University of Trás-os-Montes and Alto Douro

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

Master thesis in Biochemistry

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I, Raquel Vieira, declare that this thesis entitled, “Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development” was expressly elaborated as Original Dissertation for the purpose of obtaining a Master's Degree in Biochemistry at the University of Trás-os-Montes and Alto Douro.

Signed: Raquel Susana Fernandes Vieira

Date: 18 / December / 2018

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Resumo

A produção agrícola é frequentemente afetada pelas condições ambientais inerentes às mudanças climatéricas. Esta situação acarreta o aumento da utilização de pesticidas, como é o exemplo de fungicidas para controlar o míldio e oídio na vitivinicultura. Neste sentido, a utilização dos fungicidas têm sido associados ao aparecimento dos seus resíduos no solo e lençóis de água com possíveis efeitos tóxicos nos ecossistemas. Contudo, o estudo e o conhecimento toxicológico acerca dos resíduos e efeitos destes compostos são limitados ou inexistentes. Assim este trabalho teve como objetivo avaliar a toxicidade de fungicidas sintéticos (azoxistrobina, tebuconazole e mancozeb) e de compostos de origem natural (extrato de *Mimosa tenuiflora*, extrato de *Equisetum arvense* e timol) recorrendo à utilização de embriões de peixe-zebra como modelo.

Numa primeira abordagem procedeu-se ao cálculo dos valores das concentrações médias letais para 96h de exposição (96h-LC_{50}) iniciada em embriões na fase de blástula (~2 horas pós-fertilização). Com base no valor de 96h-LC_{50}, os embriões foram expostos a concentrações que variaram entre 0.001 e 0.1 mgL^{-1} para a azoxistrobina (LC_{50} = 1.15 mgL^{-1}), 0.0005 e 0.05 mgL^{-1} para o mancozebe (LC_{50} = 5.13 mgL^{-1}) e 0.05 e 5 mgL^{-1} para o tebuconazole (LC_{50} = 7.25 mgL^{-1}). Para os compostos de origem natural, os embriões foram expostos a concentrações que variaram entre 0.00625 e 0.625 mgL^{-1} para o extrato aquoso de *Equisetum arvense* (LC_{50} = 435.31 mgL^{-1}), 0.008 e 0.8 mgL^{-1} para o extrato etanólico de *Mimosa tenuiflora* (LC_{50} = 123.87 mgL^{-1}) e 0.01 e 1 mgL^{-1} para o timol (LC_{50} = 32.67 mgL^{-1}). Durante a exposição foram avaliados diversos parâmetros letais (mortalidade, destacamento da cauda e da cabeça), sub-letais (desenvolvimento dos somitos, olhos, otólitos, edema, pigmentação, movimentos espontâneos, sistema circulatório e eclosão), teratogénicos (malformações), bem como a análise morfométrica das larvas. Outros parâmetros bioquímicos como enzimas ligadas ao stresse oxidativo (SOD, CAT, GPx e GR), níveis de ROS, níveis de glutationas (GSH e GSSG), enzimas relativas a degradação de compostos (GST e CarE), ligadas a neurotransmissão (AcHE) e respiração anaeróbia (LDH), foram também analisados.

Os embriões expostos a compostos sintéticos apresentaram uma maior percentagem de efeitos letais, sub-letais e a nível enzimático. Os embriões expostos a mancozebe apresentam um decréscimo na sua taxa de eclosão muito acentuado para todas as concentrações avaliadas, e um grande número de malformações (edemas cardíacos e do saco vitelino, bem como torções na coluna) apresentavam uma maior prevalência na

concentração mais elevada, sendo assim efeitos dose-dependentes. Já os expostos a azoxistrobina na concentração mais baixa apresentam um aumento dos ROS bem como um aumento da SOD, GST, CarE e AcHE, bem como uma diminuição da atividade da CAT e dos níveis de GSH e GSSG. Não se verificaram diferenças no desenvolvimento dos embriões expostos a tebuconazole, bem como a fungicidas à base dos compostos naturais.

Conclui-se assim que a presença de compostos sintéticos no ambiente pode causar alterações significativas nos ecossistemas aquáticos, podendo estes serem substituídos pelos compostos naturais. Contudo, mais estudo são necessários para comprovar na totalidade a segurança e sensibilidade dos compostos testados.

Palavras chave: Toxicologia; Fungicidas; Peixe-Zebra; Desenvolvimento embrionário; Stresse oxidativo

Abstract

Agriculture is affected by the environmental conditions inherent in climate change. This phenomenon is associated with increased use of pesticides in order to control fungi and pests (eg, mildew and powdery mildew) that attack agricultural, especially wine production. In this sense, the use of fungicides have been associated with the appearance of their residues in the soil and water sheets with possible toxic effects on ecosystems

However, the study and toxicological knowledge about the residues and effects of these compounds are limited or non-existent. The objective of this work was to evaluate the toxicity of synthetic fungicides (azoxystrobin, tebuconazole and mancozeb) and compounds of natural origin (extract of *Mimosa tenuiflora*, extract of *Equisetum arvense* and thymol) using zebrafish embryos as a toxicological model.

In a first approach, we calculated the mean lethal concentration values for 96h exposure (96h-LC_{50}) in embryos in the blastula phase (~ 2 hours post-fertilization). Based on the 96h-LC_{50} value, the embryos were exposed to concentrations ranging from 0.001 to 0.1 mgL^{-1} for azoxystrobin (LC_{50} = 1.15 mgL^{-1}), 0.0005 and 0.05 mgL^{-1} for mancozeb = 5.13 mgL^{-1}) and 0.05 and 5 mgL^{-1} for tebuconazole (LC_{50} = 7.25 mgL^{-1}). For the compounds of natural origin, the embryos were exposed to concentrations ranging from 0.00625 to 0.625 mgL^{-1} for the aqueous extract of *Equisetum arvense* (LC_{50} = 435.31 mgL^{-1}), 0.008 and 0.8 mgL^{-1} for the *Mimosa tenuiflora* extract (LC_{50} = 123.87 mgL^{-1}) and 0.01 and 1 mgL^{-1} for thymol (LC_{50} = 32.67 mgL^{-1}). During the exhibition, several lethal parameters (mortality, tail and head detachment), sub-lethal (development of somites, eyes, otoliths, edema, pigmentation, spontaneous movements, circulatory system and hatching), teratogenic (malformations) such as the morphometric analysis of the larvae. Other biochemical parameters such as oxidative stress-linked enzymes (SOD, CAT, GPx and GR), ROS levels, glutathione levels (GSH and GSSG), compounds degradation enzymes (GST and CarE), neurotransmission and anaerobic respiration (LDH), were also analyzed.

Embryos exposed to synthetic compounds showed a higher percentage of lethal, sublethal and enzymatic effects. Embryos exposed to mancozeb showed a marked decrease in their hatching rate for all concentrations evaluated, and a large number of malformations (cardiac and yolk sac edema as well as spinal torsions) had a higher prevalence at the highest concentration, thus being dose-dependent effects. On the other

hand, those exposed to azoxystrobin at the lowest concentration present an increase in ROS as well as an increase in SOD, GST, CarE and AcHE, as well as a decrease in CAT activity and GSH and GSSG levels. There were no differences in the development of embryos exposed to tebuconazole, as well as fungicides based on natural compounds.

It is thus concluded that the presence of synthetic compounds in the environment can cause significant changes in aquatic ecosystems, and these can be replaced by natural compounds. However, further study is needed to fully demonstrate the safety and sensitivity of the compounds tested.

Keywords: Toxicology; Fungicides; Zebrafish; Embryonic development; Oxidative Stress

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List of abbreviations

CDNB - 1-chloro-2, 4-dinitrobenzene

CYP51A – *Cytochrome* P450 14-α sterol demethylase

DCF – 2', 7' –dichlorofluorescein

DCFH-DA - dichloro-dihydro-fluorescein diacetate

DDT – Dichlorodiphenyltrichloroethane

DMDCs – Dimethyldithiocarbamates

DTCs – Dithiocarbamates

DTNB - 5,5'-dithio-bis-2-nitrobenzoic acid

EBDCs - Ethylenebis dithiocarbamates

EBI – Ethylene bisisothiocyante

EBIS - Ethylene bisisothiocyanate sulfide

EC - European Council

ETU – Ethylenethiourea

EU – Ethyleneurea

FAO - The Food and Agriculture Organization

GRPs - glucose-regulated proteins

hpf – Hours post fertilization

HPT - hypothalamic-pituitary-thyroid

HSPs - heat shock proteins

IUPAC – International Union of Pure and Applied Chemistry

LC_{50} – Lethal concentration 50

LPO - Lipid peroxidation

MBC - Methyl-2-benzimidazole carbamate

MRL – Maximum residual limits

MTs - metallothioneins

OECD - Organization for Economic Cooperation and Development

POD – peroxidase

SDH - succinic dehydrogenase

Shh - Sonic hedgehog

TCA - trichloroacetic acid

TSH - thyroid stimulating hormone

VEGF - vascular endothelial growth factor

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List of works presented based on this dissertation

Book Chapter:

- Chapter Five – "Zebrafish Early Life Stages for Toxicological Screening: Insights From Molecular and Biochemical Markers" - Advances in Molecular Toxicology, Volume 12, 2018, Pages 151 – 179

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- "Climate change and the impact of fungicide use on the embryonic development of zebrafish"- National Congress on Climate Change 2018, UTAD, Vila Real, Portugal, 21 of February of 2018.

- "Toxicity evaluation of synthetic and natural fungicides applied in agriculture using zebrafish embryos"- XI Conference on Biochemistry - Science and Citizenship (Science and Technology Week), UTAD, Vila Real, Portugal, 21 of November of 2017.

Poster communication:

- "Developmental joint effects of selected fungicide mixtures in zebrafish" – CICTA 2018 – 11th Iberian and 8th Ibero-American Congress of Environmental Pollution and Toxicology, Madrid, Spain, 11 to 13 of July of 2018.

Chapter I

Introduction

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Overview

The damage caused to the environment by climate change, which can be associated with agricultural activities, are some of the main reasons for the intensification of pesticide use. Over the years, plant protection products are among the compounds that most contribute to high crop yields and efficient agricultural management. Among the pesticides, the fungicides represent the least studied compounds. These are used in combating fungi and are approved to control, for example, powdery mildew and mildew, in various agricultural crops. As a consequence, their residues have been documented in water sources, in complex matrices, near agricultural areas, in concentrations that often exceed the limit allowed by the 2013/39/EU Directive, as well as in a wide variety of foods regulated by Regulation (EC) No. 396/2005.

In Portugal, as well as in many other countries, one of the most attacked production areas has been viticulture which, due to the occurrence of warm and humidity outside of the season, has created an environment propitious to the development of fungi diseases. In the last years, the producers have already registered falls off in production caused by the above mentioned fungal diseases. However, recent research indicates that fungicides are potentially causing metabolic, endocrine and carcinogenic disorders in animals and humans. Taken together, this information reflects the environmental risk of these compounds that is urgent to evaluate in order to find the most appropriate solutions. Considering what has been described, this study proposes the evaluation of the toxicity of synthetic fungicides (azoxystrobin, tebuconazole and mancozeb) and natural fungicides (thymol, extract of *Equisetum* and extract of *Mimosa tenuiflora*) in zebrafish embryos. In general, the toxicological mechanism of these compounds was investigates through the studies of the developmental toxicity and biochemical changes observed in exposed zebrafish embryo/larvae.

2. The use of pesticides in agriculture

With population growth, agriculture must respond to consumption and demand for agricultural and horticultural products. In this way, there must be a control of all the parameters that can affect the production, of which it is highlighted the agricultural pests. Target pests include any living organism that causes damage, transmits or produces disease in plants, crops, and human (Copplestone 1988; Spence *et al.*, 2008). In order to control the pests and protect agricultural and horticultural crops, techniques such as gene improvement (Punja 2001), physical, chemical and biological methods (Wright 2014; Knipling 1965) are currently applied being the most common method based on the use of chemical pesticides.

In 1986, the Food and Agriculture Organization of the United Nations (FAO), defined pesticide "as any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs or which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies" (NATIONS 1986). These compounds can be naturally derived, synthetically and inorganic produced substances that includes bactericides, baits, fungicides, herbicides, insecticides, lures, rodenticides, and repellents (Ferrer 2003).

Nowadays, the European Union has tight legislation on the marketing and use of pesticides, but this has not always been the case. When dichlorodiphenyltrichloroethane (DDT) was discovered, in 1939 (Turusov, Rakitsky, and Tomatis 2002), it was used in an abusive and deregulated way, causing many environmental and atmospheric damages on the planet (van den Berg 2009). In fact, although pesticides have some benefits (improving productivity, protection of crop losses/yield reduction, vector disease control, quality of food and other areas), they have many adverse environmental impacts their persistence can make once-rich soil unusable for farming, affecting soil fertility, bioaccumulation can wipe out living creatures and sources of food, and its runoff and groundwater infiltration can contaminate water (Morgado *et al.*, 2016; Cruzeiro *et al.*, 2016) and environmental non-target organisms (Aktar, Sengupta, and Chowdhury 2009). Additionally, pesticides have the potential for various unintended negative impact on human health, ranging from respiratory issues (Kirkhorn and Schenker 2002), the

impairment to the central nervous system (Rosenstock *et al.*, 1991; Korsak and Sato 1977), developmental issues in babies and children, to different types of cancer (Daniels, Olshan, and Savitz 1997; Buckley *et al.*, 2000).

Within the pesticides group, fungicides stand out for their high use in pest control in agriculture. These compounds are applied several times at short intervals (eg less than 15 to 15 days in certain cultures). The regular use of fungicides may pose a risk to the environment, particularly if residues persist in the soil or migrate off-site and enter waterways. If this occurs it could lead to adverse impacts to the health of terrestrial and aquatic ecosystems. The way the applications are made also helps the compounds to disperse easily, since most of the fungicides used in agriculture are applied by spraying (Wightwick *et al.*, 2010). Thus, the toxicity of such pesticides should be further studied in order to be able to control the damage caused by their use and thereby allow the entry of compounds with fungicidal activity but with a lower toxicity due to their formulation based on natural compounds.

3. Fungicides

The fungicides can be classified in several ways, using different criteria, but the most commonly employed are the mode of action and the chemical group: Inorganic Compounds (eg. Copper-based fungicides); Dithiocarbamates (DTCs) which can be classified into three subclasses depending upon their carbon skeleton: dimethyldithiocarbamates (DMDCs) such as ziram, thiram, and ferbam; ethylenebis dithiocarbamates (EBDCs) such as mancozeb, maneb, zineb, and metiram; propylenebis(dithiocarbamates) such as propineb; Triazoles (eg. Tebuconazole and difeconazole) whose antifungal activity is based on their ability to inhibit *Cytochrome* P450 14-α sterol demethylase (CYP51A), a key enzyme for sterol biosynthesis in fungi, affecting the permeability of the cell membrane and, consequently, the development of the mycelium of the fungus (Konwick *et al.*, 2006; Croucher, Jewess, and Roberts 2007; Sancho *et al.*, 2010); Benzimidazoles (eg. carbendazim, benomyl, and thiophanate-methyl) that inhibit β-tubulin assembly during mitosis and were first used to control gray mold (*B. cinerea*) and apple scab (*Venturia inaequalis*); Phthalimides are one of the oldest groups of fungicides; Dicarboximides (eg. vinclozolin, iprodione, and procymidone) are inhibitors of the triglyceride biosynthesis in sclerotia-forming fungi, including *Botrytis cinerea*; Strobilurins or QoI-Fungicides, (eg. Azoxystrobin)

which have a common antifungal mode of action, inhibiting electron transfer at the Qo site in mitochondrial complex III and there are more three fungicides classes Phenylamides, Phenoxyquinolines and Phenylpyrroles.

However, fungicides can also be classified according to where they are active in the disease cycle: (1) protectant or preventive, applied before the fungus is present in order to prevent their attack on the crop, if they are effective prior to infection and the initiation of the disease cycle; (2) curative, fungicides that are effective against the fungus growing in the leaf tissue post the infecting spore germination; or (3) eradicate or antisporulant, directly applied on the fungus to eliminate it by preventing it from further contaminating the culture, that is, fungicides that are capable of stopping sporulation by the pathogen (Angelotti *et al.*, 2014; Caballero, Finglas, and Toldrá 2015).

One of the major problems with the use of these compounds is the way they are applied. The vast majority is dissolved in water and sprayed over the crops. This method is dangerous both for the performer that can be exposed directly to these compounds, and to the other crops and animals because much of it stays in the air, soil and water (Bird, Esterly, and Perry 1996). As such, there is a need to evaluate the effects of these compounds on the organisms present in the environment where they are applied. There are already toxicological studies conducted that addressed the effects of some classes of fungicides, which include reproductive effects, changes on the functioning of the thyroid (Trivedi *et al.*, 1993), act as endocrine disrupters (Yu *et al.*, 2013), and some even being cancerous and neurotoxic (Faro 2010). However, there is still a long path of research to be carried out. There are more and more alternatives of synthetic fungicides on the market, with natural products providing opportunities for new drug leads but about which very little is known.

In the present study, three synthetic fungicides of different classes (Mancozeb (Dithiocarbamates), Tebuconazole (Triazoles) and Azoxystrobin (Strobilurins)) were selected to evaluate its toxicological effects, using zebrafish as an animal model. Three other natural-based products with proven fungicide activity (plant extracts and essential oils) were evaluated since they are thought to be an alternative to the use of synthetic compounds. Yet, few or no information is known about their effects at the organism level.

3.1. Synthetic fungicides

3.1.1. Strobilurins - Azoxystrobin

Azoxystrobin, a molecule with a high effect as a fungicide, was developed by Syngenta in 1992 and was first sold in 1996 (Bartett *et al.,* 2001). It is a systemic, broad-spectrum fungicide belonging to the class of methoxyacrylates, which are derived from the strobilurins (naturally-occurring compound) (Fig. 1). Its IUPAC name is [methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3- methoxyacrylate, molecular formula $C_{22}H_{17}N_3O_5$ and its molecular weight is 403.4 g mol^{-1} (Bartlett *et al.*, 2002; Romeh 2017).

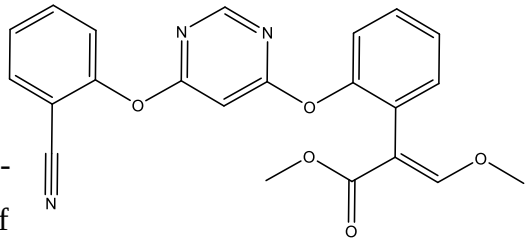

Figure 1 - Azoxystrobin molecular structure.

This compound is the active formulation of Quadris®, and others fungicide such as Ortiva®, Abound®, Priori®, Quilt® and Amistar®Opti, mostly sold by Syngenta (Mastovska 2008; Rodrigues, Lopes, and Pardal 2013). It is used in about 72 countries and is applied in a wide variety of crops (Bartlett *et al.,* 2002). It is effective against four major groups of plant pathogenic fungi including ascomycetes (e.g., powdery mildews), basidiomycetes (e.g., rusts), deuteromycetes (e.g., rice blast) and oomycetes (e.g., downy mildew) (Bartlett *et al.,* 2002; Romeh 2017).

3.1.2. Toxicological effects

The mode of action of azoxystrobin in fungi is through the disruption of mitochondrial electron transport chain, between the complex III (cytochrome c oxidoreductase or cytochrome bc1) and the complex IV (cytochrome c oxidase), preventing ATP synthesis (Rodrigues, Lopes, and Pardal 2013). As a result of this inhibition, there is a leakage of electrons from the mitochondrial chain that eventually triggers oxidative stress processes at the cellular level, since excess free electrons cause an increase in the production of reactive oxygen species (ROS) (Osellame, Blacker, and Duchen 2012; Turrens 2003; Rodrigues, Lopes, and Pardal 2013). Although it has been developed with the aim of acting as a fungicide, it also has effects on other organisms and can cause serious environmental problems. Indeed, and although its residues might affect the environmental safety and human health when they are found in amounts above the

maximum residue limits (MRL, 0.01 $mgkg^{-1}$ for a wide range of products and 70 $mgkg^{-1}$ for aromatic plants and edible flowers), there are still few studies about its toxicological effects. In a recent study carried out by Cao and his workgroup in 2016, this compound was shown to adversely reduce egg production, fertilization and to induce histological alterations in the gonads and liver of adult zebrafish. It could also significantly affect the mRNA levels of genes involved in the oding of follicle stimulating hormone (FSH), for luteinizing hormone (LH), corticosteroids and vitellogenin, which can be used as markers of estrogenicity in early stages of zebrafish development (Cao *et al.*, 2016).

This compound has also been shown to have other effects on the zebrafish embryonic development and on some enzyme activities, with a median lethal concentration (LC_{50}) of 1.18 mgL^{-1} in a study by Jia *et al.,* (2018). Azoxystrobin treatments inhibited glutathione S-transferase (GST) activity, which was the opposite response from the malondialdehyde (MDA) contents. In the early period of exposure, an increase in ROS was observed in the liver of female zebrafish, and carboxylesterase (CarE) was activated to degrade azoxystrobin as well as catalase (CAT) and peroxidase (POD). After 14 days of exposure, POD activity decreased and superoxide dismutase (SOD) activity was increased due to an increase in ROS production. With the prolonged exposure, CAT, POD, SOD and GST activities were increased due to an excess increase of ROS (Jia *et al.*, 2018).

A recent study also focused on the effects of exposure to azoxystrobin in zebrafish with a determined median lethal concentration (96 h-LC_{50}) value of 0.777 mgL^{-1} (Jiang *et al.*, 2018). The results showed a ROS accumulation, increased GST, GPX and POD activity and the transcriptions of antioxidant and stress response related genes, while the opposite trend occurred for SOD and CAT activity following a 24h or 48h exposure period. The increased E_2 and VTG levels in zebrafish larvae, and altered transcription levels of regulatory and steroidogenic genes in the hypothalamus-pituitary-gonad (HPG) axis indicated an endocrine disruption (Jiang *et al.*, 2018).

In another study conducted by Cao and colleagues (Cao *et al.*, 2018) in zebrafish larvae and adults showed short-term developmental toxicity to larval and adult zebrafish after 8 days of exposure, including inhibited mitochondrial complex III activity, reduced ATP concentration, elevated ROS and MDA concentration, activated antioxidant enzyme activities (SOD, CAT and GPx), alteration of liver histology and mitochondrial ultrastructure, and changes in the expression of genes related to mitochondrial respiratory, oxidative stress, cell apoptosis and innate immune response.

Han, in 2016, performed a study exposing adult zebrafish for 28 days to this compound and observed an accumulation of ROS, which resulted in an inhibition of SOD activity whereas the CAT and GST activities were increased. Eventually, the antioxidant system was overwhelmed, resulted in lipid peroxidation and caused DNA damage.

Although there are already some studies in the early stages of development in zebrafish, there are still pathways and mechanisms of action that remain to be explored. This is a very important and sensitive phase where the occurrence of damages can compromise the development of the animal.

3.1.3. Ecotoxicological impact

Since fungicides hit crops and animals that are not their target, they have become a major environmental problem. When accumulated in the soil, they can affect its properties (Joint 2001), since the soil has a high affinity for hydrophobic organic pollutants. However, it can also act as an aid in the natural degradation of these compounds (some bacteria present in soils) (Cai *et al.,* 2008; Arias-Estévez *et al.,* 2008). However, when in a great quantity, pesticides end up not being degraded, accumulating in the soil and affecting crops, eventually being absorbed by fruits (Hayward and Grierson 1960; Liu, Huang, *et al.,* 2016). In addition, pesticides may be leached by the water, eventually reaching groundwater, rivers and streams (Arias-Estévez *et al.,* 2008).

At the soil level, several fungicides are depleted and may cause various environmental problems. Many of the fungicides degrade when on the soil, and depending on their solubility and affinity they may be more retained in the soil, contaminating the same and other applied crops in that soil, or being leached more easily, thus reaching the rivers and seas (Arias-Estévez *et al.,* 2008; Cai *et al.,* 2008; Li *et al.,* 2015; Morgado *et al.,* 2016).

A study performed by Adetutu and co-workers (2008) showed that the metabolism of azoxystrobin in soil occurred within 21 days even in the absence of light and metabolites may persist after 21 days. The authors observed that it causes changes in the total structure of the soil fungal community, increasing or reducing the diversity of fungi in the soil. Azoxystrobin has also been shown short-term differential inhibitory effects on different fungal strains. This can damage non-target soil organisms, like nitrifying bacteria.

The azoxystrobin-based concentrations found in the environment were reported as up to 30 μgL^{-1} in Europe (Berenzen *et al.*, 2005) and 4.6 μgL^{-1} in the United States (Smalling and Orlando 2011). Some studies reported in Portugal show that traces of azoxystrobin are found in Portuguese rivers and the concentrations can fluctuate from 11.1 to 154.61 ngL^{-1} according to the season of the year. In the Iberian Douro River, it was found in concentrations in the order of 29.9 ngL^{-1} (Max) (Cruzeiro *et al.*, 2017). In the Ria Formosa Lagoon, south of Portugal, a study showed that a number of pesticides, including azoxystrobin, can be found at a maximum concentration of 154.6 ngL^{-1} (Cruzeiro *et al.*, 2015). However, until now, the concentrations that were tested in animal models ranged from 4.2 to 12 μgL^{-1}. A study by Kunz (2017) showed that azoxystrobin may have negative effects, reduce the biomass and reproduction, on aquatic invertebrates (*H. azteca, C. dilutus, and C. dubia*).

In view of these studies, and considering the persistence of this synthetic fungicide in the environment, studies should be carried out to evaluate the effects of this compound on non-target organisms.

3.2.1. Triazoles - Tebuconazole

Tebuconazole belongs to the triazoles group, one of the largest classes of fungicides. Bayer was the first to launch a triazole, namely triadimefon (Bayleton) in 1973 (Jayaratne *et al.*, 2001). With the advancement and need to respond to other problems, the research in this class of fungicides continued. Until 1986, several compounds were developed with tebuconazole being one of them. Its commercial names include HWG 1608, Bay HWG 1680, Folicur, Raxil and Elite. Bayer is a major producer of this fungicide. This compound has an enantiomeric structure, however the fungicidal activity of the (2)-R-tebuconazole is greater than (1)-S-tebuconazole),

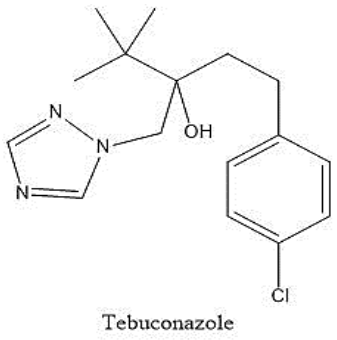

Figure 2 - Tebuconazole molecular structure

Tebuconazole is a systemic follicular fungicide that is rapidly absorbed in the vegetative parts of the plant. It is a broad-spectrum triazole fungicide with the molecular formula $C_{16}H_{22}ClN_3O$ and a molecular weight of 307.822 g mol^{-1} (fig. 2). It is used in a large number of cultures, in combating fusarium, rusts, white rot, and powdery mildew.

The MRL described by the regulation of the European Parliament and of the Council for this compound range from 0.02 to 40.0 mg kg^{-1} depending on the different foods.

3.2.2. Toxicological effects

The mode of action of this compound is based on the inhibition of the sterol 14α-demethylase. This enzyme is one of the intermediates in pathways leading to the formation of cholesterol in humans and ergosterol in fungi (Zarn, Bruschweiler, and Schlatter 2003). Due to the tebuconazole's mode of action, this compound raises suspicions about how it acts on non-target organisms and what adverse effects it will induce. However, there are still few animal toxicological studies of this compound. It has been studied in animal models such as *Daphnia magna*. A study carried out by Qi (2018) demonstrated that the racemic mixture of tebuconazole have medium toxicity to *D. magna* while *S*-tebuconazole has slightly more toxicity than the other two configurations. When exposure time increased to 14 days, rac-, *R*-, and *S*-tebuconazole could induce significant changes in the activity of chitinase and chitobiase. Moreover, a study carried out by Yu and his work group in 2013, showed that tebuconazole can function as a deregulator at the thyroid level. Larvae of zebrafish exposed to this compound presented increased T3 levels while decreased T4 concentrations. Furthermore, a series of transcription genes involved in the hypothalamic-pituitary-thyroid (HPT) axis was changed. The results demonstrated that the exposure might result in thyroid endocrine toxicity, thus affecting the normal development of zebrafish larvae. In another study conducted with adult zebrafish, it has been shown that animals take quite some time to replenish the levels of certain compounds in the body once their production has been affected by the tebuconazole. The levels of glucose, lactate, cholesterol and triglycerides increased, with cholesterol and glucose recovering after 14 days of recovery period whereas triglycerides and lactate continued to be elevated and other parameters were not stabilized (Sancho *et al.,* 2010).

Despite the above mentioned, no embryo development toxicity studies are found in the literature. Thus, it is of interest to carry out more studies in which its effects should be evaluated during the embryonic development.

3.2.3. Ecotoxicological impact

In 2005, Berezen and co-workers showed that the maximum concentration of tebuconazole found in the environment is 9.1 μgL^{-1} in Europe. This study was performed with samples from 13 streams in the region of Braunschweig (northern Germany). In Portugal, traces of tebuconazole are found in rivers and the concentrations can fluctuate from 28.7 to 198.1 ngL^{-1} according to the localization and season of the year. In the Iberian Douro River, it was found at the maximum concentration of 151.9 ngL^{-1} (Cruzeiro *et al.*, 2017) while in the Ria Formosa Lagoon, a study showed that it can be found at a maximum concentration of 62.4 ngL^{-1} (Cruzeiro *et al.*, 2015).

Although this compound does not yet have many reports in the literature, it is thought that its application poses some risks. Some studies on fungicide effects in soil have emerged over the years. In 2011, a study carried out by Herrero-Hernandez (2011) showed that dissipation was more rapid in amended soils than in unamended ones, and the processes of fungicide persistence, mobility and degradation in the soil were affected to a greater or less extent over time by solid matter.

Since some of the already existing studies deal with the theorical problems of this compound in some species, and increasingly this compound is detected in the environment, it is relevant to carry out studies on this subject. Advancement in the study of this theme will allow a clear understanding of the effects of this compound on non-target organisms and the environment that surrounds them, studying the pathways of degradation of the compounds, and their specific targets.

3.3.1. Dithiocarbamates - Mancozeb

Mancozeb, also known as dithane or 1,2-ethanedicarbamic acid, is an ethylene bisdithiocarbamate (EBDC) fungicide. In 1962, Rohm and Haas registered the zinc ion complex of maneb (mancozeb), a broad-spectrum fungicide (Runkle *et al.*, 2017). Its molecular formula is $C_4H_6N_2S_4Mn.C_4H_6N_2S_4Zn$, represented in fig. 3, and this compound has a molecular mass of 541.01 g mol^{-1} (Bolton *et al.*, 2008). In agriculture, this fungicide is used to

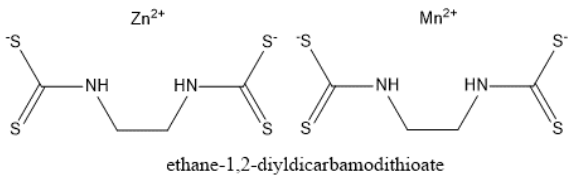

Figure 3 - Mancozeb molecular structure

protect many vegetables and fruits for roughly 400 different plant pathogens (Wexler *et al.*, 2005).

Numerous legislations on maximum residue limits for food of plant and animal origin have been established, which range from 0.05 to 25.0 mg kg^{-1} depending on the different foods. One of the major problems with the use of this compound is that in the presence of oxygen and water it can be quickly hydrolyzed and form ethylenethiourea (ETU), ethyleneurea (EU), ethylene bisisothiocyante (EBI) and ethylene bisisothiocyanate sulfide (EBIS). ETU is extremely toxic with a persistence of 5-10 weeks and has a great mobility in the soil due to its high solubility in water, unlike mancozeb that is little soluble in water (Hwang, Cash, and Zabik 2003; Hayes and Laws 1991).

3.3.2. Toxicological effects

Mancozeb itself has no fungicidal activity, only when it is exposed to water and is quickly hydrolyzed into EBIS which in turn, via the action of UV light, is then converted into EBI. These metabolites are thought to interfere with biochemical processes within the fungal cell cytoplasm and mitochondria resulting in the inhibition of spore germination. The activity of mancozeb is purely protective in that the compound remains on the leaf surface and does not penetrate through the cuticle, no systemic redistribution occurs, it does not have any curative fungicidal properties (Cryer *et al.*, 2015) .

Another metabolist of mancozeb is ETU which is extremely toxic and responsible for the toxicity associated with the class of EBDC. This compound is associated with negative effects on the thyroid, which were recognized in the 1950s by Ivanova-Chemishanska, and in the 1960s its mechanism of carcinogenicity was described as nongenomic and linked to changes in thyroid hormone levels. ETU is responsible for an increase in the thyroid stimulating hormone (TSH) (Ivanova-Chemishanska, Markov, and Dashev 1971) and increased pituitary weight (Raizada, Datta, and Dikshith 1979). However, not only the EBDC cause thyroid problems, but also the azoles class of fungicides, like tebuconazole and haxanoconazole, can cause thyroid effects, according to a study by Yu and his group in 2013, using zebrafish as an animal model.

Mancozeb has very adverse effects. A study conducted by Belpoggi and co-workers (2006), in which a long-term exposure of rats to this compound was performed, showed that mancozeb should be considered a multipotent carcinogenic agent, capable of

producing tumors of many types in various sites in treated animals. This compound also induces toxic effects on mammalian granulosa cells, according to Paro and workgroup (2012). Even though at low concentrations, this compound can negatively affect both the mouse and human and suggests that, at least concerning the reproductive system, it may affect human reproduction which is also supported by a recent study in which birth defects were detected in three infants exposed to mancozeb during pregnancy (Calvert *et al.*, 2007). Mancozeb is also related to possible mitochondrial dysfunction and neurotoxicity, being a pro-oxidant neurotoxicant and inducing ROS generation (Domico *et al.*, 2007). Moreover, this compound has also the ability to attack blood cells at DNA and chromosomal levels (Marques *et al.*, 2016).

Mancozeb degrades very rapidly, and its metabolites are even more toxic than this when in contact with the soil, water or air can easily reach the non-target organism causing damage and also affecting other crops and degrading the soil, impeding important processes.

3.3.3. Ecotoxicological impact

Mancozeb reduce the process of ammonification and nitrification (Walia *et al.*, 2014). Similar results were observed for nitrifying and ammonifying bacteria. Phosphorus solubilization was increases with the concentration of mancozeb used. In unamended soil, microbial biomass carbon and carbon mineralization were adversely affected by mancozeb. Soil enzymes, that is, amylase, invertase, and phosphatase showed an adverse and disruptive effect when mancozeb used was above 10 ppm (Walia *et al.*, 2014).

There are also studies on the effects of this compound that show that it can have impacts on carbon and nitrogen mineralization in soils. In 2009, Cernohlavkova and co-workers, realized a study to detected if this fungicide had significant toxic effects on nitrogen transformation processes. Although the adverse effects were not long lasting, the potential risk of using this pesticide on soil microorganisms may exist. In soil microbial ecotoxicology, there is often a dilemma on how to distinguish between stimulation and inhibition of tested substances. The results emphasized the focus on specific microbial parameters such as nitrification.

It is important to understand the effects of this compound on early stages of embryonic development since the animals are born in already contaminated environments. However, there are still no studies that deal with ecotoxicology from this point of view, and studies for this compound are not available, using animal models.

3.2. Natural fungicides

Taking these arguments, about synthetic fungicides, into consideration, there is the need for the use of compounds that have fewer effects on living organisms and environment but that can be effective in attacking crops and protecting against the different diseases. Thus, there has been a growing interest in introducing new products that are safe for human and non-target organisms and environmentally safe. In response to the aforementioned needs, there has been an increasing interest in natural based pesticides (Tripathi and Dubey 2004).

The optimization of plant natural compounds with proven fungicidal activity against fungal diseases for agriculture is an important research because it would permit to search some important alternatives to the use of synthetic fungicides.

Plants have some tools for combating fungi and diseases caused by them. Those tools can be physical barriers and chemical, that can be external or compounds produced by the plant itself (secondary metabolites). Plant extracts are a group of substances extracted from different parts of plants which contain a great number of compounds with fungal properties. These can be aqueous and hydroethanolic extracts (Shabana *et al.*, 2017). An example is the extract of *Equisetum* (Radulovic, Stojanovic, and Palic 2006) and the extract of *Mimosa tenuiflora* (Padilha *et al.*, 2010). Relative to the first one, a recent study by Yeganegi (2018) assessed, among other data, the antimicrobial effect on the growth of some pathogenic strain causing poisoning and infection, in which it was concluded that this stratum had a favorable activity against most of the tested microorganisms. Also, the extract of *Mimosa tenuiflora* was studied by Padilha (2010) in clinical isolates of *Staphylococcus aureus* also obtaining favorable results against the same. Overall, these studies support their antifungal properties.

Moreover, aromatic plants are especially rich in essential oil which have been used in foods as flavoring agents (Burt 2004). Most of these compounds are terpenes and have fungicidal properties (Bocquet *et al.*, 2018; Medeiros *et al.*, 2016; Thomidis and Filotheou 2016). Essential oil can be directly used or substances responsible for the antimicrobial properties can be isolated such as active terpenes, for example, eugenol (Xie *et al.*, 2017), thymol (Sharifzadeh *et al.*, 2018; Ribes *et al.*, 2017) and carvacrol (Chavan and Tupe 2014). Some of these compounds also have fungal activity. A study by Ahmand (2011) showed that thymol and carvacrol can disrupt ergosterol biosynthesis and membrane integrity against *Candida*. Another study performed by Abbaszadeh (2014),

demonstrated thymol, carvacrol, eugenol and menthol as alternative agents to control the growth of food-relevant fungi (*Aspergillus* spp., and *Cladosporium* spp.). The increase of the concentration of the tested compounds was directly related to the significant reduction of fungi growth.

As most of the essential oils with antifungal activity are peonline strata, the mechanism of phenolic compounds centers on their effects on cellular membranes. Simple phenols disrupt the cytoplasmic membrane and cause leakage of cells. Phenolics may also inhibit cellular proteins directly. However, some researchers have concluded that phenolic compounds may present many mechanisms of action and that there may be several targets which lead to inhibition of microorganisms (Davidson, Taylor, and Schmidt 2013).

Although it is thought that these compounds may be a more environmentally friendly alternative in combating fungi, there are still no sufficient toxicological studies to state that these compounds have no counterpart, and which doses can be applied without causing no problem, for the environment as well as for humans and non-target organisms.

4. Zebrafish as a model system

In general, the use of different animal models and *in vitro* cellular or tissues preparations as long been the basis for studying the toxicological and mechanistic responses induced by chemical and drug exposure in order to find clues about human diseases and disorders (Saeidnia, Manayi, and Abdollahi 2015; Doke and Dhawale 2015). However, in the last years, the international regulations and guidelines for animal experimentation require the implementation of 3Rs (replacement, refinement and reduction) (Russell, Burch, and Hume 1959). Different methods and alternative organisms have been applied to implement these principles. The use of the early life stages of zebrafish has become a model of choice as an alternative to mammalian testing for toxicology and biomedical research (Doke and Dhawale 2015) being considered as replacement or refinement methods (Lammer *et al.,* 2009). Indeed, since the work of George Streisinger suggesting this species as an animal model for basic research, based on its embryonic and genetic features (Stahl 1985), several laboratories worldwide have recognized and implemented zebrafish embryos as a major alternative model organism for science research, been currently used in the most diverse areas such as in embryonic development, genome evolution (Postlethwait *et al.,* 2004), behavior analysis (Clark, Boczek, and Ekker 2011) (eco)toxicology evaluation (Peterson and Macrae 2012), physiology (Lohr and Hammerschmidt 2011) as well as to study human-related diseases (Lieschke and Currie 2007; Santoriello and Zon 2012; McCluskey and Postlethwait 2015), among others.

4.1. Characteristics

The zebrafish (*Danio rerio*) is a freshwater teleost fish native to South-East Asia which, according to the regulatory criterion of independent feeding (up to 120 hours post fertilization, hpf), are considered as non-protected (Strahle *et al.,* 2012), surpassing the ethical issues concerning other models' embryonic studies. Moreover, zebrafish embryos present a unique combination of genetic and experimental embryologic features with approximately 70% of human genes showing at least one obvious zebrafish orthologue, making them ideal for embryonic studies and allowing the easier generation of transgenic animals (Lele and Krone 1996). It is a small teleost fish with 3-5 cm being the easiest vertebrate to keep in the laboratory, requiring a small space and reduced maintenance

costs compared to other high models (Lieschke and Currie 2007; Avdesh *et al.,* 2012). Zebrafish has a very high reproductive rate, their fertilization takes place externally (Teraoka, Dong, and Hiraga 2003), and present some similar structural and physiological characteristics with humans (Postlethwait *et al.,* 2004). The embryos are transparent and develop externally offering a complex and multicellular system in which the interaction of various tissues and developmental processes is integrated (Zon and Peterson 2005).

While the described advantages suggest this model as an ideal model for vertebrate developmental studies, it should be noted that this alternative animal model has some limitations. For instance, zebrafish lacks some organs, such as lungs, limbs and others (Ali *et al.,* 2011). In addition, it is a poikilothermic organism and the developing embryos do not have a placenta although presenting a protection structure with similar a function, the chorion (Lieschke and Currie 2007). Another specific practical concerns are the genome duplication that may affect some, but not all, genes (Spitsbergen and Kent 2003), the differences in the proliferation rates during neurodevelopmental periods of zebrafish and the lack of advance and knowledge of certain brain areas, its structures and their relation with mammalian counterparts (Stewart *et al.,* 2014). The presence of the chorion up to 48 hpf may difficult the administration of compounds; however, this can be overcome by manual or automated removing processes or using advanced techniques such as microinjection (Bugel, Tanguay, and Planchart 2014). Moreover, pharmacokinetic studies are limited in this species not only due to problems related to the administration of water-soluble compounds and the use of solvents that can impact toxicological outcomes but also due to the gap in knowledge of functional metabolic processes in zebrafish embryos (Verbueken *et al.,* 2017).

Nevertheless, despite the limitations, the characteristics present by this small teleost fish show its potential as an alternative toxicity testing strategy which is reflected by the increased number of publications using zebrafish that further underlines its growing popularity and impact in biomedical and (eco)toxicological research.

4.2. Embryonic development

Zebrafish have a notoriously fast embryonic development, and due to the fact that their eggs are transparent, their development can be accompanied- to detail with the help of a microscope.

Its development begins immediately after fertilization with the phase known as zygote (1 single cell). In this phase, the migration of cytoplasm begins in the direction of the animal pole with the purpose of giving rise to a blastodisc. There follows the cleavage period in which there is a high cell division rate. This period starts soon after the zygote phase and lasts until about two hours thereafter. At the end of this step, it is expected that there are already at least 64 cells and 4 tiers of blastomeres (Kimmel *et al.,* 1995).

The blastula period is characterized by critical changes in the embryo and the formation of more blastomeres. The formation of the blastula itself occurs when the cells migrate to the periphery and begin to delimit an internal cavity called the blastocyst (Kimmel *et al.,* 1995). The formed blastula cells begin to rearrange migrating to the internal region (endoderm and mesoderm) and more surface give rise to ectoderm. The next phase starts at 5 hpf and has the name of gastrulation and lasts for about 5 hours, until 10 hpf embryo, at which segmentation stage begins (Dahm, Geisler, and Nüsslein-Volhard 2005; Kimmel *et al.,* 1995).

By 24 hpf embryo has all the major tissues and many organ precursors formed, and it is possible to observe the beating heart, the circulating blood, the central nervous system, the eyes and optic vesicles (Kimmel *et al.,* 1995; Dahm and Geisler 2006; Peterson and Macrae 2012; Sumanas and Lin 2004).

The major organs are formed within 24-48 hpf during the period of pharyngula in which the organs become functional and the circulatory system begins to form. Between 48-72hpf hatching period begins (Kimmel *et al.,* 1995). At this point, the larva already has its internal organs with toxicological interest such as the liver (Gupta and Padilla 2014), pancreas, thyroid gland, a vascular network and a blood-brain barrier (Sumanas and Lin 2004; Scholz *et al.,* 2008).

Some of the development phases images correspondent to different development hours post fertilization, referred throughout the above description are represented in figure 4.

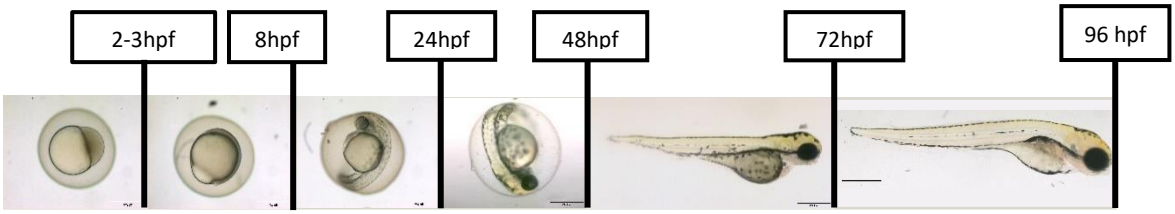

Figure 4 - Embryonic development of zebrafish at different hours post fertilization.

4.3. Effects of fungicides in early zebrafish development

The early stages of zebrafish development are increasingly used to test several compounds such as fungicides. However, only a few classes have been tested in this model. One of the classes with some studies carried out in this model is the triazole classes. A study in which zebrafish fertilized eggs were exposed to difenoconazole showed induced changes in the embryonic development including hatching inhibition, abnormal spontaneous movement, slow heart rate, growth regression and morphological deformities. The teratogenic effects on yolk sac and pericardium of the embryos as well as on the larvae notochord were described. In the subsequent phase, difeconazole at a concentration of 0.50 mgL^{-1} caused a significant body color blackening and a decrease in the heart rate of zebrafish larvae. In addition, the concentration of 0.25 mgL^{-1} inhibited the growth weight of adult zebrafish after 14 days exposure (Mu *et al.*, 2013). The effects of another triazole, fenbuconazole, in zebrafish embryos were also studied (Wu, *et al.*, 2018). The development and cardiac functioning were observed after exposure to 5, 50 and 500 ngL^{-1} nominal concentrations for 72h. The results showed that the highest concentration caused significantly increased pericardial edema rate, spine curvature rate, disturbed cardiac function and led to shortened lower jaw. The transcription of genes was also altered.

Another fungicide for which this model was used to evaluate the toxicity was Mancozeb, the EBCD fungicide. A study carried out by Costa (2018) , assessed the potential embryotoxic effects induced by mancozeb (from 5 up to 72 hpf) as well as the role of ROS in this process by the pre-treatment with a classical antioxidant (N-acetylcysteine, NAC). Markers of ROS, glutathione reduced form (GSH) levels and GST

activity were measured along with genotoxicity (comet assay), cell death (acridine orange) and behavioral parameters (spontaneous movement, touch stimulation and swimming response), in order to determine potential mechanisms of embryotoxicity. The results of this study showed that this compound was able to induce morphological abnormalities such as body axis distortion, DNA damage, cell death, increased ROS generation and changes in behavioral endpoints, during zebrafish development.

Despite the referred reports, fungicides are still little studied at the biochemical level and on vital parameters of the early stages of organism's development. It is not known what happens in aquatic and terrestrial environments to animals exposed to these compounds, since many are born in contaminated environments. There are also no reports at the ecotoxicological level of the effects of some classes of pesticides and even of the recent natural alternatives that are entering the market. Therefore, it is of interest to assess the effects on development and at the cellular level, using alternative models as is the case of zebrafish and evaluate the different LC_{50} for each compound in order to make their use safer.

5. Biochemical parameters

A number of biochemical parameters have been used under laboratory conditions to evaluate chemical toxic effects after exposure of embryonic zebrafish. Within the biochemical techniques, several drug-induced responses can be evaluated according to the type of study to be performed that underlie the basis for compounds' biological effects, biotransformation and toxicity. Sensitive cellular biomarkers of exposure have been used, providing valuable information on the cellular damage induced by compounds that can be used as early indicators of toxicity. Among them, the determination of biotransformation enzymes (phase I and II enzymes), oxidative stress parameters, stress proteins, metallothioneins and neurotoxic parameters are commonly used. These biomarkers have been successfully employed in a variety of research areas through different assays complemented with genetic approaches.

Relative to the biotransformation, this process is induced, following exposure of toxic substances to fish and occurs in two-phase steps. Phase I reactions are the major pathways for the biotransformation of compounds, rendering the xenobiotic molecule less active through the inclusion of polar groups and, thus, making it more water-soluble, enabling Phase II reactions to take place (Almeida *et al.,* 2015). While Phase I reactions include oxidation, reduction and hydrolysis reactions, Phase II detoxification involves conjugation reactions such as glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation (Jancova, Anzenbacher, and Anzenbacherova 2010). The main enzymes involved in the Phase I reactions are cytochromes P450 (CYPs) and others associated with the smooth endoplasmic reticulum (microsomes) (Bucheli and Fent 1995) performing mainly hydroxylations, hence, acting as monooxygenases, dioxygenases and hydrolases (Jancova, Anzenbacher, and Anzenbacherova 2010). This family is regulated by the aryl hydrocarbon receptor and is usually integral to the detoxification of many exogenous chemicals (Goldstone *et al.,* 2009). Studies have shown that the cytochrome P450 1A (CYP1A), a class of cytochrome P450 isozymes, is a more suitable biomarker for fish toxicity. The induction of CYP1A is usually associated with the enzyme activity of 7-ethoxyresorufin-O-deethylase (EROD) (Whyte *et al.,* 2000). The CYP1A catalysis the oxidative dealkylation of 7-ethoxyresorufin into the fluorescent resorufin which is mediated by a NADPH-cytochrome P450 reductase (Gagnon and Rawson 2017). Usually, other measured enzymatic activity in zebrafish embryos is related to the carboxylesterase enzyme. This enzyme belongs to the class of hydrolases

being part of the most effective pathway in the degradation of pesticides (Wheelock, Shan, and Ottea 2005). Although research on these enzymes is still developing, in the case of zebrafish, this enzyme hydrolyses triacylglycerides and esters, thereby, being considered a phase I enzyme due to the important role it plays in the metabolization and excretion of toxic compounds (Hatfield *et al.,* 2016). The quantification method for the activity of this enzyme family is based on a resorufin-based probe through the introduction of p-acetoxyphenylmethyloxy as a bi-functional moiety to resorufin. This substituent not only quenches the spectroscopic signal of resorufin, but also serves as a recognition unit for carboxylesterase (Zhang *et al.,* 2012).

The Phase II enzymes also play an important role in the biotransformation of endogenous compounds and xenobiotics and can also be used as a toxicity biomarker. They include mostly transferases and include reduced and oxidized glutathione and glutathione S-transferase (GST) (Jancova, Anzenbacher, and Anzenbacherova 2010). The elimination of many xenobiotic compounds is accomplished by conjugation with reduced glutathione (GSH) either spontaneously or enzymatically in reactions catalyzed by GST (Lu 2009). Besides, GSH is the dominant non-protein thiol being essential to maintain the intracellular redox balance and the thiol status of proteins (Forman, Zhang, and Rinna 2009; Lu 2009). This later is considered the ratio between reduced and oxidized glutathione (GSH/GSSG) and has been widely used as an indicator of oxidative stress following exposure to several compounds. In laboratory toxicity tests, the impact of compounds has been investigated by spectrophotometric methods using the Ellman's reagent (DTNB - (5,5'-dithio-bis-[2-nitrobenzoic acid]) (Ellman 1959) or by fluorescence quantification of the fluorescence resulting from the reaction with the probe o-phthalaldehyde (Senft, Dalton, and Shertzer 2000). Overall, changes in glutathione levels occur during early developmental stages and the GSH dynamics have been described as important for the maintenance of the redox homeostasis, thus, allowing the correct development of the embryo (Timme-Laragy *et al.,* 2013). Moreover, GSH is an important substrate for GST, an important enzyme within phase II xenobiotic metabolism (Massarsky, Kozal, and Di Giulio 2017), which can be evaluated by monitoring the formation of the conjugate of GSH of electrophiles such as 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, iodomethane, ethacrynic acid, and bromosulfophthalein (Habig *et al.,* 1974). This enzyme provides essential functions in intracellular transport, as well as defense against oxidative damage and peroxidative DNA products (Birben *et al.,* 2012). Therefore, given the importance of glutathione to the embryo development,

the evaluation of these biomarkers is useful in the evaluation of toxic responses induced by different compounds.

Furthermore, biotransformation enzymes' activity typically leads to the generation of reactive oxygen species (ROS), through several reactions taking advantage of the presence of oxygen (Klotz and Steinbrenner 2017). These ROS are known to interfere in several biological processes through changes in signaling cascades at several levels (Schieber and Chandel 2014). The formation of free radicals is continuous at the physiological level and plays important roles in developmental processes (Hansen 2006; Dennery 2007). Still, increased oxygen toxicity can lead to enzyme deactivation, lipid peroxidation, DNA damage and eventually cellular death processes. The superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) are groups of antioxidant enzymes that constitute part of a cellular defense system (Ighodaro and Akinloye 2017). SODs are redox-active metalloenzymes (requires a metal cofactor for its activity) that catalyze the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen (Sayre, Perry, and Smith 2008). CAT is a common antioxidant enzyme present in almost all living tissues and responsible for the degradation or reduction of hydrogen peroxide to water and molecular oxygen using either iron or manganese as a cofactor (Ighodaro and Akinloye 2017). GPx is an important intracellular selenocysteine peroxidase that breaks down hydrogen peroxide to water (Ighodaro and Akinloye 2017), while GR catalyzes the NADPH-driven reduction of GSSG to GSH. Overall, the interplay between these enzymes is extremely important in the defense mechanism against reactive radicals; for this reason, these enzymes have been proposed as biomarkers of toxicity. In general, in laboratory studies, the determination of antioxidant enzyme activities for SOD, CAT, GPx and GR is a common procedure and full assay procedures can be found in the literature. Briefly, SOD activity is determined based on the formation of a colorimetric dye involving xanthine oxidase while CAT activity is evaluated by following the decomposition of hydrogen peroxide. The GPx assay monitors the NADPH oxidation to $NADP^+$ mediated by GR. The GR procedure is based on the direct decomposition of NADPH (fig. 5).

Furthermore, an unbalance in these redox couples is likely to result in an overproduction of reactive free radicals, which are very unstable and are responsible for the alteration of macromolecules (Gutteridge 1995). Among them, lipids, nucleic acids and proteins are the major targets (Lobo *et al.,* 2010). The most commonly used method to assess damage induced by oxidative radicals involves the analysis of peroxidation

products of lipids, which are susceptible to be attacked by these radicals. The concentrations of resulting peroxides are often assessed by absorbance of peroxidation products or by the colorimetric complexes formed by reaction with externally added reagents (such as TBARS assay) (Dotan, Lichtenberg, and Pinchuk 2004).

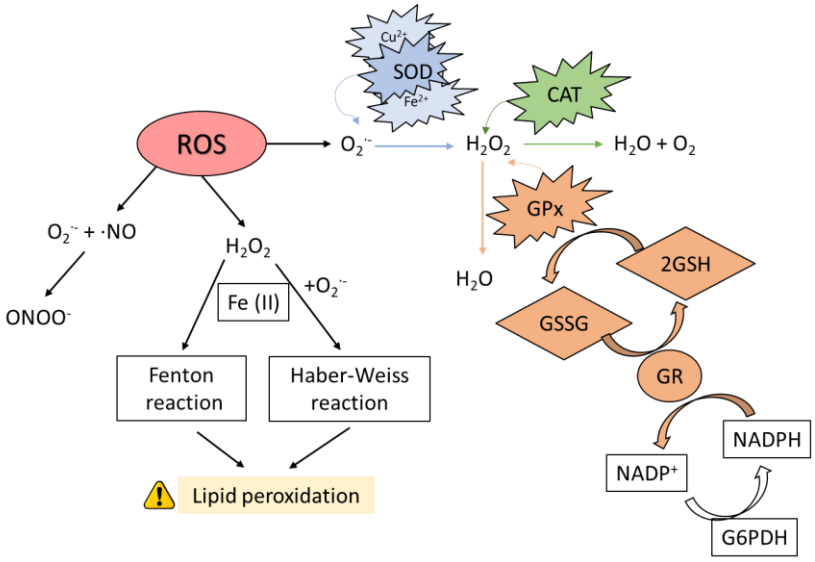

Figure 5 - Representative scheme of some enzymatic and non-enzymatic defenses related to oxidative stress. Adapted by (Morry, Ngamcherdtrakul, and Yantasee 2017).

Relative to the oxidized proteins, they are commonly evaluated trough the alteration induced in the carbonyl groups while damage induced in nucleic acids is usually assessed by a variety of subjective methods (Dotan, Lichtenberg, and Pinchuk 2004). In stress and harmful situations, the organism also responds by inducing of cytoprotective proteins that include the heat shock proteins (HSPs), the glucose-regulated proteins (GRPs) and the stressor-specific stress proteins, which include metallothioneins (MTs) (Piano, Valbonesi, and Fabbri 2004). Among these, metallothioneins and heme oxygenase proteins have been described and quantified by simple and sensitive biochemical methods, while HSPs have been quantified by manually intensive and time-consuming methods (e.g. western-blot) (Lewis *et al.*, 1999). In fact, MTs are a family of proteins essential for the regulation, sequestration and detoxification of metals and oxidant damage, metabolic regulation, sequestration and/or redox control and are one of the widely studied biomarkers for assessing metal contamination in aquatic models by using the Ellman reagent DTNB (Ellman 1959).

Besides oxidative biomarkers, additional neurotoxic parameters have been used as important biomarkers for contamination in zebrafish. An example is the acetylcholinesterase, an enzyme that, besides being involved in synaptic transmission, is

also involved in non-cholinergic functions during development (Bertrand *et al.*, 2001). Its activity is easily evaluated by the spectrophotometric Ellman assay (Worek, Eyer, and Thiermann 2012). In addition, the physiological and biochemical changes induced by exposure to compounds can be reflected by assaying the activity of enzymes intimately linked to metabolism, such as succinic dehydrogenase (SDH), an enzyme embedded in the internal membrane of mitochondria, glucose-6-phosphate dehydrogenase (G6PDH), which catalyzes the pentose phosphate pathway- and the lactate dehydrogenase (LDH), which catalyzes anaerobic glycolysis (Patrinostro *et al.*, 2013; Yang *et al.*, 2017), which are important in the energy metabolism, and can be evaluated by using selected probes and spectrophotometric methods.

Overall, these parameters have been employed as a toxicological biomarkers in zebrafish embryos, proving to be a valuable biomarkers in a number of investigations following exposure to a variety of compounds. Notwithstanding, additional parameters can also be used as possible toxicological biomarkers (immunological parameters, osmoregulatory markers, apoptosis, among others). However, due to the absence of consistent results among experiments, further studies are required to validate and recommend these biomarkers as potential biomarkers for fish development toxicity.

Chapter II

Objetives

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The purpose of this study was to evaluate the effects of various synthetic (azoxystrobin; tebuconazole; manzozebe), and natural fungicides (thymol, extract of *Equisetum* and extract of *Mimosa tenuiflora*), on zebrafish embryo development. For this, an approach to various toxicological methods was used:

i. Determination of the LC_{50} value for each compound by the study of various concentrations of fungicides;

ii. Establishment of study concentrations based on the LC_{50} for each compound;

iii. Evaluation of lethal, sub-lethal and teratogenic parameters (lethality, somite formation, tail detachment, head development, spontaneous movements, edema presence (yolk sac and cardiac), hatching rate, eyes and otoliths development, circulatory system, heartbeats and malformations);

iv. Perform biochemical analyzes to evaluate oxidative parameters - production of reactive oxygen species, and lipid peroxidation - and antioxidative parameters - superoxide dismutase, catalase, glutathione peroxidase, acetylcholinesterase, among others;

v. Analysis of results and assessment of the associated risk

The figure 6 shows a schematic representation of the experimental strategy to used achieve the proposed objectives for this study.

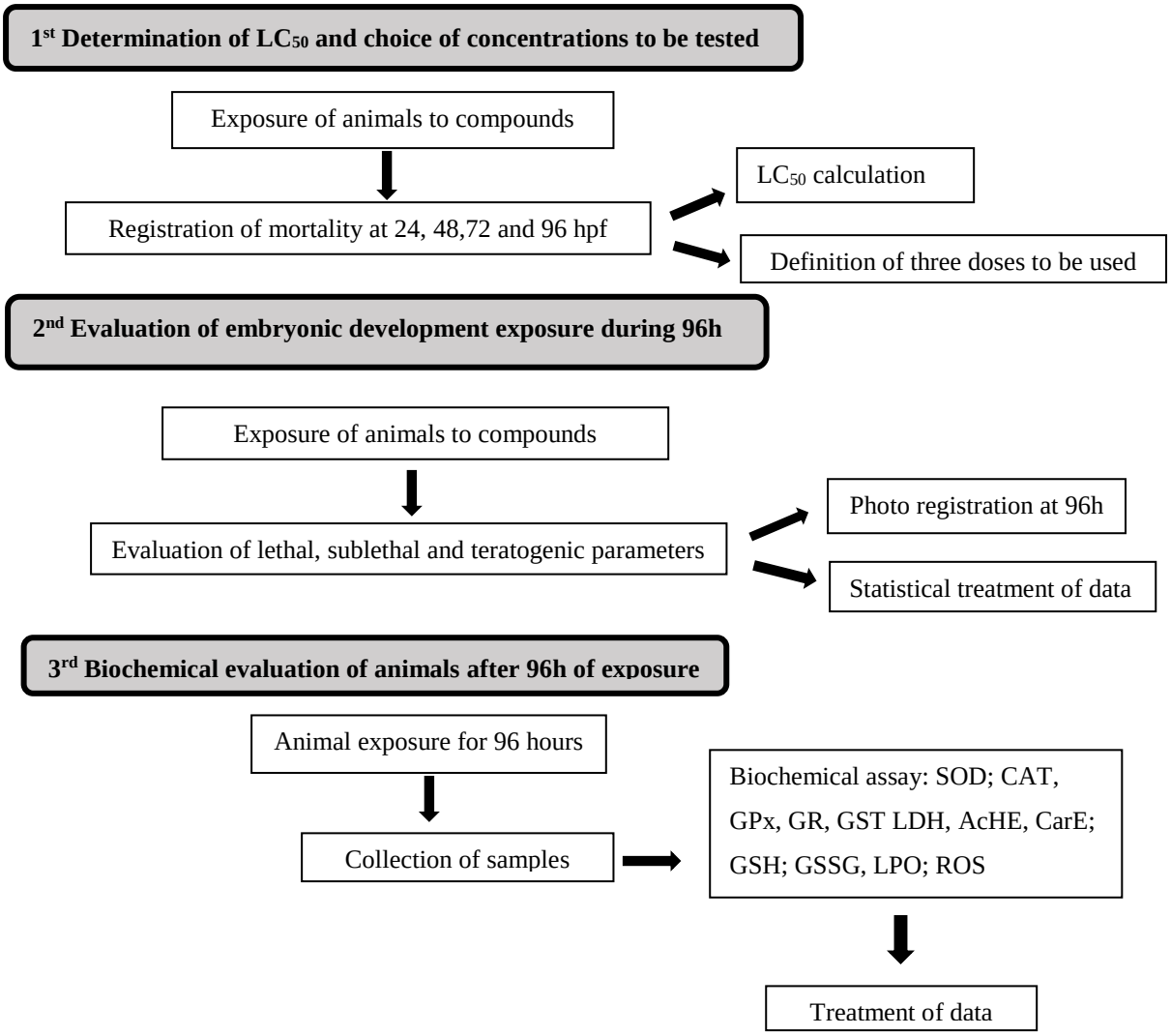

Figure 6 - Schematic representation of the proposed experimental strategy.

Chapter III

Material and Methods

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Chemicals

Azoxystrobin (Quadris, 250 g ai L^{-1}, CAS number: 131860-33-8) was purchased from Syngenta Crop Protection - Soluções para a Agricultura, Lda (Portugal), Tebuconazole (Folicur 0.25 g ai L^{-1}, CAS number: 107534-96-3) was acquired to Bayer CropScience Lda (Portugal) and Mancozeb (Mancozeb 0.80 g ai L^{-1}, CAS number: 8018-01-7) was purchased from SAPEC Agro, S.A (Portugal). Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebourg, France). *Equisetum arvense* aqueous extract (6.25%) was acquired to Aries Umweltprodukte (Horstedt, Germany), ethanolic extract of *Mimosa tenuiflora* (Matry, 80% ai) was purchased from Biagro (Valencia, Spain) and Thymol (extra pure, CAS number: 89-83-8) was acquired to EMD Millipore (Oeiras, Portugal). All chemicals used for biochemical tests were of the highest grade commercially available and purchased from Sigma-Aldrich. Except when specified, solutions were prepared with ultra-pure water purified by a Milli-Q Gradient system (Millipore, Bedford, USA). The stock solutions were prepared in water and stored at 4ºC until further dilution. All solutions were freshly made with embryo water (28 ± 0.5 °C, 200 mgL^{-1} Instant Ocean Salt and 84 100 mgL^{-1} sodium bicarbonate; UV sterilized) prepared from City of Vila Real filtered-tap water. During the exposure period the temperature was kept at 28 ± 0.5 °C.

2. Experimental design

The wild-type (AB strain) zebrafish were maintained at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal) in an open water system supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5) at 28 ± 0.5 °C in a 14:10 h light-dark cycle. Animals were fed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. nauplii.

The animals spawning was favored by diverse ambient factors and food supplementation, as the onset of lights in the morning, adornments were used to simulate favorable conditions, the temperature of the water was decrease for 25-26and the groups of animals in the breeding tanks are placed based on ratio of 2 males : 1 female (Felix, Antunes, and Coimbra 2014).

After spawning, embryos were collected, washed in embryo water and bleached according to established protocols (fig. 7 (Westerfield 2007; Varga 2011)). Fertilized embryos with normal morphology (Kimmel *et al.*, 1995), assessed using a SMZ 445 stereomicroscope (Nikon, Japan), were used for the subsequent experiments.

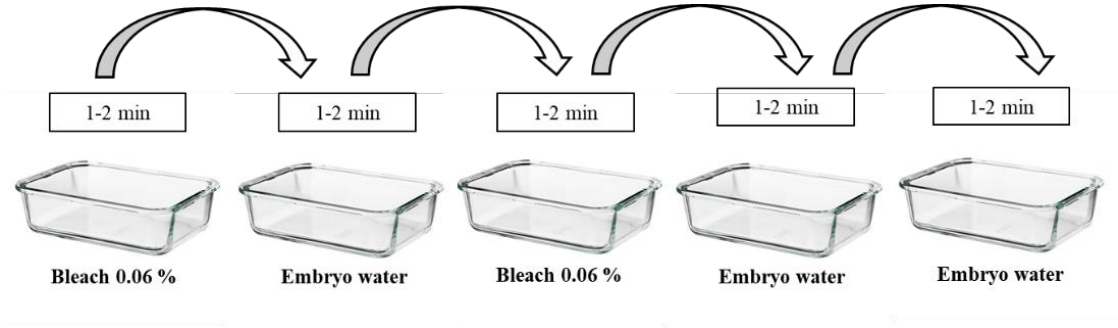

Figure 7 – Representative scheme of embryo bleaching process.

3. Selection of exposure concentrations (LC_{50}-96h)

Embryos at the early blastula stage (~2.0 hours post-fertilization – hpf) were exposed during 96h in 50 mL beakers, for 96h to different concentrations of each fungicide: Azoxystrobin (0.0025, 0.025, 0.25, 2.5 and 25 mgL^{-1}), Tebuconazole (0.0025, 0.025, 0.25, 2.5, 25 and 50 mgL^{-1}), Mancozeb (0.008, 0.08, 0.8, 5, 8, 80 and 800 mgL^{-1}), *Equisetum* aqueous extract (0.000625, 0.00625, 0.0625, 0.625, 6.25, 62.5, 625 and 6250 mgL^{-1}), ethanolic extract of *Mimosa tenuiflora* (0.008, 0.08, 0.8, 8, 80, 8000 and 80000 mgL^{-1}) and Thymol (0.001, 0.01, 0.1, 1, 10 and 100 mgL^{-1}), according to the OECD standard protocol (OECD 236) with minor modifications. These concentrations were based on preliminary studies, agriculture application doses and taking in consideration the environmental reports (Berenzen *et al.*, 2005; Cruzeiro *et al.*, 2017). All test solutions were renewed every 24h. Mortality was recorded after 24, 48, 72 and 96 h of exposure. Control sets were simultaneously prepared under the same conditions to correct mortalities. The experiments were repeated three times and the LC_{50} value was derived through probit analysis (Finney 1971). These results were used to select concentrations for the remaining experimental conditions.

Selected fungicides concentrations were 0.001, 0.01 and 0.1 mgL^{-1} for azoxystrobin (LC_{50} = 1.15 ± 0.32 mgL^{-1} – Appendices I - 1), for mancozeb (LC_{50} = 5.13 ± 2.93 mgL^{-1} – Appendices I - 2) were 0.0005, 0.005 and 0.05 mgL^{-1} and for tebuconazole (LC_{50} = 7.25 ± 2.61 mgL^{-1} – Appendices I - 3) were 0.05, 0.5 and 5 mgL^{-1}. For the natural based

fungicides, the selected concentrations were 0.00625, 0.0625 and 0.625 mgL^{-1} for *Equisetum* aqueous extract (LC_{50} = 435.31 ± 67.24 mgL^{-1} – Appendices I - 4), for ethanolic extract of *Mimosa tenuiflora* (LC_{50} = 123.87 ± 54.60 mgL^{-1} – Appendices I - 5) were 0.008, 0.08 and 0.8 mgL^{-1} and for thymol (LC_{50} = 32.67 ± 7.47 mgL^{-1} – Appendices I - 6) were 0.01, 0.1 and 1 mgL^{-1}. As well as to the water system (control group).

4. Developmental toxicity tests

The developmental toxicity test was performed according to methods outlined before, which consisted in the evaluation of observable lethal, sub-lethal and teratogenic parameters (table 1), (Girardi *et al.*, 2017; Felix *et al.*, 2017; Hallare, Kohler, and Triebskorn 2004; Santos, Matos, and Coimbra 2014). Each random 100 embryos were put into single beakers containing different concentrations of the tested fungicide and each concentration was replicated five times. A blank control was also set and tests lasted for 96 h. Test solutions were renewed every 24 h and dead eggs were removed daily from each beaker. Mortality was recorded daily and, at different time points (24, 48, 72 and 96 hpf), specific endpoints (Table 1) were evaluated as presence or absence of different characteristics, or quantified (e.g. spontaneous movements (movement per minute), heartbeat (btm – beats per minute), hatching and teratogenic parameters) in 10 animals randomly removed from each group. The analysis were performed using an inverted microscope (IX 51, Olympus, Antwerp, Belgium) (fig. 8).

Figure 8- Schematic representation of some of the lethal, sublethal and teratogenic parameters evaluated at 24, 48, 72 and 96hpf. Some representative images of embryo development status of the zebrafish are also presented at the respective evaluation times, as well as the apparatus in which they were performed.

At the end of the experimental period, at 96 hpf, larvae were evaluated for the presence of malformations (edema, spinal and notochord torsions), body length, area of yolk sac, area of heart and area of eye. For immobilization of larvae, 3% methylcellulose (Muntean *et al.*, 2010) was used and digital images were obtained in a color digital CCD camera (Color View III, Olympus, Hamburg, Germany), mounted on an inverted microscope (IX 51, Olympus, Antwerp, Belgium) using a 4× Olympus UIS-2 objective lens (Olympus Co., Ltd., Tokyo, Japan), and data were acquired using Cell A software (Olympus, Antwerp, Belgium). Images were further combined, merged and processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, USA). Measurements were taken using a digital image analysis software (Digimizer version 4.1.1.0, MedCalc Software, Mariakerke, Belgium).

Table 1 - Lethal, sublethal and teratogenic parameters to the evaluation of the embryonic development of zebrafish. X – Observed (as presence or absence) and XX- Accounted. This table was adapted from (Girardi *et al.*, 2017).

	Periodical development			
	24hpf	**48hpf**	**72hpf**	**96hpf**
Lethal				
Mortality	x	x	x	x
Tail Detachment	x	x	x	x
Head Detachment	x			
Sublethal				
Development of the somites	x	x	x	x
Eye development	x	x	x	x
Development of otoliths	x	x	x	x
Edema (cardiac and yolk sac)	x	x	x	x
Pigmentation	x	x	x	x
Spontaneous movements	xx	x	x	x
Circulation in the tail			x	x
Heartbeat		xx		
Hatching			xx	
Teratogenic				
Malformations			x	xx
Size				xx

5. Biomarker determinations

5.1. Sample preparation for biomarker analysis

For the biomarker determination, five new experimental exposures were performed containing 100 embryos per concentration and larvae were collected at 96 hpf and stored at -80ºC in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4) (Deng *et al.,* 2009). Samples were then homogenized using beads in a Tissuelyser II, the homogenates were centrifuged at 15 000×g at 4 ºC for 20 min in a refrigerated centrifuge (4ºC, Sigma 3K30), and supernatants were collected for biochemical analysis. The biomarker assessment was performed at 30 °C using a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Cary Eclipse fluorescence spectrophotometer. All samples were performed in duplicate and measured against a reagent blank in the appropriate microplate and values normalized to the total protein concentration, determined by the Bradford assay (Bradford 1976) at 595 nm, using Bovine Serum Albumin (BSA) as a standard (0-1.5 $mg.mL^{-1}$).

5.2. Reactive oxygen species (ROS) quantification

Determination of total ROS was performed at 485 nm (excitation) and 530 nm (emission) wavelengths, using the fluorescent probe DCFH-DA, as previously described (Deng *et al.,* 2009) and was estimated based on a DCF standard curve (0-50 µM). Briefly, in a 96-well microplate, 20 µL sample were added to 100 µL de PBS (pH =7.4) and 8.3 µL DCFH-DA 10 $mg.mL^{-1}$ (in DMSO) were then added. The microplate was incubated for 30 min at 37 ºC before read. Data was express as µmol DCF mg $protein^{-1}$.

5.3. Oxidative stress markers

5.3.1. Superoxide Dismutase (SOD)

The SOD activity was assayed by measuring its ability to inhibit the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm (Durak *et al.,* 1993). Briefly, in each well of the 96-well microplate, 10 µL of sample were mixed with 170 µL of potassium phosphate buffer 50 mM containing 0.6 mM hypoxanthine, 1 mM EDTA, and 0.2 mM NBT (nitrobluetetrazolium). The reaction was started by the addition of 28 mU mL^{-1} of xanthine oxidase diluted in potassium phosphate buffer 50 mM and 1 mM EDTA. The

increase in absorbance due to dismutation of O^{2-} into H_2O_2 was recorded for 3 minutes and SOD from bovine erythrocytes was used for construction of a standard curve (0-60 U mL^{-1}) and the final values were expressed in U mg $protein^{-1}$.

5.3.2. Catalase (CAT)

The CAT activity was determined based on the Claiborne method (Claiborne 1985). The assay reaction consisted of 10 µL of sample added to 90 µL sodium buffer 100 mM (pH 7.4) containing 20 mM H_2O_2 and the decrease in absorbance was monitored at 240 nm for 3 minutes. Activity was calculated as enzyme units per milligram of protein using bovine catalase as a standard (0-6 U mL^{-1}).

5.3.3. Glutathione Peroxidase (GPx)

The GPx activity was measured by the method described by Massarsky, Kozal, and Di Giulio (2017). In each well of the 96-well microplate, 10 µL sample were mixed with 200 µL of sodium phosphate buffer 100 mM (pH 7.0) containing 5 mM NaN_3 (to inhibit CAT), 0.261 mM NADPH, 0.9 U mL^{-1} Glutathione Reductase (GR) and 18 mM GSH. The reaction was started after 2 min with the addition of 10 µL H_2O_2 1.5 mM. The decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$ was observed for 3 minutes and the activity was determined using the extinction coefficient of 6.22 mM^{-1} cm^{-1}. The enzymatic activity of GPx was expressed in µmol NADPH min^1.mg $protein^{-1}$.

5.3.4. Glutathione reductase (GR)

The GR activity was measured by the method used by Massarsky, Kozal, and Di Giulio (2017). In each well of the 96-well microplate, 10 µL sample were mixed with 200 µL of potassium phosphate buffer 50 mM (pH 7.0), 0.5 mM EDTA containing 0.3 mM NADPH and the reaction was started by addition of 20 µL GSSG 11 mM. The decrease in absorbance at 340 nm due to the oxidation of NADPH to $NADP^+$ was observed for 3 minutes and the activity was determined using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. The enzymatic activity of GR was expressed in µmol NADPH min^{-1}.mg $protein^{-1}$.

5.4. Glutathione levels

The glutathione levels were determined fluorometrically by measuring both the reduced (GSH) and the oxidized states (GSSG) using the fluorochrome ortho-phthalaldehyde (OPA, prepared in methanol) at 320 nm and 420 nm for excitation and emission wavelengths, respectively, and according to the method previously outlined (Gartaganis *et al.*, 2007). For both analyses, 10 µL of sample were used and 40 µL of TCA 25% were added to precipitate proteins. For GSH, 170 µL of buffer Tris HCl 0.26 M pH 7.8, 115 µL of NaOH 0.56 N and 15 µL of OPT 1 mg mL^{-1} were added before incubation for 15 min at room temperature and read. For GSSG, 20 µL of NEM 40 mM were added and plate incubated for 30 min at room temperature. Then, 150 µL of phosphate buffer 0.23 M pH, 105 µL NaOH 0.71 N and 25 µL de OPA 1 mg mL^{-1} were added before incubation for 15 min at room temperature before read. Concentrations were estimated based on GSH and GSSG standard curves (0-500 µM) and data express as µmol GSH mg $protein^{-1}$ and µmol GSSG mg $protein^{-1}$. The ratio between GSH and GSSG was calculated as the oxidative-stress index (OSI).

5.5. Xenobiotic biotransformation

5.5.1. Glutathione-s-transferase (GST)

The GST activity was measured by the method of Habig and Jakoby (1981). In the 96-well microplate, 10 µL sample were placed and 180 µL potassium phosphate buffer 100 mM, pH 7.4 containing 1 mM CDNB were then added, after 2 min incubation, 50 µL GSH 25 mM were added to start the reaction. The increase in absorbance at 340 nm due to the oxidation of CDNB was observed for 3 minutes and the activity was determined using the extinction coefficient of 9.60 $mM^{-1}cm^{-1}$. The enzymatic activity of GST was expressed in µmol CDNB min^{-1}.mg $protein^{-1}$.

5.5.2. Carboxylesterase (CarE)

Carboxylesterase (CarE) activity was determined using the method described for microplates by Hosokawa and Satoh (2002). Briefly, on a microplate, 10 µL sample were incubated for 2 min and then 150 µL of 4-nitrophenyl acetate 1 mM were added and read for 3 min at 405 nm. CarE activity was express as µmol 4-nitrophenol min^{-1}.mg $protein^{-1}$ (ε = 16,4 $mM^{-1}cm^{-1}$).

5.6. Oxidative damage - Thiobarbituric acid reactive substances (TBARS)

The quantification of malondealdehyde (MDA) was used as an indicator of lipid peroxidation (LPO) and was determined according to the method described by Gartaganis 2007 (Gartaganis *et al.*, 2007). This method was performed at 535 nm (excitation) and 550 nm (emission) wavelengths, based on the chromogenic assay by a thiobarbituric (TBA) acid-based. Samples (20 µL) were mixed wih 130 µL of homogenization buffer, 150 µL of TBA reagent (0. 5% TBA, 20% TCA e 0.33N HCl) and 2 µL of BHT 2% (EtOH) in a microcentrifuge tube, which was incubated for 15 min at 100 ºC. Then, it was cooled in ice and 300 µL of butanol was added. Tubes were vortexed and centrifuged at 15000 xg in a centrifuge (Sigma 3K30) for 3 min and 200 µL were read. LPO was calculated based on a standard curve between 0 and 25 µM of MDA (prepared in H_2O and reacting with TBA reagent) and the value of LPO was express as µmol MDA mg $protein^{-1}$.

5.7. Neurotransmission (AChE) and anaerobic metabolism (LDH)

5.7.1. Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) activity was determined based on Ellman´s method (Ellman *et al.,* 1961) using the method described for microplates by Rodriguez-Fuentes *et al.,* (2015). On the microplate, 10 µL of sample were placed and 180 µL DTNB 0.5 mM, 10 µL acetylthiocholine iodide 20 mM were then added. The microplate was read at 405 nm for 3 minutes and data analyzed using the 5-thio-2-nitrobenzoic acid (TNB)

extinction coefficient of 13.6 mM^{-1} cm^{-1}, and data expressed as µmol TNB min^{-1}.mg $protein^{-1}$.

5.7.2. Lactate dehydrogenase (LDH)

For lactate dehydrogenase (LDH) activity, the method described by Domingues *et al.*, (2010) at 340 nm was used using the extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. On the microplate, 10 µL of sample were mixed with 200 µL of NADH 0.24 mM and the reaction started with 40 µL sodium pyruvate 10 mM and read for 3 min. LDH activity was express as µmol NADH min^{-1}.mg $protein^{-1}$.

6. Statistical analysis

The statistical analysis was performed using the statistical package SPSS 20.0 for Windows (SPSS Inc., Chicago, USA). Each beaker was considered an experimental unit, thus when 10 embryos were taken from the beaker, their values were averaged and considered as n=1. In order to decide which statistical test to apply, several assumptions were made, such as normality (Sapiro-Wilk test) and homogeneity of variance (Levene's test). When the assumptions of normality and homogeneity of variances were met, differences among groups were assessed by analysis of variance (ANOVA) followed by the Tukey multiple comparison test and data expressed as mean ± standard deviation. When these assumptions were not met, the data treatment was performed using non-parametric tests: the Kruskal-Wallis analysis of variance, followed by Dunn's test with a Bonferroni correction for multiple comparisons was used and data expressed as medians and interquartile range (25^{th}; 75^{th} percentiles) (Felix, Antunes, and Coimbra 2014).

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Chapter IV

IV.1

Results

Azoxystrobin

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at 24, 48, 72 and 96 hpf (fig. 9). At 24 hpf, no differences between concentrations tested and the control group were observed. The same occurred at 48, 72 and 96 hpf.

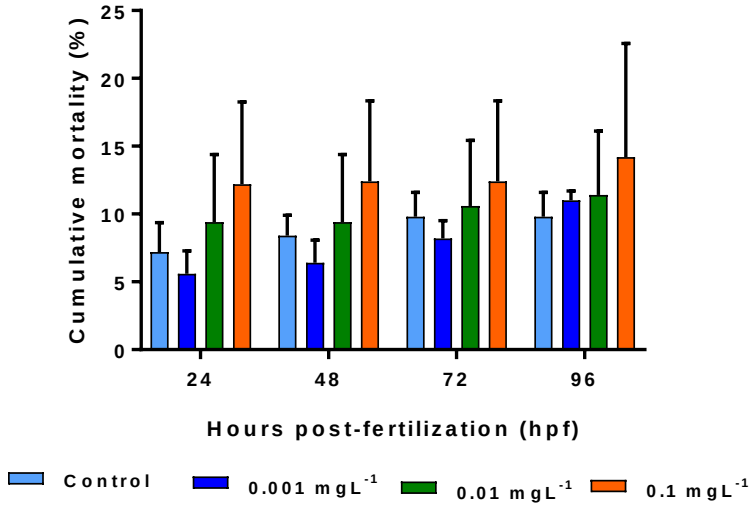

Figure 9 - Cumulative mortality of zebrafish embryos exposed to azoxystrobin. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed at 24 hpf (table 2) showed significant differences among groups ($X^2(3)=9.405$, $p=0.024$). The concentration of 0.001 mgL^{-1} presented a significant decrease in the number of spontaneous movements relative to the control group ($p=0.006$), to the 0.01 mgL^{-1} ($p=0.027$) and to the highest concentration ($p=0.014$). No other significant differences were observed.

The exposure of embryos to azoxystrobin induced a significant increase in the heartbeat (table 2), particularly between concentration 0.001 mgL^{-1} and 0.01 mgL^{-1} ($p=0.006$). A significant increase was also detected between 0.001 mgL^{-1} and 0.1 mgL^{-1} ($p=0.007$). No other significant differences among groups were observed.

The hatching rate (table 2) was evaluated at 72 hpf ($F(3,20)=6.701$, $p=0.004$) and more than 50% of the embryos hatched for all the concentration evaluated. The 0.01 mgL^{-1} ($p=0.027$) and 0.1 mgL^{-1} ($p=0.003$) presented a decrease in relation to the control group, while the 0.001 mgL^{-1} ($p=0.128$) showed no differences in relation to the remaining groups.

At 96 hpf, the malformations (table 2) observed at the animals exposed to the compound were very few although significant differences were observed between groups ($X^2(3)=9.607$, p=0.022). In this regard, and as expected, the control group showed no apparent malformations. On the other hand, exposure to the highest concentration induced higher percentage of malformed animals compared to the control group (p=0.003). No other significant differences among groups were perceived. Malformations are represented in figure 10 for the concentration of 0.1 mgL^{-1}. Exposure to this concentration induced a higher percentage of pericardium edema (pe); yolk sac edema (ye); eye (ae) or tail deviations (t).

Table 2- Sublethal parameters and malformations (%) of animals exposed to the different concentrations of azoxystrobin.

Endpoint	**24 hpf**	**48 hpf**	**72 hpf**	**96 hpf**
Treatment (mgL^{-1})	**Spontaneous movement**	**Heartbeats (bpm)**	**Hatching rate (%)**	**Malformations (%)**
0	3 (2 – 3)^{a}	127 (122 – 128)^{ab}	69 ± 3^{a}	0 (0 – 0)^{a}
0.001	1 (0 – 1)^{b}	122 (120 – 123)^{a}	63 ± 2^{ab}	0 (0 – 10)^{ab}
0.01	3 (2 – 3)^{a}	134 (131 – 134)^{b}	60 ± 6^{b}	10 (0 – 10)^{ab}
0.1	3 (2 – 3)^{a}	130 (129 – 142)^{b}	57 ± 5^{b}	10 (10 – 10)^{b}
Statistical test	$X^2(3) = 9.405$	$X^2(3) = 10.680$	F (3,20) =6.701	$X^2(3) = 9.607$
p value	0.024	0.014	0.004	0.022

Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. In the same column, different lowercase letters indicate significant differences among groups (p<0.05).

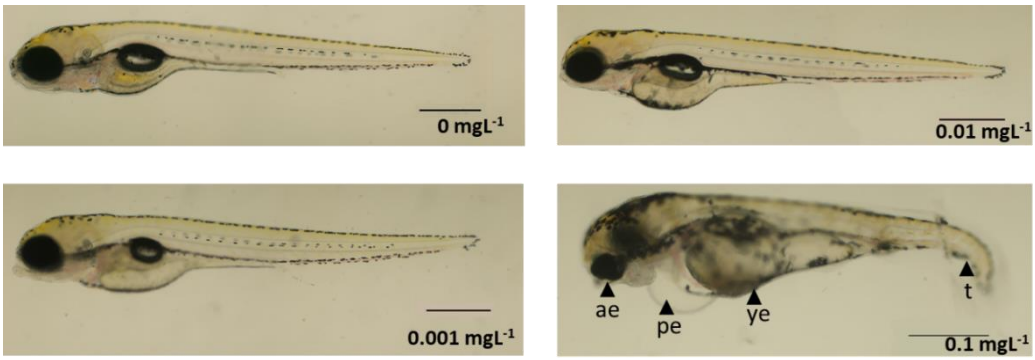

Figure 10 - Representative optical images of the zebrafish larvae after 96 h embryonic exposure to azoxystrobin. The concentration of 0.1 mgL^{-1}, induced a higher percentage of pericardium edema (pe); yolk sac edema (ye); eye (ae) or tail deviations (t). The scale bar represents 500 μm.

Other lethal (tail and head detachment) and sublethal parameters (development of the somites, eyes and otoliths development, visible blood circulation and edema) were analyzed and results are shown in table 3. These parameters were present/visible in all treatment groups at 24 and 48 hpf. At 72 hpf, the presence of edema (yolk sac and pericardia) were observed in the animals exposed to azoxystrobin although no differences were observed compared to the control group.

Table 3 -Frequency of normal embryos/larva following embryonic exposure to azoxystrobin.

Endpoint	**24hpf**			**48 hpf**			**72 hpf**
Treatment (mgL^{-1})	**Tail detach.**	**Head detach.**	**Somite**	**Eyes devel.**	**Otoliths devel.**	**Blood circulation**	**Edema***
0	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.001	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.01	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.1	10/10	10/10	10/10	10/10	10/10	10/10	1/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken and results are presented in table 4. No significant differences were observed in the body length ($X^2(3)=4.509$, p=0.212) of the exposed animals. The area of yolk sac showed differences among the evaluated groups ($X^2(3)=13.640$, p=0.003). The animals exposed to 0.001 mgL^{-1} presented a decrease in relation to 0.01 mgL^{-1} (p=0.008) and in relation to 0.1 mgL^{-1} (p <0.001). The remaining parameters showed to be similar among treatment groups.

Table 4 – Effects of azoxystrobin exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	**96 hpf**			
Treatment (mgL^{-1})	**Body Length (mm)**	**Area of yolk sac (mm^2)**	**Area of heart (mm^2)**	**Area of eye (mm^2)**
0	3.63 (3.61 – 3.67)	0.166 (0.166 – 0.170)^{ab}	0.034 ± 0.008	0.072 ± 0.008
0.001	3.55 (3.48 – 3.63)	0.156 (0.144 – 0.161)^{a}	0.032 ± 0.001	0.068 ± 0.004
0.01	3.47 (3.47 – 3.62)	0.190 (0.178 – 0.200)^{b}	0.033 ± 0.005	0.066 ± 0.006
0.1	3.39 (3.26 – 3.43)	0.202 (0.195 – 0.205)^{b}	0.035 ± 0.008	0.061 ± 0.004
Statistical test	$X^2(3) = 4.509$	$X^2(3) = 13.640$	F (3,20) = 0.146	F (3, 20) = 3.111
p value	0.212	0.003	0.931	0.056

Data from at least five independent replicates of 100 animals each. 10 animals randomly per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups (p<0.05)

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzymatic and non-enzymatic defenses were evaluated and the results are presented in figures 11, 12, 15, and 16.

3.1. Reactive oxygen species (ROS) quantification

Considering the ROS quantification (fig. 11), there were statistical differences among tested groups ($X^2(3) = 16.750$, $p=0.001$) with the lowest group showing a significant increase relative to the control group ($p <0.001$) and to the 0.1 mgL^{-1} group ($p=0.011$). The 0.01 mgL^{-1} group also presented a significative increase in relation to the control group ($p=0.018$). No other significant difference was detected.

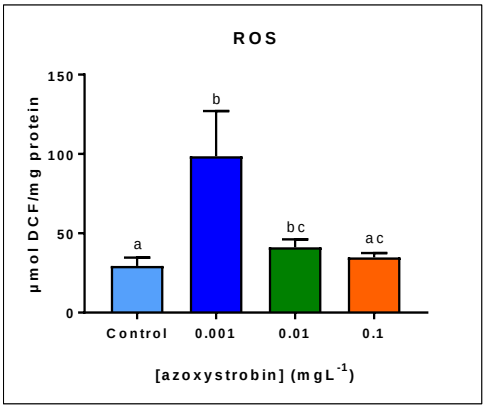

Figure 11 - Reactive oxygen species (ROS) quantification in zebrafish embryos exposed to azoxystrobin. Data from at least five independent samples (n=100/each). Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.2. Oxidative stress markers

The activity of SOD, CAT, GPx and GR showed statistical differences between the different concentrations assessed and the control group (fig. 12). As regards the statistical differences found for the SOD ($X^2(3)=12.638$, $p=0.005$), an increase in its activity in the concentration of 0.001 mgL^{-1} in relation to the control group ($p=0.01$) and in relation to other groups 0.01 mgL^{-1} ($p=0.002$) and 0.1 mgL^{-1} ($p=0.003$) was observed. The remaining concentrations did not present statistically significant differences. The activity of CAT ($X^2(3)=13,187$, $p=0.004$) shown that the lowest activity corresponded to the concentration of 0.001 mgL^{-1} ($p=0.029$) in relation to the control and in relation to the other groups 0.01 mgL^{-1} ($p=0.001$) and 0.1 mgL^{-1} ($p=0.002$). The remaining concentrations did not present statistically significant differences. GPx also showed significant differences

($X^2(3)=9,813$, p=0.02) among groups. The 0.001 mgL^{-1} (p=0.006) and 0.1 mgL^{-1} (p=0.015) groups had lower GPx activity than the control. The concentration of 0.01 mgL^{-1} did not present statistical differences in relation to the control group (p=0.132). The same situation was verified for GR ($X^2(3)=9.160$, p=0.027). A decrease in the activity of the enzyme following exposure to the concentration of 0.001 mgL^{-1} (p=0.008) and 0.1 mgL^{-1} (p=0.01) was observed in relation to the control group. No other significant difference was detected.

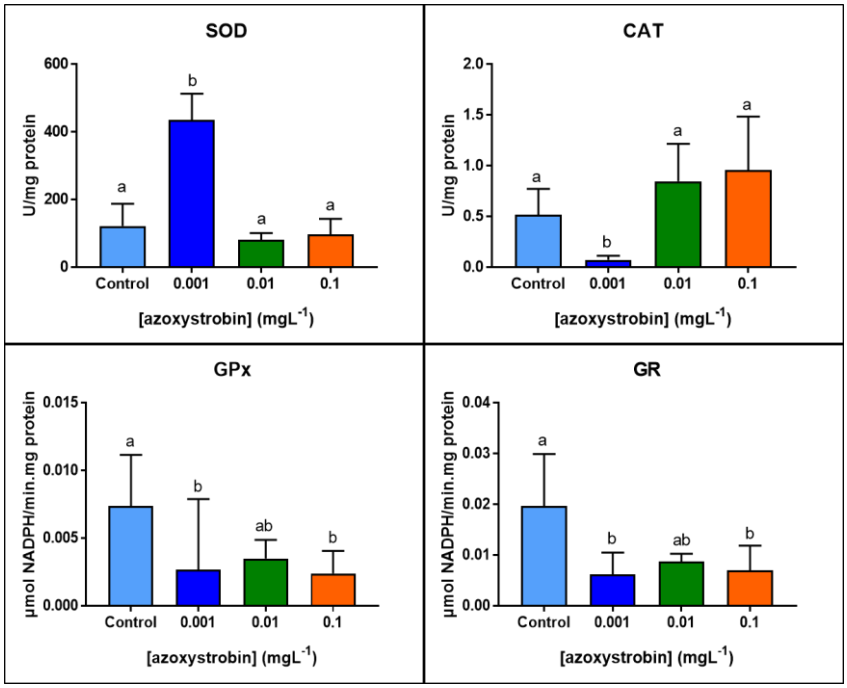

Figure 12 – Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity determination in zebrafish embryos exposed to azoxystrobin. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD .Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups (p<0.05).

3.3. Glutathione levels

The oxidative-stress index (OSI), defined as the ratio between GSH and GSSG (GSH/GSSG) is represented in figure 13. The GSH level ($X^2(3)=12.085$, p=0.007) showed a significative decrease in relation to control group (p=0.011) and the remaining concentrations (0.01 mgL^{-1}, p=0.003 and 0.1 mgL^{-1}, p=0.002). The GSSG ($X^2(3)=12.351$, p=0.006) presented a significative decrease in its level in the lowest concentration in relation to control group (p=0.001) and the remaining concentrations (0.01 mgL^{-1} and 0.1 mgL^{-1}, both, p=0.004). The ratio between GSH and GSSG (GSH/GSSG) there was no statistically significant differences ($X^2(3)=2.609$, p=0.456) for the different concentrations evaluated and the control group.

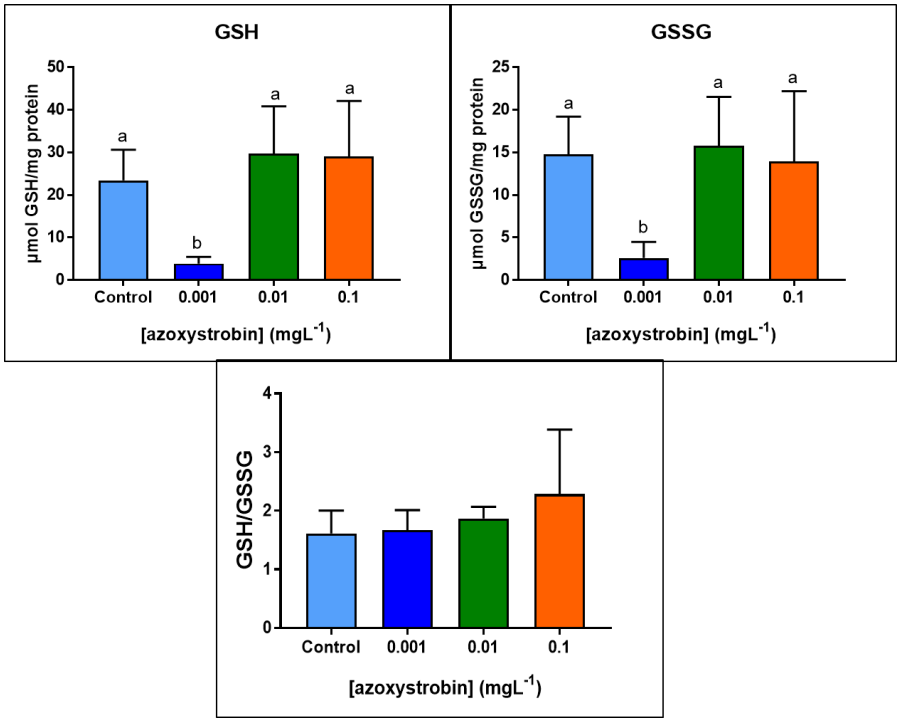

Figure 13 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ration between GSH and GSSG (OSI) level determination in zebrafish embryos exposed to azoxystrobin. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.4. Xenobiotic biotransformation

The GST and CarE enzymes, responsible for the degradation of some xenobiotics (fig. 14), showed differences in animals exposed to the different concentrations and the control. The GST activity ($F(3,23)=32.625$, $p<0.001$) in larvae form the concentration of 0.001 mgL^{-1} was very marked with an increase in relation to that of the control group ($p<0.001$) and the remaining concentrations (0.01 mgL^{-1} and 0.1 mgL^{-1}, both $p<0.001$). In relation to the other concentrations, there were no significant differences. The same situation occurred for CarE ($X^2(3)=11.347$, $p=0.01$) in which exposure to the concentration of 0.001 mgL^{-1} induced a higher activity of this enzyme when compared to the control group ($p=0.005$) and to the remaining concentrations (0.01 mgL^{-1}, $p=0.013$ and 0.1 mgL^{-1}, $p=0.003$). For the remaining concentrations, there were no significant differences.

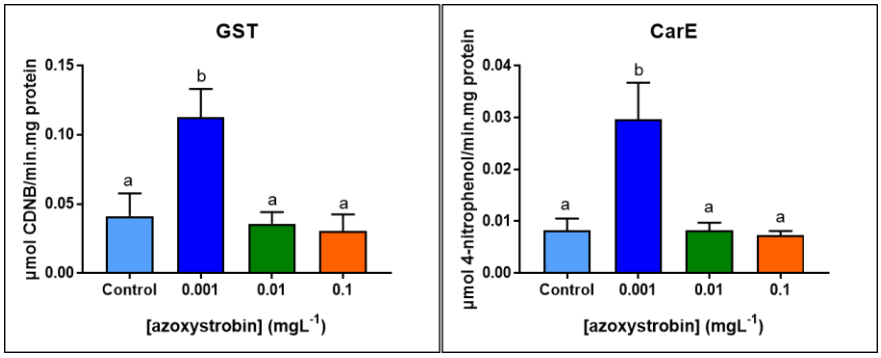

Figure 14 – Glutathione-s-transferase (GST), carboxylesterase (CarE), activity determination in zebrafish embryos exposed to azoxystrobin. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.5. Oxidative damage

Significant differences ($X^2(3)=16,261$ $p=0.004$) were observed in the lipid peroxidation (fig. 15). The concentration of 0.001 mgL^{-1} showed a significant decrease compared to the control ($p=0.02$) and the remaining concentrations evaluated 0.01 mgL^{-1} ($p=0.002$) and 0.1 mgL^{-1} ($p=0.001$). However, exposure to the concentrations of 0.01 and 0.1 mgL^{-1} showed no differences compared to the control group, or among them.

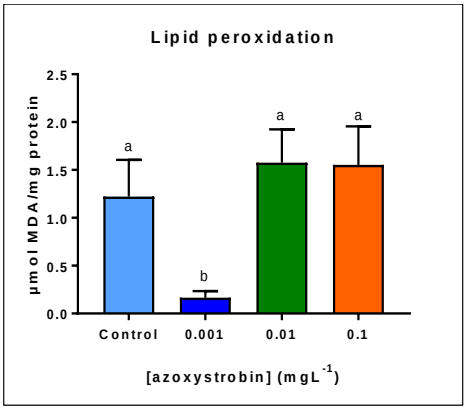

Figure 15- Lipidic peroxidation in zebrafish embryos exposed to azoxystrobin. Data from at least five independent replicates of 100 animals each. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.6. Neurotransmission and anaerobic metabolism

The effects of azoxystrobin on neurotransmission were evaluated through the AChE activity. The AChE activity (fig. 16) showed statistical differences between groups ($X^2(3)=11.255$, $p=0.01$) with an increased activity in the concentration of 0.001 mgL^{-1} compared to the control group ($p=0.003$) and the remaining concentrations (0.01 mgL^{-1}, $p=0.015$ and 0.1 mgL^{-1}, $p=0.006$). However, the concentrations groups of 0.01 and 0.1 mgL^{-1} no presented differences compared to the control group, or among them. In relation to the anaerobic metabolism enzyme evaluated by the activity of LDH ($X^2(3)=5.907$, $p=0.116$), there were no statistically significant differences among groups (fig. 16).

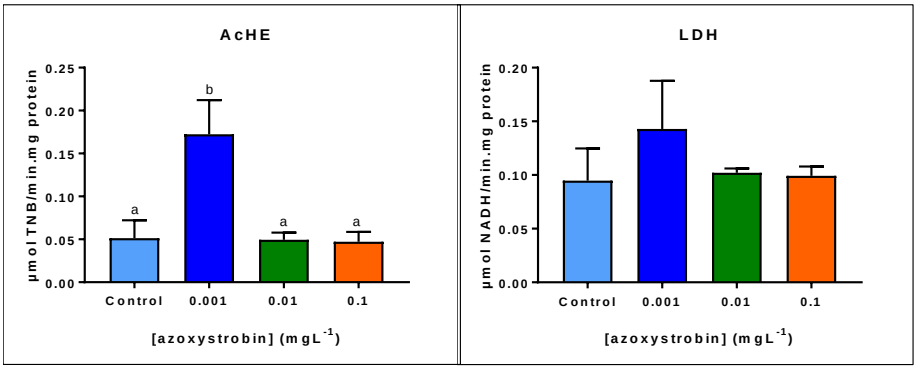

Figure 16- Acetylcholinesterase (AChE) and Lactate dehydrogenase (LHD) activity determination in zebrafish embryos exposed to azoxystrobin. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

Chapter IV

IV.2

Discussion

Azoxystrobin

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The objective of the present study was to evaluate the effect of azoxystrobin in zebrafish embryos exposed to different concentrations during a 96h period. Exposure to the lowest concentration caused a decrease in spontaneous movements and heart beats, caused oxidative stress (increase in ROS levels and in the activity of SOD), as well as increased the activity of the enzymes responsible for the metabolism of xenobiotics, GST and CarE. An increase in the AChE activity was also induced by exposure to azoxystrobin.

The process of embryonic development is a very complex process, consisting in the interplay of several pathways that relate to each other in order to complete this process without errors (Kimmel *et al.,* 1995; Lawson and Weinstein 2002)). However, exposure to some drugs can affect the embryonic development (Xin *et al.,* 2015; Girardi *et al.,* 2017; Jin *et al.,* 2016), as is the case of azoxystrobin. This fungicide has already been reported to interfere in the development of zebrafish by inducing changes in the spontaneous movements, heartbeat, hatching rate and causing several malformations (Cao *et al.,* 2018; Jia *et al.,* 2018; Jiang *et al.,* 2018). However, the results described by other authors are associated with higher doses of azoxystrobin, while in this study the effects were verified at the lowest concentration.

The first step of this work consisted on the calculation of the LC_{50} according to the OECD protocol No 236. The acute toxicity data provide information which is useful to identify the mode of action of a substance and also helps to compare the dose response among various chemical substances. The 96h-LC_{50} tests are conducted to assess the vulnerability (every 24h, up to four apical observations are recorded as indicators of lethality: coagulation of fertilized eggs, lack of somite) and survival potential of organisms to particular toxic chemical substances. Chemical agents with lower LC_{50} values are more toxic because their lower concentrations result in 50% of mortality in organisms (Tarkhani *et al.,* 2012). At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC_{50} is calculated. This parameter is considered the first step in developmental toxicity in order to define the doses to be used (Guidelines 2013). In this study, the value obtained for the 96h-LC_{50} was 1.15 mgL^{-1}. This value is similar to that of previously published studies with zebrafish, ranging from 0.78 mgL^{-1} to 1.23 mgL^{-1} (Jia *et al.,* 2018; Jiang *et al.,* 2018). Considering this LC_{50} value, three concentrations were selected (0.001, 0.01 and 0.1 mgL^{-1}) and the animals were exposed during a period of 96 hours with development changes being recorded at 24, 48, 72 and 96h.

Movements in vertebrates (walking or swimming) depends on the neural networks in the brain and spinal cord (McKeown, Downes, and Hutson 2009). Zebrafish embryos possess a very strong locomotive behavior which can be a useful tool to study the neuromuscular function (Goody *et al.,* 2012). Several key time points in the behavioral development of zebrafish have been described. Zebrafish embryos start to show spontaneous movements of the trunk and tail at 17 hours post fertilization (hpf) and has a peak of spontaneous movement at 19 hpf (Pietri *et al.,* 2009; Saint-Amant and Drapeau 1998). After hatching, around 72 hpf, they start to show movements more complex behaviors (McKeown, Downes, and Hutson 2009). As shown in previous studies, the spontaneous movements are due to uncontrolled action potential in motorneurons. Compounds are known to be involved in the reduction of the spontaneous movement frequency by limiting the spreading of the action potential and consequently the myotomal contraction rate through the blockage of membrane Na^+ channel (Fraysse, Mons, and Garric 2006). Indeed, Jin *et al.,* (2009) suggested that an increased spontaneous movements frequency is associated to the prolongation at channel opening to cause repetitive firing of action potentials. Thus, the results obtained, i.e., the decrease in spontaneous movements observed for the lowest tested dose of azoxystrobin, may be explained by the decrease in the opening time of the Na^+ channels. Still, further tests are needed to support this hypothesis namely through the evaluation of Na^+ channels.

In addition, exposure to azoxystrobin induced changes in the heartbeat of zebrafish. The heartbeat is an important development parameter (Lin, Hui, and Cheng 2007; Mersereau *et al.,* 2015) and previous studies relate AChE inhibitors with a decreased heartbeat due to the direct binding acetylcholine to muscarinic acetylcholine receptors on the sino-atrial node, affecting nitric oxide synthesis by endothelial nitric oxide synthase as well as subsequent intracellular signaling (Lin, Hui, and Cheng 2007). As a result, potassium ion channel function and action potential development are altered (Herring, Danson, and Paterson 2002; Massion *et al.,* 2003) resulting in a slower heart rate. On the other side, an increase in heart rate is mediated by an increased activity at sympathetic nerve terminals in the heart and vasculature (Mersereau *et al.,* 2015). The increases in heartbeat and blood pressure are due, in part, to peripheral effects at the synapse by blockade of epinephrine reuptake, or possibly via sympathetic stimulation of epinephrine release from the adrenal medulla (Tella, Schindler, and Goldberg 1990; Gillis 1995). In this study it was observed a decrease in the heartbeat in relation to the control at the lowest concentration, and an increase in the AChE activity. In general, and relating both, it may

be suggested that exposure to high doses of azoxystrobin have an inhibitory effect of the AChE activity and the consequent increase of the heartbeat. Still, further studies of cardiovascular effects of azoxystrobin are required to corroborate this hypothesis.

Moreover, zebrafish exposed to the concentration of 0.1 mgL^{-1} had a higher percentage of malformations (cardiac and yolk sac edema and tail) at 96 hpf, and showed a hatching inhibition, a reduction in body length, an increase of the area of yolk sac and heart. As previously mentioned, the development of zebrafish is a complex process that may be affected by the action of this compound. The embryo hatching occurs after digestion of the chorion by the hatching enzyme and violent movement of the embryo (Winnicki, Stańkowska-Radziun, and Radziun 1970; Yamagami 1981). As described in previous studies (Zhou *et al.,* 2009; Osterauer and Kohler 2008), various compounds can affect hatching, and can do so in different ways. Damage on biosynthesis of the hatching enzyme or abnormal distribution of the hatching enzyme were described as the main cause of hatching inhibition (Rosenthal and Alderdice 1976; Hagenmaier 1974), followed by the diminished activity of the embryo and inability of the emerging larvae to break through the chorion (Papiya and Kanamadi 2000). Although not been described previously, azoxystrobin may induce some similar effect, suggesting the failure of chorion digestion at higher concentrations tested. Still, further studies, particularly focusing on the uptake and metabolism of this compound in zebrafish early development may shed light on this subject. Yet, the cardiac and yolk sac edema have been related with a possible permeabilization of the first zebrafish defense barrier during its development (Hill *et al.,* 2004). These effects increase the surface permeability on the primary barrier during early development (Hill *et al.,* 2004; Guiney, Walker, and Peterson 1990). The existence of a water permeability barrier at the surface of the zebrafish embryo can be inferred from the fact that zebrafish embryos are able to maintain osmotic balance prior to the development of organs (Hill *et al.,* 2004) or it could be an indicator of metabolic or osmotic disruptions possibly caused by mitochondrial malfunction due to the chemical action (Papiya and Kanamadi 2000). One of these two hypotheses (causing permeabilization, or interfering with osmotic balance) may explain the occurrence of these processes and perhaps also be associated with the effects of azoxystrobin. In addition, Cheng *et al.,* (2000), explained malformation in tail through the inability of embryos to express the *evenskipped 1* gene, which is important during tail extension on development that occurs by altered cell migration of the precursors of the somatic mesoderm in the trunk (Ho and Kane 1990). Moreover, the decrease in body length of

animals exposed to other fungicides, such as difenoconazole, has already been described and associated to decreases in growth hormone (GH) and insulin-like growth factor 1 (IGF-1) (Mu *et al.,* 2016)(Liu *et al.,* 2014)(Meganathan *et al.,* 2015; Colao *et al.,* 2006). Still, to what extent does this situation actually represents the outcome azoxystrobin exposure deserves further investigation. Moreover, the malformations were more evident in the higher concentration, although the lowest concentration also presented malformations in small amount. This may be related to the entry of the compound in the egg during embryo development. In this sense, this work will need to be complimented with a pharmacokinetic study which may give further indications as to the possible absorption and metabolism of the compound.

Biochemical parameters were also analyzed to complement the data of developmental toxicity. Studies suggest that the toxicity of azoxystrobin is associated with the fact that it acts at the mitochondrial chain, more properly at complex III and may thus be associated with a ROS increase and consequent oxidative damage (Gao *et al.,* 2014; Affourtit, Heaney, and Moore 2000; Xiao *et al.,* 2014). The ROS increase activate the antioxidant defenses, and the first to act is SOD. Several studies claim that there is an increase in SOD associated with an increase in ROS following exposure to azoxystrobin (Jia *et al.,* 2018). Overall, these data validate the results obtained in this study in which the concentration of 0.001 mgL^{-1} induced an increase in the ROS levels and a consequent increase of the SOD activity. The CAT, GPx and GR activities decreased following exposure to the concentration of 0.001 mgL^{-1}, which shows that the first line of defense is activated to combat the ROS, a defense that is only carried out by the SOD, and the remaining defenses may not be operating in full, which may mean that there is a marked damage of oxidative stress. In fact, this has already been described for other compounds. (Wu *et al.,* 2011; Craig, Wood, and McClelland 2007).

Additionally, the GPx and GR activity were lower in concentration of 0.001 mgL^{-1} consequently the levels of GSH and GSSG were also lower. These low levels may mean that there is no oxidation/reduction, process performed by GPx and GR enzymes, so this radical elimination pathway was affected by this compound concentration. The high levels of ROS detected for this concentration were confirmed by the data previously mentioned (Wu *et al.,* 2011). The glutathione system and related enzymes are considered a second line of defense against oxidative damage (Jafari 2007) being the ratio of GSH to GSSG often used as an indicator of intracellular redox status (Timme-Laragy *et al.,* 2013). The glutathione system is a key non-enzymatic radical scavenger and antioxidant

that scavenges residual free radicals generated from oxidative metabolism and those not decomposed by antioxidant enzymes (El-Shenawy 2010). The GSH dynamics are important for the maintenance of the redox homeostasis, and suggest that there are critical time periods when embryos could be susceptible to redox perturbations (Timme-Laragy *et al.,* 2013; Massarsky, Kozal, and Di Giulio 2017). During the metabolic action of GSH, its sulfhydryl group becomes oxidized to form the corresponding GSSG disulphide compound (DeLeve and Kaplowitz 1991). The reaction of oxidation of GSH in GSSG, realized by the GR requires NADPH that gives rise to $NADP^+$ (Schafer and Buettner 2001; Wu *et al.,* 2011; Carvan Iii *et al.,* 2001). The decrease of GSH and GSSG, contents might be a combined impact of the declined GPx and GR activity.

GST and CarE collaborate in the reactions of the first phase of detoxification of xenobiotic (Barata, Solayan, and Porte 2004). GST represents a family of enzymes with a central role in the biotransformation of xenobiotics and endogenic compounds (Andrade *et al.,* 2016). Thus, GST has been considered as an indicator of stress and increasingly used as an environmental biomarker (Hyne and Maher 2003). Moreover, GST might contribute to the elimination of superoxide radicals caused by oxidative stress (Jiang *et al.,* 2018), which may explain the increase in its activity since there is a very clear accumulation of ROS following exposure to the concentration of 0.001 mgL^{-1}. CarE might serve as a secondary target sequestering organophosphates (Garcia *et al.,* 2000) and, thereby, being an important pathway of detoxification (Abbas and Hayton 1997; Kuster and Altenburger 2006). The higher CarE activity suggests a greater sensitivity to inhibit the performance of some xenobiotics in some aquatic organisms (Kuster 2005). Moreover, a recent study has proved that CarE is a good indicator for pesticide exposure (Laguerre *et al.,* 2009; Wang *et al.,* 2018; Wheelock, Shan, and Ottea 2005). In this study, this enzyme showed an increase in this activity for the concentration of 0.001 mgL^{-1}. These data could be possible due to a fast bioconcentration of azoxystrobin in short time and further evaluation of the potential for azoxystrobin to be absorbed and retained in the embryo/larvae's tissues may give further information about the potential effects of this compound in this enzyme.

The long-term damaging effects of ROS exposure *in vivo* is the formation of oxidized macromolecules which can cause oxidative damage. Lipid peroxidation (LPO), an index for measuring membrane damage, occurs with an increase in hydroxyl radical production (Ganesan *et al.,* 2016; Blokhina, Virolainen, and Fagerstedt 2003). ROS increases the lipid peroxides causing a disruption in the lipid integrity of the membranes

(Radi and Matkovics 1988). Such deterioration of membrane lipids is a prominent marker of oxidative damage (Sayeed *et al.,* 2003). The concentration that presented the least damage was 0.001 mgL^{-1}. This may be due to increased SOD, GST and CarE activity, thus reducing the impact of ROS caused by the exposure to this azoxystrobin concentration.

Moreover, it has been shown that oxidative stress plays a role in the regulation and activity of AChE (Rodriguez-Fuentes *et al.,* 2015) as well as LDH (Shi *et al.,* 2009). AChE plays an important role in neurotransmission being responsible for the hydrolysis of acetylthiocholine (Olsen *et al.,* 2001). In addition, there are evidences of the involvement of AChE in other physiological process including:1) regulation of cell proliferation (Zhang *et al.,* 2002b), apoptosis (Jiang and Zhang 2008; Ganesan *et al.,* 2016), and cell migrations (Drews 1975), 2) regulates differentiation through signaling (Falugi and Aluigi 2012) and ultimately, 3) embryonic development (Falugi 1993). Changes in the activity of this enzyme can cause failures in the previously mentioned processes and be responsible for problems during the embryonic development, eventually causing teratogenicity. However, the mechanisms that regulate AChE expression (de Lima, Roque, and de Almeida 2013) and this participation in apoptosis are not yet fully understood (Soreq and Seidman 2001; Zhang *et al.,* 2002a). The involvement of thyroid hormones in the regulation of AChE activity has been suggested by Puymirat, Etongue-Mayer, and Dussault (1995). AChE is inhibited when organisms are exposed to organophosphate and carbamate pesticides (Srain and Rudolph 2010). Lionetto *et al.,* (2005) suggested that AChE is a very useful biomarker of the biological effect of a mixture of neurotoxic pollutants in the aquatic environment. Several previous studies have found that ROS can inhibit AChE in various tissues (O'Malley *et al.,* 1966; den Hartog *et al.,* 2002). In this study, the lower concentration of azoxystrobin induced an accumulation of ROS. Some authors (Melo, Agostinho, and Oliveira 2003; Sberna *et al.,* 1997) associated this effect with a disturbance in calcium homeostasis and the increase of the AChE activity, although its role in embryo development has not yet been fully described. Further, LDH is a key enzyme in the anaerobic pathway of energy production and is involved in the carbohydrate metabolism (Diamantino *et al.,* 2001), and can be used as biochemical marker for eco-toxicity studies for hazard identification (Choudhury, Tarafdar, and Panigrahi 2017). In this study the lowest concentration presented the highest increase of ROS and SOD activity, showing also an increase in the LDH activity. The high activity of LDH may be associated with tissue damage in the heart, skeletal

muscle, and other organs (Cangemi *et al.,* 2014) induced by the increased oxidative damage, which may explain the results of this study in relation to the 72hpf for the lowest concentration, since the animals had some cardiac and yolk sac edema. Notwithstanding, the fact that the animals exposed to the lowest concentration are the most affected, this can be explained due to the theory of hormesis, described by several authors as for azoxystrobin, although in other organisms (Špalková, Beňová, and Falis 2012; Pratissoli *et al.,* 2010; Di *et al.,* 2016), or to other compounds (Li *et al.,* 2009; Tu *et al.,* 2013). This phenomenon consists of lower doses of a compound causing more effect than higher doses, yet this phenomenon is not yet fully understood as to the routes as it is processed (Weltje, vom Saal, and Oehlmann 2005).

In summary, exposure to the lowest azoxystrobin concentration can affect zebrafish embryonic development through interference with some developmental pathways that deserve further investigation. In this regard, oxidative stress and the consequent damage seems to be associated to the developmental arrest observed which may also be associated to energetic and neurotoxic effects. However, further studies are needed to fully support the hypothesis presented and clarify the underlying toxicological mechanisms.

Chapter V

V.1

Results

Mancozeb

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at, 24, 48, 72 and 96 hpf (fig. 17). At 24 hpf, no differences between concentrations tested and the control group were observed. The same occurred at 48, 72 and 96 hpf.

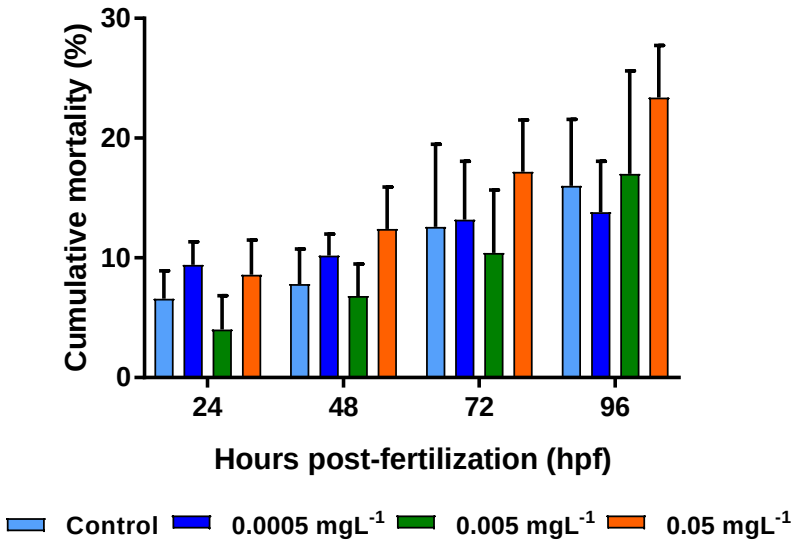

Figure 17- Cumulative mortality of zebrafish embryos exposed to mancozeb. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed at 24 hpf (table 5) showed no significant differences between groups ($X^2(3)=0.560$, $p=0.905$). The heartbeat ($X^2(3)=11.174$, $p=0.011$) showed a significant decrease in animals exposed to the concentration of 0.0005 mgL^{-1} ($p=0.008$) in relative to the control group and to the concentration of 0.005 mgL^{-1} ($p=0.002$). No other significant differences were observed.

The hatching rate (table 5) was evaluated at 72hpf ($X^2(3)=11.785$, $p=0.008$). All concentrations induced a very significative decrease in hatching rate relative to the control group (0.0005 mgL^{-1}, $p=0.013$; 0.005 mgL^{-1}, $p=0.006$ and 0.05 mgL^{-1}, $p=0.002$).

The malformations observed at 96hpf (table 5) in the animals exposed to mancozeb ($X^2(3)=13.274$, $p=0.004$), were more significative different between the highest concentration and other concentration relative to control group. In this regard, and as expected, the control group showed no apparent malformations. The highest concentration was statistically different from the control ($p=0.002$), the lowest

concentration (p=0.008) and the concentration 0.005 mgL^{-1} (p=0.002). No other significant difference was observed. All animals exposed to the concentration of 0.05 mgL^{-1} showed malformations, as mandibular deformations (m); pericardium edema (pe); yolk sac edema (ye); tail deviations (t) or spine curvatures (s) (fig. 18).

Table 5 - Sublethal parameters and malformations (%) of animals exposed to the different concentrations of mancozeb.

Endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Treatment (mgL^{-1})	Spontaneous movement	Heart beats (bpm)	Hatching rate (%)	Malformations (%)
0	2 (1 – 2)	112 (112 - 138)^{a}	62 (55 – 64)^{a}	0 (0 – 0)^{a}
0.0005	1 (1 – 2)	102 (102 - 102)^{b}	1 (0 – 6)^{b}	0 (0 – 10)^{a}
0.005	1 (1 –2)	125 (111 – 136)^{a}	0 (0 – 4)^{b}	0 (0 – 0)^{a}
0.05	2 (1 – 2)	109 (106 - 109)^{ab}	0 (0 – 2)^{b}	100 (90 – 100)^{b}
Statistical test	$X^2(3) = 0.560$	$X^2(3) = 11.174$	$X^2(3) = 11.785$	$X^2(3) = 13.274$
p value	0.905	0.011	0.008	0.004

Data from at least five independent replicates of 100 animals each. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

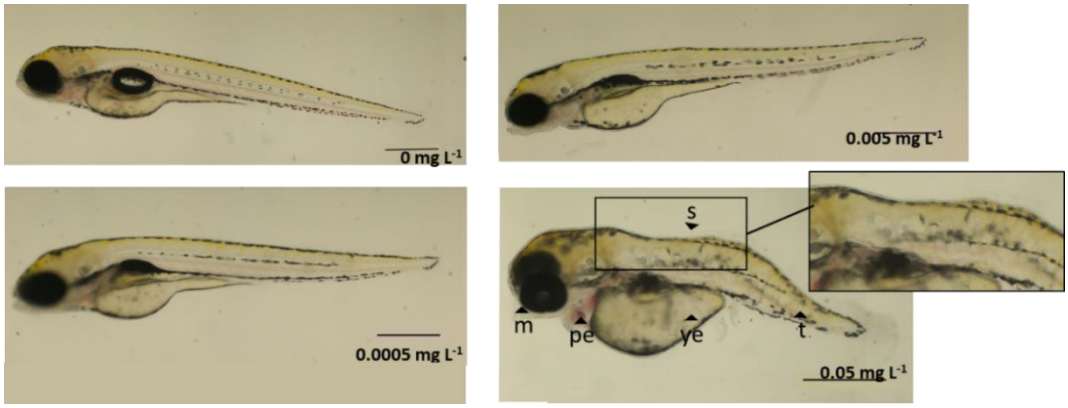

Figure 18 - Representative optical images of the zebrafish larvae after 96 h of embryonic exposure to mancozeb. Animals exposed to the concentration of 0.05 mgL-1 showed mandibular deformations (m); pericardium edema (pe); yolk sac edema (ye); tail deviations (t) or spine curvatures (s). The scale bar represents 500 μm.

Other lethal, tail and head detachment, and sublethal parameters such as development of the somites, eyes and otoliths, blood circulation and edema were analyzed and results shown in table 6. These parameters were present/visible in all treatment groups at 24 and 48 hpf. At 72 hpf, the presence of edema (yolk sac and pericardia) were observed in all group of animals exposed to mancozeb in all concentration tested. In the highest concentration, 0.05 mgL^{-1}, it was verified that 5 in 10 animals analyzed (50%) presented malformations.

Table 6 -Frequency of normal embryos/larva following embryonic exposure to mancozeb.

Endpoint	24hpf			48 hpf			72 hpf
Treatment (mgL^{-1})	Tail detach.	Head detach.	Somite	Eyes devel.	Otoliths devel.	Blood circulation	Edema*
0	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.0005	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.005	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.05	10/10	10/10	10/10	10/10	10/10	10/10	5/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken and results are presented in table 7. Significant differences ($X^2(3)=16.687$, $p=0.001$) were observed when analyzing the body length of the exposed animals that showed a significant decrease for the highest concentration of mancozeb relative to the control, 0.0005 mgL^{-1} ($p=0.025$) and 0.005 mgL^{-1} ($p=0.011$). When the area of yolk sac ($F(3,20)=6.233$, $p=0.101$) and the cardiac area ($X^2(3)=1.285$, $p=0.307$) were measured, no differences were found between the evaluated groups.

The area of the eye (table 7), showed differences between the evaluated groups ($F(3,20)=4.571$, $p=0.014$). The concentration of 0.05 mgL^{-1} showed significative decrease relative to the control group ($p=0.008$). No other significant difference was observed.

Table 7 - Effects of mancozeb exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	**96 hpf**			
Treatment (mgL^{-1})	**Body Length (mm)**	**Area of yolk sac (mm^2)**	**Area of heart (mm^2)**	**Area of eye (mm^2)**
0	3.48 (3.44 – 3.66)[a]	0.197 (0.185 – 0.199)	0.035 ± 0.005	0.069 ± 0.009[a]
0.0005	3.31 (3.26 – 3.32)[a]	0.183 (0.182 – 0.191)	0.032 ± 0.005	0.062 ± 0.004[ab]
0.005	3.28 (3.19 – 3.59[a]	0.195 (0.167 – 0.222)	0.034 ± 0.006	0.064 ± 0.007[ab]
0.05	2.622 (2.54 – 2.77)[b]	0.224 (0.197 – 0.230)	0.030 ± 0.002	0.055 ± 0.006[b]
Statistical test	$X^2(3) = 16.687$	$X^2(3) = 6.233$	F (3,20) = 1.285	$X^2(3,20) = 4.571$
p value	0.001	0.101	0.307	0.014

Data from at least five independent replicates of 100 animals each. 10 animals randomly per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzyme activity and non-enzymatic defenses were evaluated and the results are presented in figures 19, 20, 21, 22, 23 and 24.

3.1. Reactive oxygen species (ROS) quantification

Relative to the ROS quantification (fig. 19), there were no statistical differences among tested groups ($X^2(3)=5.937$, $p=0.115$).

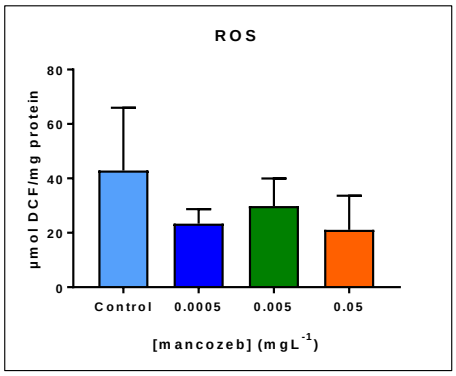

Figure 19- Reactive oxygen species (ROS) quantification in zebrafish embryos exposed to mancozeb. Data from at least five independent samples (n=100/each). Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lower-case letters indicate significant differences between groups ($p<0.05$).

3.2. Oxidative stress markers

The SOD activity ($X^2(3)=13.169$, $p=0.004$) showed statistical differences between the different concentrations assessed and the control group (figure 20). The concentrations of 0.0005 mgL^{-1} ($p=0.04$) and 0.05 mgL^{-1} ($p=0.048$) showed a decrease relative to the control group. The 0.05 mgL^{-1} showed an increase in relation to the concentration of 0.0005 mgL^{-1} ($p=0.002$) and to the concentration of 0.005 mgL^{-1} ($p=0.003$). No other differences were found between groups. The activity of CAT ($X^2(3)=3.693$, $p=0.297$), GPx ($F(3,33)=0.475$, $p=0.703$) and GR ($F(3,33)=2.662$, $p=0.067$) showed no statistically significant differences between the different concentrations evaluated and the control group.

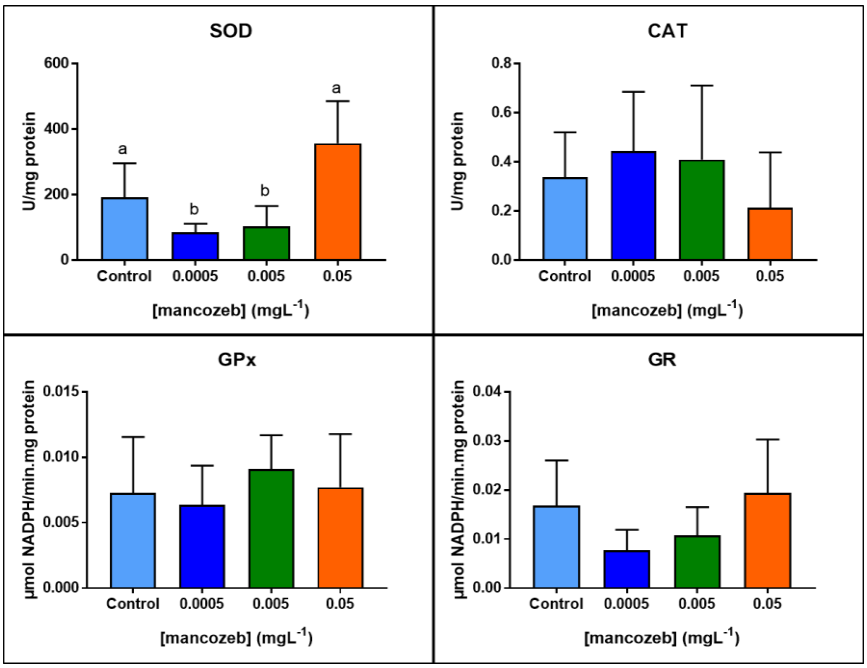

Figure 20 – Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity determination in zebrafish embryos exposed to mancozeb. Data from at least five independent replicates of 100 animals each. Data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences among groups ($p<0.05$).

3.3. Glutathione levels

The GSH level ($F(3,33)=1.254$, $p=0.309$) and GSSSG ($F(3,33)=1.516$ $p=0.232$) showed no significant differences for the different concentrations evaluated and the control group. The oxidative-stress index (OSI), the ratio between GSH and GSSG levels (GSH/GSSG), is shown in figure 21. The ratio between GSH and GSSG was statistically different among groups ($F(3,33)=3.568$, $p=0.026$): the highest concentration, 0.05 mgL^{-1}, presented an increase in relation to the control group ($p=0.015$). For the remaining concentrations, there were no significant differences.

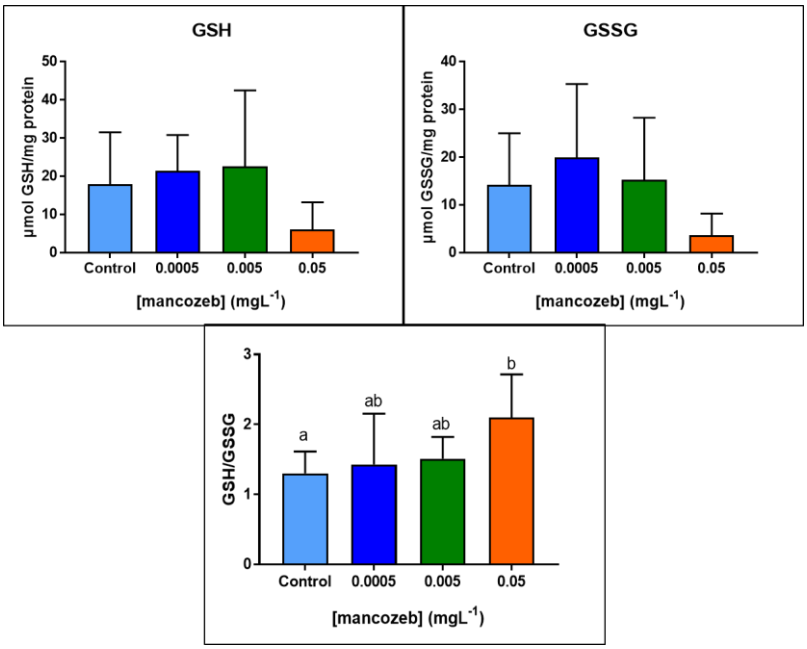

Figure 21 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ration between GSH and GSSG (OSI) level determination in zebrafish embryos exposed to mancozeb. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.4. Xenobiotic biotransformation

The results relative to the GST and CarE enzymes, responsible for the degradation of some xenobiotics, are shown in figure 22. The GST activity presented a decrease ($X^2(3)=6.783$, $p=0.01$) for all concentrations tested in relation to control group (0.0005 mgL^{-1}, $p=0.007$; 0.005 mgL^{-1}, $p=0.005$ and 0.05 mgL^{-1} $p=0.001$). No other significant difference was observed. In relation to the CarE ($F(3,33)=2.755$, $p=0.064$), there were no significant differences for the different concentrations evaluated and the control group.

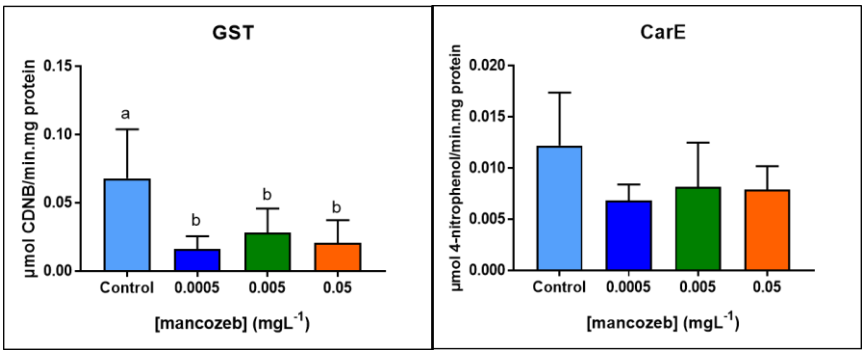

Figure 22 – Glutathione-s-transferase (GST), carboxylesterase (CarE) activity in zebrafish embryos exposed to mancozeb. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$)

3.5. Oxidative damage

No significant differences ($X^2(3)=6.906$, p= 0.075) were observed in the lipid peroxidation (fig. 23) between the different concentrations evaluated and the control group.

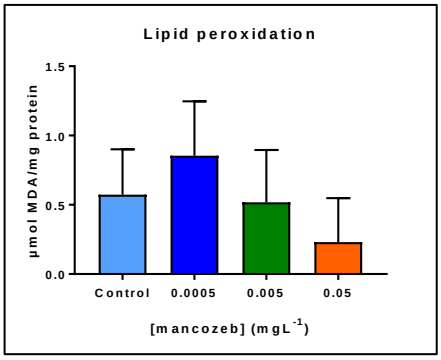

Figure 23- Lipidic peroxidation in zebrafish embryos exposed to mancozeb. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.6. Neurotransmission and anaerobic metabolism

The effects of azoxystrobin on neurotransmission were evaluated through the AChE activity. The AChE activity (fig. 24) showed statistical differences among groups ($X^2(3)=8.979$ p=0.03), with a decrease in its activity in larvae exposed to the concentration of 0.05 mgL^{-1} (p=0.043) and the 0.0005 mgL^{-1} (p=0.039) compared to the control. The lowest concentration (p=0.027) and the concentration of 0.05 mgL^{-1} (p=0.03), presented a decrease relative to the concentration of 0.005 mgL^{-1}. No other significant difference was observed.

In relation to the anaerobic metabolism, evaluated by the activity of LDH ($X^2(3)$ = 6.171 p=0.104), there were no statistically significant differences for the different concentrations evaluated and the control group (fig. 24).

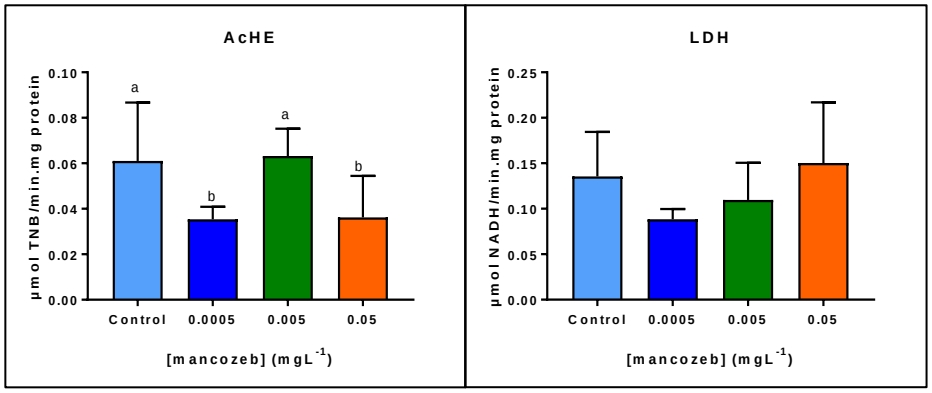

Figure 24- Acetylcholinesterase (AChE) activity and Lactate dehydrogenase (LHD) activity determination in zebrafish embryos exposed to mancozeb. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences among groups ($p<0.05$).

Chapter V

V.2

Discussion

Mancozeb

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The objective of the present study was to evaluate the effects of mancozeb in zebrafish embryos exposed to different concentrations during a 96h period. Exposure to the lowest and the highest concentrations caused a decrease in heartbeats while the effects in hatching rate was almost null in all concentrations tested. The animals exposed to the concentration of 0.05 mgL^{-1} were malformed presenting a reduction in its body length. The exposure to the concentration of 0.0005 and 0.005 mgL^{-1} caused a decrease in the activity of SOD, a decrease in the activity of GST for all concentrations and an inhibition of the AChE activity for 0.0005 and 0.05 mgL^{-1}.

The embryogenesis is an important development phase for the development of the animal in which several pathways correlate with each other, being very susceptible to the occurrence of errors (Kimmel *et al.,* 1995; Lawson and Weinstein 2002). Though, exposure to fungicides can affect the embryo development (De la Paz *et al.,* 2017; Garanzini and Menone 2015; Rajeswary *et al.,* 2007), as is the case of mancozeb or other compounds of their class, (Domico *et al.,* 2007; Marques *et al.,* 2016; Nebbia and Fink-Gremmels 1996). Mancozeb has already been reported to interfere in the development of *Astyanax jacuhiensis* by causing several malformations (cardiac and yolk sac edema, tail, spinal curvature and severely twisted notochord) (Goldoni and da Silva 2012; Santos, Simoes, and Sa-Correia 2009). The infuction of oxidative stress and neurotoxic activity was also reported in *Clarias batrachus* and zebrafish (Srivastava, Singh, and Pandey 2016; da Costa-Silva *et al.,* 2018). In addiction it has been classified as a thyroid gland function disruptor in zebrafish (Thienpont *et al.,* 2011). This compound belongs to the class of EDCS, known for the toxicity of the degradation products of the compounds that form part of it. In the case of mancozeb, it degrades very quickly in water giving rise to several products, being ETU the most known due to its already proven toxicity (Hwang, Cash, and Zabik 2003; Lopez-Fernandez *et al.,* 2016). As such, and as in current study no pharmacokinetic approach has been taken in consideration, further study to observe degradation of the compound would be necessary in order to infer if the observed results are due to mancozeb or to its degradation products.

Notwithstanding, in ecotoxicological studies, the first step of each work consists in the calculation of the $LC_{50,}$ according to the OECD protocol No 236, in order to infer the vulnerability of the embryo to the exposed compound. During the exposure period, critical indicators of lethality are evaluated (Guidelines 2013). In this study, the value obtained for the 96h-LC_{50} was 5.13 mgL^{-1}. This value is similar to that of previously published studies with fish, 3.90 mgL^{-1} for zebrafish, 2.2 mgL^{-1} for *Oncorhynchus mykiss*

and 9.0 mgL^{-1} for *Carassius auratus* (Gopi *et al.*, 2012). Considering this LC_{50} value, concentrations were selected (0.0005, 0.005 and 0.05 mgL^{-1}) and the animals were exposed during a period of 96 hours with development changes being recorded at 24, 48, 72 and 96h.

Movements in vertebrates depends on the neural networks in the brain and spinal cord (McKeown, Downes, and Hutson 2009). Zebrafish embryos keep a strong locomotive performance, with the first movements detected as early as 17 hpf (Pietri *et al.*, 2009; Saint-Amant and Drapeau 1998), being used as an early indicator for the study of the neuromuscular function (Goody *et al.*, 2012). In this study mancozeb did not induce changes in the spontaneous movement at the doses tested. Still, as no study has been found in the literature relative to neurobehavioral changes induced by this compound, further evaluation of the behavioral outcomes at later stages may elucidate possible neurotoxic effects of this compound.

Exposure to mancozeb also induced changes in the heartbeat of zebrafish. The heartbeat decrease was observed in previous studies and associated with AChE inhibitors and the direct binding acetylcholine to muscarinic acetylcholine receptors (Lin, Hui, and Cheng 2007). AChE inhibition result in the alteration of potassium ion channel function and action potential development (Herring, Danson, and Paterson 2002; Massion *et al.*, 2003) resulting in decrease of heartbeat. In this study, a decrease in heartbeat and an inhibition the AChE activity was observed at the lowest and higher concentration of mancozeb. Considering the previously reported study we can infer that exposure to 0.0005 0.05 mgL^{-1} of mancozeb have an inhibitory effect in the AChE activity and consequent decrease of the heartbeat. However, additional studies of cardiovascular effects of mancozeb are required to validate this hypothesis.

Furthermore, all animals exposed to the concentration of 0.05 mgL^{-1} had malformations (cardiac and yolk sac edema, tail, spinal curvature and severely twisted notochord) at 96 hpf as well as a reduction in body length, an increase of the area of yolk sac and decrease in area of the eye. Moreover, animals exposed to all tested concentrations showed a hatching inhibition. The development of zebrafish is a complex process that may be affected by the action of this compound (da Costa-Silva *et al.*, 2018). In particular, the hatching of the embryo is dependent on the action of the hatching enzyme as well as on the movements of the embryo (Winnicki, Stańkowska-Radziun, and Radziun 1970; Yamagami 1981). Previous studies (Zhou *et al.*, 2009; Osterauer and Kohler 2008) have reported that a variety of compounds can affect the hatching of the

embryo by interfering on the biosynthesis of the hatching enzyme and its distribution (Rosenthal and Alderdice 1976; Hagenmaier 1974) as well as by a diminished embryo activity culminating in the inability to emerge from the chorion (Papiya and Kanamadi 2000). A study with a compound belonging to the same class as mancozeb similar showed effects to those there obtained, in terms of inhibition of hatching (Haendel *et al.,* 2004). Although it has not been described previously, mancozeb or their degradation products principally ETU, may induce some similar effects, suggesting the failure of chorion digestion at all concentrations tested Still, further toxicokinetic studies may shed light on this subject. Yet, previous studies have related the occurrence of edemas with changes in the chorion permeabilization (Hill *et al.*, 2004)(Hill *et al.,* 2004; Guiney, Walker, and Peterson 1990). In fact, for the correct embryonic development, zebrafish embryos need to maintain the osmotic balance (Hill *et al.,* 2004). As such, the osmotic disruption possibly caused by mitochondrial malfunctions (Papiya and Kanamadi 2000) may perhaps be associated to the observed effects. Still, further studies are required to verify this hypothesis. Previous authors (Birch and Prahlad 1986; Van Leeuwen, Espeldoorn, and Mol 1986) reports that EBDC can be associated with disruption of collagen in fish. Collagen is clearly important for the proper formation and stability of the notochord among other developmental process, and this is the first report to link the disruption of *collagen 2a1* expression to the early developmental of zebrafish (Chandrasekar *et al.*, 2011). Mancozeb or ETU can cause disruption of *collagen 2a1* expression and consequent instability of the notochord. However, detailed analysis of gene regulation in early development will be needed to identify possible changes at the molecular level. In addition, other study with dithiocarbamates showed that this compounds class can induce cartilage and bone malformations (Strecker, Weigt, and Braunbeck 2013). The authors relate notochord malformation with an accumulation of materials around notochord and with sonic hedgehog (Shh) signaling interacts with collagen XV (essential for notochord differentiation) (Pagnon-Minot *et al.,* 2008). Dithiocarbamates are also known to alter the expression of *collagen 2a1*, an important component of the notochordal sheath (Tilton *et al.,* 2006) (Stemple 2005) and the pharyngeal cartilages (Yan *et al.,* 1995) are highly regulated by this vitamin A metabolite, among other factors (Suzuki, Srivastava, and Kurokawa 2000). The decrease in body length of animal observed may thus be related to changes at the above-mentioned collagen and cartilage level. Thus mancozeb, can have effects on cartilages that affect the normal development of the animals. In this sense, this

work will need to be complimented with a cartilage and bone development analysis with alcian blue and fluorescein.

Studies suggest that manganese and zinc ions present in the structure of mancozeb, have the potential of generating oxidative stress, through mitochondrial dysfunction and alterations in glutathione levels (Nzengue *et al.,* 2011). Accordingly, the potential of this pesticides class to induce toxicity may be related to their association with transition metals, which can generate the formation of ROS through a Fenton-like reaction (Hoffman and Hardej 2012). Several studies claim that there is an increase in SOD to control the increased ROS in organism (Han *et al.,* 2016). However, in this study, the exposure to the concentration of 0.0005 and 0.005 mgL^{-1} induced a decrease in the SOD activity. The CAT, GPx and GR activity did not show differences. On the other side, changes in the glutahtione levels were observed. The ratio between glutathione, GSH to GSSG is often used as an indicator of intracellular redox status (Timme-Laragy *et al.,* 2013), presented an increase at the highest concentrations of mancozeb tested. During early development, the GSH dynamics are important for the maintenance of the redox homeostasis being that changes in this ratio can be correlated to impared embryogenesis and embryo death (Timme-Laragy *et al.,* 2013; Massarsky, Kozal, and Di Giulio 2017). The changes observed can be related with tissue hypoxia decreasing GSH levels, which lead to a corresponding increase in GSSG levels described for human aortic endothelial cells (HAEC) (Prasai *et al.,* 2018). Previously studies demonstrated that altering basal glutathione levels affect the angiogenesis in a murine model of ischemic vascular remodeling through an increase in vascular endothelial growth factor (VEGF) production (Bir *et al.,* 2013), and showed the decreased GSH to control the increased ROS and VEGF production and to promote angiogenesis. These data suggest that glutathione levels regulate *VEGFR2* signaling. In this sense, the increase in GSH: GSSG levels may be related with the decrease in GSH levels, as a consequence of an increase in VEGF production. However, studies on *VEGFR2* signaling in zebrafish exposed to this compound would be required to infer this affirmation.

GST represents a family of enzymes with a central role in the biotransformation of xenobiotics and endogenic compounds (Andrade *et al.,* 2016)(Barata, Solayan, and Porte 2004). This enzyme has been considered as an indicator of oxidative stress and progressively used as an environmental biomarker (Hyne and Maher 2003). GST might contribute to the elimination of ROS (Jiang *et al.,* 2018). The no alteration of ROS level

in all concentrations of this compound can explain the decrease of GST activity in all concentration.

Previous studies have been shown that oxidative stress is an important part in the control and regulation of activity of AChE (Rodriguez-Fuentes *et al.*, 2015). AChE plays an important role being responsible for the hydrolysis of acetylthiocholine in neurotransmission (Olsen *et al.*, 2001), regulation of cell proliferation, apoptosis and cell migrations (Zhang *et al.*, 2002b; Jiang and Zhang 2008; Ganesan *et al.*, 2016; Drews 1975), regulates differentiation through signaling (Falugi and Aluigi 2012) and ultimately and in embryonic development (Falugi 1993). Changes in the activity of this enzyme can cause failures in the previously mentioned processes and can be responsible for problems in the embryonic development, eventually causing teratogenicity. Organophosfates and carbamate pesticides have the capacity to inhibit the activity of AChE (Srain and Rudolph 2010). Mancozeb is compound is an EDCS and several studies have already related their action to the inhibition of AChE (Kackar, Srivastava, and Raizada 1999), this inhibition can be detected in this study in animals exposed to concentrations of 0.0005 mgL^{-1} and 0.05 mgL^{-1}.

In summary, exposure to the highest mancozeb concentration can affect zebrafish embryonic development through the interference with some developmental pathways that deserve further investigation. Oxidative stress and the consequent damage seem to be associated to the developmental arrest observed which, in turn, may be associated to malformations. However, further studies are needed to fully support the hypothesis presented and clarify the underlying toxicological mechanisms. In particular, the elucidation of the pharmacokinetic properties of mancozeb are essential to further understand and fully characterize the observed effects.

Chapter VI

VI.1

Results

Tebuconazole

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at, 24, 48, 72 and 96 hpf (fig. 25). At 24 hpf, no differences among concentrations tested were observed. The same occurred at 48, 72 and 96 hpf.

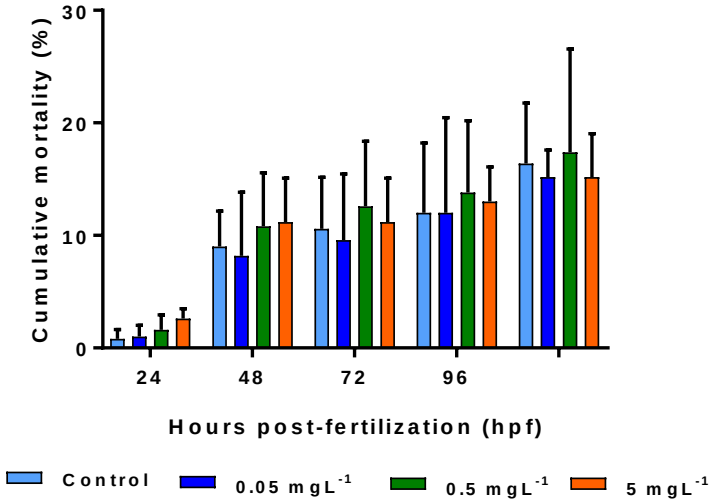

Figure 25 - Cumulative mortality of zebrafish embryos exposed to tebuconazole. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed at 24 hpf (table 8) showed a decrease ($X^2(3)=8.19$, $p=0.042$) in the concentration of 5 mgL^{-1} relative to concentrations of 0.5 mgL^{-1} ($p=0.006$). No other significant difference was observed. The heartbeat ($X^2(3)=7.022$, $p=0.071$), the hatching rate ($X^2(3)=0.869$, $p=0.833$) and the malformations ($X^2(3)=4.831$, $p=0.185$), showed no significant differences between groups. The figure 26, represent the animals exposed at different concentrations of tebuconazole.

Table 8- Sublethal parameters and malformations (%) of animals exposed to the different concentrations of tebuconazole.

Endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Treatment (mgL^{-1})	Spontaneous movement	Heart beats (bpm)	Hatching rate (%)	Malformations (%)
0	3 (3 - 3)^{ab}	122 (121 - 122)	58 (49 - 60)	0 (0 - 0)
0.05	3 (2 - 3)^{ab}	118 (118 - 119)	61 (48 - 62)	0 (0 – 0)
0.5	3 (3 - 4)^{a}	119 (118 - 121)	56 (54 - 59)	0 (0 - 10)
5	2 (2 - 2)^{b}	111 (110 - 115)	57 (47 - 63)	10 (0 - 10)
Statistical test	$X^2(3) = 8.197$	$X^2(3) = 7.022$	$X^2(3) = 0.869$	$X^2(3) = 4.831$
p value	0.042	0.071	0.833	0.185

Data from at least five independent replicates of 100 animals each. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

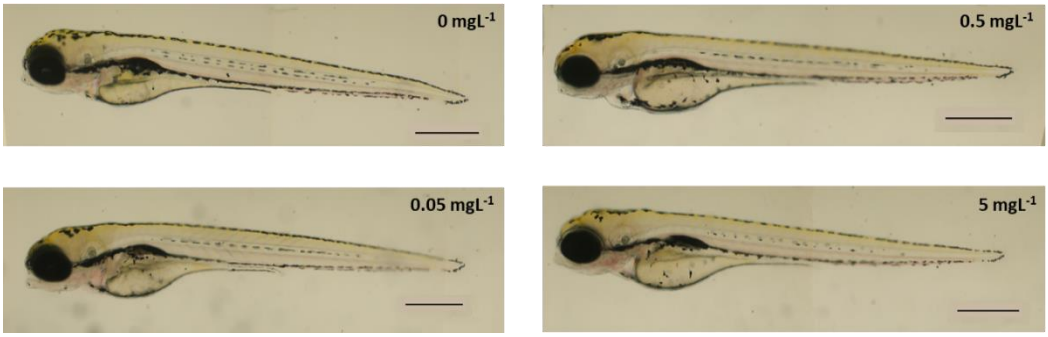

Figure 26 - Representative optical images of the zebrafish larvae after 96h of exposure to tebuconazole. The scale bar represents 500 μm.

Other lethal, tail and head detachment, and sublethal parameters such as development of the somites, eyes and otoliths development, blood circulation visible and edema were analyzed and results shown in table 9. These parameters were present/visible in all treatment groups at 24 and 48 hpf. At 72hpf, the presence of edema (yolk sac and pericardial edema) were observed in the animals exposed to concentration of 0.005 mgL^{-1} of tebuconazole.

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Table 9 -Frequency of normal embryos/larva following exposure to tebuconazole.

Endpoint	24hpf			48 hpf			72 hpf
Treatment (mgL^{-1})	Tail detach.	Head detach.	Somite	Eyes devel.	Otoliths devel.	Blood circulation	Edema *
0	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.05	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.5	10/10	10/10	10/10	10/10	10/10	10/10	0/10
5	10/10	10/10	10/10	10/10	10/10	10/10	0/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken and results are presented in table 10. The body length ($X^2(3)=4.349$, $p=0.226$, the area of yolk sac ($F(3,20)=3.008$, $p=0.061$), the area of the heart ($X^2(3)=6.429$, $p=0.093$) and the area of the eyes ($F(3,20)=1.371$, $p=0.287$) were measured, no differences were found among the evaluated groups.

Table 10 – Effects of tebuconazole exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	**96 hpf**			
Treatment (mgL^{-1})	**Body Length (mm)**	**Area of yolk sac (mm^2)**	**Area of heart (mm^2)**	**Area of eye (mm^2)**
0	3.39 (3.30 – 3.39)	0.201 ± 0.018	0.023.70 (0.021 – 0.025)	0.068 ± 0.007
0.05	3.38 (3.29 – 3.38)	0.207 ± 0.020	0.027.40 (0.026 – 0.029)	0.072 ± 0.007
0.5	3.26 (3.25 – 3.31)	0.204 ± 0.007	0.026 (0.025 – 0.026)	0.065 ± 0.010
5	3.29 (3.21 – 3.33)	0.231 ± 0.022	0.025 (0.025 – 0.026)	0.062 ± 0.008
Statistical test	$X^2(3) = 4.349$	F (3,20) = 3.008	$X^2(3) = 6.429$	F(3,20) = 1.371
p value	0.226	0.061	0.093	0.287

Data from at least five independent replicates of 100 animals each. 10 animals randomly selected per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzyme activity and non-enzymatic defenses were evaluated and the results are presented in figures 27, 28, 29, 30 and 31.

3.1. Reactive oxygen species (ROS) quantification

Concidering the ROS quantification (fig. 27), there were statistical differences among groups, ($F(3,20)=5.033$, $p=0.012$). An increase in ROS production was induced by the concentration of 0.05 mgL^{-1} ($p=0.041$) and concentration of 0.5 mgL^{-1} ($p=0.013$) relative to the control group. No other significant difference was observed.

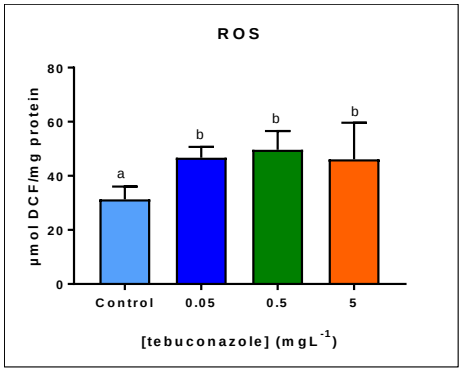

Figure 27- Reactive oxygen species (ROS) quantification in zebrafish embryos exposed to tebuconazole. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test.

3.2. Oxidative stress markers

In relation to the SOD activity ($F(3,20)=2.026$, $p=0.151$) and the CAT activity ($X^2(3)=2.531$, $p=0.47$), there were no statistically significant differences for the different concentrations evaluated and the control group (fig. 28). The activity of GPx ($F(3,20)=7.97$, $p=0.002$), sowed a decrease in all concentrations evaluated relative to the control group (0.05 mgL^{-1}, $p=0.031$; 0.5 mgL^{-1}, $p=0.005$ and 5 mgL^{-1}, $p= 0.006$). The GR activity ($F(3,20)=11.214$, $p<0.001$), presents an increase at concentration of 5 mgL^{-1} in relation to control ($p=0.003$) and other concentrations tested (0.05 mgL^{-1}, $p=0.002$ and 0.5 mgL^{-1}, $p=0.001$). No other significant differences were observed.

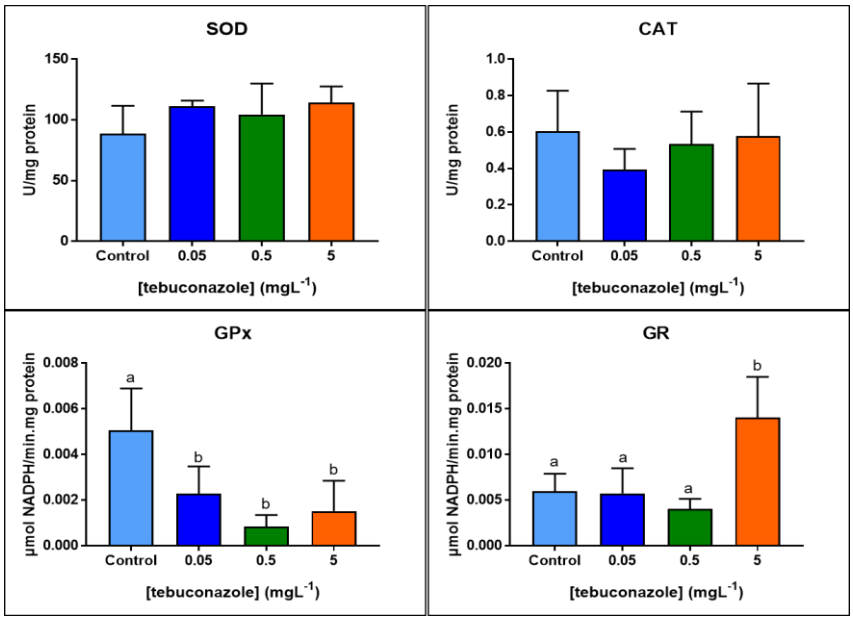

Figure 28 – Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity determination in zebrafish embryos exposed to tebuconazole. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.3. Glutathione levels

The GSH activity ($F(3,20)=2.949$, $p=0.064$) and GSSSG ($X^2(3)=6.794$, $p=0.079$) were similar among treatments (Fig. 29). The ratio between GSH and GSSG levels (GSH/GSSG) ($F(3,20) = 0.704$, $p=0.563$) also presented no significant differences for the different concentrations evaluated and the control group.

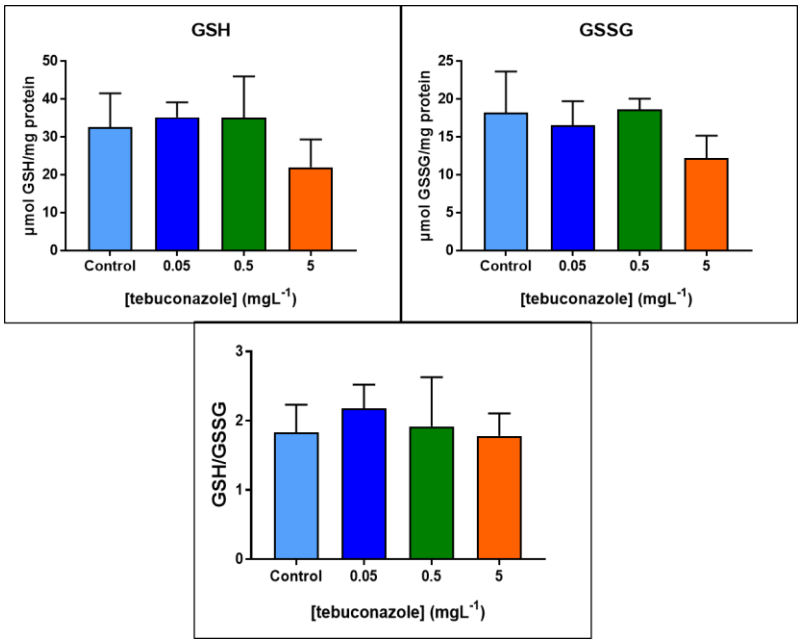

Figure 29 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ratio between GSH and GSSG (OSI) levels in zebrafish embryos exposed to tebuconazole. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.4. Xenobiotic biotransformation

The results relative to the GST and CarE enzymes, responsible for the degradation of some xenobiotics, are shown in figure 30. The GST activity ($X^2(3) = 1.537$, $p=0.674$) and the CarE activity ($F(3,20) = 2.554$, $p=0.092$) presented no significant differences for the different concentrations evaluated and the control group.

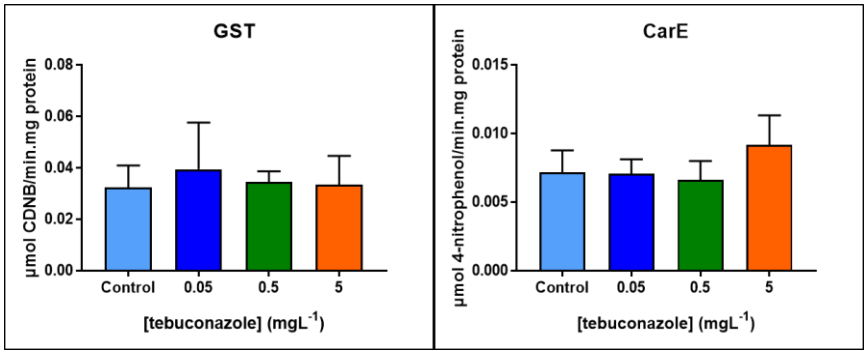

Figure 30 – Glutathione-s-transferase (GST), carboxylesterase (CarE), activity in zebrafish embryos exposed to tebuconazole. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.5. Oxidative damage, neurotransmission and anaerobic metabolism

The lipid peroxidation (fig. 31), was not statistical different (F (3,20) = 2.126, p=0.137) for the different concentrations evaluated and the control group, as the AChE activity (F (3,20) = 0.739, p=0.544), and LDH (F (3,20) = 2.652, p=0.084).

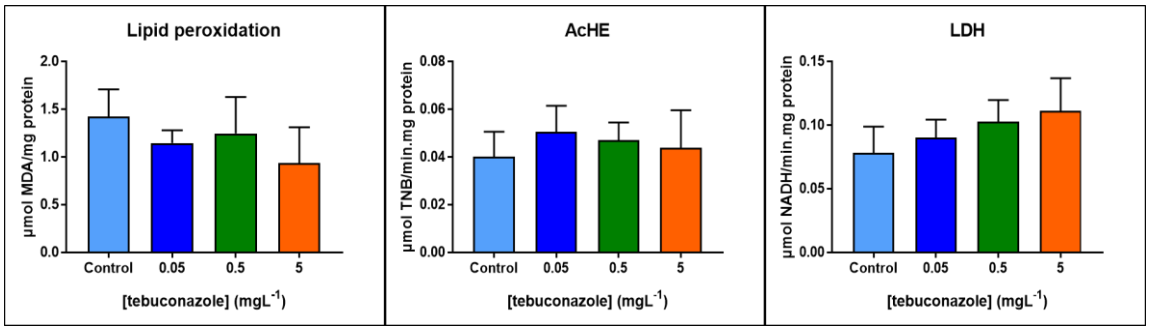

Figure 31- Lipidic peroxidation, acetylcholinesterase (AChE) and lactate dehydrogenase (LHD) activity determination in zebrafish embryos exposed to tebuconazole. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

Chapter VI

VI.2

Discussion

Tebuconazole

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The objective of the present study was to evaluate the effects of tebuconazole in zebrafish embryos exposed to different concentrations during a 96h period. Exposure to the highest concentration caused a decrease in the spontaneous movements, caused an increase in the number malformations at 72hpf and decreased the heartbeats. In addition, exposure to this concentration caused an increase of ROS and in the activity of GR while induced a decrease in the GPx activity.

Exposure to several compounds may affect embryonic development, which is a highly sensitive and controlled process in several pathways are formed and interrelated (Kimmel *et al.,* 1995; Lawson and Weinstein 2002). An example of this is exposure to fungicides such as tebuconazole (Sancho, Villarroel, and Ferrando 2016; Liu, Dong, *et al.,* 2016; Altenhofen *et al.,* 2017; Yu *et al.,* 2013).

This fungicide has already been reported to interfere in the development of zebrafish by inducing changes in the spontaneous movements, heartbeat, hatching rate and causing several malformations at concentration of 4 mgL^{-1} in zebrafish larvae exposed during 120h and 4 and 6 mgL^{-1} during 96 h in adult zebrafish (Altenhofen *et al.,* 2017; Liu, Dong, *et al.,* 2016). The first step of this work consisted on the calculation of the LC_{50} according to the OECD protocol No 236. The concentration values reported previously in studies with tebuconazole show concentrations very close to the LC_{50} value calculated in this study. In this study, the value obtained for the 96h-LC_{50} was 7.25 mgL^{-1}. This value is lower than previously published studies with zebrafish, ranging from 10.74 mgL^{-1} to 19.7 mgL^{-1} (Liu, Dong, *et al.,* 2016; Sancho *et al.,* 2010). Considering this LC_{50} value, three concentrations were selected (0.05, 0.5 and 5 mgL^{-1}) and the animals were exposed during a period of 96 hours with development changes being recorded at 24, 48, 72 and 96h.

Compounds are known to be involved in the reduction of the spontaneous movement frequency and blockage of membrane Na^+ channel (Fraysse, Mons, and Garric 2006). Although, Jin *et al.,* (2009) suggested that an increased spontaneous movements frequency can be associated to the prolongation channel opening to cause repetitive firing of action potentials, taking in consideration the results obtained, i.e., the decrease in motions observed for compounds of the tebuconazole class (Mu *et al.,* 2013; Wu *et al.,* 2018) has also been described which corroborates the results obtained in this study to the highest tested dose of tebuconazole. This may be explained by the decrease in the opening time of the Na^+ channels. Still, further tests are needed to support this hypothesis namely through the evaluation of Na^+ channels.

Moreover, zebrafish exposed to the concentration of 5 mgL^{-1} showed an increased number of malformations (cardiac and yolk sac edema and tail) at 96 hpf. Hill *et al.*, (2004) in a previous study showed that the cardiac and yolk sac edema have been related with a possible increase in the surface permeability and consequent damage of the primary zebrafish defense barrier during its development (Hill *et al.,* 2004; Guiney, Walker, and Peterson 1990). The increase of a water permeability barrier at the surface of the zebrafish embryo can be caused by a deregulation in osmotic balance during the embryonic development (Hill *et al.,* 2004) or metabolic or osmotic disruptions possibly caused by mitochondrial malfunction due to the chemical (Papiya and Kanamadi 2000). These effects may be attributed to tebuconazole, since the animals exposed to the higher concentration has edema presence, however this work will need to be complimented with a pharmacokinetic study which may give further indications as to the possible absorption and metabolism of the compound.

Moreover, studies suggest that the toxicity of tebuconazole is associated with the fact that it acts at the inhibition of the sterol 14α-demethylase. This enzyme is one of the intermediates in pathways leading to the formation of cholesterol in humans and ergosterol in fungi (Zarn, Bruschweiler, and Schlatter 2003). However, the study of this enzyme was not the objective of the current work. Instead, several other biochemical parameters were evaluated.

The ROS increase and may thus be associated with a fungicide exposed and consequent oxidative damage (Gao *et al.*, 2014; Affourtit, Heaney, and Moore 2000; Xiao *et al.,* 2014; Garanzini and Menone 2015; Domico *et al.,* 2007; Altenhofen *et al.,* 2017). The ROS increase activate the antioxidant defenses, and the first to act is SOD. Several studies claim that there is an increase in SOD associated with an increase in ROS following exposure to tebuconazole (Chen *et al.,* 2018). However, these data were not obtained in this study, although exposure to tebuconazole induced an increase in the ROS levels in all concentrations, no changes were observed in the SOD activity. The CAT activity does not appear to be affected by the exposure to this compound. This data shows that the first line of defense it is enough to combat the ROS.

Additionally, the GPx activity was decreased for all concentrations of tebuconazole tested. Yet, GR showed a high activity in the concentration of 5 mgL^{-1} but not in the rest. Moreover, the levels of GSH and GSSG were higher for the control and the two lower concentrations. These higher levels may mean that there is oxidation/reduction process performed by GPx and GR enzymes following exposure to the highest concentration. The

glutathione system and related enzymes are considered a second line of defense against oxidative damage (Jafari 2007) being the ratio of GSH to GSSG often used as an indicator of intracellular redox status (Timme-Laragy *et al.,* 2013). The glutathione system is a key nonenzymatic radical scavenger and antioxidant that scavenges residual free radicals generated from oxidative metabolism and those not decomposed by antioxidant enzymes (El-Shenawy 2010). The no alterations in GSH and GSSG, contents might be a combined impact of declined GPx activity and GR activity.

In summary, exposure to tebuconazole concentrations did not affect zebrafish embryonic development through can interference with some developmental pathways that deserve further investigation. Thus, further studies, as genetic expression of some oxidative stress and development related genes are needed to fully support the hypothesis presented and clarify the underlying toxicological mechanisms.

Chapter VII

Results

Extract of *Equisetum arvense*

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at 24, 48, 72 and 96 hpf (fig. 32). At 24 hpf, no differences between concentrations tested and the control group were observed. The same occurred at 48, 72 and 96 hpf.

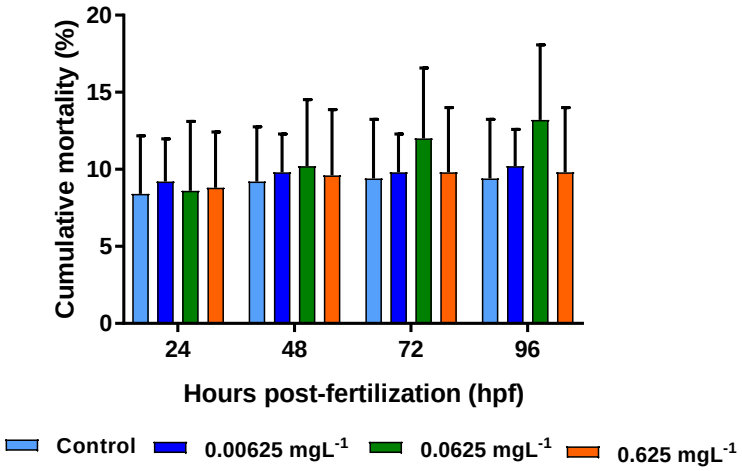

Figure 32 - Cumulative mortality of zebrafish embryos exposed to *Equisetum arvense*. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed at 24 hpf (table 11) showed significant differences between groups ($X^2(3)=7.859$, $p=0.049$). The concentration of 0.0625 mgL^{-1} showed a decrease in relation to control group ($p=0.04$), 0.00625 mgL^{-1} ($p=0.01$) and 0.625 mgL^{-1} ($p=0.04$). The heartbeat, analyzed at 48hpf showed a statistic differences ($F(3,20)=4.975$, $p=0.013$). The concentration of 0.0625 mgL^{-1} showed a decrease in relation to the control group ($p=0.025$) and 0.00625 mgL^{-1} ($p=0.016$).

The hatching rate (table 11) was evaluated at 72hpf ($X^2(3)=2.201$, $p=0.532$) and no differences relative to the control or to other concentrations were observed. The animals exposed to this compound did not presented malformations at 96hpf (fig. 33).

Table 11- Sublethal parameters and malformations (%) of animals exposed to the different concentrations of *Equisetum arvense*.

Endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Treatment (mgL^{-1})	Spontaneous movement	Heart beats (bpm)	Hatching rate (%)	Malformations (%)
0	2 (2 - 2)^{a}	123 ± 2^{a}	70 (66 - 70)	-
0.00625	2 (2 - 3)^{a}	124 ± 3^{a}	70 (68 - 70)	-
0.0625	1 (1 - 2)^{b}	115 ± 3^{b}	68 (64 - 69)	-
0.625	2 (2 - 2)^{a}	121 ±7^{ab}	68 (66 - 68)	-
Statistical test	$X^2(3) = 7.859$	F (3,20) =4.975	$X^2(3) = 2.201$	nd
p value	0.049	0.013	0.532	nd

Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. In the same column, different lowercase letters indicate significant differences between groups ($p<0.05$).

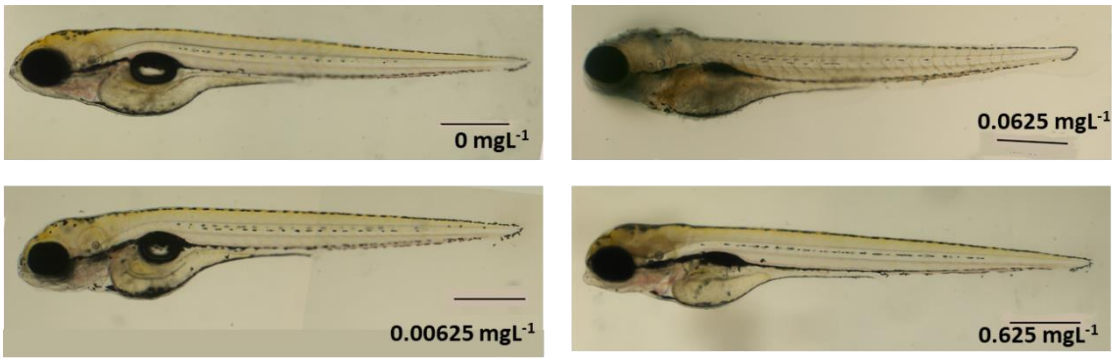

Figure 33 -Representative optical images of the zebrafish larvae after 96 h embryonic exposure to *Equisetum arvense*. The scale bar represents 500 μm.

Other sublethal parameters such as tail detachment, head detachment, somite formed, eyes developed, otoliths developed and blood circulation visible (Table 12) were analyzed and were present in all treatment groups at 24 and 48 hpf. At 72hpf, edema presence (yolk sac and pericardial edema) were no observed in the animals exposed to Equisetum in all concentrations.

Table 12 - Frequency of normal embryos/larva following embryonic exposure to *Equisetum arvense*.

Endpoint	24hpf			48 hpf			72 hpf
Treatment (mgL^{-1})	Tail detach.	Head detach.	Somite	Eyes devel.	Otoliths devel.	Blood circulation	Edema*
0	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.00625	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.0625	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.625	10/10	10/10	10/10	10/10	10/10	10/10	0/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken (table 13). Significant differences ($F(3,20) = 22.79$, $p<0.001$) were observed when analyzing the body length of the exposed animals with a significant decrease for the animals exposed to the concentration of 0.0625 mgL^{-1} in relation to control group, 0.00625 mgL^{-1} and 0.625 mgL^{-1} ($p<0.001$). When the area of yolk sac was measured, differences were found between the evaluated groups ($F(3,20) = 6.17$, $p=0.005$). The animals exposed to the concentration of 0.0625 mgL^{-1} presented superior area than the control group ($p=0.015$) and the 0.625 mgL^{-1} ($p=p=0.006$). No other significant difference was observed.

The cardiac area (table 13) was also measured and did not presented statistic differences between the studied groups ($X^2(3) = 2.634$, $p=0.452$). The area of the eye (table 13) was statistically different among groups ($F(3,20) = 26.411$, $p<0.001$). The concentration of 0.0625 mgL^{-1} showed a decrease in relation to the control, 0.00625 mgL^{-1} and 0.625 mgL^{-1} groups ($p<0.001$). No other significant difference was observed.

Table 13 – Effects of *Equisetum arvense* exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	**96 hpf**			
Treatment (mgL^{-1})	**Body Length (mm)**	**Area of yolk sac (mm^2)**	**Area of heart (mm^2)**	**Area of eye (mm^2)**
0	3.74 ± 0.04^{a}	0.206 ± 0.005^{a}	0.034 (0.034 – 0.035)	0.083 ± 0.002^{a}
0.00625	3.75 ± 0.04^{a}	0.214 ± 0.009^{ab}	0.035 (0.031 – 0.035)	0.082 ± 0.002^{a}
0.0625	3.50 ± 0.03^{b}	0.225 ± 0.012^{b}	0.035 (0.035 – 0.036)	0.074 ± 0.0007^{b}
0.625	3.72 ± 0.09^{a}	0.204 ± 0.006^{a}	0.033 (0.028 – 0.034)	0.081 ± 0.003^{a}
Statistical test	F (3,20) = 22.79	F (3,20) = 6.17	$X^2(3) = 2.634$	F (3,20) = 26.411
p value	0	0.005	0.452	0

Data from at least five independent replicates of 100 animals each. 10 animals randomly per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzyme activity and non-enzymatic defenses were evaluated and the results presented in figures 34, 35, 36, 37, 38 and 39.

3.1.Reactive oxygen species (ROS) quantification

The ROS quantification (F (3,20) = 0.646, p=0.596) was not statistically different among groups (fig. 34).

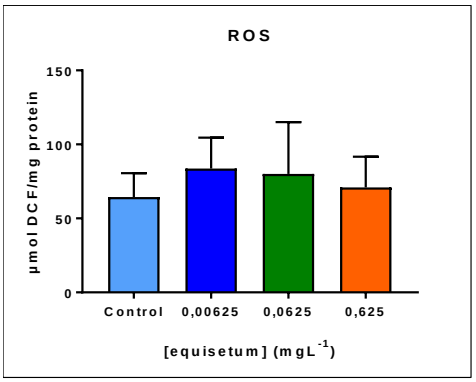

Figure 34- Reactive oxygen species (ROS) quantification in zebrafish embryos exposed to *Equisetum arvense*. Data from at least five independent samples (n=100/each). Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test.

3.2. Oxidative stress markers

The SOD activity (F (3,20) = 8.899, p=0.001) showed an increase in all concentrations tested in relation to the control group (0.00625 mgL^{-1}, p=0.001; 0.0625 mgL^{-1} p=0.028; 0.625 mgL^{-1} p=0.016). No other differences were found between groups (figure 35). The activity of CAT ($X^2(3)$ = 5.446, p=0.142), did not present statistically significant differences. The GPx activity ($X^2(3)$ = 8.623, p=0.035) showed a decrease between 0.0625 mgL^{-1} and 0.625 mgL^{-1} (p=0.004) whereas no other differences were detected between treatments. The GR activity (F (3,20) = 8.88, p=0.001) presented an increase in the concentration of 0.00625 mgL^{-1} (p=0.008) in relation to the control group and to 0.625 mgL^{-1} (p=0.002). No other differences were detected between concentrations tested and control group.

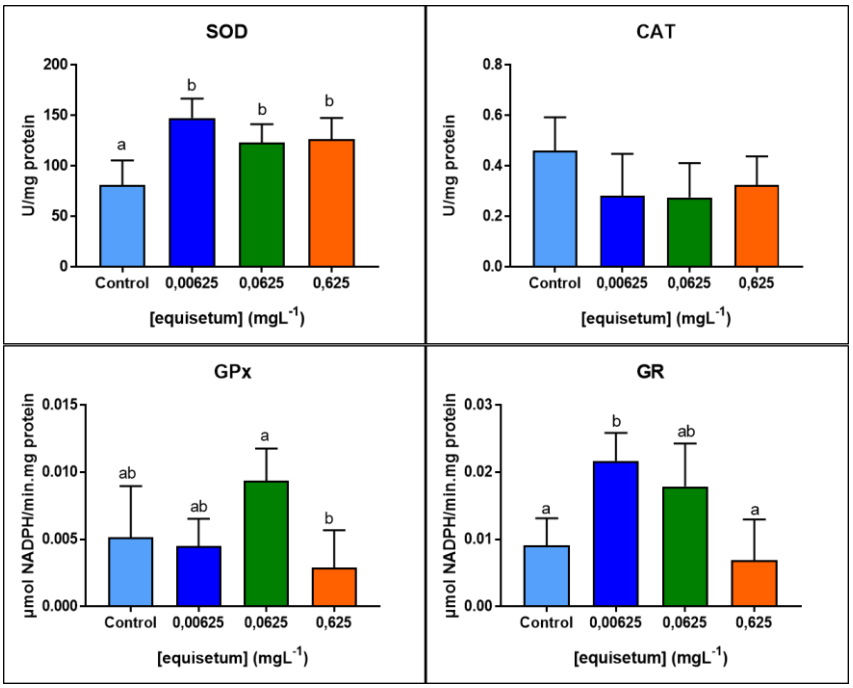

Figure 35 – Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity determination in zebrafish embryos exposed to *Equisetum arvense*. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.3. Glutathione levels and xenobiotic biotransformation

In the figure 37 is represented the GSH, GSSG levels and the ratio between both. The GSH ($F(3,20)=3.848$, $p=0.03$) in the concentration of 0.00625 mgL^{-1} showed a significative decrease in relation to the control group ($p=0.023$). The GSSG ($F(3,20)=3.708$, $p=0.034$) presented a significative decrease in its levels in the lowest concentration in relation to control group ($p=0.039$). No other differences were detected between concentrations tested and control group. The oxidative-stress index (OSI), is represented for the ratio between GSH and GSSG levels ($X^2(3) = 3.114$, $p=0.374$), the GST activity ($F(3,20) = 2.404$, $p=0.106$) and CarE activity ($F(3,20) = 1.356$, $p=0.292$) were not different among groups (figure 36).

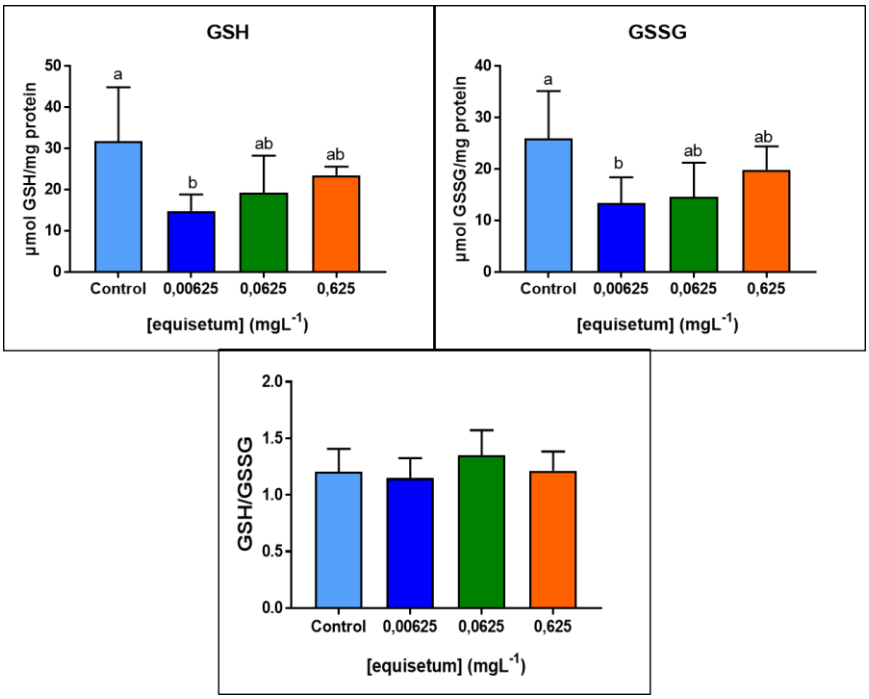

Figure 37 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ratio between GSH and GSSG (OSI) levels in zebrafish embryos exposed to *Equisetum arvense.* Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

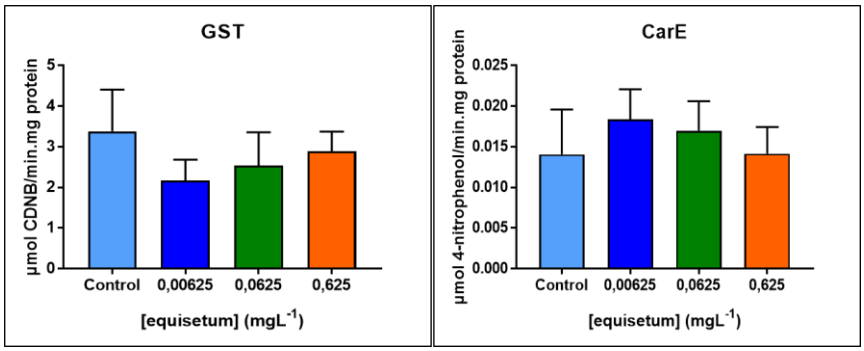

Figure 36 – Glutathione-s-transferase (GST), carboxylesterase (CarE), activity in zebrafish embryos exposed to *Equisetum arvense*. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test.

3.4. Oxidative damage

The lipid peroxidation (fig. 38) presented statistical differences ($F(3,20) = 5.539$ $p=0.008$) with a decrease in the concentration of 0.00625 mgL^{-1} ($p=0.007$) and 0.625 mgL^{-1} ($p=0.037$) relative to the control group. No other differences were detected between concentrations tested and control group.

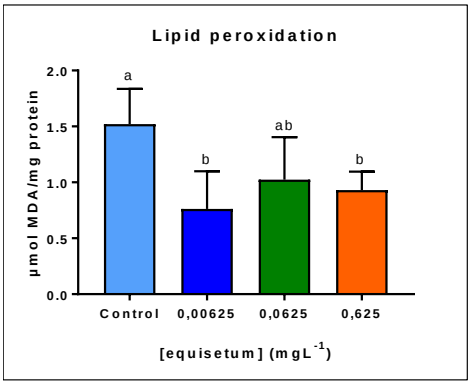

Figure 38- Lipidic peroxidation in zebrafish embryos exposed to *Equisetum arvense*. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p<0.05$)

3.5. Neurotransmission and anaerobic metabolism

The AChE activity (table 4) ($X^2(3) = 7.571$, $p=0.056$) and LDH ($X^2(3) = 6.171$ $p=0.104$) were not statistically different between evaluated concentration groups and the control group (fig. 39).

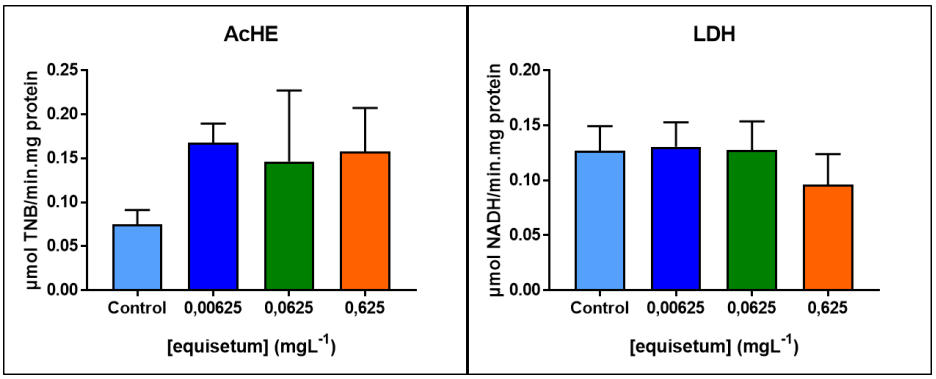

Figure 39- Acetylcholinesterase (AChE) activity and lactate dehydrogenase (LHD) activity in zebrafish embryos exposed to *Equisetum arvense*. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test.

Chapter VIII

Results

Extract of *Mimosa tenuiflora*

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at 24, 48, 72 and 96 hpf (fig. 40). At 24 hpf, no differences between concentrations tested and the control group were observed. The same occurred at 48, 72 and 96 hpf.

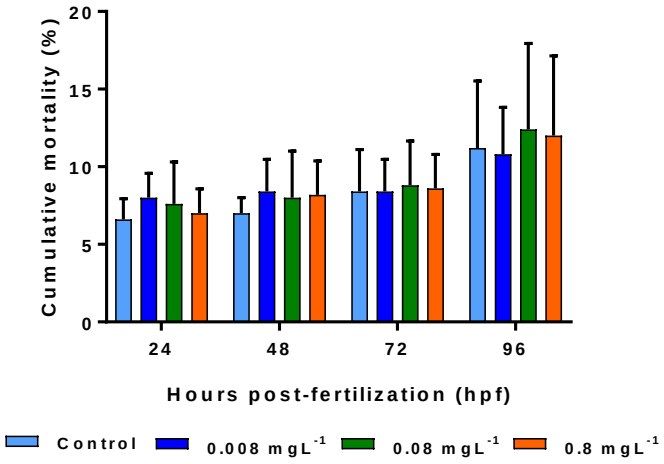

Figure 40 - Cumulative mortality of zebrafish embryos exposed to *Mimosa tenuiflora*. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed at 24hpf (table 14) showed no significant differences between groups ($X^2(3)=1.037$ p=0.792). The heart beats (F (3,20)=0.649, p=0.595), the hatching rate (F (3,20)=0.777, p=0.524) and the malformations ($X^2(3)=2.111$, p=0.55) were not significantly different between groups (fig. 41).

Table 14 - Sublethal parameters and malformations (%) of animals exposed to the different concentrations of *Mimosa tunuiflora*.

Endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Treatment (mgL^{-1})	Spontaneous movement	Heart beats (bpm)	Hatching rate (%)	Malformations (%)
0	2 (1 - 2)	113 ± 7	56 ± 15	0 (0 - 0)
0.008	1 (1 - 1)	115 ± 10	66 ± 5	0 (0 - 5)
0.08	2 (1 - 2)	120 ± 6	56 ± 13	0 (0 - 5)
0.8	1 (1 - 2)	119 ± 11	54 ± 16	0 (0 - 0)
Statistical test	$X^2(3)$ = 1.037	F (3,184) = 0.649	F (3,184) =0.777	$X^2(3)$ = 1.037
p value	0.792	0.595	0.524	0.55

Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. In the same column, different lowercase letters indicate significant differences between groups ($p<0.05$).

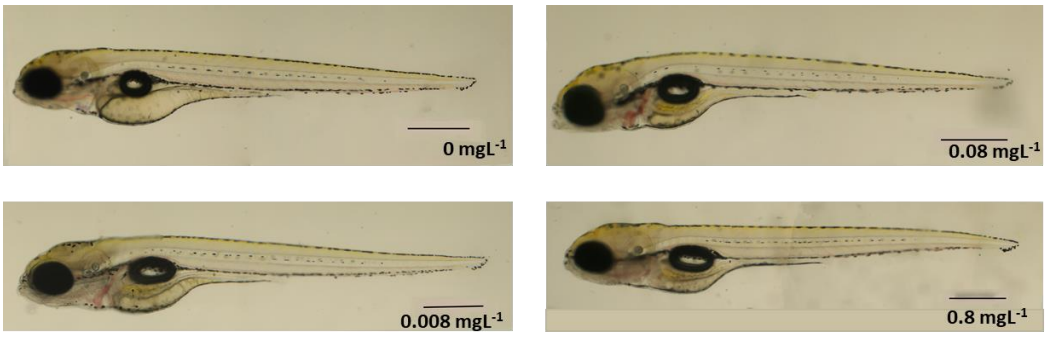

Figure 41 -Representative optical images of the zebrafish larvae after 96 h embryonic exposure to *Mimosa tunuiflora*. The scale bar represents 500 μm.

Other sublethal parameters such as tail detachment, head detachment, somite formed, eyes developed, otoliths developed and blood circulation visible (Table 15) were analyzed and were presented in all treatment groups at 24 and 48 hpf. At 72hpf, edema presence (yolk sac and pericardial edema) were no observed in the animals exposed to Equisetum in all concentrations.

Table 15 -Frequency of normal embryos/larva following embryonic exposure to *Mimosa tunuiflora*.

Endpoint	24hpf			48 hpf			72 hpf
Treatment (mgL^{-1})	Tail detach.	Head detach.	Somite	Eyes devel.	Otoliths devel.	Blood circulation	Edema*
0	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.008	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.08	10/10	10/10	10/10	10/10	10/10	10/10	0/10
0.8	10/10	10/10	10/10	10/10	10/10	10/10	0/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken (table 16). No significant differences were observed in body length ($F(3,20) = 0.637$, $p=0.602$), area of yolk sac ($F(3,20) = 1.389$, $p=0.282$), area of heart ($F(3,20) = 0.597$, $p=0.626$), or area of eyes ($F(3,20) = 0.267$, $p=0.849$).

Table 16 – Effects of *Mimosa tunuiflora* exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	**96 hpf**			
Treatment (mgL^{-1})	**Body Length (mm)**	**Area of yolk sac (mm^2)**	**Area of heart (mm^2)**	**Area of eye (mm^2)**
0	3.56 ± 0.20	0.222 ± 0.010	0.038 ± 0.005	0.081 ± 0.015
0.008	3.49 ± 0.26	0.238 ± 0.006	0.035 ± 0.006	0.086 ± 0.015
0.08	3.65 ±0.14	0.239 ± 0.021	0.039 ± 0.005	0.088 ± 0.010
0.8	3.60 ± 0.15	0.238 ± 0.019	0.038 ± 0.005	0.087 ± 0.011
Statistical test	F (3,20) =0.637	F (3,20) =1.389	F (3,20) =0.597	F (3,20) =0.267
p value	0.602	0.282	0.626	0.849

Data from at least five independent replicates of 100 animals each. 10 animals randomly per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test.Different lowercase letters indicate significant differences between groups ($p<0.05$).

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzyme activity and non-enzymatic defenses were evaluated and the results are presented in figures 42, 43, 44 and 45.

3.1. Reactive oxygen species (ROS) quantification and Oxidative stress markers

The ROS quantification (F (3,20) = 2.101, p=0.14), the SOD activity ($X^2(3) = 4.509$, p=0.212), the CAT activity (F (3,20) = 0.571, p=0.642), the GPx activity (F (3,20) = 2.96, p=0.064) and the GR activity (F (3,20) = 0.344, p=0.794) did not presented statistically differences between treatments (fig. 42).

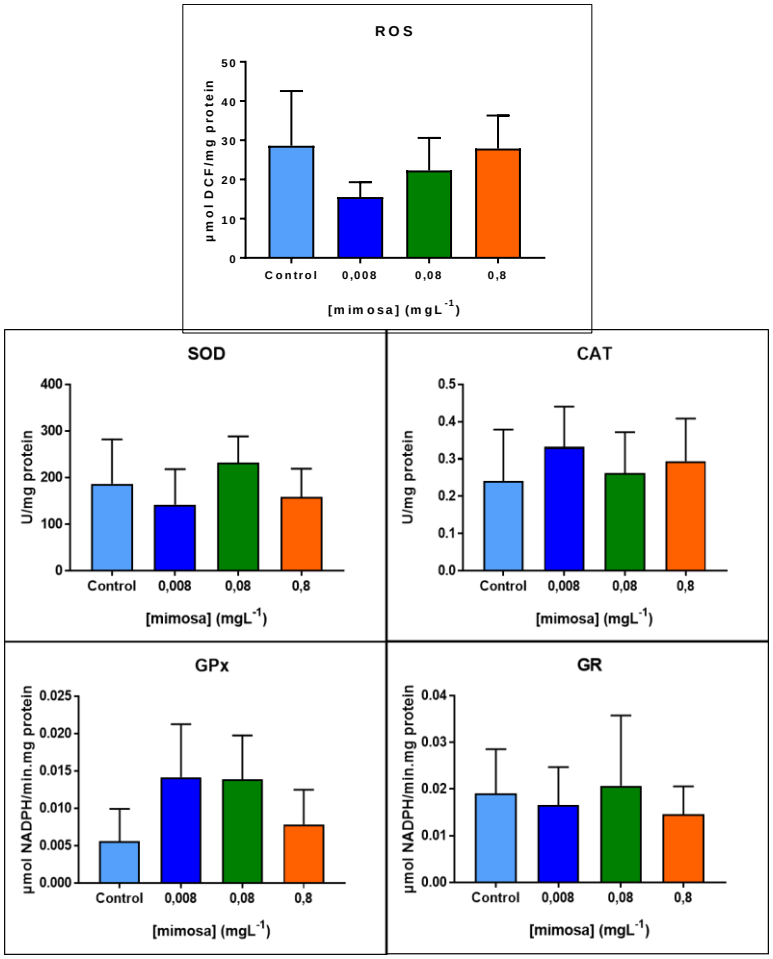

Figure 42 - Reactive oxygen species (ROS) quantification, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity determination in zebrafish embryos exposed to *Mimosa tunuiflora*. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test.

3.2. Glutathione levels

The GSH level ($X^2(3)=5.583$, p=0.134) and the GSSG level ($X^2(3)=2.154$, p=0.541) did not present statistically significant differences among evaluated concentration groups. The oxidative-stress index (OSI), represented in figure 43 for the ratio between GSH and GSSG levels (GSH/GSSG), showed significant differences between groups ($X^2(3) = 7.834$, p=0.05). The concentration of 0.008 mgL^{-1} (p=0.014) and the concentration of 0.08 mgL^{-1} (p=0.033) showed an increase in relation to the control group. For the remaining concentrations, there were no significant differences.

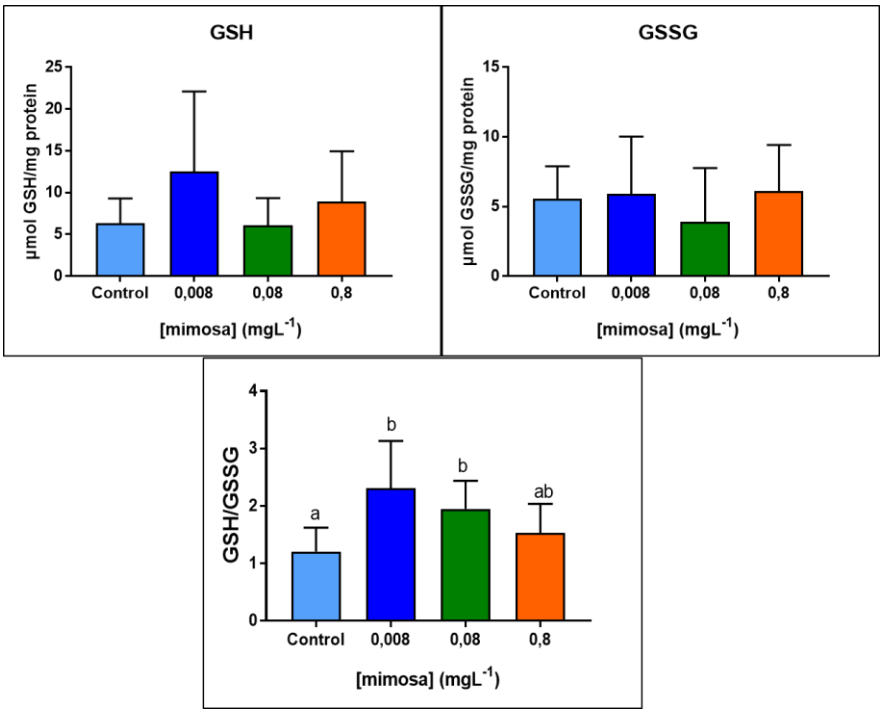

Figure 43 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ration between GSH and GSSG (OSI) level in zebrafish embryos exposed to *Mimosa tunuiflora*. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups (p<0.05).

3.3. Xenobiotic biotransformation

The GST activity ($X^2(3) = 3.537$, p=0.316) and the CarE activity ($X^2(3) = 1.196$, p=0.754) were no statistically different for the different concentrations evaluated and the control group (fig. 44).

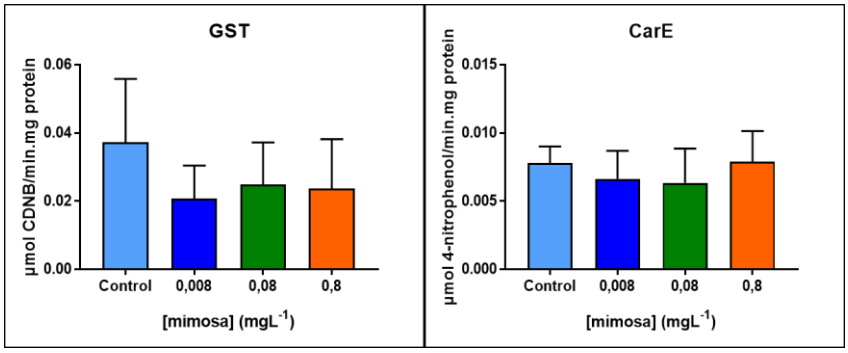

Figure 44 - Glutathione-s-transferase (GST), carboxylesterase (CarE), activity determination in zebrafish embryos exposed to *Mimosa tunuiflora*. Data from at least five independent replicates of 100 animals each. Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test.

3.4. Oxidative damage, neurotransmission and anaerobic metabolism

The lipid peroxidation (figure 45), were not showed statistical differences ($X^2(3)$ = 3.754, p=0.289) for the different concentrations evaluated and the control group, such as the AChE activity (F (3,20) = 1.829, p=0.183) and LDH activity (F (3,20) = 0.73, p=0.549).

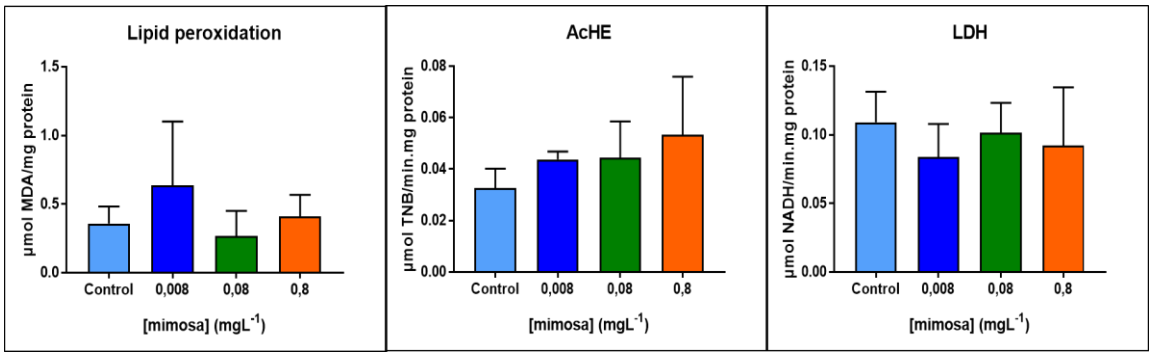

Figure 45 - Lipidic peroxidation, acetylcholinesterase (AChE) activity and lactate dehydrogenase (LHD) activity in zebrafish embryos exposed to *Mimosa tunuiflora*. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test.

Chapter IX

Results

Thymol

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Cumulative mortality

The cumulative mortality of zebrafish embryos and larvae was recorded at 24, 48, 72 and 96 hpf (fig. 46). At 24 hpf, no differences between concentrations tested and the control group were observed. The same occurred at 48, 72 and 96 hpf.

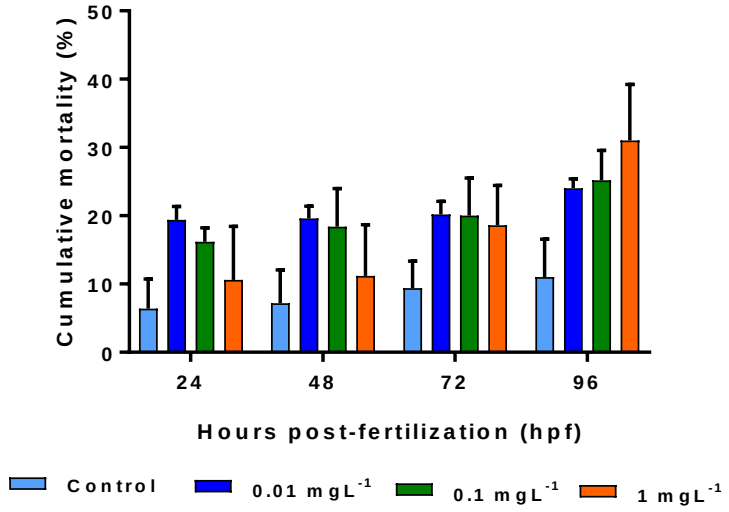

Figure 46 - Cumulative mortality of zebrafish embryos exposed to thymol. Mortality was recorded at 24, 48, 72 and 96 h post-fertilization. Data are represented as mean ± SD.

2. Developmental toxicity tests

The spontaneous movements analyzed (table 17) ($X^2(3) = 4.907$, p=0.179) the heartbeat ($F(3,20) = 2.357$, p=0.11), the hatching rate ($F(3,20) = 0.145$, p=0.931) and the malformations at 96hpf ($X^2(3) = 2.499$, p=0.476), showed no significant differences among evaluated concentration groups (fig. 47).

Table 17- Sublethal parameters and malformations (%) of animals exposed to the different concentrations of thymol.

Endpoint	24 hpf	48 hpf	72 hpf	96 hpf
Treatment (mgL^{-1})	Spontaneous movement	Heart beats (bpm)	Hatching rate (%)	Malformations (%)
0	2 (1 - 2)	144 ± 22	61 ± 8	0 (0 - 0)
0.01	0 (0 - 2)	160 ± 5	59 ± 3	0 (0 - 10)
0.1	1 (0 - 1)	166 ± 6	59 ± 5	0 (0 - 0)
1	2 (2 - 2)	145 ± 20	59 ± 5	0 (0 - 10)
Statistical test	$X^2(3) = 4.907$	F (3,20) = 2.357	F (3,20) =0.145	$X^2(3) = 2.499$
p value	0.179	0.11	0.931	0.476

Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. In the same column, different lowercase letters indicate significant differences between groups ($p<0.05$).

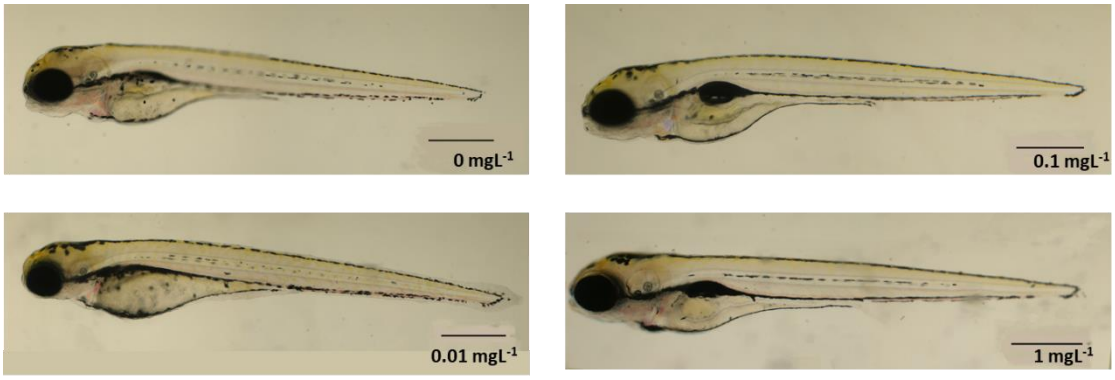

Figure 47- Representative optical images of the zebrafish larvae after 96 h embryonic exposure to thymol. The scale bar represents 500 μm.

Other sublethal parameters such as tail detachment, head detachment, somite formed, eyes developed, otoliths developed and blood circulation visible (Table 18) were analyzed and were present in all treatment groups at 24 and 48 hpf. At 72hpf, edema presence (yolk sac and pericardial edema) were observed in the animals exposed to thymol in all concentration.

Table 18 - Frequency of normal embryos/larva following embryonic exposure to thymol.

Endpoint	24hpf			48 hpf			72 hpf
Treatment (mgL^{-1})	Tail detach.	Head detach.	Somite	Eyes devel.	Otoliths devel.	Blood circulation	Edema*
0	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.01	10/10	10/10	10/10	10/10	10/10	10/10	1/10
0.1	10/10	10/10	10/10	10/10	10/10	10/10	1/10
1	10/10	10/10	10/10	10/10	10/10	10/10	1/10

Data from at least five independent replicates of 100 animals each. These parameters were present/visible, in 10 random animals. *yolk sac and cardiac edemas.

At 96 hpf, the size of the animal and other measurements (body length, area of yolk sac, area of heart and area of eyes) were taken (table 19). No significant differences were observed when analyzing the body length ($X^2(3) = 6.109$, $p=0.106$), the area of yolk sac ($F(3,20) = 1.924$, $p=0.166$) and the area of heart ($F(3,20) = 1.03$, $p=0.406$). The area of eye ($F(3,20) = 3.932$, $p=0.028$) was decreased between control group and the highest concentration of 1 mgL^{-1} ($p=0.031$).

Table 19 – Effects of thymol exposure during embryonic stage on morphological abnormalities at 96 hpf.

Endpoint	96 hpf			
Treatment (mgL^{-1})	Body Length (mm)	Area of yolk sac (mm^2)	Area of heart (mm^2)	Area of eye (mm^2)
0	3.42 (3.40 – 3.51)	0.227 ± 0.015	0.036 ± 0.005	0.080 ± 0.002^{a}
0.01	3.50 (3.44 – 3.50)	0.227 ± 0.018	0.045 ± 0.007	0.071 ± 0.009^{ab}
0.1	3.49 (3.46 – 3.49)	0.216 ± 0.014	0.042 ± 0.0013	0.069 ± 0.007^{ab}
1	3.32 (3.31 – 3.32)	0.245 ± 0.026	0.039 ± 0.009	0.067 ± 0.006^{b}
Statistical test	$X^2(3) = 6.109$	F (3,20) =1.924	F (3,20) = 1.03	F (3,20) = 3.932
p value	0.106	0.166	0.406	0.028

Data from at least five independent replicates of 100 animals each. 10 animals randomly per replicate and per concentration were evaluated. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data were presented as median (25^{th}–75^{th} quartile) and statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$)

3. Biomarker determinations

By the end of exposure, at 96 hpf, the enzyme activity and non-enzymatic defenses were evaluated and the results are presented in figures 48, 49, 50 and 51.

3.1. Reactive oxygen species (ROS) quantification

The ROS quantification ($X^2(3) = 5.583$, p=0.134) did not show significant differences among concentrations tested and the control group (fig. 48).

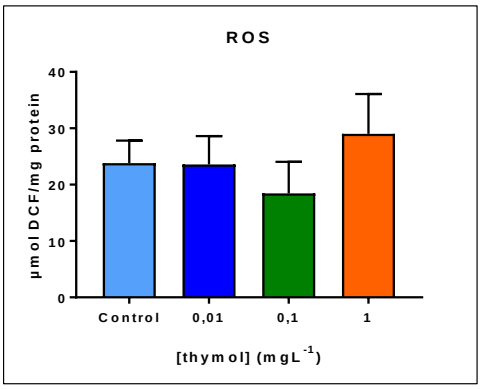

Figure 48- Reactive oxygen species (ROS) quantification in zebrafish embryos exposed to thymol. Data from at least five independent samples (n=100/each). Data are represented as mean ± SD. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.2. Oxidative stress markers

The SOD activity (F (3,20) = 2.589, p=0.089) and CAT activity (F (3,20) = 2.133, p=0.136) did not present statistically significant differences (fig. 49). The GPx activity (F (3,20) = 3.609, p=0.037) showed an increase in concentration of 0.01 mgL^{-1} in relation to control (p=0.029) and 0.01 mgL^{-1}. The GR activity (X^2 (3) = 9.103 p=0.028) showed an increase in the concentration of 0.01 mgL^{-1} (p=0.005) and in the concentration of 1 mgL^{-1} (p=0.016) in relation to the control group. No other differences were observed.

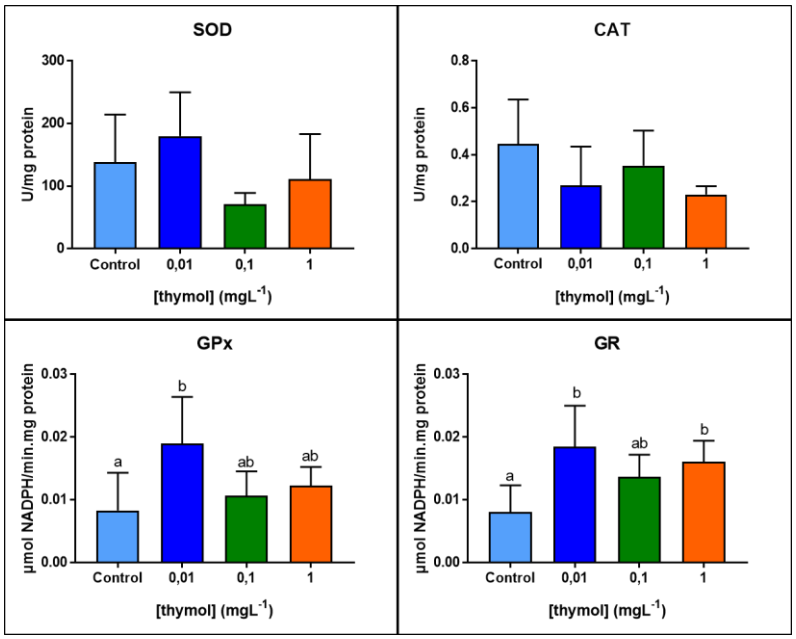

Figure 49 – Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) activity in zebrafish embryos exposed to thymol. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.3. Glutathione levels

The GSH level ($F(3,20)=3.868$, $p=0.03$) showed a significative increase in the concentration of 0.1 mgL^{-1} in relation to control group ($p=0.035$) (fig.50). The GSSG level ($F(3,20)=3.048$, $p=0.059$) showed no significative differences between concentrations tested and control group. The ratio between GSH and GSSG levels (GSH/GSSG) ($F(3,20) = 11.209$, $p <0.001$) showed an increase in all tested concentrations relative to the control group (0.01 mgL^{-1} and 0.1 mgL^{-1}, both $p=0.001$ and 1 mgL^{-1}, $p=0.025$). No other significant differences were observed.

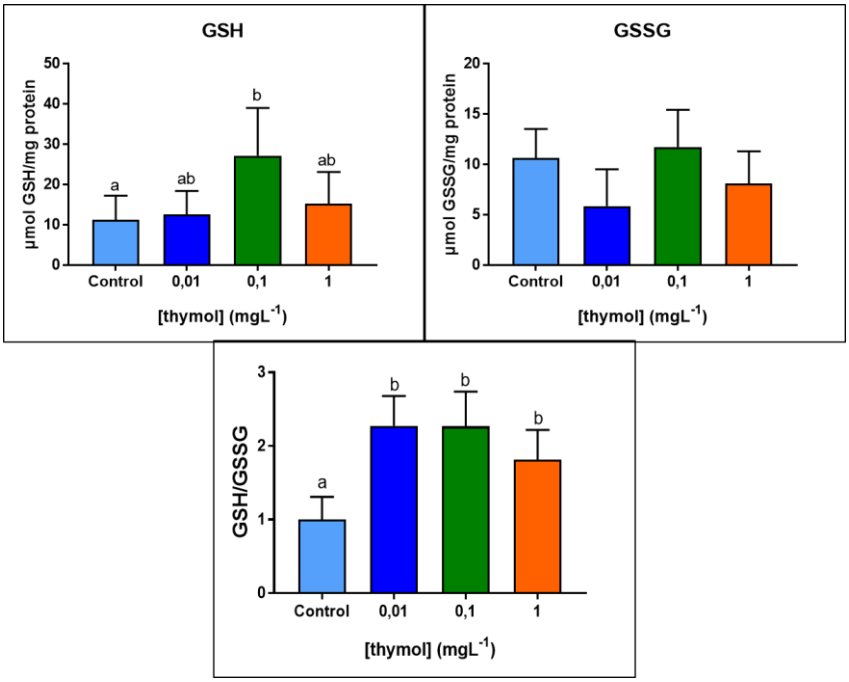

Figure 50 - Glutathione reduced form (GSH), glutathione oxidized form (GSSG), and the ration between GSH and GSSG (OSI) level in zebrafish embryos exposed to thymol. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

3.4. Xenobiotic biotransformation, oxidative damage, neurotransmission and anaerobic metabolism

The GST activity (figure 51) ($F(3,20) = 8.119$, $p=0.002$), presented an increase between the concentration of 1 mgL^{-1} ($p=0.001$) and the control group. No other significant difference was observed. The CarE ($F(3,20) = 0.654$, $p=0.592$), the lipid peroxidation ($X^2(3) = 2.726$, $p=0.436$), the AChE activity ($X^2(3) = 2.360$, $p=0.501$), and the LDH activity ($F(3,20) = 0.474$, $p=0.704$), were not statistically different for the different concentrations evaluated and the control group.

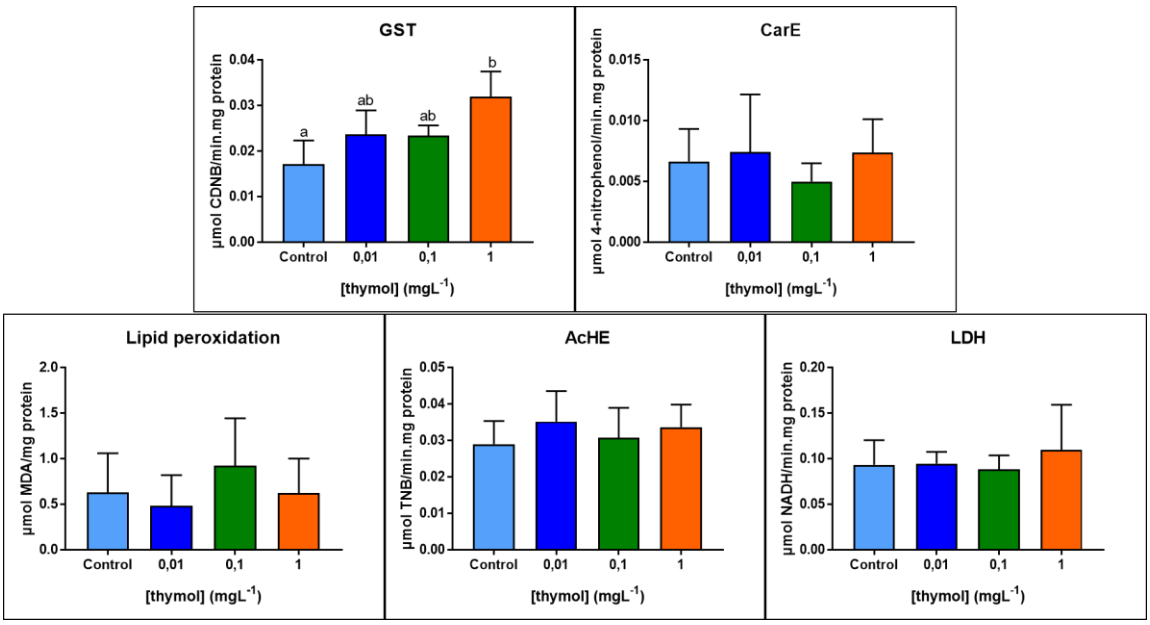

Figure 51 – Glutathione-s-transferase (GST), carboxylesterase (CarE) activity, lipidic peroxidation levels , acetylcholinesterase (AChE) activity, lactate dehydrogenase (LHD) activity in zebrafish embryos exposed to thymol. Data from at least five independent replicates of 100 animals each. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Nonparametric data statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p<0.05$).

Chapter X

Discussion

Extract of *Equisetum arvence*, extract of *Mimosa tenuiflora* and Thymol

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The objective of the present study was to evaluate the effects of Thymol as well as the extracts from *Equisetum arvense* and *Mimosa tenuiflora* in zebrafish embryos exposed to different concentrations during a 96h period. Exposure to the concentration of 0.0625 mgL^{-1} of *Equisetum* caused a decrease in the spontaneous movements and heartbeat. In addition, considering the morphological parameters, exposure to this concentration also caused a decrease in the body length and an increase in the area of yolk sac. Thymol and *Mimosa tunuiflora* had no effect on the sublethal and morphological parameters. At the biochemical parameters, thymol showed an increase in the GPx and GR activity, GSH levels, GSH/GSSG ratio and in the GST activity. The exposure to *Equisetum* caused an increase in SOD and GR activity and a decrease in the GPx activity. *Mimosa tunuiflora* did not show changes in the evaluated biochemical parameters.

According to the OECD protocol No 236 the first step in a toxicological assay is on the calculation of the LC_{50}. This protocol is useful to identify the mode of action of a substance and also helps to compare the dose response among various chemical substances. The 96h-LC_{50} tests are conducted to assess the vulnerability (every 24h) and survival potential of organisms in the presence of a compound. At the end of the exposure period, acute toxicity is determined based on a positive outcome in any of the four apical observations recorded, and the LC_{50} is calculated (Guidelines 2013). In this study, the values obtained for the 96h-LC_{50} were 32.67 mgL^{-1} for thymol, 435.31 mgL^{-1} for *Equisetum arvense* and 123.87 mgL^{-1} for *Mimosa tenuiflora*. Considering these LC_{50} values, three concentrations were selected: 0.01, 0.1 and 1 mgL^{-1} for thymol, 0.00625, 0.0625 and 0.625 mgL^{-1} for *Equisetum arvense* and 0.008, 0.08 and 0.8 mgL^{-1} for *Mimosa tenuiflora,* and the animals were exposed during a period of 96 hours with development changes being recorded at 24, 48, 72 and 96h.

Zebrafish embryos possess a very strong locomotive behavior which can be a useful tool to study the neuromuscular function (Goody *et al.,* 2012). Several key time points in the behavioral development of zebrafish have been described. Zebrafish embryos start to show spontaneous movements of the trunk and tail at 17 hours postfertilization (hpf) (Pietri *et al.,* 2009; Saint-Amant and Drapeau 1998). After hatching, around 72 hpf, they start to show movements more complex behaviors (McKeown, Downes, and Hutson 2009). As shown in previous studies, the spontaneous movements are due to uncontrolled action potential in motorneurons. Compounds are known to be involved in the reduction of the spontaneous movement frequency by limiting the spreading of the action potential and consequently the myotomal contraction rate through the blockage of membrane

Na^+ channel (Fraysse, Mons, and Garric 2006). Although, Jin *et al.,* (2009) suggested that an increased spontaneous movements frequency is associated to the prolongation channel opening to cause repetitive firing of action potentials, taking in consideration the results obtained, i.e., the decrease in movements observed for the concentrations of 0.0625 mgL^{-1} of Equisetum, may be explained by the decrease in the opening time of the Na^+ channels for some constituent present in the extract tested. Still, further tests are needed to support this hypothesis namely through the evaluation of Na^+ channels.

In addition, exposure to *Equisetum* extract induced changes in the heartbeat of zebrafish. The heartbeat is an important development parameter (Lin, Hui, and Cheng 2007; Mersereau *et al.,* 2015) and previous studies relate AChE inhibitors with a decreased heartbeat due to the direct binding acetylcholine to muscarinic acetylcholine receptors on the sino-atrial node, affecting nitric oxide synthesis by endothelial nitric oxide synthase as well as subsequent intracellular signaling (Lin, Hui, and Cheng 2007). As a result, potassium ion channel function and action potential development are altered (Herring, Danson, and Paterson 2002; Massion *et al.,* 2003) resulting in a slower heart rate. In this study it was observed a decrease in heartbeat in relation to the control at the concentration of 0.0625 mgL^{-1} of *Equisetum* extract, but no alterations on AChE activity. Still, further studies of cardiovascular effects are required to corroborate this hypothesis.

Moreover, zebrafish exposed to the concentration of 0.0625 mgL^{-1} of *Equisetum* extract had a decrease in body length. This can occur in animals exposed to synthetic fungicides, such as difenoconazole, has already described, and may be associated to decreases in growth hormone (GH) and insulin-like growth factor 1 (*IGF-1*) (Mu *et al.,* 2016) (Liu *et al.,* 2014) (Meganathan *et al.,* 2015; Colao *et al.,* 2006). Still, to what extent does this situation actually represents the outcome to natural compounds exposure deserves further investigation. Thymol and *Mimosa tenuiflora* extract did not present changes in the parameters previously mentioned.

Moreover, biochemical parameters were also analyzed to complete the data of developmental toxicity. Studies suggest that natural compounds have anti-oxidants proprieties (Yu *et al.,* 2016; Cetojevic-Simin *et al.,* 2010). In this study, exposure to natural compounds did not cause a significant increase in ROS at any of the concentrations assessed, which may be related to the anti-oxidant capacity presented by these compounds. Still, an increase in SOD activity for all concentrations of *Equisetum* extract was observed, that can be because there will be other reactive species that have not been measured and that may be influencing the results causing the increase of the

enzymes. This compound also induced an increase in GR activity for the concentration of 0.00625 mgL^{-1} and 0.0625 mgL^{-1}, consequently the levels of GSH and GSSG were decrease at lower concentration of *Equisetum* extract. Thymol also showed an increase in GR and GPx activity for the concentration of 0.01 mgL^{-1} and an increase in GSH level. Low levels of GSH mean that there is no oxidation/reduction, process performed by GPx and GR enzymes, so this radical elimination pathway was affected by this compound concentration. The glutathione system and related enzymes are considered a second line of defense against oxidative damage (Jafari 2007) being the ratio of GSH to GSSG often used as an indicator of intracellular redox status (Timme-Laragy *et al.,* 2013). The GSH activity are important for the maintenance of the redox homeostasis in organism cells (Timme-Laragy *et al.,* 2013; Massarsky, Kozal, and Di Giulio 2017). Other glutathione is GST, an enzyme that collaborates in the reactions of the first phase of detoxification of xenobiotic (Barata, Solayan, and Porte 2004; Andrade *et al.,* 2016). Moreover, GST might contribute to the elimination of superoxide radicals caused by oxidative stress (Jiang *et al.,* 2018). The decrease of GSH and GSSG contents might be a combined impact of increase in GR activity. This fact such as the increase in SOD and GST may be associated with the anti-oxidant properties of natural compound (Tabti *et al.,* 2014; Kalidindi *et al.,* 2015; Sakka Rouis-Soussi *et al.,* 2014). Santo *et al.,* (2018) showed that *Uncaria tomentosa extract* can increase a SOD level in zebrafish adult liver. However, Zhang *et al.,* (2017) through the analysis of oxidative stress-related enzyme genes and other parameters, found that Quercetin may have deregulatory effects on mitochondria and consequently increased oxidative stress in zebrafish larvae. Still further studies are required to prove such effects.

LPO, an index for measuring membrane damage, occurs with an increase in hydroxyl radical production (Ganesan *et al.,* 2016; Blokhina, Virolainen, and Fagerstedt 2003). ROS increases the lipid peroxides causing a disruption in the lipid integrity of the membranes (Radi and Matkovics 1988). Such deterioration of membrane lipids is a prominent marker of oxidative damage (Sayeed *et al.,* 2003). The concentration of 0.00625 mgL^{-1} and 0.625 mgL^{-1} showed a decrease in lipidic peroxidation. This may be due to increased SOD, GPx and GR, thus reducing the impact of ROS.

In summary, exposure to *Mimosa tenuiflora* does not affect the embryonic development of zebrafish. *Equisetum arvense* caused a decrease in the spontaneous movements and in the heart beats, induced an increase in the activity of antioxidant enzymes against the oxidative stress caused by the increase of ROS. Thymol does not

interfere at the morphological level in the embryonic development however it induced an increase in the anti-oxidant defenses. However, pathways how can that natural compound increase the anti-oxidant defenses that deserve further investigation. In addition, further studies are needed to fully support the hypothesis presented and to clarify the underlying toxicological mechanisms, e.g. evaluation of the gene expression relative to the antioxidant enzymes, in order to determine whether that the animals exposed to these compounds really had an anti-oxidant defenses increase.

Chapter XI

Conclusion

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

The purpose of this study was to evaluate the effects of various synthetic (azoxystrobin; tebuconazole; mancozeb) and natural fungicides (thymol, extract of *Equisetum* and extract of *Mimosa tenuiflora*) on zebrafish embryo development, used as toxicological model.

At the level of LC_{50}, natural fungicides presented higher values which, in toxicological terms, seems to be favorable compared to the synthetic compounds. However, considering the LC_{50} and the values applied and those found in the environment, all synthetic compounds tested had a good margin of safety. Still, although natural compounds have higher LC_{50} values, it is not advised that they be exceeded due to the complications that may arise from their use and possible accumulation.

The evaluation of lethal, sub-lethal and teratogenic parameters during zebrafish embryonic development showed that mancozeb is the one that stands out, as it causes a high inhibition of hatching and a high rate of malformations as well as the reduction of other important parameters such as the heart beats. Azoxystrobin also causes malformations at the highest dose tested, however with less incidence and severity than mancozeb. There were no significant differences in the embryonic development of zebrafish exposed to tebuconazole, *Equisetum arvense* extract, *Mimosa tenuiflora* extract and thymol.

After performing the biochemical analyzes to evaluate oxidative-related parameters it can be concluded that the compound that presents a greater concern in terms of oxidative stress is azoxystrobin, due to the sharp increase of the ROS at the lowest concentration and consequent increases of the enzymes responsible for the control of these reactive species. It is also the one that presents greater cellular damage (LPO) and the most significant alterations in the AChE level which may imply changes at the neuronal level. In addition, other processes controlled by this enzyme may be affected. The enzymes responsible for the xenobiotic biotransformation have also a higher activity in the animals exposed to azoxystrobin, which can occur due to the structure of the compound. The remaining synthetic and natural compounds, although showing an increase in some of the aforementioned parameters, were not very relevant in view of the difference with the control group. Overall, this data shows that the use of natural fungicides may be an alternative to the use of the synthetic fungicides tested in terms of the evaluated parameters.

However, future pharmacokinetic studies should be performed to evaluate and characterize how these compounds act at the level of the organism, in order to prove some

of the data obtained and to associate the effects with the respective compound/mechanism of action. Evaluation of the expression of genes related to oxidative stress (eg. *cat*, c*u/zn sod* and others), to development (eg. *EF1α*) and genes related to possible malformations in the notochord (eg. floating head (*flh*), no tail (*ntl*) and *doc*), as well as to evaluate the expression of proteins related to these parameters by western blot, among other possible techniques. Moreover, the analysis of animal behavior may uncover other neurotoxicological effects that were not visible at the physiological and organism level.

Chapter XII

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Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

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Chapter XIII

Appendice I

Effects of synthetic and plant-based fungicides on biomarkers in the early stages of zebrafish development

1. Azoxystrobin 96h-LC_{50}

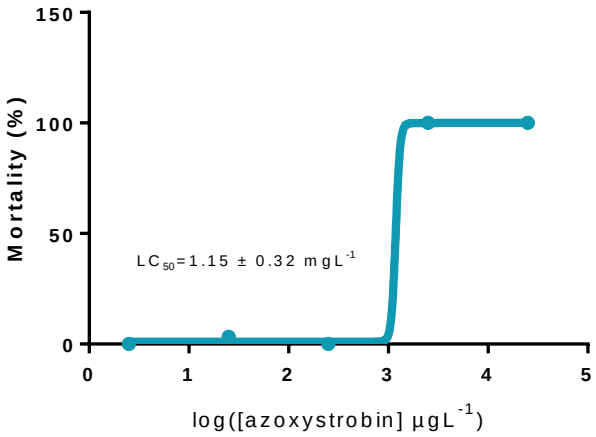

Figure 52 - Dose–response curve of mortality of zebrafish following 96h exposure to azoxystrobin. Log-transformed azoxystrobin concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 1.15 mgL^{-1} with the 95% confidence interval ranging from 0.83 to 1.47 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.

2. Mancozeb 96h-LC_{50}

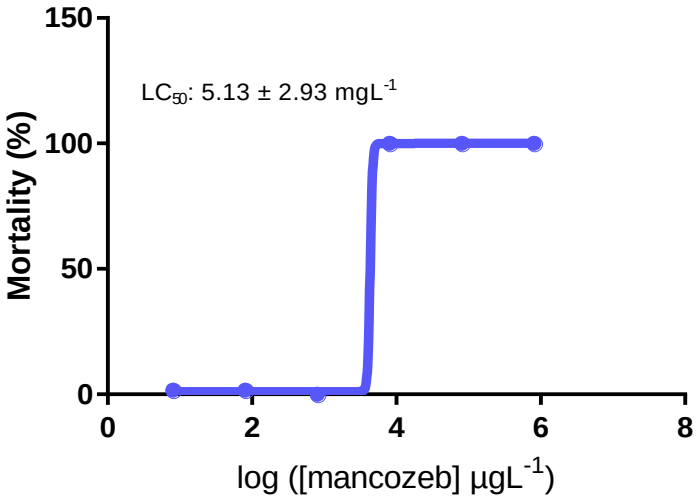

Figure 53 - Dose–response curve of mortality of zebrafish following 96h exposure to mancozeb. Log-transformed mancozeb concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 5.13 mgL^{-1} with the 95% confidence interval ranging from 2.20 to 8.06 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.

3. Tebuconazole 96h-LC_{50}

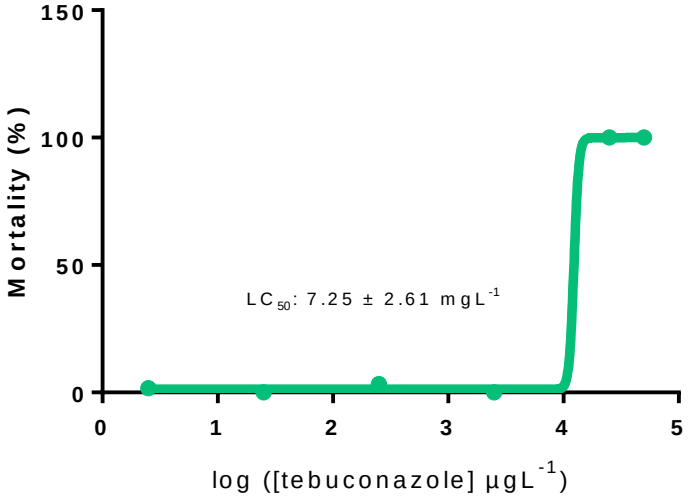

Figure 54 - Dose–response curve of mortality of zebrafish following 96h exposure to tebuconazole. Log-transformed tebuconazole concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 7.25 mgL^{-1} with the 95% confidence interval ranging from 4.64 to 9.86 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.

4. *Equisetum arvense* 96h-LC_{50}

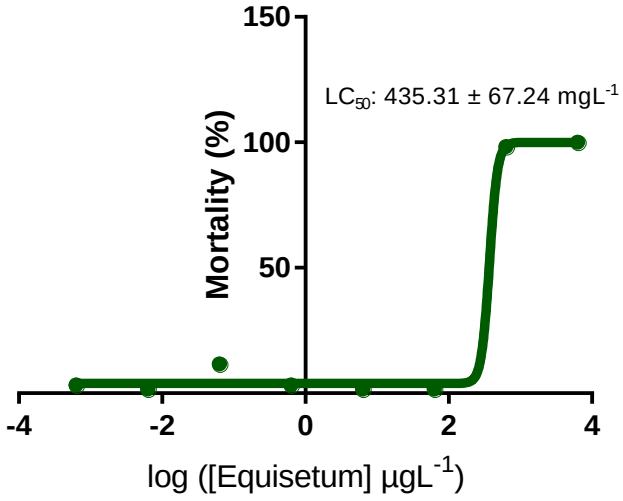

Figure 55 - Dose–response curve of mortality of zebrafish following 96h exposure to *Equisetum arvense* extract. Log-transformed *Equisetum arvense* extract concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 435.31 mgL^{-1} with the 95% confidence interval ranging from 368.07 to 502.55 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.

5. *Mimosa tunuiflora* 96h-LC_{50}

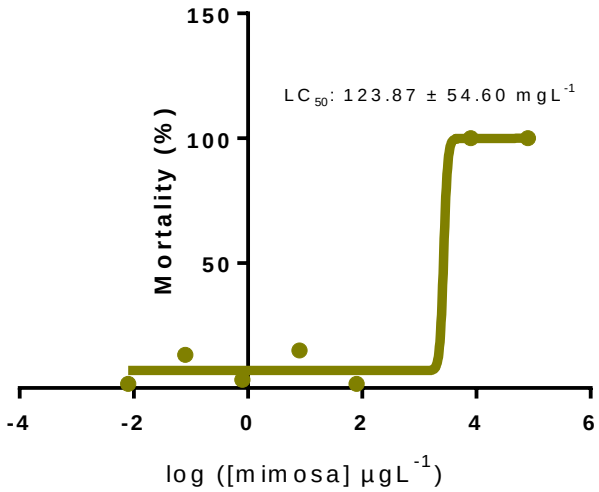

Figure 56- Dose–response curve of mortality of zebrafish following 96h exposure to *Mimosa tunuiflora* extract. Log-transformed *Mimosa tunuiflora* extract concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 1.15 mgL^{-1} with the 95% confidence interval ranging from 0.83 to 1.47 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.

6. Thymol 96h-LC_{50}

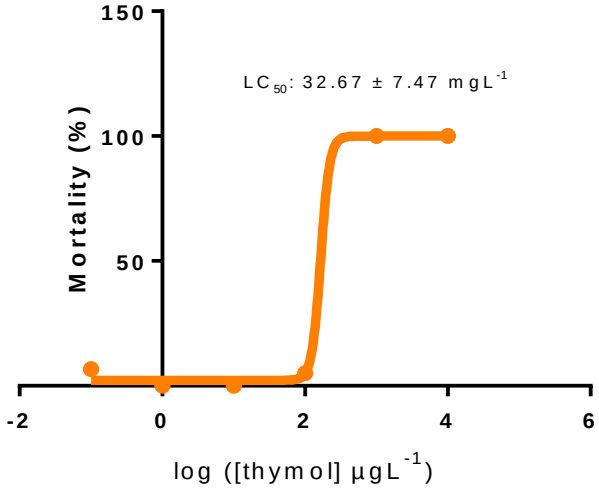

Figure 57 - Dose–response curve of mortality of zebrafish following 96h exposure to thymol. Log-transformed thymol concentrations are plotted on the x axis in function of the mortality, considered the correction of control mortality. The 96-h lethal concentration (LC_{50}) value of azoxystrobin was 1.15 mgL^{-1} with the 95% confidence interval ranging from 0.83 to 1.47 mgL^{-1}. The values are presented as mean ± SD from three independent replicate exposures.