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Abstract

Pharmaceuticals are produced and used in very large volumes and their use and diversity is increasing every year. The presence of pharmaceuticals in aquatic systems is recognized as a widespread problem and monitoring these contaminants in the environment is important since their presence can affect the ecosystems even at very low levels. Wastewater treatment plants have been identified as a major route for release of pharmaceuticals in aquatic environment. The antiepileptic drug carbamazepine (CBZ) has been detected in municipal sewage and surface water samples and it potentially poses an environmental risk.

In this thesis, the effect of environmentally relevant CBZ concentrations on lipids metabolism in zebrafish (*Danio rerio*) embryo was evaluated. CBZ exposure solutions had a final concentration of 1, 5, 10 and 50 µg/L, aquarium medium and a 0.001% DMSO solution were used as controls. Embryos were exposed for 72 h and 96 h.

Mortality at the end of each exposure time was assessed. Fluorometric assays were used to measure lipids concentration. Triglyceride, free fatty acid and total and free cholesterol were chosen to represent changes in lipid metabolism in embryos exposed to CBZ.

There was no significant difference in embryo mortality at 72 h and 96 h exposure, except for exposure group with 5 µg/L CBZ. This group showed a significant higher mortality at 72 h exposure. Overall, lipid results showed that the exposure to CBZ affected the lipid metabolism both after 72 h and 96 h of exposure. At 72 h of exposure, there were significant differences in free cholesterol and free fatty acid concentrations as well. At 96 h of exposure, there were significant differences in triglyceride concentration.

Lipids perform many important functions in the organism and changes in lipid metabolism in zebrafish embryos could cause adverse effects on their growth, behaviour and reproduction These alterations can affect fish health and impact the aquatic ecosystem. The results obtained in this thesis showed that CBZ can be a possible threat for the aquatic environment. They also confirmed the necessity of further studying the effects of pharmaceuticals on the aquatic ecosystem, to better assess their ecotoxicity.

This thesis is related to the PHARMASEA project. This project studies the occurrence, distribution, fate and biological effects of pharmaceuticals in the marine environment.

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List of acronyms

AFT – acute fish toxicity test

CBZ – carbamazepine

CECs - chemicals of emerging concern

DMSO – dimethyl sulfoxide

FELST – fish early-life stage toxicity test

FET – fish embryo toxicity test

FFA – free fatty acids

HDL – high density lipoprotein

Hpf – hours post fertilization

LDL – low density lipoprotein

OECD - Organisation for Economic Co-operation and Development

PPCPs – pharmaceuticals and personal care products

REACH - Registration, Evaluation, Authorization and Restriction of Chemicals

TG – triglyceride

VLDL – very low density lipoprotein

WWTPs – wastewater treatment plants

ZFET – zebrafish embryo toxicity test

ZFNI - The Zebrafish Information Network

1. Introduction

1.1 Pollution of aquatic environments

Aquatic ecosystems cover over two thirds of our planet. The aquatic environment is composed of marine and freshwater ecosystems. Marine environments cover approximately 71% of the Earth's surface and they include oceans, estuaries, coral reefs and coastal ecosystems. Freshwater ecosystems cover less than 1% of the Earth's surface and they are made up of lentic, lotic and wetland ecosystems (Häder et al., 2020). Aquatic ecosystems perform numerous important environmental functions. They recycle nutrients, purify water, attenuate floods, stabilize global climate, increase and maintain streamflow, restore ground water, and provide habitat for wildlife and recreation for people (Häder et al., 2020; *Restoration of Aquatic Ecosystems*, 1992).

Anthropogenic activities are severely impacting aquatic ecosystems, which are also increasingly affected by global change, urban and tourism developments and the unsustainable exploitation of aquatic resources (Häder et al., 2020). Pollution of aquatic ecosystems can arise from different sources (Fig. 1). Pollution of water bodies from waste disposal and agricultural, industrial and urban runoffs has negative impacts on marine and freshwater organisms and it has been increasing in the past decades (Häder et al., 2020; Masood et al., 2016).

Water body pollution is often characterised by mixtures of contaminants, which are present in concentration ranges from a few mg/L to ng/L in environmental samples (Weichert et al., 2017). The term chemicals of emerging concern (CECs) refer to contaminants which may cause ecological impacts and are not currently regulated. This group of substances includes pharmaceuticals and personal care products (PPCPs), pesticides, veterinary products (Li, 2014). They often have persistent, bioaccumulative and toxic properties and pose a serious risk to the environment since many of them are unregulated or inadequately regulated (Li, 2014; Weichert et al., 2017). The ecotoxicological impact of CECs is not yet fully quantified. Of special ecological relevance are contaminants that are developed and released with the aim of a certain biological activity. These include, for example, pharmaceuticals such as hormones, antibiotics, painkillers or neuropharmaceuticals as well as agrochemicals, such as insecticides, herbicides or fungicides (Weichert et al., 2017). CECs and other pollutants can enter aquatic environments through different routes depending on which function they have and in which way they are excreted or disposed after usage (Fig. 1).
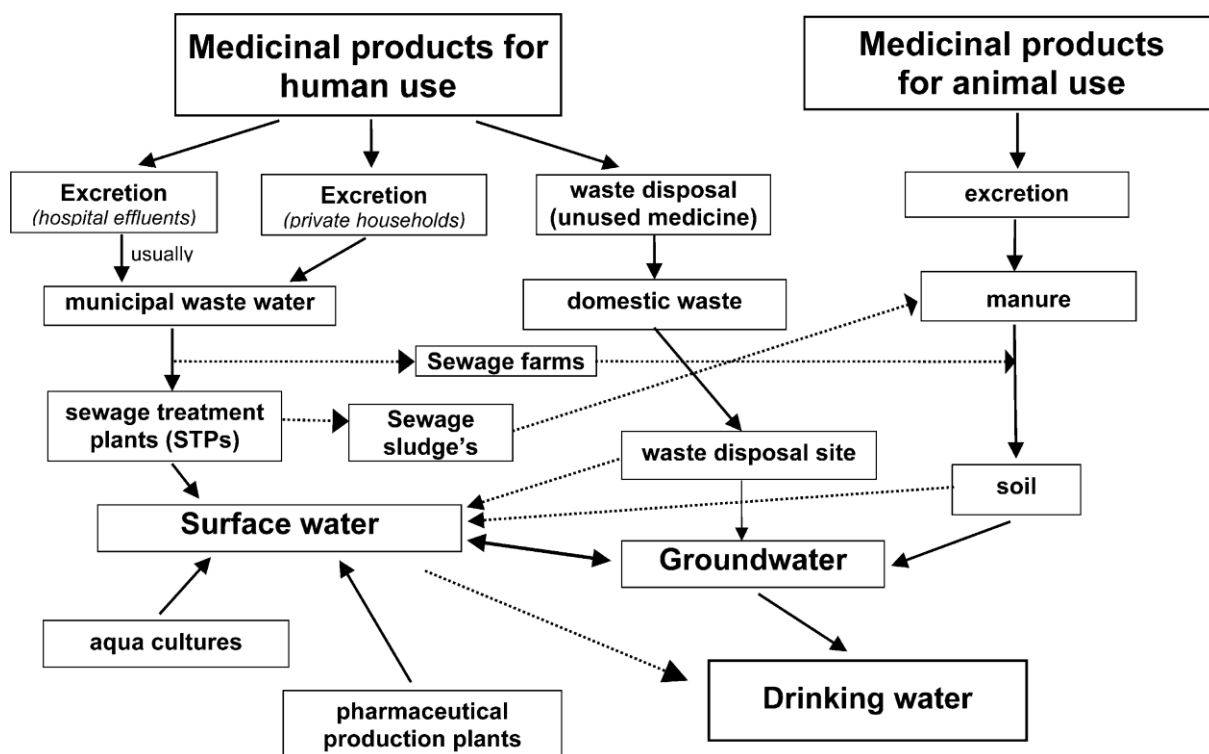
Figure 1: Scheme showing possible sources and pathways for the occurrence of pharmaceutical residues in the aquatic environment (Heberer, 2002).

1.2 Carbamazepine

CBZ was first introduced in 1963. It works by stabilizing the electrical excitability of neural membranes and it suppresses the spread of excitation to neighbouring regions of the brain (Pohlmann-Eden, 2014). CBZ is widely used in the medical field because of its remarkable efficacy (Wei et al., 2021).
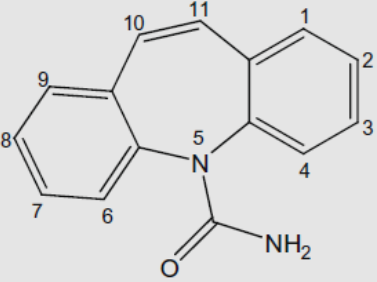
CBZ is mainly used for the treatment of epilepsy, a chronic neurological disease that affects around 50 million people worldwide, but also for the treatment of trigeminal neuralgia, bipolar disorder, chronic pain, depression and other neurological disorders, arrhythmia and alcoholism (Ferrell & McLeod, 2008; Pohlmann-Eden, 2014; Wei et al., 2021).

Multiple mechanisms of action for CBZ have been proposed. CBZ is likely to act both presynaptically, to block release of neurotransmitter by blocking firing of action potentials, and postsynaptically, by blocking the development of high-frequency repetitive discharge initiated at cell bodies (Levy et al., 2002). These combined presynaptic and postsynaptic effects are likely to form the basis of the anticonvulsant actions of CBZ (Levy et al., 2002).

CBZ is a tricyclic aromatic compound (Tab. 1). CBZ is a lipid-soluble drug that is 65% to 80% bound to plasma proteins, and its cerebrospinal fluid concentrations vary from 19% to 33% of total plasma concentrations (Levy et al., 2002). CBZ is administered to adults in doses of 10 to 20 mg/kg/day to achieve total plasma concentrations of 4 to 12 µg/mL (Levy et al., 2002). Approximately 72% of orally administered CBZ is absorbed, while 28% is unchanged and subsequently discharged through the faeces (Tab. 1) (Y. Zhang et al., 2008). After it is

absorbed, CBZ is heavily metabolized by the liver: ca. 95% of the compound is transformed while only about 1% of dosage leaves the body in an unaltered form (Rodrigues et al., 2023; Y. Zhang et al., 2008). The metabolites of this drug undergo enterohepatic cycling and are excreted with urine (Y. Zhang et al., 2008).

Table 1: Physical, chemical and pharmacological properties of carbamazepine (modified from (Y. Zhang et al., 2008))

Carbamazepine (CBZ)	
Pharmacology Structure, formula, CAS No. and molecular weight	 $C_{15}H_{12}N_2O$ 298-46-4 236.27 g mol^{-1}
Usage	Analgesic, antiepileptic
Water solubility[a]	17.7 mg L^{-1} (25 °C)
Log$P$ (octanol–water)[a]	2.45
Henry's Law Constant[a]	1.09×10^{-5} Pa m^3 mol^{-1} (25 °C)
pKa	neutral[b]
Elimination half-life[c]	25–65 h
Excretion[d]	72% of oral dosage excreted in urine, 28% in faeces

1.3 Presence of carbamazepine in the environment and wastewater treatment plants

The antiepileptic drug CBZ, frequently detected in municipal sewage and surface water samples, belongs to the medium range to high-risk class of pollutants, as it poses potential human health risk being repeatedly detected in drinking water (Heberer, 2002; Tahir et al., 2021). CBZ residues may enter aquatic environments through the effluents of wastewater treatment plants (WWTPs), urban runoff or domestic and industrial wastewater discharges (Clara et al., 2004; Masood et al., 2016). Due to its low degradation rate and high persistence, CBZ is resistant to biodegradation at low concentrations, in either salt or fresh waters, and it is very slowly degraded by sunlight (Almeida et al., 2014; Teo et al., 2016).

Excessive intake of CBZ by animals can cause tachyarrhythmias and seizures (Wei et al., 2021). Reported negative effects of CBZ on fish include disruption of locomotion, feeding and growth (Rodrigues et al., 2023).

CBZ global production and consumption are high, due to high dosage and chronic usage (Teo et al., 2016). The annual consumption of CBZ is around 1000 tons, and approximately 3% of the consumption is released into the water environment each year (Xin et al., 2021). Analysis of the effluents in different European WWTPs uncovered that more than 75% are not able to

remove this drug efficiently (Braeutigam et al., 2012). Investigations of influent and effluent samples from different WWTPs have shown that less than 10% is removed during the treatment (Heberer, 2002). Therefor CBZ has been proposed as an anthropogenic marker in water bodies (Y. Zhang et al., 2008).

The disposal of unused medication via the toilet seems to be of minor importance, however many of the pharmaceuticals applied in human medical care are not eliminated by the human body and reach aquatic recipients through wastewater. Often, they are excreted only slightly transformed or even unchanged, mostly conjugated to polar molecules. These conjugates can easily be cleaved during sewage treatment and the original chemicals will then be released into the aquatic environment mostly by effluents from WWTPs (Heberer, 2002).

WWTPs are primarily designed to reduce the amount of organic matter, nitrogen and phosphorous in wastewater. The level of technology of WWTPs vary significantly between countries and regions, but primary, secondary and tertiary treatment are the basis of how traditional WWTPs work (Csuka, 2017; Magnusson et al., 2016). In a WWTP water is cleaned using mechanical, biological and/or chemical cleaning steps (Boye, 2009). In primary treatment, sewage entering WWTP flows through a screen, which removes large solid objects. It then passes into another chamber, where sand and small stones settle to the bottom while fat and oils accumulate on the surface. Organic and inorganic matter along with other suspended solids can be removed in a sedimentation tank (Boye, 2009). Secondary treatment of wastewater use oxidation to further purify wastewater; this can be done by using biofiltration, aeration or oxidation ponds. (Csuka, 2017). Biofiltration uses sand filters, contact filters, or trickling filters to remove additional sediment from wastewater (Csuka, 2017). In aeration, sewage is pumped into an aeration tank, where it is mixed with air and bacteria rich sludge. The bacteria break down the organic matter and the sludge accumulating at the bottom of the tank can be used again (Boye, 2009). Oxidation ponds are typically used in warmer places and utilizes natural water bodies like lagoons. Wastewater pass through the water body for a period of time and is then retained for two to three weeks (Csuka, 2017). To complete secondary treatment, water is disinfected with chlorine, UV light or ozone before being discharged into receiving water (Boye, 2009). Tertiary treatment is generally used to remove phosphates and nitrates from the water. Substances like activates carbon and sand are among the most used materials in this process (Csuka, 2017).

For environmental risk assessments of CECs, it is important to utilise sensitive bioassays with ecologically relevant endpoints. Bioassays utilising adult or early life stages of fish offer a broad variety of possible endpoints. Acute lethal and sublethal effects of substances can be detected in short-term tests, such as the acute fish toxicity test (AFT) or the fish embryo toxicity test (FET). In long-term tests, like the fish early-life stage toxicity test (FELST), chronic endpoints can be assessed (Weichert et al., 2017)

1.4 Zebrafish as model organism

The introduction of chemicals into the environment by human activities represents a serious risk to environmental and human health. Therefore, current legislation needs appropriate data on risk assessment for the registration of chemicals, pesticides, biocides and pharmaceuticals. These data include information on the toxicity at various trophic levels and require testing with vertebrates, in particular with fish (Scholz et al., 2008). Acute fish toxicity data are part of the base set data requirements in the hazard and environmental risk assessment and it is usually determined according to OECD (Organisation for Economic Co-operation and Development) test guideline (Busquet et al., 2014).

The rising amount of experimentation utilizing animals throughout the last decades demands the active development of alternative methods and assays. Prerequisite to any biological assay utilizing animals is consideration about its necessity, feasibility, ethics and the potential harm caused (Bauer et al., 2021). In an effort to reduce animal tests while maintaining the required standards for the security of new chemicals, development and implementation of alternative and new approach methods has advanced within the last years, driven by modern legislations for chemical control as REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the EU Cosmetics Regulation and the current EU Animal Welfare Regulation (Brotzmann et al., 2022). Animal welfare legislation in Europe demands the incorporation of the 3Rs principles (replacement, reduction, refinement) into regulatory frameworks and emphasises the need for development and validation of alternative methods (Busquet et al., 2014). Fish embryo assays are considered pain-free *in vivo* tests and they are accepted as a replacement of animal experiments (Q. Zhang et al., 2015).

One of the first use of zebrafish (*Danio rerio*) embryos in environmental science was to develop an alternative to the 96-h AFT (Scholz et al., 2008). Zebrafish embryo toxicity test (ZFET) is suggested to replace toxicity in adult fish, since acute toxicity in fish embryos correlates very well with acute toxicity in adult fish (Delov et al., 2014). An advantage of the ZFET, as well as embryo testing in general, *versus* standard fish toxicity test is that it allows simultaneous screening of many chemical substances because embryos can be manipulated and exposed in multiwell plates, thereby reducing the time, cost, and effort (Glaberman et al., 2017). Other advantages are that offspring are produced in abundance and develop rapidly and experimentation in fish embryos at earliest life stages can be performed without being regulated as animal experiments (Bauer et al., 2021; Glaberman et al., 2017). Analysis of acute toxicity in embryos can also include the screening for developmental disorders and specific and subchronic toxicities like teratogenicity or neurotoxicity (Fig. 2) (Delov et al., 2014; Scholz et al., 2008). Fish embryos are excellent models for studies aimed at the understanding of toxic mechanisms and the indication of possible adverse and long-term effects (Fig. 2). Morphological and sub-lethal endpoints, such as changes in heartbeat, spontaneous movements and hatching rate have the potential to give information on the mode-of-action and may also indicate long-term effects of chemicals (Scholz et al., 2008). Toxicokinetic—the rate of absorption, distribution and excretion—and toxicodynamic—the (molecular) mechanism of action—are important parameters for risk assessment, since they indicate potential subtle and long-term effects and determine the effective, internal concentration of a toxic compound (Scholz et al., 2008).
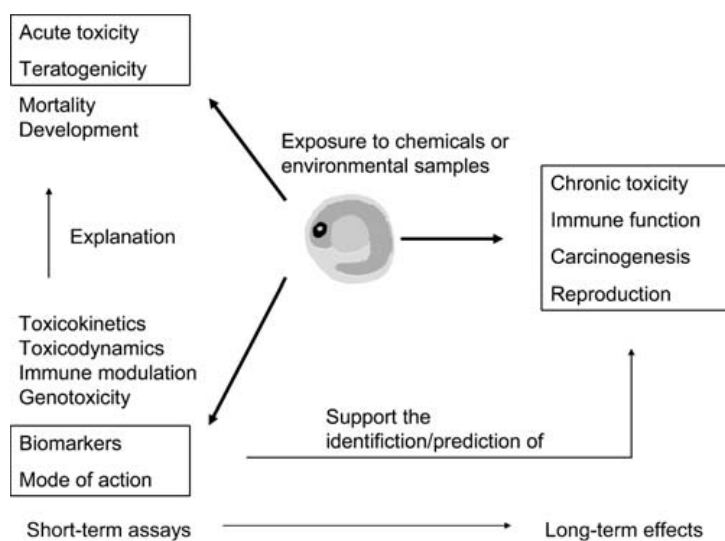
Figure 2: Summary of approaches and possible (future) applications of the fish embryo model for the integrative risk assessment of chemicals (Scholz et al., 2008)

Zebrafish is a small tropical freshwater fish which lives in river basins of India, Northern Pakistan, Nepal, Bhutan, and South Asia. Adults are about 2.5–4 cm long (Bozkurt, 2020). Males appear thinner than females with a more gold or yellow colour on their side. Females can be recognized by their bigger bellies, where they store eggs. Zebrafish have a very short reproduction cycle, and they reach maturity at the age of about 3 months. One female can spawn about 100 eggs per day which are fertilised by sperm release of the male into the water. Under laboratory conditions several thousand embryos can easily be produced daily and used for parallel experimental treatments (Scholz et al., 2008). Embryogenesis is completed in 72 h. and pigmentation in the embryos starts about 30–72 h post fertilization (Bozkurt, 2020).

The exponential growth of zebrafish as model organism in different research fields is due to several favourable features such as its fast life cycle, high fecundity, transparency of the embryonic/larval stages which facilitates the observation of the development, low maintenance cost, and easy handling (Bozkurt, 2020; Brotzmann et al., 2022). They show rapid development, hatches in less than 3 days and become mature in 90 days (Bozkurt, 2020). Moreover, they have high genetic similarity and homologous physiology with human beings and other vertebrates especially in terms of the central nervous system (Bauer et al., 2021; Bozkurt, 2020).

Zebrafish has been gaining attention for toxicological assays, either of administered compounds or those present in the environment, as well as in drug discovery due to its physiological response and histological features similar of those of mammals, making it a potentially important alternative for in vivo testing in rodents used in the laboratory routine (Bozkurt, 2020; Driessen et al., 2013). The major organs accountable to metabolize and excrete xenobiotics are the liver and the kidney. The liver of zebrafish has disorganized hepatocytes and lacks Kupffer cells accountable to phagocyte foreign bodies (Bozkurt, 2020).

1.5 Lipid metabolism

Lipids are a group of organic chemicals present in all animals and plants (Waisundara & Jovandaric, 2020). They are soluble in organic solvents and usually contain fatty acids esterified to alcohol groups in the case of the glycerides, and to amino groups in the case of the sphingolipids (Tocher, 2003). Lipids are an important source of metabolic energy for growth, reproduction and movement (Tocher, 2003). They also play essential roles in cells as signalling molecules, membrane components and sources of fuel (Anderson et al., 2011).

Lipid profiling offers a useful perspective on whole organismal fitness since these molecules serve many important functions (Dreier et al., 2021). During the first four days of development, a zebrafish embryo relies on its yolk sac for the nutrients needed to sustain its growth and survival. Yolk sac lipids are the source of essential fat-soluble vitamins, triacylglycerol and cholesterol. Once the circulatory system forms, yolk, hepatic, and intestinal lipids are transported by lipoproteins to specific target tissues via the bloodstream (Anderson et al., 2011). The liver in zebrafish begins to form at 48 hours post fertilization (hpf) and starts metabolic functions at 72 hpf, commencing to undergo molecular synthesis and metabolism (Bai et al., 2021).

The liver is the central organ for fatty acid metabolism and control of lipids homeostasis (Alves-Bezerra & Cohen, 2017). Precise control of the lipid process (including lipid accumulation, lipogenesis, fatty acid β-oxidation, cholesterol synthesis, and metabolism) is necessary to maintain lipid homeostasis. Disordered homeostasis can lead to obesity, malnutrition, endocrine disruption, or other metabolic associated diseases (Teng et al., 2019).

Triglycerides are esters of glycerol and they represent the major form of storage and transport of fatty acids within cells and in the plasma (Alves-Bezerra & Cohen, 2017; Waisundara & Jovandaric, 2020). Fatty acids can be released from triglycerides and oxidised to provide the energy required for cells life (Waisundara & Jovandaric, 2020). Free fatty acids (FFA) are lipids released from the adipose tissue and several cell types upon lipolysis (Rodríguez-Carrio et al., 2017). They are essential nutrients that contribute to various cellular functions. FFAs play an important role as energy storage molecules, structural components, signalling molecules, regulators in gene expression and energy fuel homeostasis and they can also act as mediators between metabolic conditions and the immune system (Hara et al., 2014; Kimura et al., 2020; Rodríguez-Carrio et al., 2017).

Cholesterol is a structural component of cell membranes, but it serves also as precursor molecule for making steroid hormones, bile acids and vitamin D (Mu et al., 2015; Price et al., 2022; Waisundara & Jovandaric, 2020; Zampelas & Magriplis, 2019). Steroid hormones are important for organization and development of sexual organs, behaviour, immune function, growth, and response to stress (Price et al., 2022). Cholesterol can be esterified or free. Free cholesterol is biologically active and has cytotoxic effects while cholesteryl esters are found mostly in tissue and are a protective form of cholesterol for storage in the cells and transporting in plasma (Bagheri et al., 2018; Waisundara & Jovandaric, 2020). Altered cholesterol homeostasis has been implicated in disease generation in fish and mammals. Zebrafish is often been used as a model organism for the study of cholesterol-related

diseases in vertebrates, demonstrating that appropriate regulation of cholesterol is essential for organogenesis during embryonic development (McGruer et al., 2021).

1.6 Aim of the study

The aim of this thesis was to investigate the effects of environmentally relevant concentrations of CBZ on the lipid metabolism of zebrafish embryos. Triglyceride, free fatty acid and total and free cholesterol were chosen to be analysed in order to represent lipid metabolism. To reach the thesis objective, zebrafish embryos were exposed to 4 different CBZ concentrations for 72 h and 96 h and then the lipid composition was analysed via fluorometric technique using three different assay kits from Abcam. The hypothesis to test was the significant difference in lipids concentration between embryos exposed to CBZ and the ones exposed to control solutions with aquarium medium and 0.001% DMSO. If a statistical difference was observed, it means that CBZ can affect embryo metabolism and potentially development in zebrafish.

2. Materials and methods

2.1 Zebrafish husbandry

Adult wild type zebrafish were maintained in laboratory aquaria at 28 °C with a 14:10 h light-dark photoperiod. Fish were kept in clear 4.5 L polycarbonate tanks with continuous water circulation in Iwaki Aquatic LAbREED™ ITS-Z Zebrafish System. Water that circulates the system has been filtered via reverse osmosis, UV sanitation, and mechanical (50 μM) and activated carbon filters. Conductivity, pH, temperature and water level were monitored. Water conditions were set in accordance with The Zebrafish Information Network (ZFIN), an internationally recognized resource for zebrafish rearing conditions and maintaining a healthy brood stock. Water quality parameters (ammonia, nitrate, nitrite) were monitored daily.

Zebrafish were fed twice a day once with Artemia and once with commercial dry feed (GEMMA Micro 300, Skretting).

2.2 Spawning and embryo collection

Embryos were obtained by natural spawning of the adults. Male and female individuals (1:1 sex ratio) were transferred into breeding tanks (Fig. 3) in the afternoon and kept divided until morning in an incubator at 28 °C to obtain embryos. Adults were let spawning for 1 hour before collecting the embryos. Immediately following spawning events, embryos were collected and placed into petri dish. Eggs were checked under a light microscope (WPI SMARTPZM Teaching Microscope System) with zoom range between 0.67X and 4.5X (Fig. 3). Fertilized eggs were divided from unfertilized eggs and then transferred to exposure medium in 6-wells plates.
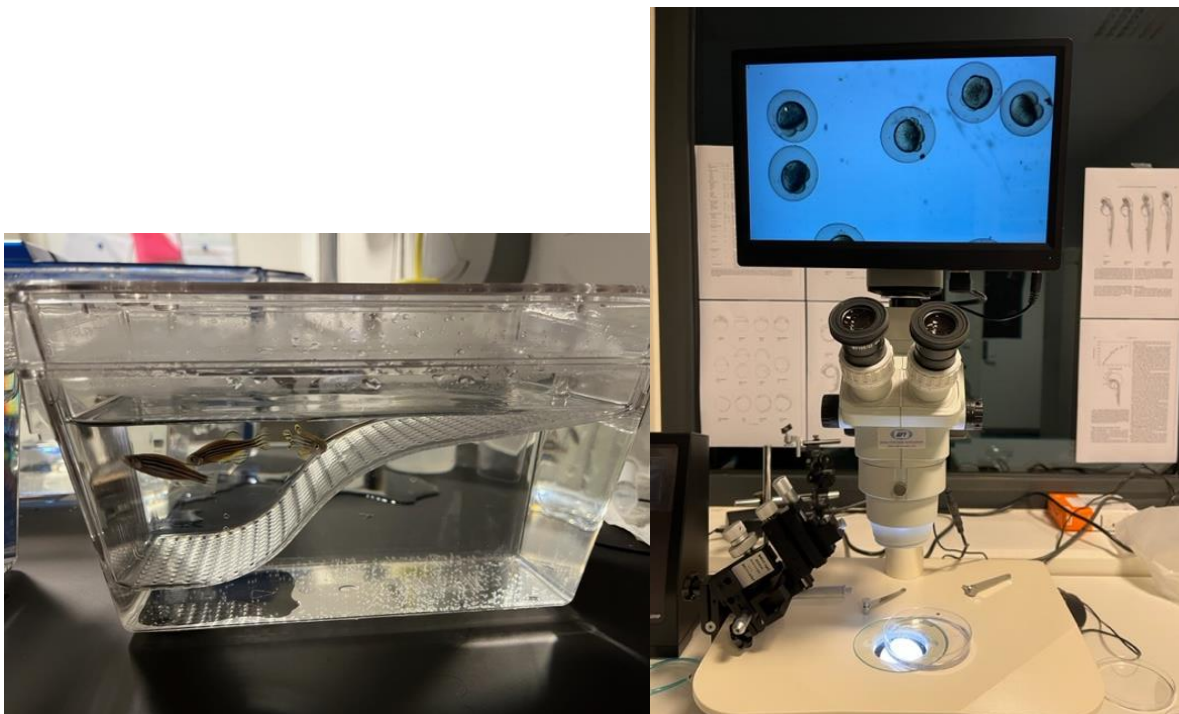
Figure 3: Breeding tank before embryos collection (left) and separation of fertilized and unfertilized eggs using a microscope (right).

2.3 Chemicals and exposure solutions

CBZ (CAS nr: 298-46-4; Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) to make a 5 mg/ml stock solution. The stock solution was diluted with water to prepare the exposure solutions.

Exposure solutions had a final concentration of 1, 5, 10 and 50 µg/L. DMSO concentration was less than 0.001% in all treatment groups.

2.4 Drug treatment

For the exposure experiment sterile non-treated 6-wells plates were used (Fig. 4). Each well contained 30 embryos and ca. 10 ml medium.

Figure 4: Sterile non-treated 6 wells plate used for the exposure experiment.

Eggs were collected immediately after spawning and fertilized eggs were stored in a clean petri dish with aquarium water. Embryos were then transferred to 6-wells plates. The exposure was initiated at < 4 hpf.

Embryos were exposed for 72 h and 96 h to different CBZ solutions.

For each experiment, 3 parallels (biological replicates) per exposure group and timeframe were made. Each well contained 30 embryos. Two control groups were present: one treated with 0.001% DMSO (the CBZ carrier) and one containing only distilled water.

Solutions were changed every 24 h using glass Pasteur pipettes. The 24 h. old medium was collected and stored at -20 °C for future chemical analysis.

After 72 h and 96 h, embryos were collected into cryo tubes and snap frozen in liquid nitrogen before being stored at -80 °C till further analysis (Fig. 5).

Figure 5: Cryo tubes after snap freezing before being stored at -80 °c. They contain embryos from all concentrations and parallels for both 72 and 96 h. exposure.

2.5 Lipid metabolism assay kits

The effect of CBZ on embryos' metabolism was investigated using three different assay kits. These kits use fluorometric techniques to assess the energetic metabolism by the quantification of four lipids: triglycerides (Triglyceride Assay Kit – Quantification, Ab65336/ K622-100, Abcam), total cholesterol and free cholesterol (Cholesterol Assay Kit – HDL and LDL/VLDL, Ab65390, Abcam) and free fatty acids (Free Fatty Acid Assay Kit – Quantification, Ab65341/K612-100, Abcam).

The protocols and reagents used for these assays were the ones given by the company. The concentration and activities of each assay were obtained by using of standards. A trial test was made for each assay before starting to analyse samples to establish the adjustments needed. Different samples volumes were tested before starting the assay to determine the best initial volume for each assay. The optimal sample volume to use in the well plate was also assessed before starting the assay.

Samples from the exposure experiment were left in ice for around an hour to allow them to warm up after being stored at -80 °c. They were then washed with 500 µL cold PBS and homogenized using an Omni tissue homogenizer for 10 s. The homogenizer was cleaned between each sample using water and ethanol. The homogenized samples were then moved

in new Eppendorf tubes and used for the assay or stored in a freezer at -80 °c. 100 µL of sample was used as start volume for every assay.

The output for the fluorometric assay was measured at Ex/Em = 535/587 nm using a SpectraMax iD5 microplate reader connected to the SoftMax Pro 7.1.2 data acquisition and analysis software from molecular devices.

2.51 Triglyceride Assay

In the triglyceride assay, triglycerides are transformed into to free fatty acids and glycerol. Glycerol is then oxidized and generates a product which reacts with leading to the production of colour (spectrophotometry at λ= 570 nm) and fluorescence (Ex/Em = 535/587 nm) (*Triglyceride Assay Kit - Quantification (Ab65336/K622-100) | Abcam*, n.d.).

Homogenized samples were diluted with 1 ml 5% NP-40 substitute/ddH2O solution. Samples were heated in a warm bath for 5 min. until the solutions became cloudy. They were then cooled down to room temperature and this step was repeated one more time to solubilize all triglycerides. Samples were centrifuged at top speed for 2 min. and the supernatant was 10-folds diluted using ddH2O. 20 µL of sample was added into each sample wells and volume was adjusted to 50 µL/well using Assay Buffer. Each sample was analysed using 3 technical replicates, while 5 technical replicates were used for standard dilutions. Average of 5 technical replicates of standard dilutions readings of 5 out of 6 standards were used to create a standard curve. Standard number 6 was removed, following Abcam's suggestion, to give a better r^2 value. r^2 value for the standard curve was 0.978 (Fig. 6), which is higher than 0.95 which is the minimum recommended value from Abcam. The trendline equation was calculated based on the standard curve data. Triglyceride amount in sample wells was found using the trendline equation. Concentration of triglyceride in nmol/µL (mM) in the test samples is calculated as:

Triglyceride concentration = (B/V) * D

Where:

B = amount of triglyceride in the sample well calculated from standard curve in nmol.
V = sample volume added in the sample wells (µL).
D = sample dilution factor if sample is diluted to fit within the standard curve range.
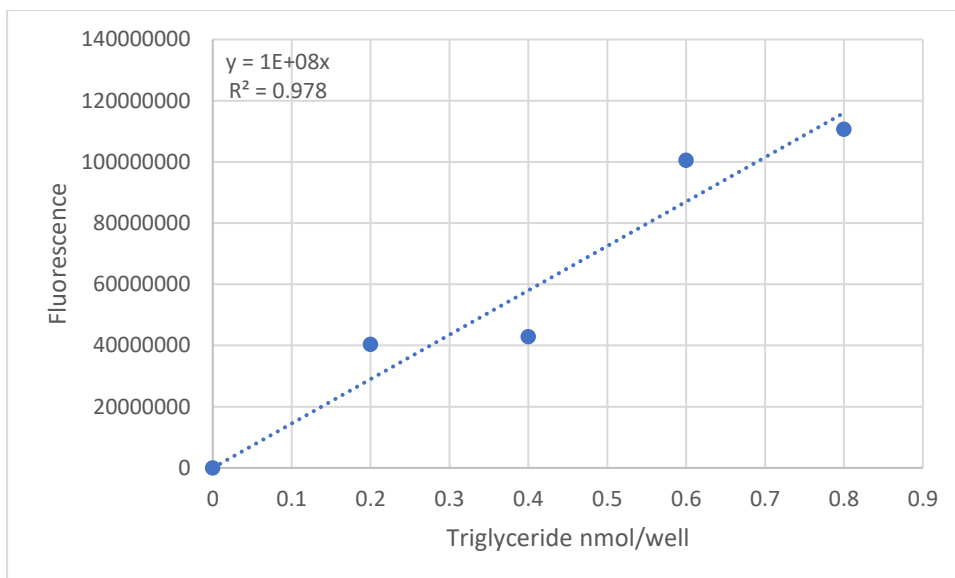
Figure 6: Triglyceride assay standard curve. Graph shows r² value and trendline equation for standard curve.

2.52 Cholesterol Assay (HDL and LDL)

In this assay, cholesterol oxidase acts on free cholesterol to produce a chemical which reacts with a probe to generate fluorescence (Ex/Em = 538/587 nm) (*Cholesterol Assay Kit - HDL and LDL/VLDL (Ab65390) | Abcam*, n.d.).

Homogenized samples were mixed with 100 µL of Cholesterol Assay Buffer and were centrifuged for 10 min. at 4 °c at 13 000 xg to remove insoluble material. Supernatant was collected in a new tube and used to measure total and free cholesterol. In the samples used it was not possible to divide HDL and LDL/VLDL fractions. 20 µL of sample was added into sample wells for free cholesterol and total cholesterol and the volume was adjusted to 50 µL/well using Assay Buffer. 50 µL of Total Cholesterol Reaction Mix was added into each standard and total cholesterol sample wells, while 50 µL of Free Cholesterol Reaction Mix was added into free cholesterol sample wells. Total Cholesterol Reaction Mix contains cholesterol esterase which hydrolyse cholesteryl ester to free cholesterol. If esterase is not added the assay does not detect cholesteryl ester and measure only free cholesterol. Each sample was analysed using 3 technical replicates for both free cholesterol and total cholesterol, while 5 technical replicates were used for standard dilutions. Average of 5 technical replicates of standard dilutions readings of 6 standards were used to create a standard curve. r² value for the standard curve was 0.999 (Fig. 7), which is higher than the minimum recommended value from Abcam. The trendline equation was calculated based on the standard curve data. Cholesterol amount in wells was calculated using the trendline equation. Concentration of cholesterol (µg/µL) in the test samples is calculated as:

Cholesterol concentration = (AV * D) * DF

Where:
A = amount of cholesterol in the sample well calculated from standard curve (µg).

V = sample volume added in the sample wells (µL).

D = sample dilution factor: for total cholesterol = 1.

DF = additional dilution factor if sample has been diluted further to fit within standard curve range.
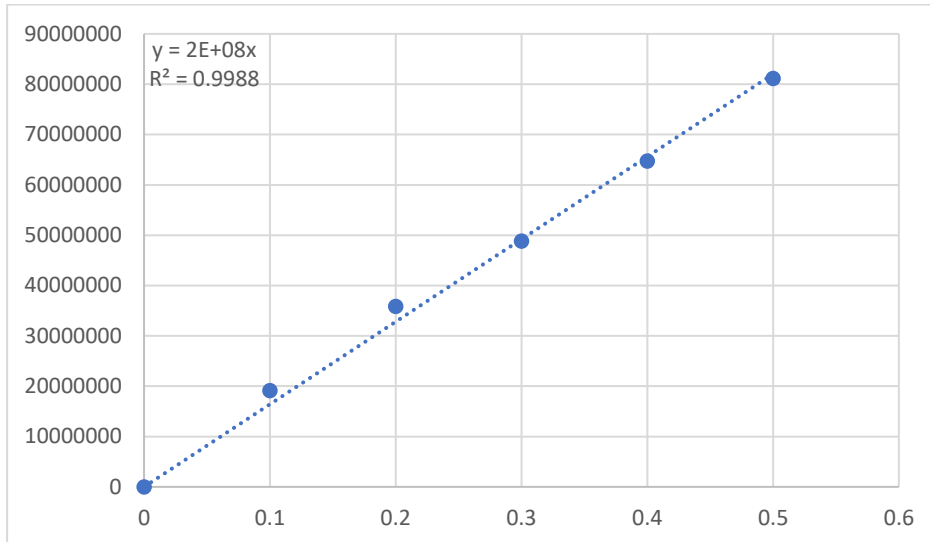
Figure 7: Cholesterol assay standard curve. Graph shows r^2 value and trendline equation for standard curve.

2.53 Free Fatty Acid Assay

In the free fatty acid assay protocol, fatty acids are converted to their CoA derivatives (coenzyme A), which are subsequently oxidized, leading to formation of colour/ fluorescence (*Free Fatty Acid Assay Kit - Quantification (Ab65341/K612-100) | Abcam*, n.d.). Fatty acids can then be quantified by either colorimetric (λ= 570 nm) or fluorometric (Ex/Em= 535/587 nm) methods. Palmitic acid is used to generate a standard curve.

Homogenized samples were dissolved in 200 µL 1% Triton X-100/chloroform and incubated on ice for 30 min. They were then centrifuge at top speed for 10 min. Organic phase (lower phase) was collected and air dried at 50 °c in a fume hood for ca. 30 min. to remove chloroform. Samples were then vacuum dried for 30 min. and the dried lipids were dissolved in 200 µL Fatty Acid Assay Buffer. 20 µL of sample was added into sample wells and the volume was adjusted to 50 µL/well using Assay Buffer. Each sample was analysed using 3 technical replicates, while 5 technical replicates were used for standard dilutions. Average of 5 technical replicates of standard dilutions readings of 6 standards were used to create a standard curve. T test confirmed that one technical replicate value for standard number 1 was outside of the confidence interval. This value was not used to calculate the mean reading for standard 1. r^2 value for the standard curve was 0.981 (Fig. 8), which is higher than the minimum recommended value from Abcam. The trendline equation was calculated based on the standard curve data. Fatty acid amount in sample wells was found using the trendline equation. Concentration of Free Fatty Acid in the test samples is calculated as:

Fatty acid concentration = (Fa/Sv) * D

Where:
Fa = amount of fatty acid in the sample well calculated from standard curve (nmol).
Sv = amount of sample volume added in sample wells (μL).
D = sample dilution factor.
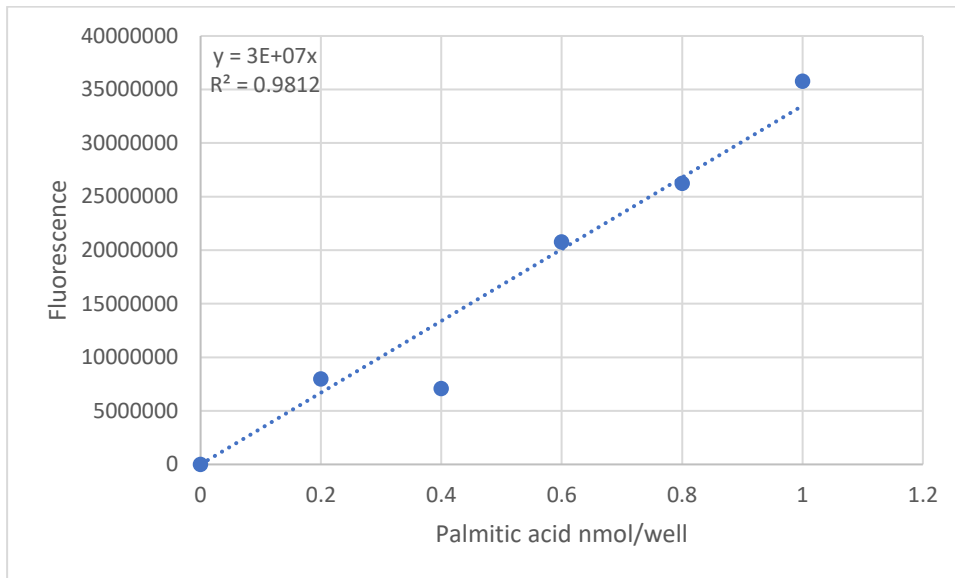
Figure 8: Free fatty acid assay standard curve. Graph shows r^2 value and trendline equation for standard curve.

2.6 Statistical analysis and data plan

SPSS (version 28) was used for running statistical analysis. Levene's test was used to assess the homogeneity of variance between samples. A two-way ANOVA was used to compare triglycerides concentration between exposure groups and time, while non-parametric independent sample Kruskal-Wallis H test was used to compare free fatty acid and cholesterol concentrations based on exposure groups. Two separate tests were run for exposure time for non-parametric data. Pairwise comparison was used to find statistically significant difference between samples. T-test was used to identify and remove outliers for all data. Boxplots are used to visualize the results. A p-value < 0.05 was used as level of statistically significant difference.

It was chosen to represent lipids concentration results in nmol/ μL and μg/μL. Results are also represented in nmol/embryo and μg/embryo so that they could be compared to other findings. Results expressed in nmol/embryo and μg/embryo take into consideration the different mortality rate and the different number of embryos present in each biological replicate. Non-parametric independent sample Kruskal-Wallis H test was used to compare lipids concentration for results expressed in nmol/embryo and μg/embryo. Pairwise comparison was used to find statistically significant difference between samples.

3. Results and discussion

3.1 Embryos mortality

The embryo mortality was recorded at the end of the first exposure (Fig. 1 and Tab. 1, Appendix). OECD guidelines establish that the mortality rate in the control group has to be ≤ 10 % (OECD, 2013). Fertilization rate is the number in percentage of fertilized eggs, while mortality rate shows the number of fertilized eggs that did not developed int embryos. The control group for the 96 h exposure had a mortality rate of 13 %, which is higher than what recommended on OECD guidelines. Therefore, the exposure was repeated.

Embryos from two different spawning days were used for the second exposure. The fertility rate was 99% and 94%, which was considered satisfactory to proceed with the planned experiment. The fertilization rate was measured by counting the number of fertilized and unfertilized eggs 1 h after spawning event. Mortality rate for the control groups for the second exposure was lower than the recommended OECD value (Fig. 9). Therefore, it was chosen to utilize only embryos from the second exposure for further analysis.

Mean mortality and standard deviation was calculated for each group (Tab. 2, Appendix). The mortality registered in this experiment resembles what was previously observed in other studies (Pohl et al., 2019; Ribbenstedt et al., 2022). In the negative control mortality of 3.33% and 0% was observed for the 72 h and 96 h exposure. In the negative control with 0.001% DMSO, mortality was 9% and 2.33% for 72 h and 96 h exposure. At 96 h exposure, there is no significant difference in mortality. At 72 h exposure the group with CBZ concentration of 5 μg/L shows the highest mortality, with a significant difference between all other groups except control group with 0.001% DMSO. Exposure group with CBZ concentration of 5 μg/L shows also a significant higher mortality at 72 h than at 96 h. Difference in mortality rate between 72 h and 96 h exposure could be caused by biological difference of embryos from different spawning days.
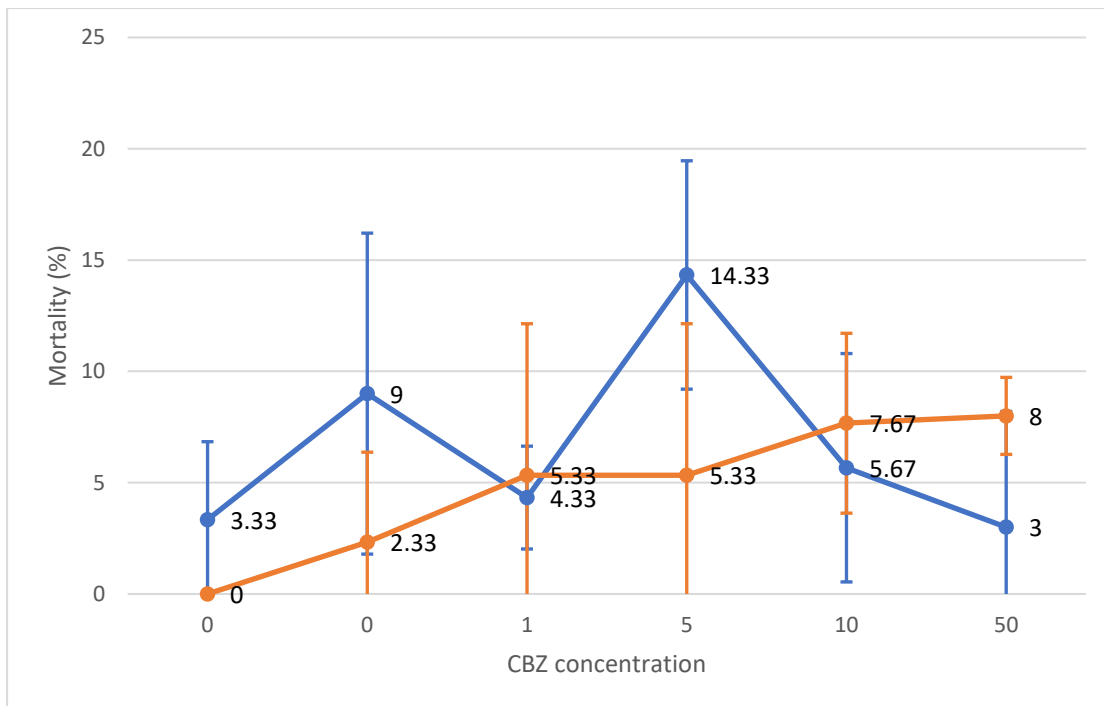
Figure 9: Embryo mortality for the second exposure group. Blue line shows mortality at 72 h while orange mortality at 96 h. Data reported as mean ± standard deviation, n = 3. *p < 0.05

Different studies have demonstrated that CBZ can induce adverse effects in aquatic organism and environmentally relevant concentrations of CBZ impacted reproduction in zebrafish and increase embryo mortality (Chen et al., 2020; Xin et al., 2021). Acute toxicity tests have revealed that CBZ is unlikely to be lethal at environmental concentrations, with reported LC50 (median lethal concentration) and EC50 (median effective concentration) values approaching mg/L levels (Chen et al., 2020).

3.2 Lipid metabolism

Many toxicity effects of CBZ on aquatic organisms have been reported, but its effects and mechanisms of lipotoxicity have not been well documented. Lipid metabolism disorder could cause adverse effects on the growth, behaviour and reproduction of fish (Xin et al., 2021). Therefor lipid metabolism of zebrafish embryos was tested after 72 h and 96 h CBZ exposure. Results are expressed in nmol/ µL and µg/µL, but they are also represented in nmol/embryo and µg/embryo. Differences between the two types of results are present. This can be due to the different mortality and number of embryos present in each biological replicate; results in nmol/embryo and µg/embryo take account of this difference.

During early embryo development, yolk lipids provide the nutrients needed to sustain growth and survival, especially triglycerides and cholesterol. Triglycerides and cholesterol are main constituents of lipids and changes in their synthesis and metabolism can affect lipid metabolism (Qian et al., 2018).

CBZ treatments (1–5µg/L) can significantly accelerate the absorption of yolk sac in the exposed zebrafish embryos at 96 hpf but not at 72 hpf. Until 120 hpf, zebrafish embryos are

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not actively feeding, so the yolk sac is the only source of nutrition for zebrafish embryos during the early developmental stage and its absorption reflects embryos' energy requirements (Halbach et al., 2020; Qiang et al., 2016).

3.21 Triglyceride assay

Figure 10: Triglyceride concentration in ng/μL in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in μg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Figure 10 reports triglyceride concentration expressed in ng/μL for zebrafish embryos exposed for 72 h and 96 h to CBZ concentrations from 1 μg/L to 50 μg/L and for zebrafish embryos exposed to negative controls with aquarium medium and aquarium medium with 0.001% DMSO. 2 biological replicates, instead of 3, were used for the 72 h exposure group with 5 μg/L CBZ concentration to find the results for the triglycerides assay test. A biological replicate was removed because the result value was an outlier.

There is no significant difference between groups at 72 h exposure. At 96 h exposure, exposure group with CBZ concentration of 1 and 5 μg/L and control group with 0.001% DMSO have significant higher triglyceride's concentration than negative control group. Exposure group with CBZ concentration of 1 μg/L also has significant higher concentration of triglycerides than exposure group with CBZ concentration of 10 and 50 μg/L. Exposure group with 1 μg/L CBZ and control group with 0.001% DMSO shows also significant difference in triglyceride concentration between exposure times.
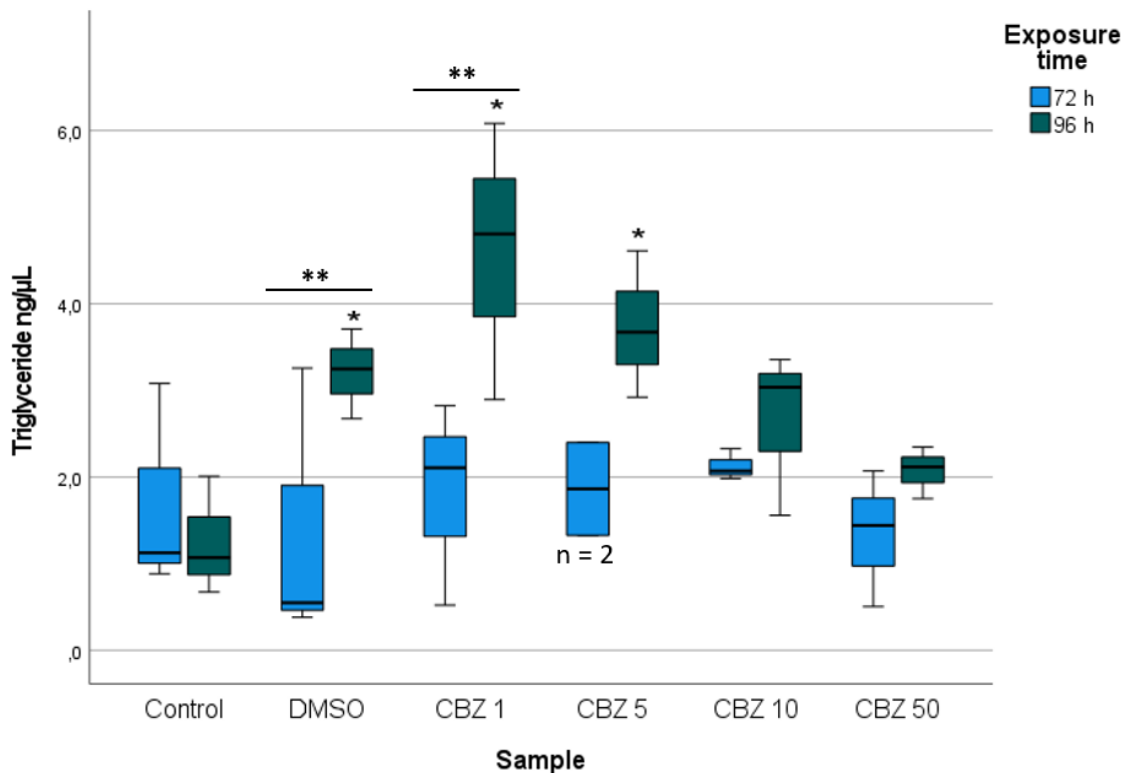
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Figure 11: Triglyceride concentration in ng/embryo in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in μg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Results for triglyceride concentrations in ng/embryo were also calculated (Fig. 11). Like for results in ng/μL, there is no significant difference between groups at 72 h exposure. At 96 h exposure, there is significant difference between control group and exposure group with CBZ concentration of 1 and 5 μg/L, while control group with 0.001% DMSO does not have significant difference. There is also significant difference between exposure group with CBZ concentration of 1 μg/L and exposure group with CBZ concentration of 50 μg/L, but not with 10 μg/L CBZ exposure group. Only exposure group with 1 μg/L CBZ shows significant difference between exposure times.

Liver in zebrafish start to undergo molecular synthesis and metabolism at 72 hpf, before that yolk lipids are the source of triglyceride (Bai et al., 2021; Qian et al., 2018). It was observed that exposure groups with 1 and 5 μg/L CBZ have significant higher triglyceride concentration than control group at 96 h exposure. Other findings shows that CBZ treatments (1–5μg/L) can significantly accelerate the absorption of yolk sac in the exposed zebrafish embryos at 96 hpf but not at 72 hpf (Qiang et al., 2016). The accelerated absorption of the yolk sack can cause an increase in triglyceride concentration in embryos.
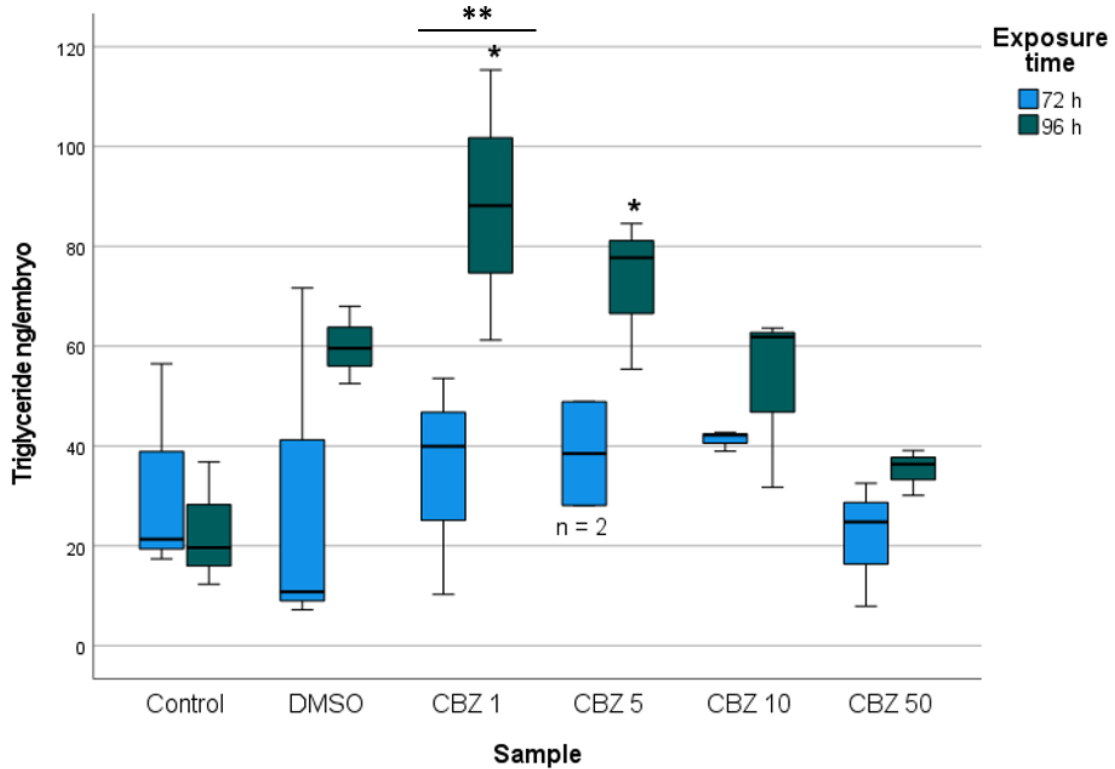
3.22 Cholesterol assay

Figure 12: Free cholesterol concentration in μg/μL in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in μg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Free cholesterol concentration in μg/μL was measured for zebrafish embryos exposed to different CBZ concentrations and for negative controls (Fig. 12). Exposure groups had CBZ concentrations from 1 μg/L to 50 μg/L, while negative controls had aquarium medium and aquarium medium with 0.001% DMSO. Exposure time was 72 h and 96 h.

At 72 h exposure, there is significant difference in free cholesterol concentration between control group and exposure groups with 1, 5 and 10 μg/L CBZ. These exposure groups show a significant lower concentration of free cholesterol compared to control group with aquarium medium. 5 μg/L CBZ exposure group has also significant lower free cholesterol concentration than control with 0.001% DMSO.

There is no significant difference between groups at 96 h exposure.

For exposure groups with 1, 5 and 10 μg/L CBZ there is also a significant difference in free cholesterol concentration between 72 h and 96 h exposure.

Figure 13: Total cholesterol concentration in μg/μL in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in μg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Figure 13 reports total cholesterol's concentration expressed in μg/μL for zebrafish embryos exposed for 72 h and 96 h to CBZ concentrations from 1 μg/L to 50 μg/L and for zebrafish exposed to negative control controls with aquarium medium and aquarium medium with 0.001% DMSO.

There is no significant difference between control groups and exposure groups at 72 h. However, there is significant difference between exposure group with 5 μg/L CBZ and exposure group with 50 μg/L CBZ. At 96 h exposure there is no significant difference between groups. For control group with aquarium medium and exposure group with 5 μg/L CBZ there is significant difference in total cholesterol concentration between 72 h and 96 h exposure.
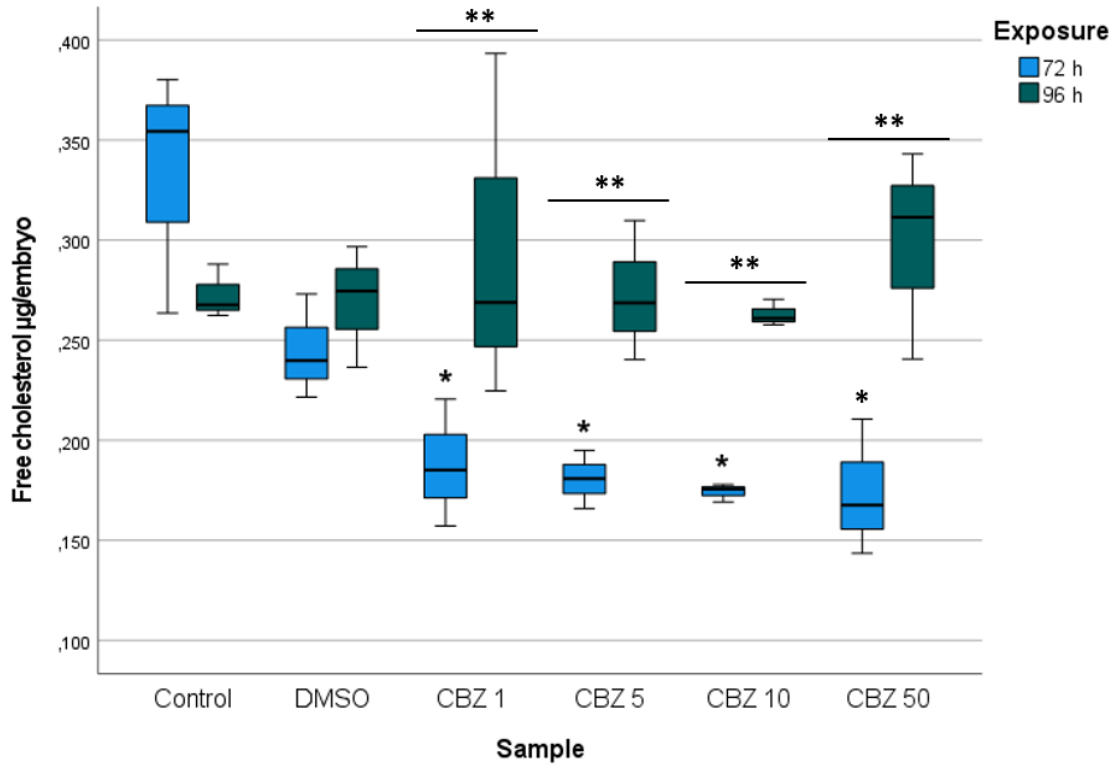
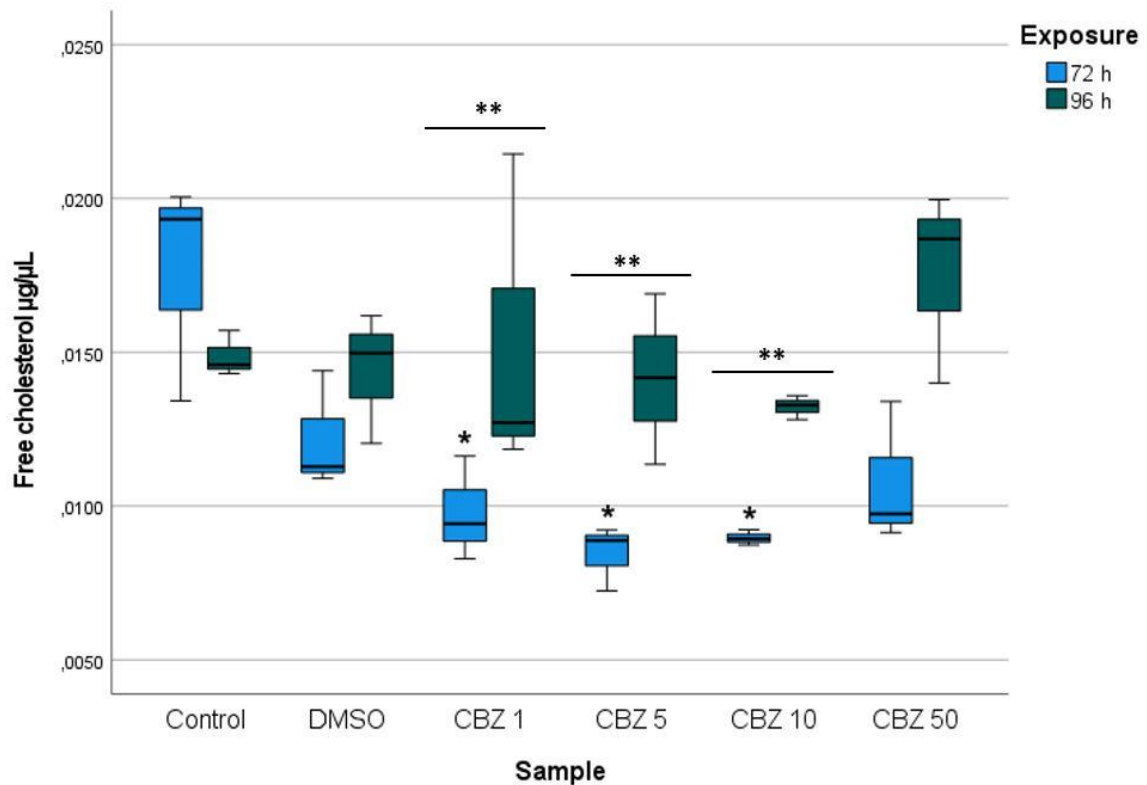*Figure 14*: Free cholesterol concentration in µg/embryo in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in µg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.
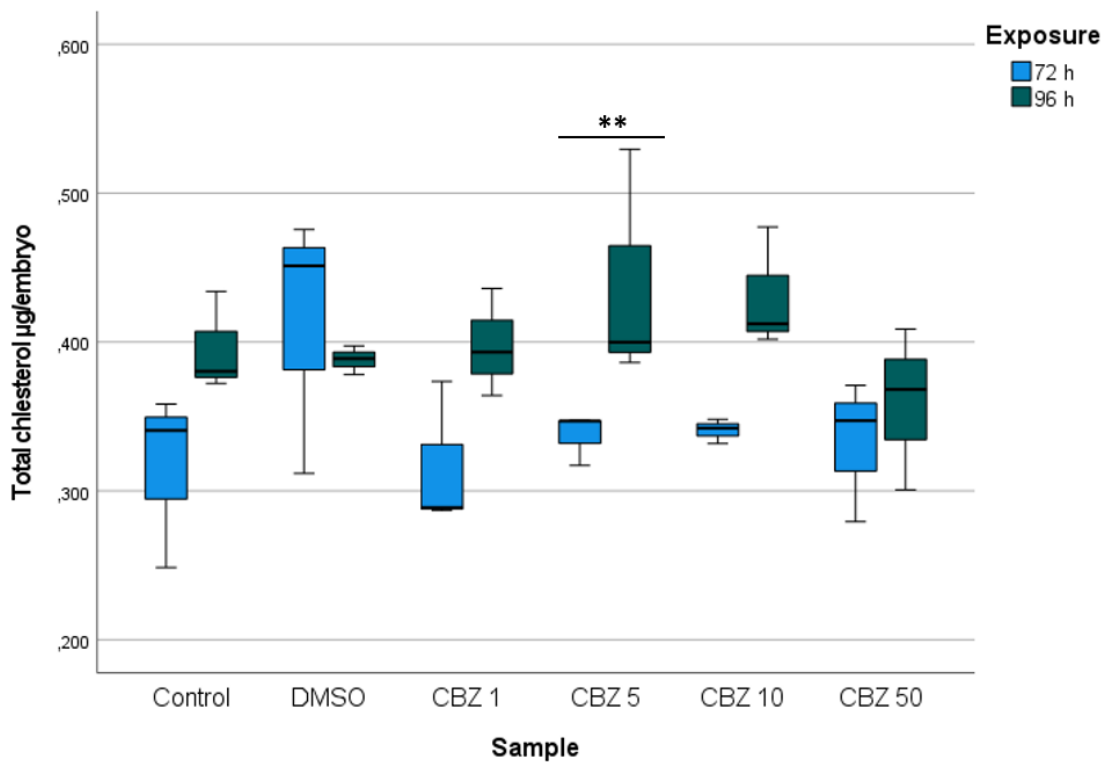
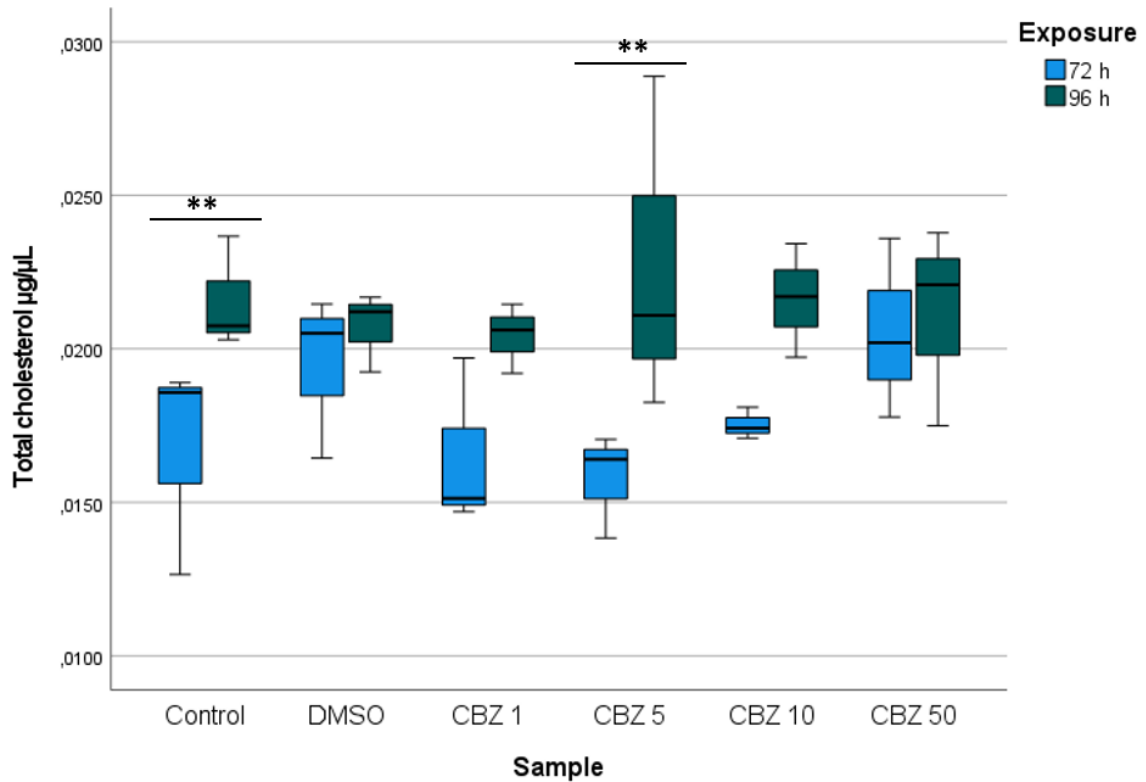*Figure 15*: Total cholesterol concentration in µg/embryo in zebrafish embryos in relation to exposure

time and carbamazepine (CBZ) concentration in µg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Results for cholesterol concentrations in µg/embryo were also calculated (Fig. 14 and 15).

Results for free cholesterol concentration are shown in figure 14. At 72 h exposure, there is significant difference in free cholesterol concentration between control group and all exposure groups. Exposure group with 50 µg/L CBZ did not show significant difference in results expressed in µg/µL. 50 µg/L CBZ exposure group has also significant lower free cholesterol concentration than control with 0.001% DMSO, while for results in µg/µL exposure group with 5 µg/L CBZ has significant difference with 0.001% DMSO control group. At 96 h exposure, there is no significant difference between groups. There is significant difference for all exposure groups between exposure times. Exposure group with 50 µg/L CBZ did not show significant difference between exposure times in results expressed in µg/µL.

Results for total cholesterol concentration are shown in figure 15. There is no significant difference between groups at 72 h exposure. At 96 h exposure, there is significant difference between 10 µg/L and 50 µg/L CBZ exposure groups. This difference was not shown in results expressed in µg/µL. For exposure group with 5 µg/L CBZ there is significant difference in total cholesterol concentration between 72 h and 96 h exposure, while control group with aquarium medium shows no significant difference in total cholesterol concentration results per embryo.

Cholesterol is a structural component of cell membranes and a precursor molecule for making steroid hormones (Mu et al., 2015; Price et al., 2022; Waisundara & Jovandaric, 2020; Zampelas & Magriplis, 2019). Steroid hormones are important for organization and development of sexual organs, behaviour, immune function, growth, and response to stress (Price et al., 2022). Altered cholesterol concentrations can cause hormones imbalance and behavioural and reproduction problems.

3.23 Free fatty acids assay

Figure 16: Free fatty acid concentration in nmol/µL in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in µg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Figure 16 shows free fatty acid concentration in nmol/µL for zebrafish embryos exposed for 72 h and 96 h to CBZ concentrations from 1 µg/L to 50 µg/L and for zebrafish exposed to negative control controls with aquarium medium and aquarium medium with 0.001% DMSO. 3 biological replicates were used to calculate mean concentration in exposure groups. For 96 h exposure group with CBZ concentration of 1, 5 and 10 µg/L 2 biological replicates were used.

At 72 h exposure, there is significant difference between negative control group and CBZ concentration of 50 µg/L. 50 µg/L CBZ exposure group has also a significant higher concentration of free fatty acid than 1 µg/L CBZ group at 72 h exposure.

At 96 h exposure there is no significant difference between control groups and CBZ exposure groups. Exposure group with CBZ concentration of 10 µg/L has significant lower free fatty acid's concentration than 50 and 1 µg/L CBZ exposure groups at 96 h exposure.

There is also significant difference in free fatty acid's concentration between 72 h and 96 h exposure in 10 µg/L CBZ exposure group.
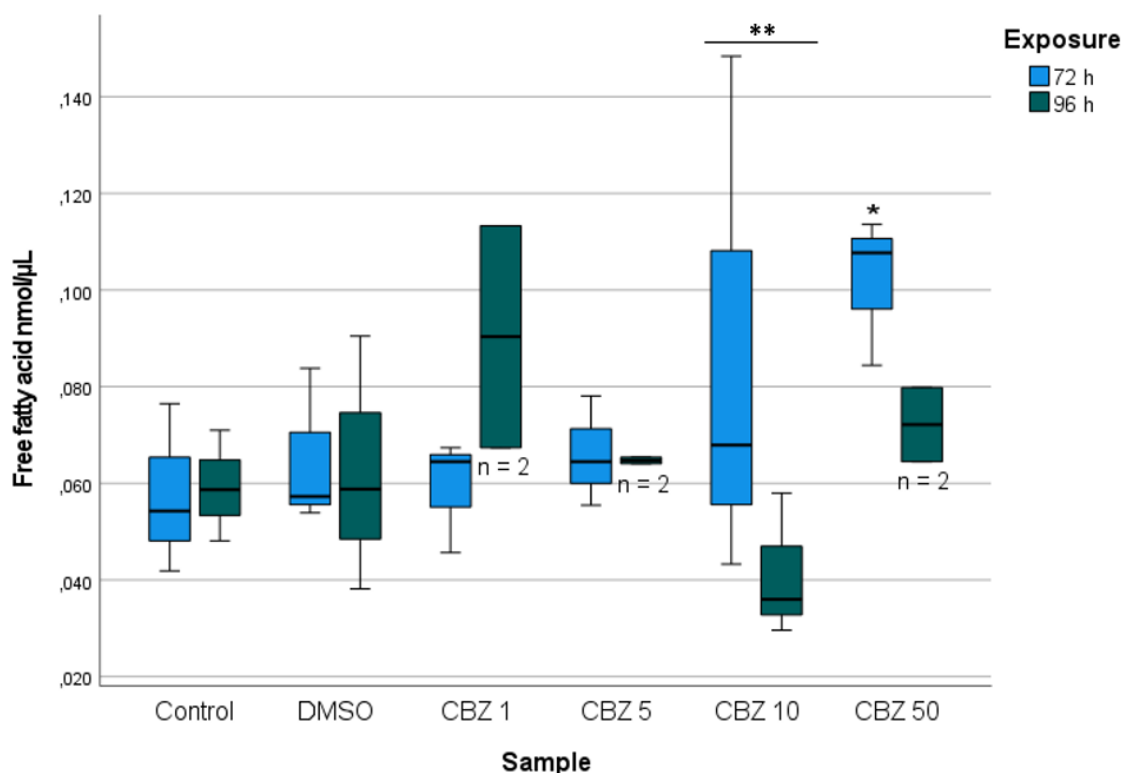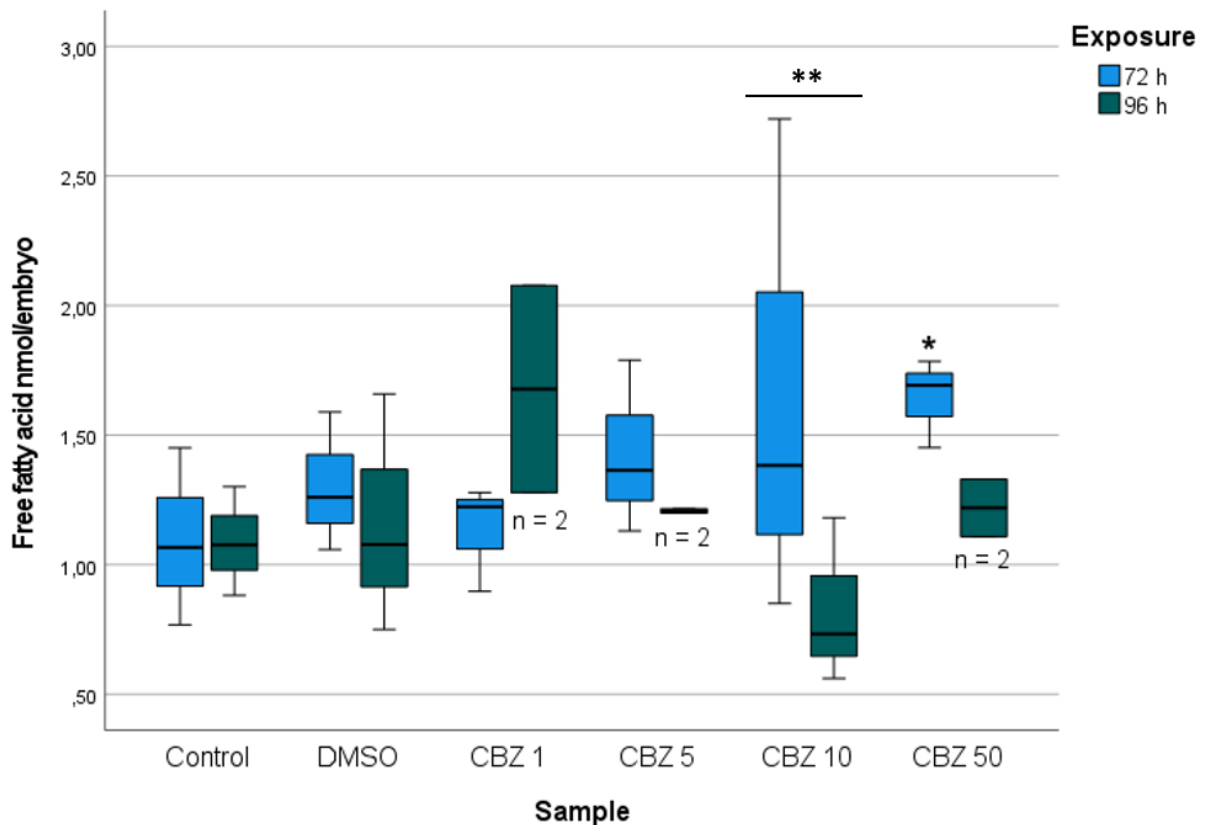
Figure 17: Free fatty acid concentration in nmol/embryo in zebrafish embryos in relation to exposure time and carbamazepine (CBZ) concentration in µg/L. 72h exposure time is represented in blue, while 96h exposure time is represented in green. Interquartile range (IQR) shows were 50% of the values lies together with median value, while whiskers show min to max values.

Results for free fatty acid concentrations in nmol/embryo were also calculated (Fig. 17). At 72 h exposure, there is significant difference only between negative control group and CBZ concentration of 50 µg/L. Opposite to results expressed in nmol/µL, 50 µg/L CBZ exposure group does not show significant difference with any other groups. At 96 h exposure, exposure group with CBZ concentration of 10 µg/L has significant lower free fatty acid concentration than 1 µg/L CBZ exposure group like for the results calculated in nmol/µL, but it has no significant difference with 50 µg/L CBZ exposure group. There is also significant difference in free fatty acid's concentration between 72 h and 96 h exposure in 10 µg/L CBZ exposure group.

Fatty acids are important components of cell membranes and play an important role in energy transport and storage, cell structure, and intermediates for hormone synthesis (Teng et al., 2019). Fatty acids are also related to cardiovascular function. Studies have shown that fatty acids are involved in growth, cognition, and stunting in the early development of organisms (Teng et al., 2019).

4. Conclusion

In this thesis, effects of exposure to environmentally relevant CBZ concentrations were evaluated. Embryos mortality was recorded after 72 h and 96 h of exposure to confirm that the chosen concentrations were sublethal. Concentrations of triglyceride, free fatty acid and total and free cholesterol were also measured to determine the influence of CBZ on the lipid metabolism in the embryos.

Mortality results showed that there was no significant different in embryos mortality after 96 h. At 72 h of exposure, 5 µg/L CBZ exposure group showed a significant higher mortality than all other groups except control group with 0.001% DMSO. Exposure group with CBZ concentration of 5 µg/L shows also a significant higher mortality at 72 h than at 96 h.

Overall results in lipids concentration show that CBZ affects lipids metabolism both at 72 h and 96 h exposure. At 72 h exposure, there was no significant effect in triglyceride and total cholesterol concentration. It was observed a significant lower concentration of free cholesterol in all exposure groups compared to controls groups at 72 h exposure. For the free fatty acids content, exposure group with the highest CBZ concentration showed higher levels compared to the control group at 72 h exposure, while there was no significant difference between groups at 96 h exposure. At 96 h exposure there was also no significant difference in free cholesterol and total cholesterol concentrations. The triglyceride concentration was significantly higher at 96 h exposure in groups treated with 1 and 5 µg/L CBZ. These results provide insight in the effects of CBZ exposure on lipids metabolism.

Environmentally relevant concentrations of CBZ have been shown to affect fish more often during chronic exposures compared to acute exposures (Chen et al., 2020). Other studies suggest that an environmentally relevant concentration of carbamazepine may speed up the normal development in exposed zebrafish embryos (Qian et al., 2018).

5. Further prospects

Pharmaceuticals are nowadays recognized as a threat for aquatic ecosystems and monitoring these contaminants in the environment is an important field of research since their presence can affect the ecosystems even at very low levels (Guilaine et al., 2020; Mezzelani et al., 2018). Pharmaceuticals consumption increases every year, leading to a continuous input of biologically active molecules in natural environments (Bound & Voulvoulis, 2004; Mezzelani et al., 2018). Pharmaceuticals enter into aquatic systems mainly through wastewater discharge and the majority of WWTPs is unable to remove efficiently pharmaceuticals and other emerging contaminants from the water before releasing it into the environment (Braeutigam et al., 2012; Clara et al., 2004; Heberer, 2002). It is therefore important to monitor the presence of pharmaceuticals in wastewater discharge and for WWTPs to develop regulations for treatment and disposal of wastewater that consider emerging contaminants. Doing so, the quality of effluent water released into the environment can be increased.

Some research regarding the effects of pharmaceuticals and CBZ on aquatic organisms are done, as shown in this thesis. However long-term effects, bioaccumulation and biomagnification are not well documented. Further studies can be done on the effects of environmentally relevant concentration of pharmaceuticals on aquatic organism behavioural response and population changes. It is also important to research the effects of pollutants present in the aquatic environment on early developing stage, since embryo and larvae development and mortality can affect the overall population by decreasing the number of individuals and create imbalance in the prey-predator relationship. Studies using zebrafish in the developing stage to assess the toxic effects of chemicals present in the aquatic ecosystem can provide data for environmental risk assessment (Garbinato et al., 2020).

In aquatic ecosystems, pharmaceuticals are not present as isolated compounds. They co-occur in complex mixtures with other drugs and/or chemical pollutants which can have additive or synergetic effects (Mezzelani et al., 2018). In addition, pollutants are also subject to changes in the environment such as oxidation, photolysis, or biotransformation. These changes can occur through both biotic and abiotic processes (Ortúzar et al., 2022). Further studies should be done on the chemical changes that CBZ can undergo in the environment as well as on the interaction of CBZ with other substances present in the aquatic ecosystem and/or with other environmental stressors.

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Appendix

Table 1: Mean embryos mortality for the second exposure group expressed in percentage for each CBZ concentration at 72 h and 96 h. Standard deviation for each group is also shown.

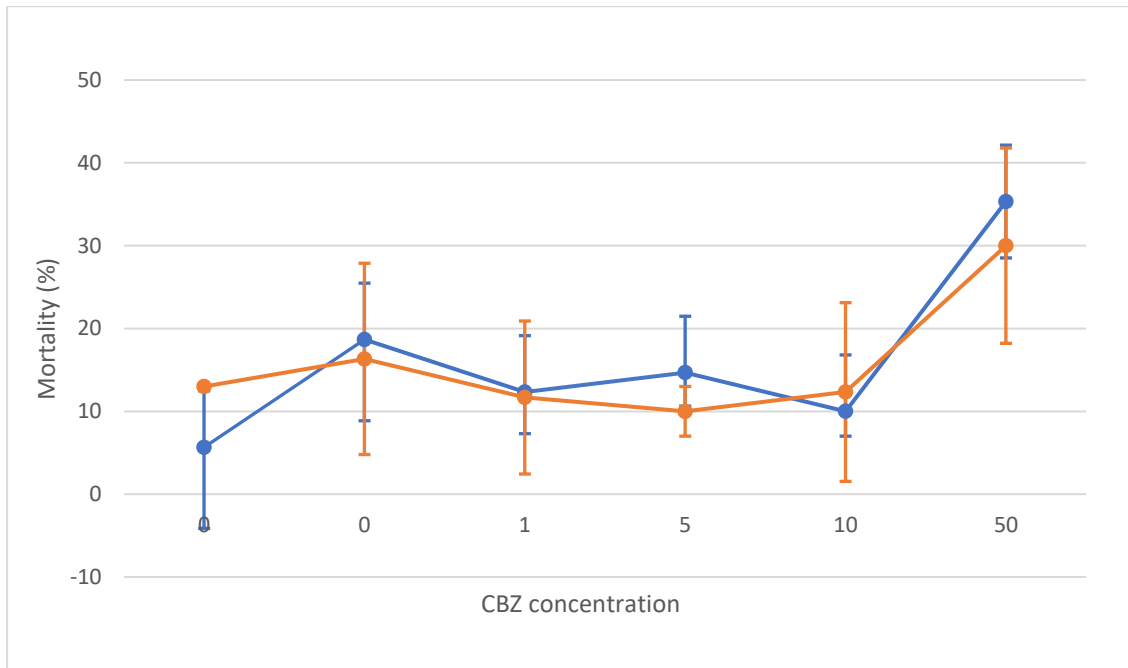| Concentration CBZ	Time of exposure	Mean mortality %	Standard deviation
0 µg/L	72 h	3.33	3.15
	96 h	0	0
0 µg/L + 0.001% DMSO	72 h	9	7.21
	96 h	2.33	4.04
1 µg/L	72 h	4.33	2.31
	96 h	5.33	7.23
5 µg/L	72 h	14.33	5.13
	96 h	5.33	6.81
10 µg/L	72 h	5.67	5.13
	96 h	7.67	4.04
50 µg/L	72 h	3	5.2
	96 h	8	1.73

Fig 1: Embryo mortality for the first exposure group. Blue line shows mortality at 72 h while orange mortality at 96 h. Data reported as mean ± standard deviation, n = 3. *p < 0.005

Table 2: Mean embryos mortality for the first exposure group expressed in percentage for each CBZ concentration at 72 and 96 h. Standard deviation for each group is also shown.

Concentration CBZ	Time of exposure	Mean mortality %	Standard deviation
0 µg/L	72 h	5.67	9.81
	96 h	13	0
0 µg/L + 0.001% DMSO	72 h	18.67	9.81
	96 h	16.33	11.55
1 µg/L	72 h	12.33	5.03
	96 h	11.67	9.24
5 µg/L	72 h	14.67	4.04
	96 h	10	3

10 µg/L	72 h	10	3
	96 h	12.33	10.79
50 µg/L	72 h	35.33	6.81
	96 h	30	11.79