metabolism of BAC by CYP 1A1 or by the activation of the Fenton process. In previous studies, fish of the species *O. mykiss* exposed to concentrations of 0.5 mg/L and 1.05 mg/L, reported an increase in CAT enzymatic activity (Antunes et al., 2016). Although not established in our work, it is known that, metabolism of BAC, in human conjunctival cells, leads to increase in O_2^- and H_2O_2 (Debbasch et al., 2001). H_2O_2, when in the presence of free iron bivalent ions, through the Fenton reaction generates hydroxyl radical (Poljsak et al., 2011; Nandi et al, 2019). HO* is responsible for protein damage, membrane disruption and lipid peroxidation. The increase in HO* promotes oxidation of proteins, leading to loss of protein function and loss of enzyme activity (Butterfield et al., 1998; Goyal and Basak, 2012). Due to the lack of an antioxidant defense for hydroxyl radicals, this ROS can lead to cellular damage and death. (Bhattacharjee, 2019; García-Caparrós et al., 2021).

Given the combination of inhibition and increment in catalase activity observed in the study of Antunes et al. (2016), the decreased activity of this parameter observed in fish exposed to 2.5 mg/L of BAC is probably a biphasic response due to the increment of ROS (Goyal and Basak, 2012). In plants exposed to BAC, a u-shaped pattern of increased and decreased activity of CAT, in a concentration dependent manner, was observed (Li et al., 2019). It is licit to suggest that the biological response to BAC exposure is dual and for higher doses other antioxidant defenses must be activated.

The enzymatic activity of GPx, an antioxidant enzyme, was inhibited in our study. Although significant changes were recorded only in fish exposed to the last two concentrations, the inhibitory trend of GPx is clear. It should be noted that both enzymes of the glutathione pathway, GSTs, and GPx, were inhibited. GPx reduces lipid hydroperoxides

and GSTs, through various reactions, detoxify oxidized lipids (Čolak and Žorić, 2019). Thus, the obtained response suggests an inability of the organism to decrease the level of free radicals produced by the metabolism of BAC. Also, despite not established in our work, the decrease in the activity of these enzymes may be due to the lower level of GSH, registered by Gheorghe et al. (2020), after exposing *Cyprinus carpio* to benzalthonium chloride.

7. Conclusion

BAC has a wide range of applications, due to its chemical and physical properties. Since previsions suggest a possible growth on the production of disinfectants as a consequence of the SARS-CoV2 pandemic, an assessment of the possible adverse effects in aquatic species is of extreme importance. In our work, we reported a significant increase in swimming activity of *D. rerio*, in all the performed assays, but especially evident in fish exposed to 2.5 mg/L of BAC. This increase in activity could be due to a possible inhibition of AChE, as reported in the literature. Detoxification pathways, with the involvement of CYP 450, had different outcomes. CYP 1A1 showed an increment in its activity following an exposure of zebrafish to 0.5 mg/L and CYP 1A2 showed a clear inhibition, with significant results after an exposure of *D. rerio* to 0.5 mg/L, and 2.5 mg/L of BAC. Also, an increment in the antioxidant defense, through the enzymatic activity of CAT, was observed. However, GPx and GSTs enzymatic activities were inhibited. Our study may reflect a metabolism of the compound by the CYP 1A1 isoenzyme, with consequent production of ROS that triggers the antioxidant defense by increasing the activity of CAT. However, this possible increase in ROS could lead to inhibition of GPx and GSTs.

The adverse effects reported in this study in animals exposed to 0.1, 0.5, and 2.5 mg/L are relevant since BAC, before the pandemic, was reported to range from 0.5 to 5 mg/L in the aquatic matrix. Given the increased use of this compound in recent years, and the predicted increase in the upcoming years, it is expected that this compound will reach the aquatic compartment in higher quantities, leading to even more potential deleterious effects than those observed here.

8. References

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

Al-Asmakh, M., Majdalawieh, A.F., Abdullah, A.M., Younes, N., Da'as, S.I., Radwan, A.B., and Nasrallah, G.K. (2020). AEO-7 surfactant is "super toxic" and induces severe cardiac, liver and locomotion damage in zebrafish embryos. *Environmental Sciences Europe*, *32*(1). https://doi.org/10.1186/s12302-020-00429-z

Almeida, A.R., Salimian, M., Ferro, M., Marques, P.A., Goncalves, G., Titus, E., and Domingues, I. (2019). Biochemical and behavioral responses of zebrafish embryos to magnetic graphene/nickel nanocomposites. *Ecotoxicology and Environmental Safety*, *186*, 109760. https://doi.org/10.1016/j.ecoenv.2019.109760

Andrade, T.S., Henriques, J.F., Almeida, A.R., Machado, A.L., Koba, O., Giang, P.T., and Domingues, I. (2016). Corrigendum to "Carbendazim exposure induces developmental, biochemical and behavioural disturbance in zebrafish embryos". *Aquatic Toxicology*, *170*, 390-399. https://doi.org/10.1016/j.aquatox.2016.01.015

Antunes, S.C., Nunes, B., Rodrigues, S., Nunes, R., Fernandes, J., and Correia, A. T. (2016). Effects of chronic exposure to benzalkonium chloride in *Oncorhynchus mykiss*: Cholinergic neurotoxicity, oxidative stress, peroxidative damage and genotoxicity. *Environmental Toxicology and Pharmacology*, *45*, 115–122. https://doi.org/10.1016/j.etap.2016.04.016

Arugonda, S. (1999). Quaternary ammonium compounds. Retrieved April 9, 2022, from International programme on Chemical Safety, Poisons information Monograph G022 Chemical website: http://www.inchem.org/documents/pims/chemical/pimg022.htm%0D

Atli, G., and Canli, M. (2010). Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. *Ecotoxicology and Environmental Safety*, *73*(8), 1884–1889. https://doi.org/10.1016/j.ecoenv.2010.09.005

Bambino, K., and Chu, J. (2017). Zebrafish in toxicology and environmental health. *Current topics in developmental biology*, *124*, 331-367. https://doi.org/https://doi.org/10.1016/bs.ctdb.2016.10.007

Bebe, F.N., and Panemangalore, M. (2003). Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. *Journal of Environmental*

Science and Health - Part B Pesticides, Food Contaminants, and Agricultural Wastes, *38*(3), 349–363. https://doi.org/10.1081/PFC-120019901

Bhattacharjee, S. (2019). ROS and Oxidative Stress: Origin and Implication. *Reactive Oxygen Species in Plant Biology*, 1–31.

Bonansea, R.I., Wunderlin, D.A., and Amé, M.V. (2016). Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures. *Ecotoxicology and Environmental Safety*, *129*, 311–319. https://doi.org/10.1016/j.ecoenv.2016.03.043

Bouwknecht, J.A., and Paylor, R. (2008). Pitfalls in the interpretation of genetic and pharmacological effects on anxiety-like behaviour in rodents. *Behavioural Pharmacology*, *19*(5–6), 385–402. https://doi.org/10.1097/FBP.0b013e32830c3658

Bradford, M M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Butterfield, D.A., Koppal, T., Howard, B., Yatin, S., Allen, K., and Aksenov, M. (1998). Structural and Functional Changes in Proteins Induced by Free Radical – mediated Oxidative Antioxidants N-tert-Butyl-phenylnitrone. *Annals of the New York Academy of Sciences*, *845*, 448–462.

Canistro, D., Melega, S., Ranieri, D., Sapone, A., Gustavino, B., Monfrinotti, M., and Paolini, M. (2012). Modulation of cytochrome P450 and induction of DNA damage in *Cyprinus carpio* exposed in situ to surface water treated with chlorine or alternative disinfectants in different seasons. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *729*(1–2), 81–89. https://doi.org/10.1016/j.mrfmmm.2011.09.008

Cao, Y., Semanchik, N., Lee, S.H., Somlo, S., Barbano, P.E., Coifman, R., and Sun, Z. (2009). Chemical modifier screen identifies HDAC inhibitors as suppressors of PKD models. *Proceedingd of the National Acadmy of Sciences of the Uniter States of America*, *106*(51), 21819–21824.

Chang, W.J., and Hwang, P.P. (2011). Development of zebrafish epidermis. *Birth Defects Research Part C - Embryo Today: Reviews*, *93*(3), 205–214. https://doi.org/10.1002/bdrc.20215

Cheah, D.M., Wright, P.F., Holdway, D., and Ahokas, J. (1995). *Octopus pallidus* cytochrome P-450: characterisation and induction studies with β-naphthoflavone and Aroclor. *Aquatic Toxicology*, *33*, 201–214.

Choi, S.M., Roh, T.H., Lim, D.S., Kacew, S., Kim, H.S., and Lee, B.M. (2018). Risk assessment of benzalkonium chloride in cosmetic products. *Journal of Toxicology and Environmental Health - Part B: Critical Reviews*, *21*(1), 8–23. https://doi.org/10.1080/10937404.2017.1408552

Clouzeau, C., Godefroy, D., Riancho, L., Rostène, W., Baudouin, C., and Brignole-Baudouin, F. (2012). Hyperosmolarity potentiates toxic effects of benzalkonium chloride on conjunctival epithelial cells in vitro. *Molecular Vision*, *18*, 851–863.

Colovic, M.B., Krstic, D.Z., Lazarevic-Pasti, T.D., Bondzic, A.M., and Vasic, V. M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology*, *11*(3), 315–335. https://doi.org/10.2174/1570159x11311030006

Čolak, E. and Žorić, L. (2019). Antioxidants and Age-Related Macular Degeneration. *Handbook of Nutrition, Diet, and the Eye, 6,* 85-106. https://doi.org/10.1016/B978-0-12-815245-4.00006-5

Cosentino, C.C., Roman, B.L., Drummond, I.A., and Hukriede, N. A. (2010). Intravenous microinjections of zebrafish larvae to study acute kidney injury. *Journal of Visualized Experiments*, (42), 3–7. https://doi.org/10.3791/2079

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

Debbasch, C., Brignole, F., Pisella, P.J., Warnet, J.M., Rat, P., and Baudouin, C. (2001). Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. *Investigative Ophthalmology and Visual Science*, *42*(3), 642–652.

Ekstrom, G., and Ingelman-Sundberg, M. (1989). Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). *Biochemical Pharmacology*, *38*(8), 1313–1319.

Eleftheriadis, H., Cheong, M., Sandeman, S., Syam, P.P., Brittain, P., Klintworth, G.K., and Tyson, S.L. (2002). Corneal toxicity secondary to inadvertent use of benzalkonium

chloride preserved viscoelastic material in cataract surgery. *Evidence-Based Eye Care*, *3*(4), 184–185. https://doi.org/10.1097/00132578-200210000-00008

European Medicines Agency. (2017). Questions and answers on benzalkonium chloride used as an excipient in medicinal products for human use. *Ema/Chmp/704195/2013*, *44*(828), 1–10.

Feng, T., Li, Z. B., Guo, X., and Guo, J. (2008). Effects of trichlorfon and sodium dodecyl sulphate on antioxidant defense system an acetylcholinesterase of *Tilapia nilotica* in vitro. *Pesticide Biochemistry and Physiology*, *92*, 107–113.

Ferreira, M., Caetano, M., Antunes, P., Costa, J., Gil, O., Bandarra, N., and Reis-Henriques, M. . (2010). Assessment of contaminants and biomarkers of exposure in wild and farmed seabass. *Ecotoxicology and Environmental Safety*, *73*, 579–588.

Flohé, L., and Gunzler, W. (1984). Assays of glutathione peroxidase. *Methods in Enzymology*, *105*, 114–120.

García-Caparrós, P., De Filippis, L., Gul, A., Hasanuzzaman, M., Ozturk, M., Altay, V., and Lao, M. T. (2021). Oxidative Stress and Antioxidant Metabolism under Adverse Environmental Conditions: a Review. *Botanical Review*, *87*(4), 421–466. https://doi.org/10.1007/s12229-020-09231-1

Gheorghe, S., Mitroi, D.N., Stan, M.S., Staicu, C.A., Cicirma, M., Lucaciu, I. E., and Dinischiotu, A. (2020). Evaluation of sub-lethal toxicity of benzethonium chloride in *Cyprinus carpio* liver. *Applied Sciences (Switzerland)*, *10*(23), 1–15. https://doi.org/10.3390/app10238485

Gomes, A. (2019). Types of disinfectants. *Octoclean*. Retrieved from: https://octoclean.com/blog/the-health-and-environmental-impacts-of-disinfectants-and-how-to-make-safe-decisions/

Gordon, W.E., Espinoza, J.A., Leerberg, D.M., Yelon, D., and Hamdoun, A. (2019). Xenobiotic transporter activity in zebrafish embryo ionocytes. *Aquatic Toxicology*, *212*, 88–97. https://doi.org/10.1016/j.aquatox.2019.04.013

Goyal, M.M., and Basak, A. (2012). Hydroxyl radical generation theory: A possible explanation of unexplained actions of mammalian catalase. *International Journal of Biochemistry and Molecular Biology*, *3*(3), 282–289. https://doi.org/10.1016/j.freeradbiomed.2012.10.050

Guh, Y.J., Lin, C.H., and Hwang, P.P. (2015). Osmoregulation in zebrafish: Ion transport mechanisms and functional regulation. *EXCLI Journal*, *14*, 627–659. https://doi.org/10.17179/excli2015-246

Guilhermino, L., Barros, P., Silva, M.C. and Soares, A.M.V.M. (1998). Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate insecticides be questioned? *Biomarkers, 3,* 157-163.

Guilhermino, L., Lacerda, M.N., Nogueira, A.J.A., and Soares, A.M.V.M. (2000). In vitro and in vivo inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: Possible implications for contamination biomonitoring. *Science of the Total Environment*, *247*(2–3), 137–141. https://doi.org/10.1016/S0048-9697(99)00485-4

Habig, W.H., Pabst, M., and Jakoby, W. (1974). Glutathione S-transferases the frist enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139.

Hofer, R., Jeney, Z., and Bucher, F. (1995). Chronic effects of linear alkylbenzene sulfonate and ammonia on rainbow trout fry at water criteria limits. *Wat.Res*, *29*(12), 2725–2729.

Hollert, H., and Keiter, S.H. (2015). *Danio rerio* as a model in aquatic toxicology and sediment research. *Environmental Science and Pollution Research*, *22*(21), 16243–16246. https://doi.org/10.1007/s11356-015-5362-1

Hora, P.I., Pati, S.G., McNamara, P.J., and Arnold, W.A. (2020). Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications. *Environmental Science and Technology Letters*, *7*(9), 622–631. https://doi.org/10.1021/acs.estlett.0c00437

Hwang, P.P., and Chou, M.Y. (2013). Zebrafish as an animal model to study ion homeostasis. *Pflugers Archiv European Journal of Physiology*, *465*(9), 1233–1247. https://doi.org/10.1007/s00424-013-1269-1

Hwang, P.P., Lee, T.H., and Lin, L.Y. (2011). Ion regulation in fish gills: Recent progress in the cellular and molecular mechanisms. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *301*(1), 28–47. https://doi.org/10.1152/ajpregu.00047.2011

Jifa, W., Zhiming, Y., Xiuxian, S., You, W., and Xihua, C. (2005). Comparative researches on effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate upon

Lateolabrax japonicus biomarker system. *Environmental Toxicology and Pharmacology*, *20*(3), 465–470. https://doi.org/10.1016/j.etap.2005.05.006

Kapelyukh, Y., Henderson, C.J., Scheer, N., Rode, A., and Wolf, C.R. (2019). Defining the contribution of CYP 1A1 and CYP 1A2 to drug metabolism using humanized CYP 1A1/1A2 and CYP 1A1/CYP 1A2 knockout mice. *Drug Metabolism and Disposition*, *47*(8), 907–918. https://doi.org/10.1124/dmd.119.087718

Karbowski, M., Kurono, C., Wozniak, M., Ostrowski, M., Teranishi, M., Nisshizawa, Y., and Wakabayashi, T. (1999). Free radical-induced megamitochondria formation and apoptosis. *Radiation Oncology*, *26*, 396–409.

Kierkegaard, A., Chen, C., Armitage, J. M., Arnot, J.A., Droge, S., and McLachlan, M.S. (2020). Tissue Distribution of Several Series of Cationic Surfactants in Rainbow Trout (*Oncorhynchus mykiss*) following Exposure via Water. *Environmental Science and Technology*, *54*(7), 4190–4199. https://doi.org/10.1021/acs.est.9b07600

Kim, S., Ji, K., Shin, H., Park, S., Kho, Y., Park, K., and Choi, K. (2020). Occurrences of benzalkonium chloride in streams near a pharmaceutical manufacturing complex in Korea and associated ecological risk. *Chemosphere*, *256*, 127084. https://doi.org/10.1016/j.chemosphere.2020.127084

Kimmel, C.B., Ballard, W.W., Kimmer, S.R., Ullmann, B., and Schilling, T.F. (1995). Zebrafish as an alternative vertebrate model for investigating developmental toxicity - the triadimefon example. *International Journal of Molecular Sciences*, *203*, 253–310. https://doi.org/10.3390/ijms18040817

Kümmerer, K., Eitel, A., Braun, U., Hubner, P., Daschner, F., Mascart, G., Milandri, M., Reinthaler, F., Verhoef, J. (1997). Analysis of benzalkonium chloride in the effluent from European hospitals by solid-phase extraction and high-performance liquid chromatography with post-column ion-pairing and fluorescence detection. *Journal of Chromatography A*, *774*(1–2), 281–286.

Lei, Y.M.K., Lekha, N., and Alegre, M.L. (2015). The Electrophysiology of Hypo- and Hyperkalemia. *Clinics and Research in Hepatology and Gastroenterology*, *39*(1), 9–19. https://doi.org/10.1161/CIRCEP.116.004667.The

Letelier, M.E., Martínez, M., González-Lira, V., Faúndez, M., and Aracena-Parks, P. (2006). Inhibition of cytosolic glutathione S-transferase activity from rat liver by copper.

Chemico-Biological Interactions, *164*(1–2), 39–48. https://doi.org/10.1016/j.cbi.2006.08.013

Li, Y., Zhou, C., Wang, S., Lin, Q., Ni, Z., Qiu, H., and Qiu, R. (2019). Phytotoxicity and oxidative effects of typical quaternary ammonium compounds on wheat (*Triticum aestivum L.*) seedlings. *Environmental Science and Pollution Research*, *26*(25), 25985–25999. https://doi.org/10.1007/s11356-019-05822-7

Liu, B., Yu, Z., Song, X., and Yang, F. (2010). Effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate on the *Mytilus galloprovincialis* biomarker system. *Ecotoxicology and Environmental Safety*, *73*(5), 835–841. https://doi.org/10.1016/j.ecoenv.2009.12.011

Lott, E., and Jone, E. (2022). *Cholinergic toxicity*. StatPearls.

Lu, C., Deng, K., Hu, C. and Lyu, L. (2020). Dual-reaction-center catalytic process continues Fenton's story. *Frontiers of Environmental Science and Engineering*, *14*(5). http://dx.doi.org/10.1007/s11783-020-1261-x

Magalhães, D.D.P., Da Cunha, R.A., Dos Santos, J.A.A., Buss, D.F., and Baptista, D.F. (2007). Behavioral response of Zebrafish *Danio rerio* Hamilton 1822 to sublethal stress by sodium hypochlorite: Ecotoxicological assay using an image analysis biomonitoring system. *Ecotoxicology*, *16*(5), 417–422. https://doi.org/10.1007/s10646-007-0144-2

Marple, B., Roland, P., and Benninger, M. (2004). Safety review of benzalkonium chloride used as a preservative in intranasal solutions: An overview of conflicting data and opinions. *Otolaryngology - Head and Neck Surgery*, *130*(1), 131–141. https://doi.org/10.1016/j.otohns.2003.07.005

Martinez-Tabche, L., Mora, B.R., Faz, C.G., Castelan, I.C, Ortiz, M.M, Gonzalez, V.U. and Flores, M.O. (1997). Toxic effect of sodium dodecylbenzenesulfonate, lead, petroleum, and their mixtures on the activity of acetylcholinesterase of *Moina macrocopa* in vitro. *Environmental Toxicology and Water Quality*, *12*(3), 211-215. http://dx.doi.org/10.1002/(sici)1098-2256(1997)12:3<211::aid-tox2>3.0.co;2-b

Matsuhashi, T., Liu, X., Karbowski, M., Wozniak, M., Antosiewicz, J., and Wakabayashi, T. (1997). Role of free radicals in the mechanism of the hydrazine-induced formation of megamitochondria. *Free Radical Biology and Medicine*, *23*(2), 285–293. https://doi.org/10.1016/S0891-5849(96)00616-8

Monteiro, D.A., Rantin, F.T., and Kalinin, A.L. (2010). Inorganic mercury exposure: Toxicological effects, oxidative stress biomarkers and bioaccumulation in the tropical freshwater fish matrinxã, *Brycon amazonicus* (Spix and Agassiz, 1829). *Ecotoxicology*, *19*(1), 105–123. https://doi.org/10.1007/s10646-009-0395-1

Mustapha, D.S., and Bawa-Allah, K.A. (2020). Differential toxicities of anionic and nonionic surfactants in fish. *Environmental Science and Pollution Research*, *27*(14), 16754–16762. https://doi.org/10.1007/s11356-020-08212-6

Nandi, A., Yan, L. J., Jana, C. K., and Das, N. (2019). Role of Catalase in Oxidative Stress- And Age-Associated Degenerative Diseases. *Oxidative Medicine and Cellular Longevity*, *2019*. https://doi.org/10.1155/2019/9613090

Nowak, M., Zawadzka, K., Szemraj, J., Góralczyk-Bińkowska, A., and Lisowska, K. (2021). Biodegradation of chloroxylenol by *Cunninghamella elegan*s im 1785/21gp and trametes versicolor im 373: Insight into ecotoxicity and metabolic pathways. *International Journal of Molecular Sciences*, *22*(9). https://doi.org/10.3390/ijms22094360

Nunes, B. (2011). The Use of cholinesterases in Ecotoxicology. In *Reviews of Environmental Contamination and Toxicology*. https://doi.org/https://doi.org/10.1007/978-1-4419-8453-1_2

Nunes, B., Carvalho, F., and Guilhermino, L. (2005). Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*. *Ecotoxicology and Environmental Safety*, *61*(3), 413–419. https://doi.org/10.1016/j.ecoenv.2004.08.010

OCDE. (2013). *OECD guidelines for testing of chemicals*, 509–511. https://doi.org/10.3109/9781841848570-66

Ogungbemi, A., Leuthold, D., Scholz, S., and Küster, E. (2019). Hypo- or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. *Environmental Sciences Europe*, *31*(1). https://doi.org/10.1186/s12302-019-0270-5

Okahara, A., and Kawazu, K. (2013). Local toxicity of benzalkonium chloride in ophthalmic solutions following repeated applications. *Journal of Toxicological Sciences*, *38*(4), 531–537. https://doi.org/10.2131/jts.38.531

Oliveira, A.C., Fascineli, M.L., Andrade, T.S., Sousa-Moura, D., Domingues, I., Camargo, N.S., and Villacis, R.A.R. (2021). Exposure to tricyclic antidepressant

nortriptyline affects early-life stages of zebrafish (*Danio rerio*). *Ecotoxicology and Environmental Safety*, *210*. https://doi.org/10.1016/j.ecoenv.2020.111868

Ostergaard, D., Engbaek, J., and Viby-Mogensen, J. (1989). Adverse reactions and interactions of neuromuscular blocking drugs. *Medical Toxicology and Adverse Drug Experience*, *4*(5), 351–368. https://doi.org/https://doi.org/10.1007/BF03259917

Park, J. W., Reed, J. R., and Backes, W. L. (2015). The localization of cytochrome P450s CYP 1A1 and CYP 1A2 into different lipid microdomains is governed by their N-terminal and internal protein regions. *Journal of Biological Chemistry*, *290*(49), 29449–29460. https://doi.org/10.1074/jbc.M115.687103

Pauloin, T., Dutot, M., Warnet, J.M., and Rat, P. (2008). In vitro modulation of preservative toxicity: High molecular weight hyaluronan decreases apoptosis and oxidative stress induced by benzalkonium chloride. *European Journal of Pharmaceutical Sciences*, *34*(4–5), 263–273. https://doi.org/10.1016/j.ejps.2008.04.006

Pereira, B.M.P., and Tagkopoulos, I. (2019). Benzalkonium chlorides: Uses, regulatory status, and microbial resistance. *Applied and Environmental Microbiology*, *85*(13), 1–13. https://doi.org/10.1128/AEM.00377-19

Phillips, T.A., Summerfelt, RC., and Atchison, G.J. (2002). Environmental, biological, and methodological factors affecting cholinesterase activity in walleye (*Stizostedion vitreum*). *Archives of Environmental Contamination and Toxicology*, *43*(1), 75–80. https://doi.org/10.1007/s00244-002-1134-2

Poljsak, B., Milisav, I., Lampe, T., and Ostan, I. (2011). Reproductive benefit of oxidative damage: An oxidative stress "malevolence"? *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2011/760978

Precision reports. (2022). Global Quaternary Ammonium Compounds Market Growth 2022-2028. Retrieved from https://www.marketwatch.com/press-release/quaternary-ammonium-compounds-market-size-2022-global-top-manufacturers-analysis-covid-19-impact-business-strategies-industry-share-demand-trend-opportunities-and-challenge-forecast-until-2028-2022-02-16

Pullaguri, N., Nema, S., Bhargava, Y., and Bhargava, A. (2020). Triclosan alters adult zebrafish behavior and targets acetylcholinesterase activity and expression. *Environmental Toxicology and Pharmacology*, *7*5, 103311. https://doi.org/10.1016/j.etap.2019.103311

Raymond, R. (2009). *Handbook of Pharmaceutical Excipients* (6th ed.). UK: Pharmaceutical Press.

Rosen, M.J., Li, F., Morrall, S.W., and Versteeg, D. J. (2001). The relationship between the interfacial properties of surfactants and their toxicity to aquatic organisms. *Environmental Science and Technology*, *35*(5), 954–959. https://doi.org/10.1021/es0015141

Saglio, P., Trijasse, S., and Azam, D. (1996). Behavioral effects of waterborne carbofuran in goldfish. *Archives of Environmental Contamination and Toxicology*, *31*(2), 232–238. https://doi.org/10.1007/BF00212371

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., and Champagne, D.L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*, *228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., and Voelker, D. (2008). The zebrafish embryo model in environmental risk assessment - Applications beyond acute toxicity testing. *Environmental Science and Pollution Research*, *15*(5), 394–404. https://doi.org/10.1007/s11356-008-0018-z

Seguin, R.P., Herron, J.M., Lopez, V., Dempsey, J.L., and Xu, L. (2019). Metabolism of Benzalkonium Chlorides by Human Hepatic Cytochromes P450. *Chemical Research in Toxicology*, *32*(12), 2466–2478. https://doi.org/10.1021/acs.chemrestox.9b00293.Metabolism

Selderslaghs, I.W.T., Blust, R., and Witters, H.E. (2012). Feasibility study of the zebrafish assay as an alternative method to screen for developmental toxicity and embryotoxicity using a training set of 27 compounds. *Reproductive Toxicology*, *33*(2), 142–154. https://doi.org/10.1016/j.reprotox.2011.08.003

Shephard, M.D.S., and Whiting, M.J. (1992). Nephelometric determination of total protein in cerebrospinal fluid and urine using benzalkonium chloride as precipitation reagent. *Annals of Clinical Biochemistry*, *29*(4), 411–417. https://doi.org/10.1177/000456329202900407

Sreevidya, V.S., Lenz, K.A., Svoboda, K.R., and Ma, H. (2018). Benzalkonium chloride, benzethonium chloride, and chloroxylenol - Three replacement antimicrobials are more toxic than triclosan and triclocarban in two model organisms. *Environmental Pollution*, *235*, 814–824. https://doi.org/10.1016/j.envpol.2017.12.108

Swanhart, L.M., Cosentino, C.C., Diep, C.Q., Davidson, A.J., Caestecker, M., and Hukriede, N.A. (2011). Zebrafish Kidney Development: Basic Science to Translational Research. *Birth Defects Research Part C: Embryo Today*, *93*(2), 141–156. https://doi.org/https://doi.org/10.1002/bdrc.20209

Swiercz, R., Halatek, T., Wasowicz, W., Kur, B., Grzelinska, Z., and Majcherek, W. (2008). Pulmonary irritation after inhalation exposure to benzalkonum chloride in rats. *International Journal of Occupational and Environmental Health*, *21*(2), 157–163. https://doi.org/10.2478/v10001-008-0020-1

Tezel, U., and Pavlostathis, S.G. (2011). Role of Quaternary Ammonium Compounds on Antimicrobial Resistance in the Environment. In Patricia L. Keen and M. H. M. . Montforts (Eds.), *Antimicrobial Resistance in the Environment* (Vol. 20). https://doi.org/https://doi.org/10.1002/9781118156247.ch20

Tilton, F.A., Bammler, T.K., and Gallagher, E.P. (2012). Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology, 153*(1), 9-16. https://doi.org/10.1016/j.cbpc. 2010.07.008.

Timmer, N., and Droge, S.T.J. (2017). Sorption of Cationic Surfactants to Artificial Cell Membranes: Comparing Phospholipid Bilayers with Monolayer Coatings and Molecular Simulations. *Environmental Science and Technology*, *51*(5), 2890–2898. https://doi.org/10.1021/acs.est.6b05662

Turrens, J. (2003). Mitochondrial formation of reactive oxygen species. *Journal of Physiology, 552*(2), 335-344. https://doi.org/10.1113/jphysiol.2003.049478

United States Environmental Protection Agency [US EPA]. (1991). *R.E.D. Facts. Sodium and Calcium Hypochlorite Salts*. (September 738-F-91-108), 1–5.

United Sates Environmental Protection Agency [US EPA]. (2022). List N: Disinfectants for Use Against SARS-CoV-2. Retrieved January 20, 2022, from https://www.epa.gov/%0Apesticide-registration/list-n-disinfectants-use-against-sars-cov-2

Van De Voorde, A., Lorgeoux, C., Gromaire, M.C., and Chebbo, G. (2012). Analysis of quaternary ammonium compounds in urban stormwater samples. *Environmental Pollution*, *164*, 150–157. https://doi.org/10.1016/j.envpol.2012.01.037

Van der Oost, R., Beyer, J., and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology*, *13*, 57–149.

Waller, D.L., Fisher, S.W., and Dabrowska, H. (1996). *FISH-CULTURIST*. *58*, 77–84.

Wang, Q., Liu, N., Wang, J., Wu, Y., and Wang, L. (2014). Physiological changes and acetylcholinesterase activity in th cladoceran *Moina macrocopa* (Straus, 1820) exposed to mercury and sodium dodecylsulphate. *Crustaceana, 87*(14), 1678–1690.

Wessels, S., and Ingmer, H. (2013). Modes of action of three disinfectant active substances: A review. *Regulatory Toxicology and Pharmacology*, *67*(3), 456–467. https://doi.org/10.1016/j.yrtph.2013.09.006

Winston, G.W. and Giulio, R.T.D. (1991). Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquatic Toxicology*, *19*(2) 137-161. https://doi.org/10.1016/0166-445X(91)90033-6

Xia, W.J., and Onyuksel, H. (2000). Mechanistic studies on surfactant-induced membrane permeability enhancement. *Pharmaceutical Research*, *17*(5), 612–618. https://doi.org/10.1023/A:1007581202873

Xue, Y., Hieda, Y., Saito, Y., Nomura, T., Fujihara, J., Takayama, K., and Takeshita, H. (2004). Distribution and disposition of benzalkonium chloride following various routes of administration in rats. *Toxicology Letters*, *148*(1–2), 113–123. https://doi.org/10.1016/j.toxlet.2003.12.068

Yi, X., Wei, Y., Zhai, W., Wang, P., Liu, D., and Zhou, Z. (2022). Effects of three surfactants on the degradation and environmental risk of metolachlor in aquatic environment. *Chemosphere*, 134295. https://doi.org/10.1016/j.chemosphere.2022.134295

Yu, X., Kamali, M., Van Aken, P., Appels, L., Van der Bruggen, B., and Dewil, R. (2021). Advanced oxidation of benzalkonium chloride in aqueous media under ozone and ozone/UV systems – Degradation kinetics and toxicity evaluation. *Chemical Engineering Journal*, *413*, 127431. https://doi.org/10.1016/j.cej.2020.127431

Zhang, B., Chen, X., Pan, R., Xu, T., Zhao, J., Huang, W., and Yin, D. (2017). Effects of three different embryonic exposure modes of 2, 2', 4, 4'-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. *Chemosphere*, *169*, 542–549. https://doi.org/10.1016/j.chemosphere.2016.11.098

Zhang, C., Cui, F., Zeng, G. ming, Jiang, M., Yang, Z. zhu, Yu, Z. gang, and Shen, L. qing. (2015). Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment. *Science of the Total Environment, 518–519*, 352–362. https://doi.org/10.1016/j.scitotenv.2015.03.007

Zhang, C., Tezel, U., Li, K., Liu, D., Ren, R., Du, J. and Pavlostathis, S.G. (2011). Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. *Water Research, 45*(3), 1238-1246. http://dx.doi.org/10.1016/j.watres.2010.09.037

Zhang, Q., Xia, Y.F. and Hong, J.M. (2016). Mechanism and toxicity research of benzalkonium chloride oxidation in aqueous solution by H_2O_2/Fe^{2+} process. *Environmental Science and Pollution Research, 23*(17), 17822-17830. http://dx.doi.org/10.1007/s11356-016-6986-5

Chapter III | Biological responses in *Danio rerio* caused by the anionic disinfectant (SDBS) in a scenario of SARS-CoV-2 pandemic

1. Abstract

The use of disinfectants has been continuously growing since the severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic swept the world. Although the pandemic scenario is now apparently controlled, the increase in production, and use of disinfectants is expected to rise. The disinfectant sodium dodecylbenzene sulfonic acid salt (SDBS) is one of the most common and produced disinfectants. Since SDS, a similar compound of SDBS, has already been reported in levels between 0.2 mg/L to 10 mg/L in municipal wastewater, the expected increase in production could lead to an increase in the presence of this compound in the environment. So, it becomes imperative to establish and understand the possible effects of SDBS in aquatic organisms. Our data reported that, after an exposure of zebrafish embryos to SDBS (0.2, 1, and 5 mg/L), there was an increase in mortality in animals exposed to 5 mg/L of SDBS after 96h. This increase in mortality rate is probably due to the disruption of the phospholipidic membrane of the embryo. In addition, an increase, and a decrease were observed in the total activity of larvae, after the organisms have been exposed to 1 and 5 mg/L of SDBS, respectively. Also, a significant increment of erratic movements was reported in our study in animals exposed to concentrations of 1 and 5 mg/L. Adverse changes in the cholinergic pathways may be the mechanism underlying our results. Previous studies show a decrease in acetylcholinesterase (AChE) in animals exposed to SDBS. No significant alterations were observed in animals exposed to 0.2, 1, and 5 mg/L of SDBS, in terms of the thigmotaxic behavior, so we could not infer if SDBS can lead to anxious behavior. Finally, our assessment of enzymatic biomarkers, showed inhibition in the activities of CYP 1A1, and CYP 1A2. Given the evidence reported in previous studies, of the metabolism of SDBS by CYP 450 isoenzymes, we suggest that the here observed inhibition of both CYP1A forms could be due to isoenzyme specificities or by the formation of toxic metabolites that could lead to an inhibition of the enzyme. The xenobiotic in question can be metabolized by other CYP 450 isoenzymes, resulting in an increase of reactive oxygen species, promoting oxidation of proteins, leading to a loss of protein function and loss of enzyme activity. Thus, the inhibition observed in the here tested isoenzymes may result from the metabolism by other enzymes. In addition, we also reported a significant increase in CAT activity. The H_2O_2 generated through the metabolism of the compound is used by CAT, reflecting its antioxidant nature by reducing the amount of ROS, such as H_2O_2,

in the organism Concerning GPx and GSTs activities, we observed an inhibitory trend, which could be due to the increment in ROS, due to the metabolism of SDBS. However, this compound can also be metabolized through conjugation with GSH. Therefore, the metabolism of the compound must take place via other alternative pathways that were not evaluated in this study. Nevertheless, this metabolism resulted in an excess of ROS, since the antioxidant defense, namely CAT, was activated. Finally, the inhibition observed for both GPx and GSTs might be due to a possible increase in ROS. In summary, we conclude with our study that animals exposed to environmental concentrations of SDBS, similar to those already reported before the pandemic, exhibited adverse embryonic, behavioral and metabolic effects. Given the increased use of this compound, it is anticipated that environmental concentrations will rise, leading to additional negative effects in animals exposed to it.

2. Keywords

Zebrafish; Behavior; FET; Oxidative stress; Antioxidant defense; CYP 450.

3. Introduction

Production and marketing of a certain chemical product is ruled by the market demands. When, in 2019, the world faced the severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic, the growing need for disinfectants, as a measure for the prevention of the infection, led to a sudden increase in their demand and production. Although some new compounds have been developed to mitigate COVID-19, the increase in the production of existing products was the greatest measure implemented. Surfactants are a large class of products destined to clean and disinfect, given their properties in reducing the surface tension (liquid-liquid, solid-liquid, and liquid-air), and detergency (Mao and Wang, 2000; Rebello et al., 2014; Steber, 2007). Due to the conditions imposed by the pandemic, a market that was of 41.3 billion dollars in 2019 is projected to reach 58.5 billion dollars by 2027 (Research, 2020).

Surfactants cover a wide variety of compounds, and can be divided into anionic, cationic, nonionic, and amphoteric surfactants (Huang et al., 2014). The class of anionic

surfactants is the most commonly used on a daily basis (Parhizgar et al., 2017). Also due to their cheap cost, and foaming properties, anionic surfactants are the disinfectant agents with the highest predicted growth (Research, 2020). Among the vast diversity of anionic surfactants, Sodium Dodecylbenzene Sulfonic acid (SDBS), a linear alkylbenzene sulfonate (LAS), stands out for its abundance and versatility of application (Steber, 2007). SDBS is used in formulations for laundry, car and floor detergents, engine degreasers, personal care items (such as toothpaste, shaving cream, shampoos, and bath salts), and is also a disinfection product used in multiple contexts (healthcare, institutional, and residential), and in medical settings (Niraula et al., 2012). Due to the widespread of SDBS or LAS, concentrations up to 30 mg/kg dry weight, and of 416 µg/L, have been found in treated sludge and surface waters, respectively (Berna et al., 1989; Fox et al., 2000). Over the years, environmental concentrations of SDS, a similar compound to SDBS, have been reported between 0.2 mg/L and 10 mg/L in municipal wastewater (Dizer, 1990). The different concentrations mentioned previously are far above the concentrations reported to be toxic (5.2 to 36 mg/L), which again prompts the need to explore the toxicity of this compound(Safety data sheet [SDS], 2008).The toxicity of this xenobiotic is mediated by the generation of reactive oxygen species (ROS) by phase I enzymes such cytochrome P450 (CYP 450) (Stegeman, 1981; Jifa et al., 2005; Nunes et al., 2008; Wibbertmann et al., 2011). SDBS, when absorbed by gills (in fish and bivalves), is metabolized and bio-transformed in the liver by phase I enzymes. The metabolism of the xenobiotic by CYP 450 can result in ROS leading to enzymatic activation of antioxidant enzymes, such as catalase (CAT), and glutathione peroxidase (GPx). Glutathione S-transferases (GSTs) can also promote detoxification of SDBS (Nunes, 2005; Álvarez-Muñoz et al., 2006; Shukla and Trivedi, 2018; Sobrino-Figueroa, 2013). When compared to other anionic detergents, SDBS is likely to be more toxic, given the presence of the benzene ring, for the same environmental concentrations (Rebello et al., 2014).

The biotransformation of this compound into a more hydrophilic one increases its excretion rate (Zhang et al., 2005; Monferran et al., 2007; Shukla and Trivedi, 2018). Despite the absence of reports, to date, of the involvement of CYP 450 in metabolizing SDBS in fish, some reports have presented evidence of the involvement of CYP 450 after exposure to surfactants (Mountfield et al., 2000; Ren et al., 2008; Christiansen et al., 2011). Since CYP 450 is responsible for the oxidation of several surfactants (anionic, cationic, and nonionic)

it is possible that these enzymes are involved in the metabolism of SDBS (Mountfield et al., 2000; Ren et al., 2008; Christiansen et al., 2011; Vliegenthart et al., 2014). Phase II enzymes such as GSTs conjugates GSH with xenobiotics to further increase their hydrophilicity. Jifa et al. (2005) reported an increment in GSTs activity after exposing individuals of *Lateolabrax japonicus* to 1 mg/L of SDS and SDBS. SDBS is known to promote the generation of ROS, which may be scavenged by GSTs (Hayes et al., 2005). The possible rising amount of ROS content is balanced by antioxidant enzymes such as CAT, and GPx (Cserháti et al., 2002; Jifa et al., 2005; Wu et al., 2010). In the study by Jifa (2005), CAT, and GPx, activity measured in *L. japonicus* after being exposed to 1 mg/L of SDBS, increased along the exposure. Also, an increment in CAT was reported by Shukla and Trivedi (2018). However, some studies report an increase in CAT activity in animals of the species *Hydrocharis dubis* when exposed to concentrations up to 10 mg/L, in a chronic trial, followed by a decrease in the activity of this biomarker (Wu et al., 2010).

Behavior is also a key point to assess toxicity, since it plays a critical function on the fight or flight response, mating, and food seeking behavior (Little and Brewer, 2001). Given the chemical properties of anionic surfactants and their ability to disrupt normal membrane function, behavior can give us indications of other metabolic impairments, such as neurotoxicity (Zhang et al., 2005; Schnörr et al., 2012). Thigmotaxis is a stereotypic behavior where, fish stay in close proximity to the outer boundaries of a novel environment. Alterations in this behavior are implicated in stress and anxiety of the organism (Schnörr et al., 2012). Multiple xenobiotics are known to enhance or inhibit this behavior. Exposure to a variety of compounds at the early stages of development, could also lead to impairments and increased mortality. A Fish Embryo Toxicity Test (FET), performed by Wang et al. (2015), showed no significant alterations after an exposure to an anionic surfactant. Despite this and compared to the concentrations found in the rivers, the concentrations tested for the behavior and FET trials were only in the ug/L range (Wang et al., 2015).

Danio rerio (Zebrafish) is an important model organism used in the research areas of genetics, neurophysiology, and developmental biology (Vascotto et al., 1997; Grunwald and Eisen, 2002; Rubistein, 2003). Among the many key features of this animal, its small body frame, large number of offspring, easy maintenance in laboratory conditions, and short life span, are some of the most important ones (Kimmel et al., 1995). Also, their transparent embryos allow us to identify any impairments at their first development stages. Zebrafish

gained strength as a model organism due to their similarity to other vertebrates, which allows a more comprehensive perspective of human drug metabolism, in comparison to invertebrate models (Dooley and Zon, 2000; Shin and Fishman, 2002).

This study aimed to elucidate the metabolic pathways involved in the metabolism and detoxification of SDBS in *D. rerio*, especially focusing on the analysis of phase I enzymes (CYP 450: CYP 1A1, and CYP 1A2), GSTs, a phase II enzyme, and enzymes of the antioxidant defense, such as CAT, and GPx. Also, it is our intention to explore the potential effects of SDBS in locomotor behavior and the embryonic development in zebrafish larvae.

4. Materials and methods

4.1 Chemicals

Dodecylbenzene sulfonic acid sodium salt (CAS: 25155-30-0, purity 80 - 85%) was purchased from Thermo Fisher Scientific®. All chemicals used for enzymatic buffers were obtained from Sigma-Aldrich®, apart from Bradford reagent, which was obtain from Biorad®, UK.

4.2 Test Organisms

D. rerio eggs and larvae with 5 days post-hatching (dpf) were obtained from a culture established at the Department of Biology, in the University of Aveiro. Zebrafish adults were kept in a recirculating system with adjusted pH and conductivity through application of instant ocean synthetic salt, Spectrum Brands. The animals were kept under optimal conditions of temperature 27 ± 1ºC, with a 12:12h light/dark photoperiod cycle, a conductivity of 750 ± 50 µS/cm, and dissolved oxygen at 95%. All fish were fed daily with Gemma Micro 500 (Skretting®, Spain).

In the day before of the collection of the eggs, reproductive groups were placed in breeding tanks with a divider, separating the males from the females. The next day, at the onset of illumination, the divider was removed. After 1h30 eggs were removed and raised in system water. Zebrafish eggs with normal development were selected using a Stereoscopic

Zoom Microscope – SMZ 1500 Nikon, for all tests. Unfertilized eggs, or eggs with irregularities, were discarded.

In all trials, larvae with 5-dpf or embryos were exposed to 3 concentrations: 0.2 mg/L, 1 mg/L and, 5 mg/L of SDBS. These concentrations were selected based on levels already found in the environment (0.2 – 10 mg/L), and also on concentrations involved in documented effects (1 mg/L), below the LC_{50}, at 96h (5.6 mg/L in *Oncorhynchus mykiss*) (Jifa, 2005; Lenga, 2021).

4.3 FET

Embryo acute toxicity test was performed according the OCDE guideline 236 (OCDE, 2013). Embryos collected in the cleavage phase were randomly assigned and distributed (20 eggs per concentration) in 24 well plates. Each plate had an internal control. FET was initiated immediately after the selection. The medium was renewed every 24h, and coagulation, cardiac rhythm, tail detachment, hatching delay, presence of somites, edema, and pigmentation were assessed. Test organisms were considered dead according to the guideline (coagulation, no detachment of the tails, absence of cardiac rhythm, or somites). During the 96h, the tests were kept under optimal conditions described previously.

4.4 Behavioral Tests

Larvae with 5-dph, with no deformities or abnormalities, were placed, one per each well, in 24-well microplates. Randomly, 96 larvae were distributed along the 3 concentrations. After 24h of exposure to SDBS, the movement of the organisms were tracked using the Zebrabox video-tracking system equipped with a 25 frame per second infrared camera (Viewpoint, Lyon, France). The endpoints that were analyzed were the total distance traveled, thigmotaxis, and turn angles, to measure total activity, thigmotaxis behavior, and erratic swimming, respectively. The movement of larvae was recorded during a 6 min habituation period, in the light, and a 4 min period in the dark, after abruptly turning off the lights (Schnörr et al., 2012). To record the movements in both areas, as stated by the standard thigmotaxis test, an inner and an outer area were created with a 12.25 mm radius, and 4 mm radius after establishing the inner area, respectively. The angles produced by larvae, when

changing direction were measured, and grouped into 4 distinct classes as described in Zhang et al. (2017). Angles with a range of 0° to ± 10° were classified in class 4. Turns with ranges between ± 10°, and ± 30° were grouped in class 3. Class 2 comprises the angles produced between ± 30°, and ± 90°. Finally, class 1 included angles in the range of ± 90° to ± 180°. To analyze thigmotaxis, the protocol proposed by Schnörr et al. (2012) was followed. In order to reduce individual variability, distance traveled, time spent swimming in the peripheral zone, and turn angles, were converted to percentage values according to the equations proposed by Bouwknecht and Paylor (2008). Therefore, the values for these parameters are presented from now on as the percentage of total distance traveled (%TDM), and as the percentage of total time traveled (%TTM). After the movement was recorded, the data were saved, and the larvae were frozen for later biochemical analysis.

4.5 Enzymatic determinations

The enzymatic activity of phase I enzymes CYP 450 (CYP 1A1, and CYP 1A2), the phase II enzyme GSTs, and antioxidant defense enzymes, such as CAT, and GPx, , were analyzed with 12 replicates per concentration, each one with 8 larvae with 5-dpf, obtained after an acute exposure to SDBS. Meanwhile, for GPx, 10 larvae with 5-dpf, were used per sample. After a 24h acute exposure to SDBS, the samples were frozen with 1 mL of 50 mM phosphate buffer, pH=7.0, with X-100 0.1%, to determine CAT, GPx, and GSTs. On the other hand, for the preparation of samples destined to evaluate CYP 1A1, and CYP 1A2, 1 mL of 50 nM Tris HCL, pH=8.0, 0.15M KCL, 1mM dithiothreitol solution was added. The samples were then frozen and stored at -80ºC. After homogenization, the samples were centrifuged at 1500 *g*, or 1000 *g* (Megafuge 8R, Thermo Scientific) for CYP 1A1, and CYP 1A2, for 10 min, at 4ºC.

The activities of both CYP 1A1 and CYP 1A2 were determined using the method proposed by Cheah et al. (1995). After the first centrifugation step (1000 *g*, for 10 min, at 4ºC), the supernatant was removed and placed in new Eppendorfs microtubes with a solution of 12.5 mM of sucrose, with 8 mM $CaC1_2$, at pH= 7.4. Following a 1696 *g* centrifugation for 10 min, at 4º C, the supernatant was discarded and the microsomes formed, were resuspended with 0.1 M of phosphate buffer, pH=7.4, with 1 mM EDTA, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate and 0.4% (w/v), and Triton X-100. Once the samples were

prepared, they were placed in 96-well plates with 7-ethoxyresorufin solution, for the determination of CYP 1A1, and methoxyresorufin for CYP 1A2. Finally, nicotinamide adenine dinucleotide phosphate (NADPH) was added to start the reaction. According to Ferreira et al. (2010), the activity measured using the fluorometer (λex=540nm and λem=590nm) was expressed in pmol per min per milligram of protein.

To assess the phase II metabolic biomarker GSTs was selected. According to the method described by Habig et al., (1974), the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, is catalyzed by glutathione S-transferases. This reaction gives rise to the formation of a thioether, which can be measured at 340 nm. After the collection of the data, enzymatic activity was expressed as nanomoles of thioether produced per min per milligram of protein.

As described by Aebi (1984), the enzymatic activity of CAT was measured by the decomposition of hydrogen peroxide (H_2O_2). The activity was then registered at a wavelength of 240 nm, using a microplate reader (Multiskan Spetrum, Thermo Scientific) with SkanIt ^{TM} software, and was expressed as a unit of activity being equal to the number of moles of H_2O_2 consumed per minute per milligram of protein.

The activity of GPx was measured at 340 nm, by following the oxidation of NADPH, which occurs when oxidized glutathione (GSSG) is reduced to GSGH. Glutathione peroxidase can be classified as GPx selenium dependent or total GPx. For our purposes only the total activity was measured. As described by Flohé and Gumzler (1984) the enzymatic activity was expressed as a unit of millimoles of oxidized NADPH per minute per milligram of protein.

The total protein concentration, for all the enzymatic determinations, was assessed through the methodology proposed by Bradford (1976). A standard of 1 mg/ml of y-globulin was used as standard.

4.6 Statistical analysis

For all determinations performed, Kolmogorov-Smirnov tests were applied to verify the normality of the data. A one-way ANOVA (or a Kruskal Wallis test when the normality test failed) was performed to verify the existence of differences between treatments. To infer statistically significant differences between treatments and the control, a Dunnett test was performed. The level of significance was set as 0.05, GraphPad was used for all statistics.

5. Results

5.1 FET

The acute exposure showed an increase in mortality between the 72h until 96h, in animals exposed to 5 mg/L of SDBS. At concentration of 0.2 mg/L, 10% of the embryos exposed were considered dead, according to the OCDE guideline, at 96h. At the highest concentrations of 1 mg/L, and 5 mg/L, the percentage of dead embryos was 12%, and 80%, respectively (Figure 10). No abnormalities were observed in exposed embryos.

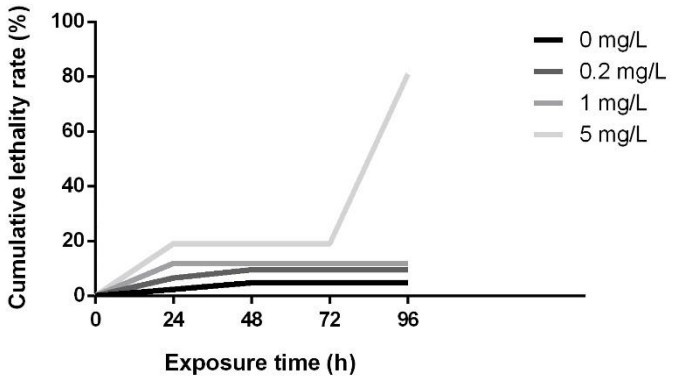

Figure 10 - Cumulative lethality rate (%), after a 96h exposure to SDBS. N=96.

5.2 Behavioral Tests

A significant increase, and a decrease in the total distance travelled (mm) were reported in organisms exposed to the last two concentrations (1 mg/L and 5 mg/L), respectively ($F_{[3, 377]} = 12.20$, $p < 0.0001$) (Figure 11).

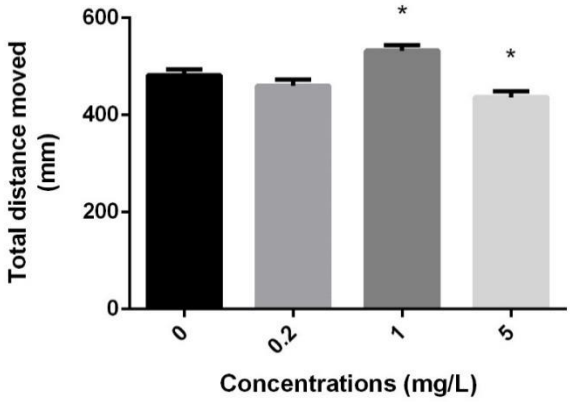

Figure 11 - Total distance moved (mm) after an acute exposure of *D. rerio* to different concentrations of SDBS. Results are represented as mean standard error. n= 96. Significant differences from the control, p< 0.05.

Concerning the %TDM, no significant results were observed in all concentrations ($F_{[3, 380]} = 0.9969$, $p = 0.3943$) (Figure 12A), and at the %TTM ($F_{[3, 380]} = 1.356$, $p = 0.2560$) (Figure 12B).

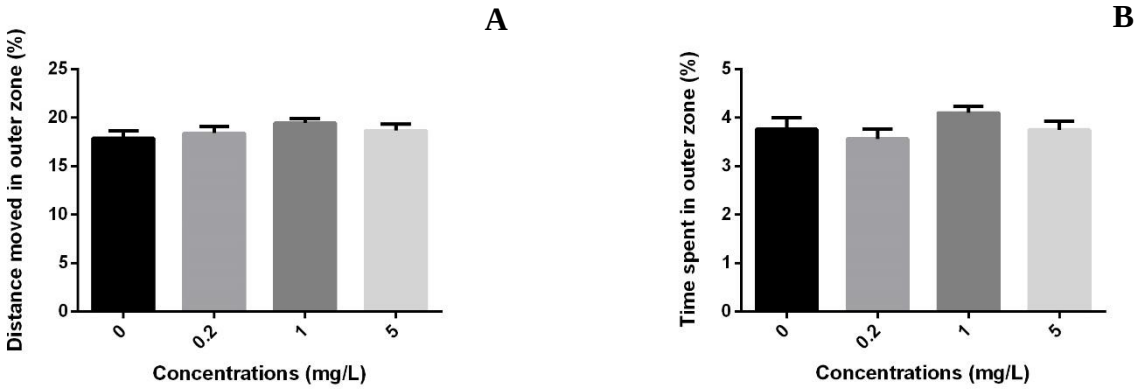

Figure 12 - (A) Effects on distanced travel in the outer zone, and (B) time spent in the outer zone, made after an acute exposure of *D. rerio* to SDBS. Data are represented as mean ± standard error. n= 96. Significant differences from the control, p<0.05.

In terms of the type of angles recorded during the movement of the larvae, significant results were found in the organisms exposed to the last two concentrations of 1 mg/L, and 5 mg/L ($F_{[3, 48]} = 2.025$, $p < 0.05$), where the proportion of class 1 angles increased when compared to control (Figure 13).

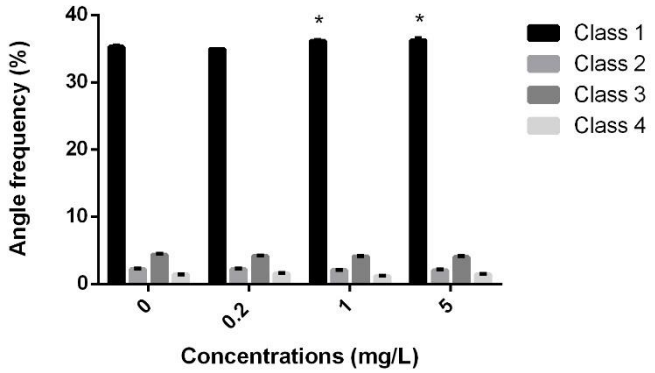

Figure 13 - Results of the angle frequency determined in *D. rerio*, after an acute exposure to SDBS. Data are represented as mean ± standard error. n=96. Significant differences form control, $p < 0.05$.

5.3 Enzymatic determinations

5.3.1 Phase I metabolic biomarkers

Regarding the activity of the phase I enzymes, a significant decrease where reported for fish exposed to 1 mg/L, and 5 mg/L concentrations, in terms of CYP 1A1 ($F_{[3, 35]} = 5.571$, $p = 0.0031$) (Figure 14A). A decrease in terms of CYP 1A2 wase also reported in organisms exposed to the last two concentrations ($F_{[3, 34]} = 11.45$, $p < 0.0001$) (Fig 14B).

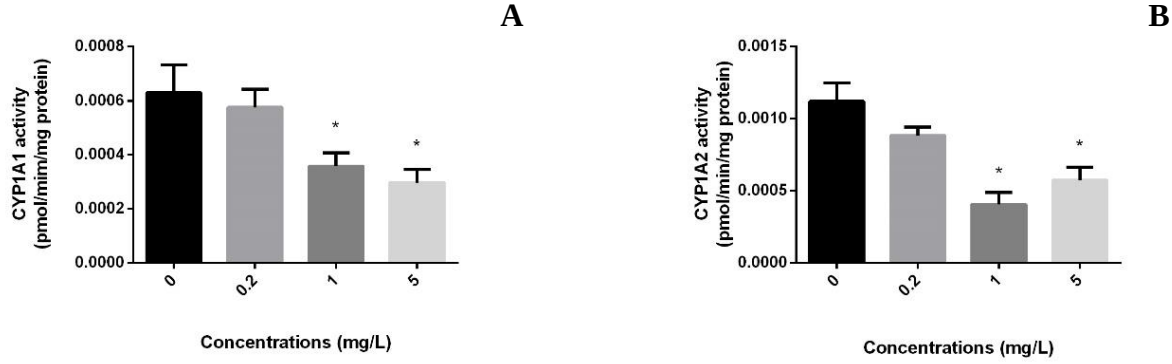

Figure 14 - Results of CYP 450 enzymes determined in *D. rerio*, after an acute exposure to SDBS. A) CYP 1A1 activity; B) CYP 1A2 activity; Data are represented as mean ± standard error. n=12 Significant differences from the control, $p < 0.05$..

5.3.2 Phase II metabolic biomarker

In our study, GSTs activity was significantly inhibited in fish exposed to all concentrations when compared to the control ($F_{[3, 39]} = 11.95$, $p < 0.001$) (Figure 15).

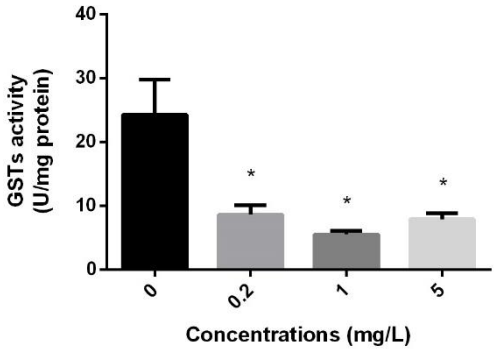

Figure 15 - GSTs activity determined in individuals of *D. rerio*, after an acute exposure to SDBS; Data are represented as mean ± standard error. n=12. Significant differences from the control, $p<0.05$.

5.3.3 Oxidative stress biomarkers

CAT activity had a tendency to decrease along the concentrations, with the exception of fish exposed to the last concentration, where CAT activity spiked to a level which was statistically significant ($F_{[3, 36]} = 54.82$, $p < 0.0001$) (Figure 16A). GPx activity was inhibited in organisms exposed to all concentrations of SDBS. However, significant results were only attained for animals exposed to a concentration of 0.2 mg/L ($F_{[3, 27]} = 2,693$, $p = 0.0660$) (Figure 16B).

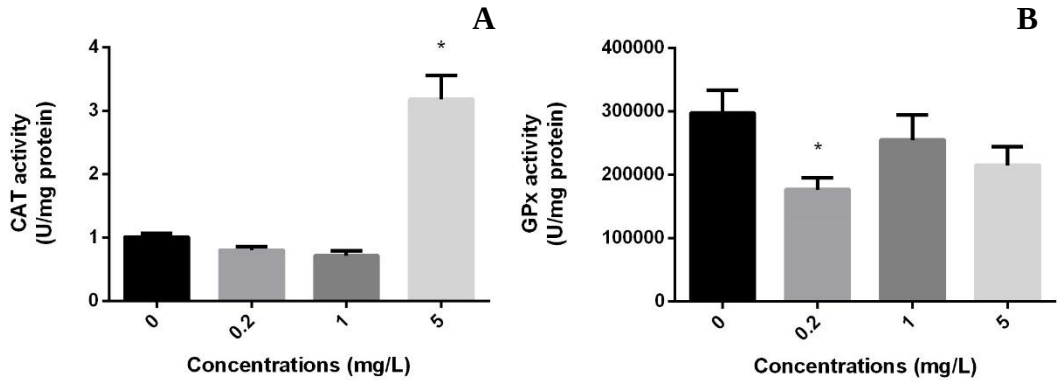

Figure 16 - Results of oxidative stress after an acute exposure of *D. rerio* to SDBS. (A) CAT activity; (B) GPx activity; Data are represented as mean ± standard error. n=12. Significant differences from the control, $p<0.05$.

6. Discussion

6.1 FET

The results found in our study do not indicate any morphological changes at the embryonic stages. These results are in line with those reported by Wang et al., (2015), in zebrafish upon an exposure to SDS at the µg/L level. Although no morphological changes were found, mortality rate spiked in the first 24h at the highest concentrations, stabilizing for 48h. At 96h another mortality peak occurred, reaching 80% at the 5 mg/L concentration. Although no studies have been found with SDBS, we expected a higher mortality since the LC_{50} for SDS, in *Gambusia holbrooki*, is of 15.1 mg/L, and the $LC_{50,}$ for SDBS, calculated in *Oncorhynchus mykiss*, was of 5.6 mg/L (Nunes et al., 2005; Lenga, 2021). A similar LC_{50} was calculated for *D. rerio* embryos, of 5.77 mg/L (Yi et al., 2022). The values found in our study go in agreement with the ones reported in previous studies (Nunes et al., 2005; Lenga, 2021; Yi et al., 2022). Nevertheless, the high mortality reported in *D. rerio* exposed to 5.28, 26.4, and 132 µg/ml of SDS (Funfak et al., 2007), despite the clear differences in the protocol (time, concentration, and compound), are somewhat similar to ours. The high number of dead embryos, reported in the study of Funfak et al. (2017) was related to the sudden coagulation of the zebrafish embryos. Also, regarding the effect of SDS, Ali et al. (2011) recorded a 94% mortality after at 96h. We assume that this high mortality rate can be

associated to the possible impairments in the embryonic membrane (Zhang et al., 2005). The toxicity of anionic surfactants is linked to the hydrophobic groups, the length of the hydrocarbon chain, and the charge of the molecule. Surfactants penetrate the phospholipidic membrane, leading to the weakening of the integrity and structures of the cellular membrane. This disruption leads to extravasation of the cell contents, thereby disrupting the normal function of the cell, which in turn can lead to the death of the zebrafish embryo, since the embryonic membrane protecting the embryo has a similar constitution and structure as of a cell membrane (Kimmel et al., 1995; Mustapha and Bawa-Allah, 2020).

6.2 Behavioral analysis

Alterations in the normal behavioral patterns are considered potential red flags to assess toxicity (Little andand Brewer, 2001). In our study we observed significant differences in the total distance traveled, in animals exposed to 1 mg/L, and 5 mg/L, although in opposite directions. In organisms exposed to 1 mg/L there was an increment, and in organisms exposed to 5 mg/L there was a decrease in the total distance traveled. The dual response obtained was also reported in previous studies, where at low concentrations an increase in the swimming activity was reported, followed by a decrease (Zhang et al., 2015). As observed in previous studies, there is a direct correlation between the levels of acetylcholinesterase (AChE) and the swimming activity (Saglio et al., 1996; Phillips et al., 2002; Jifa et al., 2005; Antunes et al., 2016). The neurotransmission acetylcholine (ACh) pathways can be impaired by anionic surfactants (Feng et al., 2008; Wang et al., 2014). When ACh is released to the synaptic cleft, binds to the ACh receptors in the post-synaptic membrane, leading to the transmission of the nerve impulse (Colovic et al., 2013). The main goal of AChE is to hydrolyze ACh in the synaptic clef to prevent hyperstimulation of the post-synaptic nerve (Colovic et al., 2013). When an inhibition or activation of this enzyme takes place, impairments in the motor function can occur (Tierney et al., 2008; Bonansea et al., 2016; Pullaguri et al., 2020). In the work of Nunes et al. (2016), after an exposure to SDS, at a range of concentrations of 0.05 to 0.8 mg/L, no effects of the activity of AChE were reported in *G. holbrooki*, but in the study of Feng et al. (2008), in animals exposed to 0.5 to 1 g/L, the hydrolytic action of AChE was impaired in *Tilapia nilotica*. Also, Wang et al. (2014) reported a significant inhibition of AChE in *Moina macrocopa* exposed to

concentrations of 0.8 to 4 mg/L of SDS. So, the inhibition of AChE found in previous studies, after exposure to SDS, can lead to an increase in the swimming activity, or a decrease, at different concentrations. When an inhibition of AChE is in place, the ACh released remains in the synaptic cleft (Lott and Jone, 2022). This accumulation generates an hyperstimulation of the post-synaptic nerve (Lott and Jone, 2022). The possible hyperstimulation could increase the swimming activity, reported in our study. However, the AChE inhibition could ultimately lead to paralysis, or a decrease in the swimming activity (Colovic et al., 2013; Lott and Jone, 2022). This dual response is not strange to the action of SDS, as reported in other studies (Zhang et al., 2015; Nunes et al., 2016). Thigmotaxis behavior refers to the tendency of an animal to remain close to the walls when placed in a new environment, which can be considered as a measure of anxiety (Champagne et all, 2010; Best et al., 2017). However, in our study, we observed no significant differences in the time spent or in the distance moved, in the outer area, but significant differences were observed in the total distance swam. In the dark period, the fish were found to swim smoothly through the area, so we can assume that the fish were not anxious. In other studies from the literature, conducted on zebrafish, despite using other tests, no behavioral changes were also recorded, in animals exposed to concentrations of 1µg/L, and 10 mg/L of SDBS, and SDS, respectively (Wang et al., 2015; Yi et al., 2022). The total distance traveled, reported in our study, suggests both hyperactivity and hypoactivity when compared to the control. In addition, our results reported significant statistical differences in the frequency of angles measured (%), in class 1, at 1 mg/L, and 5 mg/L. As described in the literature, the increase in class 1 angles (angles of 90º to 180º) shows a pattern of locomotion with zig-zag movements, suggesting an erratic behavior of the organism (Zhang et al., 2017). Roy (1988) also demonstrated the prominent erratic behavior upon exposure to SDBS. These erratic movements suggest anxiety-like behaviors (Correia et al., 2019). This last parameter is in agreement with our results obtained for the total distance swam. Therefore, we suggest that the absence of significant changes in the thigmotaxis test can be due to the lack of precision of the measuring apparatus. If the organisms produced a more erratic swimming, which could translate to a higher number of entries and exits between the two areas, not an increase in the time spent and distance traveled in the outer area, that is what the machine reads (Figure17).

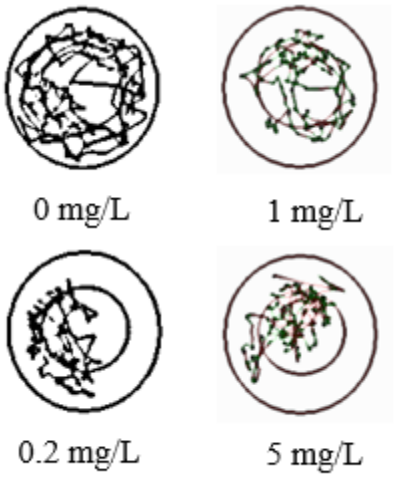

Figure 17 - Thigmotaxis behavior, traveling path, after an exposure of *D. rerio* to SDBS.

6.3 Biomarkers

Toxicity of SDBS is caused by a) destabilization of cell membranes, namely the phosphatidylcholine monolayers, altering the permeability of membranes; and b) increase of ROS production through mono-oxygenases, and dehydrogenases (Cserháti et al., 2002; Zhang et al., 2005; Monferran et al., 2007). The increase in ROS (O_2^-, HO_2, OH^*, and H_2O_2) creates an unbalance in the metabolism, which can in turn be compensated by the activation of antioxidant enzymes (Barata et al., 2005; Lushchak, 2011). Since we obtained a comprehensive inhibition of all measured biomarkers, we will perform a combined analysis of the results.

At the time of this study, data for the effects of SDBS on the CYP 450 enzymatic activity, in zebrafish, are unknown. However, recent studies show the ability of surfactants to interfere with the normal functioning of CYP 450 (Seguin et al., 2019). Our results showed a clear inhibition along the gradient of concentrations, in terms of CYP 1A1, and CYP 1A2, with a significant decrease in the animals exposed to the last two concentrations (1 mg/L, and 5 mg/L). In the study of Christiansen et al. (2011), they reported a surfactant-dependent inhibition regarding the evaluated isoenzymes (CYP 3A4, and CYP 2C9). Various types of enzymatic inhibition can occur, such as competitive, non-competitive, and mechanism-based inhibition (Deodhar et al., 2020). These inhibitions are always dependent on the affinity, and concentration of the substrate (Wen et al., 2001; Shitara et al., 2004; Lilja et al., 2005). In a

mechanism-based inhibition, the substrate forms a reactive intermediate that in turn leads to irreversible reduction of the enzyme (Deodhar et al., 2020). When we talk about competitive inhibition, irreversible or reversible, we talk about the ability of a single CYP 450 isoform to metabolize a series of substrates (Deodhar et al., 2020). A non-competitive inhibition operates by changing the structure of its active site so that the substrate loses its affinity (Deodhar et al., 2020). We propose two possible factors that might be leading to our inhibition. The first is the possible formation of metabolites that inhibit the enzyme itself when metabolized. However, to date, no information has been found regarding the possible formation of reactive metabolites. Therefore, it is imperative to understand how this compound is metabolized. The second factor is the affinity binding. All compounds require a higher or lower affinity for isoenzymes, depending on their chemical and physical properties (Gustafsson et al., 2004; Axarli et al., 2005). We suggest that SDBS could have a lower affinity towards CYP 1A1, and CYP 1A2. In previous studies, this compound has been found to be metabolized by CYP10A2 and CYP102A1 in bacteria (Axarli et al., 2010). Shukla and Trivedi (2018) also trace the increment in ROS by the metabolism of the compound in this complex. In summary, we propose that the inhibition observed in our study, given that there is evidence of ROS production by the metabolism of this compound, that SDBS has a higher affinity for other isoenzymes of this complex or that its metabolism forms reactive metabolites that led to its enzymatic inhibition.

GSTs catalyses the conjugation of GSH with xenobiotics to increase their hydrophilicity and be more easily excreted. However, GSTs can also act as an antioxidant enzyme, when GSH is conjugated with intracellular molecules damaged by reactive oxygen species, to prevent oxidative stress (Liu et al., 2010). Ultimately, the GSTs action promotes cell protection, from xenobiotics, and from ROS (Hayes and Pulford, 1995; Hayes and McLellan, 1999; Hayes et al., 2005; Deavall et al., 2012). Our results show a significant decrease in animals exposed to all concentrations, expressing an inhibition of the enzymatic activity. At the time of our experiments, no data were available of GSTs activity after an exposure to SDBS, in zebrafish. However, in the study of Nunes et al. (2008), a decrease in GSTs activity were reported after an exposure to SDS, in *G. holbrooki.* Also, in the study of Jifa et al. (2005), *L. japonicus* exposed to 1 mg/L of SDBS, in a chronic trial, reported no significant alterations in GSTs activity, after the 18 days of exposure. The same authors found an inhibition of the enzymatic activity after 6 days of exposure to SDS, accompanied

by a significant increase after 18 days of exposure (Jifa et al., 2005). Also, an increase in the activity was observed after an exposure to SDS, at concentrations of 0.34 and 1.02 mg/L, in *C. carpio,* after 15 days of exposure (Bhattacharya et al., 2022). However, in the same experiment, a decrease in GSTs activity was reported after 45 days of exposure (Bhattacharya et al., 2022). The different results found may be due to the different experimental designs, and different compounds, as reported in the study of Jifa et al. (2005), where a comparation between SDS and SDBS was made. Notwithstanding, the inhibition reported in our study could be due to an excess of ROS produced by the metabolism of SDBS, as observed in previous studies (Jifa et al., 2005; Wibbertmann et al., 2011). The goal of antioxidant enzymes, such as GSTs, is to actively scavenge the excess of ROS generated by xenobiotics. However, when there is an excess of ROS, the enzyme cannot scavenge the entire amount of ROS. This unbalance between the enzymatic activity and ROS, can lead to a reduction or even an inhibition of GSTs (Brendler-Schwaab et al., 2005). An inhibition of GSTs through the excess of ROS produced during the metabolism of surfactants has been already documented, and in other antioxidant enzymes (Jifa et al., 2005; Li, 2008; Atli and Canli, 2010; Shukla and Trivedi, 2018; Franco-Belussi et al., 2021).

The hydrocarbon metabolism of surfactants intensifies the production of ROS, which in turn can disrupt enzymatic structures, and ultimately altering the enzyme activity, leading to impairments in the defence system implicated in the detoxification metabolism (Cserháti et al., 2002; Jee and Kang, 2005; Li, 2008). The majority of the compounds from the class of surfactants are known to promote ROS formation (Jifa et al., 2005; Wibbertmann et al., 2011; Seguin et al., 2019). Hydrogen peroxide produced due to the biotransformation of SDBS can be compensated by the activation of enzymes, such as CAT (Jifa et al., 2005; Wibbertmann et al., 2011). CAT is a key enzyme that converts H_2O_2 to molecular oxygen and water (Ighodaro and Akinloye, 2018). Shukla et al. (2018), after an exposure of the fish *Channa Punctatus* to SDBS, found a considerable activation in the antioxidant defence, starting at a concentration starting of 3.4 mg/L. On the other hand, a decrease was reported in animals exposed to 1.2 mg/L, although no statistical differences were found (Shukla et al., 2018). The results obtained in our study are in agreement with those reported by Shukla et al. (2018), since animals exposed to the lowest concentrations presented no statistical differences, when compared to the control, while animals exposed to the highest concentration had increased activity of this enzyme. Also in the study of Bhattacharya et al.

(2022) an increase in CAT activity was reported in *Cyprinus carpio* exposed to lower concentrations (0.34 and 1.02 mg/L), although the activity was only measured after an exposure of 15 days. The increase in CAT enzymatic activity could indicate the possible neutralization of H_2O_2, produced by the metabolism of the surfactant (Kumari et all, 2014; Bhattacharya et al., 2022). Since previous studies have demonstrated the ability of anionic surfactants, specially SDBS, to induce the generation of H_2O_2, we suggest that the increment reported in CAT activity in our study, could be a response to the high amount of H_2O_2 in the organism (Shukla and Trivedi, 2018; Bhattacharjee, 2019; Seguin et al., 2019; Wibbertmann et al., 2011; Bhattacharya et al., 2022). Regarding the other antioxidant enzyme analysed in our study, GPx, a clear inhibition was also observed, with significant differences in animals exposed to 0.1 mg/L. GPx catalyses the conversion of H_2O_2, a reactive oxygen species, into water and oxygen. These results are in agreement with the ones found after exposing *G. holbrooki* to SDS (Nunes et al., 2008). Also in the study of Bhattacharya et al. (2022), a decrease in GPx activity was observed at concentrations of 0.34 and 1.02 mg/l of SDS, in *C. carpio*. This enzyme, along with CAT, is an important antioxidant enzyme, preventing oxidative stress (Ighodaro and Akinloye, 2018). When a diminished activity of GPx is in place, we may suggest a failure of the antioxidant defence system, due to the increase in ROS (Yeldandi et al., 2000; Ozok, 2020). However, in other studies, an increment in the GPx activity was reported. Jifa et al. (2005) saw an increment of the activity of this enzyme after an exposure of 1 mg/L of SDBS, after 6 days, in *L. japonicus*. Also, after an exposure of 3 mg/L SDBS, in *Mytilus galloprovincialis*, after a period of 72 days, an increase in GPx activity was observed (Liu et al., 2010). So, we propose that the different effects found in the literature, are due to the different concentrations, organisms chosen, and time of exposure. Notwithstanding, the inhibition observed in our study, after 24h of exposure to SDBS, in animals exposed to concentrations between 0.1 to 5 mg/L, can be a consequence of H_2O_2 accumulation, already reported in other studies for this specific compound and other related surfactants (Jifa et al., 2005; Shukla and Trivedi, 2018; Wibbertmann et al., 2011; Bhattacharjee, 2019; Seguin et al., 2019).

7. Conclusion

Anionic surfactants are known to disturb phospholipid membranes, influence enzyme activities, and increase ROS production. As the production of these compounds tends to increase due to the increasing trend in disinfection products production, specially SDBS, due to the affordable price, serious environmental concerns may arise. Although some information is now available concerning the effects of anionic surfactants, to our knowledge, no study yet provided a wider perspective of the metabolic pathways of SDBS and their potential effects at environmental concentrations. Concerning the FET assay preformed, no predominant malformations were observed; however, an increased mortality rate was reported at 96 h, in animals exposed to the highest concentrations. As demonstrated by our study, impairments in behaviour are observed through increased and decreased swimming, and erratic movements performed, in animals exposed to 1 mg/L, and 5 mg/L. This increment in activity followed by a decrease, and in the presence of erratic movements, suggests impairments in the zebrafish larvae, probably due to alterations, such as an inhibition of the AChE levels, reported in other studies. Also, possible effects of membrane disruption and protein disturbance are in place. Our work also demonstrated a possible specificity towards CYP 450 isoenzymes or the formation of reactive metabolites, given the reported inhibition of CYP 1A1 and CYP 1A2 in our study. However, an increase in CAT activity was observed, which we can assume is the organisms response to the increasing amount of H_2O_2. We also observed an inhibition of GPx and GSTs activity, which we propose is due to the possible increased in ROS. In summary, we found no evidence that SDBS is metabolized by CYP 1A1 and CYP 1A2, in fish. However, metabolism of this compound does occur, forming ROS, as the antioxidant activity of the enzyme CAT was increased. Given the presence and increase of ROS, we suggest that the inhibition of GPx and GSTs results from this increase, as the body is no longer able to detoxify these ROS. The lack of information on this compound, and their increasing amount of production, derived from the SARS-CoV-2 pandemic, raises the emergent necessity for additional studies on the possible impairments of the metabolic pathways.

8. References

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

Ali, S., van Mil, H.G.J., and Richardson, M.K. (2011). Large-Scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. *PLOS ONE*, *6*(6). https://doi.org/10.1371/journal.pone.0021076

Álvarez-Muñoz, D., Sáez, M., Blasco, J., Gómez-Parra, A., and González-Mazo, E. (2006). Enzymatic activity of acid and alkaline phosphatases and catalase in *Ruditapes philippinarum* as biomarkers of stress caused by anionic (C11-LAS) and non-ionic (NPEO2.8) surfactants. *Ciencias Marinas*, *32*(2 B), 447–455. https://doi.org/10.7773/cm.v32i22.1082

Ankley, G., and Burkhard, L. (1992). Identification of surfactants as toxicants in a primary effluent. *Environmental Toxicology and Chemistry*, *11*(9), 1235–1248.

Antunes, S.C., Nunes, B., Rodrigues, S., Nunes, R., Fernandes, J., and Correia, A.T. (2016). Effects of chronic exposure to benzalkonium chloride in *Oncorhynchus mykiss*: Cholinergic neurotoxicity, oxidative stress, peroxidative damage and genotoxicity. *Environmental Toxicology and Pharmacology*, *45*, 115–122. https://doi.org/10.1016/j.etap.2016.04.016

Atli, G., and Canli, M. (2010). Response of antioxidant system of freshwater fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. *Ecotoxicology and Environmental Safety*, *73*(8), 1884–1889. https://doi.org/10.1016/j.ecoenv.2010.09.005

Axarli, I., Prigipaki, A., and Labrou, N.E. (2005). Engineering the substrate specificity of cytochrome P450 CYP102A2 by directed evolution: production of an efficient enzyme for bioconversion of fine chemicals. *Biomolecular Engineering*, 22(1–3), 81–88. https://doi.org/10.1016/j.bioeng.2004.11.003

Barata, C., Varo, I., Navarro, J.C., Arun, S., and Porte, C. (2005). Antioxidant enzyme activities and lipid peroxidation in the freshwater cladoceran *Daphnia magna* exposed to redox cycling compounds. *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology*, *140*(2), 175–186. https://doi.org/10.1016/j.cca.2005.01.013

Berna, J., Ferrer, J., Moreno, A., Prats, D., and Ruiz Bevia, F. (1989). The fate of LAS in the environment. *Tenside Surfactants Detergents*, *26*, 101–107.

Best, C., Kurrasch, D.M., and Vijayan, M.M. (2017). Maternal cortisol stimulates neurogenesis and affects larval behaviour in zebrafish. *Scientific Reports*, *7*, 1–10. https://doi.org/10.1038/srep40905

Bhattacharjee, S. (2019). ROS and Oxidative Stress: Origin and Implication. *Reactive Oxygen Species in Plant Biology*, 1–31.

Bhattacharya, R., Chatterjee, A., Chatterjee, S., and Saha, N.C. (2022). Commonly used surfactants sodium dodecyl sulphate, cetylpyridinium chloride and sodium laureth sulphate and their effects on antioxidant defence system and oxidative stress indices in *Cyprinus carpio L.*: an integrated in silico and in vivo approach. *Environmental Science and Pollution Research*, *29*(20), 30622–30637. https://doi.org/10.1007/s11356-021-17864-x

Bonansea, R.I., Wunderlin, D.A., and Amé, M.V. (2016). Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures. *Ecotoxicology and Environmental Safety*, *129*, 311–319. https://doi.org/10.1016/j.ecoenv.2016.03.043

Bouwknecht, J.A., and Paylor, R. (2008). Pitfalls in the interpretation of genetic and pharmacological effects on anxiety-like behaviour in rodents. *Behavioural Pharmacology*, *19*(5–6), 385–402. https://doi.org/10.1097/FBP.0b013e32830c3658

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Brendler-Schwaab, S., Hartmann, A., Pfuhler, S., and Speit, G. (2005). The in vivo comet assay: Use and status in genotoxicity testing. *Mutagenesis*, *20*(4), 245–254. https://doi.org/10.1093/mutage/gei033

Champagne, D., Hoefnagels, C., Kloet, R., and Richardson, M. (2010). Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. *Behavioral Brain Research*, *214*, 332–342.

Cheah, D.M., Wright, P.F., Holdway, D., and Ahokas, J. (1995). *Octopus pallidus* cytochrome P-450: characterisation and induction studies with β-naphthoflavone and Aroclor. *Aquatic Toxicology*, *33*, 201–214.

Christiansen, A., Backensfeld, T., Denner, K., and Weitschies, W. (2011). Effects of non-ionic surfactants on cytochrome P450-mediated metabolism in vitro. *European Journal*

of Pharmaceutics and Biopharmaceutics, *78*(1), 166–172. https://doi.org/10.1016/j.ejpb.2010.12.033

Colovic, M.B., Krstic, D.Z., Lazarevic-Pasti, T.D., Bondzic, A.M., and Vasic, V.M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology*, *11*(3), 315–335. https://doi.org/10.2174/1570159x11311030006

Correia, D., Almeida, A.R., Santos, J., Machado, A.L., Koba U.O., Žlábek, V., and Domingues, I. (2019). Behavioral effects in adult zebrafish after developmental exposure to carbaryl. *Chemosphere*, *235*, 1022–1029. https://doi.org/10.1016/j.chemosphere.2019.07.029

Cserháti, T., Forgács, E., and Oros, G. (2002). Biological activity and environmental impact of anionic surfactants. *Environment International*, *28*, 337–348. https://doi.org/10.1016/j.memsci.2004.04.007

Deavall, D.G., Martin, E.A., Horner, J.M., and Roberts, R. (2012). Drug-induced oxidative stress and toxicity. *Journal of Toxicology*, *2012*. https://doi.org/10.1155/2012/645460

Deodhar, M., Al Rihani, S.B., Arwood, M.J., Darakjian, L., Dow, P., Turgeon, J., and Michaud, V. (2020). Mechanisms of cyp450 inhibition: Understanding drug-drug interactions due to mechanism-based inhibition in clinical practice. *Pharmaceutics*, *12*(9), 1–18. https://doi.org/10.3390/pharmaceutics12090846

Dizer, H. (1990). Influence of anionic detergents SDS and alkybenzolsufonate on adsorption and transport behavior of several enteretropic virus in soil under stimulated conditions. *International journal of hygiene and environmental medicine*, *190*, 257–274.

Dooley, K., and Zon, L.I. (2000). Zebrafish: A model system for the study of human disease. *Current Opinion in Genetics and Development*, *10*(3), 252–256. https://doi.org/10.1016/S0959-437X(00)00074-5

Feng, T., Li, Z.B., Guo, X., and Guo, J. (2008). Effects of trichlorfon and sodium dodecyl sulphate on antioxidant defense system an acetylcholinesterase of *Tilapia nilotica* in vitro. *Pesticide Biochemistry and Physiology*, *92*, 107–113.

Ferreira, M., Caetano, M., Antunes, P., Costa, J., Gil, O., Bandarra, N., and Reis-Henriques, M. (2010). Assessment of contaminants and biomarkers of exposure in wild and farmed seabass. *Ecotoxicology and Environmental Safety*, *73*, 579–588.

Flohé, L., and Gunzler, W. (1984). Assays of glutathione peroxidase. *Methods in Enzymology, 105*, 114–120.

Fox, K., Holt, M., Daniel, M., Buckland, H., and Guymer, I. (2000). Removal of linear alkylbenzene sulfonate from a small Yorkshire stream: Contribution to GREAT-ER project 7. *Science of the Total Environment*, *251–252*, 265–275. https://doi.org/10.1016/S0048-9697(00)00389-2

Franco-Belussi, L., Jones-Costa, M., Salla, R.F., Souza, B.F.S., Pinto-Vidal, F.A., Oliveira, C.R., and Oliveira, C. (2021). Hepatotoxicity of the anionic surfactant linear alkylbenzene sulphonate (LAS) in bullfrog tadpoles. *Chemosphere*, *266*, 129014. https://doi.org/10.1016/j.chemosphere.2020.129014

Funfak, A., Brösing, A., Brand, M., and Köhler, J. M. (2007). Micro fluid segment technique for screening and development studies on *Danio rerio* embryos. *Lab on a Chip*, *7*(9), 1132–1138. https://doi.org/10.1039/b701116d

Grunwald, D.J., and Eisen, J.S. (2002). Headwaters of zebrafish - emergence of a new model vertebrate. *Nature Reviews Genetics*, *3*, 717–724.

Gustafsson, M.C.U., Roitel, O., Marshall, K.R., Noble, M.A., Chapman, S.K., Pessegueiro, A., Fulco, A.J., Cheesman, M.R., von Wachenfeldt, C. and Munro, A.W. (2004). Expression, purification, and characterization of *Bacillus subtilis* cytochromes P450 CYP102A2 and CYP102A3: flavocytochrome homologues of P450 BM3 from *Bacillus megaterium*. *Biochemistry, 43*(18), 5474–5487. https://doi.org/10.1021/bi035904m

Habig, W.H., Pabst, M., and Jakoby, W. (1974). Glutathione S-transferases the frist enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139.

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

Hayes, J.D., Flanagan, J.U., and Jowsey, I.R. (2005). Glutathione transferases. *Annual Review of Pharmacology and Toxicology*, *45*, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857

Hayes, J.D., and McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research*, *31*(4), 273–300. https://doi.org/10.1080/10715769900300851

Huang, J., Peng, L., Zeng, G., Li, X., Zhao, Y., Liu, L., and Chai, Q. (2014). Evaluation of micellar enhanced ultrafiltration for removing methylene blue and cadmium ion simultaneously with mixed surfactants. *Separation and Purification Technology*, *125*, 83–89. https://doi.org/10.1016/j.seppur.2014.01.020

Ighodaro, O.M., and Akinloye, O.A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria Journal of Medicine*, *54*(4), 287–293. https://doi.org/10.1016/j.ajme.2017.09.001

Jee, J.H., and Kang, J.C. (2005). Biochemical changes of enzymatic defense system after phenanthrene exposure in olive flounder, *Paralichthys olivaceus*. *Physiological Research*, *54*(6), 585–591.

Jifa, W., Zhiming, Y., Xiuxian, S., You, W., and Xihua, C. (2005). Comparative researches on effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate upon *Lateolabrax japonicus* biomarker system. *Environmental Toxicology and Pharmacology*, *20*(3), 465–470. https://doi.org/10.1016/j.etap.2005.05.006

Kimmel, C.B., Ballard, W.W., Kimmer, S.R., Ullmann, B., and Schilling, T.F. (1995). Zebrafish as an alternative vertebrate model for investigating developmental toxicity - the triadimefon example. *International Journal of Molecular Sciences*, *203*, 253–310. https://doi.org/10.3390/ijms18040817

Kumari, K., Khare, A., and Dange, S. (2014). The Applicability of Oxidative Stress Biomarkers in Assessing Chromium Induced Toxicity in the Fish *Labeo rohita*. *BioMed Research International*, *2014*. https://doi.org/10.1155/2014/782493

Lenga, R.E. (2021). *The Sigma-Aldrich library of chemical safety data*. Retrieved from https://www.sigmaaldrich.com/PT/pt/sds/aldrich/289957

Li, M.H. (2008). Effects of nonionic and ionic surfactants on survival, oxidative stress, and cholinesterase activity of planarian. *Chemosphere*, *70*(10), 1796–1803. https://doi.org/10.1016/j.chemosphere.2007.08.032

Lilja, J.J., Backman, J.T., and Neuvonen, P.J. (2005). Effect of gemfibrozil on the pharmacokinetics and pharmacodynamics of racemic warfarin in healthy subjects. *British*

Journal of Clinical Pharmacology, *59*(4), 433–439. https://doi.org/10.1111/j.1365-2125.2004.02323.x

Little, E.E., and Brewer, S.K. (2001). Neurobehavioral toxicity in fish. *Toxicol. Enviroment*, *2*, 139–174.

Liu, B., Yu, Z., Song, X., and Yang, F. (2010). Effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate on the *Mytilus galloprovincialis* biomarker system. *Ecotoxicology and Environmental Safety*, *73*(5), 835–841. https://doi.org/10.1016/j.ecoenv.2009.12.011

Lott, E., and Jone, E. (2022). *Cholinergic toxicity*. StatPearls.

Lushchak, V.I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, *101*(1), 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006

Mao, L., and Wang, J.Q. (2000). Distinct inhibition of acute cocaine-stimulated motor activity following microinjection of a group III metabotropic glutamate receptor agonist into the dorsal striatum of rats. *Pharmacology Biochemistry and Behavior*, *67*(1), 93–101. https://doi.org/10.1016/S0091-3057(00)00307-5

Monferran, M.V., Wunderlin, D.A., Nimptsch, J., and Pflugmacher, S. (2007). Biotransformation and antioxidant response in *Ceratophyllum demersum* experimentally exposed to 1,2- and 1,4-dichlorobenzene. *Chemosphere*, *68*(11), 2073–2079. https://doi.org/10.1016/j.chemosphere.2007.02.016

Mountfield, R.J., Senepin, S., Schleimer, M., Walter, I., and Bittner, B. (2000). Potential inhibitory effects of formulation ingredients on intestinal cytochrome P450. *International Journal of Pharmaceutics*, *211*(1–2), 89–92. https://doi.org/10.1016/S0378-5173(00)00586-X

Mustapha, D.S., and Bawa-Allah, K.A. (2020). Differential toxicities of anionic and nonionic surfactants in fish. *Environmental Science and Pollution Research*, *27*(14), 16754–16762. https://doi.org/10.1007/s11356-020-08212-6

Niraula, T.P., Shah, S.K., Bhattarai, A., and Chatterjee, S. (2012). Cationic Surfactants and their uses in different fields. *TUTA Journal*, 33–37.

Nunes, B., Carvalho, F., and Guilhermino, L. (2005). Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*. *Ecotoxicology and Environmental Safety*, *61*(3), 413–419. https://doi.org/10.1016/j.ecoenv.2004.08.010

Nunes, B., Gaio, A.R., Carvalho, F., and Guilhermino, L. (2008). Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent. *Ecotoxicology and Environmental Safety*, *71*(2), 341–354. https://doi.org/10.1016/j.ecoenv.2007.12.006

Nunes, B., Miranda, M.T., and Correia, A.T. (2016). Absence of effects of different types of detergents on the cholinesterasic activity and histological markers of mosquitofish (*Gambusia holbrooki*) after a sub-lethal chronic exposure. *Environmental Science and Pollution Research*, *23*(15), 14937–14944. https://doi.org/10.1007/s11356-016-6608-2

OCDE. (2013). *OECD guidelines for testing of chemicals*, 509–511. https://doi.org/10.3109/9781841848570-66

Ozok, N. (2020). Effects of cypermethrim on antioxidant enzymes and lipid proxidation of Lake Van fish (*Alburnus tarichi*). *Drug and Chemical Toxicology*, *43*, 51–56. https://doi.org/https://doi.org/10.1080/01480545.2019.1660363

Parhizgar, F., Alishahi, A., Varasteh, H., and Rezaee, H. (2017). Removing Sodium Dodecyl Benzene Sulfonate (SDBS) from Aqueous Solutions Using Chitosan. *Journal of Polymers and the Environment*, *25*(3), 836–843. https://doi.org/10.1007/s10924-016-0855-7

Phillips, T.A., Summerfelt, R.C., and Atchison, G.J. (2002). Environmental, biological, and methodological factors affecting cholinesterase activity in walleye (*Stizostedion vitreum*). *Archives of Environmental Contamination and Toxicology*, *43*(1), 75–80. https://doi.org/10.1007/s00244-002-1134-2

Pullaguri, N., Nema, S., Bhargava, Y., and Bhargava, A. (2020). Triclosan alters adult zebrafish behavior and targets acetylcholinesterase activity and expression. *Environmental Toxicology and Pharmacology*, *75*, 103311. https://doi.org/10.1016/j.etap.2019.103311

Rebello, S., Asok, A.K., Mundayoor, S., and Jisha, M.S. (2014). Surfactants: Toxicity, remediation and green surfactants. *Environmental Chemistry Letters*, *12*(2), 275–287. https://doi.org/10.1007/s10311-014-0466-2

Ren, X., Mao, X., Si, L., Cao, L., Xiong, H., Qiu, J., and Li, G. (2008). Pharmaceutical excipients inhibit cytochrome P450 activity in cell free systems and after systemic administration. *European Journal of Pharmaceutics and Biopharmaceutics*, *70*(1), 279–288. https://doi.org/10.1016/j.ejpb.2008.03.019

Research, A.M. (2020). Surfactants Market by Type (Anionic, Non-ionic, Cationic, Amphoteric, and Others) and Application (Household Detergents, Personal Care, Industrial and Institutional Cleaners, Food Processing, Oilfield Chemicals, Agricultural Chemicals, Textiles, Emulsion Polymerization (Plastics), Paints and Coatings, Construction, and Others): Global Opportunity Analysis and Industry Forecast, 2020–2027. Retrieved March 28, 2022, from https://www.alliedmarketresearch.com/surfactant-market

Roy, D. (1988). Toxicity of an anionic detergent, dodeecylbenzene sodium sulfonate, to a freshwater fish, *Rita rita*: Determination of LC50 values by different methods. *Ecotoxicology and Environmental Safety*, *15*(2), 186–194.

Rubistein, A.L. (2003). Zebrafish : From disease modeling to drug discovery Amy L Rubinstein. *Current Opinion in Drug Discovery and Development*, *6*(2), 218–223.

Saglio, P., Trijasse, S., and Azam, D. (1996). Behavioral effects of waterborne carbofuran in goldfish. *Archives of Environmental Contamination and Toxicology*, *31*(2), 232–238. https://doi.org/10.1007/BF00212371

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., and Champagne, D.L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*, *228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Safety data sheet [SDS]. (2008). Dodecylbenzene sulfonic acid, mixture of C10-C13 isomers, version 4; CAS No 85536-14-7. Thermo Fisher Scientific.

Seguin, R.P., Herron, J.M., Lopez, V., Dempsey, J.L., and Xu, L. (2019). Metabolism of Benzalkonium Chlorides by Human Hepatic Cytochromes P450. *Chemical Research in Toxicology*, *32*(12), 2466–2478. https://doi.org/10.1021/acs.chemrestox.9b00293.Metabolism

Shin, J.T., and Fishman, M.C. (2002). From zebrafish to human: Modular medical models. *Annual Review of Genomics and Human Genetics*, *3*(24), 311–340. https://doi.org/10.1146/annurev.genom.3.031402.131506

Shitara, Y., Hirano, M., Sato, H., and Sugiyama, Y. (2004). Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (OATP2/OATP1B1:SLC21A6)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin: analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. *The Journal of pharmacology and experimental therapeutics*, *311*(1), 228–236. https://doi.org/10.1124/jpet.104.068536

Shukla, A., and Trivedi, S.P. (2018). Anionic Surfactant, Linear Alkyl Benzene Sulphonate Induced Oxidative Stress and Hepatic Impairments in Fish *Channa punctatus*. *Proceedings of the Zoological Society*, *71*(4), 382–389. https://doi.org/10.1007/s12595-017-0223-1

Sobrino-Figueroa, A.S. (2013). Evaluation of oxidative stress and genetic damage caused by detergents in the zebrafish *Danio rerio* (*Cyprinidae*). *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *165*(4), 528–532. https://doi.org/10.1016/j.cbpa.2013.03.026

Steber, J. (2007). The Ecotoxicity of Cleaning Product Ingredients. In *Handbook for Cleaning / Decontamination of Surfaces*, 721–746. https://doi.org/https://doi.org/10.1016/B978-044451664-0/50022-X

Stegeman, J.J. (1981). Polynuclear aromatic hydrocarbons and their metabolism in the marine environment. In *Gelboin*, 1–59. Academic Press, New York.

Tierney, K.B., Sampson, J.L., Ross, P.S., Sekela, M.A., and Kennedy, C.J. (2008). Salmon olfaction is impaired by an environmentally realistic pesticide mixture. *Environmental Science and Technology*, *42*(13), 4996–5001. https://doi.org/10.1021/es800240u

Vascotto, S.G., Beckham, Y., and Kelly, G.M. (1997). The zebrafish's swim to fame as an experimental model in biology. *Biochemistry and Cell Biology*. https://doi.org/https://doi.org/10.1139/o97-081

Vliegenthart, A.D.B., Tucker, C.S., Del Pozo, J., and Dear, J.W. (2014). Zebrafish as model organisms for studying drug-induced liver injury. *British Journal of Clinical Pharmacology*, *78*(6), 1217–1227. https://doi.org/10.1111/bcp.12408

Wang, Q., Liu, N., Wang, J., Wu, Y., and Wang, L. (2014). Physiological changes and acetylcholinesterase activity in th cladoceran *Moina macrocopa* (Straus, 1820) exposed to mercury and sodium dodecylsulphate. *Crustaceana*, *87*(14), 1678–1690.

Wang, Y., Zhang, Y., Li, X., Sun, M., Wei, Z., Wang, Y., and Gao, A. (2015). Exploring the Effects of Different Types of Surfactants on Zebrafish Embryos and Larvae. *Nature Publishing Group*, 1–10. https://doi.org/10.1038/srep10107

Wibbertmann, A., Mangelsdorf, I., Gamon, K., and Sedlak, R. (2011). Toxicological properties and risk assessment of the anionic surfactants category: Alkyl sulfates, primary

alkane sulfonates, and α-olefin sulfonates. *Ecotoxicology and Environmental Safety*, *74*(5), 1089–1106. https://doi.org/10.1016/j.ecoenv.2011.02.007

Wu, Z., Yu, D., Li, J., Wu, G., and Niu, X. (2010). Growth and antioxidant response in *Hydrocharis dubis* (Bl.) Backer exposed to linear alkylbenzene sulfonate. *Ecotoxicology*, *19*(4), 761–769. https://doi.org/10.1007/s10646-009-0453-8

Yeldandi, A.V., Rao, M.S., and Reddy, J.K. (2000). Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *448*(2), 159–177. https://doi.org/10.1016/S0027-5107(99)00234-1

Yi, X., Wei, Y., Zhai, W., Wang, P., Liu, D., and Zhou, Z. (2022). Effects of three surfactants on the degradation and environmental risk of metolachlor in aquatic environment. *Chemosphere*, 134295. https://doi.org/10.1016/j.chemosphere.2022.134295

Zhang, B., Chen, X., Pan, R., Xu, T., Zhao, J., Huang, W., and Yin, D. (2017). Effects of three different embryonic exposure modes of 2, 2', 4, 4'-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. *Chemosphere*, *169*, 542–549. https://doi.org/10.1016/j.chemosphere.2016.11.098

Zhang, Ying, Ma, J., Zhou, S., and Ma, F. (2015). Concentration-dependent toxicity effect of SDBS on swimming behavior of freshwater fishes. *Environmental Toxicology and Pharmacology*, *40*(1), 77–85. https://doi.org/10.1016/j.etap.2015.05.005

Zhang, Y., Tan, Y., Stormer, H.L. and Kim, P. (2005). Experimental observation of the quantum Hall effect and Berry's phase in graphene. *Nature*, *438*, 201–204

Chapter IV | Effects of thymol disinfectant related to SARS-CoV-2 - An assessment of embryonic, behavioral, and enzymatic biomarkers in the freshwater model fish *Danio rerio*

1. Abstract

Natural compounds have a high potential in our daily life. Since the beginning of the severe acute respiratory syndrome coronavirus (SARS-CoV-2), natural phenols such as Thymol (THY), due to their antiviral and antimicrobial properties, have made the list of disinfectants approved to contradict the spread of the virus. Despite the wide application of THY, little information is available regarding the possible effects of this phenol on aquatic organisms. Given the expected increase in consumption of this compound over the next few years, it is imperative to know the potential hazards of THY. To this end, embryonic tests were performed, such as the Fish Embryo Toxicity Test (FET), behavioral tests, where the total distance traveled (mm), thigmotactic behavior and erratic movement were evaluated, after an acute exposure of *Danio rerio.* Finally, the enzymatic activities of the CYP 1A1, CYP 1A2, GSTs, CAT, and GPx enzymes were assessed. Our results report an increased mortality after 96h of exposure to animals exposed to all concentrations. We suggest that the mortality observed in our study could be connected to malformations, such as pericardial oedema, prior to the 96h evaluation. Also, a decrease in the total activity was observed in animals exposed to the highest concentration. This decrease could result from a possible inhibition of acetylcholinesterase (AChE), reported in previous studies. When it comes to the thigmotaxis test, we cannot draw any conclusions given the lack of significant results for the total time spent in the outer zone parameter. However, we can say that animals exposed to all concentrations showed a higher number of erratic movements. Finally, regarding the metabolic biomarkers, our study demonstrates a transversal decrease of all measured enzymatic activities, with the exception of GPx, in which we did not report any significant results. Thus, we observed a decrease in phase I enzymes, CYP 1A1 and CYP 1A2, and in phase II, GSTs. We also observed a decrease in the activity of the antioxidant defense CAT. These results demonstrate the antioxidant potential of this compound, which has been previously described in other works. Given the increasing consumption of this product, we can expect that the here documented adverse effects will be increased, leading to a pro-oxidative action of the compound.

2. Keywords

FET; Covid-19; SDBS; THY; Antioxidant defence.

3. Introduction

Since the severe acute respiratory syndrome coronavirus (SARS-CoV-2) pandemic outbreak swept the world, plenty of research has been directed towards creating measures to prevent the spread of the virus by using disinfectant agents. According to the List N Tool: COVID-19 Disinfectants by the United States Environmental Protection Agency (US EPA), in 2020, when the pandemic initially broke out, there were 430 products registered that were effective against Covid-19. However, after only two years, the number of products available increased by 43% (Hora et al., 2020; United States Environmental Protection Agency [US EPA], 2022). Although the pandemic is now at the end, the use of disinfectants is not expected to decrease, but it is likely to increase in 10% until 2027 (Hora et al., 2020).

Among the various categories presented in this list (active ingredient, surface type, contact time, and use), Thymol (THY) appears as one of the active ingredients to be less toxic (US EPA, 2022). The use of THY, chemically known as 2-isopropyl-5-methylphenol, has been around since ancient times, since it is present in herbs (Nagoor-Meeran et al., 2017). Along the centuries, several cultures, such as the Greeks, Romans and Egyptians, have use thyme in food preparations, and as a preservative, in medicine and in mummification processes (Zarzuelo and Crespo, 2002). This monoterpene phenol has powerful antimicrobial, antifungal, antiseptic, antioxidant, and antiviral properties (Didry et al., 1994; Mahmoud, 1994; Yanishlieva et al., 1999). Alongside with these various applications and qualities, THY is also present in soaps, toothpastes, mouthwash, shampoos, in treatment solutions for aquaculture, and in pesticides (Shapiro et al., 1994; Yilmaz et al, 2011; Carayon et al., 2014; Manou et al., 2020).

The wider application of THY led to a detection of this compound in a range of 0.5 to 3.0 ng/L in the Tamagawa river, between 2004 and 2005 (Nakada et al., 2008). THY seems to be bioaccumulated by exposed biota, since it was detected in fish fillets of Walley pike (*Stizostedion vitreum*) and in the Northern pike (*Esox lucius*) (Heil and Lindsay, 1989). Since several of its uses, such as in aquaculture, protection against plagues, and as

disinfectants, came in the past decades, it is expected that higher concentrations can now be detected in the environment, and in our food.

Although THY has received a lot of attention along the years, almost no information of its toxicity in aquatic organisms is known. In humans, this phenolic compound undergoes hydroxylation of the aromatic ring and the iso-propyl side chain probably through reactions carried out by CYP 450, as reported in *Candida tropicalis*. (Stiborová et al., 2003; Thalhamer et al., 2011). This reaction leads to the formation of unstable products such as p-cymene-3-ol-8-ene, and p-cymene-3,8-diol (Thalhamer et al., 2011). Oxidation processes, by the CYP 450 complex, were also identified in human, and rabbit metabolisms (Placha et al., 2022). Phase II enzymes such as uridine 5'-diphospho-glucuronosyltransferase (UDP) and sulfotransferases (SULTs) detoxifies THY (Thalhamer et al., 2011). Besides the vast information concerning the human metabolism, differences have been found in different animal models (Austgulen, et al., 1987; Thalhamer et al., 2011). Viran et al. (2003) suggested the necessity of monitoring the possible impairments of this compound in fish. Due to its lipophilic characteristics, THY is highly absorbed by the fish gills, which could contribute to a higher sensitivity of these organisms (Viran et al., 2003). Due to the phenolic hydroxyl group, THY is known to protect against the impairments caused by free radicals and increase the enzymatic activity of endogenous antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione-S-transferase (GSTs), when the organism has a high content of reactive oxygen species (ROS) (Wojdyło et al., 2007; Nagoor-Meeran and Prince, 2012). Also, although observed in *in vitro* studies, thymol has a biphasic effect on the blockage of the ion exchange of calcium and potassium ions (Ebashi, 1965; Szentandrássy et al., 2003). After an acute exposure in *Poecilia reticulata*, to levels between 1-20 mg/L, the calculated LC_{50} was of 12.51 mg/L and 10.99 mg/L for females and for males, respectively (Bullangpoti et al., 2018). Also, Vieira et al. (2021) reported a calculated LC_{50} for zebrafish embryos of 2.35 mg/L. So, we could assume that THY toxicity varies among species. Studies conducted with zebrafish showed high levels of ROS production at concentrations of 42 µM (Krishnan et al., 2019). Also, Krishnan et al. (2019) reported a diminished activity of CAT, and an increased expression of the overall activity of CYP 450. Phase II enzymes are also involved in the detoxification process of THY in fish. Vieira et al. (2021) observed a significant increase in the zebrafish GSTs activity after an exposure to 1 mg/L of THY, after a 96h exposure.

Alongside metabolic alterations, embryonic alterations and malformations were also reported in zebrafish when expose to THY. Reduced yolk sack and development of pericardial edemas were found in fish exposed for 96h to 1 mg/L of THY (Vieira et al., 2021). After 96h, in the previously mentioned study, a significant number of dead organisms were documented after an exposure to 1 mg/L (Vieira et al., 2021).

Thigmotaxis behavior assay is an important experiment to evaluate anxiety-like behaviors in organisms. In fish this stereotypic behavior is associated with the fish staying in close proximity to the outer boundaries of a novel environment, so alterations in the time spent or in the distance traveled in close proximity to the boundaries are indicators or stress (Schnörr et al., 2012). No changes in behavior of zebrafish larvae, when presented with an adverse stimulus (red bouncing ball), were documented following exposure of 1 mg/L of THY, for 96h. Increasing hyperactivity in zebrafish larvae was observed in a thigmotaxis test, after an initial exposure in the early stages of development to 1 mg/L of THY (Vieira et al., 2021).

Danio rerio (zebrafish) is an important organism model due to its similarities to other vertebrate models (Dooley and Zon, 2000; Shin and Fishman, 2002). Innumerous diseases can be studied, at a first stage, with zebrafish, since this organism is easier to handle in laboratory studies than others (Nagoor-Meeran et al., 2017). The small body frame, high offspring, easy genetic manipulation, short life span, easy maintenance, and low price, are some of the characteristics that favor this organism in comparison to others (Kimmel et al., 1995).

Our study aimed to elucidate the possible impairments caused by THY in zebrafish, after an acute exposure to environmentally relevant concentrations, in terms of phase I metabolic enzymes, such as CYP 1A1, and CYP 1A2, phase II metabolic enzyme GSTs, and finally the antioxidant enzymes CAT and GPx. Additionally, embryonic and behavioral alterations were evaluated to elucidate about the occurrence of early malformations and impairments in an important environmental factor such as behavior.

4. Materials and methods

4.1 Chemicals

Thymol (CAS: 89-83-8, purity >98.5%) and chemicals used for enzymatic determinations were purchased from Sigma Aldrich®. Bradford reagent was obtained from Biorad®, UK.

4.2 Test organism

D. rerio eggs were obtained from an established bioterium at the Department of Biology, in the University of Aveiro. Organisms were kept under optimal conditions, such as 95% of dissolved oxygen, conductivity of 750 ± 50 µS/cm, 12:12 h light/dark period, and a temperature of 27 ± 1º C. The system water was reconstituted with the help of instant ocean synthetic salt, Spectrum Brands. Zebrafish were fed daily with Gemma Micro 500 (Skretting®, Spain).

Reproductive couples were placed the day before the collection in breeding tanks. These animals were divided by sex and separated in the breeding tanks by a divider. In the following day, the divider was removed, and the fish were allowed to breed for at least 1h30 the eggs were collected and cleaned in system water. Afterwards, the eggs were checked under a Stereoscopic Zoom Microscope – SMZ 1500, Nikon. All eggs with deformations were discarded. For the purposes of behavioral trials and enzymatic evaluations, the obtained eggs were allowed to grow until 5 days post-fertilization (dpf), and then exposed to 3 concentrations of thymol (5, 50, and 500 ng/L), for a period of 24h, before being frozen and stored. The concentrations were established by multiplying by a factor of 10 the minimal environmental concentration reported (0.5 ng/L). The second and third concentrations were obtained by also multiplying the concentration established before by a factor of 10 (5, 50, and 500 ng/L).

4.3 FET

Embryos were selected, as previously mentioned, and the recommendations of the OCDE 236 guideline (OCDE, 2013) were followed. Embryos were randomly distributed (20 eggs per treatment), and immediately transferred into a 24 well plate. The media in which the test compound was dissolved, and the media of the internal plate controls, were renewed each 24h, and conditions were kept under the normal conditions mentioned above. Every 24h, coagulation, existence of cardiac rhythm, tail detachment, presence of somites, edema and pigmentation, and hatching delay, were monitored for 96h. According to the OCDE 236 guideline, if a positive outcome in any of the parameters appeared, the embryo was considered dead.

4.4 Behavioral Tests

Larvae with 5-dpf were exposed to the 3 different concentrations (5, 50, and 500 ng/L), in 24 well plates. After the 24h exposure period, the movement of 96 larvae per concentration was registered with the help of Zebrabox 25 frame per second infrared camera, Viewpoint, Lyon, France. Three different locomotor parameters were assessed during the test. Total activity of the fish was the first parameter to be measured, by calculating the total distance travelled. The assay contemplated 2 segments, the first with the purpose of acclimating the larvae. After this period of 6 min, the lights were abruptly turned off for 4 min, as described by Schnörr et al. (2012). An inner area was created with a 12.25 mm radius, and an outer area was set as 4 mm radius These measures ensured that the size of the larvae (4 mm with 5-dph) was totally in the outer zone. The data, concerning the thigmotaxis evaluation were pre-treated to obtain, according to Schnörr et al. (2012), the percentage of distanced traveled (%TDM), and time spent in the outer zone (%TTM), in the dark period. Finally, the last parameter for locomotor behavior was the frequency of turn angles, as a measure of erratic movements (Zhang et al., 2017). The angles produced during the dark period were also pre-treated to obtain a percentage to reduce individual variability (Bouwknecht and Paylor, 2008). Angles were grouped into 4 distinct classes as described in Zhang et al. (2017). Turn ranges between 0º to ± 10º constituted class 4. Angles between ±

10º and ± 30º, and between ± 30º and ± 90º, were summed up in class 3 and 2, respectively. Finally, turn angles in the range of ± 90º to ± 180º, were grouped in class 1.

4.5 Enzymatic determinations

To assess the enzymatic activity of phase I, phase II, and antioxidant defense biomarkers, samples were composed by 12 replicates per concentration, each with 8 larvae, with 5-dpf. For the quantification of GPx activity, samples were composed of 10 larvae. After an acute exposure to 3 different concentrations of THY, 1 mL of 50 mM phosphate buffer, pH=7.0, with X-100 0.1% was added to determine CAT, GPx, and GSTs activities. Afterwards, the samples were frozen, and stored at -80ºC in Eppendorf microtubes. Before the determination of enzymatic activities, samples were centrifuged at 1500 *g*, for 10 min, at 4ºC (Megafuge 8R, Thermo Scientific).

Cytochrome P450 activity was measured through the evaluation of CYP 1A1, and CYP 1A2 activities. For the purposes of these assays, 12 replicates of 8 larvae with 5-dpf each were exposed to THY for 24h. Then the samples were frozen, and 1 mL of 50 nM Tris HCL, pH=8.0, 0.15M KCL, 1mM dithiothreitol solution was added. Immediately before the determinations, samples were homogenized and centrifuged at 1000 *g*, for 10 min, at 4ºC. Afterwards, the supernatants were removed and placed in a new set of Eppendorf microtubes, with 8 mM CaC12, at pH=7.4. Subsequent centrifugation at 1696 *g* for 10 min at 4ºC was performed to obtain microsomes, which were in turn resuspended with 0.1 M of phosphate buffer, pH=7.4, with 1 mM EDTA, 20% (v/v) glycerol, 0.5% (w/v) sodium cholate and 0.4% (w/v), and Triton X-100. To determine CYP 1A1, 7-ethoxyresorufin solution was added to the 96-well plates; methoxyresorufin was used to determine CYP 1A2. The final step to measure the enzymatic activity was to add nicotinamide adenine dinucleotide phosphate (NADPH) to start the reaction. Before assessing the activity through the fluorometer (Megafuge 8R, Thermo Scientific) (λex=540nm and λem=590nm), the plates destined to CYP 1A1 incubated for 10 min. The method proposed by Cheah et al. (1995) was followed and results expressed in pmol per mim/mg of protein.

To assess GSTs, a phase II metabolic biomarker, the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was conjugated with glutathione, by the isoenzymes glutathione S-transferases, to allow the formation of the thioether. The presence of this thioether was then

measured at a wavelength of 340 nm. This method was performed according to Habig et al. (1974), and expressed as nanomoles of thioether formed per mim/mg of protein.

CAT activity was measured through the decomposition of hydrogen peroxide (H_2O_2), as described by Aebi (1984). The activity was measured at a wavelength of 240 nm, using a microplate reader (Multiskan Spetrum, Thermo Scientific) with SkanIt ^{TM} software, and was expressed as the number of moles of H_2O_2 consumed per min/mg of protein.

GPx activity was measured as described by Flohé and Grumzler (1984), at a wavelength of 340 nm. Oxidation of NADPH allowed us to obtain an indirect measure of GPx activity, since NADPH is oxidized in the process of reduction glutathione. For the purposes of this work, only total GPx was measured by using cumene hydroperoxide as a substrate. The GPx activity was expressed as nmol of oxidized NADPH per minute per milligram of protein.

For all enzymatic determinations, the total protein concentration was assessed through the method proposed by Bradford (1976). A standard curve of 1 mg/mL of globulin was used.

4.6 Statistical analysis

For all obtained data, the normality was verified through a Kolmogorov-Smirnov test. Then an ANOVA or a Kruskal Wallis (when the normality test failed) was made. Finally, a Dunnett test was used to infer possible significant differences between the concentrations and the control treatment. The level of significance was set as $p< 0.05$, by using GraphPad software.

5. Results

5.1 FET

After an acute exposure, an increase in mortality was observed at 96h, in animals exposed to all concentrations. At a concentration of 5 ng/L the cumulative lethality was of 16,67%. In the last two concentrations of 50, and 500 ng/L, we observed a cumulative mortality of 14,6%, and a 18,75% respectively (Figure 18).

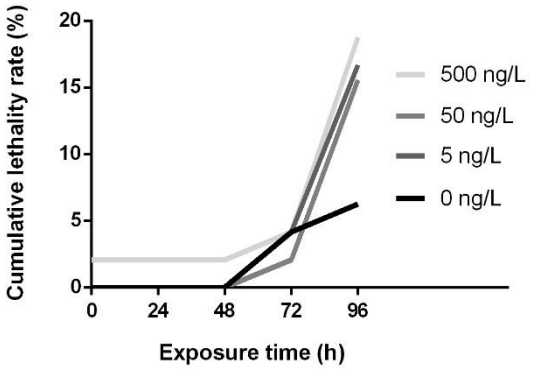

Figure 18 - Cumulative lethality rate (%), after a 96h exposure to THY. N=96.

In our study, animals exposed to all concentrations developed tail malformations (Figure 19 A2), and pericardial edemas were observed at 72 hours of exposure. The incidence of pericardial edema (Figure 19 A1, A3, B1, C1) in the embryos, after 72h of exposure, is the most predominantly malformation, with 6,25%, 10.4%, and 8.33%, at the 5, 50 and 500 ng/L, respectively.

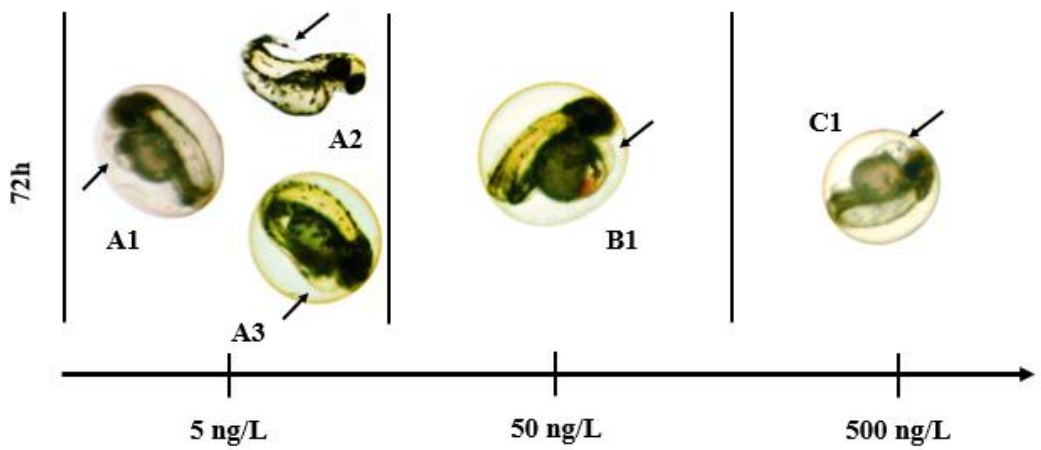

Figure 19 - Malformations observed after 72h exposure to THY. A1, A3, B1 and, C1) Pericardial edema; A2) Tail malformation.

5.2 Locomotion assays

5.2.1 Swimming activity

First, the total distanced moved was assessed, and a significant decrease was found in fish exposed to the highest concentration (500 ng/L) compared to the control ($F_{[3, 346]}$ = 10.48, $p < 0.0001$) (Figure 20).

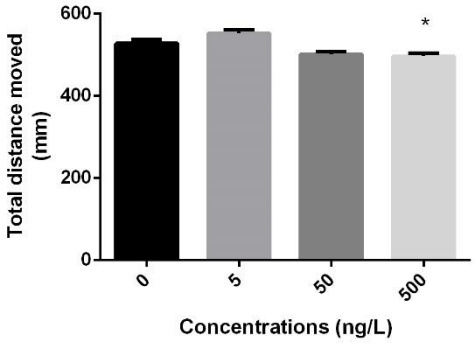

Figure 20 - Total distance moved (mm) after an acute exposure of *D. rerio* to different concentrations of THY. Results are represented as mean standard error. n= 96. Significant differences from the control, p< 0.05.

5.2.2 Thigmotaxis

Thigmotaxis revealed a significant decrease in animals exposed to 5 ng/L, and 500 ng/L, in the distanced moved parameter ($F_{[3, 524]}$ = 4.665, p = 0.0032) (Figure 21A). Regarding the time spent in the outer zone no significant diferences were found ($F_{[3, 524]}$ = 3.248, p = 0.0216) (Figure 21B).

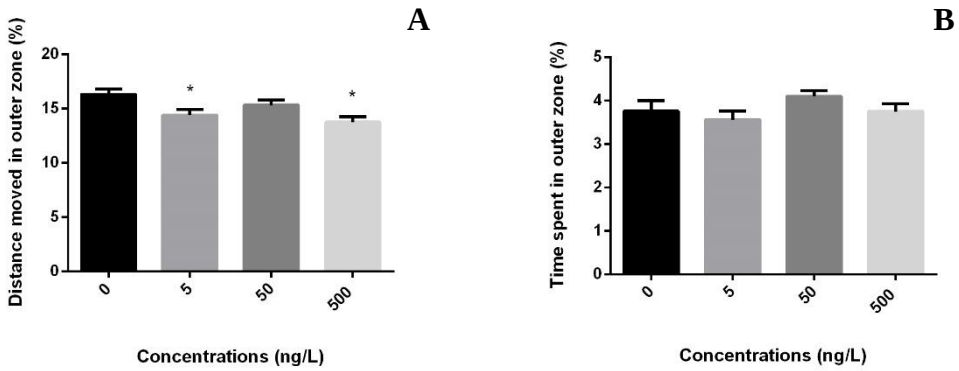

Figure 21 - (A) Effects on distanced travel in the outer zone, and (B) time spent in the outer zone, made after an acute exposure of *D. rerio* to THY. Data are represented as mean ± standard error. n= 96. Significant differences from the control, $p<0.05$..

5.2.3 Assessment of erratic movements

Compared to the control, turn angles showed a significant increase in class 1, for all organisms exposed to the three tested concentrations ($F_{[9, 48]} = 13.80$, $p < 0.0001$) (Figure 22).

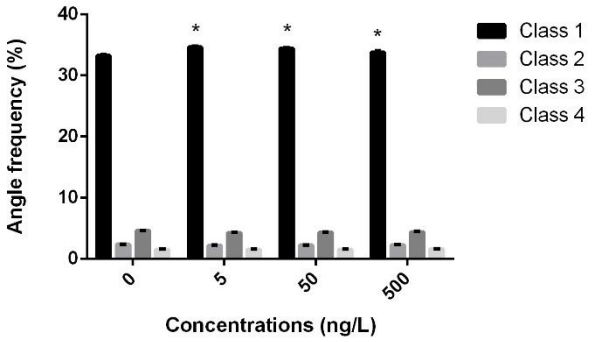

Figure 22 - Results of the angle frequency determined in *D. rerio*, after an acute exposure to THY. Data are represented as mean ± standard error. n=96. Significant differences form control, $p< 0.05$.

5.3 Enzymatic determinations

5.3.1 Phase I metabolic biomarkers

CYP 1A1 showed a significant decrease in activity of animals exposed to 5 ng/L, and 500 ng/L, when compared to the control ($F_{[3,\ 34]}$ = 10.19, p <0.0001) (Figure 23A). Concerning CYP 1A2 activity, a significant decrease was also observed but only in the two highest concentrations ($F_{[3,\ 37]}$ = 8.282, p = 0.0002) (Figure 23B).

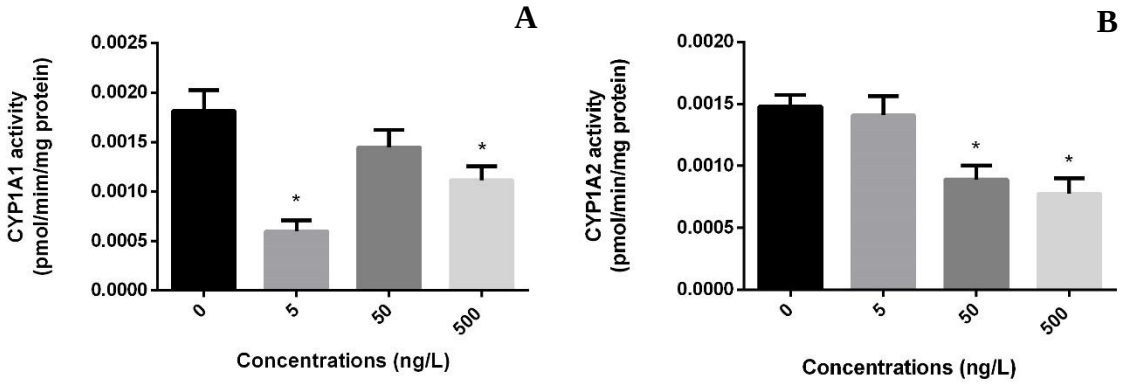

Figure 23 - Results of CYP 450 enzymes determined in *D. rerio*, after an acute exposure to THY. A) CYP 1A1 activity; B) CYP 1A2 activity; Data are represented as mean ± standard error. n=12 Significant differences from the control, p< 0.05.

5.3.2 Phase II metabolic biomarker

A similar result was also observed in GSTs activity, where a decrease in enzymatic activity in organisms exposed to the last two concentrations was observed. ($F_{[3,\ 39]}$ = 14.16, p <0.0001) (Figure 24).

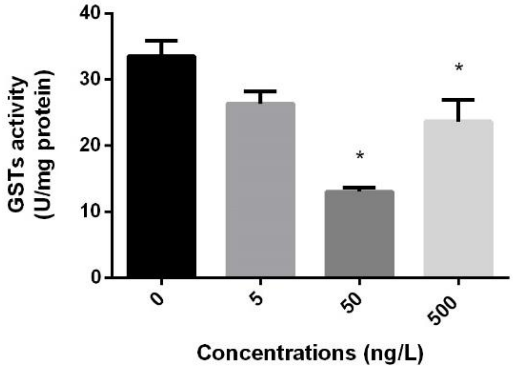

Figure 24 - GSTs activity determined in individuals of *D. rerio*, after an acute exposure to THY; Data are represented as mean ± standard error. n=12. Significant differences from the control, $p<0.05$.

5.3.3 Oxidative stress biomarkers

Compared to the control, CAT activity showed a significant inhibition in fish exposed to the highest two concentrations ($F_{[3, 35]} = 11.55$, $p < 0.0001$) (Figure 25A). GPx activity did not yield any significant alterations, for all concentrations, when compared to the control, after the acute exposure to THY ($F_{[3, 30]} = 0.7113$, $p = 0.5529$) (Figure 25B).

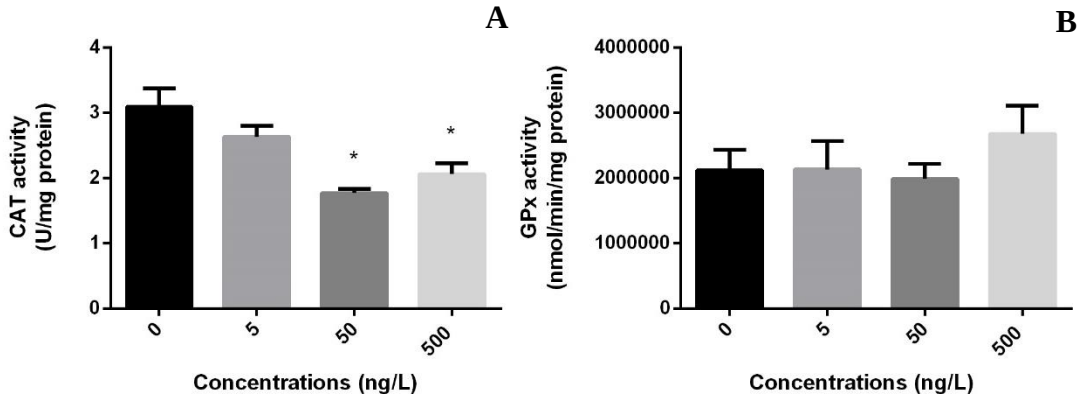

Figure 25 - Results of oxidative stress after an acute exposure of *D. rerio* to THY. (A) CAT activity; (B) GPx activity; Data are represented as mean ± standard error. n=12. Significant differences from control, $p< 0.05$.

6. Discussion

6.1 FET

The results found in our study indicate a predominant malformation of pericardial edema in *D. rerio*, after 72h of exposure to 500 ng/L, which resulted in a higher number of dead organisms after 96h of exposure. These results are in agreement with the ones reported by Vieira et al. (2021), where zebrafish were submitted to an exposure to 1 mg/L of THY for 96h. The incidence of this malformation was also observed, in zebrafish, after an exposure for 96h to 42µM of THY (Krishnan et al., 2019). The results in our study showed a higher cardiac toxicity. During embryogenesis, multiple mechanisms are in place to ensure the normal development of the embryo (Kimmel et al., 1995; Schier, 2001). The teratogenic effect on embryogenesis can be related to impairments in the PI3K/Akt and ERK pathways (Lv and Chen, 2017). THY is known to promote alterations in these pathways, which are involved in cell differentiation, growth, survival, and could cause pericardial edema (Finkielsztein and Kelly, 2009; Hikasa and Sokol, 2013; Yu and Cui, 2016; Krishnan et al., 2019). Also, activation of Nrf2, a regulator of antioxidative response, has been linked to developmental malformations on zebrafish embryos (Krishnan et al., 2019; He et al., 2020; Vieira et al., 2021). In the study of Krishnan et al. (2019), an increase in the Nrf2, after an exposure to zebrafish embryos of 42µM, indicated the possible impairments in the antioxidant response in this early life stages, which could lead to malformations along the development. Pericardial edema could also be a result of cardiotoxicity resulting from the blockage of Ca^{2+} channels caused by THY (Aftab et al., 1995). The blockage of these channels may lead to hypotension and bradycardia (Aftab et al., 1995; Dorta et al., 2003). The administration of THY in dogs induced cardiac arrhythmias, due to the inhibition of K^{+} and Ca^{2+} channels (Magyar et al., 2004). Many studies reported the development of cardiac problems, in canine, human, and guinea pigs hearts, and heart cells, due to the adverse effects that THY has on Ca^{2+} channels and pumps (Aftab et al., 1995; Magyar et al., 2002; Magyar et al., 2004; Szentandrassy et al., 2004). Deregulation of these ionic currents are linked to changes in membrane depolarization in muscular and nervous cells that constitutes cardiac tissue, so early alterations, in the embryonic stages, in these ionic channels can promote cardiac toxicity leading to the formation of pericardial edema (Ebashi, 1965; Nagoor-Meeran

et al., 2015; Yu and Cui, 2016). Thus, the formation of pericardial edema can be one possible indicator of teratogenic alterations or from disturbances in the normal functioning of the cardiac system, such as disruption of ionic currents.

6.2 Behavioral assays

Our study showed a tendency for a diminished total distance traveled by the exposed organisms, although only significant differences were reported in animals exposed to the highest concentration (500ng/L). In the thigmotaxis test, the total distance traveled in the peripheral zone also showed a significant decrease in fish exposed to concentrations of 5, and 500ng/L. The %TTM parameter did not show any significant change or a clear trend, when compared to the control. So, no assumptions concerning the anxiety-like behavior of the organism can be made. However, our study shows a significant increase in class 1 of turn angles in all concentrations. An increase in the number of erratic movements goes in agreement with the results found by Bullangpoti et al. (2018).

THY is known for its capacity to alter the homeostasis of calcium and potassium currents (Ebashi, 1965; Szentandrássy et al., 2003). These two ions are responsible for altering the action potential, and carrying out the transmission of the nerve impulse to other nerve cell. When Na^+ enters the cellular membrane, depolarization occurs, which is followed by a rush of K^+ ions to the external of the cellular membrane to reestablish the membrane potential (Sah and Davies, 2000). In the study of Bullangpoti et al. (2018), a decrease in body activity in *P. reticulata* was observed, and a paralytic effect was also registered after an exposure to 10 mg/L. However, in Vieira et al. (2021), a concentration of 1.175 mg/L of THY lead to hyperactivity in thigmotaxis tests, with zebrafish larvae. Notwithstanding the possible impairments due to alterations in ion currents, alterations in the acetylcholinesterase (AChE) activity can also lead to alterations in behavior, such as locomotor activity and erratic swimming (Tierney et al., 2008; Colovic et al., 2013; Bonansea et al., 2016; Pullaguri et al., 2020). AChE is responsible for hydrolyzing the excess of acetylcholine (ACh) from the synaptic clef. The inhibition of AChE results in hyperstimulation of the pos-synaptic neurons, which first can increase in the nerve impulse, and ultimately cause paralysis and dead (Tierney et al., 2008; Colovic et al., 2013; Bonansea et al., 2016; Pullaguri et al., 2020). In the study of Jukic et al. (2007) a 50% inhibition of AChE was registered after an exposure

to 0.74 mg/L of THY essential oil in *in vitro* conditions. An inhibition was also reported in male *P. reticulata,* after being exposed to 10.99 mg/L of THY (Bullangpoti et al., 2018). So, a combination effect of impairments in these two pathways can be at place, and be dependent on the concentration (Ebashi, 1965; Szentandrássy et al., 2003; Jukic et al., 2007).

6.3 Enzymatic determinations

The breakdown of THY in the human organism occurs via hydroxylation reactions of the isopropyl chain, originating p-cyme-3,8-diol, and p-cyme-3-ol-8-ene, and hydroxylation of the aromatic ring, giving rise to p-cyme-2,5-diol, which by oxidation reactions creates p-cyme-2,5-dione, and p-cyme-2,3-diol (Thalhamer et al., 2011). Although no study on fish discriminating the different metabolites from the metabolism of THY was found, most of them report the antioxidant activity of the compound (Cai et al., 2006; Capatina et al., 2020; El-Nekeety et al., 2011; Thalhamer et al., 2011). In general, phenolic rings, also present in THY, display an active role on the scavenging of free radicals (Antolovich et al., 2002). THY exerts its antioxidant capacities by preventing the formation of free radicals or by scavenging reactive oxygen species (ROS) through endogenous antioxidant enzymes (Brewer, 2011). Ahmadifar et al. (2011) suggested that adding THY to the food would translate into beneficial effects in the growth of *Oncorhynchus mykiss* juveniles. Positive effects on Alzheimer's disease in reverting oxidative damage of other compounds, were also reported (El-Nekeety et al., 2011; Capatina et al., 2020). Despite the common knowledge of the antioxidant properties of phenols, including THY, auto-oxidation can also occur, leading to oxidative stress (Shalaby and Horwitz, 2015; Bayliak et al., 2016; Krishnan et al., 2019). Oxidative stress can occur when there is an excess of ROS. Normal levels of ROS and free radicals such as superoxide and hydrogen peroxide are produced, and effectively degraded to prevent an excess of ROS in the cell, although some xenobiotics can lead to alterations in this balance, causing oxidative stress (Bhattacharjee, 2019).

CYP 450 enzymes metabolize a variety of xenobiotics to promote their successful elimination (Holth et al., 2008). Krishnan et al. (2019) also reported the involvement of CYP 450 in the metabolism of THY in zebrafish larvae, suggesting the metabolism of THY through this phase I enzyme. However, the overexpression of the CYP 450 complex observed in Krishnan et al. (2019) study could also be a sign that the metabolites of THY

can promote toxicity to the zebrafish organism (Krishnan et al., 2019). In human studies, the involvement of CYP 1A2, CYP 2A6, and CYP 2B6 in metabolizing THY has been shown, with preferential use of CYP 2A6 (Dong et al., 2012). In our study, the CYP 450 isoenzymes were generally significantly inhibited in animals exposed to the two highest concentrations for CYP 1A2 (50, 500ng/L), and also in fish exposed both to the lowest and highest concentration of THY (5, 500ng/L) for CYP 1A1. Anitha et al. (2018) showed a marked inhibition of CYP 3A4 isoenzyme after a co-exposure to 5-100 µg/L thyme oleoresin, in an *in vitro* study. Misaka et al. (2013), also showed CYP 450 inhibition in rats, after an exposure to phenolic compounds in green tea, which has THY in its composition. However, Krishanan et al. (2019) reported an increase in the relative protein expression of CYP 450, after an exposure to 42µM of THY. To our knowledge, as previously mentioned, phenolic compounds can present pro-oxidant or antioxidant activities in a dose dependent manner (Poljsak et al., 2013; Zehetner et al., 2019). On one hand, THY presents antioxidant properties, which means that it reduces ROS generated by external or internal factors. Meaning that antioxidants can remove ROS generated or even beneficial ROS, such as the ones involved in signaling molecules for cell proliferation, and apoptosis (Murrell et al., 1990; Kroemer, 1999; Kim et al., 2002). On the other hand, regarding the well-established antioxidant properties of phenols, it is worth noting that phenols can act as prooxidants, by increasing oxidative stress (Palozza, 1998; Podmore et al., 1998). The phenol radical can react with oxygen, producing quinones and superoxide anion (Shahidi et al., 1992; Cotelle, 2001). The increase in superoxide and hydroxyl radicals can thus be activated by CYP 450, inhibiting its activity (Ekstrom and Ingelman-Sundberg, 1989). Llana-Ruiz-Cabello et al. (2015) showed the simultaneous antioxidant and oxidant activities of THY and carvacrol. So, an induction or inhibition of CYP 450 can be observed, after an exposure to THY (Ferguson, 2001; Llana-Ruiz-Cabello et al., 2015). Another possible reason for the decrease observed in our study is the preference of this substrate for other isoenzymes (Powley and Carlson, 2001; Anitha et al., 2018; Placha et al., 2022). As reported in other animal models, such as rabbit and mouse, the phenolic ring present in THY could also be metabolized by other isoenzymes such as CYP 2C9, and CYP 3A4 (Anitha et al., 2018; Placha et al., 2022). The preference for other isoenzymes is also reported in the study of Powley and Carlson (2001), where an inhibition of CYP 2E1 and CYP 2F2 was observed and was dependent on the samples (lung or liver). Also, in the study of Robert et al. (1993), a preference towards

the CYP 2E1 isoenzyme compared to CYP 2B1 was reported, adding further evidence for the preference of this phenol to be metabolized by other isoenzymes than those addressed in our study. Additionally, Dong et al. (2012), also reported a marked preference for CYP 2A6 in relation to CYP 1A2 and CYP 2B6.

The phase II enzymes GSTs conjugates GSH with xenobiotics to increase their hydrophilicity, thereby increasing their excretion rate. GSTs can also conjugate GSH with ROS to prevent oxidative stress, thus acting as an antioxidant enzyme (Hayes and Pulford, 1995; Hayes and McLellan, 1999; Hayes et al., 2005; Liu et al., 2009; Deavall et al., 2012). Our results showed a significant decrease in GSTs activity in animals exposed to 50, 500ng/L of THY. A decrease was also reported in *Chironomus kiinensis* larvae after an acute exposure to phenol of 1 to 100 mg/L (Sun et al., 2019). In the study by Vieira et al. (2021), an increase in GSTs activity was reported after an exposure to 1 mg/L of THY. However, the observed increase was not due to the increase in ROS, since in the study of Vieira et al. (2021) the parameters measured (ROS, SOD, and LPO) did not report oxidative stress. We hypothesize that the phase II enzyme, GSTs, might not be the primary detoxification pathway but rather the UDP, and SULTs pathways (Thalhamer et al., 2011).

Finally, the antioxidant enzymes such CAT and GPx constitute a line of defense against these ROS. CAT transforms H_2O_2 into water; and GPx reduces H_2O_2 and hydroperoxides with the help of glutathione (GSH) (Willcox et al., 2004). In our study, CAT, an oxidative stress biomarker, had its activities significantly decreased, for animals exposed to the two highest concentrations (50, 500ng/L). An inhibition of catalase was also observed in zebrafish larvae after a 96h exposure to 42µM of thymol (Krishnan et al., 2019). Rana et al. (2008) showed a significant decrease in CAT activity after an exposure of thyme extract to rats. Also, Krych and Gebicka (2013) saw a significant inhibition in *in vitro* with flavonoids. However, there are also some studies that observed an increment in catalase activity measured in *Candida albicans* after an exposure to a range of concentrations (5 µg/mL to 20 µg/mL) (Khan et al., 2015). GPx showed no significant alterations in animals exposed to all treatments when compared to the control. In other studies, GPx activity was impaired after an administration of THY to rats (Youdim and Deans, 1999; Rana and Soni, 2008; Oliveras-López et al., 2013; Pieszka et al.,, 2013). However, the absence of alterations in the enzymatic activity cannot be considered an absence of ROS or an antioxidant effect,

since the activities of CAT and GPx do not depend on one each other (Bukowska, 2004; Oliveras-López et al., 2013).

Raskovic et al. (2015) demonstrated in albino wistar rats a general decrease in several oxidative stress biomarkers, such as CAT, GPx, and GSH, after a co-treatment of thymol in liver exposed to carbon tetrachloride. Since contradictory results were found in the literature, we propose that the differences observed in our results compared to others can be attributed to differences in the organism models (Mondet et al., 2011; Paul and Kang, 2011; Kim et al., 2017). Differences in radical scavenging are connected to the different structures resulting from the first reactions (e.g., hydroxylation). High antioxidative effects are connected to the ortho-dihydroxy groups present in phenolic compounds (Cai et al., 2006). The attack on the *ortho* position is more energy favorable, and promotes a higher antioxidant power than an attack on the *para* position (Nagoor-Meeran et al., 2017). Another possible reason for the differences observed is the compound selected for the exposure. Most of the studies uses thyme oil as reference, which for all purposes has a high content of THY (~40 %), but not only thymol (Manou et al., 1998). Other compounds, such as carvacrol and flavonoids are present in exposures to thyme oil, and can thus produce different outcomes (Manou et al., 1998; Capatina et al., 2020). Krych and Gebicka (2013) showed an inhibition of catalase activity by the presence of flavonoids, so impairments related to the exposure to thyme oils could be linked to secondary products. Additional evidence also indicate that the possible differences could be due to the different concentrations used (Nagoor-Meeran et al., 2017). In low concentrations or with a co-exposure of a substrate known to produce ROS, THY promotes an antioxidant defense (Foti, 2007). Antioxidants can act as preventive, degrading hydroperoxides and H_2O_2, through enzymes, or can act by neutralizing peroxide radicals, transferring the H ion to the ROO* (Foti, 2007). In higher concentrations, THY could inhibit the activity of such enzymes. Thymol is also not entirely metabolized by the enzymes present in this study. In humans, thymol is found in blood samples as thymol sulfate, so preferential routes of metabolism can also be a possible variable in the outcomes (Thalhamer et al., 2011; Nagoor-Meeran et al., 2017). Finally, we propose that, given our concentrations, organism model, and the purity of the tested compound, the inhibition of all biomarkers explored in this study reflects the antioxidant power at environmental relevant concentrations, and not the effect of oxidative damage due to rising levels of ROS. At this

range of concentrations, and without co-exposures that can promote oxidative stress, our results support the antioxidant properties of this compound.

7. Conclusion

According to the Environmental Protection Agency, THY is a safe compound for humans and animals, with negligible toxicity (Nagoor-Meeran et al., 2017). However, over the years, several studies have pointed out its benefits but also its possible negative effects. Since its application was already widespread, it is expected that environmental concentrations of THY will increase due to its application as a disinfection agent against SARS-CoV-2. Our study reported a decrease activity in *D. rerio* exposed to the highest concentration. We proposed that the downward trend observed could be related to an inhibition of AChE. No assumptions could be made from the thigmotaxis assay, so we could not infer anxious-like behavior. Finally, our study demonstrated that animals exposed to all concentrations produced more erratic movements. THY is known for its antioxidant properties, but also for its pro-oxidant properties. Their capacity to act as an antioxidant or as a pro-oxidant is dependent on the organism model chosen, the concentration, co-exposures, or other factors. In our study we report an inhibition of the enzymatic activity of CYP 1A1, CYP 1A2, CAT, and GSTs. This transversal reduction of enzymatic activity is in agreement with several studies (Youdim and Deans, 1999; Rana and Soni, 2008; Krych and Gebicka, 2013; Oliveras-López et al., 2013; Pieszka et al., 2013; Raskovic et al., 2015; Sun et al., 2019). However, others report an increase activity of phase I, and II enzymes, and antioxidant enzymes (Khan et al., 2015; Vieira et al., 2021). However, the increase activity of these enzymes is reported in higher concentrations of THY, and after a co-exposer to a substance known to produce ROS (Khan et al., 2015; Vieira et al., 2021). Since the compound is an antioxidant, it actively scavenges ROS, produced by the metabolism of THY, probably resulting from phase I enzymes, leading to an increase in the activity of antioxidant enzymes such as CAT or GPx. But this increase occurs only when there is an excess production of ROS. When there is no ROS production, a decrease in the activity of the antioxidant enzymes occurs. Therefore, given that the concentrations here tested are higher than those found in the environment (0.5 to 3.0 ng/L), we can conclude that the toxicity of this disinfectant is considerably lower, and therefore consistent with the

designation given by the US EPA. However, the obtained data, and the perspective of the expected increase of these compounds in aquatic environments in the following years, makes an assessment based on post-pandemic levels imperative to further clarify the metabolism and the adverse environmental effects of thymol.

8. References

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

Aftab, K., Atta-Ur-Rahman, and Usmanghani, K. (1995). Blood pressure lowering action of active principle from *Trachyspermum ammi* (L.) Sprague. *Phytomedicine 2*, 35–40. https://doi.org/10.1016/S0944-7113(11)80046-2

Ahmadifar, E., Falahatkar, B., and Akrami, R. (2011). Effects of dietary thymol-carvacrol on growth performance, hematological parameters and tissue composition of juvenile rainbow trout, *Oncorhynchus mykiss*. *Journal of Applied Ichthyology, 27*(4), 1057–1060. https://doi.org/10.1111/j.1439-0426.2011.01763.x

Anitha, R.O.Y., Adeline P.R., and Lakshmi, T. (2018). Evaluation of the cytochrome p450 inhibitory effect of thyme oleoresin from thymus vulgaris l -an in vitro study. *Asian Journal of Pharmaceutical and Clinical Research, 11*(9), 149–151. https://doi.org/10.22159/ajpcr.2018.v11i9.26759

Antolovich, M., Prenzler, P.D., Patsalides, E., McDonald, S., and Robards, K. (2002). Methods for testing antioxidant activity. *Analyst, 127*(1), 183–198. https://doi.org/10.1039/b009171p

Austgulen, L., Solheim, E., and Scheline, R. (1987). Metabolism in rats of p-cymene derivatives: carvacrol and thymol. *Pharmacology and Toxicology, 61*, 98–102.

Bayliak, M.M., Burdyliuk, N.I., and Lushchak, V.I. (2016). Effects of pH on antioxidant and prooxidant properties of common medicinal herbs. *Open Life Sciences, 11*(1), 298–307. https://doi.org/10.1515/biol-2016-0040

Bhattacharjee, S. (2019). ROS and Oxidative Stress: Origin and Implication. *Reactive Oxygen Species in Plant Biology*, 1–31.

Bonansea, R.I., Wunderlin, D.A., and Amé, M.V. (2016). Behavioral swimming effects and acetylcholinesterase activity changes in *Jenynsia multidentata* exposed to chlorpyrifos and cypermethrin individually and in mixtures. *Ecotoxicology and Environmental Safety, 129*, 311–319. https://doi.org/10.1016/j.ecoenv.2016.03.043

Bouwknecht, J.A., and Paylor, R. (2008). Pitfalls in the interpretation of genetic and pharmacological effects on anxiety-like behavior in rodents. *Behavioural Pharmacology, 19*(5–6), 385–402. https://doi.org/10.1097/FBP.0b013e32830c3658

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1–2), 248–254.

Brewer, M.S. (2011). Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Comprehensive Reviews in Food Science and Food Safety*, *10*(4), 221–247. https://doi.org/10.1111/j.1541-4337.2011.00156.x

Bukowska, B. (2004). 2,4,5-T and 2,4,5-TCP induce oxidative damage in human erythrocytes: The role of glutathione. *Cell Biology International*, *28*(7), 557–563. https://doi.org/10.1016/j.cellbi.2004.04.013

Bullangpoti, V., Mujchariyakul, W., Laksanavilat, N., and Junhirun, P. (2018). Acute toxicity of essential oil compounds (thymol and 1,8-cineole) to insectivorous guppy, *Poecilia reticulata* Peters, 1859. *Agriculture and Natural Resources*, *52*(2), 190–194. https://doi.org/10.1016/j.anres.2018.06.011

Cai, Y.Z., Sun, M., Xing, J., Luo, Q., and Corke, H. (2006). Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences*, *78*(25), 2872–2888. https://doi.org/10.1016/j.lfs.2005.11.004

Capatina, L., Todirascu-Ciornea, E., Napoli, E.M., Ruberto, G., Hritcu, L., and Dumitru, G. (2020). Thymus vulgaris essential oil protects zebrafish against cognitive dysfunction by regulating cholinergic and antioxidants systems. *Antioxidants*, *9*(11), 1–18. https://doi.org/10.3390/antiox9111083

Carayon, J.L., Téné, N., Bonnafé, E., Alayrangues, J., Hotier, L., Armengaud, C., and Treilhou, M. (2014). Thymol as an alternative to pesticides: Persistence and effects of Apilife Var on the phototactic behavior of the honeybee *Apis mellifera*. *Environmental Science and Pollution Research*, *21*(7), 4934–4939. https://doi.org/10.1007/s11356-013-2143-6

Cheah, D.M., Wright, P.F., Holdway, D., and Ahokas, J. (1995). *Octopus pallidus* cytochrome P-450: characterization and induction studies with β-naphthoflavone and Aroclor. *Aquatic Toxicology*, *33*, 201–214.

Colovic, M.B., Krstic, D.Z., Lazarevic-Pasti, T.D., Bondzic, A.M., and Vasic, V.M. (2013). Acetylcholinesterase Inhibitors: Pharmacology and Toxicology. *Current Neuropharmacology*, *11*(3), 315–335. https://doi.org/10.2174/1570159x11311030006

Cotelle, N. (2001). Role of Flavonoids in Oxidative Stress. *Current Topics in Medicinal Chemistry*, *1*(6), 569–590. https://doi.org/https://doi.org/10.2174/1568026013394750

Deavall, D.G., Martin, E.A., Horner, J.M., and Roberts, R. (2012). Drug-induced oxidative stress and toxicity. *Journal of Toxicolog*y. https://doi.org/10.1155/2012/645460

Didry, N., Dubreuil, L., and Pinkas, M. (1994). Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharmaceutica Acta Helvetiae*, *69*(1), 25–28. https://doi.org/https://doi.org/10.1016/0031-6865(94)90027-2

Dong, R.H., Fang, Z.Z.E., Zhu, L.L., Ge, G.B., Cao, Y.F., Li, X.B., and Liu, Z.Y. (2012). Identification of CYP isoforms involved in the metabolism of thymol and carvacrol in human liver microsomes (HLMs). *Pharmazie*, *67*(12), 1002–1006. https://doi.org/10.1691/ph.2012.2534

Dooley, K., and Zon, L.I. (2000). Zebrafish: A model system for the study of human disease. *Current Opinion in Genetics and Development*, *10*(3), 252–256. https://doi.org/10.1016/S0959-437X(00)00074-5

Dorta, D.J., Leite, S., De Marco, K.C., Prado, I.M.R., Rodrigues, T., Mingatto, F.E., and Curti, C. (2003). A proposed sequence of events for cadmium-induced mitochondrial impairment. *Journal of Inorganic Biochemistry*, *97*(3), 251–257. https://doi.org/10.1016/S0162-0134(03)00314-3

Ebashi, E. (1965). The sarcoplasmic reticulum and excitation-concentration coupling. *Molecular Biology of Muscular Contraction*, 197–206.

Ekstrom, G., and Ingelman-Sundberg, M. (1989). Rat liver microsomal NADPH-supported oxidase activity and lipid peroxidation dependent on ethanol-inducible cytochrome P-450 (P-450IIE1). *Biochemical Pharmacology*, *38*(8), 1313–1319.

El-Nekeety, A.A., Mohamed, S.R., Hathout, A.S., Hassan, N.S., Aly, S.E., and Abdel-Wahhab, M.A. (2011). Antioxidant properties of Thymus vulgaris oil against aflatoxin-induce oxidative stress in male rats. *Toxicon*, *57*(7–8), 984–991. https://doi.org/10.1016/j.toxicon.2011.03.021

Ferguson, L.R. (2001). Role of plant polyphenols in genomic stability. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *475*(1–2), 89–111. https://doi.org/10.1016/S0027-5107(01)00073-2

Finkielsztein, A., and Kelly, G.M. (2009). Altering PI3K-Akt signaling in zebrafish embryos affects PTEN phosphorylation and gastrulation. *Biology of the Cell, 101*(11), 661–682. https://doi.org/10.1042/bc20090034

Flohé, L., and Gunzler, W. (1984). Assays of glutathione peroxidase. *Methods in Enzymology, 105*, 114–120.

Foti M. C. (2007). Antioxidant properties of phenols. *The Journal of pharmacy and pharmacology, 59*(12), 1673–1685. https://doi.org/10.1211/jpp.59.12.0010

Habig, W.H., Pabst, M., and Jakoby, W. (1974). Glutathione S-transferases the frist enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, *249*(22), 7130–7139.

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

Hayes, J.D., Flanagan, J.U., and Jowsey, I.R. (2005). Glutathione transferases. *Annual Review of Pharmacology and Toxicology, 45*, 51–88. https://doi.org/10.1146/annurev.pharmtox.45.120403.095857

Hayes, J.D., and McLellan, L.I. (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defense against oxidative stress. *Free Radical Research, 31*(4), 273–300. https://doi.org/10.1080/10715769900300851

He, T., Li, X., Wang, X., Xu, X., Yan, X., Li, X., and She, G. (2020). Chemical composition and antioxidant potential on essential oils of *Thymus quinquecostatus* Celak. from Loess Plateau in China, regulating Nrf2/Keap1 signaling pathway in zebrafish. *Scientific Reports, 10*(1), 1–18. https://doi.org/10.1038/s41598-020-68188-8

Heil, T.P., and Lindsay, R.C. (1989). Environmental and industrial factors relating to flavor tainting of fish in the upper Wiscosin river. *Journal of Environmental Science and Health*, 527–552.

Hikasa, H., and Sokol, S.Y. (2013). Wnt signaling in vertebrate axis specification. *Cold Spring Harbor Perspectives in Biology, 5*(1). https://doi.org/10.1101/cshperspect.a007955

Holth, T.F., Nourizadeh-Lillabadi, R., Blaesbjerg, M., Grung, M., Holbech, H., Petersen, G.I., and Hylland, K. (2008). Differential gene expression and biomarkers in zebrafish (*Danio rerio*) following exposure to produced water components. *Aquatic Toxicology, 90*(4), 277–291. https://doi.org/10.1016/j.aquatox.2008.08.020

Hora, P.I., Pati, S.G., McNamara, P.J., and Arnold, W.A. (2020). Increased Use of Quaternary Ammonium Compounds during the SARS-CoV-2 Pandemic and Beyond: Consideration of Environmental Implications. *Environmental Science and Technology* Letters, *7*(9), 622–631. https://doi.org/10.1021/acs.estlett.0c00437

Jukic, M., Politeo, O., Maksimovic, M., Milos, M., and Milos, M. (2007). In Vitro Acetylcholinesterase inhibitory properties of Thymol, Carvacrol and their derivatives thymoquinone and thymohydroquinone. *Phytotherapy Research*, *21*, 259–261. https://doi.org/10.1002/ptr.2063

Khan, A., Ahmad, A., Ahmad Khan, L., Padoa, C. J., van Vuuren, S., and Manzoor, N. (2015). Effect of two monoterpene phenols on antioxidant defense system in *Candida albicans*. *Microbial Pathogenesis, 80*, 50–56. https://doi.org/10.1016/j.micpath.2015.02.004

Kim, K.M., Kim, P.K.M., Kwon, Y.G., Bai, S.K., Nam, W.D., and Kim, Y.M. (2002). Regulation of apoptosis by nitrosative stress. *Journal of Biochemistry and Molecular Biology, 35*(1), 127–133. https://doi.org/10.5483/bmbrep.2002.35.1.127

Kim, S.H., Sharma, C., and Kang, S.C. (2017). Ajowan Oil Potentiates Ros-mediated Teratogenic Effect in Zebrafish Embryos. *Journal of Essential Oil-Bearing Plants, 20*(4), 883–896. https://doi.org/10.1080/0972060X.2017.1383193

Kimmel, C.B., Ballard, W.W., Kimmer, S.R., Ullmann, B., and Schilling, T.F. (1995). Zebrafish as an alternative vertebrate model for investigating developmental toxicity - the triadimefon example. *International Journal of Molecular Sciences*, *203*, 253–310. https://doi.org/10.3390/ijms18040817

Krishnan, M., Kim, D.K., Gie K.S., and Kang, S.C. (2019). Thymol exposure mediates pro-oxidant shift by regulating Nrf2 and apoptotic events in zebrafish (*Danio rerio*) embryos. *Environmental Toxicology and Pharmacology, 65*, 1–8. https://doi.org/10.1016/j.etap.2018.11.001

Kroemer, G. (1999). Mitochondrial control of apoptosis: An overview. *Biochemical Society Symposium, 66*, 1–15. https://doi.org/10.1042/bss0660001

Krych, J., and Gebicka, L. (2013). Catalase is inhibited by flavonoids. *International Journal of Biological Macromolecules*, *58*, 148–153. https://doi.org/10.1016/j.ijbiomac.2013.03.070

Liu, G.J., Nagarajah, R., Banati, R.B., and Bennett, M.R. (2009). Glutamate induces directed chemotaxis of microglia. *European Journal of Neuroscience, 29*(6), 1108–1118. https://doi.org/10.1111/j.1460-9568.2009.06659.x

Llana-Ruiz-Cabello, M., Gutiérrez-Praena, D., Puerto, M., Pichardo, S., Jos, Á., and Cameán, A.M. (2015). In vitro pro-oxidant/antioxidant role of carvacrol, thymol and their mixture in the intestinal Caco-2 cell line. *Toxicology in Vitro, 29*(4), 647–656. https://doi.org/10.1016/j.tiv.2015.02.006

Lv, R., and Chen, Z. (2017). Thymol inhibits cell migration and invasion by downregulating the activation of PI3K/AKT and ERK pathways in human colon cancer cells. *Tropical Journal of Pharmaceutical Research, 16*(12), 2895–2901. https://doi.org/10.4314/tjpr.v16i12.13

Magyar, J., Szentandrassy, N., Banyasz, T., Fulop, L., Varro, A., and Nanasi, P. P. (2002). Effects of thymol on calcium and potassium currents in canine and human ventricular cardiomyocytes. B*ritish Journal of Pharmacology, 136*, 330–338.https://doi.org/10.1038/sj.bjp.0704718

Magyar, J., Szentandrassy, N., Banyasz, T., Fulop, L., Varro, A., and Nanasi, P. P. (2004). Effects of terpenoid phenol derivatives on calcium current in canine and human ventricular cardiomyocytes. *European Journal of Pharmacology, 487*, 29–36. https://doi.org/10.1016/j.ejphar.2004.01.011

Mahmoud, A.L. (1994). Antifungal action and antiaflatoxigenic properties of some essential oil constituents. *Letters in Applied Microbiology*. https://doi.org/https://doi.org/10.1111/j.1472-765X.1994.tb00918.x

Manou, I., Bouillard, L., Devleeschouwer, M.J., and Barel, A.O. (1998). Evaluation of the preservative properties of *Thymus vulgaris* essential oil in a topically applied formulation under a challenge test. *Journal of Applied Microbiology, 84*, 368–376. https://doi.org/10.3166/phyto-2018-0067

Misaka, S., Kawabe, K., Onoue, S., Werba, J.P., Giroli, M., Watanabe, H., and Yamada, S. (2013). Green tea extract affects the cytochrome P450 3A activity and

pharmacokinetics of simvastatin in rats. *Drug Metabolism and Pharmacokinetics*, *28*(6), 514–518. https://doi.org/10.2133/dmpk.DMPK-13-NT-006

Mondet, F., Goodwin, M., and Mercer, A. (2011). Age-related changes in the behavioral response of honeybees to Apiguard ®, a thymol-based treatment used to control the mite *Varroa destructor*. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *197*(11), 1055–1062. https://doi.org/10.1007/s00359-011-0666-1

Murrell, G.A.C., Francis, M.J.O., and Bromley, L. (1990). Modulation of fibroblast proliferation by oxygen free radicals. *Biochemical Journal*, *265*(3), 659–665. https://doi.org/https://doi.org/10.1042/bj2650659

Nagoor-Meeran, M.F., Jagadeesh, G.S., and Selvaraj, P. (2015). Thymol attenuates inflammation in isoproterenol induced myocardial infarcted rats by inhibiting the release of lysosomal enzymes and downregulating the expressions of proinflammatory cytokines. *European Journal of Pharmacology*, *754*, 153–161. https://doi.org/10.1016/j.ejphar.2015.02.028

Nagoor-Meeran, M.F., Javed, H., Taee, H. Al, Azimullah, S., and Ojha, S.K. (2017). Pharmacological properties and molecular mechanisms of thymol: Prospects for its therapeutic potential and pharmaceutical development. Frontiers in Pharmacology, 8, 1–34. https://doi.org/10.3389/fphar.2017.00380

Nagoor-Meeran, M.F., and Prince, P.S.M. (2012). Protective effects of thymol on altered plasma lipid peroxidation and nonenzymic antioxidants in isoproterenol-induced myocardial infarcted rats. *Journal of Biochemical and Molecular Toxicology*, *26*(9), 368–373. https://doi.org/10.1002/jbt.21431

Nakada, N., Kiri, K., Shinohara, H., Harada, A., Kuroda, K., Takizawa, S., and Takada, H. (2008). Evaluation of pharmaceuticals and personal care products as water-soluble molecular markers of sewage. *Environmental Science and Technology*, *42*(17), 6347–6353. https://doi.org/10.1021/es7030856

OCDE. (2013). *OECD guidelines for testing of chemicals*, 509–511. https://doi.org/10.3109/9781841848570-66

Oliveras-López, M., Molina, J.J., Mir, M., Rey, E.F., Martín, F., and De la Serrana, H. (2013). Extra virgin olive oil (EVOO) consumption and antioxidant status in healthy institutionalized elderly humans. *Archives of Gerontology and Geriatrics*, *57*(2), 234–242.

Palozza, P. (1998). Prooxidant actions of carotenoids in biologic systems. *Nutrition Reviews, 56*(9), 257–265. https://doi.org/10.1111/j.1753-4887.1998.tb01762.x

Paul, S., and Kang, S.C. (2011). In vitro determination of the contraceptive spermicidal activity of essential oil of *Trachyspermum ammi* (L.) Sprague ex Turrill fruits. *New Biotechnology, 28*(6), 684–690. https://doi.org/10.1016/j.nbt.2011.02.008

Pieszka, M., Tombarkiewicz, B., Roman, A., Migdal, W., and Niedziólka, J. (2013). Effect of bioactive substances found in rapeseed, raspberry and strawberry seed oils on blood lipid profile and selected parameters of oxidative status in rats. *Environmental Toxicology and Pharmacology*, *36*(3), 1055–1062. https://doi.org/https://doi.org/10.1016/j.etap.2013.09.007

Placha, I., Bacova, K., and Plachy, L. (2022). Current Knowledge on the Bioavailability of Thymol as a Feed Additive in Humans and Animals with a Focus on Rabbit Metabolic Processes. *Animals: an open access journal from MDPI, 12*(9), 1131. https://doi.org/10.3390/ani12091131

Podmore, I.D., Griffiths, H.R., Herbert, K.E., Mistry, N., Mistry, P., and Lunec, J. (1998). Vitamin C exhibits pro-oxidant properties. *Nature, 392*(6676), 559. https://doi.org/10.1038/33308

Poljsak, B., Šuput, D., and Milisav, I. (2013). Achieving the balance between ROS and antioxidants: When to use the synthetic antioxidants. *Oxidative Medicine and Cellular Longevity*. https://doi.org/10.1155/2013/95679

Robert, S., Thomas, C., Chung S.Y., Helmut, T., Karl, P., and Franz, O. (1993). Benzene metabolism by reconstituted cytochromes p450 2b1 and 2e1 and its modulation by cytochrome b_5, microsomal epoxide hydrolase, and glutathione transferases: Evidence for an important role of microsomal epoxide hydrolase in the formation of hydroquinone. *Toxicology and Applied Pharmacology, 20*(2), 172, 181.
https://doi.org/10.1006/taap.1993.1185

Pullaguri, N., Nema, S., Bhargava, Y., and Bhargava, A. (2020). Triclosan alters adult zebrafish behavior and targets acetylcholinesterase activity and expression. *Environmental Toxicology and Pharmacology, 75*, 103311. https://doi.org/10.1016/j.etap.2019.103311

Rana, P., and Soni, G. (2008). Antioxidant potential of thyme extract: Alleviation of N- nitrosodiethylamine-induced oxidative stress. *Human and Experimental Toxicology, 27*(3), 215–221. https://doi.org/10.1177/0960327108088970

Rašković, A., Pavlović, N., Kvrgić, M., Sudji, J., Mitić, G., Čapo, I., and Mikov, M. (2015). Effects of pharmaceutical formulations containing thyme on carbon tetrachloride-induced liver injury in rats. *BMC Complementary and Alternative Medicine, 15*(1), 442. https://doi.org/10.1186/s12906-015-0966-z

Sah, P., and Davies, P. (2000). Calcium-activated potassium currents in mammalian neurons. *Clinical and Experimental Pharmacology and Physiology, 27*(9), 657–663. https://doi.org/10.1046/j.1440-1681.2000.03317.x

Schier, A.F. (2001). Axis formation and patterning in zebrafish. *Current Opinion in Genetics and Development, 11*(4), 393–404. https://doi.org/10.1016/S0959-437X(00)00209-4

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., and Champagne, D.L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioral Brain Research, 228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Shahidi, F., Janitha, P., and Wanasundara, P.D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*(1), 67–103. https://doi.org/https://doi.org/10.1080/10408399209527581

Shalaby, S., and Horwitz, B.A. (2015). Plant phenolic compounds and oxidative stress: integrated signals in fungal–plant interactions. *Current Genetics, 61*(3), 347–357. https://doi.org/10.1007/s00294-014-0458-6

Shapiro, S., Meier, A., and Guggenheim, B. (1994). The antimicrobial activity of essential oils and essential oil components towards oral bacteria. *Molecular Oral Microbiology*. https://doi.org/https://doi.org/10.1111/j.1399-302X.1994.tb00059.x

Shin, J.T., and Fishman, M.C. (2002). From zebrafish to human: Modular medical models. *Annual Review of Genomics and Human Genetics, 3*(24), 311–340. https://doi.org/10.1146/annurev.genom.3.031402.131506

Stiborová, M., Suchá, V., Miksanová, M., Páca, J., and Páca, J. (2003). Hydroxylation of phenol to catechol by *Candida tropicalis*: involvement of cytochrome P450. *General physiology and biophysics*, *22*(2), 167–179.

Sun, L., Wang, J., Li, X., and Cao, C. (2019). Effects of phenol on glutathione S-transferase expression and enzyme activity in *Chironomus kiiensis* larvae. *Ecotoxicology, 28*(7), 754–762. https://doi.org/10.1007/s10646-019-02071-9

Szentandrássy, N., Szentesi, P., Magyar, J., Nánási, P., and Csernoch, L. (2003). Effect of thymol on kinetic properties of Ca^{2+} and K^{+} currents in rat skeletal muscle. *BMC Pharmacology, 3*, 1–8. https://doi.org/10.1186/1471-2210-3-9

Thalhamer, B., Buchberger, W., and Waser, M. (2011). Identification of thymol phase I metabolites in human urine by headspace sorptive extraction combined with thermal desorption and gas chromatography mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis, 56*(1), 64–69. https://doi.org/10.1016/j.jpba.2011.04.014

Tierney, K.B., Sampson, J.L., Ross, P.S., Sekela, M.A., and Kennedy, C.J. (2008). Salmon olfaction is impaired by an environmentally realistic pesticide mixture. *Environmental Science and Technology, 42*(13), 4996–5001. https://doi.org/10.1021/es800240u

United Sates Environmental Protection Agency [US EPA]. (2022). List N: Disinfectants for Use Against SARS-CoV-2. Retrieved January 20, 2022, from https://www.epa.gov/%0Apesticide-registration/list-n-disinfectants-use-against-sars-cov-2

Vieira, R., Venâncio, C., and Félix, L. (2021). Teratogenic, oxidative stress and behavioral outcomes of three fungicides of natural origin (*Equisetum arvense, Mimosa tenuiflora, thymol*) on zebrafish (*Danio rerio*). *Toxics, 9*(1), 1–15. https://doi.org/10.3390/toxics9010008

Viran, R., Erkoç, F. Ü., Polat, H., and Koçak, O. (2003). Investigation of acute toxicity of deltamethrin on guppies (Poecilia reticulata). *Ecotoxicology and Environmental Safety, 55*(1), 82–85. https://doi.org/10.1016/S0147-6513(02)00096-9

Willcox, J.K., Ash, S.L., and Catignani, G.L. (2004). Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition, 44*(4), 275–295. https://doi.org/DOI: 10.1080/10408690490468489

Wojdyło, A., Oszmiański, J., and Czemerys, R. (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chemistry, 105*(3), 940–949. https://doi.org/10.1016/j.foodchem.2007.04.038

Yanishlieva, N.V., Marinova, E.M., Gordon, M.H., and Raneva, V.G. (1999). Antioxidant activity and mechanism of action of thymol and carvacrol in two lipid systems. *Food* Chemistry, 64(1), 59–66. https://doi.org/10.1016/S0308-8146(98)00086-7

Yilmaz, E., Çoban, D., Kirim, B., Guller, M., and Ozturk, S. (2011). The effects of the Carvacrol and Thymol addition to *Pangasius hypophthalmus* feeds in different amount on the growth performance.

Youdim, K., and Deans, S. (1999). Dietary supplementation of thyme (*Thymus vulgaris L.*) essential oil during the lifetime of the rat: its effects on the antioxidant status in liver, kidney and heart tissues. *Mechanisms of Ageing and Development.* https://doi.org/10.1016/S0047-6374(99)00033-0

Yu, J.S.L., and Cui, W. (2016). Proliferation, survival and metabolism: The role of PI3K/AKT/ mTOR signaling in pluripotency and cell fate determination. *Development, 143*(17), 3050–3060. https://doi.org/10.1242/dev.137075

Zarzuelo, A., and Crespo, E. (2002). The medicinal and non-medicinal uses of thyme. In Thyme: The Genus Thymus. *Medicinal and Aromatic Plants- Industrial profiles*, 263–292

Zehetner, P., Höferl, M., and Buchbauer, G. (2019). Essential oil components and cytochrome P450 enzymes: a review. *Flavour and Fragrance Journal, 34*(4), 223–240. https://doi.org/10.1002/ffj.3496

Zhang, B., Chen, X., Pan, R., Xu, T., Zhao, J., Huang, W., and Yin, D. (2017). Effects of three different embryonic exposure modes of 2, 2', 4, 4'-tetrabromodiphenyl ether on the path angle and social activity of zebrafish larvae. *Chemosphere, 169,* 542–549. https://doi.org/10.1016/j.chemosphere.2016.11.098

Chapter V | Final remarks

The present work was intended to evaluate the possible effects arising from the increased use of disinfectants during the pandemic, and in the years to come. Given the extensive use of disinfectants as a preventive measure during the pandemic and considering that the market for disinfectants will continue to grow, it is imperative that the effects of these compounds are understood in the various species that they may affect when in the environment. Several news reports over the past two years have highlighted the adverse effects on birds, mammals, and aquatic organisms arising from the excessive loads of chemical agents used to prevent the dissemination of the SARS-CoV-2 virus. Therefore, we intended to evaluate the possible effects of three different disinfectants on three main endpoints: embryonic, behavioral, and biochemical. The results from the Fish Embryo Acute Toxicity Test (FET) showed possible malformations, delayed hatching, and mortality. Regarding the behavioral analysis, in terms of the three parameters measured, we observed an increment in the total distance traveled in animals exposed to Benzalkonium Chloride (BAC), and sodium dodecylbenzene sulfonic acid (SDBS), and a decrease in animals exposed to Thymol (THY). Concerning the thigmotactic behavior, only animals exposed to BAC reported a more anxious behavior. Finally, considering the number of angles performed, a general increase in class 1 angles was observed in animals exposed to BAC, SDBS, and THY. Finally, metabolic biomarkers were also evaluated, and evidenced a general decrease of the enzymatic activity of phase I and II enzymes. Our results reported an increase in CYP 1A1 activity in animals exposed to BAC. In animals exposed to SDBS and THY a decrease in CYP 1A1 activity was observed. Finally, the CYP 1A2 enzyme showed an inhibition in every animal exposed to all compounds. Regarding the enzymes of antioxidant defense, our study reported an increase in catalase (CAT) activity in animals exposed to BAC, and to SDBS. A decrease was also observed in CAT activity in animals exposed to THY, and in the activity of glutathione peroxidase (GPx) in organisms exposed to the three compounds. The CYP 1A1 and CYP 1A2 isoenzymes of the cytochrome P450 complex (CYP 450) were evaluated to assess the possible effects on phase I enzymes. We conclude that the metabolism of BAC was carried out by CYP 1A1 in the organisms exposed to BAC, while for the rest of the exposures no differences in the metabolism of the compounds by these phase I enzymes were observed, possibly due to specificity of the chosen isoenzymes. Phase II enzymes, namely glutathione-S-transferases (GSTs) reported a decreasing enzymatic activity in all organisms exposed to THY.

The first disinfectant evaluated was BAC, a cationic surfactant, given its widespread production, application, and expected growth. In our study we observed an increase in pericardial edema, and tail deformities. Our study also reported a high mortality rate in animals exposed to 2.5 mg/L BAC at 96h. Regarding behavioral parameters, we observed an increase in total activity in animals exposed to 2.5 mg/L of BAC. Also, an increase in animals exposed to the same concentration was observed in the thigmotaxis test, suggesting an increase in anxiety. At the same concentration, our study also reported an increase in the number of class 1 angles, translating into a higher number of erratic movements. This transversal increase might be connected to a disruption of the ionic balance of the membrane, and a possible inhibition of acetylcholinesterase (AChE). Finally, regarding the metabolic biomarkers evaluated, we observed an increase in CYP 1A1 enzyme activity, suggesting that BAC may be metabolized by this enzyme. However, CYP 1A2 showed a decrease in activity, suggesting a difference in the metabolism of this compound by zebrafish. This observed increase in the CYP 1A1 isoenzyme and decrease in the CYP 1A2 isoenzyme may be due to the compounds preference for one isoenzyme over the other, given the structural and activity differences of the two enzymes. The metabolism of BAC leads to the possible production of hydrogen peroxide (H_2O_2), since we observed an increase in CAT. However, the activity of GPx and GSTs was inhibited, possibly by increased reactive oxygen species (ROS).

The second compound studied was SDBS, an anionic surfactant. Like BAC, SDBS was selected due to its low production cost, wide application, and large-scale production. Our study showed a higher mortality in animals exposed to 5 mg/L of SDBS, after 96h. Given the mechanisms of action of SDBS on the membranes, possible alterations in the phospholipid membrane of the yolk sac might have caused a higher mortality. Regarding behavioral parameters, namely total activity, we found an increase in total activity in animals exposed to 1 mg/L, and a decrease in animals exposed to 5 mg/L. We propose that this increase, and subsequent decrease could be a result from an AChE inhibition (as reported in other studies), resulting in a larger amount of acetylcholine (ACh) remaining in the synaptic cleft, leading to hyperstimulation of the postsynaptic nerves. This hyperstimulation may result in increased swimming activity, and consequently also in paralysis. No changes in thigmotactic behavior were observed, so we were unable to conclude whether the compound, in the tested concentrations, promoted anxiety-like behaviors in zebrafish. Finally, our study showed a decrease in CYP 1A1 and CYP 1A2 enzymatic activities. Despite these results,

previous studies have shown that SDBS can be metabolized by CYP 450, and our study reported an increase in CAT activity, presumably due to the increment on H_2O_2 produced CYP 450 during the SDBS metabolism. Given this evidence, we suggest that the decrease in the activities of these enzymes may be due to preference for other isoenzymes of this complex, or by the formation of toxic metabolites that inhibit the enzyme itself. Other studies indicate the possible metabolism via CYP 2D6 and the CYP 4 family. Therefore, the lack of increased activity of this enzyme in our study, pointing to its metabolism, may indicate that SDBS could be metabolized by other isoenzymes, leading to a decrease in the activity of the enzymes explored in this work. Although in our study we found no evidence of metabolism of SDBS by the selected phase I enzymes, the activity of the enzyme CAT was increased. This response reflects an activation of the antioxidant defense, so we can conclude that H_2O_2 results from the metabolism of SDBS. Regarding GPx and GSTs activity, an inhibition was observed possibly due to increased ROS resulting from the possible metabolism of the compound by phase I enzymes.

The last compound was selected considering its natural occurrence in plants. These compounds, being natural, are classified as environmentally friendly disinfectants, but many studies have brought to light many of the adverse effects that these compounds can exert. Thus, thymol (THY) was the compound selected given its longstanding use, origin, wide distribution, and application. In our study, we observed a higher mortality in all organisms after 96h of exposure. We suggest that the higher mortality rate could be due to the high incidence of malformations, namely pericardial edema, observed at 72h. A decrease in total activity was observed in animals exposed at the highest concentrations. This decrease was also reported in studies from the literature, being associated with an inhibition of AChE. As for the thigmotaxis test, we cannot draw any conclusion from our data, since there were no significant differences in the time spent in the peripheral zone parameter. Regarding the evaluation of the angles made during the test, we found an increase in class 1 angles, in animals exposed to all concentrations, translating into a more erratic behavior. In the enzymatic evaluation, all the enzymes evaluated exhibited a decrease, except GPx activity. Given the widely known antioxidant properties of this compound, we propose that this enzymatic decrease of phase I, phase II and antioxidant defense enzymes, might be explained by scavenging of free radicals promoted by THY. Thus, we can infer that THY promotes a beneficial effect in animals at the concentrations tested. However, since the increased use of

THY, this beneficial antioxidant effect can produce a prooxidant action, leading to negative effects.

In conclusion, our study highlighted a number of potential deleterious effects upon exposure of our model organism, zebrafish, to three different disinfectants to relevant concentrations in different aquatic matrices (effluent, municipal wastewater and rivers). In the case of BAC, alterations were observed mainly in animals exposed to 0.5 mg/L, and 2.5 mg/L, concentrations already reported in the environment before the pandemic. Also, in the case of SDBS, our study shows changes in all animals exposed to 0.2, 1, and 5 mg/L, concentrations also already reported before the pandemic. Regarding THY, the concentrations tested were slightly above the values found in the environment in 2004/2005, but well below the concentrations tested in previous studies, thus increasing the relevance of the obtained data. Thus, we conclude that the increased use of disinfectants given the pandemic scenario, and the predicted growth of these compounds, may lead to negative effects on several parameters (embryonic, behavioral, and metabolic), in several different organisms. Therefore, and keeping in mind that the concentrations tested in our study were concentrations already reported in the environment before the pandemic, the continuous evaluation of the potential effects of disinfectants should be considered.