Universidade de Aveiro Departamento de Biologia
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Niedja
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Efeitos crónicos de carbamazepina em *Danio rerio:* uma avaliação multi-paramétrica

Chronic effects of carbamazepine on *Danio rerio*: a multi-parametric evaluation

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Paula Inês Borralho Domingues, Bolseira de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e sob coorientação do Doutor Marcelino Miguel Oliveira, Bolseiro de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e do Doutor Cesar Koppe Grisólia, Professor Titular do Departamento de Biologia da Universidade de Brasília.

À minha mãe com todo amor, pela força e garra que representa e pelos ensinamentos que me disponibilizou ao longo da vida.

O júri

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Agradecimentos

Agradeço a Deus porque a pesar de parecer sorte acredito muito que foi ele quem me deu excelentes condições de trabalho em um momento não favorável da minha vida. Foi melhor que ganhar no euro milhões ou mega-sena, pois recebi a dádiva de ter maravilhosos seres humanos sob o pseudónimo de: orientadores (obrigada Universidade de Aveiro), coorientadores, um oficial e outro extraoficial (obrigada Universidade de Brasília), e diversos colegas durante a elaboração deste trabalho, assim sem mais delongas vamos aos nomes 😊.

Agradeço primeiramente à minha orientadora Dra. Inês Domingues, que com muita sabedoria, dedicação e paciência tem conduzido este trabalho. Agradeço por todo o seu arsenal de conhecimento que inspira confiança. Agradeço porque a Dra. Inês esteve sempre disponível e com boa vontade para me corrigir os erros e me ensinar. Inês, agradeço porque tu és um grande ser humano, estejas certa de que, além do reconhecimento da tua dedicação, em termos científicos, tenho muitíssimo respeito e admiração por si.

Agradeço ao Dr. Miguel Oliveira que sempre demonstrou empenho, força de vontade e disponibilidade em me apoiar neste trabalho, muitas vezes fazendo críticas e me conduzindo a reflexões científicas. Dr. Miguel, obrigada por todo o cuidado e dedicação que desprendestes, foste incansável como orientador e quando eu mesma desacreditavas tu estavas lá 😊. Por fim, agradeço a Dra. Inês e ao Dr. Miguel em conjunto, que não mediram esforços em me ajudar quando sérios problemas de saúde e pessoais atingiram a minha família e mesmo distante mantiveram os olhos em mim com reuniões periódicas, acompanhando de perto aquilo que eu estava a fazer, o meu muitíssimo obrigada aos dois por todo o acompanhamento e, por nunca me terem deixado caminhar sozinha nesta etapa.

O meu muito obrigada vai também ao meu coorientador Dr. César Grisólia que com muito carinho me recebeu no seu laboratório, me dando plena liberdade para trabalhar e aos poucos me permitiu estar inserida no seu grupo de trabalho. Por muitas vezes ouviu "professor preciso de kit disso ou daquilo" e nunca reclamou, muito pelo contrário, foi mais que solicito. Professor, agradeço por todas as vezes que, juntamente com outros colegas, o senhor nos levou à estação de ónibus/autocarro quando os experimentos terminavam mais tarde que o esperado, o meu muito obrigada sei que não cabia ao senhor tal função foi simplesmente zelo da sua parte para connosco, sinceramente torço por mais parcerias assim.

Ao meu coorientador extraoficial Dr. Rhaul Oliveira que de início estava desconfiado e sem muitas alegrias em me ver, mas que com o tempo deu o seu melhor e mostrou que quando está envolvido em algo não oferece nada além do seu melhor. Dr. Rhaul obrigada pelo apoio científico que foi indispensável para este trabalho. Rhaulzito, meu muito obrigada por todas as vezes que fostes para a bancada pôr a mão na massa juntamente comigo, pelos lanches que me oferecestes todas as vezes que eu era parte da experiência, pelas nossas aventuras até a estação de ónibus/autocarro após longas horas de trabalho quase madrugada e, pela companhia agradabilíssima que me oferecestes, muitíssimo obrigada.

Sinceramente, espero que hajam incontáveis Inês, Miguel, Cesar e Rhauls na vida académica distribuídos mundo a fora.

Agradeço a minha mãe, que é simplesmente a melhor mãe do mundo, agradeço por sua parceria e por seus incentivos em todos os sentidos, pois me permitiu cumprir mais esta etapa académica, te desejo saúde e nada além. Agradeço à minha irmã, por ser o meu apoio, afinal "a faxina deste fim de semana é tua" (taurina reage 😊).

Agradeço aos colegas de laboratório porque sempre que precisei contei convosco, só aos melhores brasileiros: Vitória Perillo obrigada pelo apoio nas trocas de água e pelas vezes que me apoiaste na coleta de ovos, por comprares o nosso almoço e pela companhia, estava escrito e o universo conspirou nos dando o 30/09, portanto és a minha libriana preferida, depois de mim mesma; Diego, Carol, Bryan, Pedro, obrigada pelo apoio inicial na leitura dos FETs foi de suma importância, Juliana (técnica do laboratório) obrigada por me ajudares com os peixes, por me ouvir sempre que precisei e pelas orações; Alisson, obrigada pela tentativa do cometa (teste que fracassam também contam); Muriel, Naty e Ana meu muito obrigada pela companhia. As melhores colegas portuguesas: Sara Esteves obrigada por todos os momentos partilhados na biblioteca, cafés e jantares, és chata, mas te perdoo 😊 afinal foste minha dupla estes 2 anos. Obrigada às três mais giras: Beatriz Cruz, Marta Fraga e Maria Inês (em ordem alfabética), este último período se tornou mais leve na vossa companhia, vocês fazem parte da surpresa boa deste mestrado. E não esquecendo, o ser mais fofo do Dbio, Ângela Barreto, obrigada pelo apoio que sempre me oferecestes, serás uma excelente orientadora. Obrigada a Daniela Correia, Joana Santos e Rita Almeida pela companhia no curto período de laboratório.

Palavras-chave

Carbamazepina, peixe-zebra, contaminantes emergentes, ecossistema aquático, toxicidade crónica, efeitos biológicos, comportamento alimentar, marcadores bioquímicos, genotoxicidade, stress oxidativo, reprodução, propranolol, efeitos transgeracionais.

Resumo

Os fármacos são atualmente considerados contaminantes ambientais emergentes, devido à sua constante deteção nos ecossistemas aquáticos, consequência do aumento na sua produção, diversificação e consumo. A carbamazepina (Cbz) é um fármaco humano utilizado para tratamento de epilepsia, distúrbios bipolares e neuralgia trigeminal estando entre os fármacos mais prescritos no mundo e sendo considerado um marcador de poluição antropogénica. Para uma correta avaliação de risco ambiental é essencial avaliar os efeitos a longo termo dos compostos em vários níveis de organização biológica. Assim, os objetivos deste trabalho foram: i) avaliar a toxicidade crónica da Cbz para o peixe-zebra (*Danio rerio*) adulto, numa gama de concentrações que inclui uma concentração ambientalmente relevante (10 $\mu g.L^{-1}$) e uma concentração correspondente a 5% do valor de concentração letal mediana (CL_{50}) (10000 $\mu g.L^{-1}$). Foram estudados efeitos no crescimento, comportamento alimentar, reprodução (número total de ovos e viabilidade), defesas antioxidantes (atividade da catalase - CAT e Glutationa *S*- transferase - GST), neurotransmissão (atividade da acetilcolinesterase - AChE), atividade metabólica (atividade da lactato desidrogenase - LDH) e anomalias nucleares eritrocíticas nos adultos expostos a Cbz; ii) avaliar a toxicidade aguda do propranolol (Prop) (um fármaco cardiovascular utilizado no controlo da hipertensão, angina pectoris e arritmia após enfarte agudo do miocárdio) para adulto *D. rerio* após previa exposição a Cbz, iii) avaliar alterações na suscetibilidade de embriões decorrente da exposição aguda ao Prop após exposição parental a Cbz. A exposição crónica do peixe-zebra a Cbz induziu alterações no tempo total de ingestão de alimento nas duas concentrações testadas e induziu inibição da CAT no fígado e brânquias (10 e 10000 $\mu g.L^{-1}$). A GST apresentou uma resposta dependente do órgão, com um aumento de atividade nas brânquias (10 e 10000 $\mu g.L^{-1}$) e fígado (10000 $\mu g.L^{-1}$) e diminuição no intestino (10000 $\mu g.L^{-1}$). A atividade da AChE aumentou na cabeça (10 e 10000 $\mu g.L^{-1}$) e músculo (10000 $\mu g.L^{-1}$), enquanto a atividade da LDH apresentou-se aumentada no fígado (10000 $\mu g.L^{-1}$) e diminuída no músculo e brânquias na concentração de Cbz mais elevada (10000 $\mu g.L^{-1}$). Em termos reprodutivos, a Cbz (10 e 10000 $\mu g.L^{-1}$) diminuiu o número de ovos viáveis produzidos por peixe-zebra. A prévia exposição de adulto *D. rerio* a baixas concentrações de Cbz (0 e 10 $\mu g.L^{-1}$) induziu 100% de mortalidade quando os organismos foram expostos ao Prop (1000 e 5000 $\mu g.L^{-1}$) enquanto, organismos expostos a maior concentração de Cbz (10000 $\mu g.L^{-1}$) apresentaram capacidade para tolerar o Prop. A exposição crónica a Cbz não teve no entanto efeitos significativos na taxa de crescimento dos organismos, não tendo igualmente sido detectadas anomalias nucleares eritrocíticas, indicadores de cito e genotoxicidade. Os descendentes não demonstraram alterações na susceptibilidade ao Prop, quando comparados com os organismos controlo. De uma forma geral, os dados obtidos neste trabalho sugerem que a exposição crónica a baixas concentrações de Cbz afeta o comportamento e processos bioquímicos no peixe-zebra com possíveis consequências a nível da reprodução.

Keywords

Carbamazepine, zebrafish, emerging contaminants, aquatic ecosystem, chronic toxicity, biological effects, feeding behavior, biochemical markers, genotoxicity, oxidative stress, reproduction, propranolol, transgenerational effects.

Abstract

Pharmaceuticals are emerging environmental contaminants due to their constant detection into aquatic ecosystems, as a response to the increase in the rate of production, high diversity, and high consumption. Carbamazepine (Cbz) is used to epilepsy treatment, bipolar disorders, trigeminal neuralgia, being among the most prescribed drugs in the world and is considered a marker of anthropogenic pollution. For a correct environmental risk assessment, it is essential to evaluate the long-term effects of the compounds in various levels of biological organization. The objectives of this study were: i) evaluate the chronic toxicity (63 days) of Cbz for adult *Danio rerio* at an environmentally relevant concentration (10 $\mu g.L^{-1}$) and at a concentration close to 5% of the median lethal concentration (LC_{50}) value (10000 $\mu g.L^{-1}$). Effects were studied on growth, feeding behavior, reproduction (total number of eggs and viability), antioxidant defences (Catalase - CAT and Glutathione-S-Transferase - GST), neurotransmission (acetylcholinesterase activity – AChE), metabolic activity (lactate dehydrogenase - LDH) and nuclear abnormalities in adults exposed to Cbz; ii) to evaluate the acute toxicity of propranolol (Prop) (a cardiovascular pharmaceutical used to hypertension control, angina pectoris and arrhythmia after acute myocardial infarction) for adult *D. rerio* after chronic exposure to Cbz, iii) to evaluate changes in offspring susceptibility to acute exposure to Prop after parental exposure to Cbz and, in offspring. Chronic exposure of zebrafish to Cbz induced changes in total time for food intake at the two concentrations tested. Cbz exposure induced changes in enzymatic activity: CAT was inhibited in the liver and gills (10 and 10000 $\mu g.L^{-1}$); GST presented an organ-dependent response with increased activity in the gills (10 and 10000 $\mu g.L^{-1}$) and in the liver (10000 $\mu g.L^{-1}$) and a decrease in enzyme activity in the intestine (10000 $\mu g.L^{-1}$); the activity of AChE was increased in head (10 and 10000 $\mu g.L^{-1}$) and muscle (10000 $\mu g.L^{-1}$); while LDH showed increased activity in the liver and decreased in muscle and gills, these effects were observed in the highest concentration of Cbz (10000 $\mu g.L^{-1}$). The number of eggs produced did not suffer changes; however, the number of viable eggs produced by zebrafish, exposed to Cbz was reduced (10 and 10000 $\mu g.L^{-1}$). The previous exposure of adult *D. rerio* to low concentrations of Cbz (0 and 10 $\mu g.L^{-1}$) induced mortality of 100% when these organisms were exposed to Prop (1000 and 5000 $\mu g.L^{-1}$) while organisms exposed to a higher concentration of Cbz (10000 µg.L-1) were able to tolerate the Prop.The chronic exposure to Cbz did not induced significant effects in growth rate of the organisms and no erythrocytic nuclear abnormalities, cito and genotoxicity indicators were detected. The offspring did not show changes in Prop susceptibility, when compared with the control organisms. Overall, the data obtained in this work suggest that chronic exposure to low concentration of Cbz affects feeding behaviour and biochemical processes in zebrafish with possible consequences at reproduction level.

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List of abbreviations and acronyms

AChE – acetylcholinesterase

ANOVA – analysis of variance

Cbz – carbamazepine

Ca^{+2} – calcium ion

CAT – catalase

DDT – dichlorodiphenyltrichloroethane

DTNB – 5,5–dithiobis–2–nitrobenzoic acid

ERA – environmental risk assessment

EMEA – European medicines agency

EC_{50} – median effect concentration

EROD – ethoxyresorufin–O–deethylase

FET – Fish Embryo Acute Toxicity

g - grams

GPx – glutathione peroxidase

GR – glutathione reductase

GST – glutathione–S–transferase

h – hours

Hpf – hours post fertilization

K^+ – potassium ion

LC_{50} – median lethal concentration

LPO – lipid peroxidation

LDH – lactate dehydrogenase

LOEC – lowest observed effect concentration

M – mol

mM – millimolar

min – minute (s)

mL – milliliters

MN – micronucleus

$mg.L^{-1}$ – milligrams per liter

NA – nuclear abnormalities

$ng.L^{-1}$ – nanograms per liter

nm – nanometer

Na^+ – sodium ion

NADH – nicotinamide adenine dinucleotide (reduced)

NOEC – no observed effect concentration

OECD – Organization for Economic Co–operation and Development

PEC – predicted environmental concentration

PBS – phosphate–buffered saline

PE – stands for parental exposure

PNEC – predictive no effect concentration

Prop – propranolol

PMS – post – mitochondrial supernatant

r- specific growth rate

3Rs – refinement, reduction and, replacement

ROS – reactive oxygen species

SOD – superoxide dismutase

Sec – seconds

U – amount of enzyme that catalyzes the reaction of 1 nanomoles of substrate per minute per milligrams of protein

WWTP – wastewater treatment plant

$\mu g.L^{-1}$ – micrograms per liter

µL- microliter

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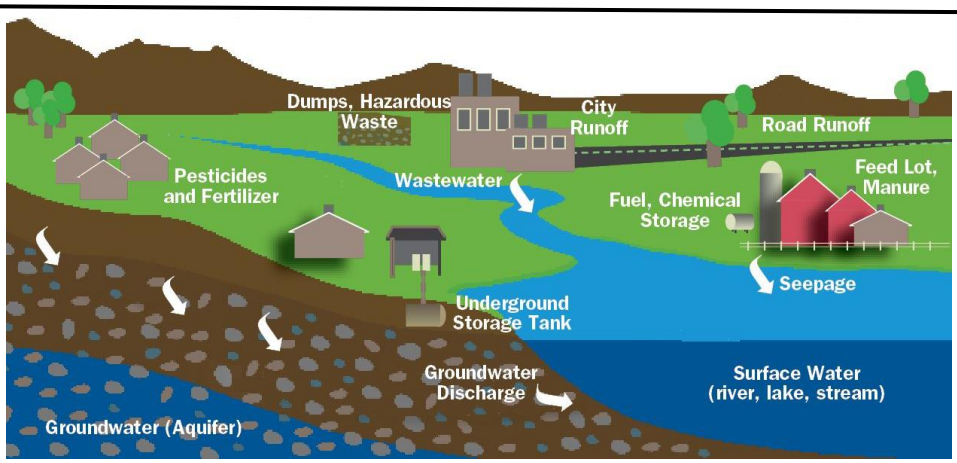
General introduction



Source: https://amwater.com/wvaw/water-information/water-learning-center/source-water

1.1. CONTAMINATION OF THE AQUATIC ENVIRONMENT

Water is essential to life, nonetheless, several anthropogenic activities lead to the production of large amounts of substances that are intentionally or accidently released into the environment and eventually reach water systems (e.g. underground waters, rivers, and sea) where they may have pernicious effects to the biota and ultimately to humans. The consequences of the presence of these foreign substances (xenobiotics) to the aquatic systems may include direct effects on the biota and/or alterations on the environment where they live. Taking into account that the presence of these substances may alter physical, chemical and biological characteristics, these parameters can be used to assess the overall quality of aquatic systems (Tyagi et al., 2013).

The anthropogenic pressure in the aquatic environment (e.g. release of pollutants) has been increasingly studied in the last decades due to the need to promote the conservation of aquatic species and the protection of water systems (European Commission, 2008). Among the main known sources of environmental pollution are industries (which release a variety of chemicals such as metals, polycyclic aromatic hydrocarbons, and persistent organic pollutants); agriculture (contributing with pesticides input) and human and animal health protection activities (responsible for the input of pharmaceuticals residues in the aquatic environment and others). Although sewage treatment plants can reduce the input of certain types of contaminants in the aquatic systems, many of them are able to remain in the water systems for decades, affecting flora and fauna (OECD, 2005). On account of this situation, the European Union established the Directive 96/61/CE (European Union Council 1996), a set of rules aiming to prevent, control or eliminate dangerous substances from the environment. To attain a classification of good condition in terms of chemical status of water, it is necessary to comply with established maximum allowed levels of substances that may pose high risk to the aquatic environment (Commission of the European Communities, 2006) .

Among chemical substances currently identified as a risk factor to the aquatic environment, according with their persistence, bioaccumulation, and toxicity, are aromatics hydrocarbons and polycyclic aromatics hydrocarbons, chlorinated hydrocarbons, organochlorine pesticides and metals (Table 1). But more recently, several types of

micropollutants have been found in aquatic systems such as nanomaterials, pharmaceuticals, industrial additives, personal care products, water treatment by-products, flame/fire retardants, some hormones, caffeine and nicotine (Alvarez-Cohen and Sedlak, 2003).

Many compounds may affect biota directly by leading to a reduction on the abundance of species and increased infertility or indirectly by inducing effects on tolerant species (Fleeger et al., 2003). Such effects may vary according to the concentration of the chemical, its persistence in the environment, presence of other substances and physical-chemicals characteristics of the water.

Currently, environmental risk assessment (ERA) is required for the authorisation of new substances like pharmaceuticals. The knowledge of the properties and chemical structure of compounds is highly important in the ERA. The process involves hazard identification, characterization, exposure assessment and risk characterization (Kroes, 2005). ERA involves two phases. The first one consists in the estimation of the environmental exposure to the chemical by calculating a predicted environmental concentration- PEC, considering the persistence, bioaccumulation, and toxicity. PEC values below 0.01 $\mu g.L^{-1}$ are considered as representing insignificant risk to aquatic environment. PEC values higher than 0.01 $\mu g.L^{-1}$ involve a Phase 2 analysis that takes into account the value of PEC and a predictive no-effect concentration (PNEC), calculated by dividing the lowest short-term or long term no observed effect concentration (NOEC) by an appropriate factor (Long et al., 1995). A risk quotient is calculated by dividing PEC by PNEC. Therefore, estimated risk and permissible levels of contamination are defined according to the response of various organisms such as fish (early life stage toxicity test) , *Daphnia sp* (reproduction test) and algae (growth inhibition test) to the tested compound (Long et al., 1995).

Table 1. List of substances considered environmentally dangerous according to European environmental legislation adapted from the Directive (2012).

Chemical Class		Compounds
Aromatic hydrocarbons		Anthracene, Benzene, Naphthalene
Chlorinated hydrocarbon		Dichloromethane
Drugs		Cybutryne, Diclofenac
Hormones		17 α-Etinilestradiol, 17β-Estradiol
Metals		Lead and its compounds, Cadmium and its compounds; Mercury and its compounds; Nickel and its compounds
Organic compounds	**Bromoorganic**	Hexabromocyclododecanes Triclorobenzenes; Brominated diphenylethers
	Chloroorganic	Heptachlor and Hepatachor apoxide; Chloroalkanes; Trichol; Trichloromethane (chloroform); Dioxins and dioxin-like compounds; C10-13
	Fluoro	Perfluorooctane, Sulfonic acid and its derivatives
	Others	Tributyltin compounds; Nonylphenols X6; Octylphenols7
Pesticides	**Organophosphastes**	Dicofol (e.g.Dichlorodiphenyltrichloroethane-DDT), Dichlorvos
	Fungicide	Hexaclorobenzene, Pentachlorobenzene, Pentachlorophenol, Quinoxyfen.
	Herbicides	Alachlor, Atrazine, Aclonifen Diuron, Isoproturon, Simazine, Trifluralin, Bifenox, Terbutryn.
	Insecticides and acaricide	Chlorfenvinphos, Chlorpyrifos (Chlorpyrifos-ethyl), 1,2-dichloroethane, Endosulfan, Hexaclorociclo-hexano, Cypermethrin11

1.2. EMERGING CONTAMINANTS

In recent years, new contaminants have been detected in water systems (e.g. microplastics, nanoparticles and pharmaceuticals), due to ineffective removal by the conventional water treatment plants. Thus, there is a high concern on how these contaminants may affect aquatic environment in the short- and long-term. Although a considerable amount of research has been performed with “classical contaminants”, the

available research for these emerging contaminants may be considered scarce, particularly in terms of long term exposures and combined exposures (Arnold et al., 2013).

Emerging contaminants are compounds that were not considered dangerous attending to their distribution and concentration in the environment but that are now increasingly detected (Alvarez-Cohen and Sedlak, 2003). Nanomaterials, pharmaceuticals, industrial additives and by products, fragrances and personal care products, food additives, water treatment by products, flame/fire retardants, surfactants, hormones are organics pollutants with difficult removal efficacy in conventional drinking water treatment plants using filtration granulated active carbon, due to their size (small molecules) and polarity.

Pharmaceuticals have emerged in the past decade as emerging contaminants (Sauvé and Desrosiers, 2014). The development of sensitive analytical technique allowed their detection in the range of nanograms per liter ($ng.L^{-1}$) and micrograms per liter ($\mu g.L^{-1}$) (Santos et al. 2010) as well as the distinction of parent compounds and metabolites (Fent et al., 2006; Ternes, 1998). Once administered, pharmaceuticals absorbed by humans are excreted through urine or faeces and reach the wastewater treatment plants. Although some pharmaceuticals may be degraded in sewage treatment plants, for most of them an incomplete or inadequate treatment results in their release into the environment (Almeida et al., 2014; Carter et al., 2014; Arnold et al., 2014; Kotchen et al., 2008). Thus, parent compounds and their metabolites (Clara et al., 2004) reach in this way the different ecosystem compartments such as streams, rivers, underground waters, surface waters, sea, soil (Oetken et al., 2005) and even drinking waters (Stackelberg et al., 2004). Once in the environment, they may induce a variety of effects such as alterations in reproduction, growth and may cause endocrine disruption (Brausch et al., 2012).

Pharmaceuticals have been classified as contaminants of emerging concern, due to their increased annual production, high consumption (hundreds of tons per year), diversity (Santos et al., 2010) and the fact that they are designed/used to interact with biological systems and have specific mechanisms in the target organisms (Oliveira et al., 2015). They are classified as persistent compounds due to its continuous release into the environment (Ferrari et al., 2003). The Directive 93/67/EEC classified pharmaceuticals according to their half maximum effective concentration EC_{50}-value as: very toxic to aquatic organism (<1

milligrams per liter ($mg.L^{-1}$)); toxic to aquatic organism (1 – 10 $mg.L^{-1}$) and harmful to aquatic organism (10-100 $mg.L^{-1}$) (Cleuvers, 2003; European Union Council, 1996). Effects on biota at the individual or population level may then be expected due to their capacity to induce biological effects even at small concentrations (Arnold et al., 2013). Some of the pharmaceutical classes with higher consumption are analgesics and anti-inflammatory drugs, contraceptives, antibiotics, beta-blockers, lipid regulators and neuroactive drugs (Fent et al., 2006). Table 2 presents a list of priority pharmaceuticals found in the surface and ground waters.

Although an increasing number of studies have focused on the effects of pharmaceuticals to non-target organisms the actual risk of pharmaceuticals to aquatic environments is still unknown (Fent et al., 2006; Oliveira et al., 2015). Harmful effects of chemicals are usually evaluated through acute toxicity tests which assess effects of a single short exposure. However, effects of low concentrations of pharmaceuticals, such as the ones found in the environment, are better evaluated using chronic toxicity tests where longer exposures are used and sublethal effects are evaluated. Some effects elicited include carcinogenicity (formation of tumours), mutagenicity (alterations in the genetic material) and teratogenicity (embryo development) (Alvarez-Cohen and Sedlak, 2003).

The monitoring of pharmaceuticals in the environmental has been conducted in different countries (Table 3). For instance, in the United Kingdom, despite of a restriction in the use of sulphonamide since 1995, it is still detected in the environment (Luo et al., 2014; Svensson, 2008) and its presence has been associated with bacterial resistance to antibiotics (Svensson, 2008). In Germany, 60 pharmaceuticals and metabolites were detected in wastewater and groundwater and in the Netherlands, carbamazepine (Cbz) was detected at concentrations of 0.87 $\mu g.L^{-1}$ in sewage treatment plants and of 0.198 $\mu g.L^{-1}$ in surface waters (van den Brandhof and Montforts, 2010).

Table 2. List of priority pharmaceuticals found the surface and ground waters. Adapted from Svensson (2008).

Pharmaceutical	Class	Surface water	Reference
Atenolol	Anti-hypertensive	0.24 $\mu g.L^{-1}$ 0.241 $\mu g.L^{-1}$ 0.00344 – 0241 $\mu g.L^{-1}$	Cleuvers (2005) Fraysse & Garric (2005)

Table 2. List of priority pharmaceuticals found the surface and ground waters. Adapted from Svensson (2008).

Pharmaceutical	Class	Surface water	Reference
		257.000 µg.L^{-1}.day	Calamari et al. (2003) Castiglioni et al. (2006)
Bezafibrate	Lipid regulators	0.00158-0.05715 µg.L^{-1}	Calamari et al. (2003)
Carbamazepine	Anticonvulsant/ antidepressant	1.075 µg.L^{-1} 0.230-1.110 µg.L^{-1} 0.25 µg.L^{-1} 28.000 µg.L^{-1}.day 0.198 µg.L^{-1} 11.3 µg.L^{-1}	Heberer (2002) Chen et al. (2014) Ternes (1988) Castiglioni et al. (2006) Van den Brandhof and Montforts (2010) Gunthert et al. (2014)
Ciprofloxacin	Antibacterial	0.144- 0.0261 µg.L^{-1}	Calamari et al. (2003) Sacher et al. (2001)
Cyclophosphamide	Cytotoxic	0.0022-0.010 µg.L^{-1}	Zuccato et al. (2000)
Diclofenac	Nonsteroidal anti-inflammatory	0.010 µg.L^{-1} 1.030 µg.L^{-1}	Fent et al. (2006) Heberer (2002) Ferrari et al. (2004)
Furosemide	Diuretic	0.00172-0.255 µg.L^{-1} 66.000 µg.L^{-1}.day	Calamari et al. (2003) Castiglioni et al. (2006)
Gemfibrozil	Lipid regulators	1.5 µg.L^{-1} 0.0018-0.0091 µg.L^{-1}	Ternes (1998) Santos et al. (2010)
Hydroclorothiazide	Diuretic	0.0244 – 0.256 µg.L^{-1} 197.000 µg.L^{-1}.day	Calamari et al. (2003) Castiglioni et al. (2006)
Ibuprofen	Nonsteroidal anti-inflammatory	0.091 – 0.092 µg.L^{-1} 0.07-0.53 µg.L^{-1}µg.L^{-1}	Zuccato et al. (2000) Ternes (1998)
Lincomycin	Antibacterial	0.0031 – 0.249 µg.L^{-1} 60 µg.L^{-1}	Calamari et al. (2003) Santos et al. (2010)
Ofloxacin	Antibacterial	94. 000 µg.L^{-1}.day	Castiglioni et al. (2004) Ferrari et al. (2004)
Raditidine	Ulcer Healing	0.0007 – 0.039 µg.L^{-1}	Calamari et al. (2003)
Paracetamol	Nonsteroidal anti-inflammatory	10 µg.L^{-1}	Fent et al. (2006) Rabiet et al. (2006) Bound & Voulvoulis (2005)
Sabultamol	Bronchodilator	0.003 – 0.005 µg.L^{-1} 2.000 µg.L^{-1}.day	Zuccato et al. (2000) Castiglioni et al. (2006)
Sulfamethoxazole	Antibacterial	122.000 µg.L^{-1}.day	Castiglioni et al. (2004) Heberer (2002)

Different levels may be found among compounds of the same class. A good example can be presented by beta-blockers (propranolol, metoprolol, and atenolol). Propranolol is

detected in sewage discharges at concentrations between 0.01 µg.L^{-1} and 1.9 µg.L^{-1} and in surface waters in the range of 0.01 µg.L^{-1} to 0.5 µg.L^{-1}. Metoprolol levels in sewage discharges are in a similar range of propranolol, 0.1 µg.L^{-1} to 2.2 µg.L^{-1} whereas in surface waters levels have been detected up to 2.2 µg.L^{-1} (Owen et al., 2007). Although beta-blockers have been detected at high concentration in sewage discharges, in surface waters they are found at low concentrations (e.g., acebutolol: 0.01-0.13 µg.L^{-1} betaxolol: 0.19 µg.L^{-1}) (Owen et al., 2007).

In Netherlands the pharmaceutical most frequently detected in drinking water has been Cbz with values up to 0.025 µg.L^{-1} (van den Brandhof and Montforts, 2010).

Table 3: List of countries where levels of pharmaceuticals were reported in aquatic environmental.

Countries	**Pharmaceuticals**	**References**
United Kingdom	Ibuprofen Metformin hydrochloride Paracetamol	Bound & Voulvoulis (2005)
	Naproxen	Fent et al. (2006)
	Aspirin Atenolol Carbamazepine Diclofenac Erythromycin	Alvarez & Seldak (2003)
	Oxytetracycline Sulphonamide	Svensson (2008)
	Colorphobic acid Erythromycin Metoprolol Sulfamethoxazole	Luo et al. (2014)

Table 3: List of countries where levels of pharmaceuticals were reported in aquatic environmental.

Countries	Pharmaceuticals	References
Germany	Acetylsalicylic acid Albutamol Carbamazepine Clenbuterol Clorifibric acid Cyclophosphamide Diclofenac Diazepam Etinylestradiol Gemfibrozil Ibuprofen Ifosfamide Ketopren Macrolides Metoprolol Penicillin Pentoxifylline Propranolol Sulphonamides Terbutaline	Heberer (2002)
Netherlands	Diclofenac Carbamazepine Metoprolol	Van den Brandhof & Montforts (2010)
	Etinylestradiol	Heberer (2002)
Canada	Naproxen	Ternes (1998)
	Etinylestradiol	Heberer (2002)
Australia	Diclofenac Ibuprofen Clorifibric acid	Heberer (2002)
Brazil	Diclofenac Etynylestradiol	Heberer, 2002
Greece	Carbamazepine Diclofenac Gemfibrozil	Luo et al. (2014)
	Diclofenac	Heberer (2002)
Spain	Erythromycin Atenolol Metoprolol Bezafibrate Carbamazepine Mefenamic acid Naproxen Salicylic acid Sulfamethoxazole	Luo et al. (2014)

Table 3: List of countries where levels of pharmaceuticals were reported in aquatic environmental.

Countries	Pharmaceuticals	References
United States	Etinylestradiol Tetracycline	Heberer (2002)
	Benzafibrate Carbamazepine Gemfibrozil Ketoprofen	Luo et al. (2014)
Switzerland	Ciprofloxacin Norfloxacin	Heberer (2002)
	Atenolol Diclofenac Metoprolol Sulfamethoxazole	Luo et al. (2014)
India	Diclofenac	Fent (2002)
Pakistan	Diclofenac	

1.2.1. CARBAMAZEPINE

Cbz is an anticonvulsant pharmaceutical used in the treatment of trigeminal neuralgia and to control epilepsy and bipolar disorders (Ambrósio et al., 2002). Its mechanism of action has been associated with effects on voltage-dependent sodium channels in humans (Ambrósio et al., 2002; Macdonald and Kelly, 1995). There is also evidence that Cbz interacts with other types of channels and receptors, such as voltage-gated sodium (Na^{+}) channels, leading to channel switching from inactivated to closed state, it may inhibit calcium (Ca^{+2}) channel with reversible voltage, suppressing the Ca^{+2} current and inhibiting the secretion of catecholamine. Cbz may act on the glutamatergic system by suppressing glutamate release, may interact with the serotonergic system by increasing the level of serotonin in the extracellular environment, decreasing the basal level of cyclic adenosine monophosphate and may lead to inhibition of potassium (K^{+}) (Ambrósio et al., 2002).

In humans, Cbz has been reported to cause adverse effects such as sedation, ataxia, nausea and, in longer administration periods, modify plasma lipids, levels of sex hormones, and reduce the number of blood cells (Luis et al., 2016). This pharmaceutical is metabolised in the liver to Cbz 10,11- epoxide which still has pharmacological activity. Only 2% to 3% is eliminated in an unchanged form (Clara et al., 2004). The Cbz 10,11-epoxide inhibited the

catecholamine production in bovine cells culture through its interaction with N-type voltage-sensitive Ca^{+2} channels. Cbz presents a low biodegradability and high persistence (Oetken et al., 2005), its solubility, dissolution and bioavailability depends on its crystalline forms, Cbz may bind to other groups through its amine group thus facilitating its solubility; in water, it is found in the dehydrate form which is less soluble than the anhydrate form (Shayanfar et al., 2014) making it one of the most commonly detected pharmaceutical in water systems (Luis et al., 2016). Regardless of the time spent in water treatment stations, the removal of Cbz is around 10%. Thus, it can be found in effluents at high concentrations (e.g., 0.12 $\mu g.L^{-1}$ and 0.250 $\mu g.L^{-1}$ in Canada and Germany, respectively) (Mcdowell et al., 2005). In surface water, collected at 100 places in the United States, the average concentration of Cbz reached 0.35 $\mu g.L^{-1}$ in surface water and in China, levels between 0.23 to 1.11 $\mu g.L^{-1}$ have been found (Chen et al., 2014). The widespread distribution of the pharmaceutical in the water system led to its classification as marker of anthropogenic contamination in aquatic environment (Clara et al., 2004).

The highest environmental concentration of Cbz have been found in effluents at 0.98-1.20 $\mu g.L^{-1}$ in France and 6.3 $\mu g.L^{-1}$ in Germany (Ferrari et al., 2004).

The LC_{50} of the Cbz on aquatic organisms vary according to the tested species (Cleuvers, 2003) (table 4). Cbz has been reported to induce chronic effects in non-target organisms even at environmentally relevant concentration (Chen et al., 2014). Nassef et al. (2010) evaluated the effects of Cbz in the embryonic development of Japanese medaka (*Oryzias latipes*) through eggs nanoinjection. One-day post fertilization survival was affected and embryos haemorrhage was observed. After 4 days, development was delayed. All changes were found at the concentration of 0.012 $\mu g.L^{-1}$ per egg, and the estimated median effective concentration (EC_{50}) value after incubation was 0.131 $\mu g.L^{-1}$ per egg.

Table 4: LC_{50} of Cbz from different aquatic organisms

Species	Endpoint	Concentration ($\mu g.L^{-1}$)	Time	References
Cyprinus carpio	LC_{50}	59700	24 h	Malarvizhi et al. (2012)
Danio rerio	LC_{50}	>=245000	72 h	Van den Brandhof et al. (2010)
Danio rerio	LC_{50}	118000	72 h	Weigt et al. (2011)
Danio rerio	LC_{50}	50000	24 h	Pruvot et al. (2012)

Table 4: LC_{50} of Cbz from different aquatic organisms

Species	Endpoint	Concentration (µg.L^{-1})	Time	References
Oryzias latipes	LC_{50}	15000-35400	48 h	Kim et al. (2007)
Oryzias latipes	LC_{50}	45870	96 h	Kim et al. (2009)
Oryzias latipes	LC_{50}	61500	96 h	Nassef et al. (2009)
Oncorhynchus mykiss	LC_{50}	19900	96 h	Li et al. (2011)
Pimephales promelas	[1]LC_{50}	>862	28 dph	Overturf et al. (2012)

[1] Effect observed: Survival

In a study looking at the acute toxicity of the most used pharmaceuticals in Korea, Kim et al. (2007) reported 52500 µg.L^{-1} as the EC_{50} value of Cbz to *Vibrio fischeri* after 5 minutes (min) of exposure, 76300 µg.L^{-1} as the 96 h-EC_{50} to *Daphnia magna.*

Triebskorn et al. (2007) analysed ultrastructural effects of Cbz in *Cyprinus carpio* liver, kidney, and gills after 28 days exposure. This study reported an increased number of macrophages and cytoplasm membrane material in liver, without a clear concentration-effect response. In kidney vesiculation, and dilation of endoplasmic reticulum, increased number of macrophages and of cell debris into the intracellular medium was found in fish exposed to 50 µg.L^{-1} of Cbz. In gills, epithelial lifting and oedema were observed at 5 µg.L^{-1}; in mucus cells hyperplasia and hypertrophy was observed at 20 µg.L^{-1} Cbz.

Li et al. (2010) in a study with spermatozoa of common carp (*Cyprinus carpio*) reported reduction on the motility and velocity of sperm after 2 hours (h) exposure to 2000 and 20000 µg.L^{-1} of Cbz respectively. Lipid peroxidation increased between the concentrations of 200 at 20000 µg.L^{-1} whereas carbonyl protein increased at the highest concentration. Superoxide dismutase (SOD) activity was induced at 200 µg.L^{-1} of Cbz but was inhibited at 20000 µg.L^{-1}. Glutathione reductase (GR) was inhibited at 20000 µg.L^{-1} and the glutathione peroxidase (GPx) activity was reduced with increasing concentrations.

In a study performed with zebrafish (*D. rerio*) embryos exposed to Cbz for 72 h, a NOEC of 30600 µg.L^{-1} and EC_{50} of 86500 µg.L^{-1} were calculated. After 72 h exposure to Cbz no hatching and a growth delay occurred in embryos exposed to 122000 µg.L^{-1} of Cbz. Moreover at 244500 µg.L^{-1} of Cbz heart abnormalities (pericardial oedema) were observed (van den Brandhof and Montforts, 2010).

Madureira et al. (2011) showed that *D. rerio* males exposed to 1780 µg.L^{-1} of Cbz had an increase in hepatic mass and suggests an increase of metabolic demand. On the other hand, fish exposed to Cbz (10 and 50 µg.L^{-1}) for 14 days, showed a significant reduction in mean speed compared to control group and in the time spent in the bottom (10 µg.L^{-1}) although at the highest concentration (200 µg.L^{-1}) in *Jenynsia multidentata* this behavior is not maintained (Calcagno et al., 2016).

1.2.4. PROPRANOLOL

Propranolol (Prop) is a cardiovascular pharmaceutical used in the treatment of hypertension, angina pectoris and arrhythmia after acute myocardial infarction (Fent et al., 2006; Oetken et al., 2005). Prop assists in reducing the cardiac frequency (Fraysse et al., 2006) once it has the ability to stabilize the cell membrane and as consequence reduce its permeability to several ions like Na^{+}, k^{+} and Ca^{+2} (Fonseca, 2010; Fraysse and Garric, 2005). Prop is a beta-blocker; this class of compounds is divided in two principal categories, pharmaceuticals that are metabolise in the liver (e.g. Prop, metoprolol) and those eliminated predominantly in unchanged form (e.g. atenolol, nadolol) (Owen et al., 2007). Prop, atenolol and metoprolol can interact with carbohydrate and lipids metabolism (Fonseca, 2010). Beta-blockers can bind to the proteins according to their hydrophilicity. Prop, for example, has high capacity (96%) to bind to proteins whereas metoprolol presents a much lower ability 12% (Owen et al., 2007).

Prop has short half-life into the organism remaining available between 2 to 4 h. Its metabolites are eliminated through the urine (Owen et al., 2007) while 1-4% of Prop is excreted through faeces as parent compound (Alder et al., 2010).

Increasing usage of the beta-blockers has been reported and hence they are now, e.g.: Prop has been found in influents of sewage treatment plants at 520 000 000 µg. day (Cleuvers, 2005). The Prop can be detected in surface and ground water at concentrations up to 0.59 µg.L^{-1} (Ternes, 1998). The removal of Prop in sewage treatment plant is relatively high (around 96%) (Fent et al., 2006). It is considered photodegradable and its transformation products are considered less toxic due to high polarity and hydrophilicity (Liu et al., 2009).

The presence of beta-blockers in aquatic ecosystem can trigger physiological responses on species that have adrenergic receptors similar to the mammalian (e.g. fish) thus potentiating the hazards on non-target organisms (Brooks et al., 2009).

The effects of Prop on aquatic organisms is described for fresh and salt water organisms in table 4.

Table 5: EC_{50} and LC_{50} to Prop in different organisms

Species	Endpoint	Concentration (µg.L^{-1})	Time	References
Synechococcus leoponensis	LC_{50}	668	24 h	Ferrari et al. (2004)
Ceriodaphnia dubia	[1]EC_{50}	1400/ 8000	48 h	Cleuvers (2005)
			48 h	Hugget et al. (2002)
Cyiclotella meneghiniana	[2]EC_{50}	244	96 h	Ferrari et al. (2004)
Danio rerio	LC_{50}	21600	48 h	Mitchel (2015)
	[3]EC_{50}	>14	24 h	Fraysse et al. (2006)
Daphnia magna	[4]EC_{50}	7700/1600	48	Cleuvers (2005)
			48 h	Hugget et al. (2002)
Desmodemus subspicatus	[2]EC_{50}	730	48 h	Cleuvers (2005)
Fucus vesiculosus	[5]EC_{50}	1000	1344 days	Oskarsson et al. (2012)
Lemna minor	[2]EC_{50}	113000	168 h	Cleuvers (2005)
Oncorhynchus mykiss	[2]EC_{50}	10000	10 days	Owen et al. (2009)
Oryzias latipes	LC_{50}	11400/ 24300/100000	24 h	Kim et al. (2009)
			240 h	Ferrari et al. (2004)
			48 h	Hugget et al. (2002)
Pimephales promelas	LC_{50}	3400	72 h	Mitchel (2015)
Thamnocephalus platyurus	LC_{50}	10310	24 h	Kim et al. (2009)

[1]Effect observed: Immobilization
[2]Effect observed: Growth inhibition
[3]Effect observed: Decrease movements (embryos)
[4]Effect observed Immobilization and /or Survival
[5]Effect observed: Photosynthesis Inhibition

Liu et al. (2009) observed mortality of 100% in *Brachionus calyciflorus* after exposure for 48 h at 10000 µg.L^{-1} of Prop. Fraysse et al. (2006) reported decrease of spontaneous movements in zebrafish after 24 h exposure to 32000 µg.L^{-1} of Prop; tail shortening, lack of circulation caudal, reduced of pigmentation and pericardial oedema in

40% of the embryos exposed to 32000 µg.L^{-1} in 48 h, and at 80 h reduction of pericardial area after exposure to 1500 µg.L^{-1}.

Diatom and fish have been considered the most sensitive organisms to Prop with risk ratio values for surface water around 0.244 µg.L^{-1} ($PNEC_{acute}$) and 0.01 µg.L^{-1} ($PNEC_{chronic}$), respectively (Ferrari et al., 2004).

Nonetheless the number of studies about Prop effects in aquatic organism and risk assessment are limited (Cleuvers, 2005).

2. BIOMARKERS

Biomarkers are selected biological endpoints, measured in species of interest and may be biochemical (e.g. AChE inhibition), physiological, biomarkers of oxidative stress, histological indicators or adverse health effects evaluated after exposure to an environmental contaminant (Forbes et al., 2006; Hook et al., 2014). Correspond to primary responses of a biological system after exposure to a chemical or other stressor that anticipate responses at higher hierarchical levels. Thus, biological responses may contribute to understand mechanisms of chemical impacts (Forbes et al., 2006).

For example, pharmaceutical compounds may induce an increase in the production of reactive oxygen (ROS) and result in oxidative damage to aquatic organisms (Nunes et al., 2008). ROS are produced under normal conditions by living organisms as result of cellular metabolism, however are highly reactive molecules and may damage cell structures (e.g. carbohydrates, nucleic acids, lipids, and proteins) (Birben et al., 2012). Thus, aerobic organisms have antioxidant systems with enzymatic (SOD, CAT, GST and GPx) and non-enzymatic antioxidants (e.g. vitamins C and E, beta carotene and glutathione (GSH)), and under stress conditions use the antioxidant system as a mechanism to adapt to environmental stress (Birben et. al, 2012; Gravato et al. 2006) and LDH is evaluated as an enzyme important in red-ox maintenance. Among the most investigated biomarkers are enzymes involved in the detoxification of xenobiotics and their metabolites (biotransformation enzymes, antioxidant enzymes) (van der Oost et al., 2003). Besides indicators of oxidative stress, behavioural changes are also toxicity indicators, since provide integrative measures of neurotoxicity (AChE). Generally, behavioural changes are linked to disturbances at the biochemical level (e.g. altered neurotransmitters and thyroid

hormones) and may induce effects at the population level, such as direct effects linked to the search for a sexual partner, avoidance of predators or pollutants, or indirect effects such as altered reproductive success due to compromised feeding and thus to energy metabolism (Amiard-Triquet, 2009).

2. FISH ECOTOXICITY TESTS

Studies on the occurrence and fate of micropollutants in aquatic environment have been performed with the purpose of assessing the presence of substances in water with the potential to generate harmful effects on biota. Standard tests using fish, daphnia and algae can be conducted to determine safe concentrations for non-target organisms (European Medicines Agency (EMEA), 2006).

Due to similar physiological and developmental characteristics to mammals (Owen et al., 2007), fish have been introduced in the laboratory routines all over the world to replace mammals models (Parker, 2015).Therefore, *Oncorchynchus mykiss* (rainbow trout), *Pimephales promelas* (fathead minnow), *Brachydanio rerio* (zebrafish) and *Oryzias latipes* (ricefish) are standard freshwater fish species while *Cyprinodon variegatus* (sheepshead minnow) standard saltwater fish species (Parker, 2015). Moreover, fish embryos are experimental models considered as an alternative to experimental animals replacing very often adult testing (Braunbeck et al., 2005).

2.1. ZEBRAFISH (*DANIO RERIO*)

The zebrafish is a benthopelagic cyprinid freshwater fish original from South Asia (Truong et al., 2011; Weigt et al., 2011). It can be kept in laboratory with easy and cheap maintenance. It has an average size between 3 and 5 cm, short reproductive cycle and females can to produce hundreds of eggs per week (high fecundity). Zebrafish eggs are optically transparent which allows monitoring throughout the stages of development (Kimmel et al., 1995). This allows observation of all cells and instantaneous analysis of tissues (Chakraborty et al., 2016). Zebrafish has a quick growth at 26 ºC, it has neuronal plate formed 10 h post fertilization (Braunbeck et al., 2005; Truong et al., 2011) and its organs are fully developed after 5 and 6 days after fertilization (Chakraborty et al., 2016).

Zebrafish is considered one important vertebrate model in genetics, developmental biology and neurophysiology (Spence et al., 2007). It has cardiovascular system, nervous system and digestive system similar to mammals (Chakraborty et al., 2016). Moreover it has a sequenced genome (Zhang et al., 2008) and high similarity to human (about of 75% of the genome are homologous genes), a characteristic that allows its use in the genetic evaluation of specific functions and in the study of various human diseases (Chakraborty et al., 2016). Zebrafish embryos allow the analysis of various endpoints of acute and chronic toxicity and have a wide literature scientific base available (Braunbeck et al., 2005; Zhang et al., 2008).

Although there are a considerable number of studies on the ecotoxicological effects of pharmaceuticals, there is a lack of studies concerning long term exposures, combined exposure, and sequential exposures. Thus, the evaluation of long term exposures and epigenetic responses in the offspring of parents exposed to a chemical compound can be a realistic scenario, since it allows us to evaluate the changes in the descendants, acquired by the creation of memory signal after parenteral exposure to chemical products, for example changes in the ability to adaptation and susceptibility of offspring to pharmaceuticals.

3. OBJECTIVES

Many fish studies tend to investigate acute effects of pharmaceuticals at high concentrations but low environmental relevance. However, in the environment, fish are exposed over their life time to xenobiotics to much lower concentrations than those commonly used in laboratory assays. Although chronic trials are more realistic for health assessment of populations present in aquatic ecosystems, the available studies are extremely scarce, particularly are few studies on the effects of Cbz on health fish in environmentally relevant concentrations. Thus, this work aimed at evaluating:

1. Chronic effects of Cbz on adult *D. rerio* during 63 days of exposure evaluating different endpoints like growth rate, feeding behaviour, alterations in reproduction, biochemical responses, and histological alterations.
2. Acute effects of Prop on adult *D. rerio* after 63 days of exposure a Cbz.
3. Susceptibility of the progeny to Prop assessed through ontogenetic development alterations and survival rate.

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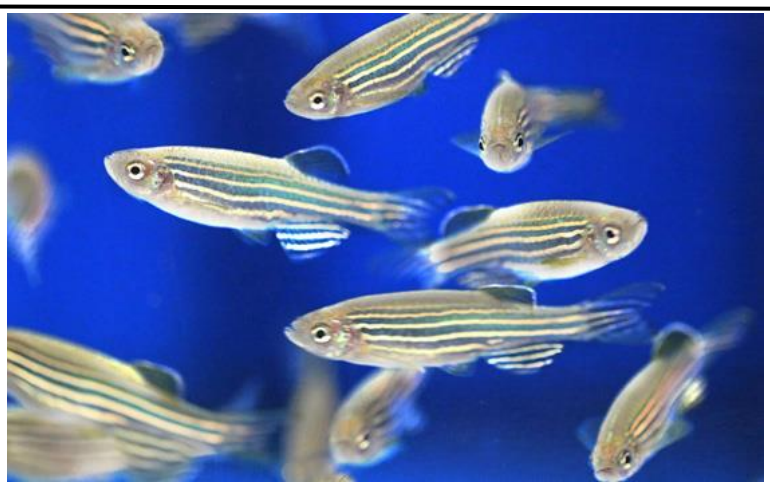
CHAPTER 2

Chronic exposure of adult *Danio rerio* to carbamazepine



Source: http://www.curearthritis.org/new-osteoarthritis-drug-targets/

Chronic exposure of adult *Danio rerio* to carbamazepine

ABSTRACT

Carbamazepine (Cbz), is among the drugs most commonly detected in the aquatic environment. Although this pharmaceutical has been proposed as an anthropogenic marker of urban pollution, its effects to aquatic organisms after chronic exposure are still poorly studied as is the susceptibility of the offspring to additional stressors. In this study, adult zebrafish (*Danio rerio*) were exposed for 63 days to 10 and 10000 $\mu g.L^{-1}$ of Cbz plus a control, and its offspring exposed to a concentration range (0, 0.6, 1.9, 5.6, 16.7 and 50 $mg.L^{-1}$) of propranolol (Prop) another human pharmaceutical (beta-blocker), for 96 h. Sub-blethal parameters analyzed in *D. rerio* adults were growth rate; feeding behavior; biochemical markers to assess effects on neurotransmission (acetylcholinesterase - AChE), antioxidant defenses (catalase- CAT, glutathione-S-transferase- GST); metabolic activity (lactate dehydrogenase - LDH); nuclear anomalies, total number of eggs and their viability. Understand how the chronic exposure of adult *D. rerio* the Cbz may determine the physiological response these organisms against acute exposure to Prop. To assess the susceptibility of offspring to Prop, survival rates were analyzed.

Cbz disrupted fish feeding behavior at both tested concentrations. AChE activity in the head increased after exposure to both Cbz concentrations whereas in the muscle activity was increased in the highest concentration. CAT activity decreased in liver and gills at the two concentrations tested whereas GST activity had an organ specific response, being increased in gills in two concentrations tested and inhibited in the liver and after exposure 10000 $\mu g.L^{-1}$. LDH activity increased in liver and decreased in muscle and gills in the highest concentration tested. Cbz had no effect on *D. rerio* growth rate nor induced cytogenotoxic effects. The total number of eggs produced was decreased in Cbz exposed organisms. The offspring of the exposed animals did not display altered susceptibility to Prop. The present study shows that exposure to chronic exposure to Cbz, even at low concentrations, is sufficient to induce behavioral alterations, alterations in antioxidant defenses, metabolism, and reproduction in *D. rerio*. However, Cbz did not induce altered susceptibility to Prop in the offspring.

(Keywords: Pharmaceuticals, chronic exposure, fish, reproduction, offspring susceptibility)

1. INTRODUCTION

The observed increase in the production, consumption and variety of pharmaceuticals available in the market, allied with its biological active nature and known release in the environment, mainly through sewage treatment plants effluents (Clara et al., 2004) led to its inclusion in the emerging contaminants of concern (Santos et al., 2010). These compounds and their biologically active metabolites are not conveniently removed or biodegraded in the treatment plants (Martins et al., 2012) and once in the environment may undergo bioaccumulation (Deblonde et al., 2011). Different classes of human pharmaceuticals (e.g. analgesics, beta-blockers, anticonvulsants, anti-cancer, hormones and lipid-regulators) have been identified in the aquatic environment (Deblonde et al., 2011).

Carbamazepine (Cbz) is among the most commonly detected pharmaceuticals in the environment, having been proposed as an anthropogenic marker of urban pollution (Clara et al., 2004) since it has low degradation rate in the environment (Pires et al., 2016). Only 10 % of Cbz is removed in waste water treatment plants (Chen et al., 2014) and the permanency time in the aquatic environment may be around of 82 days (Brandão et al., 2013). It may be found in wastewater treatment plants, surface waters, and soils (Oliveira et al., 2015). This human pharmaceutical is an anticonvulsant prescribed for the treatment of psychomotor epilepsy, bipolar disorder, and trigeminal neuralgia, known to interact with potassium (k^+) and sodium (Na^+) channels and several signaling pathways (Ambrósio et al., 2002) and to modulate voltage gated Na^+ channels that will decrease the neuronal activity (Galus et al., 2014). In the liver, the main biotransformation organ, it is converted into Cbz 10,11- epoxide and other derivatives (Clara et al., 2004). Cbz is one of the pharmaceuticals with the highest distribution and abundance in the aquatic environment in the world (Oropesa et al., 2016; Pires et al., 2016) and once in the aquatic environment keeps its biological ability and may be bioaccumulated and bioconcentrated (Oropesa et al., 2016).

Chronic effects of Cbz on behavior, growth and fecundity have been detected at several trophic level (Oropesa et al., 2016). Galus et al. (2013) performed a study to evaluate effects of the chronic exposure (42 days) to low concentrations (environmentally relevant) of Cbz on zebrafish (*Danio rerio*). This study reported decreased embryo

production in adult zebrafish exposed at 0.5 and 10 µg.L^{-1}. The exposure of zebrafish to 0.5 µg.L^{-1} of Cbz yielded irregularities in oocytes, somatic stromal tissue and decreased plasma sex steroids. The authors suggested that these changes could be due to the mode of action of Cbz in fish that may be similar to mammals. In a study conducted by Brandão et al. (2013) on different anticonvulsant drugs present in environment the effects of Cbz were assessed in terms of oxidative stress (in hepatic, digestive and gill tissues) and behavioral parameters in fish *Lepomis gibbosus* during 96 h. Organism exposure to 250 to 1000 µg.L^{-1} of Cbz had a significant increase of glutathione-S-transferase (GST) activity in the digestive tract and significant alterations were recorded in the glutathione reductase (GR) activity when measured in gills and digestive tract at all tests concentrations (62.5, 125, 500, 1000 µg.L^{-1}). Li et al. (2010a) evaluated effects of Cbz in spermatozoa of *Cyprinus carpio* after 2 hours (h) of exposure. The authors concluded that fish exposed to 2000 and 20000 µg.L^{-1} of Cbz had significant motility and sperm velocity decrease when compared to control group. Lipid peroxidation in sperm was significantly increased after 2 h of exposure to Cbz in all treatments (200, 2000 and 1000 µg.L^{-1}) compared with control group. Activities of superoxide dismutase (SOD) was induced in fish exposed to 200 µg.L^{-1} of Cbz, while GR activity was significantly reduced in sperm of fish exposed to 20000 µg.L^{-1} of Cbz and the glutathione peroxidase (GPx) activity was reduced in a dose-dependent response after 2 h of exposure.

Propranolol (Prop) is a non-selective beta-blocker that connects to beta 1 and beta 2- adrenergic receptors; it acts by reducing the heart rate and force cardiac contraction. Although there are more than more than 30 types of beta-blockers, Prop is one of the most commonly prescribed due to the increase cardiovascular disease and hypertension (Sun et al., 2015), making it the most commonly found beta-blocker in the environment (Ding et al., 2015). Once in aquatic systems it may undergo photolysis, with a reported half-life of 6.7 days (Alder et al., 2010). Despite the high metabolization and photodegradation, Prop has been detected in sewage effluents and surface water (Owen et al., 2009) at concentrations up to 0.59 µg.L^{-1} (Ternes, 1998) and, compared to other beta-blockers, Prop has a high capacity for bioaccumulation (Owen et al., 2007). Prop effects were previously evaluated in several aquatic organisms. Ding et al. (Ding et al., 2015) study using

Scenedesmus obliquus, *Daphnia magna* and *Carassius auratus*, evaluated the trophic transfer of this compound. The algae (*S. obliquus*) exposed the 10, 100 and 1000 µg.L^{-1} of Prop during 48 h, accumulated the compound but, when *D. magna* was fed with algae previously contaminated, no bioaccumulation occurred. However, in fish that was fed with *D. magna* an increase in hepatic SOD activity after days 1 and 2 of exposure was verified in all treatments (0.31, 0.83 and 1.66 µg.L^{-1}). In the liver of fish fed for 4 days with *D. magna* previously exposed to Prop (1.66 µg.L^{-1}) an increase of Ethoxyresorufin -O- deethylase (EROD) activity occurred. Finn et al. (2012) evaluated the effects of Prop on *Oryzias latipes* and *D. rerio* in a study performed with the purpose of investigating the effects of parental and embryonic exposure on embryonic heart rate and cardiac morphology. This study demonstrated that embryos of *O. latipes* from parents exposed for 24 h to Prop (0.1, 1 and 10 µg.L^{-1}) presented significantly decreased heart rate at 68 h post fertilization (hpf). In *D. rerio*, a significant reduction of heart rate in all treated groups and at all periods (44, 54 and 64 hpf) occurred. Moreover, in embryos *D. rerio* exposed to 0.1 µg.L^{-1} of Prop elongation of heart tubes occurred when compared with control embryos. In a study performed by Fraysse et al. (2006), zebrafish embryos exposed to Prop presented a reduction in the number of spontaneous movements after 24 h of exposure in all treatments (1500, 2000 and 32000 µg.L^{-1}) except at 14000 µg.L^{-1}. Embryos exposed for 48 h to 32000 µg.L^{-1} of Prop presented pericardial edema, tail curvatures, lack of caudal blood circulation. In organism exposed to 1500 µg.L^{-1} of Prop for 80 h a significant reduction (around 18%) in the pericardial area occurred when compared to control pericardial area.

Although many studies on the interaction of Cbz with aquatics organisms like algae, cladocerans and fish have been performed in the last years to understand lethal and sublethal effects of this compound (Parker, 2015) there is still a lack of knowledge about the impact of Cbz on non-target organisms at long-term (Deblonde et al., 2011), including trans-generational effects (Galus et al., 2014).Therefore, the aim of the study was to evaluate chronic effects of Cbz on adult zebrafish at different levels of assessment: growth, reproduction, feeding behavior, biochemistry, genotoxicity and effects on F1 generation. At the end of the chronic assay fish were used to assess the acute toxic of Prop, with the objective to determine if sensitivity of organisms changed after exposure to Cbz.

2. MATERIALS AND METHODS

2.1 TEST CHEMICALS

Carbamazepine (Cbz) ($C_{15}H_{12}N_2O$, CAS 298-46-4) and Propranolol (Prop) ($C_{16}H_{21}NO_2$·HCl=295.81, CAS 318-98-9) were purchased from Sigma-Aldrich. All other reagents were analytical grade.

2.2 TEST ORGANISMS

Adult zebrafish (*D. rerio)* were purchased from ZAIA (Brazilia) and acclimated for 40 days to laboratory conditions. Culture water was obtained by reverse osmosis and was the conductivity adjusted to 550 (± 100) µS, by adding salt ‘‘Aquarium Sistems’’ (USA). Water temperature was kept at 26.0 (± 1) ºC, pH at 7.5 (± 0.5), and dissolved oxygen equal or above 99 % saturation. A 12:12 h (light:dark) photoperiod cycle was maintained. Nitrite and ammonia compounds were measured every three days following the OECD guideline 215 (OECD, 2000). Fish were fed twice a day with commercially diet (TetraMin fish food, EUA).

All experimental procedures involving fish were performed following the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). Animal handling was performed with accredited researchers.

2.3 CHRONIC EXPOSURE

The test procedure was based on the OECD guideline 215 (OECD, 2000) and performed under conditions similar to acclimation.

Sexually mature fish (180), with similar ages (approximately 6 months) were weighed and their sex determined. Average fish weight was of 0.51 g for females and 0.34 g for males. Males and females (90 of each sex), were randomly distributed in nine tanks, containing 5 L of test solution (control, 10 and 10000 $\mu g.L^{-1}$ of Cbz). Three replicates were used per treatment. The Cbz concentration of 10 $\mu g.L^{-1}$ was selected as an ecologically relevant concentration (e.g. 11.3 $\mu g.L^{-1}$ of Cbz were measured in wastewater treatment plant (WWTP) in lake Paranoá (Gunthert et al., 2014). The concentration of 10000 $\mu g.L^{-1}$ corresponds approximately to 5% of Cbz lethal concentration value (LC_{50}) reported (Madureira et al., 2012; van den Brandhof and Montforts, 2010; Weigt et al., 2011). Animals were exposed for 63 days.

Test solutions were obtained by dilution of a stock solution of Cbz with culture water. The exposure medium was renewed every three days. Fish were fed once a day with a quantity of TetraMin fish food (EUA) corresponding to 2% of the fish weight in the aquarium.

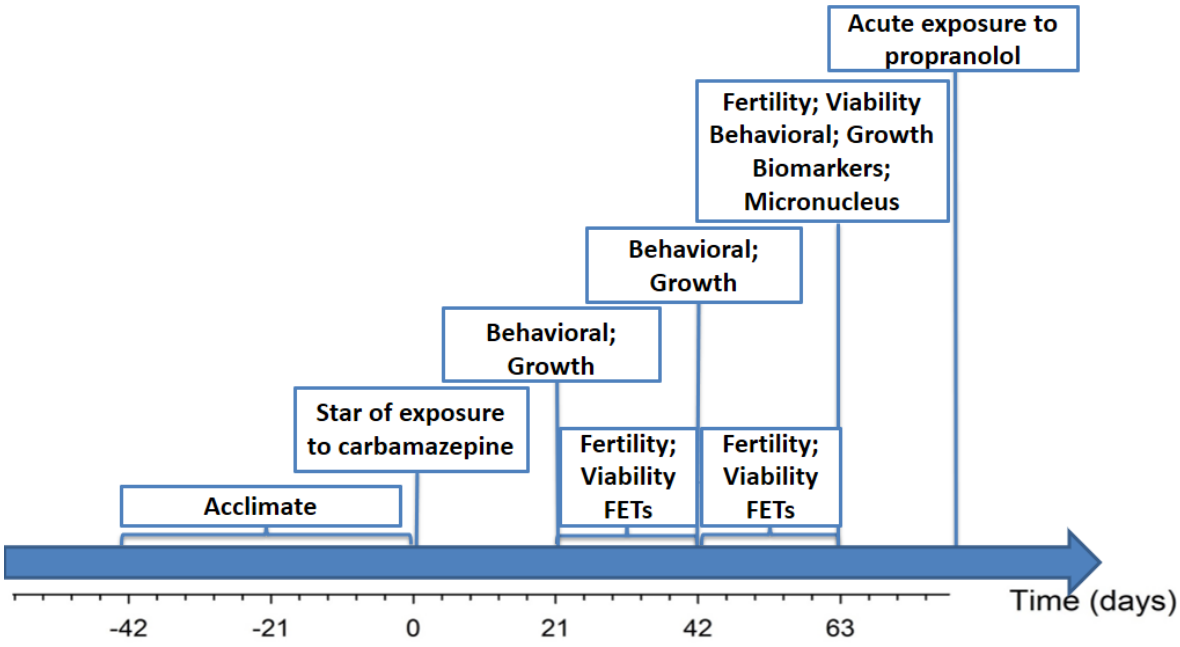


Figure *1*. Endpoints assessed along the experiment. The reproductive outcome of fish was assessed after 21 days exposure and thereafter weekly. FETs mean "Fish Embryo Acute Toxicity (FET) Test".

The endpoints assessed in this research work and its timeframe are presented in Figure 1. Fish weight and feeding behavior were evaluated (Section 2.3.1) at days 21, 42 and 63 of exposure. At days 23, 27, 35, 42, 47, 54 and 61, fish were allowed to reproduce to assess reproductive output (number of eggs and viability) and perform an embryo toxicity assay. To obtain eggs, in the morning, an hour before the lights turn on, fish were transferred from the exposure vessels to aquarium with culture water, marbles and plants in the bottom and allowed to remain in these condition for two hours after the lights turn on. Fish were then returned to the exposure vessels, eggs collected and examined under a stereomicroscope to determine the total number of eggs and fertilized eggs. Fertilized eggs were used in the fish embryo toxicity (FET) test (section 2.3.4).

At the end of the test, animals from each experimental condition were used for micronucleus analysis (12 animals; section 2.3.2), biochemical analyses (20 animals; section 2.3.3). The remaining fish were used as test organisms in an acute exposure to Prop (section 2.4).

2.3.1 Feeding behavior

Feeding behavior was assessed by transferring 5 fish of each exposure tank to an aquarium containing culture water and by adding 6 granules of TetraMin. The time taken for the first feeding action and for the total intake of food (maximum of 20 minutes (min)) was recorded.

2.3.2 Sampling for biochemical analysis

At the end of the exposure the fish were sacrificed in ice by decapitation. Peripheral blood, head, gills, liver, muscle, and intestine were sampled for posterior analysis.

2.3.3 Micronucleus (MN) and others nuclear abnormalities (NA) assessment

The test was performed following the methodology established by Hooftman & de Raat (1982) for fish erythrocytes. Approximately 20 µL of peripheral blood were collected with a heparinized pipette tip and a smear immediately performed. The slides were fixed in pure ethanol for 20 min, allowed to dry and stained in Giemsa (20%).The stained slides were observed under an optic microscope with a magnification of 1000 x and evaluated for the presence of micronuclei and other nuclear abnormalities analyzed (i.e. binucleated cells, blebbed nuclei, lobed nuclei and notched nuclei (Carrasco et al., 1990; Fenech et al., 2003) in 1000 cells per slide.

2.3.4 Biochemical determinations

Head, gill, liver, muscle, and intestine of each fish were isolated and frozen in microtubes containing phosphate buffered saline (PBS), pH 7.4. Samples were stored at -80 °C until analysis. On the analysis day, samples were thawed, homogenized by an ultrasonic cell disruptor (*Bronson Ultrassonic Sonifier 450*, Danbury, US) and centrifuged (4°C, 10000 *g*, 20 min) to isolate the post-mitochondrial supernatant (PMS).

All the enzymatic activities were measured using a microplate reader (Spectra Max M2 - Molecular Devices) and expressed as nanomoles of substrate per min per milligrams

of protein. The protein content of the samples was determined by the Bradford method (1976) using γ-globulin as standard.

AChE activity was determined in muscle and head using acetylcholine as substrate and measuring the conjugation product between thiocholine (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (DTNB) (absorbance increase), at 414 nanometers (nm), every 40 seconds (sec), for 5 min, according to the method of Ellman et al. (1961). Activity was determined using 50 µL of PMS and 250 µL of reaction mixture (acetylthiocholine (7.5 micromolar (mM)), DTNB (10 mM) in potassium-phosphate buffer (0.1 M, pH 7.2).

CAT was determined in PMS obtained from gills and liver. CAT activity was determined by monitoring at 240 nm (every 40 sec, for 5 min) the decrease in absorbance due to degradation of H_2O_2, as described in Claiborne (1985). PMS volumes used were 50 µL for gill and 20 µL for liver which were mixed with 250 µL and 130 µL respectively of reaction mixture (666 µL H_2O_2 (30 mM) in 200 ml of potassium -phosphate buffer (0.05 M, pH 7.0).

GST activity was determined in the gills, liver and intestine according to the methodology described by Habig & Jakoby (1981) and adapted by Frasco & Guilhermino (2002). The GST activity was measured at 340 nm, monitoring the increase in absorbance in 40 sec intervals for 5 min. To determine the GST activity in the liver and intestine 50 µL of PMS were mixed with 150 µL d of reaction mixture, whereas for gills 50 µL of PMS were mixed with 250 µL of reaction mixture (reduced glutathione (10 mM) and 1-chloro- 2.4-dinitrobenzene (60 mM) in potassium-phosphate buffer (0.05 M, pH 6.5)).

LDH was measured in gills, liver and muscle according to the methodology described by Vassault (1983), adapted to microplate by Diamantino et al. (2001). For analysis in the liver and intestine 15 µL of PMS, 25 µL of buffer Tris-NaCl (0.1M, pH 7,2) and 125 µL of nicotinamide adenine dinucleotide reduced (NADH) (300 µM) were added to 20 µL of pyruvate (4.5 mM). For gills and muscle, the test was performed with 25 µL of PMS, 25 µL of buffer Tris-NaCl (0.1M, pH 7,2), 250 µL NADH (300 µM) and 40 µL pyruvate (4.5 mM). Reading was performed at 340 nm at intervals of 40 sec for 5 min, following a decrease of absorbance resulting from oxidation of NADH.

2.3.5 Fish embryo toxicity assay with Prop

D. rerio embryos were exposed to Prop in 24-well plates following the OECD Guideline 236 (OECD, 2013).

The conditions of water temperature, conductivity and dissolved oxygen were similar to the culture water. A 12:12 h (light:dark) photoperiod cycle was maintained. Newly fertilized eggs were collected and distributed in 24-well microplates (1 egg per well with 2 mL of test solution) with a minimum of 4 eggs per concentration. The concentrations of Prop used were 0, 0.6, 1.9, 5.6, 16.7 and 50 $mg.L^{-1}$. Solutions were prepared from dilutions of a stock solution (200 $mg.L^{-1}$) with culture water. The test duration was 96 h. Embryos and larvae were observed under a stereoscopic microscope every 24 h. Survival were observed and reported.

2.4 Acute test with Prop

Adult zebrafish previously exposed to Cbz (0, 10 and 10000 $\mu g.L^{-1}$) were exposed to for 96 h, to 0, 1000 and 5000 $\mu g.L^{-1}$ of Prop, in a semi-static experimental design, generally following the methodologies described in the OECD Guideline 203 (OECD, 1992). All test solutions were prepared by successive dilution of a stock solution in culture water. Temperature and photoperiod conditions were like the culture conditions. Six fish per concentration were exposed in containers with 4 L of test solution. The organisms were not fed during the test period. Mortality and behavior were daily evaluated.

2.5 Statistical analysis

Growth rate was calculated and presented as “pseudo” specific growth rate (r) according to the equation (OECD 2000):

$$r:\frac{\log e\, w2-\overline{\log e\, w1}}{t2-t1}\times 100$$

where:

$\overline{\log e\ w1}$= average of the logarithms of the values w1 for the fish in the tank at the start of the experiment;

$\log e\ w2$= logarithm of the weight of an individual fish at the end of the experiment;

t1, t2 = time (days) at start and end of study period;

The Sigma plot 12.5 statistical package was used for all statistical analyses.

A Two-way ANOVA was used to detect the occurrence of changes in the "pseudo" specific growth rate using Cbz concentration and sex as factors. Feeding behavior, genotoxicity, biochemistry, and reproduction data sets were analyzed using a one-way ANOVA followed by a multiple comparison test to assess differences towards control. When normality and homoscedasticity of data were not verified a Kruskall-Wallis was done. All statistical analysis were performed with a significance level of 0.05. Lethal concentration values (LC_{50}) for early-life stages was calculated using a 4 parameters regression (Sigma plot 12.5 statistical package).

3. RESULTS

No mortality was found during the experimental assay.

3.1. GROWTH RATE

"Pseudo" specific growth rates of males and females regarding the entire period of exposure (63 days) are presented in Figure 2. The two-way ANOVA indicated that growth rates did not differ among Cbz treatments ($p=0.967$) but were different between male and female ($p<0.001$), with males presenting consistently higher growth rates than females. Growth rates were also calculated for three partial exposure periods (0 to 21; 21 to 42 and 42 to 63 days) but the same trend was verified: no effects of Cbz exposure were detected on growth (data not shown).

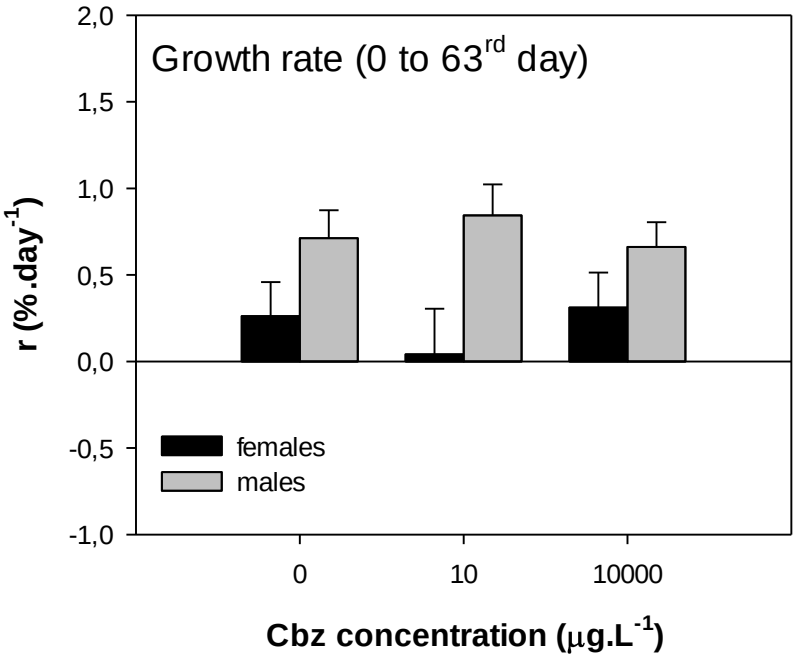


Figure 2. "Pseudo" specific growth rates (r) of *D. rerio* males (grey bars) and females (black bars) during exposure to Cbz. Results are expressed as mean ± standard error.

3.2. FEEDING BEHAVIOR

The results indicate that Cbz had no effect on the time to the first feeding action in none of the exposure periods evaluated (Figure 3). However, Cbz exposure increased the time taken to the total ingestion of food as observed in the white bars of Figure 3. This trend, however, was not statistically significant in the assessment performed at the 42 day of exposure (Figure 3 B). At 63 day of exposure, fish exposed to 10 and 10000 µg.L^{-1}

of Cbz exceeded the maximum time pre-established for total ingestion (20 min).

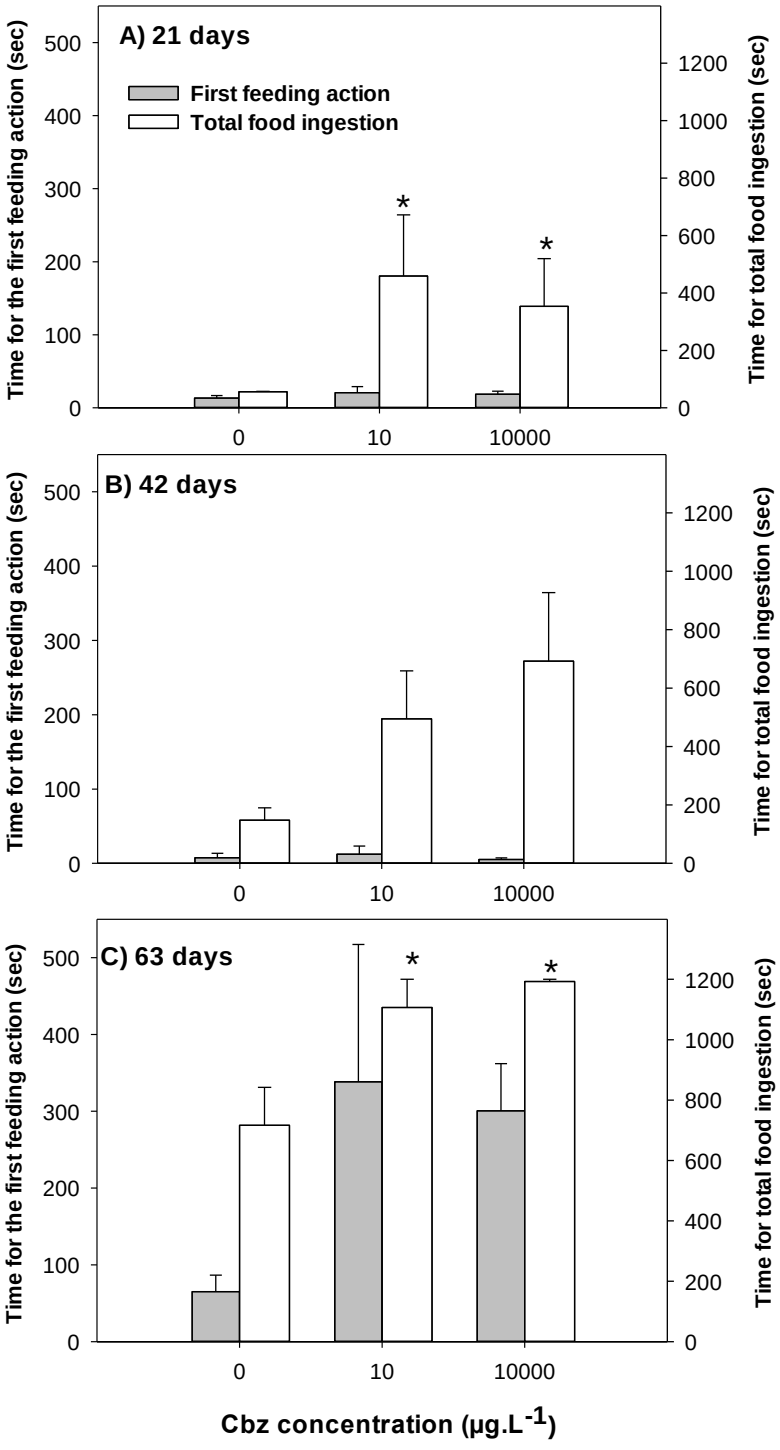


Figure 3: Feeding behavior of adult *D. rerio* recorded after 21 (A), 42 (B) and 63 days (C) of exposure to Cbz. The results are shown as time for first feeding action (grey bars) and time for total food ingestion (white bars). Asterisks (*) above the bars represents significant differences relative to the control (Dunnett's Method; $p<0.05$).

3.3. MICRONUCLEUS (MN) AND OTHERS NUCLEAR ABNORMALITIES (NA) ASSESSMENT

No micronuclei were found in any of the treatments. The incidence of other nuclear abnormalities after Cbz exposure was not statistically different from the control treatment (Figure 4).

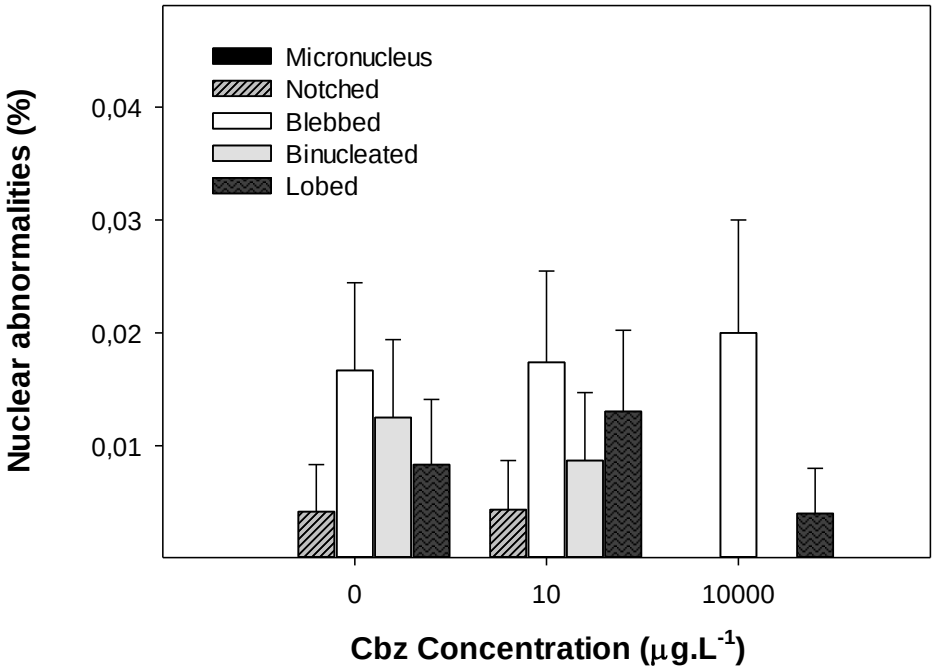


Figure 4: Endpoint of cytotoxicity and genotoxicity after chronic exposure of adult *D. rerio* to Cbz (0, 10 and 10000 µg.L^{-1}). Values represent mean ± standard error of micronucleus and other nuclear abnormalities (notched nuclei, blebbed nuclei, binucleated cells, lobed nuclei) found per treatment group.

3.4. EFFECTS OF CBZ ON BIOCHEMICAL BIOMARKERS

The effects of Cbz on AChE, CAT, GST and LDH activities in *D. rerio* are presented in Figure 5. AChE activity was affected by Cbz exposure both in muscle and head (Figure 5A). In the muscle (Figure 5A), AChE activity was increased at 10000 µg.L^{-1} ($p<0.05$) whereas in the head (Figure 5A) activity was increased at both Cbz concentrations in a dose dependent pattern ($p<0.05$).

CAT activity (Figure 5B) was inhibited both tissues in liver and gills at the two Cbz concentrations tested. However, in liver the two Cbz concentrations elicited the same degree of CAT inhibition, in gills a concentration dependent inhibition was observed.

GST activity was assessed in the liver, intestine and gill (Figure 5C). GST response was organ dependent: while increased activities were recorded in liver ($p<0.05$) and gills ($p<0.001$) an inhibition was observed in the intestine ($p<0.001$). Gills presented higher basal levels (100 U) when compared with the other tissues and the enzyme activity was increased around 50 % regardless of the Cbz concentration. In the liver and intestine only the highest concentration affected significantly the GST activity showed an induction and inhibition respectively.

LDH basal levels were higher in the muscle, followed by gills and liver. For all tissues, a significant effect of Cbz was detected for the highest Cbz concentration, however the pattern of response was different (Figure 5D). In the liver, an increase of enzymatic activity was measured (Figure 5D)($p<0.001$) whereas in muscle (Figure 5D)($p<0.001$) and gills (Figure5D)($p<0.001$) an inhibition was observed.

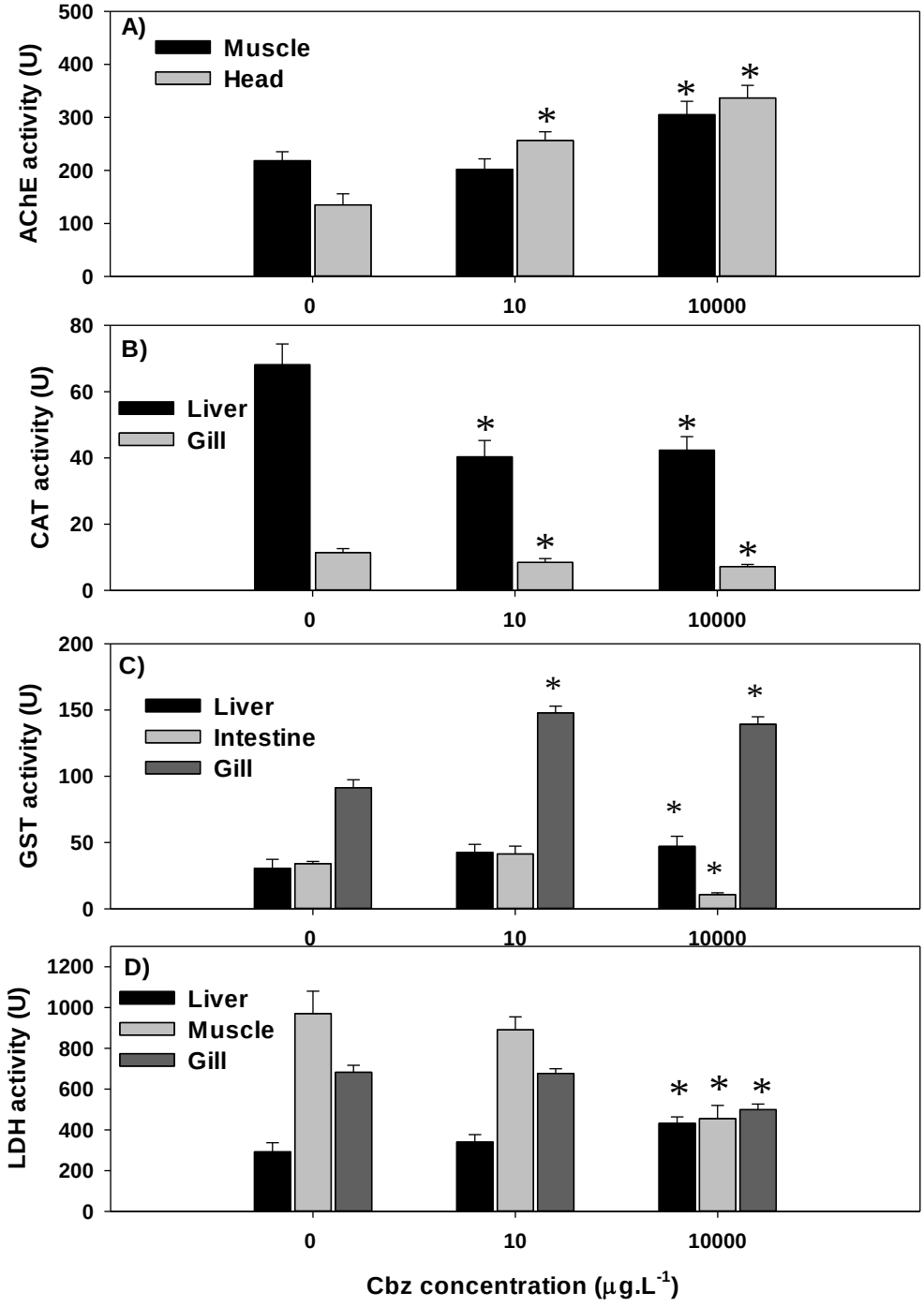


Figure 5: Enzymatic activities of adult *D. rerio* after 63 days exposure to Cbz (mean value ± standard error): A - AChE activity, B - CAT activity; C - GST activity and D - LDH activity. Asterisks mean significantly different from control treatment (AChE, CAT, LDH, Dunnett`s test $p<0.05$ after 1-way ANOVA, and GST liver Dunn's test after one-way ANOVA on Ranks). "U" mean 1 nanomol of substrate per min per mg of protein.

3.5. REPRODUCTION

During the 63 days of exposure, 7 breeding events were conducted, to assess reproductive output parameters and to obtain eggs for the embryo toxicity test. Figure 6 depicts the total number of eggs in each breeding episode (Figure 6A) and viability of those eggs (Figure 6B). The one-way ANOVA indicated that the total number of eggs produced during the experiment was not significantly different ($p=0.110$) among treatments. However, when considering the viability of eggs (Figure 6B) there was a significant decrease ($p<0.001$) between of the control and the exposed parents (10 and 10.000 $\mu g.L^{-1}$).

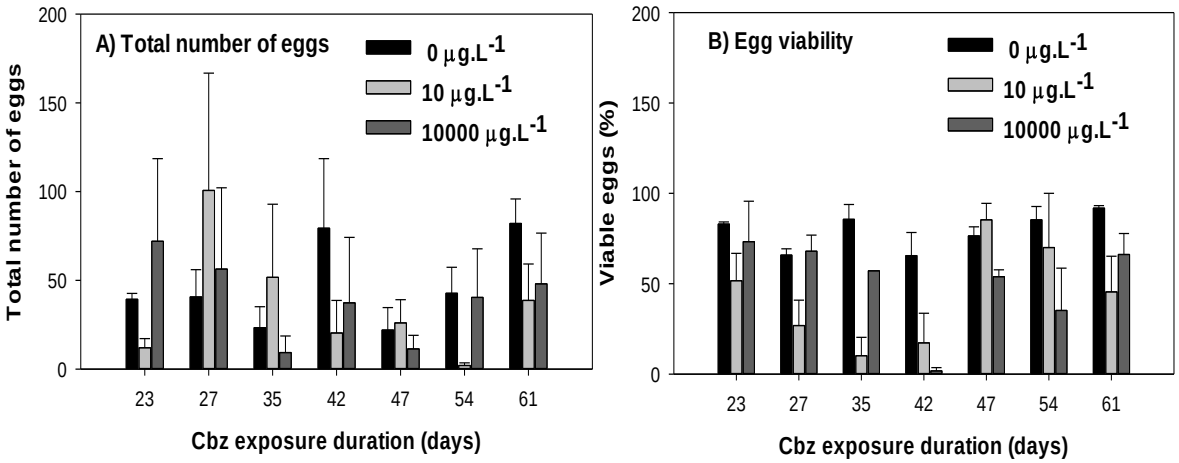


Figure 6: Total production of eggs (A) and percentage of viable eggs (B) from *D. rerio* exposed to different Cbz treatments at the different reproductive check points along the exposure period. Results are expressed as means ± standard error.

3.6. PROPRANOLOL (PROP) TOXICITY FOR D. RERIO EMBRYOS

Fertilized eggs obtained from the adults exposed to Cbz were used in FET tests to assess their sensitivity to Prop. A total of 7 FET tests were performed. No embryos/larvae died in the control groups during the 96 h exposure periods. Figure 7 shows the LC_{50} values obtained in the several tests at 48 h (Figure 7A) and 96 h (Figure 7B). The parental exposure to Cbz does not seem to significantly change the LC_{50} value calculated for embryos exposure to Prop.

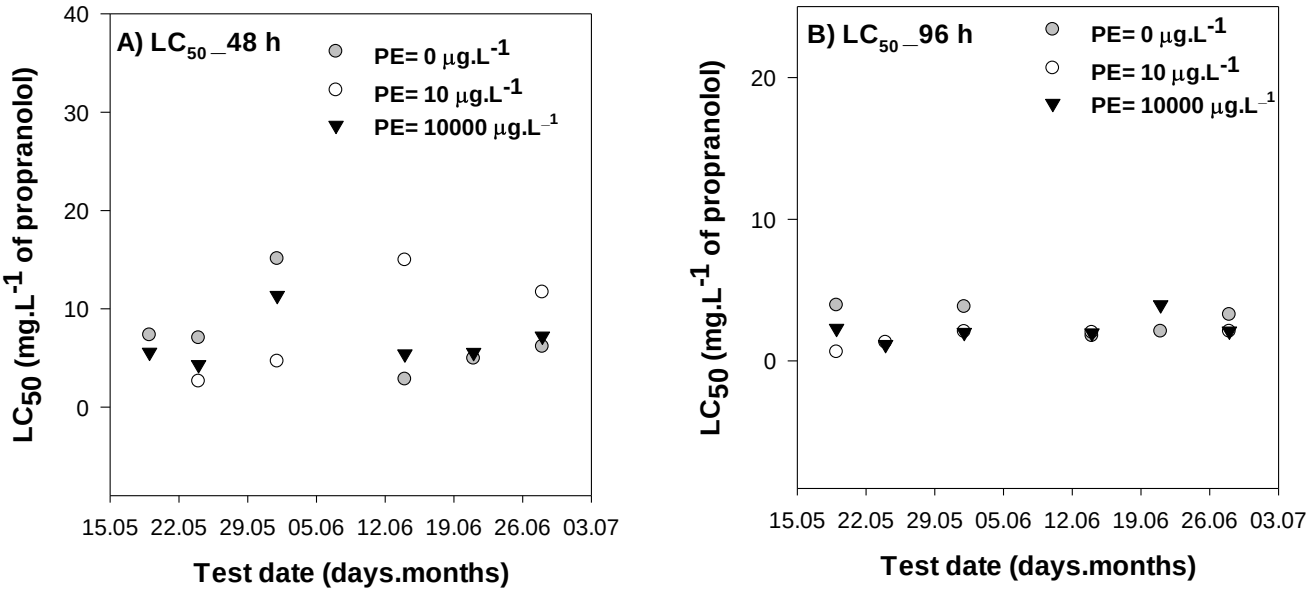


Figure 1: LC_{50} values at 48 h (A) and 96 h (B) of Prop on zebrafish embryos after exposure of parents to several concentrations of Cbz."PE" stands for Parental exposure.

For an overview of effects, Figure 8 presents the data from the several FET tests pooled together. The data shows that previous exposure of parents to Cbz did not increase the susceptibility of zebrafish embryos to Prop.

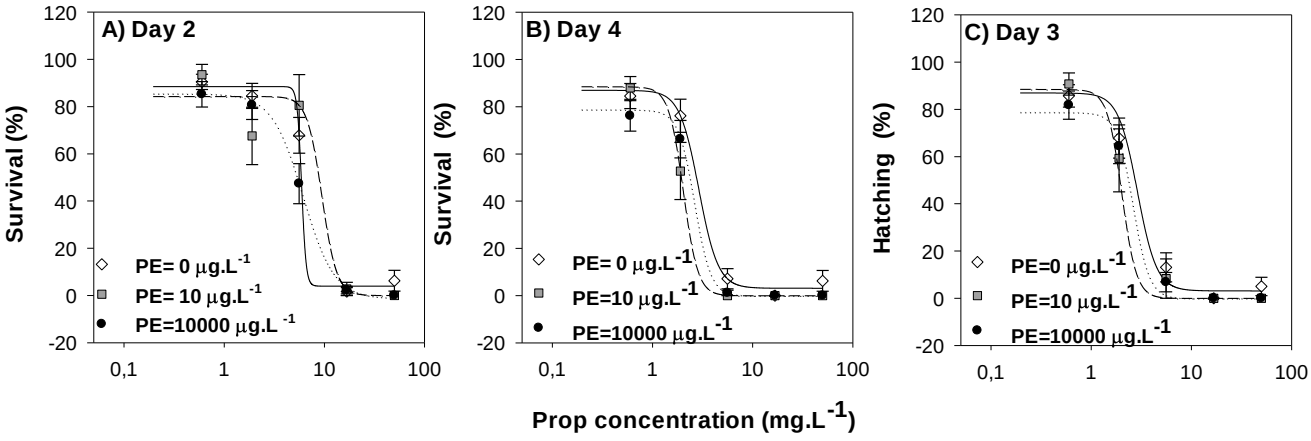


Figure 8: Susceptibility of embryos to Prop after previous exposure of parents to Cbz. Combination of all the data acquired in 8 FETs for the parameters: survival rate after 2 (A) and 4 days (B) and number of embryos hatched (C) at 3 day of exposure to Prop (0, 0.6, 1.9, 5.6, 16. 7, 50 $mg.L^{-1}$).

3.7. Acute test with Prop on adult *D. rerio*

After the 63 days exposure to Cbz, adult fish were used in an acute test (96 h) with Prop, to assess if pre-exposure changed the susceptibility of fish to a subsequent exposure to a different compound. Most notorious results (Table 1) were observed at the highest

Prop concentration (5000 µg.L^{-1}) where 100 % mortality was observed for fish previously exposed to 0 and 10 µg.L^{-1} of Cbz and 0% mortality was observed for fish previously exposed to the highest Cbz concentration (10000 µg.L^{-1}).

Table 1: Mortality (%) of fish exposed to Prop after the chronic exposure to Cbz.

Cbz pre-treatment (µg.L^{-1})	Prop (µg.L^{-1})		
	0	**1000**	**5000**
0	0	0	100
10	0	33,3	100
10000	0	0	0

4. Discussion

The occurrence of pharmaceuticals residues in the aquatic environment has raised concerns regarding potential chronic/long term effects to aquatic life. Even very low concentrations of pharmaceuticals may exert toxicity through unexpected mode of action in non-target organisms (Malarvizhi et al., 2012). In the present study, a chronic exposure to Cbz, a pharmaceutical commonly found in the aquatic environment, was performed using adult zebrafish. The effects observed at different levels (including progeny and altered susceptibility to a second chemical exposure) allow the recognition of the long-term and persistent effects of Cbz in the aquatic environment.

4.1. Effects on growth rate

In this study, Cbz induced no significant changes on fish growth regardless of the tested concentration. Similar findings were reported by Madureira et al. (2012) study that reported the lack of effects of Cbz (1780 µg.L^{-1}) on adult zebrafish weight and length after 21 days. Despite in rainbow trout (*Oncorhynchus mykiss*) has occurred a significantly decrease in fish growth exposed to Cbz (2000 µg.L^{-1}) for 42 days, which assumes that direct metabolic effect on fish and a depletion of energy resources (Li et al., 2010b). However, in our study, males showed a higher growth rate during the exposure period when compared to the growth rate in females in all groups. Growth relates feeding, assimilation and energy expenditure (Fang et al., 2009). Thus, larger fish of a certain length indicate that they are in better condition (Fang et al., 2009), while, stress conditions such as polluted aquatic

environment result in fish growth decrease (Stancova et al., 2014). Among the different factors that may influence the growth rate of zebrafish are density and behavioral interactions. High status individuals or in low density populations grow faster than low status individuals or in high density, since dominance interactions involve access resources that mediate growth (Lorenzen and Enberg, 2002; Palstra and Planas, 2012). Under non-stressful conditions female zebrafish have been reported to have a higher growth rate than males when they are in low densities or in colder waters, whereas males, in turn, develop better in warmer waters (> 35ºC) (Lawrence et al., 2008). However, in the present study, growth results more likely support the hypothesis of hierarchy behavior, since, the density and temperature were not a factor for a higher growth rate in males (See section 2.2).

4.2. Feeding behavior

In this study, the feeding behavior was evaluated as the time for the first feeding action and for total food intake after 21, 42 and 63 days of exposure to Cbz. The results show that Cbz did not change the time for the first feeding action, but increased the time for the total food intake. This result is in agreement with a study reporting the increase of the total time needed for adult *Oryzias latipes* to eat, after a 9 days exposure to 6150 $\mu g.L^{-1}$ of Cbz (Nassef et al., 2010). This effect may be related to the alteration of serotonin levels by Cbz which influences swimming speed and feeding behavior (Nassef et al., 2010). Serotonin is an important neuromodulator that interferes with hormonal and neuronal mechanisms and plays regulatory and endocrine functions. Altered levels of serotonin can induce changes in appetite, immune system, behavior and reproduction (Fent et al., 2006; Santos et al., 2010). In this context, has been reported that Cbz increases the extracellular levels of serotonin (Ambrósio et al., 2002; Yan et al., 1992) although, changes in serotonin concentrations following exposure to Cbz may vary. For instance, a therapeutic dose (25 $mg.kg^{-1}.day$) of Cbz increases the serotonergic function of the in rat hippocampus after 21 days whereas a supratherapeutic concentration (100 $mg.kg^{-1}.day$) reduces serotonin levels. Although these findings could explain changes in the feeding behavior of zebrafish after long-term exposure (63 days) to Cbz, this is a hypothesis that must be confirmed since serotonin levels were not analyzed in the present study. Alternative or complementary biological mechanisms that may be related to the feeding inhibition observed, involve the

hormone pro-opiomelanocortin which acts as a regulator of feeding and energy balance, among others. In *Salmo salar* mRNA levels of this hormone increased after 5 days treatment with Cbz 7.85 $\mu g.L^{-1}$ (Hampel et al., 2014). Changes in feeding behavior were also observed in invertebrates after chronic exposure to Cbz where decreased filtration rates (siphoning behavior) without significant change in valve closure were reported after chronic exposure (30 days) of *Corbicula fluminea* to 50 $\mu g.L^{-1}$ of Cbz (Chen et al., 2014).

Behavior parameters have been considered an important tool in monitoring the effects of chemicals and their ecological impacts. Changes in feeding and swimming behavior may have important ecological consequences due to the alteration of prey-predator relationship and decreased competitive advantage (Domingues et al., 2016). For further clarification of the effects of Cbz on the predator prey relationship associated with feeding behavior, serotonin levels may be evaluated with auxiliary tests to behavioral tests.

4.3. NUCLEAR ABNORMALITIES ASSAY

In the present study, the exposure of adult *D. rerio* to Cbz, did not increase the incidence of micronuclei or other nuclear abnormalities. In a study performed with *D. rerio* (Rocco et al., 2011), loss of DNA integrity (assessed as DNA strand breaks, was observed after 3 and 7 days exposure to 0.31 $\mu g.L^{-1}$ of Cbz, corresponding to a 65.5 and 61.0 % decreased integrity, respectively. However, after 15 days exposure a 32.9 % decrease in DNA damage was observed. The authors also evaluated the formation of apoptotic cells recording a high rate of apoptotic cells after 3 and 7 days (9.2 % and 8.9%, receptively) and a reduction (4.6%) after 15 days of exposure to Cbz (Rocco et al., 2011). Reduction of DNA damage in longer exposures suggest reparation or recovery mechanisms and justify the absence of genotoxicity verified in this work (Bolognesi and Hayashi, 2011).

These results demonstrate that as the exposure time to Cbz increases the organisms can remove from circulation the cells with damage, so the results found in this work agree with this hypothesis of recovery of organisms. The detection of genotoxicity related to acute exposure rather than chronic exposure of different organisms to Cbz can be interpreted as an adaptive process after initial stress which may result in cellular repair.

4.4. Effects of Cbz on biochemical biomarkers

AChE was increased after Cbz exposure both in the muscle and in the head samples. AChE is involved in the hydrolysis of the neurotransmitter acetylcholine into choline and acetate in the central and peripheral nervous systems (Colovic et al., 2013).

Although most frequently AChE is used as a biomarker for assessment of environmental pollution caused by neurotoxic compounds (which cause an activity inhibition) (Pfeifer et al., 2005), some chemicals cause an increase in the activity of the enzyme. In these cases, the activity increase has been related to apoptotic processes which can occur during cell development and differentiation, and after exposure insults (Zhang et al., 2012, 2002). Apoptotic processes after exposure to Cbz have already been reported in *Atlantic salmon* exposed to 7.85 $\mu g.L^{-1}$ of Cbz during 5 days (Hampel et al., 2014) and in *D. rerio* exposed to 0.31 $\mu g.L^{-1}$ of Cbz for 3 and 7 days (Rocco et al., 2011). Although AChE plays an important role in the termination of neurotransmission in cholinergic synapses, it may also participate in development, cell differentiation and pathogenic processes, however, more recently an increase in its activity in different cell lines has been detected after the apoptotic process (Zhang et al., 2002).Therefore, in the present study, AChE results more likely support the hypothesis of apoptosis due to increased activity of the enzyme in the tissues in studies.

Reactive oxygen species (ROS) are produced at low concentrations by living organisms as result of normal metabolic processes but, in the presence of environmental pollutants high concentrations can be generated, with adverse effects on cellular components such as lipids, proteins, carbohydrates, and DNA. Therefore, the equilibrium of the redox state is fundamental for the proper functioning of organisms (Birben et al., 2012). A group of antioxidant enzymes (e.g. SOD, CAT and GPx) and non-enzymatic antioxidants (e.g. GSH) play a fundamental role in the balance between oxidant/antioxidant states (Birben et al., 2012). CAT acts as first line of defense against ROS, degrading hydrogen peroxide into water and oxygen (Birben et al., 2012). In the present study, CAT activity was inhibited both in liver and gills, at the two Cbz concentrations tested, suggesting an effect in the antioxidant system. Effects of Cbz on CAT activity in fish were previously reported for rainbow trout (*Oncorhynchus mykiss*) that displayed increase in CAT

activity after 7 days exposure to 2000 µg.L^{-1}, followed by a decrease after 21 and 42 days of exposure also induced by 200 µg.L^{-1} of Cbz (Li, Z.H et al., 2010c). The authors understood that the initial increase in CAT activity may be a stress response induced by Cbz in order to neutralize ROS formation. The following reduction of CAT activity after prolonged exposure was associated to lipid peroxidation and the direct attack of ROS to proteins. The present study results suggest that in a prolonged exposure, CAT may not able to cope with the level of oxidative stress posed by Cbz. However, the present data does not allow to exclude the CAT activity may lead to the activation of other defense pathways responsible for the reduction of hydrogen peroxide, such as the GPx (Birben et al., 2012). Different results have been found in invertebrates. In the freshwater clam (*Corbicula fluminea*) exposed to 0.5, 5 and 50 µg.L^{-1} of Cbz. CAT activity was increased in the gills and digestive gland after 30 days, suggesting the increase in ROS production at concentrations of Cbz with environmental relevance (Chen et al., 2014) and supporting the hypothesis of oxidative stress as a major mode of action of Cbz in vertebrates and invertebrates.

In the present study, GST activity was increased in liver and gills of *D. rerio* while an inhibition was observed in the intestine. The liver is the main metabolic organ where antioxidant defenses are highly developed; the gills carry innumerable physiological functions such as exchange of respiratory gases, osmoregulation, excretion of nitrogen residues and maintenance of acid-base balance (Rombough, 2002) and the intestine is important for nutrient digestion and absorption (Wu et al., 2017). The inhibition of GST activity in the intestine, could be a result of decreased levels of glutathione that may be a first line of defense against ROS or a result of enzyme inhibition by high levels of ROS; suggests a higher sensitivity to inhibition in this organ whereas organ like liver and gills have an high biotransformation role and availability of non-enzymatic antioxidants like glutathione. GST belongs to the Phase II of the detoxification mechanism and is implicated in the conjugation of xenobiotics with glutathione, increasing their solubility and excretion (Vernouillet et al., 2010). GST response seems to be tissue-dependent. Like in this work (Martin-Diaz et al., 2009) found that *Mytilus galloprovincialis* exposed to 0.1 or 10 µg.L^{-1} of Cbz for 7 days showed a significant increase of GST activity in the digestive gland, whereas in the mantle and gonads no changes were recorded. In a study with *Venerupis decussate*,

an increase in GST activity was observed after exposure to 0.03 and 3 $\mu g.L^{-1}$ a decrease for 9.00 $\mu g.L^{-1}$. In the present work, the gills were the most responsive organ to Cbz in terms of GST which may be explained by its direct contact waterborne contaminants and its role in respiration, osmoregulation and in acid base balance (Li et al., 2009).

The cytoplasmic LDH is widely distributed and used as a marker of organ or tissue damage in toxicology and clinical chemistry, as it reflects the metabolic activity of a tissue and morphological and structural alterations that have an high association with pathological processes (Osman et al., 2010). Therefore, it is a powerful biomarker that is used to evaluate patterns of primary and secondary diseases in fish populations (Osman et al., 2010). In adult *D. rerio* exposed to Cbz LDH was differently affected in the tested organs. In the liver, an induction of enzymatic activity was observed while in muscle and gills an inhibition was observed. Elevation of LDH activity in gill, liver and muscle was previously observed in *Cyprinus carpio* after exposure to 5700 $\mu g.L^{-1}$ of Cbz for 35 days. The authors suggest that this increased activity could be due to the metabolic changes induced by Cbz (Malarvizhi et al., 2012). LDH activity was also increased in the blood plasma of rainbow trout (*Oncorhynchus mykiss*) after 7 days of exposure to Cbz (1, 200 and 2000 $\mu g.L^{-1}$) (Li, Z.H et al., 2010d). In the present study, results obtained in the liver samples agree with the above mentioned studies suggesting changes in the histological structure of the hepatic and extrahepatic tissues and represent damages in the tissue (Li, Z.H et al., 2010).

4.5. Reproduction

Cbz treatments had an effect in the viability but not in the number of eggs produced by zebrafish. The decreased viability of eggs supports other studies reporting decreased fecundity of fish after exposure to Cbz even trough different modes of action. Li et al. (2010a) observed decreased motility and velocity of sperm of *Cyprinus carpio* after 2 h of exposure to 2000 and 20000 $\mu g.L^{-1}$ of Cbz. Embryo production in zebrafish exposed to Cbz (0.5 and 10 $\mu g.L^{-1}$) for 6 weeks was reduced (Galus et al., 2013). The authors state that this effect may be a result of the mechanism of action of Cbz, since the Cbz causes stability in voltage gated sodium channels, reducing the excitability of neurons, this way lead to a consequently reduction of neuronal stimulation in reproductive organs and synthesis of gonadal steroids (Galus et al., 2013). However, other results have been observed, crosses

between males and females of parents exposed to Cbz indicate that exposure in males had deeper reproductive effects. In the study referenced males of F1 generation reduced the number of embryos (less 50%) when they crossed with control. Aggressive behavior of males *D. rerio* during breeding was also pointed as factor for fertility decrease rate after 6 weeks exposure to 10 $\mu g.L^{-1}$ of Cbz (Galus et al, 2014). Although fish exposure to Cbz reduced sperm quality this was not evaluated in the present study, the results found by this present agree with the one found by Galus et al. (2013) that suggests that the number and the quality of the sperms was not affected, since they were enough for the fertilization to occur, thus reducing the number of viable embryos a consequence of Cbz on reproductive organs.

The decrease in the rate of viable eggs is an important parameter supporting ecological consequences of long-term exposure of aquatic organisms to Cbz, since it may lead to a reduction in population growth.

4.6. PROPANOLOL TOXICITY FOR *D. RERIO* EMBRYOS

Embryos from zebrafish exposed to Cbz treatments present the same sensitivity towards Prop, suggesting that at the tested conditions Cbz did not elicit any trans-generational effect in zebrafish. Further studies should focus on the investigation of reproductive effects in the next generations given that effects may not be immediately detected. As an example, in the work of (Bhandari et al., 2015) transgenerational effects of Bisphenol A and 17 α-ethinyl estradiol were not detected at phenotypic level in F0 or F1 generations, but a decrease in fertilization rate was detected in the following two generations and a reduction in embryo survival was found three generations later.

In a study performed with *D. rerio* larvae, the LC_{50} values of Prop after 96 h was 2.48 $mg.L^{-1}$ (Sun et al., 2014). Huggett et al. (2002) observed LC_{50} values of 24.3 $mg.L^{-1}$ at 48 hpf in *O. latipes*. The LC_{50} values found in this study did not differ between groups of fish that were exposed to the chemicals (Cbz and Prop) and the control group, these results are in agreement with a study with *O. latipes* (Bhandari et al., 2015) where no phenotypic abnormalities after paternal exposure to toxicants was found in F1. In the study conducted by Finn et al. (2012) with *O. latipes* and *D. rerio*, adult fish exposed for 24 h had their offspring exposed after fertilization for 96 h, to the same concentrations of Prop (0.09, 1.1

and 8.3 µg.L^{-1}), to mimic environmental conditions. The lowest observed effect concentration (LOEC) concerning heart rate reduction in a study with *O. latipes* and *D. rerio* was 87 ng.L^{-1} for embryos resulting from 24 h parental exposure combined with embryonic exposure whereas embryos of unexposed parents showed no reduction in heart rate. Developmental alterations were found in *D. rerio* exposed to 0.09 µg.L^{-1} and 8.3 µg.L^{-1} Prop but not after 1.1 µg.L^{-1} concentration, whereas in *O. latipes* cardiac development was not affected (Finn et al., 2012). Overall, the present study, did not detect effects of parental exposure to 63 days in the susceptibility to Prop, in terms of survival and early life stages development. However, other endpoints should be considered in future studies (e.g. hormonal alterations, fertility).

4.7. Acute test with Prop on adult *D. rerio*

Fish exposed to the lowest concentrations of Cbz (0 and 10 µg.L^{-1}) were more susceptible when exposed to Prop (1000 and 5000 µg.L^{-1}), whereas fish previously exposed to the highest concentration of Cbz (10000 µg.L^{-1}) were able to tolerate to Prop. Exposure of fish (*Pimephales promelas*) to Prop in concentration of 3400 µg.L^{-1} over 3 days caused 100% mortality or severe toxicity requiring euthanasia because they were disorientated and, with a change in swimming behavior, with no appetite or were scenting the bottom of the tank. The authors suggest that the observed changes are like those occurring in central nervous system after exposure to toxins, and that these effects are due to the high Prop ability to cross the blood-brain barrier (Giltrow et al., 2009). Similar results was found in present study, where 100% mortality has been observed in zebrafish but only in organism previously exposed to lower concentration of Cbz (0 and 10 µg.L^{-1}). Responses of genes encoding antioxidant proteins (e.g. Cu/Zn-SOD, Mn-SOD, Cat, and GPx), genes involved in the stress response and detoxification mechanism (e.g. *hsp70, mt1, mt2 and tap*) were induced in zebrafish exposed to Prop (30, 300 and 3000 µg.L^{-1}) for 96 h, results show Prop may activate the detoxification process concentration-dependent (Sun et al., 2015). Therefore, it is possible that in present study the differences in susceptibility of adult *D. rerio* to Prop may be a consequence of the increase in GST activity detected in the liver and gills of zebrafish exposed to Cbz, as previously demonstrated in this study, which would allow better metabolization of the Prop by these organisms. On the other hand, this

increase associated with the induction of genes involved in the processes of detoxification by Prop provided to these fish better metabolic capacity, thus reducing mortality in this group.

5. Conclusion

The chronic effects of Cbz on adult zebrafish were analyzed. Cbz exerted a negative effect at the sublethal level, inducing alterations on the feeding behavior (increased time to total food intake), AChE, CAT, GST, LDH activities, reproduction (decreases in the number of viable eggs) and increased the susceptibility of *D. rerio* adults to Prop when exposed to low concentrations of Cbz. However, no significant effects were observed on parameters such as nuclear anomalies, growth rate, mortality, and susceptibility of F1 generation to Prop. Using environmentally relevant concentrations of Cbz, this study shows that Cbz may have serious consequences on the long term as demonstrated by the altered behavior and reproduction associated with the ability to alter neurotransmission endpoints and antioxidant status.

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CHAPTER 3

General Discussion

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3.1. GENERAL DISCUSSION

Pharmaceuticals have been increasingly used and are among the chemicals with higher potential to affect aquatic organisms. This capacity is related to their physico-chemical characteristics that allow the interaction of these substances with different organisms. Although a considerable amount of research is being performed on the potential impact of these substances on non-target organisms, most studies have focused on short-term exposures. Chronic studies are more realistic and allow the visualization of harmful effects at relatively low doses, since it is virtually impossible to predict the fate of xenobiotic substances with simple partitioning models (van der Oost et al., 2003).

The results from the present work confirm that Cbz can trigger different responses in adults *D. rerio* and that these organisms are excellent models for the detection of long term effects. Changes observed during this work occurred at a concentration with high ecological relevance and effects were detected at several levels of biological organization. *D. rerio* revealed high sensitivity in feeding tests, increasing the time for total food intake. This behavior is common in pathogenic conditions or during fish exposure to psychotropic agents such as appetite suppressants (Kalueff et al., 2013).

While Cbz seems to have no effect on the development of micronucleus and other nuclear abnormalities neither in the body weight of fish, biochemical biomarkers were very responsive. An increase in AChE activity was observed, possibly correlated with apoptosis occurring in the evaluated organs (Zhang et al., 2012). Inhibition of CAT activity was also observed, which is related to activation of other defense pathways responsible for the reduction of hydrogen peroxide (Birben et al., 2012). Although also responsive, effects on GST activity were organ-dependent, with an increment in liver and gills and a decrease in the intestine. LDH also presented a tissue-specific response with decreased activity in the gills and muscle, and increased activity in liver (corresponding to an activation of the anaerobic energy production pathway). Regarding reproduction effects, Cbz did not change the total number of eggs produced, but has changed the number of viable eggs. The mechanisms by which Cbz has affected the reproductive success are not yet well known.

Furthermore, Cbz exposure affected the ability of adults *D. rerio* to tolerate Prop. According with our results, in high doses of Cbz there is an increase in antioxidant activities which gives organisms the ability to tolerate exposure to Prop. This is consistent with studies performed with zebrafish where Cbz increasing the synthesis of genes involved in stress response and detoxification mechanism (Sun et al., 2015).

Altered susceptibility of F1 generation to other substances was evaluated using Prop. Results showed that the previous exposure of parents to Cbz did not change toxicity of Prop, assessed as mortality or embryo development abnormalities. This is the first analysis on the effects of prior exposure of adults *D. rerio* Cbz, to evaluate the effects of this pharmaceutical on the offspring.

In summary, data obtained in this study contribute to a better understanding of the effects of Cbz on zebrafish with numerous applications for ecotoxicological evaluation, effects were detected at environmentally relevant concentrations. Based on these results, it would be important to establish links between effects observed in the early stages of life and possible deficiencies in adult. Furthermore, taking into consideration that in the environment organisms will be exposed to contaminants their entire lifetime as will their progeny, multi-generational exposures should be performed, assessing ecologically relevant endpoints like mortality, reproduction output and behavior (e.g. swimming performance). Furthermore, epigenetic alterations should also be studied.

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