Pesticides Induce Oxidative Stress in Zebrafish Embryo

Kjetil Løtveit Thorstensen

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Institute of Molecular Biology
University of Bergen
NIFES

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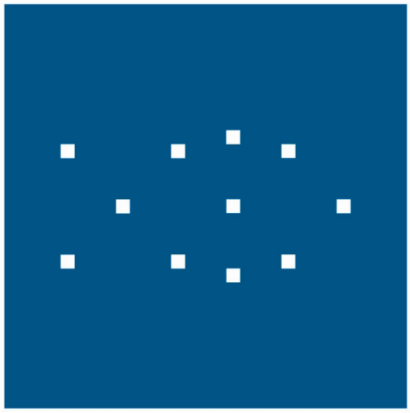

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Abbreviations

ADI – Accepted daily intake
CAT - Catalase
CPF – Chlorpyrifos
DMSO – Dimethyl Sulfoxide
DPF – Days post fertilization
ESF – Endosulfan
GPx – Glutathione peroxidase
GSH – Glutathione
GSSG – Glutathione disulfide
GST – Glutathione S-transferase
HPF – Hours post fertilization
LOAEL – Lowest observed adverse effects level
MNE – Mean Normalized Expression
NOAEL – No observed adverse effects level
ROS – Reactive oxygen species
SOD – Superoxide dismutase

1. Abstract

Aquaculture is an industry in rapid global growth. The increased demand and therefore production of aquacultural goods is bound to drain natural marine resources if fish are to continue being fed feeds based solely on marine products. To compensate for the lack of traditional marine based feed, a mixture of agricultural and marine based feed can be used, replacing some of the fish oil with vegetable oil. However, the vegetable feed may include traces of pollutants such as pesticides commonly used in agriculture. Several pesticides are more toxic to marine animals than to terrestrial animals, and bioaccumulate to a higher degree in marine animals. Thus, introduction of pesticides through fish feed can have negative effects for both the fish and consumers of aquaculture products. Some of the most used pesticides, such as endosulfan (ESF) and chlorpyrifos (CPF) are neurotoxins designed to kill insects by interacting with their nervous system. Several pesticides also have a secondary toxic ability to induce reactive oxygen species (ROS), which can lead to oxidative stress in the fish.

To gain more knowledge about the ROS inducing effects of ESF and CPF, toxicology tests were done using embryos of zebrafish (*Danio rerio*) as a model system. From a 6 hours post fertilization (hpf) to 72 hpf dose response test, the lethal dosage was found to be 500,000 µg/L ESF and 20,000 µg/L CPF. A 14 days post fertilization survival test using the exposure time from 6 hpf – 72 hpf, indicated 50,000 µg/L ESF being a lethal concentration. ESF caused negative effects, such as malformations, late hatching and reduced activity in concentrations above 20,000 µg/L and all of these were dead by day 14. CPF exposure did not seem to affect development at concentrations up to 10,000 µg/L. H_2DCFDA can be used to determine ROS generation in living cells. H_2DCFDA freely diffuse into exposed cells and will produce an increasing green fluorescence signal with increasing levels of ROS in the cells. ESF caused an increasing fluorescence gradient from 10,000 µg/L to 50,000 µg/L. This increase was also seen in CPF, but the signal was much weaker and harder to differentiate. qPCR on isolated RNA from exposed embryos indicated that ESF had mostly a downregulatory effect on genes related to antioxidants and the gene *cyp1a1*, but might stimulate *vtg1* expression at high levels. CPF had mostly stable expression, except for *gclc*, which had a significant upregulation. ESF induces more ROS than CPF at high concentrations, but CPF is more acutely lethal then ESF.

2. Introduction

2.1 Aquaculture and food safety

Aquaculture is one of the fastest growing markets in the food sectors, and its products are important export items for several countries (Hempel et al., 1999). For example, in 2013 Norway produced 1,184,631 tons of Salmon (*Salmo salar*) and Rainbow Trout (*Salmo gairdueri*), while in 2005 it was 605,327 tons (Fiskeridirektoratet, 26.11.09). The quality of the fish is important for both sales value and consumer food safety. Important factors within the food safety of fish are water quality and environmental factors. Environmental factors include biohazard risks (parasites, bacteria and viruses), chemical hazards (agrochemicals and chemotherapeutants), metals, feed ingredients/additives and organic pollutants (WHO, 2000, Tacon and Metian, 2008). It is difficult to control all of the factors named above in the open sea closely, therefore particular focus has to be paid to the quality control of the final product.

Biohazard risks can be debilitating for humans, as several microorganisms present in seafood are pathogenic (WHO, 2000). Most of these biohazards however can be reduced with parasiticides and antimicrobial treatments. The use of these chemicals is controversial since an increase in microbial resistance to the different compounds may be introduced, and worse, may be carried over to reducing the efficiency of pharmaceuticals in animals and humans (Quesada et al., 2013, WHO, 2000).

Another major concern to public health is heavy metals and arsenic as they can disrupt normal reproduction and early development. There are also other possible pollutants including oil from oil spills, paint and cleaning agents from boats and general littering. Recently, an increasing number of agrochemicals have been detected in the seafood web, which by definition are a broad range of chemicals that affect water quality such as pH, fertilizers, disinfectants and pesticides (WHO, 2000). Most important for the present work however, is the fact that many chemicals are introduced directly to cultured food fish through the fish feed, since many aquaculture diets have been shown to contain traces of pollutants (Petri et al., 2006).

2.2 Agricultural feed

In 2013, Norway used 1,532,809 tons of fish feed (Fiskeridirektoratet, 26.11.09). Fish not used for human consumption, known as “industry fish” or small pelagic fish, have traditionally been preferred as the base for the feed due to naturally high amounts of omega-3. These fish have also been used to make fish oil and fish meal (Fiskeridirektoratet, 2009, Torstensen et al., 2008). However, with the large amounts of fish being cultured, marine feed would be needed in growing amounts and this trend will eventually lead to a draining of the resources (Glover et al., 2007). Agricultural based feed is therefore increasingly used to compensate for the lack of marine feed. The fish oil in the marine feed is partly exchanged with vegetable oils (Torstensen et al., 2008). This will induce a difference in the cultured fish due to the lack of omega-3-fatty acid chains and introduction of plant specific proteins such as phytoestrogens and saponins (Krøvel et al., 2010), but may not have an overall negative effect on the fish itself. In fact, Torstensen et al. (2005) found that 100% vegetable oil seemed to increase the salmons weight and protein utilization. Replacing the fish meal does not seem to have the same effect, and seems to reduce the feed intake (Espe et al., 2006). The presence of vegetable oils however may contain traces of pollutants not normally found in the marine feed. The United States Department of Agriculture annually runs several tests on a range of food products, to determine traces of pesticide. In 2012, they tested 10,801 fruit and vegetable products; of these, 47.4 % were clean, 23.6 % had traces of one pesticide and 29 % had two or more pesticides (PDP-Program, 2012). Thus, fish feed containing vegetable oils is likely to carry traces of pesticides.

2.3 Pesticides

A pesticide is a substance or mix intending to prevent, destroy, repel or mitigate a pest, wherein a pest is an organism that is unwanted or can hurt animals, plants or humans (Lah, 2009). The use of pesticides started after the Second World War, and is widely used all over the world in large amounts (Osteen and Fernandez-Cornejo, 2013). It is estimated that 45% of food made annually is lost to pests (Abhilash and Singh, 2009), thus pesticides are essential for growing enough food to support the world’s rapidly growing population. Pesticides are also used to keep people safe from diseases carried by insects such as malaria, dengue fever and human sleeping sickness.

Pesticides are divided to several groups depending on target organism. Insecticides are specifically catered to target insects, and often include ovicides and larvicides which will kill

insect eggs and larvae, respectively (EPA, 2012). Misuse and overuse of pesticides has led to increased resistance, requiring new or stronger concentrations of the pesticides. Most pesticides are general purpose and do not target a specific kind of organism. This may cause issues for animals with habitats close to crops, and may especially affect bodies of water. Most pesticides are harmful for humans and animals (Hernández et al., 2013, Abhilash and Singh, 2009), and the use of pesticides is despite the above named benefits still a very controversial subject. Pesticides are designed to harm, so bioaccumulation can induce sickness, alterations in physiology or have fatal consequences. Some pesticides mimic hormones and may affect the hormone balance of exposed organisms. These substances, also referred to as endocrine disruptors, may affect growth and sex determination and may cause cancer and developmental disorders (Uggini et al., 2012, WHO, 2002, Hernández et al., 2013).

2.4 Toxicology of pesticides

Pesticides work by interfering with biological mechanisms in the pests. To understand better the toxic effects of pesticides, knowledge about target mechanisms, metabolic pathways, chemical interactions and toxicokinetics is needed (Hernández et al., 2013). Exposure rate is a major factor determining the toxicity of a pesticide to an organism. Within toxicology, acute exposure is an exposure of two weeks or less in duration (often less than 24 hours), while chronic exposure is a continued exposure occurring over an extended period of time (usually from weeks to years), or a significant fraction of the test species life-time (Mergel, 2009). There are also categories such as sub-acute and sub-chronic exposure, where exposure happens several times over a set period, either as once a day or for longer times during some months or years. Another main toxicity factor is the exposure route. Ingestion, inhalation or skin absorption will affect how critical the pesticide exposure would be. Normally, ingestion and inhalation would have more adverse effects than skin contact (Monosson, 2008). The last factors depend on the organism's individual characteristics such as species, health, age, sex and environment, but also the concentration of the pesticide and the type of pesticide. LC50 is a value within toxicology that indicates the lethal concentration where 50% of the test population is dead. In the same way, EC50 is the effective concentration where at least one effect is happening to 50% of the population tested. There is also NOAEL and LOAEL which mean the "No Observed Adverse Effect Level" and "Lowest Observed Adverse Effect Level" (Mergel, 2009). Most of the insecticides are neurotoxins that will kill insects by affecting the nervous system by inhibiting vital signalization within or between neurons. These neurotoxins are not target specific. There is a

big concern about the pesticides affecting animal and human development of the brain (Bjørling-Poulsen et al., 2008). Several studies also show induction of ROS from insecticides. The insecticides leads to increased lipid peroxidation due to increased ROS levels and have several antioxidants level reduced (Gupta, 2011, Jia and Misra, 2007).

2.5 Endosulfan

Endosulfan (6,7,8,9,10,10–hexachloro–1,5,5a,6,9,9a–hexahydro–6,9–methano–2,4,3–benzodioxathiepin–3–oxide) is an insecticide and a water insoluble organochlorine mixture with two isomers α- and β-endosulfan in ratios of 2:1 to 7:3 when used in crops (Silva and Gammon, 2009, Stanley et al., 2009, Han et al., 2011). The α-isomer is more toxic to insects and mammals than the β-isomer (Li and Macdonald, 2005).

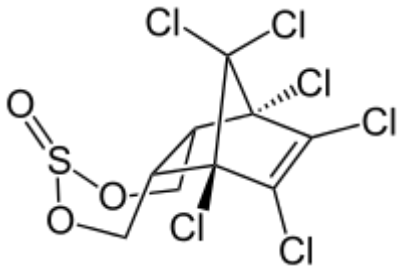

Figure 2.1: The structure of endosulfan (figure from Wikipedia).

Endosulfan (ESF) has been globally used since the 1950's, with USA starting in 1954. In April 2011 a globally ban was determined at the Stockholm Convention, suggesting ESF to be phased out within 5 years. Its efficiency against mites and insects is used for the protection of several kinds of crops, such as cotton, fruit, coffee, tea, vegetables and also as a wood conservative and as means against the tsetse fly in Africa (Weber et al., 2010, Hempel et al., 1999, Naqvi and Vaishnavi, 1993). From the 1950's to 2000, a cumulative of 338 kilotons is estimated to have been used worldwide. 113 of them was from India alone, while USA was the second largest user with 26 kilotons (Li and Macdonald, 2005). According to WHO, the ADI for ESF in humans was about 0.006 µg/kg or 20 µg/L, assuming a 60 kg adult drinking 2 liters daily for two years (WHO, 2004b). The acceptable limit in feed in the EU is 1 mg/kg. Food contaminants with ESF are the main reason for human exposure. Particularly susceptible to ESF are the unborn and neonates, the elderly and people with liver, kidney, immunodeficiency or neurological diseases (Naqvi and Vaishnavi, 1993).

Fish is also very sensitive to this pesticide and several incidences have occurred where ESF pollution has killed large amounts of fish by leakage. The lipophilic nature of ESF enables it to be deposited in fatty tissue, liver and kidney, though ESF is mostly metabolized and excreted fast in human bodies. In marine animals, ESF seems to have a larger bio accumulative potential (Naqvi and Vaishnavi, 1993). ESF usually breaks down to a diol in water through hydrolysis or a sulfate in soil or sediments through oxidation. ESF Sulfate is toxic and can last for years in water (Stanley et al., 2009). ESF is within the chemical group

cyclodienes. Cyclodienes are potent inhibitors of Na^+/K^+ and Ca^{2+}/Mg^{2+} ATPase, essential for transport of ions across membranes. More specifically in neurotoxicity, ESF is a non-competitive GABA antagonist, which means that it will bind to the Cl^- channels linked to the γ-amino-butyric acid ($GABA_A$) receptor and blocks it (Silva and Gammon, 2009, Dorval et al., 2003). ESF is also a known endocrine disruptor and may therefore affect hormone homeostasis (Dorval et al., 2003).

2.6 Chlorpyrifos

The organophosphate Chlorpyrifos (O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothioate) is a broad-spectrum insecticide. Chlorpyrifos (CPF) kills insects upon contact by affecting the normal function of the nervous system.

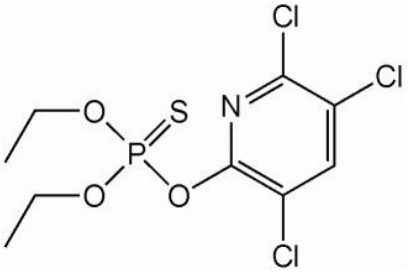

Figure 2.2: The structure of chlorpyrifos (figure from Wikipedia).

It is the most used pesticide in Europe without particular restrictions, but Dow AgroSciences, the major manufacturer of chlorpyrifos, began phasing the chemical out in 2006. In the United States it is restricted to outdoor appliances such as crops and golf courses. CPF has replaced several organochlorides and carbamates as it is a very effective pesticide with little persistence in the environment. (EPA, 2006, Bernabò et al., 2011, EUROSTAT, 2007, Eaton et al., 2008). CPF was introduced into the market in 1965 and has become the second largest selling organophosphate in the world (Deb and Das, 2013).

There have been several studies that claim that CPF affects the nervous system, especially during development, and is believed to increase the risk of attention deficit hyperactivity disorder (ADHD) and other neurological symptoms (Sledge et al., 2011, Saulsbury et al., 2009). According to EPA and WHO, the maximum tolerated concentration is 30 µg/L in water, while the ADI should be less than 0.01 mg/kg/day (WHO, 2004a). This pesticide is insoluble in water, and it is more toxic to fish when compared to organochlorine compounds. CPF directly interacts with the acetylcholine (AChE) receptors, can interfere with signaling cascades from cell surface to intracellular events and it elicit oxidative stress (Slotkin et al., 2007). For CPF to be an active oxidative agent, it needs to be metabolized. CYP2B6 of the cytochrome p450 complex will replace the sulfur group with an oxygen atom, making CPF into chlorpyrifos-oxon (Costa, 2006). The direct interaction with AChE receptors is due to the thiophosphate backbone being metabolized by cytochrome p450, which then becomes an inhibitor of the receptors (Yen et al., 2011). This will lead to hyperstimulation of AChE and

can induce several physiological damages, behavior impairment and death (Tilton et al., 2011).

2.7 Pesticide induced ROS damage

Several pesticides induce reactive oxygen species (ROS) damage, also known as oxidative stress (Abdollahi et al., 2004, Deb and Das, 2013). In addition to the target mechanisms of the pesticide the ROS damage will induce a secondary toxic effect (Abdollahi et al., 2004). ROS are chemically reactive ions, radicals or molecules with a free oxygen that may interact with proteins, nucleic acids, lipids or other molecules through oxidation. With a protein or nucleic acid interaction, this will induce a conformation change in the structure and charge of the protein or nucleic acids. This would alter its properties and therefore may disrupt its function or reading frames. DNA repair will most probably fix damaged DNA, but at times damage will occur and can lead to mutagenesis and cancer (Fridovich, 2001). Oxidation from ROS occurs naturally in the cell at several pathways, for instance in signalizing and for homeostasis, especially in the mitochondria (Fridovich, 2001, Morel and Barouki, 1998).

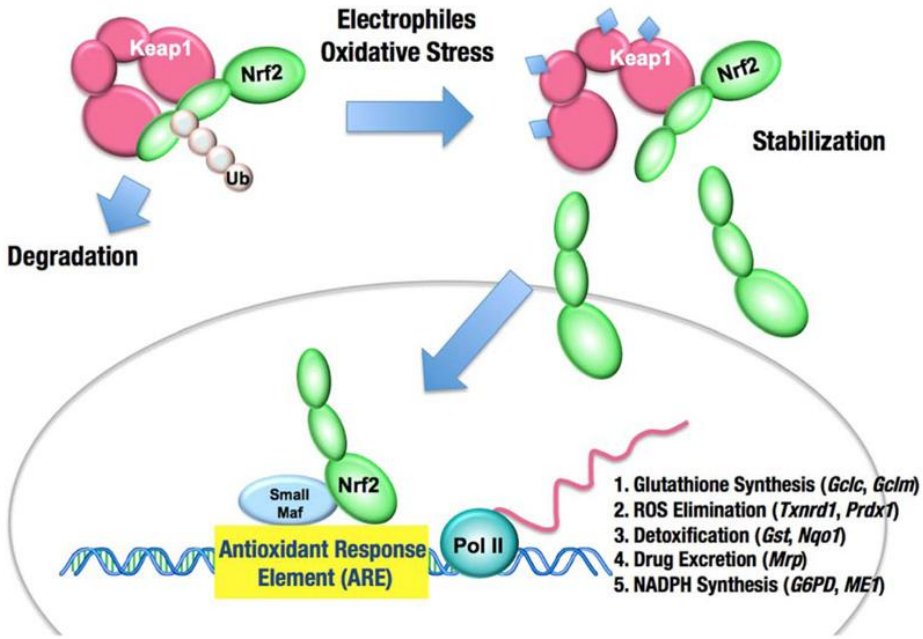

Figure 2.3: The Keap1–Nrf2 system. Under normal conditions, Nrf2 is constantly ubiquitinated through Keap1 and rapidly degraded in the proteasome. Electrophiles or oxidative stress inactivates Keap1 by oxidation of free cysteins on Keap1 and releases Nrf2. Nrf2 accumulates in the nucleus and activates many cytoprotective genes. Figure is from (Mitsuishi et al., 2012).

Oxidative stress occurs when the cell reaches an imbalance of ROS and potential detoxifiers of intermediates. This can occur by an increase in ROS, damage of antioxidant defense systems or the incapacity of oxidative damage repair. The body has developed several

countermeasures for oxidative stress. Some of them are the proteins superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx), which basically work by neutralizing superoxides, hydroxyl radicals and H_2O_2, by turning them into water (Shao et al., 2012) (see figure 2.4). The expression of several of these genes is regulated by the transcription factor Nrf2, which is normally bound to Keap1. Keap1 inhibits Nrf2, leading the complex to degradation as long as Keap1 is ubiquitinated (see figure 2.3). Keap1 has several cysteines, which upon contact with ROS will be oxidized and release Nrf2, enabling it to activate antioxidant-response elements (ARE) (Harvey et al., 2009, Mitsuishi et al., 2012). The body can cope with already induced ROS damage, through means of repair mechanisms such as DNA repair. If the damage is too great, it can greatly affect the hormone biosynthesis and/or immune system and will probably lead to cell death either through apoptosis or necrosis (Sumimoto et al., 2005, Dunyaporn et al., 2008). Oxidative stress is involved in several diseases such as Alzheimer's Disease, Parkinson's Disease, cataracts, atherosclerosis, neoplastic diseases, diabetes, chronic inflammatory diseases of the gastrointestinal tract, aging of skin and asthma (Abdollahi et al., 2004).

2.8 Glutathione peroxidase

Glutathione (GSH) is an important factor in cellular defense against ROS, as it nonenzymatically gathers both oxygen and hydroxyl radicals, and is utilized by glutathione peroxidase GPx and GST to limit the levels of reactive aldehydes and peroxides within the cell (Cole et al., 2011). The SOD family plays an important part of making hydrogen peroxide from free radicals, which the GPx then will convert to water with the help of selenium (Irwin, 2014). The SOD family uses different cofactors depending on the location in the cell, with for instance CuZn-SOD in the cytosol and Mn-SOD in the mitochondria (Lubos et al., 2011). GPxs are a family of enzymes, where GPx-1 is the most abundant in mammals. It is present in all cells, in both the cytosol and mitochondria and in some cases even in the peroxisomal space. According to the Entrez gene pages, there are eight known GPxs in mammals and according to ZFIN; it is believed to be eight in zebrafish as well. Some of the zebrafish genes seem more likely to be duplications of itself when comparing homology, as they are closer to each other than the human homologs. These are GPx1a - GPx1b and GPx4a - GPx4b. GPx4 in humans is widely expressed, but has a different substrate specificity than the other family members (Lubos et al., 2011, Entrez, Kryukov and Gladyshev, 2000).

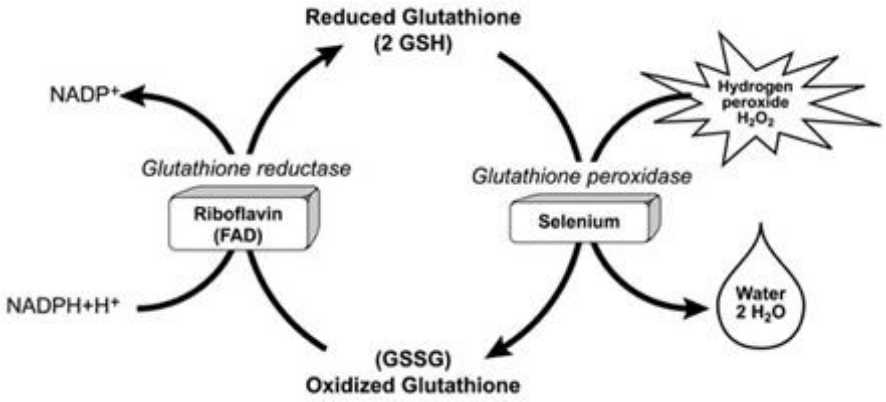

Figure 2.4: One molecule of hydrogen peroxide is reduced to two molecules of water, while two molecules of glutathione (GSH) are oxidized in a reaction catalyzed by the selenoenzyme, glutathione peroxidase. Oxidized glutathione may be reduced by the flavin adenine dinucleotide (FAD)-dependent enzyme, glutathione reductase. *(Figure from Linus Pauling institute, University of Oregon)*

GPx1 and GPx4 are both selenoproteins and reduce H_2O_2 to water, oxidizing GSH to glutathione disulfide (GSSG) (figure 2.4). The rate of GPx is affected by the rate of synthesis of GSH and the prevailing oxidative state, as well as of the export of GSH and GSSG from the cell. Most cell types do not import GSH, but produce GSH themselves. GSH synthesis is ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase and GSH synthetase. The GCLholo, a complex with glutathione cysteine ligase catalytic (GCLC) and glutathione cysteine ligase modifier (GCLM), does the first step of GSH synthesis. The rate of GSH synthesis is mainly influenced by the availability of cysteine, but also by feedback inhibition of synthesis by GSH (Li et al., 2000, Chen et al., 2005).

2.9 Zebrafish as a model system

Zebrafish (*Danio rerio*) is a tropical fish endemic to shallow waters, rivers and paddles in south Asia. They are characterized due to their black stripes along the body, however several patterns, such as dots or spots, can be found in addition. Zebrafish is a popular aquarium fish as it is a quite sturdy fish and easy to take care of. Adults are usually around 2-4 cm. A female can spawn between 100-200 embryos within a week, the embryos have all major organs developed by 5 to 6 days and have reached maturation by about three months (Bailey et al., 2013, Basu and Sachidanandan, 2013). The ideal water temperature for zebrafish is about 28-29 ºC. The high number of embryos, short generation time and fast development has lead the zebrafish to become a popular genetic model organism. In addition, the relative recently established targeted gene knock-out technology adds to this model's attractiveness.

Zebrafish is also popular because the embryos are found outside of the mother's body and the fact that they are transparent and can continuously be kept transparent while they are embryos/larvae. Zebrafish is a very well studied organism and morphological, biochemical, and physiological information of the stages of early development and in juveniles and adults of both sexes are well known(Kimmel et al., 1995, Bailey et al., 2013, Dai et al., 2014, Howe et al., 2013, Hill et al., 2005).. Thus zebrafish is ideal for toxicology as adverse effects of chemical exposure can easily be determined and the zebrafish genome has been sequenced (Postlethwait, 2006). The wild types used in this experiment is the AB and TLF, so-called after George Streisinger purchased two lines, A and B, at different times from a pet shop in Albany, Oregon and the TLF (Tupfel long fin) from their long fins caused by a mutation. These two lines are quite easy to distinguish from each other as the AB line has short fins and black stripes along the body, while the TLF lines has, as mentioned, long fins and also spots along the body instead of stripes (Sprague, 2006).

2.10 Development stages

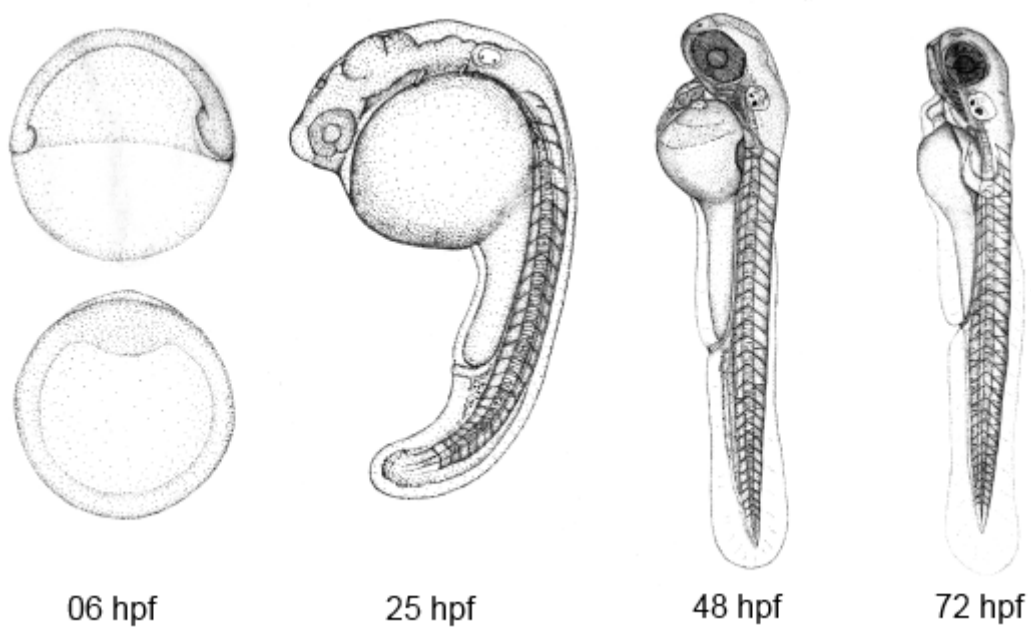

Figure 2.5: Selected stages of zebrafish development. The sketched forms is the stages mainly used/studied in this experiment. Pesticide exposure is at 6 hpf, and treatment of H_2DCFDA at 24 hpf, 48 hpf and 72 hpf. ***Modified from: Kimmel et al., 1995. Developmental Dynamics 203:253-310. Copyright © 1995 Wiley-Liss, Inc.***

The start of pesticide exposure began at 6 hours post fertilization (hpf) (first image of Figure 2,5). At 6 hpf, the embryo has developed from a one cell to a 64 cell embryo (at approximately 2 hpf) in the cleavage period, then goes into the blastula period (until

approximately 5.25 hpf) and has just started the gastrulation period, which will last until 10.3 hpf. In the blastula period, the embryos go through mid-blastula transition, which is when zygotic gene transcription occurs and epiboly starts. The gastrulation period marks completion of the epiboly and at 6 hpf, the shield has been made. There are many cell migrations occurring, making sure that the germ layers is in the right place, and the shield marks the dorsal side of the embryo, starting the determination of cells location and differentiation. After the gastrulation period, the segmentation period starts and lasts until about 24 hpf. In the segmentation period, primary organs start to develop, dermis, muscles, neural cord and notochord are forming and the body starts to elongate. Somites are being made along the trunk and tail. From 24 hpf to 48 hpf (sketch 2 and 3 in Figure 2.5), the embryo is in the pharyngula period. Here the embryos brain develops while the nervous system expands, body axis straightens and dorsal and ventral stripe are formed. It is also the point where the circulatory system is developed and the heartbeat starts for the first time. Between 48 hpf and 72 hpf is called the hatching period, where usually embryo hatches from the chorion. Primary organs continue to develop at this point and so do jaws, gills and fins, but fins are more elongated now. Its mouth is wide open, and hair cells have differentiated. Then the embryo enters the larvae period until it reaches about 30 days old. In this period, the development is still ongoing, the organs become more complex and its swim bladder is inflated (Kimmel et al., 1995).

2.11 H_2DCFDA, a fluorescent ROS dye

2',7'-Dichlorodihydrofluorescein diacetate (also called 2',7'-dichlorofluorescin diacetate) works by hydrolization of cellular esterases to dichlorodihydrofluorescein, which then will be oxidized to dichlorodifluorescein by oxidative factors (see figure 2.6). This method is mainly an *in vivo* detection technique of ROS damage in cells, where the cells with ROS damage will give a green color when the broad wavelength is between 440-600 nm (Hempel et al., 1999). It is a common method used on both eukaryote and prokaryote cells (Rastogi et al., 2010, Shen et al., 2013, Kristiansen et al., 2009). In this experiment, however, we will try to transfer this method to use it on whole zebrafish embryos to detect where the ROS damage is most likely to occur in the presence of the pesticides. This should be applicable considering that; 1) the dye is easily transferred in-between membranes and 2) the embryos transparency, which also can be kept longer with 1-phenyl 2-thiourea (PTU) in the solutions.

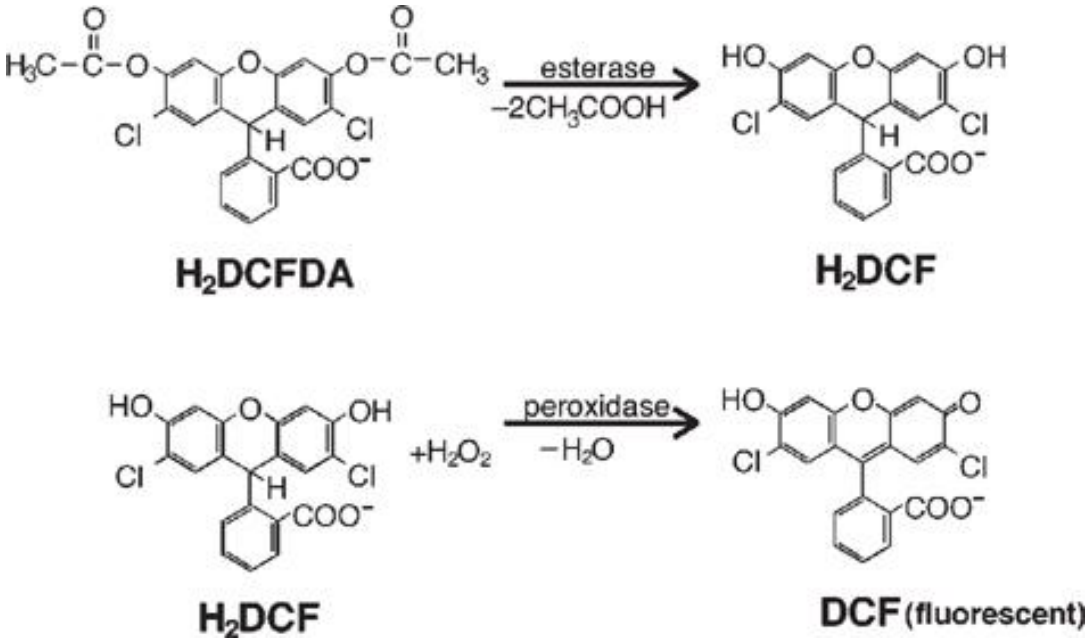

Figure 2.6: How H_2DCFDA is activated, using H_2O_2 as a terminal electron acceptor (Figure taken from (Hensley et al., 2003))

2.12 Aims and objectives

The aim of this study is to gain more knowledge about how ESF and CPF induces ROS toxicity and affects development. A dose-response test of the pesticides will be done to determine sub-lethal levels, indicating working concentrations. The survival rate after exposure will be analyzed to determine how development is affected. Pesticide induced ROS will be determined using a H_2DCFDA essay, which may show where in the embryos oxidative stress occurs. In addition, qPCR will be used to determine alteration of mRNA levels in genes related to ROS homeostasis.

3. Materials

Table 3.1: Chemicals and solutions

Chemical/solution	Concentration	Supplier
$MgCl_2$	25 mM	Applied Biosystems
Chloroform		VWR
Chlorpyrifos	250 mg (50mg/mL)	Sigma-Aldrich
deoxyNTPs		Applied Biosystems
Dimethyl sulfoxide (DMSO)	100%	Sigma
E3 medium for zebrafish embryo (ddH_2O with NACl, KCl, CaCl and $MgSO_4$)		
Endosulfan (Alpha+Beta=2+1)	250 mg (50mg/mL)	Sigma-Aldrich
Ethanol		Arcus
Glycerol		Sigma-Aldrich
H_2DCFDA (2',7'-Dichlorodihydrofluorescein diacetate)	100 mg (21 mM)	Invitrogen
Isopropanol		Arcus
Multiscribe reverse transcriptase	50 U/µL	Applied Biosystems
***N*-Phenylthiourea (PTU**		Sigma-Aldrich
Oligo d(T)16 primer		Applied Biosystems
RNase inhibitor		Applied Biosystems
SYBR GREEN Master		Roche Norge
TaqMan RT buffer 10X		Applied Biosystems
Trizol		Invitrogen

Table 3.2: Primers used. Forward (F) primers upper line, reverse (R) primer lower line.

Primers	
CuZn-sod	F: CCGGCACCGTCTATTTTCAAT
	R: GCCGTTTGTGTTGTCACCAA
Cyp1a1	F: GGTGTTGGTTTTCGGTTTGG
	R: GGCATCCCGGTGAACTTTAA
gclc	F: AGTGGAGTTCAGGCCAATGG
	R: CTTCGACAACGGAATGAGGAA
gpx1a	F: TACGTCCGTCCTGGAAAGTGG
	R: GTCACTGGGCTGAGGAAGCT
gpx4a	F: AAACGTTGCCTCCAAATGAG
	R: ATGACTTGGCGAATTCCTTG
rpl13a	F: TCTGGAGGACTGTAAGAGGTATGC
	R: AGACGCACAATCTTGAGAGCAG
uba52	F: CGAGCCTTCTCTCCGTCAGT
	R: TTGTTGGTGTGTCCGCACTT
vtg1	F: GTGCGTCGTATCTTGCCAACT
	R: AGTGGAGTTCAGGCCAATGG

Table 3.3: Instruments

Instrument	Provider
Eppendorf Cenrifuge	VWR international
Nano-Drop ND-1000 Spectrophotometer	Isogen Life Science
Gene Amp PCR system 9700	Applied biosystems
Light Cycler 480	Roche Norge
Agilent 2100 Bioanalyzer	Agilent Technologies
Ice machine	
Biomek 3000 Laboratory Automation Worksatation	Beckman Coulter
Grant Scientific Digital Dry Block Heater-BTD	Grant internationals
Binder CB 53 incubator	Binder
Olympus SZX12 Stereomicroscope	Olympus
MS2 minishaker	IKA Labortechnik
Lab Dancer S40	VWR international

Table 3.3: Software

Software
Lightcycler 480
GeNorm Excel add-on
Statistica

4. Methods

4.1 Zebrafish

4.1.1 Laboratory handling and conditions

Zebrafish were kept in 3L tanks with either 12 females and 2 males per tank or males in individual tanks, at 28.5ºC (±1ºC) and a pH of 7.5 (±0.3). The light cycle of the room is 14 hours of light and 10 hours of dark. Two generations of zebrafish were used, due to the age of the first generation. The zebrafish were fed GemmaMicro Series (75, 150, 300 or 500) from Skretting twice a day with Artemia separately once a day.

4.1.2 Crossing zebrafish

The afternoon before crossing, the sexes were separated in the tank by a mesh, with a male to female ratio of 1:2. AB or TLF lines were intercrossed for easier separation of sexes. At 8:30 in the morning, the fish were placed together in the upper part of the separating mesh. The mesh was slightly tilted to make the water shallow which stimulates the mating instincts of the fish. After 30 minutes, the fish were transferred to a new tank for an additional 30 minutes. During this time, embryos from the former tank were collected. The embryos were collected using a sieve and transferred to petridishes filled with E3 medium. After three to four hours of development, the fertilized embryos were separated from unfertilized embryos and transferred to 24-wells plates, with 15 embryos in each well.

4.1.3 Exposure with pesticides

From already prepared stock solutions of 50 mg/mL in 100% DMSO, a serial dilution with the following concentrations was made:

Table 4.1: Main concentrations used for exposure. Total of five exposure ranges.

Pesticide solution	A	B	C	D	E
CPF concentration	200 µg/L	20 µg/L	2 µg/L	0.2 µg/L	0.02 µg/L
	10,000 µg/L	5000 µg/L	2000 µg/L	1000 µg/L	200 µg/L
ESF concentration	50 µg/L	10 µg/L	2 µg/L	1 µg/L	0.1 µg/L
	500,000 µg/L	50,000 µg/L	5000 µg/L	500 µg/L	50 µg/L
	50,000 µg/L	40,000 µg/L	30,000 µg/L	20,000 µg/L	10,000 µg/L

The pesticides were dissolved in 100 % DMSO. The final pesticide solutions used in the exposure experiment contained 1 % (v/v) DMSO in E3 medium.

E3 medium was removed from the wells and 1 mL of pesticide solution or control was added to each well. The control groups were E3 and E3 with 1% DMSO (E3/DMSO). See Figure 4.1 for general layout.

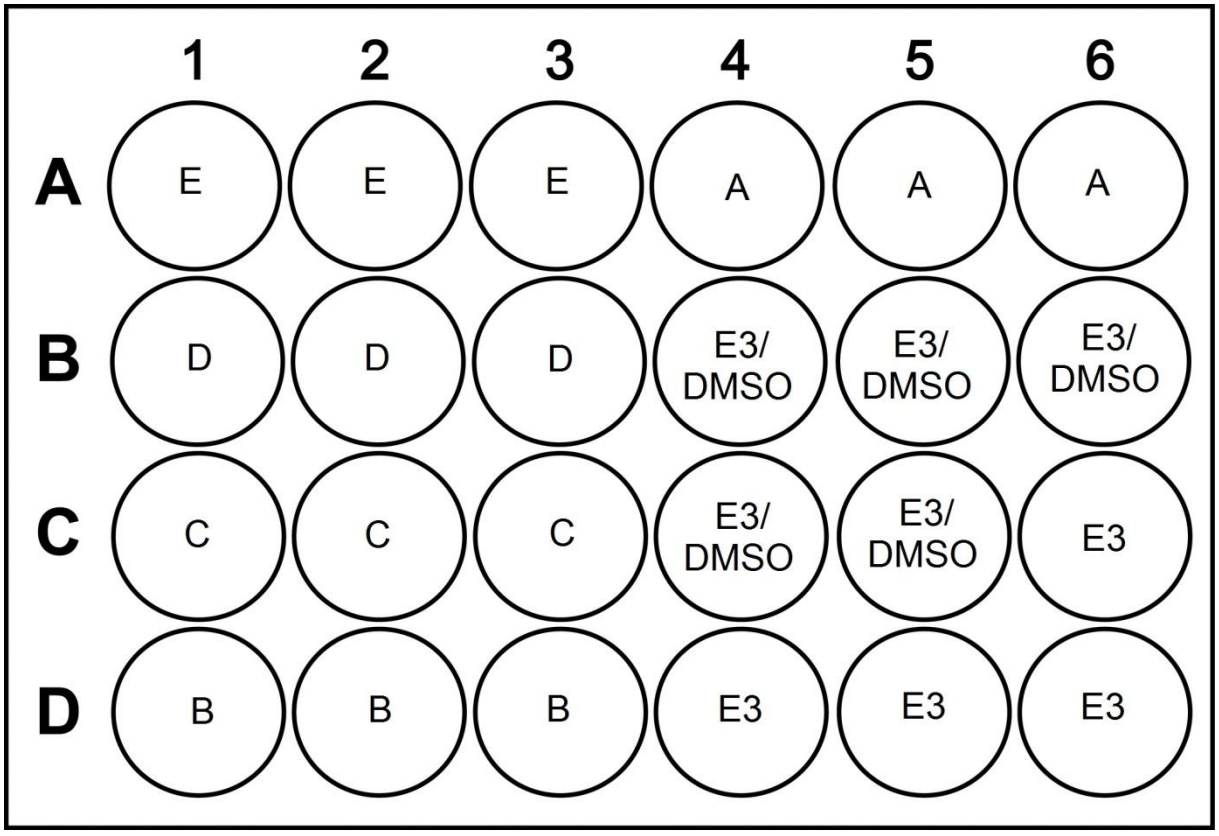

Figure 4.1: General layout of pesticides exposure in wells. Starting with the lowest concentration (E) to the highest concentration (A), with E3/1% DMSO and E3 as controls, all the pesticides exposed embryos were run in triplicates.

4.1.4 Animal care

Animal care and welfare complied within the regulations and legislations according to the Norwegian animal welfare law.

4.2 H_2DCFDA

4.2.1 Optimizing H_2DCFDA protocol

100 mg H_2DCFDA were dissolved in 10 mL 100% DMSO, giving a stock solution of 21 mM H_2DCFDA. By dilution with 100% DMSO, 200 µL 10 mM H_2DCFDA was made. From the 10 mM H_2DCFDA dilution, 50 mL of 10 µM H_2DCFDA in E3/1% DMSO was made with 100% DMSO and E3 medium.

10 embryos per three wells for each concentration (Table 4.1) were exposed from 6 hpf to 48 hpf. Five embryos were then removed, while the last five embryos continued to be exposed until 72 hpf. The removed embryos were placed in a new 24 well plate. The 48 and 72 hpf exposed embryos had the pesticide solution removed, and were incubated in 1 mL 10 µM H_2DCFDA per well. The embryos were observed after one and two hours of incubation through a stereomicroscope (Olympus SZX12 Stereomicroscope). This microscope has a 0.7- 9X zoom range, 0.11 in numerical aperture, and has a field number of 22. It has a fluorescence unit with 100W mercury lamp. Embryos were excited with 488 nm light, generating fluorescent emission of 510 nm. Mounting was done by transferring an embryo to a customized glass slide, removing as much solution as possible and then adding three-four drops of 100% glycerol on the larvae, before adding a cover glass. The glass slide was customized with 3 glass slides glued on top of each other at each side, so the larvae would not be squished between the glass slide and the cover glass.

Optimization of the protocol led to using 5 µM or 1 µM H_2DCFDA instead of the initial 10 µM. The H_2DCFDA solutions were made using the stock solution to make 50 mL 1 mM H_2DCFDA in E3/1% DMSO and 50 mL 5 mM H_2DCFDA in E3/DMSO. In addition 15 embryos per well were used, and the embryos were observed at 24 hpf in addition to 48 hpf and 72 hpf. After 20 minutes, 1 hour and 2 hours H_2DCFDA incubation, the embryos were observed through the microscope. Negative controls were embryos grown in E3 and E3/1% DMSO. As a positive control, 10 µL 50 mM H_2O_2 were added in the 1 mL E3 or E3/DMSO with 5 embryos in each well.

4.2.2 Determining pesticides sub-lethal concentration

Several alterations in the concentration range were performed to find the ranges ideal for the H_2DCFDA method. Using the set-up shown in Figure 4.1, and the set-up shown in Figure 5.4, all exposures mentioned in Table (4.1), were tested using the following concentrations; CPF concentration of 200 µg/L, 1000 µg/L and 10000 µg/L and ESF concentrations of 50 µg/L, 500 µg/L and 5000 µg/L. These were tested with 1 µM and 5 µM H_2DCFDA using 15 embryos per well and with triplicates of the concentrations and the controls.

4.3 Survival rate of zebrafish embryos after pesticide exposure

4.3.1 Endosulfan exposure

A total of 60 embryos per concentration were exposed to 10,000 µg/L, 20,000 µg/L, 30,000 µg/L, 40,000 µg/L and 50,000 µg/L ESF from 6 hpf to 72hpf, before returning them to optimal circumstances for further development until 14 days post fertilization (dpf). The set-up used was two plates as in Figure 4.1 with 10 embryos in each well. Media was changed daily while embryos were kept in the 24 well plates. After 72 hpf the embryos were washed 3x times in E3 medium before transfer to 250 mL beakers filled with 50 mL E3. At this time the embryos/larvae from the two plates used were joined together with the corresponding wells, giving a beaker of close to 20 embryos instead of two wells with 10 embryos each. Dead embryos/larvae were removed daily and their number was recorded. Within the control groups 99 embryos were exposed to E3 with 1% DMSO and 86 embryos in normal E3 handled in the same way as the embryos exposed to ESF. At 5 dpf, feeding started with gemma 75 feed. At 7 dpf, 50 mL additional E3 was added in the beakers and artemia in addition to gemma 75 was given to the larvae.

4.3.2 Chlorpyrifos exposure

CPF samples were treated in the same way as the endosulfan samples; using concentrations of 200 µg/L, 1000 µg/L, 2000 µg/L, 5000 µg/L and 10,000 µg/L CPF on approximately 60 embryos per group. Control groups were the same groups with 101 embryos exposed to E3 with 1% DMSO and 80 embryos in normal E3.

4.4 RealTime quantitative PCR

4.4.1 RNA extraction

The embryos were transferred from wells to 1.5 mL Eppendorf tubes, where the pesticide/control solution was removed and 500 µL Trizol added. Using a syringe with an attached needle, the embryos were homogenized. After homogenization, the samples were either frozen at -80^{o}C overnight or directly used. The next day the samples were centrifuged for 15 minutes at 12,000 G in 4^{o}C and the liquid transferred to 2 mL Eppendorf tubes. After being incubated at room temperature (RT) for 5 minutes, 100 µL chloroform was added to each sample and shaken for 15 seconds by hand. After 2 to 3 minutes incubation at RT, the samples were centrifuged for 15 minutes at 12,000 G in 4^{o}C again and the top layer in each sample was transferred over to new 1.5 mL Eppendorf tubes. The 2 mL tubes were discarded and 250 µL Isopropanol was added to the transferred samples. These samples were incubated for 10 minutes at RT and then between 10 minutes and up to one hour in a fridge. The samples were centrifuged for 10 minutes at 12000 G at 4^{o}C before the supernatant was removed with vacuum aspiration using an RNAse free micropipette. 500 µL ice-cold 75% ethanol was added and the samples were vortexed until the pellet was dissolved. The samples were centrifuged for 5 minutes at 10,000 G in 4^{o}C and the supernatant was removed again with vacuum. To purify the RNA, 62.5 µL 100% ethanol, 2.5 µL sodium acetate and 25 µL MilliQ water were added and left overnight in the -80^{o}C freezer. The next day the samples were centrifuged for 20 minutes at 12000 G in 4^{o}C and the supernatant removed by vacuum. The tubes were incubated for 2-3 minutes on ice and then at RT with open caps to air dry the tubes and pellets. The pellets were resuspended in 20 µL MilliQ water and the concentrations were determined using nanodrop (Isogen Life Science).

RNA was extracted from embryos exposed to 0.02 µg/L, 0.2 µg/L, 2 µg/L, 20 µg/L, 200 µg/L, 1000 µg/L, 2000 µg/L 5000 µg/L and 10,000 µg/L CPF solutions.

From ESF exposures RNA was extracted from solutions of 0.1 µg/L, 1 µg/L, 2 µg/L, 10 µg/L, 50 µg/L, 500 µg/L, 5000 µg/L, 10,000 µg/L, 20,000 µg/L, 30,000 µg/L, 40,000 µg/L and 50,000 µg/L ESF.

4.4.2 BioAnalyzer

Before making a cDNA plate, 6-12 RNA samples were selected for integrity analyses. . The integrity was checked using the RNA 6000 LabChip Kit (Agilent) and the BioAnalyzer (Agilent). Samples that had similar RNA concentration (± 50 ng/µL), were chosen as the representative samples. 1 µL of the sample was transferred to a new tube and mixed with 1 µL ddH_2O before the tubes were set to denature at 70ºC on a block heater for 2 minutes. 9 µL agarose gel was pressed onto the chip, and a total of 18 µL more agarose gel was added in 2 other wells afterwards. 5 µL marker was added in each well, except for those filled with agarose gel. 1 µL ladder mix was added to the well designated for the ladder, and 1 µL of the denatured samples were added in their respective wells. The chip were vortexed for a minute and analyzed by BioAnalyzer, giving RNA Integrity Number (RIN) scores from 0-10, where 10 is the best quality.

4.4.3 cDNA plates

RNA samples were thawed on ice. Using the concentrations obtained from the Nanodrop, the RNA samples was calculated and diluted to 50 ± 5% ng/µL with ddH_2O in new Eppendorf tubes. To make a standard curve, 2-3 µL of each extracted, undiluted RNA sample were pooled together to a RNA mix. The RNA mix was calibrated to 100 ng/µL, and by means of serial dilution a standard curve was made by adding 40 µL sample and 40 µL ddH_2O, giving a series with a concentrations of 100 ng/µL, 50 ng/µL, 25 ng/µL, 12.5 ng/µL, 6.25 ng/µL and 3.125 ng/µL.

In a clean environment, a RT mix was made according to the Table 4.2 and by taking into account how many wells of a 96 well PCR plate used. After transferring 19 µL of the mix to the control well “nac”, Multiscribe Reverse Transcriptase was added. Then 20 µL of the RT mix were transferred to each well of the 96 well PCR plate containing samples, with the exception of control wells. Triplicates of each extracted RNA sample were analyzed per cDNA plate.

Table 4.2: Ingredients of RT mix pr well in 96 well PCR plate. Multiscribe Reverse Transcriptase is not included in the control "nac".

Reagent	30 µL	End concentration
RNase free water	1.3	
10X TaqMan RT Buffer	3.0	1X
25 mM $MgCl_2$	6.6	5.5 mM
10 mM deoxyNTPs Mixture (2.5 mM of each dNTP)	6.0	500 µM per dNTP
50 µM oligo $d(T)_{16}$	1.5	2.5 µM
RNase Inhibitor (20U/µL)	0.6	0.4 U/µL
Multiscribe Reverse Transcriptase (50U/µL)	1.0	1.67 U/µL

In the 96 well plate (see Table 4.4), the RT mix was distributed according to the number of samples. For preparation of a standard curve diluted standards were added to the plate, using 10 µL per well and three wells per sample, starting from the highest concentration. The RNA samples were then added to the plate, 10 µL per well and three wells per sample, giving triplicates of each sample. 30 µL ddH_2O were added in the non-template control well "ntc" and 10 µL of a random excess RNA sample was added in the "nac" control well. A 96 well plate cover was put on the plate and the plate was centrifuged for a minute at 50 G, before running it in the Gene Amp PCR system 9700 (Applied biosystems). After the PCR steps were completed, the plate was centrifuged for a minute at 800 G. A tape pad was put on and the plate was stored at -20ºC until use.

Table 4.3: The PCR machine program for Reverse Transcriptase.

Step	Incubation	RT	Reverse transcriptase inactivation	End
Temperature (ºC)	25	48	95	4
Minutes	10	60	5	∞
Volume	30 µL			

Table 4.4: General layout of a 96 well cDNA plate. Yellow boxes indicate standard curve, made from the mixed RNA samples, diluted with ddH_2O. Red boxes are the negative control groups, "nac" without Multiscripe Reverse transcriptase and "ntc" was only water. Except for in the standard curve, where the concentration ranges from 3,125 ± 5 % ng/µL to 100 ± 5 % ng/µL, all the samples were at 50 ± 5 % ng/µL

A	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve
	100 ± 5% ng/µl	100 ± 5% ng/µl	100 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	25± 5% ng/µl	25± 5% ng/µl	25± 5% ng/µl	12,5 ± 5% ng/µl	12,5 ± 5% ng/µl	12,5 ± 5% ng/µl
B	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	Dilution curve	1	1	1	2	2	2
	6,25 ± 5% ng/µl	6,25 ± 5% ng/µl	6,25 ± 5% ng/µl	3,125± 5% ng/µl	3,125± 5% ng/µl	3,125± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
C	3	3	3	4	4	4	5	5	5	6	6	6
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
D	7	7	7	8	8	8	9	9	9	10	10	10
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
E	11	11	11	12	12	12	13	13	13	14	14	14
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
F	15	15	15	16	16	16	17	17	17	18	18	18
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
G	19	19	19	20	20	20	21	21	21	22	22	22
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl
H	23	23	23	24	24	24	-	-	-	-	nac	ntc
	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl	50 ± 5% ng/µl					50 ± 5% ng/µl	ddH_2O

4.4.4 Realtime quantitative PCR

cDNA samples were thawed and the 96 well plates were centrifuged at 1000 G for a minute. After vortexing the plates at 1300 rpm for 3 minutes the plates were again subjected to centrifugation at 1000 G for a minute. The primer solution was made according to the Table 4.5 and the number of samples in the cDNA plate. A full plate would be 114 times the values in Table 4.5 due to the use of a pipette robot (Beckman Coulter) and slightly higher volume than needed will ensure that the robot pipettes the right amount. 110 µL reaction mix was added in each well of an 8-strips tube. Using the pipette robot, 8 µL of the reaction mix were mixed with 2 µL cDNA in 384 well plates. The 384 well plate was centrifuged for 2 minutes at 1500 G before running, and it was analyzed using Light Cycler 480. The primers for reference genes amplify the uba52 and rpl13 genes, while the genes analyzed were *cyp1a1*, *vtg1*, *gclc*, *gpx1a*, *gpx4a* and *CuZn-sod*.

Table 4.5: Ingredients of SYBRGreen reaction mixture for Light Cycler 480

Reagent	Volume per sample (µL)	End concentration
ddH_2O	2.8	
Primer I (50µM)	0.1	
Primer II (50µM)	0.1	
SYBRGreen PCR Master Mix (2x)	5	1 X

4.4.5 Data analysis and statistics

qPCR data was analyzed using Genorm and one-way ANOVA (fisher LSD, POST HOC test) in Statistica; heatmaps of the data were generated using Qlucore Omics Explorer.

5. Results

5.1 Survival rate of zebrafish embryos after pesticide exposure

5.1.1 Determining sub-lethal concentration range

Dose-response tests were performed to determine sub-lethal concentrations of ESF and CPF. The 200,000 µg/L CPF exposure killed the embryos within 8 hours of exposure, while at the 20,000 µg/L CPF died within 18 hours after exposure. In the present work the NOAEL of the CPF concentrations investigated was determined to be 10,000 µg/L. In the ESF concentrations tested embryos died at 500,000 µg/L within 18 hours of exposure, while at 50,000 µg/L embryos died shortly after ended exposure at 72 hpf, within 78 hpf. The highest NOAEL for ESF values was 10,000 µg/L and LOAEL was determined as 20,000 µg/L.

During the first dose-response test, the concentrations were 20 µg/L, 200 µg/L, 2000 µg/L, 20,000 µg/L and 200,000 µg/L in the CPF solutions. In the ESF solutions the concentrations were 100 µg/L, 1000 µg/L, 2000 µg/L, 10,000 µg/L and 50,000 µg/L.

The first exposure tests had low concentrations of the pesticides. However, using the values of 0.02 µg/L, 0.2 µg/L 2 µg/L, 20 µg/L and 200 µg/L CPF and 0.1 µg/L, 1 µg/L, 2 µg/L, 10 µg/L and 20 µg/L ESF, the H_2DCFDA method did not distinguish controls and exposed groups. The concentrations of the pesticides were therefore altered to be much closer to the lethal concentrations as discovered in the dose-response test, to determine if these levels induce ROS. CPF values were now set to 200 µg/L, 1000 µg/L and 10,000 µg/L, using the layout depicted in Figure 5.4. ESF values were set to 50 µg/L, 500 µg/L and 5000 µg/L. The ESF method did still not induce enough ROS to differ from the controls.

With no clearly induced mortality from ESF within 72 hpf, the range was set even higher to determine the lethal concentration. A plate was made ranging a tenfold higher at each concentration from 5000 µg/L to 500,000 µg/L ESF. 100% Mortality was observed at 500,000 µg/L. A new plate with the concentrations 5000 µg/L, 10,000 µg/L, 20,000 µg/L, 30,000 µg/L, 40,000 µg/L and 50,000 µg/L ESF was made and used for further experiments.

5.1.2 Endosulfan exposed embryos

A 14 days survival rate test of embryos exposed from 6 hpf to 72 hpf was measured to see how the ESF exposure affected the development of the embryos (see section 4.3.1). At 78 hpf all the embryos that had been exposed to 50,000 µg/L ESF had died. The group exposed to 40,000 µg/L ESF, had a very low heart rate immediately after exposure and looked

malformed, but most survived up to 7-8 dpf after transfer to E3 medium. Then they rapidly started to die. Groups exposed to 20,000 µg/L – 50,000 µg/L had a much slower hatching rate than the lower exposed groups and controls. Malformation was observed in all groups exposed to 20,000 - 50,000 µg/L, but with higher severity and individual affectation with the increasing concentration of ESF. The larvae also were less reactive to outside stimuli in these ranges, as several did not swim away from the pipette used to clean the beakers. In the two highest concentrations, several did not react at all, even when directly touched with the pipette. Several embryos and larvae in the concentrations from 30,000 - 50,000 µg/L also got edema around the heart region. At 8 and 9 dpf a general high mortality was observed. All individuals in the group at 40,000 µg/L ESF died within day 11. In the 30,000 µg/L group all died within day 13. At the end of the experiment period, day 14, there was only one survivor in a total of 60 individuals in the 20,000 µg/L ESF group. In the 10,000 µg/L ESF group there were 19 survivors of 61 individuals. In the control groups 48 of 99 individuals survived of the embryos with 1% DMSO in the solution. 54 of 86 individuals survived of the only E3 water control group. Figure 5.1 shows the average survivors left pr day for each concentration.

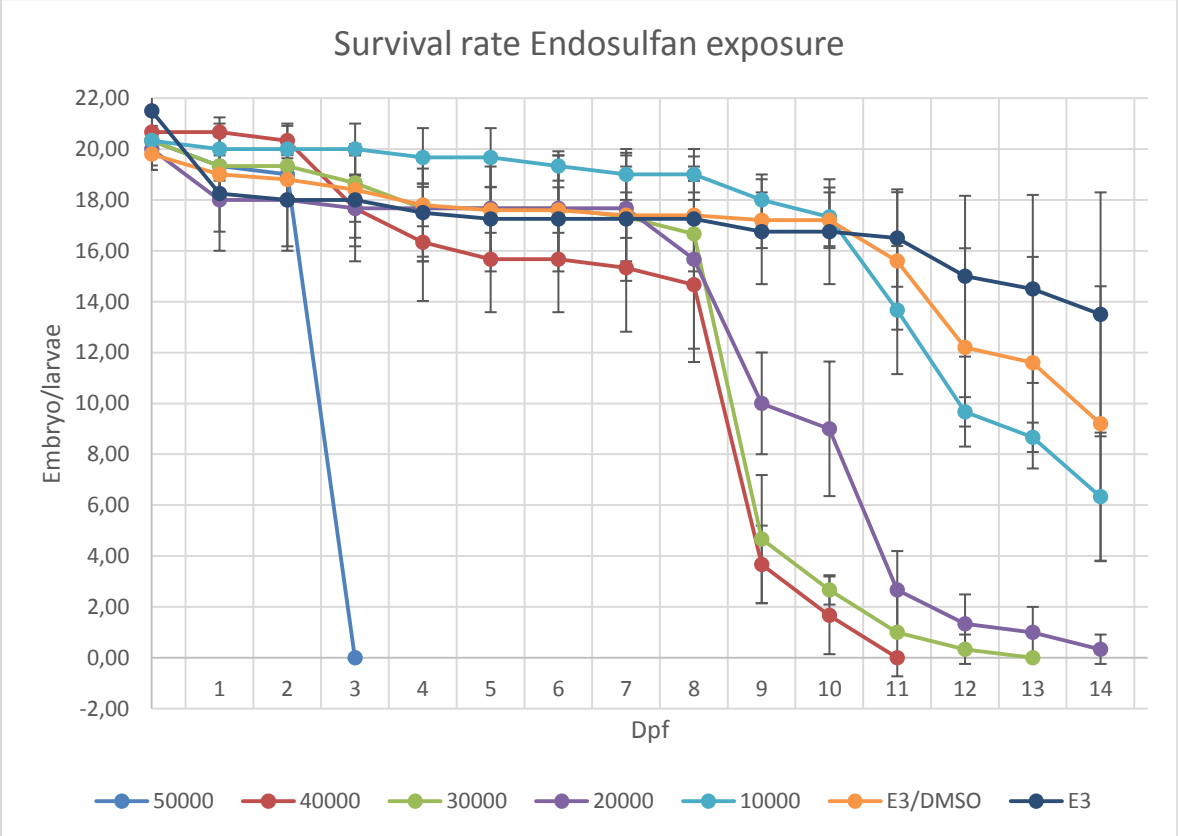

Figure 5.1: The survival rate of embryos/larvae exposed to different endosulfan concentrations. This shows the average survivors of the triplicates for each concentration and of the control groups, as indicated at the bottom.

5.1.3 Chlorpyrifos exposed embryos/larvae

The same test was done for the CPF concentrations (section 4.3.2). Unlike in the high ESF concentrations, the high CPF concentrations induced no visible malformations, and all but six embryos of all the groups had hatched within day 3. Until the last days, the general survival rate was steady. Around 12 dpf, 70 individuals had suddenly died across the beakers and there were a total of 115 dead embryos across the beakers at the end of the trial. Of 60 individuals exposed to 10,000 µg/L CPF, 48 were alive at day 14. 49 individuals of a total of 60 embryos exposed to 5000 µg/L survived. 62 embryos were exposed to 2000 µg/L and 52 survived. 47survived of a total of 62 embryos exposed to 1000 µg/L. Of the 63 embryos exposed to 200 µg/L there were 35 survivors at day 14. In the control groups 79 of a total of 101 embryos that developed in E3/1% DMSO survived, while 63 of 80 embryos developing in normal E3 survived. Figure 5.2 shows the average surviving embryos left per day for each concentration during the 14 days.

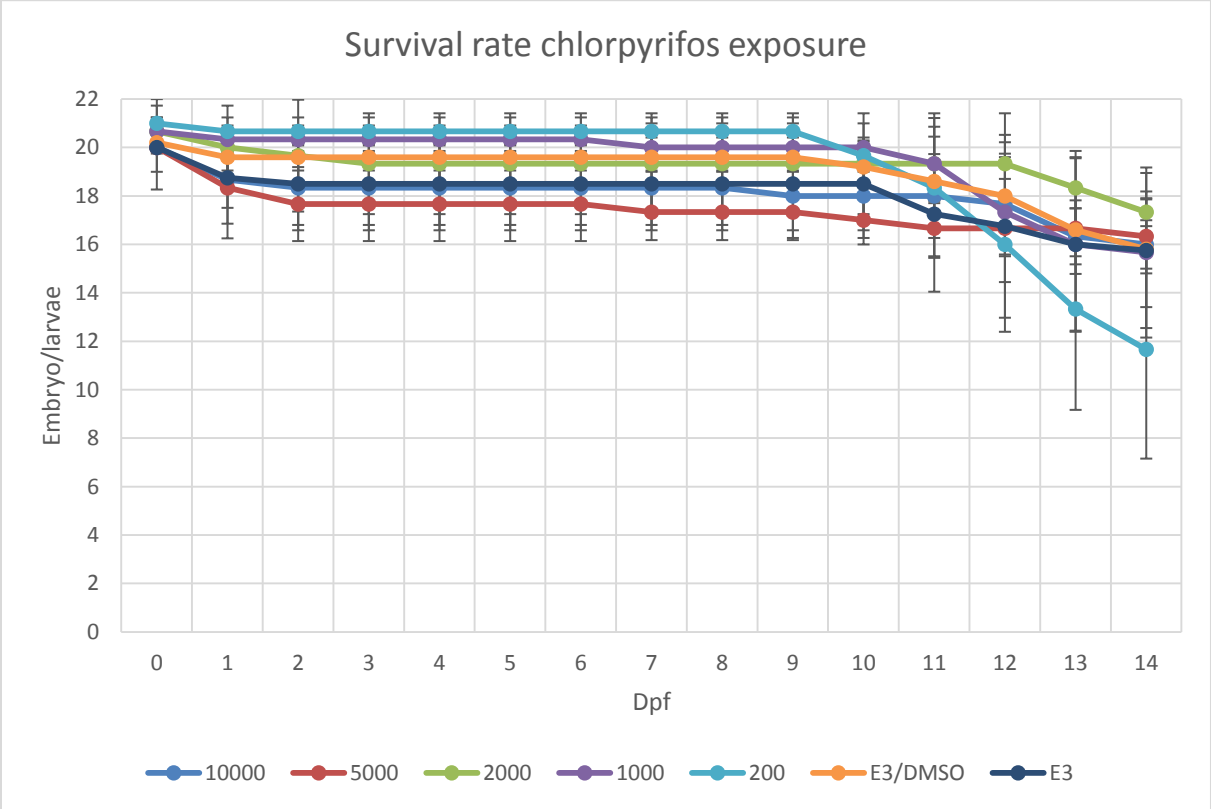

Figure 5.2: The survival rate of embryos/larvae exposed to different chlorpyrifos concentrations. This shows the average survivors of the triplicates for each concentration and of the control groups, as indicated at the bottom.

5.2 H_2DCFDA

5.2.1 Fluorescence imaging

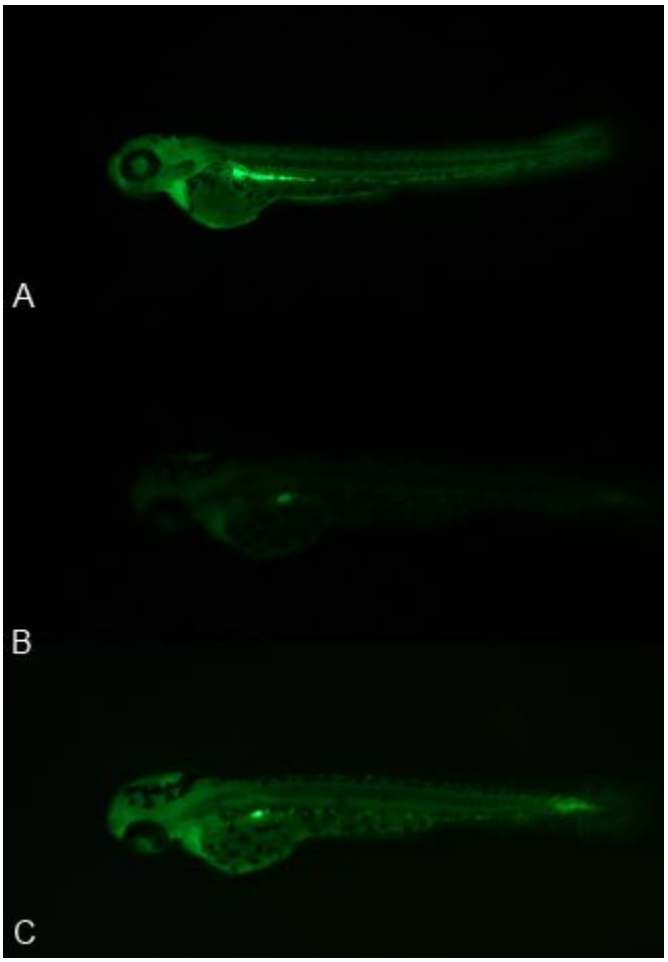

Figure 5.3: Comparison of H_2DCFDA concentration. The three pictures are all of 10,000 µg/L CPF exposed larvae. Larvae A is treated with 5 µM H_2DCFDA, while B and C is the same larvae treated with 1 µM H_2DCFDA. Picture A and B is taken with 1 second exposure with the camera, while C is with 4 seconds exposure.

With the H_2DCFDA method (section 4.2), oxidative stress can be visualized through the fluorescent signal induced from reacting with oxidative agents. With this method, inducement of oxidative stress from pesticides can be analyzed.

The protocol for this method was altered several times to determine the concentrations if the pesticides. The initial concentrations used were; 0.02 µg/L, 0.2 µg/L, 2 µg/L, 20 µg/L and 200 µg/L CPF, and 0.1 µg/L, 1 µg/L, 2 µg/L, 10 µg/L and 50 µg/L ESF. After these initial values showed no difference between exposed groups and control groups, the concentration strength was increased. Using the layout with either 5 µM or 1 µM H_2DCFDA (see Figure 5.4) the strength of H_2DCFDA was determined. CPF values were set to 200 µg/L, 1000 µg/L and 10,000 µg/L, while ESF was 50 µg/L, 500 µg/L and 5000 µg/L. While there was some

difference in CPF, the concentrations for ESF were still not distinguishable. A series from 5000 µg/L to 500,000 µg/L was made, where 50,000 µg/L was more fluorescent than the other groups. A final concentration range was set at 5000 µg/L, 10,000 µg/L, 20,000 µg/L, 30,000 µg/L, 40,000 µg/L and 50,000 µg/L. This time 15 embryos were added in each well, to give the opportunity to observe them after 24 hpf exposure as well as 48 hpf and 72 hpf exposures. After exposure, the embryos were incubated in 1 mL 5 µM or 1 µM H_2DCFDA in E3/1% DMSO. Pictures were taken of the embryos exposed 48 hpf and 72 hpf.

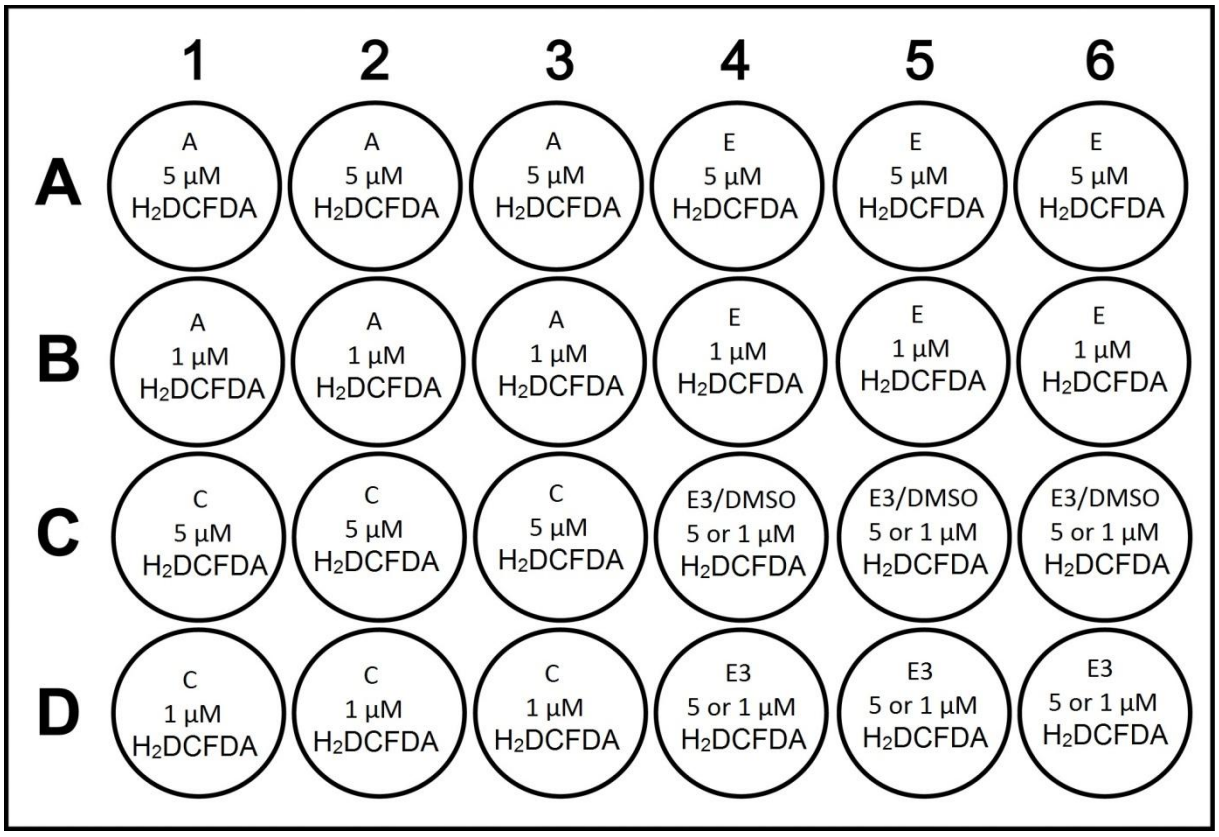

Figure 5.4: The second layout of the 24 well plates used. A is the strongest concentration of the pesticides while E is the weakest. Controls were divided between the two plates, so the chlorpyrifos (CPF) plate had 1 µM H_2DCFDA range while endosulfan (ESF) had the 5 µM range. A was 200 µg/L CPF or 50 µg/L ESF, C was 2 µg/L CPF or ESF and E was 0,02 µg/L CPF or 0,1 µg/L ESF.

Comparing the amount of ROS induced by pesticide exposure, the 24 hpf embryos usually showed no or very low fluorescence. The 48 hpf embryos had some fluorescence signal. The 72 hpf usually showed higher fluorescence signal than the 48 hpf did. This was a general consideration to both pesticides, but embryos exposed to ESF showed much stronger fluorescence than CPF exposed embryos. Though H_2DCFDA easily crosses membranes, the chorion may reduce the permeability and effect of the dye. To distinguish the embryos from

the different concentrations it was necessary to incubate the treated embryos for one hour. Within two hours the fluorescence difference was easily observed. The 10 µM solution resulted in too strong fluorescent signal. 1 µM worked on ESF exposed embryos, but not to the embryos exposed to CPF. The 5 µM concentration made the fluorescence signal generally strong. The difference in fluorescence strength can be seen in Figure 5.3.

There was some auto fluorescence from the embryos/larvae, irrespective of the concentration of pesticide or the control group. Usually the auto fluorescence is located in or at the yolk, or along the body towards the tail. This auto fluorescence can clearly be seen in the ESF 72 hpf E3/DMSO picture and is even stronger in the ESF 72 hpf 30,000 picture in Figure 5.7K, N.

To control the H_2DCFDA, a known strong oxidizer was chosen as a positive control; H_2O_2. H_2O_2 did not penetrate the chorion in the same rate as H_2DCFDA, and it made several green spots on the chorion, not seen in other embryos at 48 hours or less. The 72 hpf larvae were very green as indicated in Figure 5.5. Fluorescence signals induced from H_2O_2 was ubiquitously distributed in the embryos.

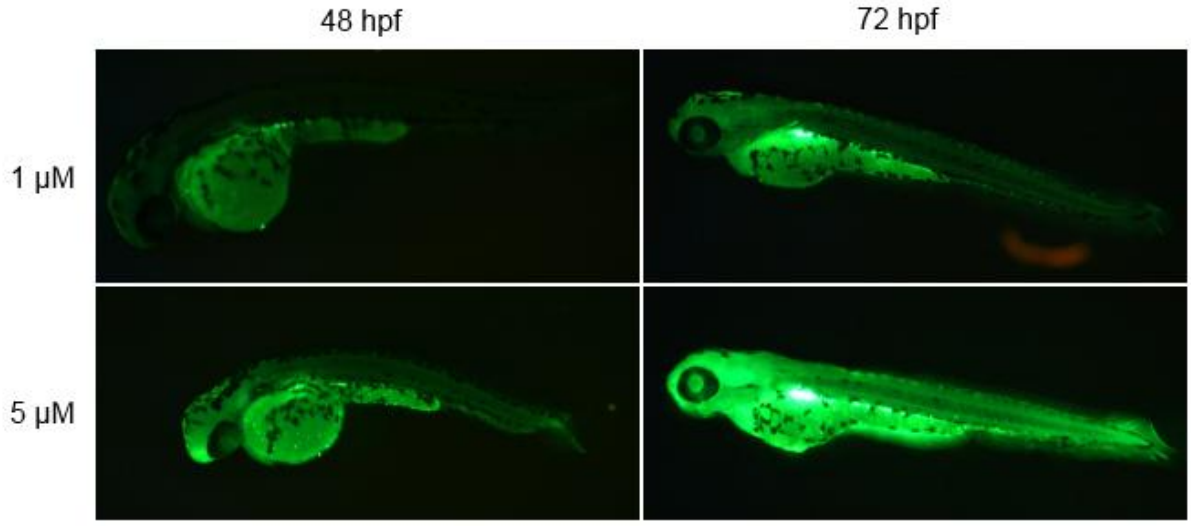

Figure 5.5: Positive control of H_2O_2. Embryos exposed to 10 µL 50mM H_2O_2 and either 1 µM H_2DCFDA or 5 µM H_2DCFDA for up to two hours.

5.2.2 Fluorescent imaging of endosulfan exposed zebrafish larvae

In embryos exposed to ESF, nearly no embryos exposed to concentrations below 10,000 µg/L were distinguishable from controls. Between 10,000 µg/L to 50,000 µg/L a gradient of

fluorescence was observed in correspondence to the higher concentrations, mainly localized in the head region of the embryos. In general, the embryos incubated in the 1 µM H_2DCFDA solution had much weaker fluorescence signal than that of 5 µM solution. The same trend is observed at both groups (Figure 5.6 and 5.7).

In the 5000 µg/L groups (Figure 5.6A, H and 5.7A, H) the embryos in 48 hpf and 72 hpf looked generally similar to the control groups (Figure 5.6G, N and 5.7G, N); with little fluorescence except for the auto fluorescence. This applied for both 1 µM and 5 µM.

In the 10,000 µg/L group, the 48 hpf embryos (Figure 5.6B and 5.7B) were also similar the control groups, but the 72 hpf (Figure 5.6I and 5.7I) shows an increase of fluorescence than from the 5000 µg/L groups (Figure 5.6H and 5.7H).

At 20,000 µg/L the 48 hpf (Figure 5.6C and 5.7C) had a slight increase in the amount of fluorescence, while the 72 hpf (Figure 5.6J and 5.7J) showed only a little stronger fluorescence than the 10,000 group.

The 30,000 µg/L 48 hpf group (Figure 5.6D and 5.7D) was nearly similar to the 20,000 µg/L group, but the 72 hpf group (Figure 5.6K and 5.7K) showed stronger fluorescence.

The 5 µM 40,000 µg/L group (Figure 5.6E) was only slightly stronger than the former concentrations with the same treatment. The larvae in the 1 µM (Figure 5.7E) showed an increased fluorescence in the larva from the 20,000 µg/L and 30,000 µg/L groups. In the 72 hpf, both 1 µM (Figure 5.7L) and 5 µM (Figure 5.6L) shows an increase of fluorescence.

At 50,000 µg/L (Figure 5.6F, M and 5.7F, M) the embryos showed the strongest fluorescence in both time rates and H_2DCFDA concentration. However, some embryos showed a much stronger fluorescence than others in a group and some showed lower fluorescence. So there were variations that may not be as indicative as the general overview. Referring to Figure 5.7, the 50,000 µg/L is less fluorescent than the 30,000 µg/L and 40,000 µg/L groups at 72 hpf. The 50,000 µg/L embryos did not elongate after dechorionation as the other embryos at lower concentrations did at 48 hpf as can be seen by the more circular larva in Figure 5.7F.

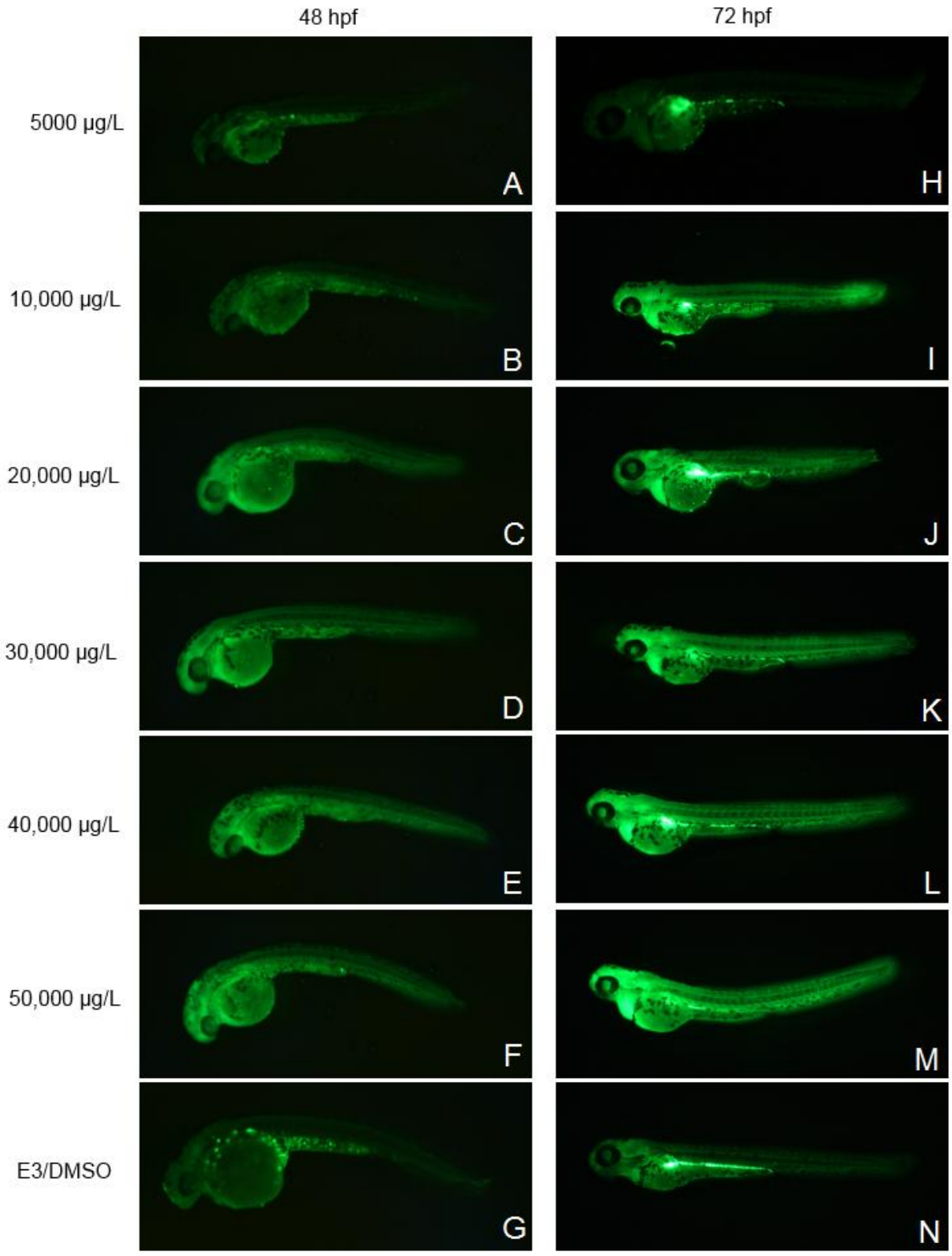

Figure 5.6: Comparison of embryos exposed to ESF according to time and dosage using 5 µM H_2DCFDA. The pictures were taken at 1-second exposure with gain at 1x. Treatment was up to two hours before pictures were taken.

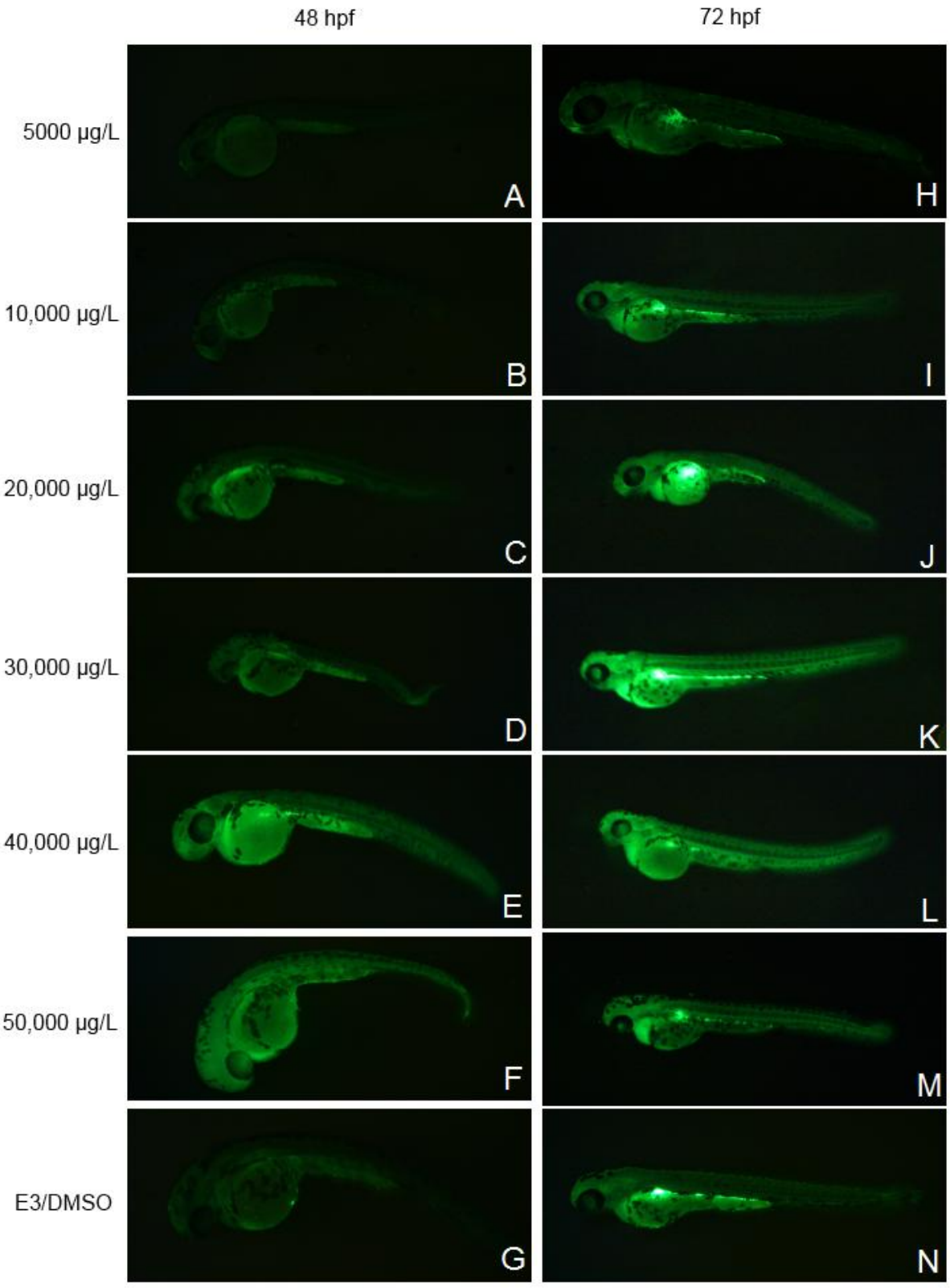

Figure 5.7: Comparison of embryos exposed to ESF according to time and dosage using 1 µM H_2DCFDA. The pictures were taken at 1-second exposure with gain at 1x. Treatment was up to two hours before the pictures were taken.

5.2.3 Fluorescent imaging of chlorpyrifos exposed zebrafish larvae

Embryos exposed to CPF had a much closer dosage interval as the lethal dose was at a lower concentration when compared to ESF. A small gradient was seen, but it was generally difficult to differentiate between the embryos exposed until 72 hpf. The embryos exposed until 48 hpf, which had to be dechorionated before mounting, showed a visible gradient (see Figure 5.8). In the CPF the ROS damage indicated by H_2DCFDA showed most often around the hindbrain area and the tail.

In the groups exposed to 200 µg/L (Figure 5.8A, G), the embryos looked similar to the control groups (Figure 5.8F, L). In the 1000 µg/L (Figure 5.8B, H), there was a slight increase of fluorescence. It was mostly seen in the heart region. At the 72 hpf (Figure 5.8H) the tails and also behind the eyes in the head were slightly more glowing. In the 2000 µg/L, the embryos at 48 hpf (Figure 5.8C) was slightly more fluorescent in the heart and behind the eyes head. The tail was also more fluorescent. The 72 hpf larvae (Figure 5.8I) showed a little increase of fluorescence compared to the 1000 µg/L.

The 5000 µg/L groups (Figure 5.8D, J) looked almost identical to the 2000 µg/L groups, with slightly more fluorescence. The strongest fluorescence observed in the 48 hpf groups, was at 10,000 µg/L (Figure 5.8E), which was the strongest concentration tested. The tail and head were more distinctly fluorescent than those from other concentrations were. In the 72 hpf group (Figure 5.8K), there was in general a slight increase from the 5000 µg/L, but the difference was hard to tell. The selected larva in the Figure 5.8K had a bit weaker fluorescence than the 3000 µg/L and 5000 µg/L groups, but this was not necessary the trend for the whole group.

The embryos here were from another batch than the ones used in the ESF exposures, but there is quite a difference in fluorescence between this control and the ESF controls. The individual representing the CPF control group had a fairly strong fluorescence, especially in the tail, and seems to be stronger than in the 72 hpf 1000 µg/L CPF group. However, the ROS damage observed in these embryos seemed quite low compared to the ESF fluorescence.

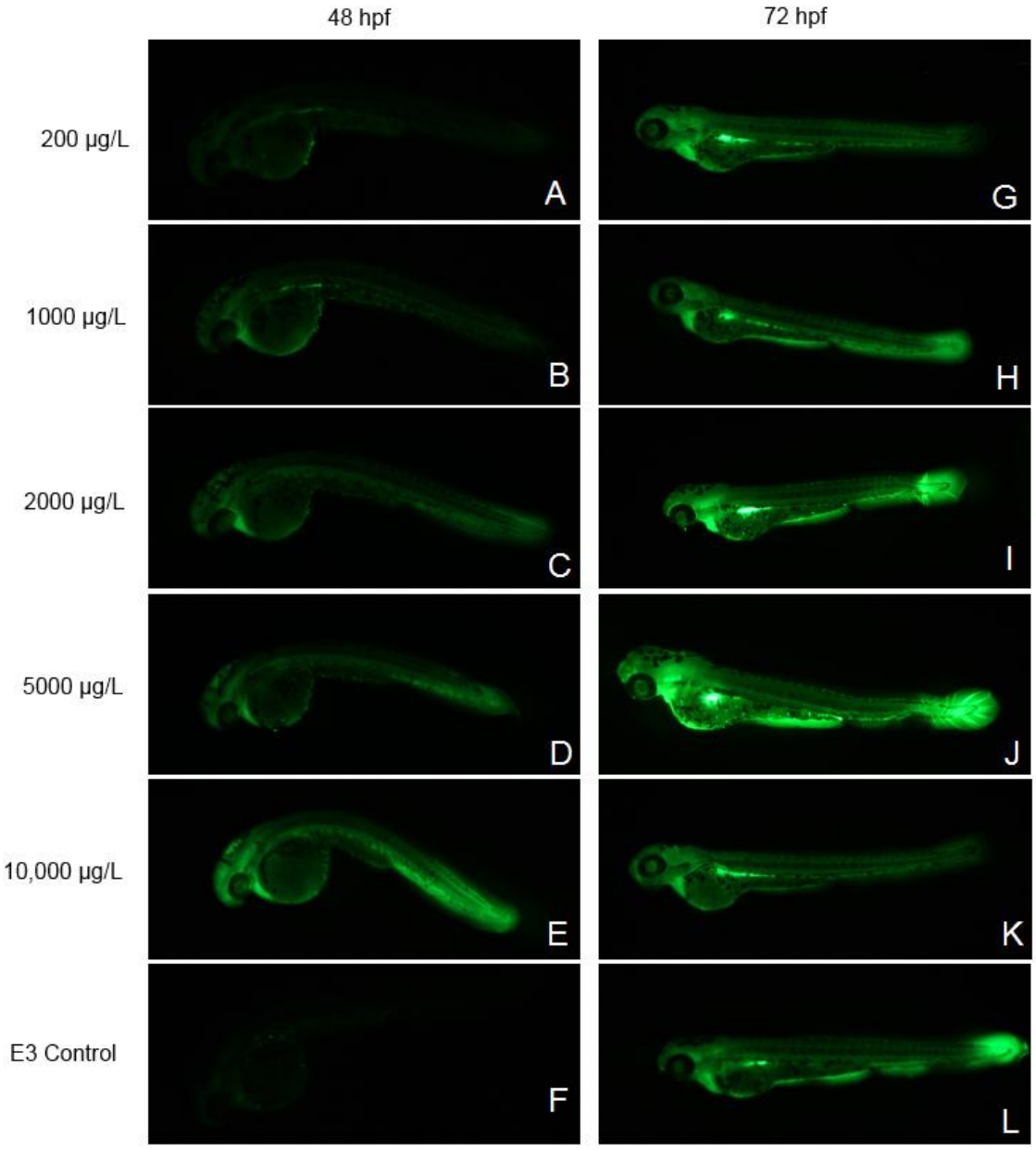

Figure 5.8: Comparison of embryos exposed to CPF according to time and dosage. The pictures were taken at 1 second exposure with gain at 1x. Treatment was by 5 µM H_2DCFDA for up to two hours.

5.3 RealTime quantitative PCR

5.3.1 RNA extraction and Bioanalyzer

To verify the oxidatitive stress response, expression of genes related to antioxidation was tested. To do this RNA had to be extracted and the RNA quality determined by Bioanalyzer (see section 4.4.1-4.4.2). Bioanalyzer scored values of 9.8-10 RIN points for every sample tested, indicating good integrity of RNA.

The cDNA plates were made with the original values; CPF – 0.02 µg/L, 0.2 µg/L, 2 µg/L, 20 µg/L and 200 µg/L and ESF – 0.1 µg/L, 1 µg/L, 2 µg/L, 10 µg/L, 50 µg/L and 500 µg/L, but they had standard curves where the Ct scores were outside of the samples Ct score range. This generated inconclusive results and the plates had to be discarded. Only the cDNA plates with results from concentration ranges where H_2DCFDA method was applied were used for further analysis. The cDNA plate with ESF 50 µg/L, 500 µg/L, 5000 µg/L, 50,000 µg/L and 500,000 µg/L was discarded as well, due to errors and inconclusive results. Thus the plates kept and used was CPF: 200 µg/L, 1000 µg/L, 2000 µg/L, 5000 µg/L and 10,000 µg/L and ESF: 5000 µg/L, 10,000 µg/L, 20,000 µg/L, 30,000 µg/L, 40,000 µg/L and 50,000 µg/L.

5.3.2 Endosulfan exposure leads to downregulation of *gpx1a, CuZn-sod* and *gclc. Gpx4a* gets a temporarily upregulation.

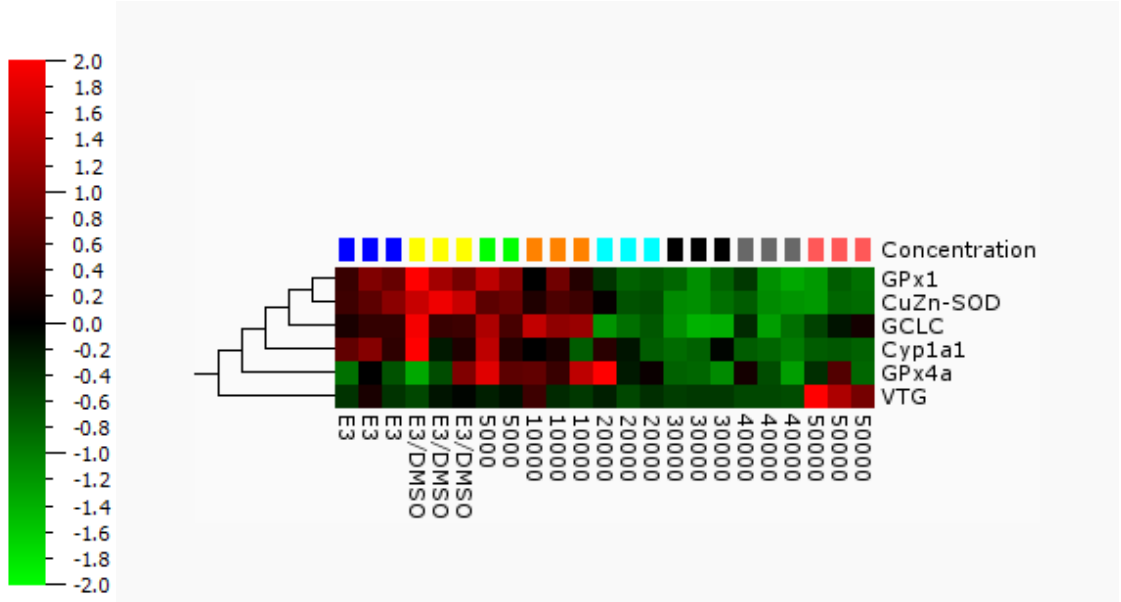

Figure 5.9: Heat map for genes tested on ESF exposed embryos. Green indicates reduced expression, while red indicates increased expression.

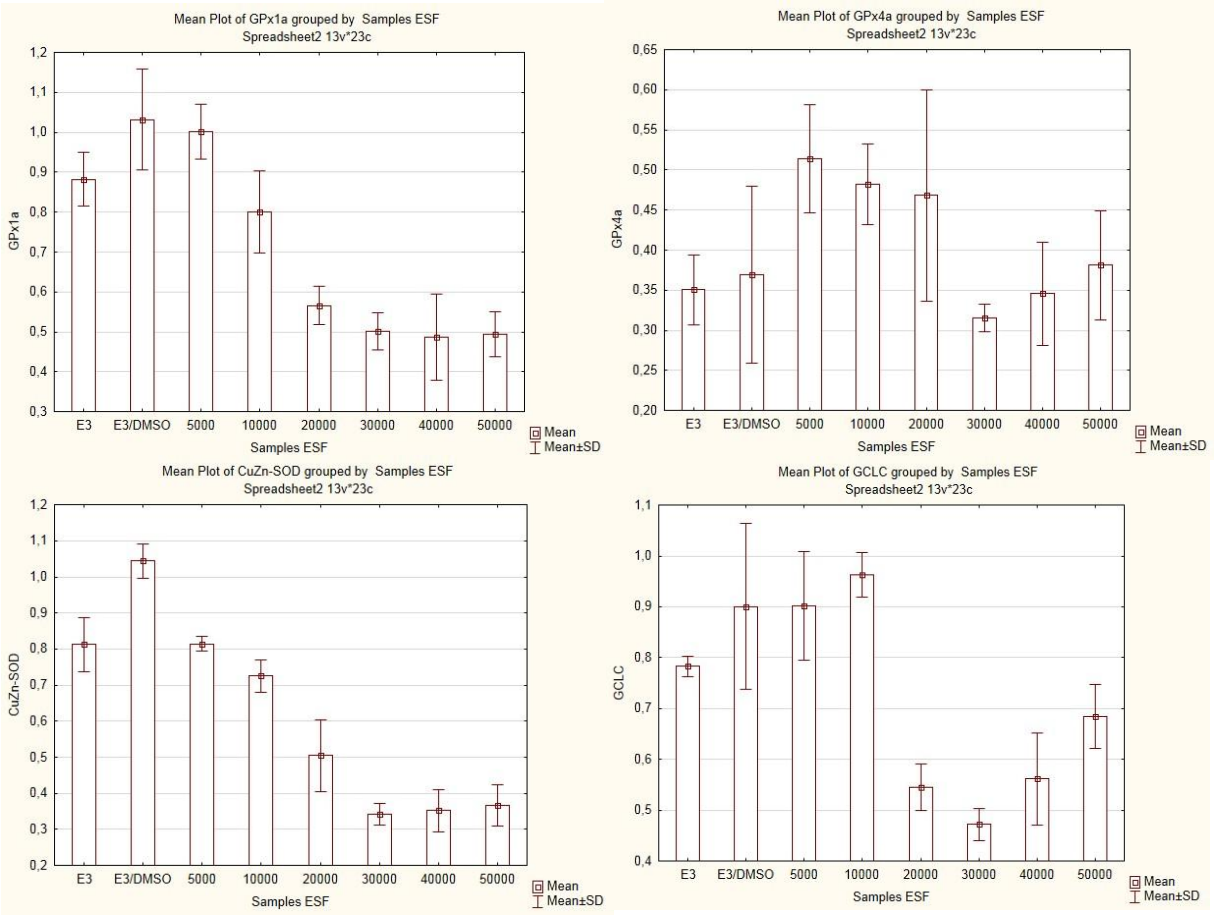

Figure 5.10: Realtime quantitative PCR results of the genes *gpx1a*, *gpx4a*, *CuZn-sod* and *gclc* on ESF exposed embryos/larvae.

The figures (5.10) of qPCR results, shows the mean normalized expression (MNE) levels of the genes *gpx1a*, *gpx4a*, *gclc*, *CuZn-sod*, *vtg1* and *cyp1a1*. The ESF exposure affects *gpx1a* showing a clear downregulation from the control groups E3 and E3/DMSO. The average of the control groups is set to 1, indicating fold increase or reduction. The MNE levels of the controls and the 5000 µg/L was at 0.9-1. There is a fold reduction down to 0.8 at 10,000 µg/L. At 20,000 µg/L, the fold reduction is 0.59 and the MNE level at 0.5. At the higher concentrations the expression begins to stabilize about 0.3-0.4 MNE, giving a fold reduction to 0.5. *CuZn-sod* had the same trend as *gpx1a*. The controls was close to 0.8 and 1 MNE, with a fold reduction at 0.88 to 0.8 MNE at 5000 µg/L. 10,000 had a fold reduction to 0.78 close to 0.7 MNE, while in 20,000 µg/L the fold reduction is 0.54 at 0.5 MNE. The 30,000 µg/L was down between 0.3-0.4 MNE where it continues to stay at the two higher concentrations. The fold reduction ends close to 0.4. In *gpx4a*, there is a 1.4 fold increase from the control groups to the 5000 µg/L and 20,000 µg/L groups. The MNE had increased from 0.35 to about 0.50 MNE. At the 30,000 µg/L the levels suddenly drop 0.87 fold, but then

there is a slight upregulation until the concentrations is barely above the control groups level at 50,000 µg/L. In *gclc* there is a slight upregulation from 0.8-0.9 to 0.9-1 MNE in the 5000 µg/L and 10,000 µg/L groups comparing to the controls. At 20,000 µg/L, the mRNA level suddenly drops 0.65 fold to 0.5 MNE. The 30,000 µg/L group shows the lowest MNE level, slightly below 0.5 at 0.56 fold reduction. At 40,000 µg/L the MNE level had a fold reduction at 0.67 or at 0.55 MNE. The 50,000 µg/L group is at 0.7 MNE with a fold reduction at 0.81, indicating a slight increase from the 30,000 µg/L and 40,000 µg/L. One way ANOVA, indicates significant ($p<0.05$) downregulation of *gpx1a*, *CuZn-sod* and *gclc*.

The heat map (Figure 5.9), shows that most of the genes had a similar trend, where only *gpx4a* and *vtg1* was different. Green values indicate decreased expression values, while red values indicate increased expression values. It is indicated which genes are correlated in their expression. This allows for an easier overall comparison than in the gene qPCR Figures. The most similar gene expression is between *gpx1a* and *CuZn-sod*. *Gclc* shows an almost similar expression level to *gpx1a* and *CuZn-sod*, with *cyp1a1* having a trend slightly less similar than that. *Gpx4a* is the more similar of the two genes with a different expression level, while *vtg1* being the furthest away from the trend. The heat map shows similar to the MNE level Figures, there is a reduction from 10,000 µg/L ESF in the four similar genes, with the least expression levels being close to 30,000 µg/L or 40,000 µg/L. *Gpx4a* shows a strong expression in 5000 µg/L to 20,000 µg/L, while otherwise a negative expression level. *Vtg1* is only strongly expressed at 50,000 µg/L.

5.3.3 Endosulfan exposure downregulates *cyp1a1*. *Vtg1* is unaffected at sub-lethal levels, but upregulates at lethal levels.

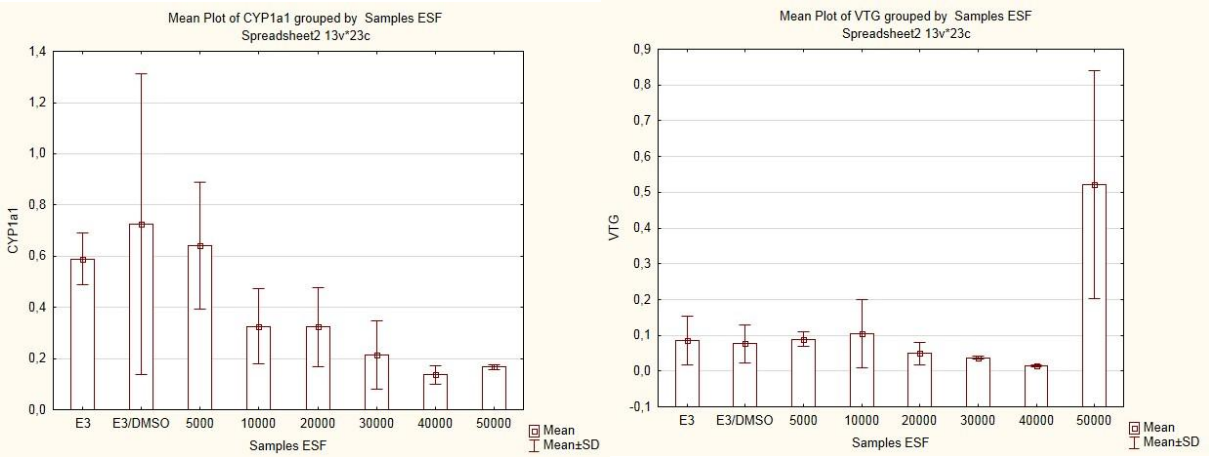

Figure 5.11: Realtime quantitative PCR of the genes *cyp1a1* and *vtg1* on endosulfan exposed embryos.

Søfteland et al. (data adapted from submitted article to Food and Chemical toxicology 2014) tested ESF and CPF exposure on hepatocytes from Atlantic salmon hepatocytes. By using their data and testing the same genes they got an effect from, a comparison can be made (see section 6.1.4).

The one way ANOVA indicated a general significant downregulation of *cyp1a1*. As seen in Figure 5.11, the 5000 µg/L group showed a MNE level close to the control groups at 0.6-0.7 MNE, but the 10,000 µg/L and 20,000 µg/L at 0.3 MNE showed a fold reduction to 0.5. In the 30,000 µg/L group, the MNE level had decreased to about 0.2 and the fold reduction to 0.33, while the two last groups had a fold reduction to 0.25 or 0.1-0.2 of MNE.

For *vtg*, MNE levels were stable between 0.1-0.2 MNE until a 6.4 fold increase at 50,000 µg/L, which was about 0.5 MNE.

5.3.4 Chlorpyrifos exposure leads to upregulation of *gclc*

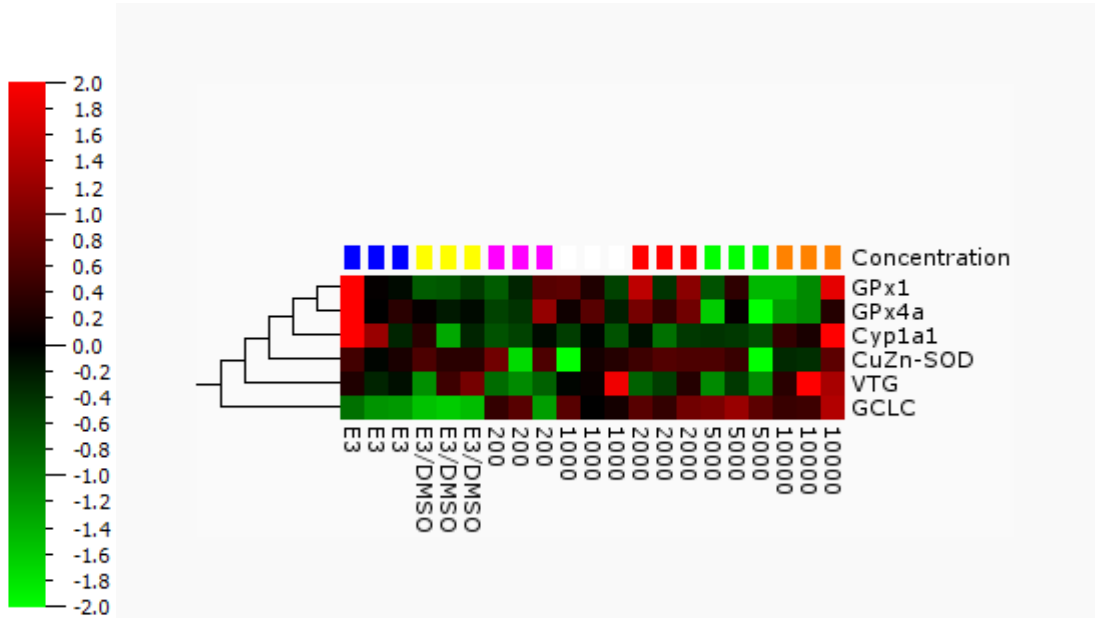

Figure 5.12: Heat map for embryos exposed to CPF. Green indicates reduced expression, while red indicates increased expression.

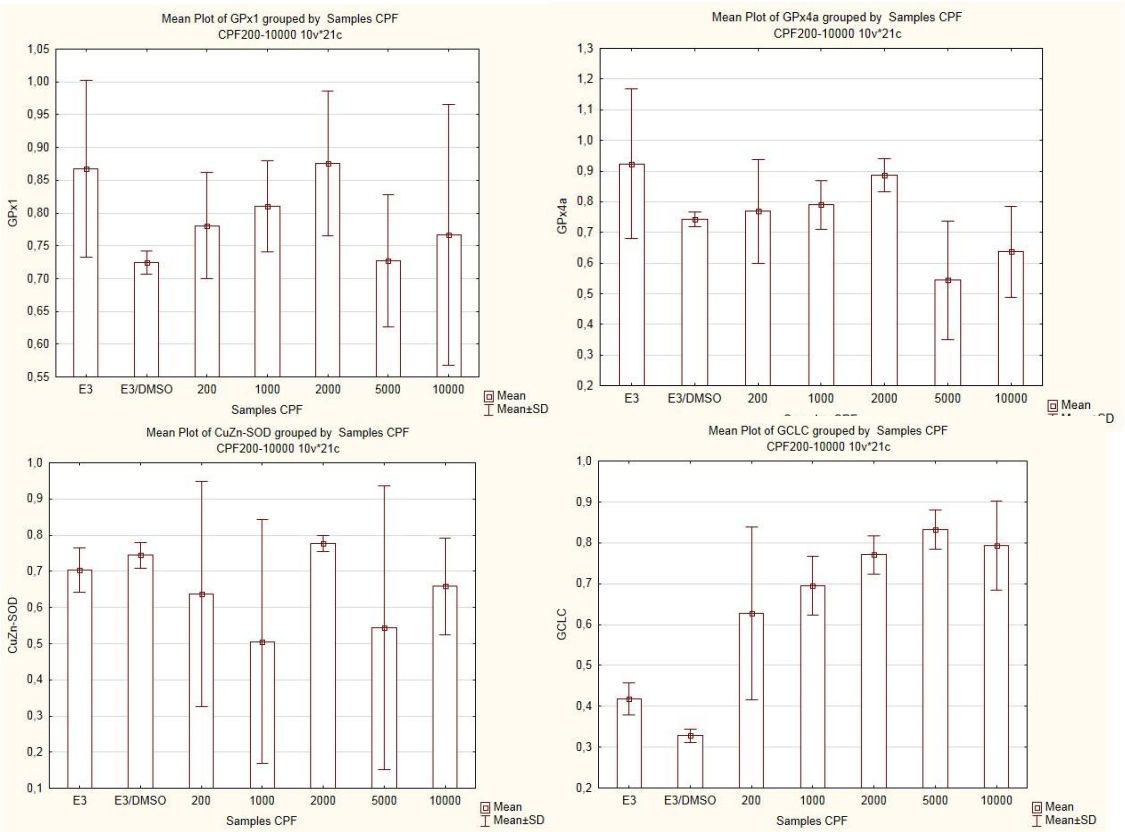

Figure 5.13: Realtime quantitative PCR results of the genes *gpx1a*, *gpx4a*, *CuZn-sod* and *gclc* on CPF exposed embryos/larvae.

As seen in Figure 5.13, *gpx1a* did not have any clear indication of regulation depending on CPF concentration. The levels look accidentally, but all the means was within 0.15 MNE. This makes the expression stable. *Gpx4a* might indicate a slight downregulation at 5000 µg/L and 10,000 µg/L as the fold was reduced to 0.64 and 0.75. The other levels were closer within 0.7 to 0.9 MNE and the fold between 0.90-1.05. *CuZn-sod* shows as *gpx1a* no clear indication of regulation depending on CPF concentration. The MNE levels were varying between 0.5 to 0.8. In *gclc* there was a significant ($p<0.05$) upregulation from the lowest concentration of CPF at about 0.6 MNE, which was 1.65 fold increased. The highest level was at 5000 µg/L with 10,000 µg/L at nearly the same level at 0.8 MNE and had a 2.2 fold increase.

The heat map of CPF (Figure 5.12) confirms the random values observed in the MNE level Figures. The expression level in most of the concentrations varies a lot between the different concentrations, but also within the same concentrations at times. Several of these genes were pretty close to each other in the MNE levels though. The Figure 5.13 shows that *gpx1a* and *gpx4a* makes a similar expression trend, with *cyp1a1* not being far off this trend. *CuZn-sod* shows only 3 strong green squares, while the rest being at almost the same level, but shows the closest trend of the remaining genes including *vtg* and *gclc*. *vtg* was a bit close to *CuZn-sod*, but with almost inverted colorization showing strong red squares instead of green. *Gclc* had a clear trend, different from all the others, with low expression values in the control groups, while showing a red squares already at 200 µg/L, and turning slightly redder at each concentration after that.

5.3.5 Chlorpyrifos exposure does not induce *cyp1a1* and *vtg1* regulation

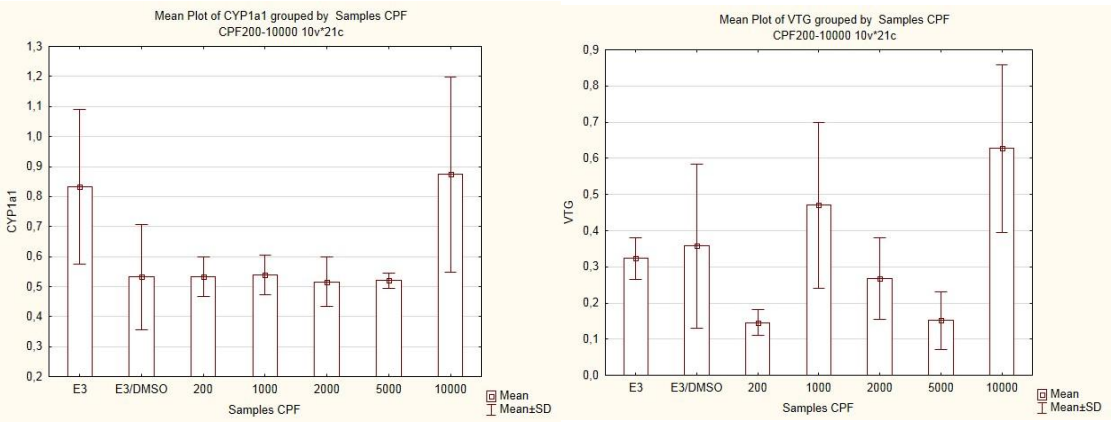

Figure 5.14: Realtime quantitative PCR of the genes *cyp1a1* and *vtg1* on chlorpyrifos exposed embryos.

As seen in figure 5.14, the E3 control group in *cyp1a1* had a higher MNE level than all the other concentrations except for the highest concentration at 10,000 µg/L CPF. These levels were at about 0.8 MNE. Then the MNE levels keep stable at 0.5 until the 10,000 µg/L CPF concentration, where it had a higher level again. As the fold was an average of the controls, the fold would indicate 0.75 reduction at all samples except at 10,000 µg/L, which would be 1.26 in increase.

For *vtg*, CPF induced no specific trend, with the MNE level having a 0.43 fold reduction from 0.35 MNE to 0.15 MNE at 200 µg/L. Then there was a 1.55 fold increase in the controls with 0.45 MNE at 1000 µg/L, followed by a 0.88 fold decrease to 0.3 MNE at 2000 µg/L. This decrease continues to 0.15 MNE at 5000 µg/L, which was a 0.5 fold reduction. In the highest concentration at 10,000 µg/L, the MNE level was higher than in any of the other concentrations, being around 0.65 and having a 2 fold increase.

6. Discussion

6.1 Discussion of results

6.1.1 Survival rates after pesticide exposure

The 14 days survivability test for ESF acts like a classic dose-response test. The higher the concentration, the earlier lethality is observed and at a higher rate; meanwhile the controls had the highest survival rate. Though a bit more surprising, after finding the approximate lethal concentrations, the CPF exposed embryos was not affected by concentrations close to the lethal dose. The embryos exposed to ESF became malformed in several degrees, in concentrations from 30,000 µg/L to 50,000 µg/L, and also got edema and hatched up until 3 days later than in controls and CPF exposed embryos. The larvae showed swimming impairment because of this malformation. This is probably the main reason why many larvae started to die after a week, as they lacked the ability to swim properly to the feed, while not having more yolk as a nutrition source. From these tests, CPF does not seem to affect the development, but kills them acutely at 20,000 µg/L, while ESF increasingly affects the development making them malformed and weaker until lethal dose is obtained. For this reason, CPF is more lethal at a lower concentration, about 20,000 µg/L, while ESF physically seems to be more hazardous even before lethal dose.

After 12 dpf, it is not uncommon that larvae can die without an apparent reason, perhaps due to internal errors, sickness or mold. Water quality is also very important. The larvae may not have properly fed yet. These may be different reasons for the big variances observed between some of the groups, and the control groups for each pesticide. However, when several larvae die at the same time in one beaker, it is more likely to be a disease or mold than lack of feed intake. This probably occurred in the control groups of the ESF exposed larvae, when more larvae died in the control groups of ESF than in all of the CPF exposed groups. Maybe it is what happened in the 200 µg/L CPF group as well, where suddenly several embryos died at the same time in two of the three beakers. It is not likely that the concentration was a causative agent here, considering the survivability of the other CPF groups and control groups.

According to WHO, the ADI for ESF for humans is about 0.006 µg/kg or 20 µg/L and tests of lake surfaces is measured to 1 µg/L or less (WHO, 2004b). According to Quinete et al., EPA set a value of water quality criteria at 56 ng/L and 8 ng/L for chronic marine wildlife exposure, and realized that several national parks in Florida showed values 2-3 times of what the EPA recommended (Quinete et al., 2013). The European Union (EU) has a limit of 1 µg/L of these pesticides. In certain rivers of India, the pesticide concentration in the water is above EUs,

but beneath the limit of WHO. ESF values in these rivers ranged between 0-0.42 µg/L in the surface water and 0.6-0.78 µg/L in the ground water (Lari et al., 2014). For CPF the ADI for is at 0.01 mg/kg, with a water quality decided at 30 µg/L (WHO, 2004a). The same rivers in India showed surface waters with a CPF range from 0.26-0.24 µg/L and ground water from 0.11-0.25 µg/L (Lari et al., 2014). In Canada there has been reported CPF values from 0.08-22 ng/L, up to 250 ng/L in the United States, while an ice cap at Svalbard which was formed between 1979 and 1986 had 16.2 ng/L CPF in it (Mackay et al., 2014). These values found in the environment are much lower than the concentrations tested in this experiment and they are low concentration chronic exposures, not high concentration acute exposures. This low concentration chronic exposure may show another effect than the acute exposure, but this would take some time to ascertain. So, a high concentration acute exposure may indicate what can happen during a sustained low concentration exposure from the pesticides.

6.1.2 Antioxidant regulation induced by pesticides

ESF downregulates the expression of genes coding for proteins requiring glutathione, which fits well according to Lu (2013), who says that the cell becomes depleted of GSH at high ROS levels. This is due to GSSG not being reduced to GSH (as seen in figure 2.4), but it is transported from the cell or it starts interacting with a sulfhydryl group, making a mixed disulfide. Otherwise, sulfur-containing amino acids, such as cysteine and methionine are susceptible to either reversible or irreversible oxidation (Dunyaporn et al., 2008) and as cysteine is required to make GSH, ROS might reduce the amount of cysteine available to make GSH.

CuZn-SOD converts superoxides (O_2^-) in the cytoplasma into H_2O_2 using Cu and Zn as cofactors. This is a fast reaction, and SOD should actively remove O_2^- until there is a lack of cofactors available to dismutate the superoxides. Thus, the decrease of *CuZn-sod* expression is probably due to excess ROS or depleted levels of cofactors. The cell may also be using resources on other vital functions in the cell. High amount of ROS affects the mitochondria as well and can lead to a decrease of ATP production (Zhang et al., 2006, Tiwari et al., 2002). This may affect GCLC as it requires ATP to produce more GSH.

For CPF exposed embryos the *gclc* gene shows an increase in upregulation when increasing the CPF concentration strength.

6.1.3 *Gclc* regulation contrast between endosulfan and chlorpyrifos exposure

There is an interesting contrast in the regulation induced by ESF and CPF, as ESF causes a clear downregulation of the gene *gclc*, while CPF leads to a clear upregulation of *gclc*. Thus, ESF leads to a reduction of the first factor of the of GSH synthesis, the Glutathione-Cysteine Ligase, while for CPF there is an increased expression rate of this factor. A consequence of this may be that the cell more actively defends against the ROS damage induced by CPF and is more able to cope with the levels of the ROS toxicity. *Gclc* is regulated by the transcription factor Nrf2, which will upon oxidation of the Keap1-Nrf2 complex, split off and activate *gclc* expression (see figure 2.3) (Harvey et al., 2009). This is most likely what is seen in the CPF exposed embryos, and thus ROS is being more or less under control. This might be plausible as the CPF exposed embryos from the H_2DCFDA had a weaker fluorescence signal and the embryos had a better survivability; looking more healthy than embryos exposed to ESF. *Gclc* is regulated by the amount of GSH and availability of cysteine. If there is a lack of cysteine due to oxidation of cystine, GCLC will not be very functional. Clearly in ESF, the induced ROS level is greater than the cells capabilities to produce enough antioxidants. This is most likely overwhelming the cells, oxidizing DNA, RNA, proteins and lipids and triggering cell death. This could influence survival rates, where the high concentrations clearly induced malformations on the larvae.

6.1.4 *Cyp1a1* is downregulated by endosulfan while *vtg1* seems mostly unaffected

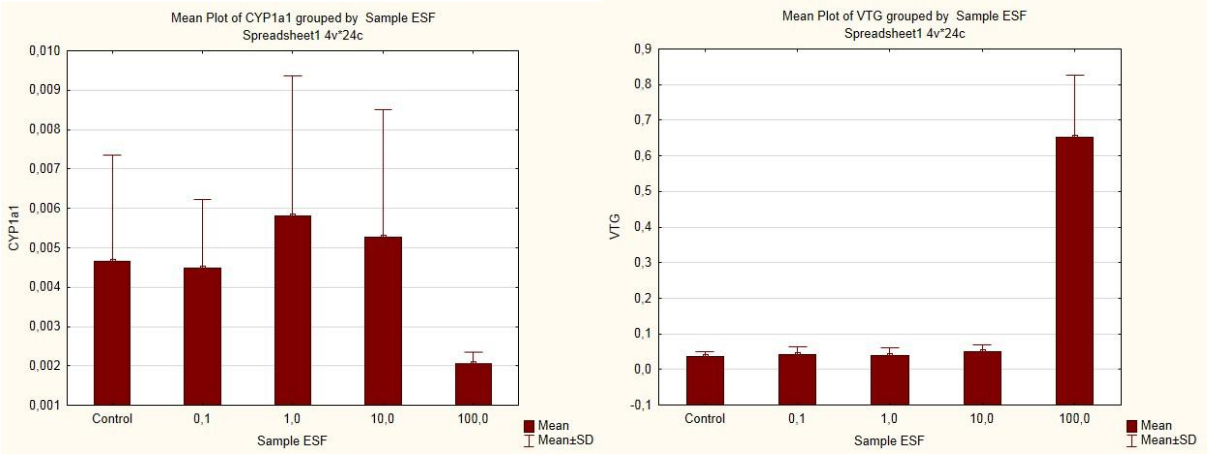

Figure 6.1: Expression of *cyp1a1* and *vtg* in endosulfan exposed Atlantic salmon hepatocytes. This figure is based data adapted from Søfteland et al., submitted to Food and Chemical toxicology 2014. The concentrations with the zebrafish was in µg/L, while the cell experiment was µM and 1 µM = 406.93 µg/L.

Søfteland et al. (article submitted to Food and Chemical toxicology 2014) compared the same pesticides on hepatocytes extracted from Atlantic salmon. They found that the pesticides had a regulatory effect on *cyp1a1* and *vtg*. Using their data, a comparison can be made. However, the hepatocytes were treated with concentrations in µM, not µg/L. For comparison in the Figures 5.11 and 6.1, 1 µM ESF equals 406.93 µg/L and in the Figures 5.14 and 6.2, 1 µM CPF equals 350.59 µg/L. The hepatocytes were cultured until 36-40 hours before exposure to pesticides for 24 hours. The zebrafish embryos were exposed to stronger ESF concentrations than the cells, but the cells have a wider exposure range due to lower concentrations.

For ESF the trends in the zebrafish embryos (Figure 5.11) and the hepatocytes (Figure 6.1) look similar. In the hepatocytes, there was a slight fold increase to 1.2 before a significant ($p<0.05$) fold reduction down to 0.44 at the highest concentration at 100 µM (=40693 µg/L). In both experiments, the *vtg* looks similar, as there was a massive spike of *vtg* in the highest concentrations tested. For *vtg* in the hepatocytes, the level was stable around 0.03 MNE until the 100 µM concentration where there was a 17.3 fold increase with the level was around 0.65 MNE.

In ESF exposed embryos, the *cyp1a1* expression is clearly downregulated. According to Morel and Barouki (1998), this is due to oxidation on nuclear factor 1 (NF1) which affects regulation of Tumor necrosis factor α (TNFα) through the antioxidant pyrroldine dithiocarbamate. Without this antioxidant acting as an inhibitor for TNFα, TNFα will inhibit the expression of Cyp1a1. *Vtg1* does not seem to be affected until the highest concentration of ESF tested, where the amount of *vtg1* is significantly higher. As VTG1 is not normally produced and detected in males and is at very low levels in immature females, the detected levels of mRNA might be quite low. *Vtg1* is a biomarker for endocrine disruption as it is known to be stimulated by ER interactions and is only expressed in juveniles and males when stimulated (Matozzo et al., 2008, Muncke and Eggen, 2006). The high level of *vtg1* in high concentration of ESF might be due to the ESF endocrine disruptor abilities, as it is a known endocrine disruptor (Briz et al., 2011).

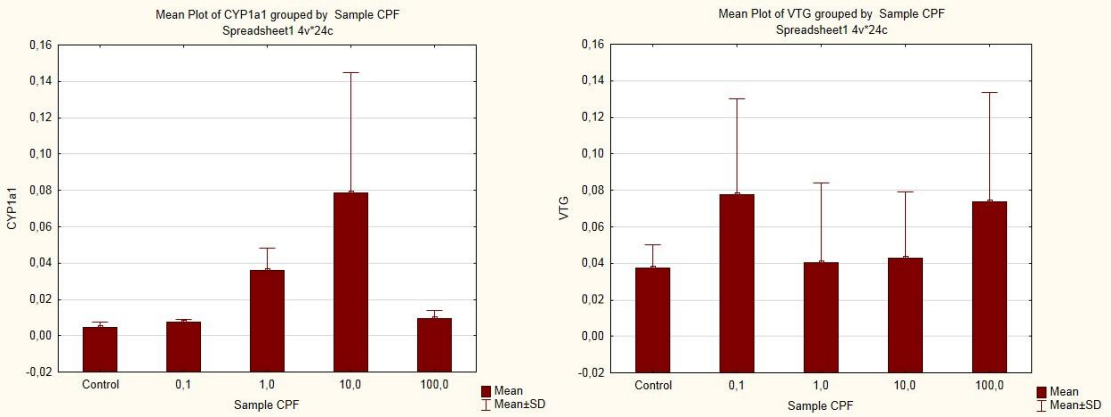

Figure 6.2: Expression of *cyp1a1* and *vtg* based on chlorpyrifos exposed Atlantic salmon hepatocytes. This figure is based on data adapted from Søfteland et al., submitted to Food and Chemical toxicology 2014. The concentrations with the zebrafish was in µg/L, while the cell experiment was µM and in 1 µM = 350.59 µg/L.

The CPF exposed embryos (Figure 5.14) did not have the same trend as the hepatocytes had (figure 6.2). In Søftelands data, *cyp1a1* had a clear upregulation between 0.1 µM at 0.01 MNE and 1 µM with 0.04 MNE, with a 7.7 fold increase. At 10 µM the MNE level was about 0.08 which was a 16.8 fold increase, but at 100 µM, the mRNA level was down to only a 2 fold increase. In both the zebrafish and hepatocyte experiment, *vtg* shows no regular pattern. In the hepatocytes at 1 µM and 10 µM, the levels was close to the control groups at 0.4 MNE, but at 0.1 µM and 100 µM the MNE level was a 2 fold increase to around 0.8 MNE.

The embryos died at 20,000 µg/L in the dose-response test of CPF, so 100 µM (=35,059 µg/L) would have been fatal for the embryos. In CPF exposed embryos, there was not a clear indication of any regulation of the genes. According to Figure 5.14, the DMSO had an effect on the expression of *cyp1a1* compared to the E3 control group. In Søftelands data though, there was a clear upregulation of *cyp1a1*, and it may be what is happening in the embryos as well, but the concentration range may be outside of the ideal range and the upregulation would be even closer to the lethal dose. CPF does not show any special influence on *vtg1* neither in the zebrafish nor in the hepatocytes cells.

6.1.5 H_2DCFDA treatment on pesticide-exposed embryos indicates general ROS

Treatment with H_2DCFDA on whole embryos did work well. In ESF exposed embryos there is a generally stronger fluorescent signal in the head region, but location in the head region varies between individuals. In CPF exposed embryos the fluorescent signal usually seemed to be stronger around the back of its head and in their tails, but auto fluorescence in the tail can also occurred, and was observed in some control groups. In general, the heart region always become very fluorescent with the treatment of the embryos exposed to these pesticides. Thus the larva's blood has probably become oxidized.

6.1.6 Zebrafish embryos as an *in vivo* toxicology model system

The zebrafish is an ideal model system for toxicology; and it is excellent to determine development effects of these pesticides, though there are specific differences among the species. Therefore, these obtained values will not be the same as for other organisms. Zebrafish are very well studied with its genome characterized. This makes it easier to determine what kind of adverse effects that can occur due to the pesticides, and allows work to be done in a much shorter timeframe. These tests will make a good indicative effect on how the pesticides may affect other fish, animals and humans.

In other aspects, some important factors for this experiment has been the timeframe chosen for the pesticide exposure, how well the compounds are transported through the chorion and the fact that the embryos had been submerged in the pesticide solutions and the exposure has not been through dietary means. The timeframe in this experiment has been 6 hpf to 72 hpf. Duration change or start of exposure during other development stages could have another effect than seen in this experiment. The experiment has been based on embryos being submerged in the pesticide solutions and not trough the diet. However, a dietary pesticide test would not show immediate effects of the pesticides, would require more time of testing and would not allow us to determine developmental effects before the larvae is developed enough for it to feed. Thus, alterations of development due to the pesticides from a feeding trial would have to be compared to offspring from parents fed on pesticides. For the embryos, the chorion is a good shield for unwanted particles at its critical states of development. The toxicity induced from a compound might be altered due to this shield, depending on how easily it is transported across the membrane (Braunbeck et al., 2005).

6.1.7 Future studies

Several questions about the effects of ESF and CPF remains unanswered and some further studies could be done to try determining more about them. Some of these studies could look at the neurotoxicity aspects of ESF and CPF and how they affect zebrafish development. In addition, one could try to determine in what tissues the pesticides mostly affect, using *in situ* hybridization and genes related to antioxidants or the neurotoxic targets like AChE for CPF.

A vital study for the future would lie within the fact that there is quite a difference in the toxicity from the exposure of a diet and by being submerged in a pesticide solution. A feeding trial would be able to tell us more about the chronic effects of the pesticides and how the zebrafish and their offspring would handle the exposure. By the feeding trial, the feed may also have antioxidants added to them, to see if the antioxidants would be able to alleviate the ROS effects induced by the pesticides. The role of the antioxidants would be an important factor as a counteragent to reduce the risks of the pesticide exposure.

6.2 Conclusions

Through the dose-response test and the survival rate test, chlorpyrifos is the more lethal pesticide for zebrafish embryos as it requires a lower concentration to kill the embryos acutely, seen at 20,000 µg/L. However, these tests did not show any visible effects on the sub-lethal concentrations like malformations and observed behavior did not seem to differ from control groups. Endosulfan had a higher concentration where it killed acutely, and at 50,000 µg/L was still alive after exposure from 6 hpf to 72 hpf. However, at the same day as exposure ended the embryos died. ESF induced malformations in concentrations above 20,000 µg/L and larvae seemed less respondent to stimuli at higher concentrations. ESF also showed a much higher response to the H_2DCFDA than CPF, indicating that ESF induces more oxidative stress. H_2DCFDA indicated general oxidative stress that was dependent on concentrations and fluorescence was usually strongest in the head region and in the tail in the larva's tissue. That ESF is more oxidative is backed up by qPCR results, where ESF caused a down regulation on genes related to antioxidants. Embryos exposed to CPF had stable expression of antioxidant related genes with the exception of an upregulation of *gclc* that might indicate more synthesis of glutathione.

7. References

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