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UNIVERSIDADE DO PORTO

CHEMOSENSITISATION ASSESSMENT OF EMERGENT AND ORGANIC POLLUTANTS USING ZEBRAFISH (*DANIO RERIO*) EMBRYO TESTS

Virgínia Filipa Pereira Monteiro da Cunha

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Chemosensitisation assessment of emergent and organic pollutants using zebrafish (*Danio rerio*) embryo tests

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Orientador – Doutora Marta Sofia Sá Ferreira

Categoria – Investigadora Auxiliar

Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)

Coorientador – Doutor Miguel Santos

Categoria – Professor Auxiliar Convidado

Afiliação – Faculdade de Ciências da Universidade do Porto.

Coorientador – Professor Doutor Pedro Moradas-Ferreira

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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“ Um sonho é um escrito,
e muitos escritos não são mais do que sonhos.“
(Umberto Eco)

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Constam nesta tese os artigos já publicados, que a seguir se discriminam:

1. Cunha V, Santos MM, Moradas-Ferreira P, Ferreira M. Simvastatin effects on detoxification mechanisms in Danio rerio embryos. Environ Sci Pollut Res Int 2016 DOI: 10.1007/s11356-016-6547-y

ABSTRACT

In the aquatic environment fish and other aquatic organism are likely to be exposed and impacted by different chemicals like pharmaceuticals and personal care products (PPCPs). Nevertheless, organisms have detoxification mechanisms that play a key role in the response to anthropogenic chemicals. Therefore, characterising these detoxification systems is of high ecotoxicological importance as a step towards understanding species-specific sensitivity and the chemical's mode of action. Fish and other vertebrate have developed different defences that biotransform (phase I and II biotransformation enzymes and antioxidant enzymes) and eliminates (phase 0 and III ATP-Binding Cassette (ABC) proteins) potentially toxic chemicals impacting their effects, accumulation and excretion. However, some compounds can constrain the efflux transporters inhibiting or decreasing the multixenobiotic resistance mechanism (MXR) activity leading to an increase in the accumulation of xenobiotics normally effluxed from the cells, in a process called chemosensitisation. It is also important to understand the regulation of genes encoding for these proteins involved in the detoxification system. In mammals, several nuclear receptors (NRs) have been identified as regulators of these defence mechanisms, but in fish little is known about its regulation. Given the importance of the detoxification system and their regulation by NRs as well as the potential chemosentisation effect of numerous environmental chemicals, this study was designed to better understand the interaction of emerging compounds with these defence mechanisms. This was approached using a) *in vivo* exposure of *Danio rerio* embryos with two highly prescribed pharmaceuticals (simvastatin, SIM and fluoxetine, FLU); and, b) in *vitro* assays with recombinant Abcb4 zebrafish protein exposure to different classes of pharmaceuticals and PCPs.

The transcription of genes encoding for NRs, biotransformation and antioxidant enzymes and ABC efflux transporters in zebrafish embryos, was determined in different development stages. With the exception of *abcc2*, mRNA levels of all target genes were detected at all development stages and therefore seem to be from maternal origin.

The development of zebrafish embryo was disrupted by FLU at environmentally relevant concentrations, while SIM showed to be more toxic to zebrafish embryo in the presence of ABC transporters inhibitors.

To assess the effect of SIM and FLU in the detoxification system, transcription of NRs (*raraa, rarab, rarga, pparαa, pparβ1, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb* and *ahr2)*, ABC proteins (*abcb4, abcc1, abcc2, abcg2a)*, biotransformation (*cyp1a1, cyp3a65, gstπ)* and antioxidant enzymes (*Cu/Zn sod, cat)* was evaluated after embryo exposure.

After 80 h exposure *pxr* and *ahr* were down regulated by SIM, while *rarab* was up regulated. Transcription of *abcb4, abcc1, gstπ* and *Cu/Zn sod* were up regulated and *cyp3a65* and *cat* were down regulated. FLU exposure led to a down regulation of all NRs, biotransformation and antioxidant enzymes and efflux protein genes with exception of *pxr* and *rxrga.* The activity of EROD, GST, SOD and CAT was also evaluated. Exposure to SIM increased EROD and GST activity and decreased SOD and CAT activity, while FLU also decreased SOD but increased CAT activity. These results show an effect of these pharmaceuticals in the transcription of NRs, especially *pxr* and *ahr* known to regulate expression of genes involved in the detoxification mechanism, mainly biotransformation genes and consequently its enzyme activity, as shown by the increased EROD activity after SIM exposure. NRs are regulators of embryo development and the disruption of their expression, as observed in the presence of these pharmaceuticals could be linked to the abnormal development and increased mortality observed. To evaluate if SIM and FLU act in conserved pathways between fish and mammals, specific interactions with lipid and neurotransmitters systems were also evaluated. SIM changed the transcription of *ppars* nonetheless it did not affect the total Chol levels. FLU decreases the transcription of important neurotransmitters transporters and receptors (*sert, dat, 5-ht2c, drd2b adra2b* and *adra2c*) even at concentrations found in the environment.

The chemosensitisation potential of different classes of PPCPs was evaluated by measuring the MXR activity on embryos and the ATPase activity of recombinant zebrafish Abcb4. The ATPase assay is a highly specific and artificial assay that examine chemical interaction with Abcb4, distinguishing between substrates and inhibitors whereas the accumulation of Rhodamine 123 - RH123, a fluorescent ABC protein substrate, is a less specific assay since RH123 is substrate to different efflux transporters. Despite being less specific the MXR assay is an *in vivo* assay therefore gives more information about relevant effect concentrations. Sertraline, fluoxetine, diclofenac, simvastatin and isoeugenol inhibited Abcb4 ATPase activity. Musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde and α-hexylcinnamaldehyde and simvastatin demonstrated Abcb4 substrates properties. Most of the PPCPs tested displayed an interaction with the MXR mechanism and inhibited ABC proteins, although at different concentrations. Importantly, isoeugenol inhibited the MXR system at environmentally relevant concentrations. Overall, to try to identify which efflux proteins are being inhibited the accumulation assay and ATPase assay should be used together. Accumulation assays should be used for initial monitoring, followed by ATPase assay where specific interactions between the chemicals and ABC proteins can be observed to establish the mode of action of these chemicals.

Taken together, the results highlight the key protective role of the detoxification mechanism against xenobiotic insults in fish. The disruption of defence genes transcription led to development abnormalities and mortality of zebrafish embryos. Our results also provide further insights on the modulation of NRs by PPCPs and impact on transcription of detoxification genes, mainly modulation of *pxr* and *ahr* by SIM and FLU impacting *cyps* mRNA expression. PPCPs also disturb zebrafish MXR activity, and some exhibited chemosensitising potential.

The results demonstrate the importance of monitoring of the presence of PPCPs in the aquatic environments since: the increase in development abnormalities and mortality by PPCPs can have an impact in organisms at population level; the deregulation of important systems can affect the development, feeding, foraging or matting behaviours that have implications on the population survival; also the energy expenditure used by the organisms to metabolize and eliminate these compounds may decrease the animal fitness and their capability to reproduce, grow or survive in polluted environments.

RESUMO

No meio aquático peixes e outros organismos são susceptíveis de estar expostos a diferentes químicos como fármacos e produtos de uso pessoal (PPCPs). Todavia, estes organismos possuem mecanismos de destoxificação que desempenham um papel fundamental na resposta contra químicos antropogénicos. Portanto, a caracterização destes sistemas de destoxificação é de grande importância ecotoxicológica sendo mais um passo para a compreensão espécie-sensibilidade específica e o modo de ação dos químicos. Peixes e outros vertebrados desenvolveram diferentes defesas que biotransformam (enzimas de biotransformação de fase I e II e enzimas antioxidantes) e eliminam (proteínas ATP-Binding Cassette (ABC) de fase 0 e III) químicos potencialmente tóxicos tendo impacto nos seus efeitos, acumulação e excreção. Contudo, alguns compostos podem constranger os transportadores de efluxo inibindo ou diminuindo a actividade deste mecanismo de resistência multi-xenobiótica (MXR) tornando possível a acumulação de xenobióticos que normalmente são efluxados das células, num processo chamado de quimiosensibilização. De igual forma é importante entender a regulação de genes que codificam proteínas envolvidas no sistema de destoxificação. Em mamíferos, muitos recetores nucleares (NRs) foram identificados como reguladores destes mecanismos de defesa, mas nos peixes pouco se sabe sobra a sua regulação. Dada a importância do sistema de destoxificação e a sua regulação por NRs assim como o potencial efeito de quimiosensibilização de inúmeros químicos ambientais, este estudo foi delineado para melhor se entender a interacção entre compostos emergentes e estes mecanismos de defesa. Para isto a) exposições *in vivo* de embriões de *Danio* rerio a dois fármacos altamente prescritos (sinvastatina, SIM e fluoxetina, FLU; e b) ensaios *in vitro* com proteína recombinante Abcb4 de peixe zebra com exposição a diferentes classes de fármacos e PCPs.

A transcrição de genes que codificam para os NRs, enzimas de biotransformação e antioxidantes e os transportadores de efluxo ABC em embriões de peixe zebra foram determinados em diferentes estádios de desenvolvimento. Com a exeção do *abcc2*, foi detetado mRNA de todos os genes alvo em todas as fases de desenvolvimento e por isso parecem ser de origem materna.

O desenvolvimento dos embriões de peixe zebra foi afectado pela FLU em concentrações que são ambientalmente relevantes, enquanto a SIM mostrou ser mais tóxica para os embriões na presença de inibidores dos transportadores ABC.

Para avaliar os efeitos da SIM e da FLU no sistema de destoxificação, a transcrição dos NRs (*raraa, rarab, rarga, pparαa, pparβ1, pparγ, pxr, rxraa, rxrab, rxrbb,*

rxrga, rxrgb e ahr2), proteínas ABC (*abcb4, abcc1, abcc2, abcg2a*), enzimas de biotransformação (*cyp1a1, cyp3a65, gstπ*) e antioxidantes (*Cu/Zn sod, cat*) foi avaliada em embriões após a exposição. Após 80 h de exposição à SIM, a expressão génica do *pxr* e *ahr* foi diminuída, enquanto que se observou um aumento do *rarab*. A transcrição de *abcb4, abcc1, gstπ* e *Cu/Zn sod* foi aumentada e a do *cyp3a65* e da *cat* decresceu. A exposição à FLU levou à diminuição dos níveis de expressão de todos os genes que codificam para NRs, enzimas de biotransformação e antioxidantes e das proteínas de efluxo com a exceção do *pxr* e do *rxrga*. A atividade das enzimas EROD, GST, SOD e CAT também foi avaliada. A exposição à SIM aumentou a actividade da EROD e da GST e diminuiu a actividade da SOD e da CAT, enquanto a presença da FLU também diminuiu a atividade da SOD mas aumentou a atividade da CAT. Estes resultados mostram um efeito destes fármacos na transcrição de NRs, especialmente *pxr* e *ahr* reconhecidos por modular a expressão de genes envolvidos no mecanismo de destoxificação. Principalmente ao nível da expressão dos genes de biotransformação e subsequente actividade enzimática, conforme observado pelo aumento da actividade da EROD. Os NRs são reguladores do desenvolvimento embrionário e a disrupção da expressão observada na presença destes fármacos podem estar correlacionadas com o desenvolvimento anormal e aumento de mortalidade observada nos embriões. Para avaliar o efeito da SIM e da FLU nas vias conservadas entre peixes e mamíferos, foram avaliadas interações específicas com o sistema lipídico e neurotransmissor. A SIM alterou a transcrição dos ppars contudo não afetou o níveis totais de colesterol. A FLU diminuiu a transcrição de importantes transportadores e receptores neurotransmissores (*sert, dat, 5-ht2c, drd2b adra2b* and *adra2c*) mesmo em concentrações encontradas no meio ambiente.

O potencial de quimiosensibilização das diferentes classes de PPCPs foi avaliada medindo a actividade de MXR nos embriões e a atividade de ATPase com proteína recombinante Abcb4 de peixe zebra. O ensaio da ATPase é um ensaio muito específico e artificial que avalia a interacção com a Abcb4, distinguindo entre substratos e inibidores enquanto que a acumulação de Rodamina 123 - RH123, um substrato fluorescente das proteínas ABC, é um ensaio menos especifico uma vez que a RH123 é substrato de diferentes transportadores de efluxo. Apesar de ser menos específico o ensaio MXR é um ensaio in vivo e por isso é mais informativo sobre a relevância das concentrações de efeito. Sertraline, fluoxetine, diclofenac, simvastatin e isoeugenol inibiram a atividade da Abcb4 ATPase. Musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde, α-hexylcinnamaldehyde demonstraram propriedades de substrato para a Abcb4. A maioria dos PPCPs testados mostraram a capacidade de interagir com o mecanismo MXR e

inibiram proteínas ABC embora em concentrações diferentes. Um resultado relevante foi a capacidade do isoeugenol inibir o sistema MXR em concentrações ambientalmente relevantes. No geral, para tentar identificar quais proteínas de efluxo estão a ser inibidas ensaios de acumulação e ATPase devem ser usados em simultâneo. Os ensaios de acumulação devem ser usados para uma monotorização inicial, seguidos dos ensaios de ATPase onde interacções mais específicas entre os químicos e as proteínas ABC podem ser detectadas e o modo de acção destes químicos estabelecido.

Em conjunto, estes resultados destacam o papel importante do mecanismo de destoxificação nos efeitos dos xenobióticos em peixes. A disrupção da transcrição dos genes de defesa levaram ao desenvolvimento de anomalias e aumento da mortalidade nos embriões de peixe zebra. Os nossos dados também fornecem novas perspetivas sobre a modulação dos NRs pelos PPCPs e o seu impacto na transcrição nos genes de destoxificação, principalmente na modulação do *pxr* e *ahr* pela SIM e FLU e o seu efeito na expressão génica dos *cyps*. Os PPCPs também alteraram a atividade de MXR dos peixes zebra, e que alguns exibem potencial de quimiosensibilização.

Os resultados demonstram a importância de monitorização da presença de PPCPs no meio aquático uma vez que: o aumento de anomalias no desenvolvimento e mortalidade dos organismos pelos PPCPs pode ter impacto a nível populacional; a desregulação de sistemas vitais pode afetar o desenvolvimento, comportamentos de alimentação, procura e de acasalamento, que tem implicações na sobrevivência da população; também o dispêndio de energia usada pelo organismo para metabolizar e eliminar estes compostos pode decrescer o *fitness* do animal e a sua capacidade de reproduzir, crescer ou sobreviver em ambientes poluídos.

OUTLINE OF THE THESIS

The thesis is organized in 8 chapters.

Chapter I is an introduction to contextualize the state of art of the key topics within the thesis.

Chapter II comprises the aims of the thesis and explains how these articulate with the subsequent experimental results presented.

Chapter III to VII contain the main studies performed, including materials, methods, results and discussion. For each study, information concerning the journal and date of publication (for published papers) / co-authors is provided.

Chapters VIII include a general discussion and main conclusions of the thesis, highlighting the most relevant achievements and also the presenting prospects for future work.

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ABC Adenosine tris-phosphate Binding Cassette

AhR Aryl-hydrocarbon Receptor

ANOVA Analysis of variance

Arnt Aryl-hydrocarbon Receptor translocator

ATP Adenosine Tris-Phosphate

BCRP Breast Cancer Resistance Associated Protein

CAR Constitutive Androstane Receptor

CAT catalase

cDNA Complementar Deoxyribonucleic Acid

CDNB 1-chloro-2,4-dinitrobenzene

CYP Cytochrome P-450

DBD DNA-binding C-domain

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DNTPs Deoxyribonucleotide

EDTA Ethylenediamine tetraacetic acid

EF1 Elongation factor 1

EPA United States Environmental Protection Agency

EROD Ethoxyresorufin-O-deethylase

FET embryo toxic assay

FLU fluoxetine

FXR Farnesoid X Receptor

GSH Glutathione

GSTs Glutathione- S-Transferases

HMG-CoA 3-hydroxy-3-methylglutaryl-coenzymeA

hpf hours post-fertilization

K_{ow} Octanol-Water Partition Coefficient

LBD C-terminal ligand-binding E domain

LOEC Lowest Observed Effect Concentration

LXR Liver X Receptor

MAO Monoamine oxidase

MDR Multidrug Resistance

mRNA Messenger Ribonucleic Acid

MRPs Multiresistance Associated Proteins

MSDs Membrane Spawning Domains

MW Molecular Weight

MXR Multixenobiotic Resistance

MZT Maternal to zygote transition

NADPH Nicotinamide Adenine Dinucleotide Phosphate-Oxidase

NBDs Nucleotide Binding Domains

NCBI National Centre for Biotechnology Information

NET Norephinephrine Transporter

NSAIDs Non-steroidal anti-inflammatory drugs

PCR Polymerase Chain Reaction

P-gp Permeability glycoprotein

PPAR Proliferator activated Receptor

PPCPs Pharmaceuticals and Personal Care Products

PXR Pregnane X Receptor

qRT-PCR Quantitative reverse transcription polymerase chain reaction

RNA Ribonucleic acid

RAR Retinoid acid Receptor

RXR Retinoid X Receptor

SER sertraline

SERT sertraline transporter

SIM simvastatin

SOD superoxide dismutase

SSRIs Selective Serotonin Reuptake Inhibitors

UV Ultraviolet

VMAT Vesicular monoamine transporter

WWTP waste water treatment plants

XRE Xenobiotic Responsive Elements

CHAPTER I

INTRODUCTION

1.1 General introduction

The aquatic environment is constantly being loaded with different chemicals, exposing the organisms to their possible deleterious effects. These chemicals appear in the environment from several routes such as surface runoff and treated and non-treated waste waters from households and industry that are directed into streams. Even though waste waters are mostly treated in sewage treatment plants, the elimination of many contaminants, such as pharmaceuticals compounds and personal care products (PPCPs), is still an incomplete process (Zuccato et al., 2006, Larsen et al., 2004, Soares et al., 2008). Once these compounds reach the aquatic ecosystem, the main concern is how these xenobiotics will impact aquatic organisms. This will depend on the physico-chemical and toxicological properties of the compounds but also the organisms ability to deal with these compounds and its defences. To contradict the unwanted effects of these chemicals, vertebrate organisms have well developed cellular detoxification mechanisms. Proteins involved in these detoxification processes include efflux transporters (ATP – binding cassette (ABC) transporters), phase I (cytochrome P450 family, eg. CYP1A, CYP3A) and phase II (glutathione-S-transferase, (GST), UDP-glucuronlytransferases (UGTs), Sulfotransferases (SULTs)) biotransformation enzymes, and antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT)) (Van der Oost et al., 2003; Bard, 2000; Stegeman et al., 1992; Lopez-Torres et al., 1993). In addition to these defence systems it is also relevant to understand the regulation of genes encoding for the proteins involved in the detoxification system. Nuclear receptors (NRs) are a family of key regulators, modulating several biological pathways including cellular detoxification, lipid metabolism, reproduction or development (Xu et al., 2005; Chai et al., 2013). In mammals pregnane X receptor (PXR), peroxisome proliferated activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), retinoid X receptor (RXR), constitutive androstane receptor (CAR) and glucocorticoid receptor (GR), among others, have been identified as key regulators of this important defence mechanism (Xu et al., 2005; Pavek and Smutny, 2014). Nonetheless, in fish there is still scarce information on NRs and their role in regulation of the detoxification system even though the evidences points to similar regulatory mechanisms as in mammals. There are numerous contaminants typically present in complex environmental mixtures that might interfere with regulatory mechanisms or NRs. These interactions can disturb the regulation of different defence and metabolic processes which can impair fish survival and ecosystems regeneration for long term. For this reason is of crucial importance to study the mode of action of emerging environmental contaminants, like pharmaceuticals (Fent et al., 2006), personal care

products (PCP) (Peck, 2006) and hazard and noxious substances (HNS), being the last two the least studied. Testing the toxicity of contaminants requires the use of vertebrates and particularly fish, when it concerns aquatic toxicology. For ethical reasons, the replacement of animal testing will be required nevertheless various alternative approaches for predicting environmental risks of chemicals are available, including analyses of structure-activity relationships or toxicity tests with cell-based systems and fish embryos (Scholz et al., 2008). In contrast to cellular replacement methods, such as fish cell lines (Schirmer, 2006), the embryo model offers a complex, multicellular system integrating the interaction of various tissues and differentiation processes. According to current European Union legislation for the protection of animal used for experimental and other scientific purposes, the use of embryonic stages of vertebrates is not regulated (Commission of the European Communities Council, Directive 2010/63/EU). Hence, experiments with embryos are considered as alternative to animal experiments (Fleming, 2007). Zebrafish, *Danio rerio*, is widely accepted as an ecotoxicological model specie, showing interesting features such as short life-cycle, embryo transparency and fully sequenced genome (Baker and Hardiman, 2014).

1.2 Pharmaceuticals and personal care products origins in the aquatic environment

New and unknown chemicals reach the aquatic systems and risk assessment has to be performed in order to protect the organisms, aquatic communities and the ecosystem. In recent years as a consequence of new and better analytical chemistry and technology advances, many emergent compounds have been detected and identified in water at low concentrations (ranging from ng/L to µg/L), including pharmaceuticals and personal care products (PPCPs) (Kim et al., 2009; Lapworth et al., 2012; Stuart et al., 2012; Jiang et al., 2013; Díaz-Cruz and Barceló, 2015). These compounds are called emerging pollutants because are usually found in low concentrations and only in the last decades have been detected in the environment, have high production and daily consumption and pose a risk to health and the environment. PPCPs found in the environment result from agricultural activities, household and industrial effluents, pharmaceutical production, and hospital, veterinary and aquaculture waste treatment stations. Waste waters ultimately will undergo purification in waste water treatment plants (WWTP), however a complete and efficient removal of contaminants is not guaranteed and PPCPs can end up in effluents, groundwater and surface water and also in drinking water (Santos et al., 2010; Lapworth et al., 2012; Jiang et al., 2013). The growing data about the presence of these compounds in aquatic ecosystems increased the number of studies focusing on the effects in non-target organisms and their environmental fate,

demonstrating their detrimental effects which make it important to further research their impact and effects on these organisms, trophic populations and the overall affected aquatic ecosystem (Ferrari et al., 2003; Brausch and Rand, 2011).

1.2.1 Pharmaceuticals

Pharmaceuticals are detected mostly in WWTPs effluents but also in surface and ground waters (Heberer, 2002; Santos et al., 2010; Verlicchi et al., 2012). Different classes of therapeutic drugs have been detected in water, including analgesic, anti-inflammatory drugs, β-blockers, antibiotics, hypolipidemics, antiepileptics and psychopharmaceuticals (Santos et al., 2010) (Fig. 1.1). Pharmaceuticals are designed to act and target metabolic pathways in humans, nonetheless, they can have the same effect in aquatic organisms with conserved pathways (Bound and Voulvoulis, 2004; Castro and Santos, 2014). The manufactures of these drugs are subject to various safety controls and approval processes, but studies with non-target organisms are often not included. Mortality, anomalies, reproduction and behavioural effects (Ferrari et al., 2003; Fent et al. 2006; Corcoran et al. 2010; Ribeiro et al., 2015; Ortiz de García et al., 2014) are detrimental effects already associated with exposure to pharmaceuticals. Ethinyl estradiol, a synthetic estrogen used in contraceptive pills, caused one of the most alarming effects which included feminization of fish (Folmar et al., 1996; Larsson et al., 1999; Jobling et al., 2002), also TBT can cause masculinization in female invertebrates (Matthiessen et al., 1998; Santos et al., 2005) as examples of reproductive disruption. Other example is the exposure to diclofenac, a non-steroid anti-inflammatory that had a lethal toxicological impact in the vulture's community in India (Oaks et al., 2004; Swan et al., 2006). Taking this information into account and the lack of studies with certain classes of pharmaceuticals, in this study the ones selected were based on their increasing consumption, detection in aquatic environments and their expected effects on conserved pathways. Thus, diclofenac (Non-steroidal anti-inflammatory drug), simvastatin (hypolipidemic drug), sertraline and fluoxetine (antidepressants) were chosen for this study (Table1).

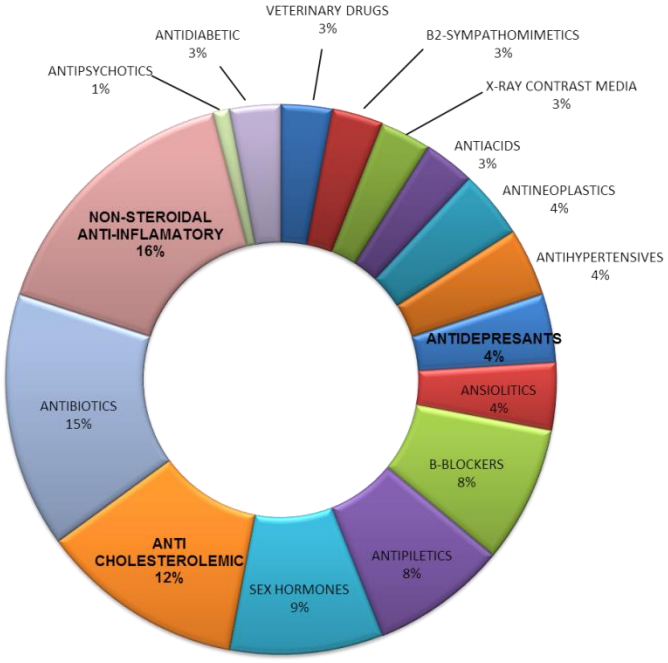
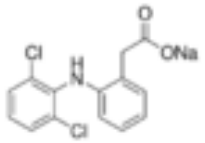
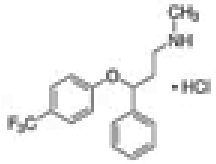
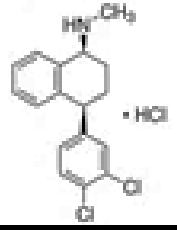
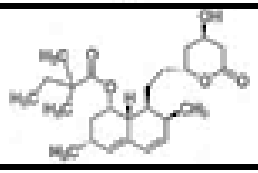


Figure 1.1: Pharmaceutical classes detected in the environment. Adapted from Santos et al., 2010. Classes at bold represent the pharmaceuticals studied in this thesis

Non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed to reduce inflammation and manage pain. They act by the inhibition of prostaglandin synthesis by inhibiting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) reducing pain and inflammation. Diclofenac is one of the most prescribed NSAIDs drugs and maybe the first one to be detected in the aquatic environment (Buser et al., 1998). NSAIDs that enter WWTPs are poorly eliminated and have been detected in water systems worldwide (Carballa et al., 2004; Santos et al., 2010). Portugal is not different from other countries and diclofenac is also one of the NSAIDs identified in water systems ranging from 3.1 to 1597 ng/L (Sousa et al., 2011; Pereira et al., 2015). In aquatic organisms, invertebrates and vertebrates, diclofenac has demonstrated the ability to induce deleterious effects like tissue damage, impaired reproduction, abnormal embryo development or oxidative stress (Schwaiger et al., 2004; Mehinto et al., 2010; Ribeiro et al., 2015; Guiloski et al., 2015; González-Ortegón et al., 2015). The changes on these parameters can in the long term compromise aquatic organisms survival, and to what extent those impacts will damage ecosystems services is yet to be determined.

Antidepressant drugs are an important group of pharmaceuticals and Selective Serotonin Reuptake Inhibitors (SSRIs) are a common class used to treat human depression, anxiety, obsessive-compulsive disorders, and panic disorders (Silverstone, 2004). SSRIs like, fluoxetine (FLU) and sertraline (SER) inhibit the reuptake of serotonin

Table 1 Names, molecular structure, molecular weights, CAS numbers, log Kow values, aqueous solubility of test pharmaceuticals

Compound	Molecular structure	MW	CAS number	Log K_{ow}	Aqueous solubility (mg/L) [chemspider.com]
diclofenac		318.1	15307-79-6	0.7	15906.5
fluoxetine		345.8	56296-78-7	4.17	3457.9
sertraline		342.7	79559-97-0	5.15	1713.5
simvastatin		418.6	79902-63-9	4.68	0.7653

by their carriers (serotonin transporter or SERT) in the presynaptic membrane, increasing serotonin concentration in the synaptic clefts, stimulating serotonergic neurotransmission (Kreke and Dietrich, 2008; Valenti et al., 2012). FLU and SER are commonly prescribed psychopharmaceuticals that can be found at low concentrations in aquatic environments (Santos et al., 2010; Metcalfe et al., 2010; Silva et al., 2014). In Portugal FLU has been detected in influents and effluents of different WWTPs in concentrations ranging from 4 to 157.4 ng/L (Silva et al., 2014). Despite the low concentrations found, studies evaluating the effect of this drug in non-target organisms are important as it prescription is increasing worldwide (Silva et al., 2012). Fish exposure to psychopharmaceuticals revealed behavioural changes such as decreased aggressiveness and feeding responses however it also impaired reproduction and mortality have been overserved (Lister et al., 2009; Santos et al., 2010; Schultz et al., 2011). Furthermore, behavioural changes in invertebrates has been reported, like reduced locomotor activity or camouflage ability impairing their ability to escape or to foraging (Fong et al., 2014; Chen et al., 2015) and in addition it was also shown to be embryotoxic (Minguez et al., 2014; Di Poi et al., 2014). Behavioural changes can have a significant impact in population dynamics that ultimately could alter the functioning of the ecosystem.

Hypolipidemic drugs are divided in two groups, statins and fibrates. Statins are one of the most prescribed pharmaceuticals for reduction of cholesterol. It acts by inhibiting 3-hydroxy-3-methylglutaryl-coenzymeA reductase (HMG-CoA) limiting the HMG-CoA conversion into mevalonate and increasing low density lipoproteins cholesterol (LDL-C) receptors and LDL-C uptake (reviewed in Fent et al., 2006).

Simvastatin (SIM) is one statin frequently prescribed to reduce mortality and morbidity from coronary heart diseases (Yang et al., 2011) and the use of this pharmaceutical is increasing in western societies due to feeding and sedentary habits. In aquatic environments SIM has been found at low concentrations in the order of ng/L (Santos et al., 2010; Ottmar et al., 2012). In Portugal SIM concentration levels found in the aquatic environment are similar to those found in other European countries ranging from 0.7 to 2052 ng/L (Sousa et al., 2011; Pereira et al., 2015). Only a few studies have addressed statins toxicity in aquatic organisms (Key et al., 2008; Ellesat et al., 2011; Neuparth et al., 2014; Ribeiro et al., 2015). Nevertheless, in fish SIM showed toxic effects, causing abnormalities in zebrafish embryos and cytotoxicity in hepatocyte cultures of marine fish (Ellesat et al., 2011; Ribeiro et al., 2015). Also to aquatic invertebrates SIM displayed toxic effects impairing growth, reproduction and gonad maturation (Neuparth et

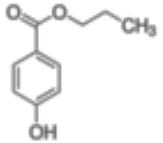
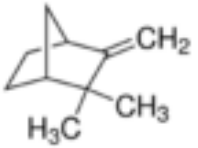
al 2014; Key et al., 2008). The impairing of organisms development and overall survival can in the long term compromise aquatic populations and damage ecosystems.

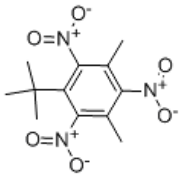
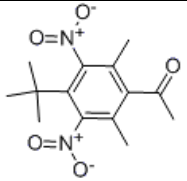
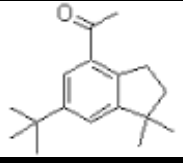
1.2.2 Personal care products

Personal care products (PCPs) are synthetic organic compounds present in different common products such as lotions, tooth paste, detergents, cosmetics, (Daughton, 2004; Ellis, 2006; Brausch and Rand, 2011). PCPs reach the aquatic environment mainly through household's waste waters. WWTPs usually are not prepared to eliminate these chemicals hence PCPs can enter the water systems (Daughton, 2004; Ellis, 2006). Some studies indicate that these compounds once present in surface waters can be eliminated naturally through volatilization, photolysis, biodegradation and/or sorption (Buerge et al., 2003, Chase et al., 2012). Nonetheless, PCPs have been detected in aquatic ecosystems (Button and Juttner, 1989; Peck and Hornbuckle, 2004; Díaz-Cruz and Barceló, 2015) and the EU Water Framework Directive has identified some of these chemicals as future emerging priority candidates for regulation and monitoring (EPA, 2012; European Comission, 2012). Additionally, some studies demonstrated that PCPs may lead to toxicity effects, and some mimic natural hormones, which can induce endocrine-disruption in aquatic organisms (Daughton and Jones-Lepp, 2001; Daughton, 2004; Ternes et al., 2004; Grung et al., 2007; Brausch and Rand, 2011), highlighting the need for a more extensive monitoring of their biological impacts.

The PCPs selected for this study were chosen based on their increasing consumption, detection in aquatic environments and lack of risk assessment information; synthetic musks (celestolide, musk xylene, musk ketone), essential oils (nerol, citronellol, isoeugenol, camphene, α-amylcinnamaldehyd, α-hexylcinnamaldehyd) and preservatives (propylparaben) (Table 2).

Table 2 Names, molecular structure, molecular weights, CAS numbers, log Kow values, aqueous solubility of test PCPs.

Compound	Molecular structure	MW	CAS number	Log K_{ow}	Aqueous solubility (mg/L) [chemspider.com]
propylparabene		180.2	94-13-3	2.71	529.3
α-amylcinnamaldehyde		202.2	122-40-7	4.33	9
α-hexylcinnamaldehyde		216.3	101-86-0	4.82	2.75
citronellol		156.3	106-22-6	3.91	105.5
camphene		136.2	79-92-5	4.22	6
isouegenol		164.2	97-54-1	3.04	165.9
nerol		154.2	106-25-2	3.47	255.8

musk xylene		297.3	81-15-2	2.25	61.35
musk ketone		294.3	81-14-1	2.11	83.59
celestolide		244.4	13171-00-1	5.93	0.22

Synthetic musks are widely used fragrances and fixative compounds found mostly in detergents, air fresheners and cosmetic and hygienic products (Luckenbach and Epel, 2005; Mottaleb et al., 2012). These musks are divided into polycyclic, nitro, macrocyclic and alicyclic musks (Arbulu et al., 2011), and although they are structurally distinct molecules, they all have the same typical musk odour. Nitro musks (such as musk xylene, musk ketone) consist of dinitro- and trinitro- substituted benzene derivatives. In Europe musk xylene and musk ketone usage is restricted but it is allowed in North America, although excluded from products for oral uptake (Luckenbach and Epel, 2005). Polycyclic musks (e.g. Celestolide) comprise indane and tetraline derivatives substituted mainly by methyl groups. Macrocyclic musks are more recent fragrances with large ringed ketones and lactones and are chemically similar to animal and plant musk odorants (Sumner et al., 2010). Alicyclic musks are also a novel class of musks and are trisubstituted cyclopentene derivative that differ from the other musk classes because they are modified akyl esters (Eh, 2004). Studies showed the presence of synthetic musks in aquatic environments ranging from 0.02 up to 5000 ng/L, as well as in aquatic species tissues such as cetaceans, fish and invertebrate organisms (Gattermann et al., 2002; Nakata, 2005; Chen et al., 2007; Chase et al., 2012; Paxeus, 1996; Peck and Hornbuckle, 2004). Studies in fish revealed musks can inhibit components of cell detoxification mechanisms, such as MXR and biotransformation enzymes (Fischer et al., 2013; Fernandes et al., 2013). In invertebrates, musks impaired larval development, reduced the growth rate and compromised multixenobiotic defence systems (Breitholtz et al., 2003; Luckenbach et al., 2004; Gooding et al., 2006).

Essential oils are concentrated, hydrophobic chemicals containing volatile aromatic compounds from several components of plants and exert diverse biological and pharmacological properties, such as antimicrobial activity (Teixeira et al., 2013). These compounds can be easily obtained by simple processes of maceration and water solution or steam distillation and are used in food preservation, fragrance industry and aromatherapy (Brophy and Doran 1996). The most important active compounds are included in two chemical groups: terpenoids (nerol, citronellol, camphene) and phenylpropanoids (isoeugenol, α-amylcinnamaldehyd, α-hexylcinnamaldehyd). Essential oils have been detected in aquatic environments in concentrations ranging from 0.38 to 121 x 103 ng/L (Button and Juttner, 1989; Belknap et al., 2006; Švestková and Vávrová, 2015). Not many studies were performed evaluating the effects of essential oils in non-target organisms, even though some results showed that these chemicals exhibited toxicity levels in Daphnia magna or Artemia salina (Conti et al., 2014; Rodrigues et al., 2013).

Parabens are antimicrobial preservatives of large spectrum, high solubility in water and stability across a wide pH range and are found in creams, lotion, shampoos, pharmaceuticals and food products (Brausch and Rand, 2011; Yamamoto et al., 2011). Some parabens species are rapidly absorbed through the skin and can accumulate in fatty components of tissues like other lipophilic compounds (Darbre et al., 2004; Tavares et al., 2009) and due to this some studies related the prolonged dermal exposure to parabens with breast cancer (Darbre et al., 2004). Among paraben species, propylparaben is one of the most commonly used in cosmetics (Brausch and Rand, 2011) and is frequently detected in the aquatic environment ranging from 7.9 to 20000 ng/L (González-Mariño et al., 2012; Albero et al., 2012; Haman et al., 2015). In aquatic organisms some studies reported a potential estrogenic activity of parabens (Yamamoto et al., 2011; Albero et al., 2012).

The frequent detection and presence of these PPCPs in the aquatic environment as well as the toxic effects already reported highlight the need for further studies regarding their effects in aquatic organisms.

1.3 Nuclear receptors and AhR – pathways for homeostasis and cellular detoxification

Aquatic organisms as other vertebrate have developed a complex biochemical defence that biotransform and eliminate potentially toxic chemicals in order to minimise associated deleterious effects (van der Oost et al., 2003; Bard, 2000; Stegeman et al., 1992; Lopez-Torres et al., 1993). Crucial to the regulation of genes involved in these detoxification mechanisms are a number of ligand-activated nuclear receptors (NRs) and the Aryl hydrocarbon receptor (AhR) (Xu et al., 2005; Chai et al., 2013). The NRs can regulate key biological functions such as development, reproduction, homeostasis, metabolism and immune function (Francis et al, 2003; Xu et al., 2005). In mammals several important regulators (xenobiotics receptors) of the detoxification system have been identified, such as, nuclear factor-erythroid 2 p45- related factor (Nrf2), pregnane X receptor (PXR), peroxisome proliferated activated receptor (PPAR), liver X receptor (LXR), farnesoid X receptor (FXR), retinoid X receptor (RXR), constitutive androstane receptor (CAR) and glucocorticoid receptor (GR) (Xu et al., 2005; Ferreira et al., 2014). In fish the information is still scarce but there are some studies that indicate some of these NRs are related with downstream genes and their role in detoxification mechanism, nevertheless more studies have to be performed to understand which NRs regulate which defence protein (Corcoran et al., 2012; Aleksunes and Klaassen, 2012; Chang et al., 2013; Wassmur et al., 2010; Cocci et al., 2013)

In humans NRs have been identified which are activated by endogenous and exogenous compounds like sex hormones, fatty acids, vitamins and drugs (le Maire et al, 2010) and since many pathways are conserved in fish one can assume that the same occurs in these organisms. NRs are divided in subfamilies depending on their dimerization and DNA-binding properties (Mangelsdorf et al., 1995; Laudet, 1997). However, they all share a common domain structure that comprehends three main domains: an N-terminal A/B domain, a central DNA-binding C-domain (DBD), linked via a hinge region to a C-terminal ligand-binding E domain (LBD) (Aranda and Pascual, 2001) (Fig. 1.2). The A/B domains are relatively low conserved and it has been suggested to have a transactivation function, activation function (AF-1) in many receptors (Juge-Aubry et al., 1999; Chamberlain et al., 1996; Sturm et al., 2010). The DBD domain is a highly conserved region composed of two zinc finger structures (Huckaby et al., 1987; Luisi et al., 1991) that mediate the binding of the NRs to a specific DNA sequence, termed response element, a prerequisite for the regulation of target genes by NRs (Mangelsdorf et al., 1995).

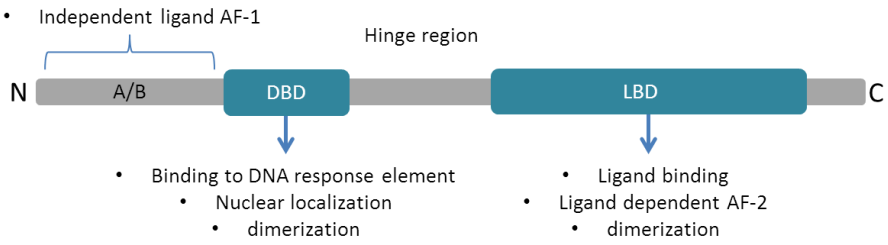


Figure 1.2: Schematic structure of a nuclear receptor

The first zinc finger is responsible for the high-affinity recognition of the "core half-site" of the response element while the second zinc finger mediates the receptor dimerization and nuclear localization of the receptor (reviewed in Jiang, 2004). NRs can bind to DNA as heterodimers, homodimers, or monomers, depending on the class (Franco et al., 2003). The hinge region is located between DBD and LBD and seems to function in association of coregulatory proteins (cofactors) with the ligand binding region in several nuclear hormone receptors. LBD domain has the second ligand (AF-2) whose role in receptor activation is dependent on a conformational change of the LBD, induced by interactions with an activating ligand (reviewed in Jiang, 2004). Has been reported that deletion of AF-2 leads to a constitutively active receptor, scientists assume that AF-1 functions in a ligand-independent manner (Hollenberg et al, 1987; Tora et al, 1989).

Moreover LBD contacts both coactivator and co-repressor proteins when active and inactive conformations, and together with the DBD has a role in dimerization.

In this study we focused our attention to some of the known mammalian xenobiotics receptors that have been identified in different fish species, such as PXR, PPAR, RXR and RAR as well as AhR, and with evidences pointing to a role in the regulation of genes from the detoxification mechanism (Corcoran et al., 2012; Aleksunes and Klaassen, 2012; Chang et al., 2013; Wassmur et al., 2010; Cocci et al., 2013; Hessel and Lampen, 2010; Stromskaya et al., 1998).

1.3.1 Pregnane X receptor (PXR)

PXR (NR1I2) in mammals mediates biological functions such as immune and inflammatory responses or lipid metabolism (di Masi et al., 2009; Smutny et al., 2013; Ma et al., 2015). This NR has been commonly designated as a xenobiotic sensor that regulates a variety of genes involved in metabolism and elimination of exogenous chemicals and endogenous molecules (Xu et al., 2005; Wang et al., 2012). It has been identified in several vertebrates like mammals, birds, fish and amphibians (Reschly & Krasowski 2006), and in mammals PXR is predominantly expressed in liver, intestine and kidney (Kliewer et al., 1998). PXR requires heterodimerization with RXR for high-affinity to pregnane x receptor response element (PXRRE) and modulate the transcription of adjacent genes (di Masi et al., 2009). Due to it large LBD this NR demonstrate a high flexibility during ligand-binding allowing it to be activated by a wide range of ligands which include bile acids, steroid hormones or synthetic drugs (di Masi et al., 2009). In fish PXR has been associated to the modulatation of key genes from the detoxification system, like *cyp3a, gst, abcb1* or *abcc2*, when exposed to different xenobiotics (Wassmur et al., 2010; Corcoran et al., 2012; Kubota et al., 2015).

1.3.2 Peroxisome proliferated activated receptor (PPAR)

PPARs are members of the nuclear receptor superfamily with functions in lipid, carbohydrate and protein metabolism, adipogenesis, development, inflammatory response and xenobiotics metabolism (Janani and Ranjitha Kumari, 2015; Ipseiz et al., 2014; Xu et al., 2005). These NRs include three subtypes members (PPARα, PPARβ and PPARγ) and where identified in several species such as fish, birds and amphibians (Krey et al., 1993; Diot and Douaire, 1999; Andersen et al., 2000). PPARs also need to heterodimerize with RXR to bind to the peroxisome proliferator responsive elements (PPREs) and regulate transcription of their target genes (Marcus et al., 1993). The LBD of PPAR is unusually large making this receptor relatively promiscuous and activated by different

natural or synthetic ligands, such as fatty acids, eicosanoids, synthetic hypolipidemic and antidiabetic agents (O'Sullivan, 2007). In mammals PPAR/RXR regulates the transcription of phase I and II biotransformation genes and antioxidant genes but also genes involved in lipid metabolism inflammatory and immune homeostasis (Xu et al., 2005; Janani and Ranjitha Kumari, 2015). Previous studies found interactions between xenobiotics and PPARs, like TBT and phthalates, suggesting that peroxisome proliferation could be used as a biomarker of environmental pollution (Cajaraville et al., 2003; Desvergne et al., 2009). In a recent study, with juvenile sole exposed to 4-nonyphenol pparα mRNA was associated with *cyp1a1* and *cyp3a4* mRNA, suggesting that PPARα can modulate phase I biotransformation enzymes (Cocci et al., 2013).

1.3.3 Retinoid acid receptor (RAR) and retinoid x receptors (RXR)

There are two families of retinoid receptors: RAR and RXR with a similar structure that consist in three isotypes α, β and γ. RXRs are unique NRs because they can form heterodimers with PXR, PPAR and RAR but also form homodimers, hence participate in several regulatory pathways depending on the partner (reviewed in Lengqvist et al., 2004). There are two types of heterodimers: nonpermissive, where the complex is activated only by the other partner's ligand (Kurokawa et al., 1994) or permissive, where ligands from both NRs can activate the complex (Kliewer et al., 1992). RAR requires binding to RXR to regulate transcriptional activity, but generally the RXR activity is silenced (Theodosiou et al., 2010). RARs have high affinity to retinoid acid (RA) in the all-trans conformation, whereas RXR can be activated by RA but also other natural and synthetic ligands such as phytanic acid or methoprene (Giguére, 1999). Detoxification genes, like efflux proteins (*mdr1* and *abcg2*) and phase I (*cyps*) where found to be induced via RAR/RXR in mammals (Stromskaya et al., 1998; Howel et al., 1998; Hessel and Lampen, 2010). The presence of these NRs in fish is already recognized, however the modulation of downstream genes via RAR/RXR is not well understood. Nonetheless, RXR seem to be involved, through the RXR-NRs complex, in the regulation of several biotransformation and elimination genes (Traber, 2004; Xu et al., 2005; Pérez et al., 2012).

1.3.4 Aryl hydrocarbon receptor (AhR)

AhR is related to functions, such as the regulation of immune response, cell cycle, proliferation and tumour promotion (Vondracek et al., 2011). In mammals AhR modulates many genes responsible for cellular detoxification, like phase I, II, antioxidant enzymes and efflux transporters (Xu et al., 2005; Kawajiri and Fujii-Kuriyama, 2007; Tompkins et al., 2010). Their high-affinity to polycyclic aromatic hydrocarbon compounds (PAH) is the

best characterized but it is also activated by other chemicals like aflatoxins or omeprazole (Stegeman and Hahn, 1994; Denison et al., 2011). In mammals, it is mainly expressed in lungs, placenta, spleen and ovaries (Yamamoto et al., 2004). The inactive AhR is located in the cytosol as a multi-protein complex with chaperone proteins (e.g. heat shock protein 90) (Beischlag et al., 2008; Denison et al., 2011). After ligand binding AhR suffers a conformational change facilitating the translocation of the complex into the nucleus and heterodimerize with the aryl hydrocarbon receptor nuclear translocator (ARNT). This heterodimer (AhR-ARNT) then binds to xenobiotic response elements (XRE) in the promoter region of target genes, activating their transcription (Beischlag et al., 2008; Denison et al., 2011). In fish, both AhR and ARNT have been identified (Andreasen et al., 2002) and the signalling pathway is believed to be analogous to mammals, regulating cyp1a and cyp3a65 (Chang et al., 2013; Andreasen et al., 2002). Some studies described that exposure to benzo[a]pyrene (BaP) a known ligand of AhR induced the expression of phase I and II enzymes and ABC transporter mRNA in fish (Costa et al., 2011; Yuan et al., 2014).

1.4 Cell detoxification mechanism

The combined actions of efflux proteins and biotransformation enzymes increase the cell's capability to eliminate the xenobiotics and inhibit their accumulation inside the cell and the organism. The detoxification mechanism involve four phases of action, which involve biotransformation enzymes active in phase I and II, and efflux transporters that are active in phase 0 or III (Xu et al., 2005) (Fig. 1.3). A better description and function of the components detoxification mechanism are described in the next sections.

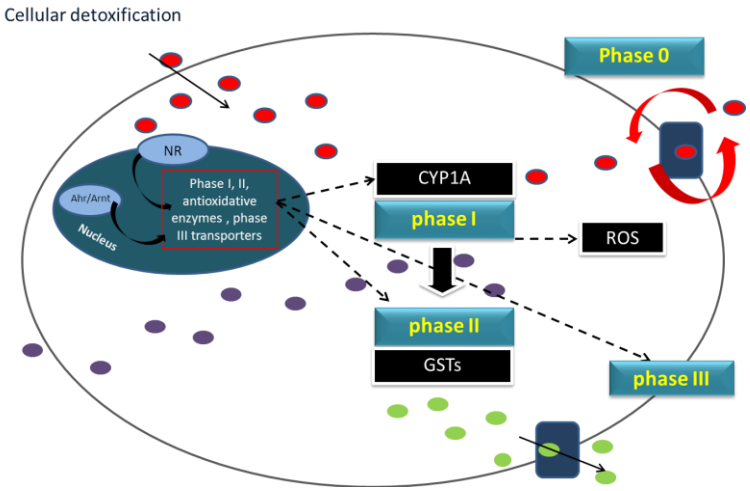


Figure 1.3: Schematic representation of the mechanisms involved in detoxification of xenobiotic compounds in cells. Xenobiotics induced the activation of specific receptor-mediated gene expression of phase I enzymes, phase II enzymes, other stress enzymes and phase 0 and phase III transporters, which result in the enhancement of xenobiotic detoxification

1.4.1 ABC transport proteins – phase 0 and III

Research on ATP – binding cassette (ABC) transporters has been mostly directed to chemotherapy resistance studies, due to a phenomenon called MDR (multidrug resistance) mediated by ABC proteins characterized by the low cellular accumulation of anticancer drugs (reviewed in Gottesman and Ling, 2006). MDR was initially related to the high levels of a membrane glycoprotein, denominated P-glycoprotein (Pgp) belonging to ABCB subfamily (Juliano & Ling, 1976; Sparreboom et al., 2003). In aquatic organisms, a mechanism analogous to the MDR, was proposed as a defence system allowing them to survive in contaminated environments with anthropogenic or natural toxic compounds, termed multixenobiotic resistance (MXR) (Kurelec, 1992). In aquatic species ABC proteins efflux toxicants and their metabolites out of the cells and are therefore denominated as a first line of defence (Bard, 2000; Epel et al., 2008). MXR was identified in different aquatic species including crabs (Kohler et al., 1998), molluscs (Luckenbach and Epel, 2008; Faria et al., 2011) and fish (Costa et al., 2012; Ferreira et al.,2014; Zaja et al., 2008) including zebrafish (Long et al., 2011; Fischer et al., 2013).

These proteins transport a large variety of endogenous and/or exogenous molecules (including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, drugs and xenobiotics) across cellular membranes using ATP as driving energy (Higgins, 1992; Dean et al., 2001).

ABC proteins are grouped into eight subfamilies in eukaryotes, ABCA to ABCH (H subfamily has been only found in Danio rerio, and also some invertebrates like Daphnia pulex, Tigriopus japonicus and Apis mellifera) (Table 3), (Dean and Annilo, 2005; Popovic et al., 2010; Jeong et al., 2014). Among all efflux proteins families the most relevant in a toxicological context are the ABCB, ABCC and ABCG families (Epel et al., 2008). Usually, a functional protein contains two nucleotide binding domains (NBDs) and two membrane spawning domains (MSDs) (Locher 2009) (Fig. 1.4). The NBDs are located in the cytoplasm and are responsible for ATP binding and hydrolysis, providing the required energy to transport substrate compounds across the cellular membrane. The NBDs are characterized by highly conserved Walker A, Walker B and C-motifs and the so-called ABC signature (Walker et al., 1982; Hyde et al., 1990). MSDs are composed by 6-10 membrane spawning α-helices that confer the substrate specificity (Locher, 2009) and enable transport due to conformational changes within the MSD (Linton and Higgins, 2007). Eukaryotic ABC proteins can be designated as full transporters (containing two NBDs and two or three MSDs), or as half transporters (containing one NBD and one MSD), that have to form homo- or heterodimers in order to constitute a functional protein

(Dean et al., 2001). The study of efflux proteins started due to the recognition of ABCB1 implication in the multidrug resistance (MDR) phenomenon preventing the action of chemotherapy in cancer cells (Juliano and Ling, 1976; Gottesman and Ling, 2006). In the beginning of the 90's also ABCC and ABCG proteins were associated with MDR (Cole et al., 1992; Kool et al., 1997; Doyle et al., 1998). The three ABC transporter subfamilies have been associated with different phases of the detoxification mechanism. ABCB1 is so called the "first line of defence" directly effluxing unmodified compounds when they cross the membrane, therefore named phase 0 of cellular detoxification. ABCC and ABCG transporters efflux mainly metabolites of phase I and II from the cell, such as oxidized molecules and glutathione-, glucuronic acid- or sulphate-conjugates, hence described as phase III of cellular detoxification (Bard, 2000; Epel et al., 2008).

ABCB1, ABCC and ABCG-like-transporters have been identified in aquatic invertebrates (Chen et al., 2015; Faria et al., 2011) and vertebrates including several teleost fish species (Hamdoun et al., 2002, Luckenbach and Epel, 2008; Costa et al., 2012). In mammals the presence of these proteins in the blood-tissue barriers, like blood-brain barrier, protects specific tissues against toxicants (Schinkel, 1999). In aquatic organisms, efflux transporters can act as selective permeable "environment-tissue barriers" with the surrounding aqueous phase, allowing them to survive and reproduce in polluted environments (Luckenbach and Epel, 2008).

Table 3: Members of the human ABC transporter superfamily and function (based on Dean and Annilo, 2005, Szakacs et al., 2008, Slot et al., 2011)

Subfamily	Members	Functions
ABCA	ABCA1-ABCA13	Cholesterol efflux, phosphatidil choline efflux, Nretinylidiene-PE efflux , Skin Barrier Function
ABCB	ABCB1 to ABCB11	Peptide transport; iron transport; Fe/S cluster transport; bile salt transport; xenobiotics transport
ABCC	ABCC1 to ABCC13	Organic anion efflux, nucleoside transport, chloride ion channel, sulfonylurea receptor, potassium channel regulation, toxin transport
ABCD	ABCD1 to ABCD4	Very long chain fatty acids transport regulation
ABCE	ABCE1	Elongation factor complex
ABCF	ABCF1 to ABCF3	Translation Initiation
ABCG	ABCG1 to ABCG5	Cholesterol transport, sterol transport, toxin transport
ABCH	ABCH1	Unknown function

Superfamily ABCB

This family contain the most studied and characterized protein of the ABC transporters, the ABCB1 (MDR1, Pgp) (Fig. 1.4a). Pgp is a protein ranging from 130 to 170 kDa, depending on the degree of glycosylation (Dong et al., 1996). It is expressed in cancer cells, plasma membranes on the apical side of cells of intestine, kidney, liver, lung, placenta, testis, uterus tissues and the blood-brain barrier (Szakács et al., 2008). ABCB1 is a promiscuous protein that transport exogenous and endogenous compounds and its substrates are mostly hydrophobic cations, amphiphilic drugs, antibiotics, insecticides, herbicides or fungicides (Leslie et al., 2005; Szakacs et al., 2008). The reason for the promiscuity of this protein is still unclear but some studies indicated that characteristics like moderate hydrophobicity and positively charged or neutral domains are cause for this fact (Schinkel and Jonker, 2003). In zebrafish, our research model, the *abcb1* orthologue is absent, but a recent study demonstrated that *abcb4* and *abcb5* are structurally similar to *abcb1* in mammals, and that Abcb4 protein has the same functional properties of ABCB1 (Fischer et al., 2013). Hence Abcb4 can play a role in the bioavailability and toxicology of contaminants in this animal model.

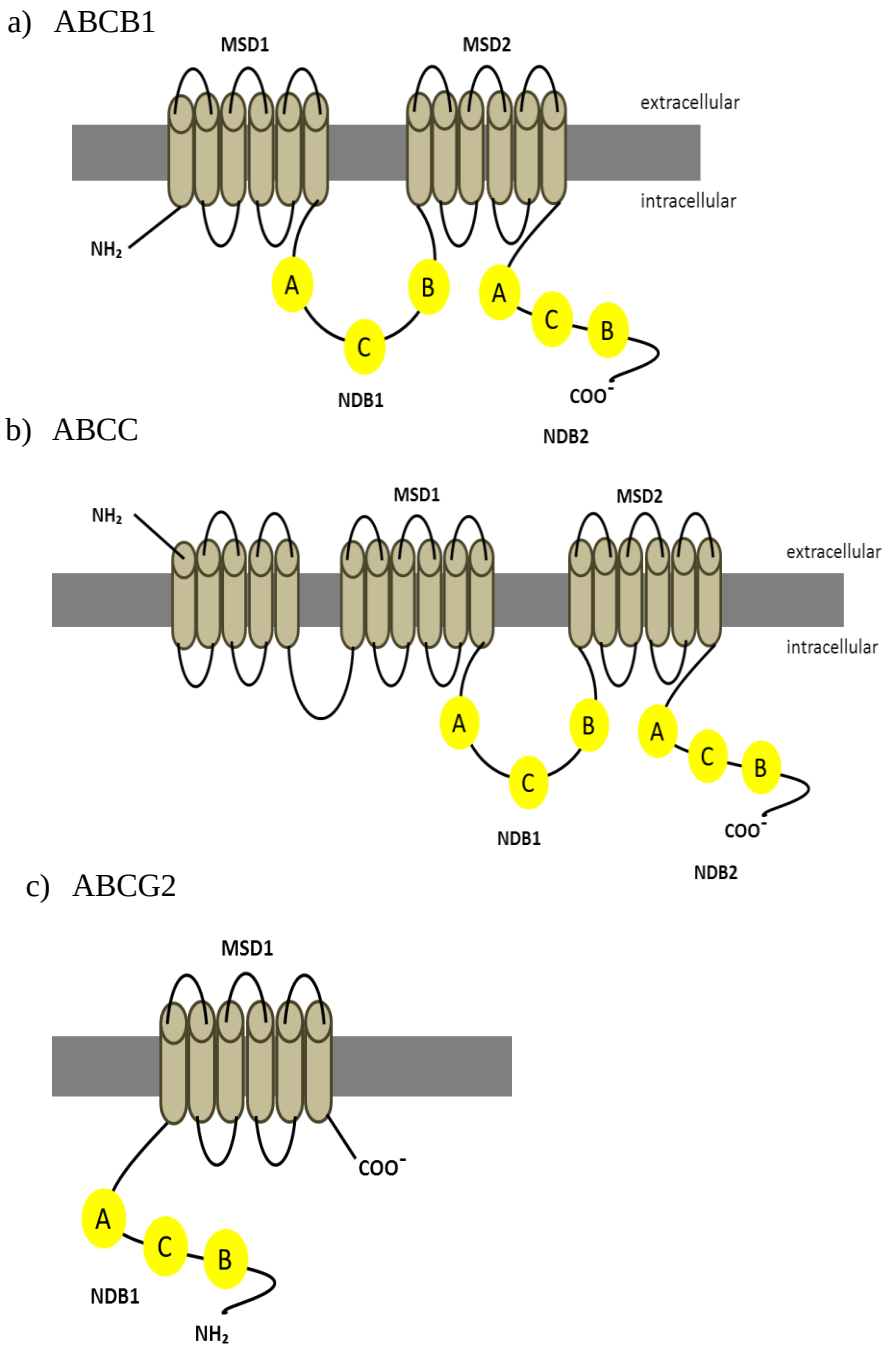


Figure 1.4: Predicted structures of ABC transporters. a) predicted structure of ABCB1; b) predicted structure of "long-chain" ABCC proteins; c) predicted structure of ABCG2. Lipidic bilayer is shown in grey, membrane spawning domains (MSDs) in light brown and nucleotide binding domains (NBDs) in yellow (A - Walker A, B - Walker B, C - C-motif). Adapted from Ferreira et al., 2014.

Superfamily ABCC (MRPs – Multiresistance associated proteins)

ABCC subfamily (Fig. 1.4b) comprises a total of 13 members, and includes 10 "multi-drug resistance associated proteins" (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, ABCC12 and ABCC13) but also cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) and ATP-dependent potassium channel regulators, such as the sulfonylurea receptors SUR1/ABCC8 and SUR2/ABCC9 (Cole et al., 1992; Deeley et al., 2006). The members of the ABCC subfamily can be divided in two different subclasses, "short" and "long" proteins (Deeley et al., 2006; Honorat et al., 2009). The "long" ABCC transporters (ABCC1, 2, 3, 6, 10 and 13) have an additional N-terminal MSD (MSD0) in comparison to the "short" transporters (ABCC4, 5, 11 and 12). These transporters are ~190kDa proteins, and share a small percentage (14-25%) amino acid identity with ABCB proteins (Cole et al., 1992; Keppler and Konig, 1997). There are evidences that suggest that ABCB and ABCC can operate either as homodimers or heterodimers (Graf et al., 2003; Xu et al., 2004). Several studies performed with different animal models indicate that ABCC1, ABCC2, ABCC3, ABCC4 and ABCC5 are the most relevant MRPs in a toxicological level in mammalian tissues although ABCC1 and ABCC2 are the best characterized transporters in this family (Szakacs et al., 2008). ABCC1 (MRP1) shows ubiquitous tissue distribution with higher expression levels in the kidney, lung, testis, skeletal muscle and peripheral blood mononuclear cells in mammals (Cole et al., 1992; Flens et al., 1996) and is located on the basolateral membrane of epithelial cell layers (Cole et al., 1992) effluxing its substrates into the blood (Evers et al., 1996). ABCC2 (MRP2) is localized in the apical membrane of cells from liver, intestine and kidney, placenta, lungs, intestine and blood-brain barrier (Schinkel and Jonker, 2003). ABCC2 is essential for cellular detoxification effluxing diverse metabolites into bile, intestinal lumen and urine (Haimeur et al., 2004). ABCC1 and ABCC2 substrates are very similar and include glutathione-, glucuronate- and sulfate-conjugates, bile acids and different drugs and their metabolites (Cekic et al., 2003; Nies and Keppler, 2007) therefore their role associated with phase III of the detoxification process.

Superfamily ABCG (ABCG2, BCRP - breast cancer resistance protein)

ABCG2 protein, a member of the ABCG subfamily also known as breast resistance associated protein (BCRP) (Doyle et al., 1998), is a half-transporter consisting of one single NBD followed by one MSD (Wakabayashi et al., 2006). ABCG2 presents a different configuration of ABCB and ABCC whereas the MSD is followed by the NBD (Fig. 1.4c). This protein seems to operate as a homodimer (Doyle et al., 1998; Miyake et al., 1999;

Kage et al., 2002) or homotetramer (Xu et al., 2004). ABCG2 is mainly expressed in apical membranes of polarized epithelial cells of different organs, like intestine, placenta, blood brain barrier and liver (Rocchi et al., 2000; Scheffer et al., 2000). The physiological functions of ABCG2 involves the transport of a wide range of substrates like porphyrins, folic acids, bile salts, heme sulfated conjugates of steroids (Doyle and Ross, 2003, Haimeur et al., 2004). Studies with ABCG2 transfected mammalian cell lines have demonstrated high resistance of these cells against a wide variety of anticancer drugs, such as topotecan, mitoxantrone, doxorubicin, and daunorubicin (Sarkadi et al., 2006).

1.4.1.1 Chemosensitisation

Drugs that could interact with Pgp and other ABC proteins and reverse MDR by blocking or saturating binding sites (for example verapamil and cyclosporine A) (Barrand et al., 1993; Zaman et al., 1994) are called chemosensitisers. In aquatic organisms, MXR act as a defence system against contaminated environments (Kurelec, 1992), that can be compromised by chemosensitisers making the cells more sensitive to other xenobiotics (Kurelec, 1992) (Fig. 1.5). Efflux proteins interact with a wide range of compounds including environmental relevant pollutants that can be classified as substrates or inhibitors (chemosensitisers), however they are not mutually exclusive; some inhibitors are themselves transported by ABC proteins (Luckenbach et al., 2014). Due to ABC transporters un-specificity to chemicals, it is important to study the interaction of emerging compounds with these proteins to identify possible chemosensitisers that may compromise cell's defence system. The chemosensitisers can block the ABC transporters by competitive or non-competitive inhibition binding to substrate binding sites or to other binding sites causing allosteric changes in the protein (Ford, 1996; Robert, 1999).

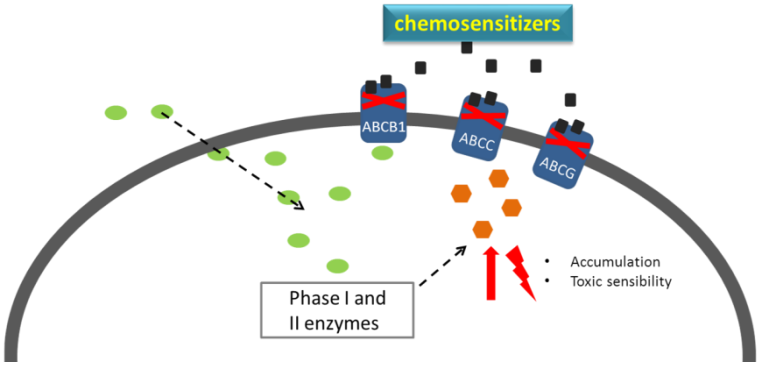


Figure 1.5: Schematics of inhibition of efflux proteins by chemosensitizers. Chemicals (green circles) can enter cells by passive diffusion (dashed arrows). These chemicals then may undergo biotransformation metabolism (phase I and II) in metabolites (orange hexagons) that ultimately could be extruded by ABC transporters. In the presence of chemosensitizers (black rectangles) ABC proteins are inhibited, increasing the parent compound and it metabolite inside the cell, making the cells sensitive to their toxicity.

1.4.2 Phase I and II biotransformation enzymes

Biotransformation of xenobiotics is a strategy adopted by organisms to cope with the presence of xenobiotics present in their habitats. It is defined by the transformation of both endogenous and xenobiotic compounds in the organism (Parkinson 1996). The biotransformation mechanism consist on a two-phase (phase I and II) process of enzymatic reactions that modify non-polar lipophilic chemicals into more polar water soluble metabolites, leading to the detoxification and elimination of the parent compound (Black and Coon, 1987; Buhler and Williams, 1989). Phase I is a non-synthetic alteration that include oxidation, reduction or hydrolysis transforming the parent compounds into metabolites which can then be conjugated in phase II to be effluxed by ABCC and ABCG proteins in phase III (Bard, 2000; Sturm & Segner, 2005). First discovered in mammalian liver, these enzymes were later found in several other animals, plants, bacteria and fungi (Nelson et al., 1996). These enzymes became important in ecotoxicology studies because they biotransform xenobiotics so that they could be excreted or transforming them in of more reactive metabolites, which leads to increased toxicity (reviewed in section 1.4.3) (van der Oost et al., 2003). Measurement of biotransformation enzymes have been also applied as biomarkers of biochemical effects, and as a complement to chemical analysis (Richardson et al., 2001; Ferreira et al., 2008). Recent studies have correlated ABC transporters expression and phase I and phase II enzymes in aquatic organisms exposed to pollutants and suggest that these systems work in a coordinated manner in xenobiotic detoxification (Paetzold et al., 2009, Zucchi et al., 2010; Costa et al., 2012a).

Phase I enzymes

Phase I enzymes introduce a functional group (e.g., -OH, -COOH, -NO2) into the chemical through different chemical reactions (oxidation, reduction, hydration, hydrolysis), providing a non-synthetic alteration of the parent compound (Commandeur et al., 1995). Most of phase I reactions are catalysed by microsomal monooxygenase (MO) enzymes, also known as the mixed-function oxidase (MFO) system (cytochrome P450, cytochrome *b5* and NADPH cytochrome reductase). The first isoenzyme of this CYP superfamily was described in 1962 as a new cytochrome, a membrane-bound haemoprotein (Omura & Sato, 1962). When this protein binds carbon monoxide, it absorbs light at 450 nm, hence the suffix P450 (Omura & Sato, 1962). The cytochromes P450 (CYPs) are predominantly located in the endoplasmic reticulum and catalyse biological oxidation and reduction reactions (Stegeman et al., 1992). A recent study of the zebrafish genome revealed 94 CYP genes which can be divided into the 18 CYP gene families that are also present in human (Goldstone et al., 2010). These proteins are involved in the metabolism of endogenous substrates as well as exogenous compounds like drugs, chemicals carcinogens and pollutants (Nebert et al., 2004). CYP1, CYP2, CYP3 and CYP4 are the members of the CYP family responsible for xenobiotics metabolism and in this thesis will focus on the xenobiotic- and steroid-metabolizing CYP1A and CYP3A subfamilies.

In fish, the most studied CYP isoform is CYP1A and has been used as a biomarker to monitor aquatic pollution (Fent, 2003; Uno et al., 2012). CYP1A-dependent ethoxyresorufin O-deethylase (EROD) activity and *cyp1a* mRNA expression have been used as biomarkers for the presence of and exposure to polycyclic aromatic hydrocarbons and dioxins in the environment (Stegeman and Hahn, 1994; Parente et al., 2008). Previous studies, in mammals and fish, showed that *cyp1a* transcription is regulated by AhR (Denison and Nagy 2003; Schlenk et al., 2008). CYP3A isoform is the predominant form in the liver in both fish and mammals (Celander et al., 1996; Thummel and Wilkinson, 1998). In humans drugs are metabolized mostly by CYP enzymes and almost half of these reactions are catalysed by CYP3A4 (Guengerich, 2008). However, CYP3A4 homolog is absence in zebrafish (Chng et al., 2012), so this activity was suggested to be carried out by other proteins, like CYP3A65 or CYP1A. In mammal's the transcription of *cyp3a* is regulated by PXR that is very promiscuous and activated by a large number of lipophilic chemicals, including many pharmaceuticals (Wassmur et al., 2010; Kubota et al., 2015). Also in zebrafish, exposure to the PXR ligand rifampicin and to the AhR ligand TCDD induced CYP3A65 transcription (Tseng et al., 2005), illustrating the intricacies in AhR and PXR receptor signalling pathways.

Phase II enzymes

Phase II process involves the conjugation of an endogenous ligand (large and often polar chemical groups or compounds, such as sugars or amino acids) to the parent compound or the phase I metabolites facilitating their excretion (Lech and Vodicnik, 1985). These synthetic conjugations are catalysed by gluthathione S-transferases (GSTs), UDP-glucuronyl transferases (UGTs) and sulfotransferases (SULT). In this study GST expression and/or activity will be used as indicative of phase II biotransformation therefore the description in more detail.

GSTs belong to a multigene superfamily of dimeric, multifunctional enzymes (Hayes and Pulford, 1995b) that catalyse the conjugation of glutathione (GSH) with compounds that have an electrophilic centre, through the formation of a thioether bound between the sulphur atom of GSH and the substrate (Chasseaud, 1979; Mannervik, 1985). In mammals GST can be divided in two superfamilies, cytosolic and membrane-bound (Hayes and Strange, 2000), and based on their sequence homology, cytosolic enzymes can be divided into eight families or classes: α, μ, κ, π, ω, σ, θ and ζ (Meyer et al., 1991; Pemble et al., 1996; Board et al., 1997; Board et al., 2000). The membrane-bound transferases were more recently discovered and are composed by at least six families (Jacobsson et al., 2000). The cytosolic GSTs are primarily involved in the metabolism of exogenous chemicals, such as environmental pollutants, cancer chemotherapeutic drugs but also potentially dangerous endogenously derived reactive compounds (Hayes and Pulford, 1995). Other GST substrates are endogenous substances formed as a consequence of reactive oxygen species making these transferases to act also as antioxidant enzymes (Hayes and McLellan, 1999). In aquatic organisms, GST activity and/or expression have been used as a biomarker in several studies to evaluate environmental contamination levels and effects (Ferreira et al., 2008; Della Torre et al., 2010).

1.4.3 Oxidative stress enzymes

Oxidative stress is the result of a disturbance in the balance between the prooxidants and antioxidants leading to negative biochemical and physiological effects. This imbalance increases free radical production or reactive oxygen species (ROS) ($O_2^{\bullet-}$ - superoxide radical, H_2O_2 - hydrogen peroxide, OH• **-** hydroxyl radical), and/or decreases antioxidant defences that can be potentially damaging to the cells. When antioxidant defences decrease damages can happen to macromolecules such as DNA, proteins and lipids resulting in damaged DNA bases, protein oxidation products, and lipid peroxidation. Sources of ROS include aerobic respiration, irradiation, UV light, production of H_2O_2, NO

and $O_2^{\bullet-}$ by activated macrophages and phagocytes, metal catalysed oxidation systems, autooxidation of electron transport carriers and xenobiotics (Stadtman and Levine 2000). Likewise xenobiotics may cause oxidative stress through the action of enzymes such as CYP1A, NAD(P)H oxidases, and flavoprotein oxidases, via metal-catalysed oxidation systems, or via redox cycling (Livingstone et al. 2000, Stadtman and Levine 2000). Biotransformation of xenobiotics by phase I enzymes activity can increase ROS production as unwanted products (van der Oost et al., 2003), causing enzyme inactivation, lipid peroxidation, DNA damage and eventually cell death (Winston and Di Giulio, 1991). Nevertheless, cells have defence mechanisms which include enzymatic inactivation of redox cycling compounds and peroxides, metabolism of ROS to water and oxygen by antioxidant enzymes (such as superoxide dismutase (SOD), Catalase (CAT)) or molecules that eliminate free radicals that can bind to transition metals ions and be converted in more detrimental ROS (Stegeman et al., 1992; Lopez-Torres et al., 1993) (Fig. 1.6).

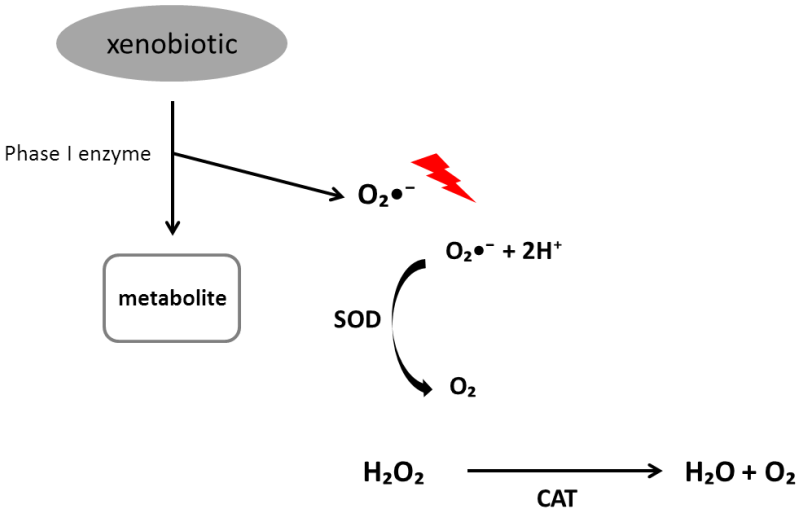


Figure 1.6: Schematics of antioxidant enzymatic removal of oxygen by-products

The increasing number of chemicals found in water systems, can consequently increase the risk of toxicity through oxidative stress. For this reason biomarkers of oxidative stress such as antioxidant enzyme activity or levels of damaged molecules, have been used as early warning of exposure effects to environmental pollution in aquatic environments (Livingstone, 2001; van der Oost et al., 2003) and can be useful for risk assessment of emering compounds. In fish the use of oxidative stress biomarkers, like measurement of SOD, CAT, GST activities and mRNA expression have become useful in risk assessment studies (van der Oost et al., 1998; Ferreira et al., 2008; Ellesat et al. 2012).

1.6 Model specie- zebrafish (*Danio rerio*)

Danio rerio, zebrafish (Fig. 1.7), is a freshwater tropical fish of the Cyprinidae family, native to India and south Asia. This specie has been broadly used as a model for toxicological studies due to a large number of advantages. Zebrafish have easy and low maintenance costs, short life-cycle (10-12 weeks), high fecundity, fully described embryonic development and the genome fully sequenced, regulatory sequences and expression profiles (Kimmel et al., 1995; Chow et al., 2012) (http://www.ensembl.org/index.html). In laboratory zebrafish can breed all year round producing a large number of transparent embryos that allows a good and quick observation of embryonic development. It has been suggested that during critical stages in their development embryos might be more sensible to xenobiotics exposure (Versonnen and Janssen, 2004), making them a helpful model in toxicological research. Zebrafish embryo toxic assay (FET) has been proposed as an alternative to animal experiments and classical acute fish toxicity testing, required for regulatory activities (Lammer et al., 2009; Kaiser et al., 2012). Also, the availability of a full genome sequence is important in genomic studies in order to understand the toxics mode of action and which pathways are being affected.

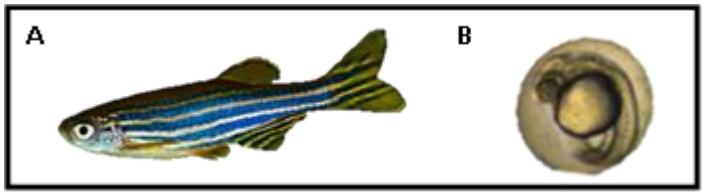


Figure 1.7: Zebrafish (*Danio rerio*) adult (A) and embryo (B).

Source of adult zebrafish: http://info.noldus.com/bid/87981/5-must-read-articles-on-zebrafish-behavioral-research

Source of zebrafish embryo: photo was taken by the author

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CHAPTER II

OBJECTIVES

The process of cellular detoxification is of paramount importance to protect the organisms inhabiting polluted environments. Biotransformation enzymes in cooperation with MXR proteins are part of the cellular detoxification system and act to reduce cellular uptake and toxicity of a wide range of chemicals. Given the important role of this defence system it is important to understand the regulation by nuclear receptors (NRs). Moreover, recent evidences indicate that MXR mechanism may be compromised by the chemosentisation effect of numerous chemicals present in aquatic ecosystem. Furthermore, the knowledge on the interaction of emergent contaminants and the defence system is scarce therefore the main goals of this thesis were the following:

1) to characterise the basal transcription profile of ABC transporters, biotransformation and antioxidant enzymes and NRs in zebrafish embryos, at different development stages and provide fundamental knowledge on components and functional characteristics of the pathways of detoxification system and their regulation by NRs after exposure to emerging contaminants
2) to address chemosensitisation potential of different contaminants classes, focusing particularly in emerging pollutants, using zebrafish embryos as model organism.

In order to achieve these goals, specific objectives have to be achieved:

1. Characterisation of the transcriptional patterns of ABC efflux transporters and biotransformation enzymes of phase I and phase II and antioxidant enzymes and NRs during the developmental stages of zebrafish;
2. Asses the toxicological effects of two pharmaceuticals (simvastatin (SIM) and fluoxetine(FLU)) in zebrafish embryo development;
3. The characterisation of the expression and response patterns of ABC transporters (*abcb4, abcc1, abcc2* and *abcg2*), biotransformation enzymes (*cyp1a, cyp3a65* and *gstπ*) and antioxidant enzymes (*cat* and *Cu/Zn sod*) after exposure to SIM and FLU at different development stages of zebrafish embryo;
4. Evaluate the effects of SIM and FLU at a biochemical level through the assessment of phase I and II catalytic activities (EROD and GST) and antioxidant activities (CAT and SOD);
5. Assess the effects of SIM and FLU in the transcription level of NRs that may regulate genes belonging to the detoxification mechanism;
6. Evaluate the interactions of SIM with lipid metabolism pathway and FLU with the neurotransmitter system in zebrafish embryos;
7. Evaluate effects of different classes of contaminants (pharmaceuticals and personal care products), in zebrafish embryo and eleutheroembryos, and assess

chemosensitisation potential of emergent compounds using dye accumulation assay in combination with standardized parameters established for fish embryo test (FET).

8. Assess chemosensitisation potential of emergent compounds in Abcb4 ATPase activity.

The goals of this thesis were explored throughout the following chapters:

- Chapter I - General Introduction
- Chapter II - Objectives
- Chapter III - Simvastatin effects on detoxification mechanisms in *Danio rerio* embryos.
- Chapter IV - Simvastatin exposure can modulate gene expression of nuclear receptors linked to detoxification process in zebrafish embryos
- Chapter V - *Danio rerio* embryos on Prozac – Effects on the detoxification mechanism and embryo development
- Chapter VI - Fluoxetine modulate transcriptional level of genes involved in the neurotransmitter system in zebrafish embryos
- Chapter VII - Effects of pharmaceuticals and personal care products (PPCPs) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (*Danio rerio*) embryos.
- Chapter VIII – integrated discussion and main conclusions of the thesis, and also the presenting prospects for future work.

CHAPTER III

SIMVASTATIN EFFECTS ON DETOXIFICATION MECHANISMS IN *DANIO RERIO* EMBRYOS.

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Simvastatin effects on detoxification mechanisms in *Danio rerio* embryos.

3.1 Abstract

The transcription and protein activity of defence mechanisms such as ABC transporters, phase I and II of cellular detoxification and antioxidant enzymes can be altered in the presence of emerging contaminants such as pharmaceuticals impacting the overall detoxification mechanism. The present work aimed to characterise the effects of simvastatin on the detoxification mechanisms of embryonic stages of *Danio rerio.* In a first approach, constitutive transcription of key genes involved in detoxification was determined. Embryos were collected at different developmental stages, and transcription patterns of genes coding for ABC transporters, phase I and II and oxidative stress were analysed. With exception of *abcc2*, all genes seem to be from maternal transfer (0 - 2 hpf). Embryos were then exposed to different concentrations of simvastatin (5 µg/L and 50 µg/L), verapamil and MK571 (10 µM) (ABC protein inhibitors) and a combination of simvastatin and ABC inhibitors. mRNA expression levels of *abcb4, abcc1, abcc2, abcg2, cyp1a, cyp3a65, gst, sod, cat* was evaluated. Accumulation assays to measure ABC proteins activity and activity of EROD, GST, CAT and Cu/ZnSOD, were also undertaken. Simvastatin acted as a weak inhibitor of ABC proteins and increased EROD and GST activity, whereas Cu/ZnSOD and CAT activity were decreased. Simvastatin up-regulated *abcb4* and *cyp3a65* transcription (both concentrations), as well as *abcc1* and *abcc2* at 50 µg/L, and down-regulated *gst, sod*, *cat* at 5 µg/L. In conclusion, our data revealed the interaction of simvastatin with detoxification mechanisms highlighting the importance of monitoring the presence of this emerging contaminant in aquatic environments.

Keywords: Simvastatin; zebrafish embryos; ABC transporters; phase I and II; biotransformation; antioxidant enzymes

3.2 Introduction

The aquatic environment is a known sink for a vast array of chemicals including emerging contaminants. Pharmaceuticals and their metabolites are often detected in surface waters in concentrations in the ng to the µg/L range (Kolpin et al. 2002; Wiegel et al. 2004). Pharmaceuticals are designed to be bioactive and act on selected signalling pathways in humans. Given the conservation of several signalling pathways across metazoans, some pharmaceuticals are expected to impact an array of *taxa* (Bound and Voulvoulis 2004; Castro and Santos 2014; Santos et al., 2016). In addition, other adverse effects can also arise, such as elevated mortality and anomalies in reproduction and behaviour (Ribeiro et al. 2015; Neuparth et al. 2014; Ortiz de García et al. 2014). Genes and proteins required for detoxifying environmental contaminants that include efflux transporters (ATP – binding cassette (ABC) transporters), phase I (cytochrome P450 family, eg. CYP1A, CYP3A) and phase II (glutathione-S-transferase, GST, UDP-glucuronlytransferases (UGTs), Sulfotransferases (SULTs)) biotransformation enzymes, and antioxidant enzymes are known to be present or genome predicted in several fish species (van der Oost et al. 2003; Bard 2000; Stegeman et al. 1992; Lopez-Torres et al. 1993). ABC transporters are considered to be the first line of defence against toxicants and are grouped in seven different families (Bard 2000; Ferreira et al. 2014a; Luckenbach et al. 2014). The most relevant in the toxicological context are the ABCB, ABCC and ABCG families (Epel et al. 2008). ABCB1 effluxes mainly unmodified compounds from the cells (phase 0 of cellular detoxification) (Epel et al. 2008). Zebrafish, *Danio rerio*, as in other fish species, have a well-developed “detoxifying machinery”, however it has some specificities such as the absence of *abcb1* orthologues. Nevertheless, it has recently been reported that Abcb4 has the same functional properties as ABCB1 (P-glycoprotein, P-gp) in mammals (Fischer et al. 2013). In a second line of defence, xenobiotics are biotransformed in phase I and II, increasing their hydrophobicity facilitating excretion (Lech and Vodicnik 1985). In phase III, the metabolites resulting from biotransformation are effluxed by transport proteins from ABCC and ABCG families (Bard 2000; Ferreira et al. 2014a). However, the biotransformation process mediated by CYPs enzymes can generate reactive oxygen species (ROS) as unwanted side products (Sapone et al. 2007). Therefore, antioxidant defences such as superoxide dismutase (SOD) and catalase (CAT) enzymes contribute to neutralize and detoxify ROS (Stegeman et al. 1992; Lopez-Torres et al. 1993). These biological responses have been widely used as biomarkers in environmental monitoring of aquatic ecosystems (van der Oost et al. 2003). Anti-cholesterolemic drugs are among the most prescribed drugs in western countries and have been found in increasing concentrations in aquatic ecosystems ranging from 0.1 and 7000 ng/L (Hernando et al. 2006; Corcoran et al. 2010; Santos et al. 2010; Pereira et al.

2015). Simvastatin (SIM) is one of the lipid regulator drugs commonly identified in water systems and previous studies have already reported that SIM can induce negative effects in non-target aquatic organisms at environmentally relevant concentrations (Ribeiro et al. 2015; Neuparth et al. 2014; Ellesat et al. 2011). Some pharmaceuticals have the potential to act as inhibitors of ABC transporters, i.e., chemosensitisers (Caminada et al. 2008), decreasing transporter activity and thus causing the accumulation of toxic chemicals inside the cells (Epel et al. 2008). P-glycoprotein (P-gp, ABCB1) is a transmembrane protein and direct correlations were found between the cholesterol content of the membranes and ATPase activity of P-gp in mammals (Kimura et al. 2007; Eckford and Sharom 2008). Interestingly, some studies have reported the interaction between statins and P-gp (Kopecka et al. 2011; Wang et al. 2001), raising the hypothesis that statins may have a chemosensitising effect on the efflux activity by lowering cholesterol in the plasma membrane (Kopecka et al. 2011). Alternatively, statins can also act as substrates and/or as direct inhibitors of this protein (Wang et al. 2001). Therefore, the reported interaction of statins with human ABC transporters and its prevalence in the aquatic environment highlights the need for more detailed studies on the chemosensitiser potential using additional animal models.

The zebrafish (*Danio rerio*) has emerged as a suitable model species in ecotoxicology and was selected for the present study given several favourable characteristics. For example, the transparency of the embryos allows for easy observation of the embryonic development and the assessment of toxicity, in addition to the extensive genomic data available. Moreover, previous studies demonstrate that zebrafish embryos are sensitive to SIM exposure (Ribeiro et al. 2015).

Hence, this preliminary study aimed to *i*) characterize the basal transcription profile of key genes coding for proteins involved in the detoxification pathways in different development stages of zebrafish embryos, *ii*) assess the potential chemosensitiser effect of SIM and the toxicity of SIM in the presence of known chemosensitisers and *iii*) evaluate the effects and interaction of SIM with the components of the detoxification mechanism on zebrafish embryos

3.3. Material and methods

3.3.1 Chemicals

β-naphthoflavone (β-NF), Rhodamine 123 (RH123), Simvastatin (SIM) (CAS # 79902-63-9), Verapamil (VER), α-Dithiothreitol (99% purity), reduced glutathione (GSH, 99% purity), 1-cloro-2,4-dinitrobenzene (CDNB, 97% purity), hydrogen peroxide (30% w/w), cytochrome *c* (95% purity), xanthine (99% purity), xanthine oxidase, bovine serum

albumin (BSA, 99% purity) were purchased from Sigma-Aldrich (Germany) and MK571 from VWR International. All the other chemicals were of analytical grade, and were purchased from local companies.

3.3.2 Parental animals

Adult wild-type zebrafish, obtained from local suppliers, were used as breeding stocks. The stock was kept at a water temperature of 27±1 ºC and in a photoperiod of 12:12 h (light:dark), in 60 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), and supplemented once a day with live brine shrimp (Artemia spp.) (Machado et al. 2014; Soares et al. 2012).

3.3.3 Rearing conditions and exposures

For reproduction, females and males (ratio 1:2) were transferred to a spawning tank, and submitted to acclimatization for 12 h in a cage with a net bottom covered with glass marbles within a 30 L aquarium. After spawning, the following day, the breeders were removed after the beginning of the light period. To assess the transcription of genes belonging to the detoxification system in zebrafish early development phases, the eggs were collected, counted, cleaned and preserved in RNALater (30 embryos per time-period) at 0, 2, 3, 6, 14, 24, 48 and 72 hours post fertilization (hpf), for transcription profiling. Different clutches were used to reduce variability in the development stages. Time-points for embryo and larvae collection were chosen based on the developmental embryonic and larval stages of *D. rerio* as described by Kimmel et al. (1995). Accordingly, embryos were grouped in 8 development stages - zygote (0 hpf), cleavage (2 hpf), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf), and larvae (72 hpf). For the toxicological assays (section 2.4), embryos (1 hpf) were transferred to a 24 well plate (10 embryos per well) and exposed to the different chemicals. For the accumulation assays (MXR activity)(section 2.6) the embryos were transferred to new aquaria (3.5 L) and were kept for 24 h, at 26 ± 1 ºC with aeration, until exposure. Prior to embryo exposures, the test solutions were placed into the plates for 24 h so that the chemicals could adsorb to the plastic, and the solution renewed after preadsorption. For the determination of the biochemical parameters, 1 hpf embryos (100 for antioxidant enzymes activities and 200 embryos for EROD activity) were placed in glass beakers at 26 ± 1 ºC with aeration, and exposed to the test solutions until 80 hpf with the test chemicals. In all assays the embryos were exposed to different concentrations of SIM (5 µg/L, 50 µg/L), chosen based on concentrations of previous studies that include the NOEC (5 µg/L) and the LOEC (50 µg/L) concentrations in

zebrafish embryos (Ribeiro et al. 2015; Torres 2013; Key et al. 2008). Exposures were also performed with two ABC transporter inhibitors (VER (10 µM) and MK571 (10 µM)) (previous accumulation tests using different concentrations of inhibitors were performed to choose the best inhibition concentration in zebrafish embryos, data not shown) and the combination of simvastatin with the two ABC transporters inhibitors.

3.3.4 Toxicological assay

Embryos were exposed from 1 hpf until 80 hpf to SIM, verapamil and MK571 (all diluted in DMSO), as previously described (section 3.3.3). The medium (water plus the tested compounds) was renewed daily during the experiment. Embryos were observed with an inverted stereoscope (Nikon Eclipse TS100) at 8 hpf, 32 hpf and 80 hpf and several parameters were recorded (mortality rate, 75% epiboly, delay/arrest of the division, abnormal cell masses, development delay, pericardia edema, head, eyes and tail abnormalities) at each time-point. The criteria such as heartbeat frequency, coagulation, pigmentation, involuntary movement and observed malformations were used to establish mortality and abnormality parameters. After the 80 h of exposure the embryos were collected and preserved in RNALater for gene expression analysis. Each assay was replicated at least six times. Data is presented as total percentage (%) of individuals that presented abnormalities or mortality.

3.3.5 EROD, GST and antioxidant enzymes activity

After 80 h of exposure, embryos were homogenized in ice-cold 100 mM potassium phosphate buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM disodic ethylenediaminetetra acetic acid (Na2EDTA) (Fernandes et al., 2008). Post-mitochondrial fractions were obtained after centrifugation at 15,000 g for 20 min at 4°C. Glutathione S-transferase (GST) activity was determined as described in Ferreira et al. (2008), using GSH 10 mM in potassium phosphate buffer 0.1 M, pH 6.5 and CDNB 60 mM in ethanol prepared just before the assay. Reaction mixture consisted of phosphate buffer, GSH solution and CDNB solution in the proportion 4.95:0.9:0.15, respectively. The reaction mixture (200 µL) was added to the sample (protein concentration 4 mg/mL) diluted in phosphate buffer, with a final concentration of 1mM GSH and 1 mM CDNB and a final of volume 300 µL. The absorbance was measured immediately every 20 s, at 340 nm, over the course of 5 min. GST activity was calculated through the slope of linear change in absorbance and expressed in nmol/min/mg protein. Since CDNB is a non-specific GST substrate total GST activity was measured. Superoxide dismutase (SOD) activity was determined by the degree of inhibition on the reduction of cytochrome *c* by superoxide anion generated by

the xanthine oxidase/xanthine system (Ferreira et al. 2005). Cytochrome *c* reduction was followed via the measurement of the absorbance at 550 nm. In the assay, concentration of the components were as follows ; sodium phosphate buffer 50 mM, pH 7.8, with Na_2EDTA 0.1 mM, xanthine 50 µM, xanthine oxidase 5.2 mU/mL and cytochrome *c* 18 µM. Two hundred and fifty µL of this mixture was reacted with the sample (protein concentration 4 mg/mL) diluted in phosphate buffer to a final volume of 300 µL. To obtain linearity, the volume of sample and phosphate buffer per well were adjusted for each sample. SOD standards were used in each assay to calculate the activity given in SOD Units (1 SOD Unit=50% inhibition of the reduction of cytochrome *c*) per mg of protein. The samples were divided in two aliquots, one to measure the total SOD activity and in the other MnSOD activity by adding to the reaction KCN 2 mM. To obtain the Cu/ZnSOD activity we then deducted the MnSOD from the total SOD activity. Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 at 240 nm (ε=40 $M^{-1}cm^{-1}$) as described in Ferreira et al. (2008) with slight modifications. The reaction mixture contained 65 mM potassium phosphate buffer, pH 7.8, 15.5 mM H_2O_2 and 0.01% TritonX-100. In the cuvette, 950 µL of reaction mixture was added to the sample (protein concentration 4 mg/mL) and diluted in phosphate buffer to a final volume of 1000 µL. To obtain linearity, the volumes of sample and phosphate buffer were adjusted for each sample. CAT activity is expressed as µmol/min/mg protein. Ethoxyresorufin O-deethylase (EROD) activity was measured in the protein fraction as described by Otte et al. (2010), with some modifications. 50 µL of each sample was incubated with ethoxyresorufin 0.5 µM for 1 min, and the enzymatic reaction was initiated by the addition of 45 µM NADPH. β-NF (10 µg/L) was used as a positive control of EROD. EROD activity was measured for 5 min at λex=530 nm and λem=585 nm, and determined by comparison to a resorufin standard curve. EROD activity was expressed in pmol/min/mg protein. All measurements were performed in triplicate for each sample. Protein determinations were performed by the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al. 1951).

3.3.6 Accumulation assay

Fluorescent dye, RH123 acts as a proxy for efflux transporter activity in the fish embryos. When this activity is absent due to transporter inhibition, accumulation of dye in the embryo increases, resulting in a stronger fluorescence signal. Prior to conducting dye accumulation assays with the test substances the best concentration of RH123 and the time of exposure to be used in the assays were determined. Different concentrations of RH123 (1, 2, 5, 8, 16 µM) and different incubation time (30 min, 1, 2 and 3 h) were tested with standard MXR transporter inhibitors (MK571 and VER). Based on the results of these assays, 8 µM of RH123 and a 2 h incubation period was used in subsequent experiments

(data not shown). ABC transporter activity was determined by means of accumulation assays using the fluorescent substrate RH123 (8 µM) in the presence of the chemicals, as previously described (section 3.3.3). This assay was performed with 24 hpf embryos because the organism has most of the organs developed and all the genes targeted in this study are transcriptionally active. Embryos (24 hpf) were incubated in the dark, at 26 ± 1 ºC, for 2 h. After the 2 h exposure period, embryos were washed three times with ice-cold water and mechanically disrupted. The fluorescence of RH123 accumulated inside the embryos was measured in the homogenate of 10 embryos using a fluorescent microplate reader (excitation/emission - 485/538 nm) reader (Fluoroskan Ascent, Labsystems). In each replicate (at least four times) RH123 accumulation in water and positive controls (MXR inhibitors) was also measured. Data is presented in percentage (%) of fluorescence in relation to the control (established as 100%). After the 2 h exposure, 30 embryos of each treatment were collected and preserved in RNALater for gene expression analysis.

3.3.7 RNA isolation and cDNA synthesis

Embryos preserved in RNALater from the toxicological and accumulation assays were used to extract total RNA according to Costa et al. (2012a). Briefly, total RNA was isolated using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. RNA quality was verified by electrophoresis in agarose gel and by the measurement of the ratio of optical density at λ260/ λ280 nm. RNA was quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a Fluoroskan Ascent, Labsystems. One microgram of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and synthesis of cDNA was performed using Iscript cDNA Synthesis (Biorad).

3.3.8 Quantitative real-time PCR (qRT-PCR)

Gene expression levels of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod, cat* were assessed by means of quantitative real time PCR (qRT-PCR). Primer pairs for each target gene were designed with Primer 3 software available at http://www.ncbi.nlm.nih.gov/, based in available sequences in GenBank, or described by others such as *abcb4* (Fischer et al. 2013), *abcc2* (Long et al. 2011b), *Cu/Zn sod* (Banni et al. 2011) and *gstπ* (Timme-Laragy et al. 2009). Identities of the amplicons were confirmed by cloning and sequencing of the DNA fragments as described by Costa et al. (2012). Primer sequences, amplicon lengths, efficiencies and Genbank accession numbers of target sequences are given in Table 1S. To determine the efficiency of the PCR reactions, standard curves were made, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves

calculated. Reactions for qRT-PCR were conducted in a iQ5 BioRad apparatus, with 10 µl of SYBR Green Supermix (BioRad), 2 µl of each primer (final concentrations ranging from 10 nM to 300 nM, in Table 1S) and 2 µl of cDNA, in a total volume of 20 µl, in duplicate. Conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 54 °C for 30 s and 72 °C for 30 s. At the end of each run a melting curve analysis was done (from 55 to 95 °C) to determine the formation of the specific products. No template controls were run to exclude contamination and the formation of primer dimers. Reference gene data for fish species suggest transcription can be significantly dependent on tissue types, life stages, or treatment (Filby and Tyler, 2007; Zhang and Hu, 2007; McCurley and Callard, 2008, Mo et al., 2014). For these reasons, commonly used reference genes (elongation factor 1 (*ef1*), RNA polymerase II (*pol II*) and ribosomal protein l8 (*rpl8*) were tested (Fig. 1S, supplementary material for more detailed information) and gene expression was quantified by normalisation with multiple reference genes using Normfinder algorithm(s) (Urbatzka et al. 2013). For characterisation of the transcription profile, *ef1* and *pol II* were used as reference genes and *ef1* and *rpl8* were used for the accumulation and toxicology assays. The relative expression ratio was calculated with efficiencies using the Pfaffl mathematical model (Pfaffl 2001). Despite this normalisation for gene transcription pattern, there were significant differences between zygote and cleavage stages (to be referred as group 1) and the other development stages (to be referred to as group 2). For this reason, no comparison was preformed of gene transcription data from cleavage to blastula. To calculate ΔΔct, the control group comprised the average of Cts from each group. Data is presented in mean of ΔΔct normalised to the reference genes.

3.3.9 Statistical analysis

Differences in mRNA transcriptional levels between treatments in the embryos from 2 h and 80 h exposure and the differences between treatments in accumulation assay and enzyme activities were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Tukey's test) at a 5% significance level. Data were log (mRNA expression and enzyme activities) or sqrt (accumulation assay) transformed in order to fit ANOVA assumptions. Correlations between gene expression of embryos exposed for 2 h and 80 h were tested by Pearson analysis. All tests were performed using software Statistica 7 (Statsoft, Inc). Differences between frequencies of treatments in the toxicological assay were evaluated by means of cross table $\chi 2$ test at 5% significance level. Tests were performed using software SPSS 22 (IBM, Inc). Data is presented as mean ± standard error.

3.4 Results

3.4.1 Characterization of the transcription profile

To establish the constitutive expression levels of genes involved in the detoxification mechanism during zebrafish embryo development, mRNA levels of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod* and *cat,* were assessed in the different development stages (Fig. 3.1). All target genes were expressed at the initial phases of development of *D. rerio*, with the exception of *abcc2* that was detected only at gastrula phase (6 hpf). In group 1 mRNA transcription levels and patterns where similar for all genes measured (Fig. 3.1) whereas in group 2 different patterns of mRNA levels were recorded (Fig. 2S-10S, supplementary material for more detailed information). *abcb4* (Fig. 2S) exhibited the highest mRNA expression in comparison to the other ABC transporters and *cyp3a65* exhibited the highest mRNA expression of all studied genes at larvae phase.

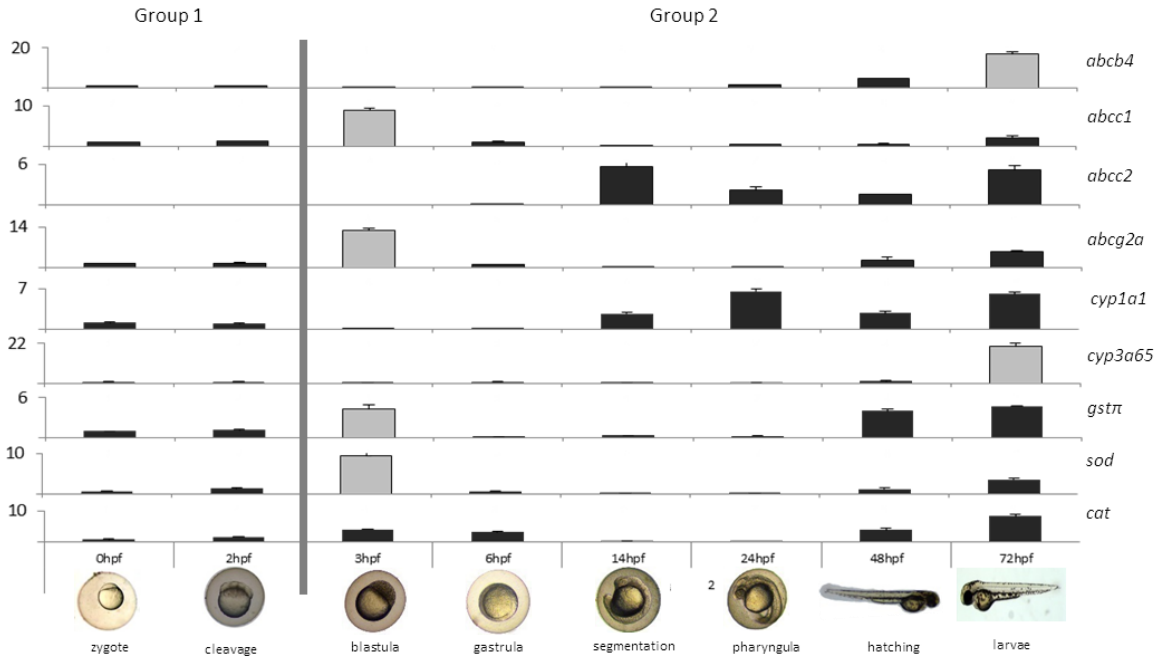

Figure 3.1: Relative mRNA expression of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod* and *cat* during the different stages of embryonic development in *D. rerio* comprising the zygote and cleavage (group 1), blastula, gastrula, segmentation pharyngula, hatching and larvae periods (group 2). The grey bars represent the major significantly differences between the development phases ($p<0.05$). Results are given as mean±SE, n=6.

3.4.2 Toxicological Assay

Cumulative mortality and abnormalities after exposure to SIM (5 and 50 µg/L), MK571 (10 µM), VER (10 µM) alone and the combination of SIM plus MK571 or VER, at different embryo development stages (8, 32 and 80 hpf), are presented in Fig. 3.2a.

Increased mortality and developmental abnormalities were recorded in embryos treated with SIM (both concentrations) alone and in combination with ABC transporter inhibitors. The highest mortality rates were observed in embryos treated with SIM plus VER ($p<0.05$), at 8 hpf. The most severe effects were observed at the delay/arrest of division in embryos exposed to SIM alone and SIM in combination with VER, the latter condition also increased significantly the presence of abnormal cell masses in exposed embryos (Fig. 11Sa, supplementary material for more detailed information).

At 32 hpf, exposure to SIM alone (5 µg/L) and the combination of SIM (50 µg/L) plus VER ($p<0.05$) significantly increased embryo mortality rate. A high percentage of abnormalities, mainly delay in development and tail abnormalities (Fig. 3.2 b2) was detected in embryos exposed to SIM (5 µg/L) plus both efflux protein inhibitors. One hundred percent of severe development delay and abnormalities ($p<0.05$) (Fig. 3.2 b3) were observed in embryos treated with SIM (50 µg/L) alone and in combination with ABC transporter inhibitors (MK571 or VER) (Fig. 11Sb, supplementary material for more detailed information).

After 80 h of exposure to SIM at 50 µg/L, alone and in combination with MK571 or VER, led to a 100 % mortality rate. Also, SIM (5 µg/L) alone or in combination with MK571 or VER has significantly increased ($p<0.05$) embryo mortality rate and abnormalities (such as tail deformities and pericardium edema) (Fig. 3.2 b6) ($p<0.05$), (Fig.11Sb, supplementary material for more detailed information).

3.4.3 EROD activity, GST and antioxidant enzymes

EROD, GST, SOD and CAT activities were measured in zebrafish embryos after 80 h exposure to SIM and the ABC transporter inhibitors at the same conditions as for the toxicology assay (Fig. 3.3). EROD activity (Fig. 3.3 a) was significantly induced by SIM and both ABC protein inhibitors (MK571 and VER) ($p<0.05$) showing a level of activity higher than the positive control (β-NF). GST activity (Fig. 3.3 b) was also significantly stimulated ($p<0.05$) in the presence of SIM and the inhibitors. Cu/ZnSOD (Fig. 3.3 c) and CAT (Fig. 3.3 d) activities were significantly inhibited in all treatments in comparison with control. No significant correlations were observed between the activity of the GST, antioxidant enzymes and EROD and the mRNA levels of the genes encoding for these enzymes.

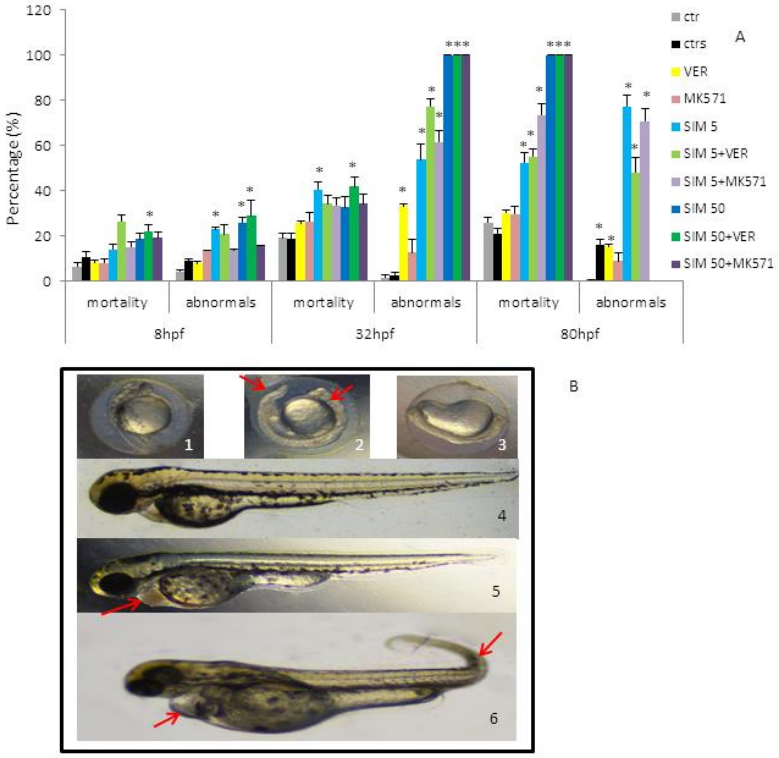

Figure 3.2: Cumulative mortality and abnormality rates at 8, 32 and 80 hpf (a), examples of abnormalities observed in the different treatments; (b) control at 32 hpf (b1), development delay with head and tail abnormalities at 32 hpf (b2), severe development delay with head abnormalities at 32 hpf (b3), control at 80 hpf (b4), blood in the pericardium (b5), multiple abnormalities (pericardia edema and tail abnormalities) at 80 hpf (b6) of *D. rerio* embryos exposed to different concentrations of SIM (SIM 5 - 5 µg/L and SIM 50 - 50 µg/L), verapamil (10 µM) and MK571 (10 µM) and the combination with SIM and ABC transporters inhibitors, for 80 h (%). Results are expressed as mean ± SE, n=6. Bars with asterisk are significantly different from the control ($p<0.05$).

3.4.4 Accumulation Assay

ABC transporter activity was determined by means of accumulation assays using the fluorescent substrate RH123, and results are presented in Fig. 3.4. As expected, an increase on RH123 accumulation inside the embryos was observed after the exposure to ABC transporter's inhibitors being significantly different in embryos exposed to MK571 ($p<0.05$). Embryos exposed to SIM showed a pattern of increased accumulation of RH123, similar to VER, but lower than MK571. Embryos exposed to the combination of SIM plus MK571, and SIM plus VER exhibited a significant increase in RH123 accumulation similar to the one observed in embryos under SIM exposure alone.

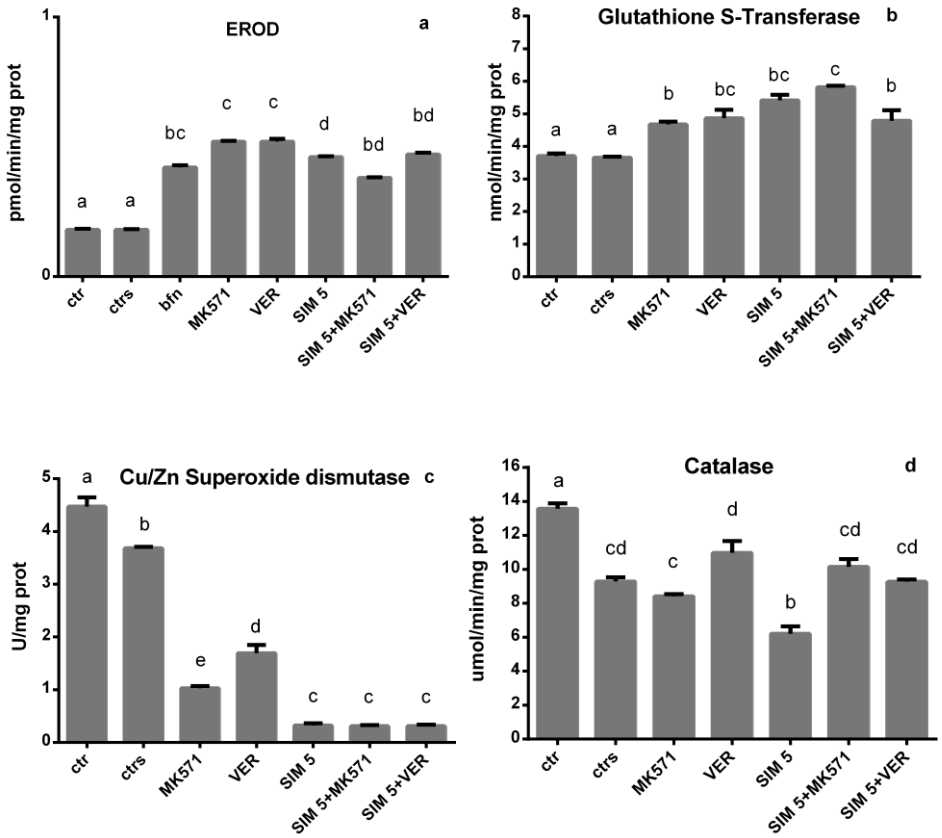

Figure 3.3: EROD (a), GST (b) and Antioxidant enzymes (c, d) activity in D. rerio embryos exposed to SIM (SIM 5 - 5 µg/L), verapamil (10 µM), MK571 (10 µM) and β-FN (10 µg/L) for 80 h. Results are given as mean±SE, n=4. Bars with different letters are significantly different from each other ($p<0.05$).

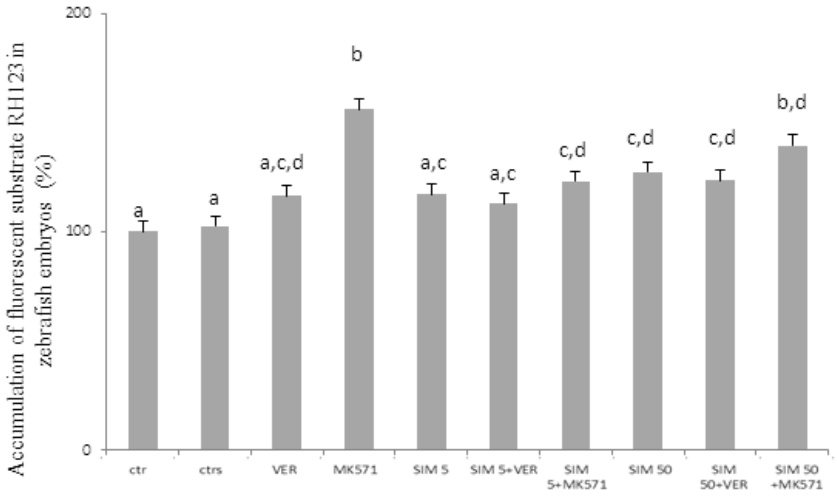

Figure 3 4: Accumulation of rhodamine 123 in D. rerio embryos (24 hpf) exposed to ABC transporters inhibitors (MK571 (10 µM) and VER (10 µM)), SIM (SIM 5 - 5 µg/L and SIM 50 - 50 µg/L), and the combination with SIM and ABC transporters inhibitors, for 2 h (% - percentage of accumulation compared to the controls). Results are given as mean±SE, n=4. Bars with different letters are significantly different from each other ($p<0.05$).

3.4.5 Transcription of target genes in exposed embryos

In order to increase our understanding on the impact of SIM in the detoxification mechanisms, we evaluated mRNA transcripts of a set of genes encoding proteins that play a key role in the detoxification mechanisms. mRNA levels of the selected genes in embryos (24 hpf) exposed for 2 h (accumulation assay) to SIM and ABC transporter inhibitors are presented in Fig. 3.5. MK571 and VER have significantly increased mRNA transcripts of *abcb4* and *abcc2* ($p<0.05$), while no changes were observed for *abcc1* and *abcg2a.* The higher concentration of SIM (50 µ/L) led to an increased mRNA expression of *abcb4, abcc1, abcc2*, *abcg2a, cyp1a1, cyp3a65* and *cat*. In embryos exposed to the combination of SIM (both concentrations) plus MK571, the mRNA transcription of *abcb4, abcc1*, *abcc2*, *abcg2a* and *cyp3a65* was up-regulated, while *cyp1a1, gstπ, Cu/Zn sod* and *cat* were down-regulated. *abcb4, abcc2* and *abcg2a* mRNA expression was down-regulated in embryos treated with SIM (5 ug/L) plus VER, while the opposite occurred with SIM at 50 ug/L. *cyp1a1, gstπ* and *Cu/Zn sod* mRNA transcription were down-regulated by the combination of SIM (5 and 50 µg/L) plus VER, whereas *cyp3a65* and *cat* were downregulated only in the combination of the lower concentration (5 µg/L) of SIM plus VER.

Gene expression at 80 hpf was assessed only in embryos exposed to the lower SIM concentration (Fig. 3.6), due to 100 % mortality in embryos exposed to SIM at 50 µg/L. In embryos exposed to MK571, mRNA expression of *abcb4*, *abcc1* and *abcc2* ($p<0.05$) was significantly up-regulated, whereas no differences were detected for *abcg2a.* In the presence of VER only *abcc2* mRNA transcription was increased, whereas for *abcb4, abcc1* and *abcg2a* no differences were observed. In embryos exposed to SIM, mRNA transcripts of *abcb4, abcc1, gstπ and Cu/Zn sod* were increased, and *cyp3a65* and *cat* were down-regulated, while no differences were observed for *abcc2*, *abcg2a, cyp1a1* transcript levels. In embryos exposed to the combination of SIM plus MK571, mRNA expression of *abcb4*, *cyp3a65* and *cat* was down-regulated, while *gstπ* was up-regulated. Cu/Zn sod and *gstπ* mRNA expression was significantly up-regulated ($p<0.05$) in embryos exposed to SIM plus VER, and *cyp3a65* and *cat* were down-regulated, while no differences were observed for the other analysed genes.

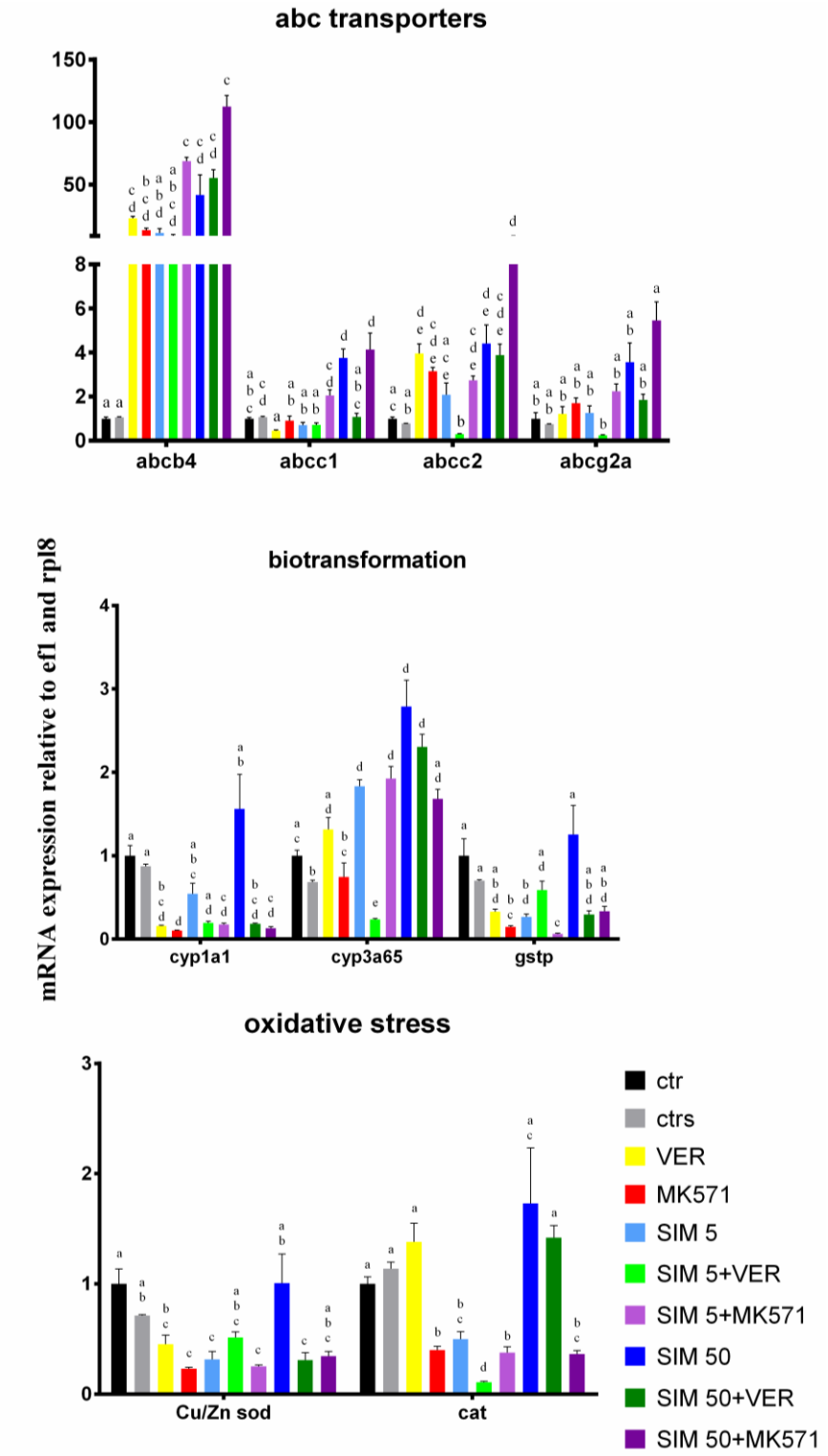

Figure 3.5: Relative mRNA expression of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ*, *Cu/Zn sod* and *cat* in *D. rerio* embryos exposed to SIM (SIM 5 - 5 µg/L and SIM 50 - 50 µg/L), verapamil (10 µM) and MK571 (10 µM) at 24 hpf for 2 h. Bars with different letters are significantly different from each other ($p<0.05$). Results are given as mean±SE, n=6

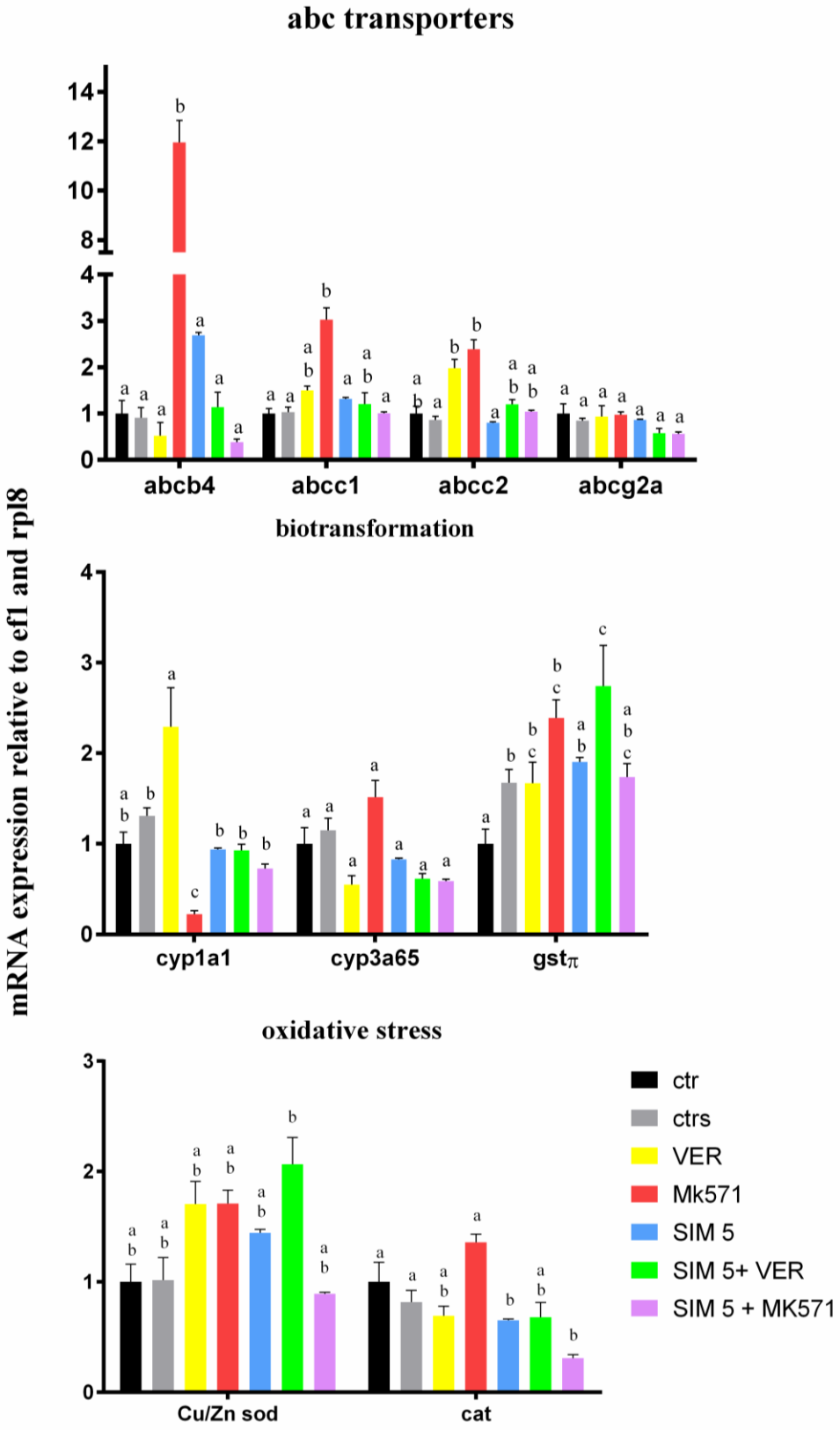

Figure 3.6: Relative mRNA expression of abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod and cat in D. rerio embryos exposed to SIM (SIM 5 - 5 µg/L), verapamil (10 µM) and MK571 (10 µM) for 80 h. Bars with different letters are significantly different from each other ($p<0.05$). Results are given as mean±SE, n=6

3.5. Discussion

3.5.1 Characterization of the transcription profile in early stages of development

The determination of constitutive transcription patterns for the detoxification components will increase background knowledge for future toxicity studies, enabling a better understanding of the interaction of chemicals with detoxification mechanisms in early embryo development stages of zebrafish.

In the two first stages of development, zygote (0 hpf) and cleavage (2 hpf), mRNA of all the selected genes (with the exception of *abcc2*) was detected indicating maternal transfer. Accordingly, Long et al. (2011b) have also shown that *abcc2* mRNA transcripts are not from maternal origin. Embryo gene transcription begins only after cleavage, in midblastula transition (Abrams and Mullins 2009), thus the presence of transcripts of these genes in such early stages of development, confirm their maternal origin. A decreased pattern of mRNA transcription was observed after blastula (3 hpf) until gastrula (6 hpf), increasing afterwards due to new transcripts of embryo origin. After blastula, primary germ layer and embryonic axis are produced and coincide with the major wave of embryo genome activation in fish (Tadros and Lipshitz 2009). Thus, the fluctuations in gene expression are related with embryo development that lead to a low mRNA expression before 6 hpf, due to the degradation of maternal mRNA, followed by an increase due to new transcripts being produced by the embryos. After hatching, mRNA expression of all the evaluated genes was increased suggesting that at this stage the larvae require additional protection against the environmental stressors. These results are in line with other studies with aquatic organisms that also reported increased transcription of genes involved in detoxification processes after hatching (Costa et al. 2012b; Faria et al. 2011; Minier et al. 2002).

3.5.2. Potential chemosensitizer effect of SIM

To evaluate the chemosensitisation potential of simvastatin, two known ABC transporter inhibitors (MK571 and VER) were used to assess the degree of inhibition of SIM in comparison to model inhibitors. MK571 has been described as an ABCC inhibitor (Navarro et al. 2012) but recent studies showed that it may also inhibit other ABC proteins (Mease et al. 2012), including Abcb4 in zebrafish (Fischer et al. 2013); while verapamil is a P-gp competitive inhibitor and substrate (Fischer et al. 2013; Ferreira et al. 2014b). SIM and/or their metabolites may have a weak inhibitory effect on the ABC proteins that is comparable to VER as shown by the increase in accumulation of RH123 inside the embryos when exposed to SIM. Indeed, an inhibitory effect of SIM on ABCB1 protein was already reported in mammals and fish cell lines (Caminada et al. 2008; Bogman et al.

2001; Wang et al. 2001; Sieczkowski et al. 2010). In contrast, other studies have demonstrated that statins can be transported by ABCC2 in humans and rats and also by ABCG2 in humans (Ellis et al. 2013; Hirano et al. 2005).To our knowledge this is the first study to evaluate the chemosensitising potential of SIM in *in vivo* fish embryos and the results also point to a potential inhibitory effect on ABC transporters by SIM. Moreover, in humans it has been shown that certain statins metabolites inhibit P-gp, increasing the systemic exposure to statins (Lemahieu et al. 2005). However, the accumulation assay does not show which efflux proteins are being inhibited and further studies should be performed in order to understand which ABC transporters can possibly be inhibited by this pharmaceutical. In mammals, statins inhibit the CYP3A and P-gp activity which was related to an increase in the bioavailability of other pharmaceuticals (Yang et al. 2011; Lee et al. 2015). This demonstrates that statins, including SIM, can diminish the cells capability to efflux other drugs. Nevertheless, to some extent statins can have a weak chemosensitiser potential on ABC transporters, and SIM and/or its metabolites might, to some extent, impact the toxicity of other pollutants and consequently have an effect in the detoxification potential in aquatic organisms. However, further studies must be performed to fully understand the potential of SIM to inhibit ABC transporters.

3.5.3. Simvastatin effects on zebrafish embryos and on transcription of genes involved in detoxification processes

The toxicity of simvastatin to *D. rerio* embryos has been previously reported (Ribeiro et al. 2015; Neuparth et al. 2014; Dahl et al. 2006). Here, MK571 and VER were used as a proxy of a chemosensitiser to test if SIM, in the presence of other chemosensitisers, could be more toxic. However, despite the use of NOEC (5 µg/L) and LOEC (50 µg/L) concentrations found in a previous study (Ribeiro et al. 2015; Torres 2013), our results revealed that embryos treated with SIM (50 µg/L) presented severe developmental delays and abnormalities that resulted in increased mortality. Nevertheless our results identify SIM as a weak inhibitor, hence SIM can act as a competitive inhibitor and/or SIM can selectively inhibit only some efflux proteins entering the cells and exerting its toxic effects. SIM plus MK571, led to a higher inhibitory effect when compared with SIM plus VER, probably related to the higher inhibitory effect of MK571. Also, in humans, the co-administration of SIM and VER increased the risk of adverse drug reactions like statin-induced toxicity (Holtzman et al. 2006; Knauer et al. 2010) which is in line with our results. mRNA of ABC transporters, mainly *abcb4*, were up-regulated after 2 h exposure to SIM, which correlated with the MXR assay where a weak chemosensitiser potential was observed. A cross-talk mechanism where the inhibition of efflux proteins triggers the transcription of ABC transporters can be the cause of this increase. In agreement, *in vitro*

studies in mammals and fish described an up-regulation and inhibition of ABCB1 (Yamasaki et al. 2009; Ellesat et al. 2012; Caminada et al. 2008; Wang et al. 2001). Additionally, an up-regulation of genes coding for biotransformation and antioxidant enzymes was also observed, mainly in embryos treated with the higher concentration of SIM (50 µg/L), demonstrating that SIM can also interact with the transcription of other genes of the detoxification mechanism. Supporting the present findings, the up-regulation of *cyp3a, cyp2b6 and sod*, under exposure to SIM and other statins such as atorvastatin, was also reported in *in vitro* studies in mammal and fish cell lines (Kocarek et al. 2002; Ellesat et al. 2012). After 80 h exposure to SIM embryos maintained the *abcb4* up-regulated pattern observed. An induction of EROD activity was observed after 80 h of exposure to SIM but no effect on the transcription of *cyp1a1* was detected. Phase II gene and enzyme activity levels were both increased in the presence of SIM. *In vitro* studies, with different fish species, also showed induction of phase I and II transcription and catalysis by different pharmaceuticals (such as rifampicin or diclofenac) (Gröner et al. 2015; Ku et al. 2014; Wassmur et al. 2013). Although to our knowledge there are no studies addressing the effects of statins, our results show that SIM, like other pharmaceuticals, can influence the biotransformation mechanism at a transcriptional and catalytic level.

In the presence of SIM, antioxidant enzyme activity decreased while a pattern of up-regulation was observed for Cu*/Zn sod* mRNA expression that can result from a transcription level response to compensate the diminished catalytic activity. In a study performed with rainbow trout gills, atorvastatin has also up-regulated *sod* expression (Ellesat et al. 2012). SIM also appears to interact with antioxidant protein synthesis and mRNA levels showing different dynamics over time. Our results showed that SIM effects on antioxidant enzymes are different than those reported in *in vivo* and *in vitro* mammalian studies where these proteins were induced in the presence of statins (Hsieh et al. 2008; Landmesser et al. 2005; Sanchez et al. 2008). These contrasting results may be due to different times of exposure and/or the use of different model organisms. Even though concentrations above the ones observed in the environment were used in the present study, the results described herein showed that statins can possibly interfere with the detoxification mechanism which can result in negative effects to the organism. Overall, our results demonstrated that transcripts of detoxification genes are present in early development stages and their transcription can be altered in the presence of xenobiotics (e.g. pharmaceuticals).Therefore the results presented in this study showed that SIM can impact gene expression and enzyme activities (such as MXR, phase I and antioxidant enzyme activities) important features for cellular defence against harmful compounds.

3.6. Conclusions

In conclusion, the majority of the target genes evaluated in these studies appear to be from maternal origin supporting their importance in the protection of the organism in early life stages of development. This preliminary study focused on the effects of SIM on the detoxification mechanisms demonstrating that, in the presence of common ABC inhibitors, SIM can be more toxic to *D. rerio* embryos. Also, SIM appears to act as a weak inhibitor of MXR, and to influence the mRNA transcription of key genes and enzymes belonging to the detoxification process which can increase the potential toxicity of other chemicals present in the water, supporting the importance of monitoring the presence of this class of substance in the aquatic environment.

Conflict of interest

The authors declare that they do not have any conflict of interest.

Contributors

Virgínia Cunha participated in the design and practical work, analysis and interpretation of data and writing of the paper. Marta Ferreira participated in the design of the work, analysis and interpretation of data and along with Miguel Santos and Pedro Moradas-Ferreira that contributed to the article preparation by revising it critically.

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SUPPLEMENTARY DATA

Table 1S: Gene list Genbank accession numbers Primer sequences and concentrations, amplicon lengths, efficiency of reaction for, ABC transporters (*abcb4, abcc2, abcc1, abcg2a*), biotransformation (*cyp1a1, cyp3a65, gstπ*) and antioxidant (*Cu/Zn sod, cat*) enzymes, *ef1*, *pol II* and *rpl8* gene expression quantification by qRT-PCR in zebrafish.

Gene	Accession number	Primers Sequence (5'→3')	Final Conc. (nM)	Amplicon length (bp)	Efficiency (%)
abcb4	JQ014001	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
cyp1a1	NM_131879.1	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102.0
cyp3a65	NM_001037438.1	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91.0
gstπ	NM_131734	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119.0
Cu/Zn sod	Y12236	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110.0
cat	NM_130912.1	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113.0
ef1	NM_131263.1	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
pol II	NM_001024461.2	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	292	96
rpl8	NM_200713.1	F: CAATGACGACCCGACCG R: CGCCAGCAACTCAGTCACT	10	136	96

Figure 1S

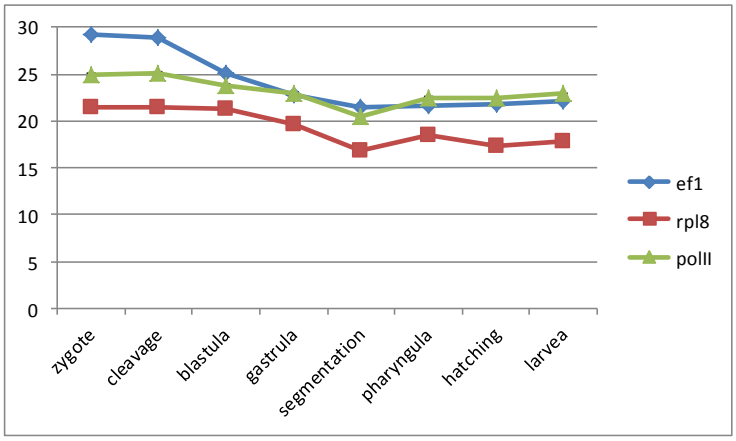

Fig 1S : Ct values of *ef1*, *rpl8* and *polII* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) periods. Results are given as mean±SE, n=6.

Figure 2S

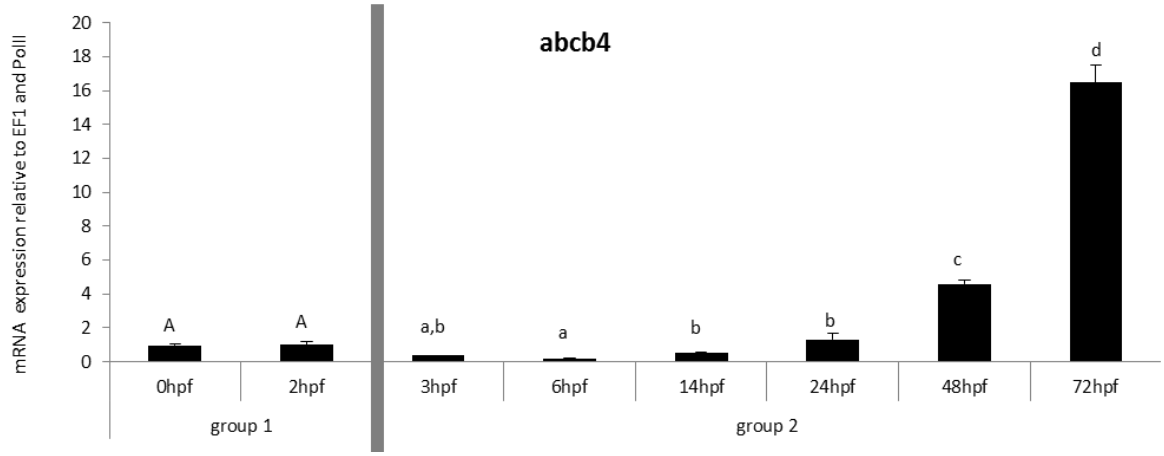

Fig 2S: Relative mRNA expression of *abcb4* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 3S

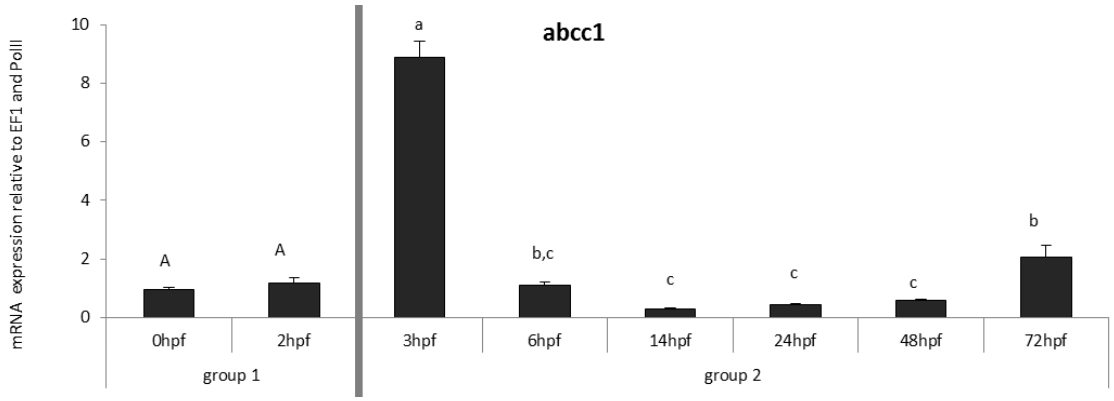

Fig 3S: Relative mRNA expression of *abcc1* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 4S

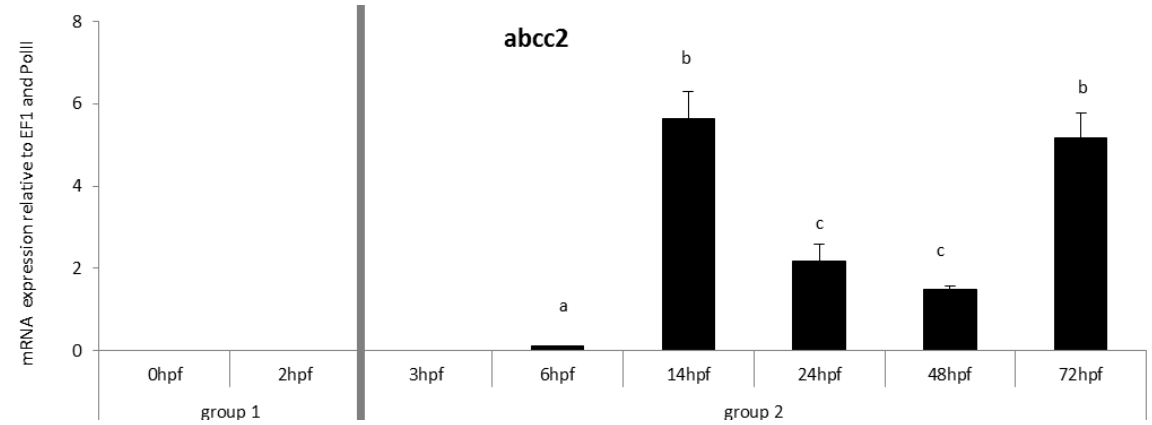

Fig 4S: Relative mRNA expression of *abcc2* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 5S

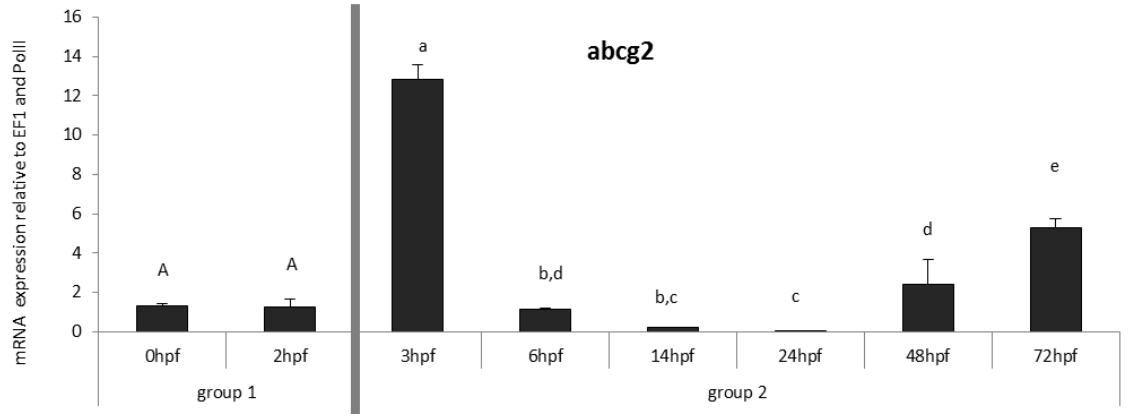

Fig 5S: Relative mRNA expression of *abcg2a* during the different stages of embryonic development *in D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 6S

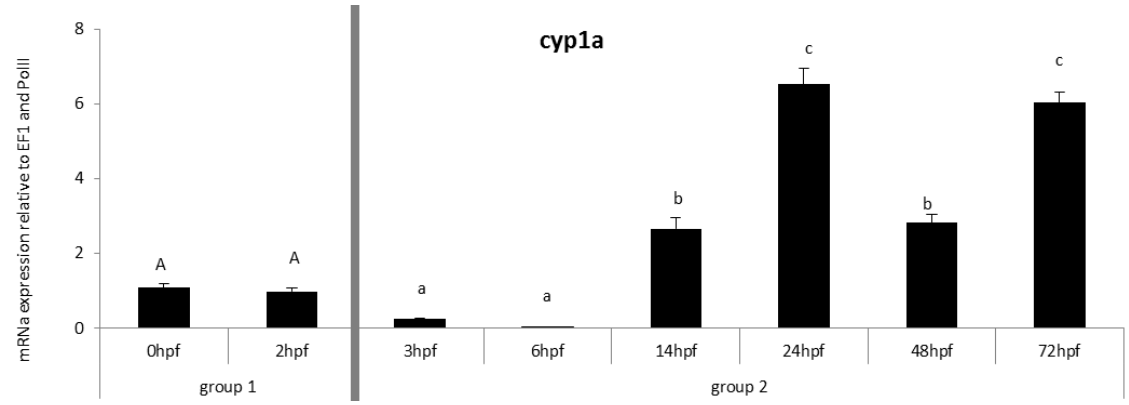

Fig 6S: Relative mRNA expression of *cyp1a1* during the different stages of embryonic development *in D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 7S

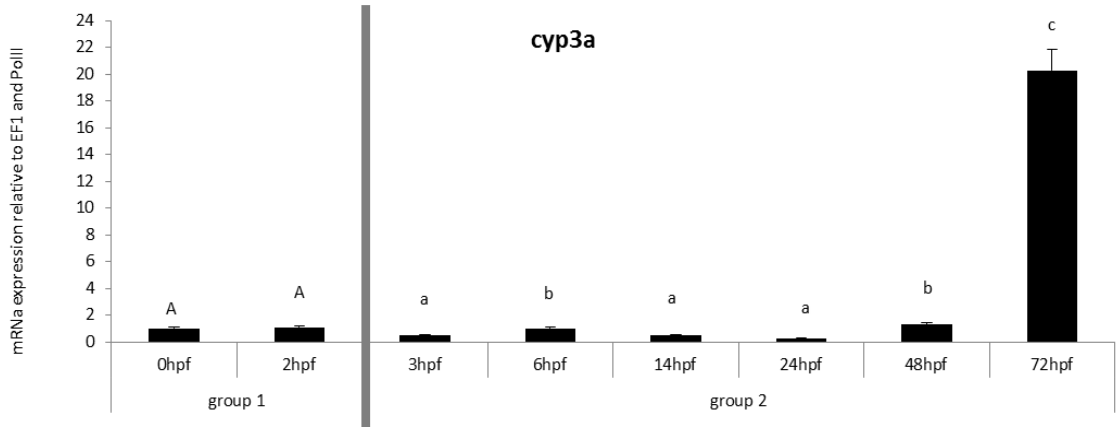

Fig 7S: Relative mRNA expression of *cyp3a65* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 8S

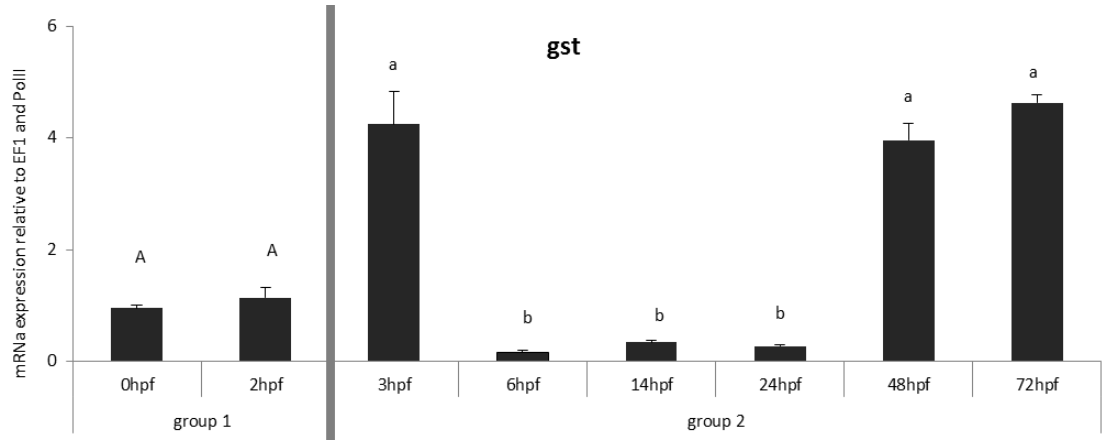

Fig 8S: Relative mRNA expression of *gstπ* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 9S

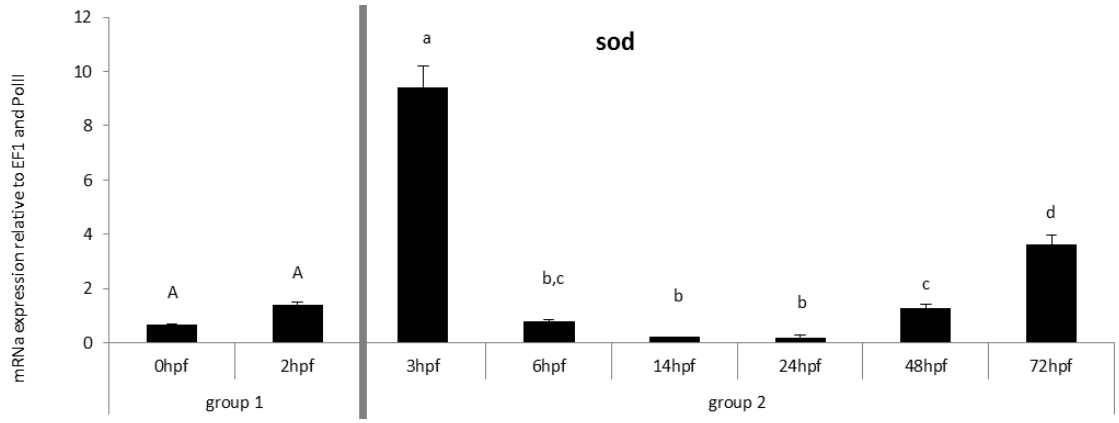

Fig 9S: Relative mRNA expression of *Cu/Zn sod* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases in group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 10S

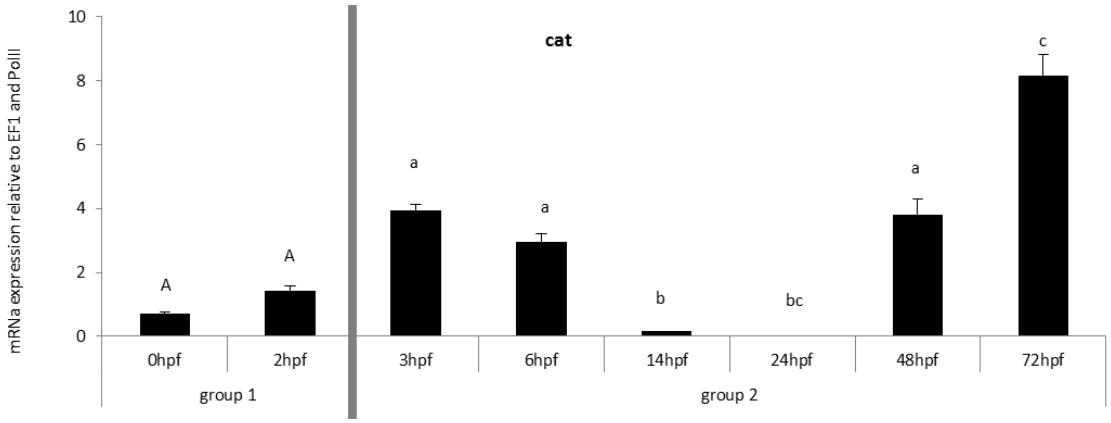

Fig 10S: Relative mRNA expression of *cat* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases in group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 11S

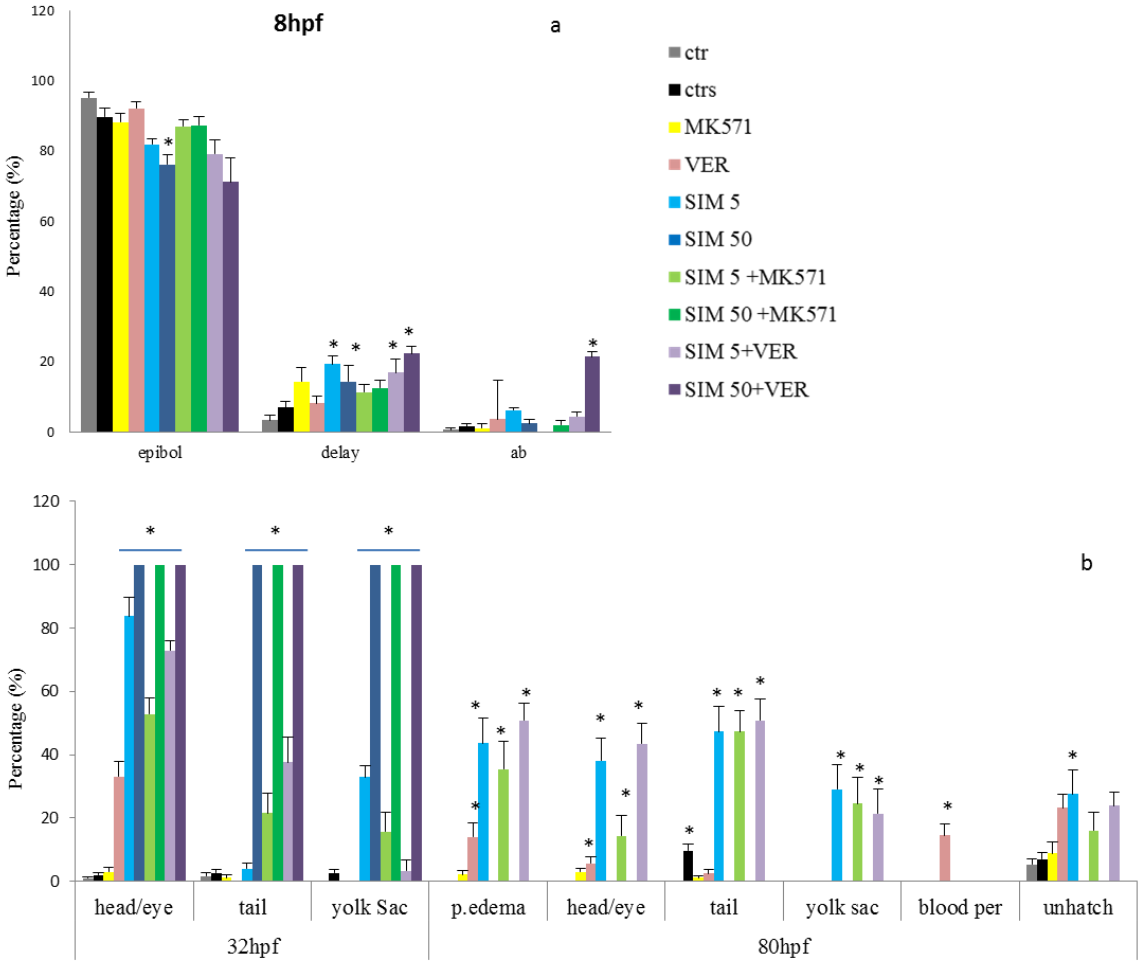

Fig 11S : Percentage of 75% epiboly, delay/arrest of the division, abnormal cell masses (ab) in *D. rerio* embryos at 8 hpf (a), and percentage of different anomalies presented by the embryos at 32 hpf and 80 hpf (b) exposed to different concentrations of SIM (SIM 5 - 5 µg/L and SIM 50 - 50 µg/L), verapamil (10µM) and MK571 (10µM) and the combination with SIM and ABC transporters inhibitors. Results are expressed as mean ± SE. Bars with asterisk are significantly different from the control.

CHAPTER IV

SIMVASTATIN EXPOSURE CAN MODULATE GENE EXPRESSION OF NUCLEAR RECEPTORS LINKED TO DETOXIFICATION PROCESS IN ZEBRAFISH EMBRYO

This chapter has been submitted as:

Cunha V, Santos MM, Moradas-Ferreira P, Castro LFC, Ferreira M. Nuclear receptors expression profile in Danio rerio embryos exposed to simvastatin.

Simvastatin exposure can modulate gene expression of nuclear receptors linked to detoxification process in zebrafish embryos

4.1 Abstract

Nuclear receptors (NRs) are involved in the regulation of several metabolic processes, and can be modulated by different chemicals, including emerging contaminants. This work aimed at characterizing the effect of an emerging contaminant the simvastatin (SIM), a regulator of cholesterol (Chol) levels, on selected NRs and aryl hydrocarbon receptor (AhR) transcription during embryonic and larvae stages of zebrafish. Embryos were collected at different development stages (0, 2, 6, 14, 24, 48 and 72 hpf) and transcription patterns of genes coding for NRs evaluated. Embryos were exposed to different concentrations of simvastatin (5 µg/L and 50 µg/L) at different development stages (0 hpf and 24 hpf) and exposure times (2 h and 80 h). In mammals, pharmaceuticals have been found to modulate the transcription of *Pxr, Ppars* and *Ahr* affecting the mRNA expression of genes belonging to different regulatory pathways, such as lipid metabolism and detoxification mechanism. The transcription levels of *ahr2, raraa, rarab, rarga, pparαa, pparβ1, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb*, as well as the levels of Chol were evaluated. All target genes seem to be from maternal transfer as revealed by their mRNA presence at 0 hpf. SIM did not change the levels of Chol, in embryos or larvae, but altered the mRNA expression of NRs and AhR in a time and NR-specific manner. After 2 h exposure to SIM in 24 hpf embryos, the transcription of ppars, pxr and ahr was up regulated, while after an 80 h exposure mRNA levels of pxr and ahr were decreased. Our results demonstrate a possible impact of SIM in the transcription regulation of NRs that play a key role in several downstream pathways involved in different physiologic functions.

Keywords: statins, cholesterol, mRNA levels, emerging contaminants, nuclear receptors, detoxification

4.2 Introduction

Nuclear receptors (NRs) comprise a large group of transcription factors, involved in the regulation of several biological processes such as reproduction, cell differentiation, metabolism, and development (Gronemeyer et al., 2004). The disruption of these NRs and their controlled signalling cascades can alter the regulation of genes involved in a variety of metabolic mechanisms, ultimately impacting the physiology of the organisms. NRs are activated by the binding of endogenous or exogenous small lipophilic compounds (Mangelsdorf et al., 1995; Pardee et al., 2011). Different chemicals, of which pharmaceuticals are a prime example, are present in the aquatic environment and can mimic bona fide ligands (Delfosse et al., 2015). Numerous studies have shown that some of these compounds have the ability to activate NR-mediated signals leading to toxic responses (Janosek et al., 2006; Grun and Blumberg, 2006; Castro and Santos, 2014).

Pharmaceutical compounds are among the emerging contaminants detected in aquatic environments in increasing concentrations. Simvastatin (SIM), an anticholesterolemic drug from the statins group, is one of the top prescribed pharmaceutical and has been detected in aquatic environments (Hernando et al., 2006; Santos et al., 2010). In humans, SIM reduces cholesterol (Chol) inhibiting 3-hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA) reductase limiting HMG-CoA conversion into mevalonate and increasing low density lipoproteins cholesterol (LDL-C) receptors and LDL-C uptake (Rodwell et al., 1976; Fent et al., 2006). SIM also exerts significant immunomodulatory and anti-inflammatory effects autonomous of lipid lowering (Alvarez de Sotomayor et al., 2008; Fraunberger et al., 2009). Previous studies reported negative effects of SIM in non-target aquatic organism (Ribeiro et al., 2015; Neuparth et al., 2014; Ellesat et al., 2011). However the knowledge is still scarce on its ability to regulate and affect NRs in particular in fish early stages of development.

This study focused in NRs which can be involved in the lipid metabolism, inflammatory and immune homeostasis and in xenobiotic metabolism. The peroxisome proliferator activated receptor subfamily (PPAR) includes three subtypes (PPARα, PPARβ and PPARγ), which were identified in the genome of various vertebrate species including fish (Ruyter et al., 1997; Cajaraville et al., 2003; Ibabe et al., 2002; Zhao et al., 2015). In mammals, PPARs were shown to be involved in the regulation of inflammatory and immune homeostasis and lipid, glucose and xenobiotic metabolism (Janani and Ranjitha Kumari, 2015; Ipseiz et al., 2014; Xu et al., 2005). The Pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are known to regulate different gene cascades involved in the transport, metabolism and elimination of xenobiotics in mammals (Xu et al.,

2005; Wang et al., 2012). The Retinoid X receptor (RXR) can form homodimers and heterodimers with other NRs, including PXR, PPAR and retinoid acid receptors (RAR) (Yu et al., 1991; Mangelsdorf and Evans, 1995; Wang and LeCluyse, 2003). Other receptor relevant in cell detoxification processes is the aryl hydrocarbon receptor (AhR) that is transactivated by aromatic and halogenated compounds (Elferink, 2003; Hahn, 2002).

In fish, the regulation of several of these mechanisms by NRs is not fully understood. Nevertheless, given the phylogenetic proximity between fish and mammals, several NR-dependent signalling pathways are phylogenetically conserved (Corcoran et al., 2012; Cocci et al., 2015; Bou et al., 2014; Carmona-Antoñanzas et al., 2014; Lima et al., 2015). In mammals gene expression of NRs such as *Pxr, Ppar* and the transcription of *Ahr* were found to be modulated by different anthropogenic chemicals, including pharmaceuticals that subsequently affected gene expression of drug-metabolizing genes (Aleksunes and Klaassen, 2012). Similar findings were reported for teleosts, including changes in the transcription levels of genes involved in drug metabolism such as *cyp1a* or *cyp3a* (Wassmur et al., 2010; Mortensen and Arukwe, 2007). Given the paucity of toxicological data for several classes of pharmaceuticals, it is essential to evaluate their effects in non-target organisms, such as teleost fish.

Zebrafish, *Danio rerio*, is widely used as a model species in several research areas, showing interesting features such as the fully sequenced genome, available molecular tools, and a short life-cycle and amenable laboratory maintenance (Baker and Hardiman, 2014). Furthermore it is an interesting species for comparative studies with mammals showing a high degree of conservation of several signalling pathways. Therefore, this study aims at *i*) characterize the basal transcription profile of NRs in zebrafish embryos, at different development stages and *ii)* evaluate the effects of SIM in mRNA transcription of NRs involved in different regulatory pathways including lipid regulation and ahr gene transcription at different stages of development and different exposure times.

4.3 Material and methods

4.3.1 Maintenance of zebrafish, rearing conditions and exposure assays

Adult wild-type zebrafish, obtained from local suppliers, were used as breeding stocks. Fish were kept at a water temperature of 27±1 ºC and in a photoperiod of 12:12 h (light:dark) in 60 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), supplemented once a day with live brine shrimp (*Artemia spp*.). For reproduction, females and males (ratio 1:2) were

transferred to a maternity, and submitted to acclimatization for 12 h in a cage with a net bottom covered with glass marbles within a 30 L aquarium. After spawning on the following day, the breeders were removed after the beginning of the light period. The spawned eggs were collected, counted, cleaned and stored in RNALater (30 embryos per time-point) at 0, 2, 3, 6, 14, 24, 48 and 72 hours post fertilization (hpf), for further transcription profiling. Different clutches were used to reduce variability in the development stages. Time-points for embryo and larvae collection were chosen based on the developmental embryonic and larval stages of *D. rerio* as described by Kimmel et al. (1995). Accordingly, embryos were grouped in 8 development stages - zygote (0 hpf), cleavage (2 hpf), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf), and larvae (72 hpf).

Two assays were performed with zebrafish embryos exposed to SIM (5, 50 µg/L) chosen according to previous studies (Ribeiro et al., 2015; Torres, 2013; Key et al., 2008). Briefly, for assay 1, after reproduction, 1 hpf embryos were transferred to new aquaria (3.5 L) and were kept for 24 h, at 26 ± 1 ºC with aeration, and then exposed to SIM in a 24 well plate (10 embryos per well), for 2 hours and then collected for lipid extraction and the remaining stored in RNALater. In assay 2, after spwanning, embryos (10 per well) were transferred to a 24 well plate and exposed to SIM for 80h. Throughout the experiment, the medium was renewed every day and at 80 hpf, 60 embryos per treatment were collected for lipid extraction and 30 embryos stored in RNALater. The two assays were performed according to a previous study (Cunha et al., 2016), in order to evaluate the effects of short-term exposure to SIM (2 h) and exposure throughout the embryonic development (80 h).

4.3.2 RNA isolation and cDNA synthesis

Embryos stored in RNALater from both assays were used to isolate total RNA. RNA isolation was performed according to Costa et al., 2012. Briefly, total RNA was isolated using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. RNA quality was verified by electrophoresis in agarose gel and by measurement of the ratio of optical density at λ260/280 nm. RNA was quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a Fluoroskan Ascent, Labsystems. One microgram of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and synthesis of cDNA was performed using Iscript cDNA Synthesis (Biorad).

4.3.3 Quantitative real-time PCR (qRT-PCR)

Gene expression of *ahr2, raraa, rarab, rarga, pparαa, pparβ1, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb,* was assessed in embryo and larvae at the different stages of development by means of quantitative real time PCR (qRT-PCR). Primer pairs for each target gene were designed with Primer 3 software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), based on available sequences in GenBank. Identities of the amplicons were confirmed by cloning and sequencing of the DNA fragments as described by Costa et al. (2012). Primer sequences, amplicon lengths, efficiencies and Genbank accession numbers of target sequences are given in Table 1S. To determine the efficiency of the PCR reactions, standard curves were made, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated (Table 1S). Reactions for qRT-PCR were conducted in an iQ5 BioRad, with 10 µl of SYBR Green Supermix (BioRad), 2 µl of each primer (final concentrations ranging from 0.001µM to 0.6µM) and 2 µl of cDNA, in a total volume of 20 µl, in duplicate. Conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 54 °C with exception of *rxr* and *rar* (60°C) (for 30 s and 72 °C for 30 s. At the end of each run a melting curve analysis was done (from 55 to 95 °C) to determine the formation of the specific products. No template controls were run to exclude contamination and the formation of primer dimers.

Gene expression was quantified according to Cunha et al., 2016, by normalizing with multiple reference genes using Normfinder algorithm (Andersen et al., 2004; Urbatzka et al., 2013). For characterization of the transcription profile the elongation factor 1 (*ef1*) and RNA polymerase II (*pol II*) were used while *ef1* and ribosomal protein l8 (*rpl8*) collected from the two assays. The relative expression ratio was calculated with PCR efficiencies using the Pfaffl mathematical model (Pfaffl, 2001). Despite this normalization for gene transcription pattern, there were significantly differences between zygote and cleavage stages (to be considered as group 1) and the following development stages (to be considered as group 2). For this reason, no comparison was performed for gene transcription data from cleavage to blastula. To calculate ΔΔCt for gene transcription pattern, the reference or control Ct was assigned as the average of Cts from each group. Data is presented in mean of mRNA expression in relation to the reference genes.

4.3.4 Lipid extraction

Lipids were extracted from zebrafish embryos (60 embryos) exposed to SIM, in both assays, using the Folch method, with some modifications (Folch et al., 1957). Briefly, embryos were homogenized in chloroform:methanol solution (2:1). The homogenate was

transferred to a glass vial and incubated at room temperature, in the dark, in an orbital shaker (100 rpm) for 30 min. To avoid lipid oxidation, a nitrogen atmosphere was always insured inside the glass vials before closing. The solution containing the lipids was then washed with 0.9 % NaCl and vortexed for 1 min and centrifuged for 10 min at 2000 rpm (15 ºC). The two phases were then separated and the upper phase removed. The solution was again washed with methanol:water (1:1), centrifuged at 2000 rpm and the upper phase removed. The lower phase containing the lipids was evaporated under nitrogen stream, and the dried extracts stored at -20ºC until quantification. Recovery rate of the extraction method was performed by spike procedure with Chol (1 mg/ml) and calculated to be 70%.

4.3.5 Cholesterol quantification

Dry extracts containing the lipids were re-suspended in 500 µL methanol and dissolved with ultrasound for at least 2 hours at 35ºC until complete dissolution. Determination of cholesterol (Chol) was performed by using a colourmetric assay, InfinityTM Cholesterol Liquid Stable Reagent (Thermo Scientific, Biognóstica, Portugal) following the manufacture's protocol. The absorbance was measured at 490 nm and 630 nm at 37 ºC. Standard curves using cholesterol dissolved in metanol were performed in every run and each assay was replicated at least three times. Data is expressed in µg/µl.

4.3.6 Statistical analysis

Differences in mRNA gene transcription and Chol quantification among treatments in the two assays were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Tukey's test) at a 5% significant level. Data were log transformed in order to fit ANOVA assumptions. All tests were performed using software Statistica 7 (Statsoft, Inc). Data is presented as mean ± standard error.

4.4. Results

4.4.1 Characterization of the transcription profile

The mRNA transcription of *ahr2, raraa, rarab, rarga, pparαa, pparβ1, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb* in the eight development stages of *D. rerio* were assessed and are presented in Fig. 4.1. All target genes were expressed at the initial phases (zygote and cleavage phases) of development showing similar transcription levels (Fig. 1S-13S, supplementary material for more detailed information). In group 2, different patterns of mRNA transcripts of the target genes were observed (Fig. 4.1). The mRNA expression pattern of *pxr, rxraa* (Fig. 1S and 2S) was similar, increasing from blastula until pharyngula phase (24 hpf), decreasing at hatching (48 hpf) ($p<0.05$), increasing again in

the larvae phase (72 hpf) ($p<0.05$). *rxrab, pparα* (Fig. 3S and 10S, respectively) mRNA decreased from blastula until gastrula (6 hpf) ($p<0.05$), gradually increasing after gastrula up to larvae phase. *rxrbb, pparβ, pparγ, ahr2* (Fig. 4S, 8S, 11S, 12S and 13S) mRNA levels decreased from blastula until segmentation phase (14 hpf), increasing after segmentation until larvae phase. Regarding *rxrga* (Fig. 5S) the mRNA expression increased gradually from blastula (3 hpf) until larvae phase. Transcription of *rxrgb* (Fig. 6S) was up regulated from blastula until gastrula phase (6 hpf), decreasing at segmentation phase, increasing again after segmentation until larvae stage. mRNA levels of *raraa, rarga* (Fig. 9S) increased from blastula until the segmentation phase (14 hpf), decreasing at 24 hpf, increasing again the transcription in the later phases (48 and 72 hpf). *rarga* displayed the highest transcripts levels of all NRs at larvae phase. mRNA expression of *rarab* decreased from blastula until segmentation phase maintaining the expression levels in the following phases.

4.4.2 Gene expression in embryos exposed to simvastatin

The mRNA levels on embryos in the assay 1 are presented in Fig. 4.2. The mRNA level of *ppar's, pxr* and *ahr* was significantly up regulated in embryos exposed to the highest concentration of SIM (50 µg/L) ($p<0.05$), whereas no differences were observed at the lowest SIM concentration (5 µg/L). An mRNA downward pattern was observed for *rar's* when the embryos were treated with SIM (at both concentrations). This down regulation was more evident in the embryos exposed to the highest concentration of SIM, being significant different for *raraa, rarab* and *rarga* ($p<0.05$). The transcription of *rxr's* was also down regulated in the presence of SIM (at both concentrations) ($p<0.05$), being significant at the lowest concentration.

In assay 2 (Fig. 4.3) the mRNA expression of the target genes was evaluated only in the lowest concentration of SIM due to a 100% mortality registered in embryos treated with 50 µg/L at 32 hpf. No significant changes were observed for ppars in embryos treated with SIM. Transcription of *pxr* and *ahr* was significantly down regulated in the presence of SIM ($p<0.05$). The mRNA of *rarab,* was up regulated ($p<0.05$), and like *pxr* and ahr exhibited an opposite pattern from the embryos in assay 1. No differences in mRNA levels were observed for *rxrab, rxrbb and rxrga*, only *rxrgb* was significantly up regulated with SIM exposure.

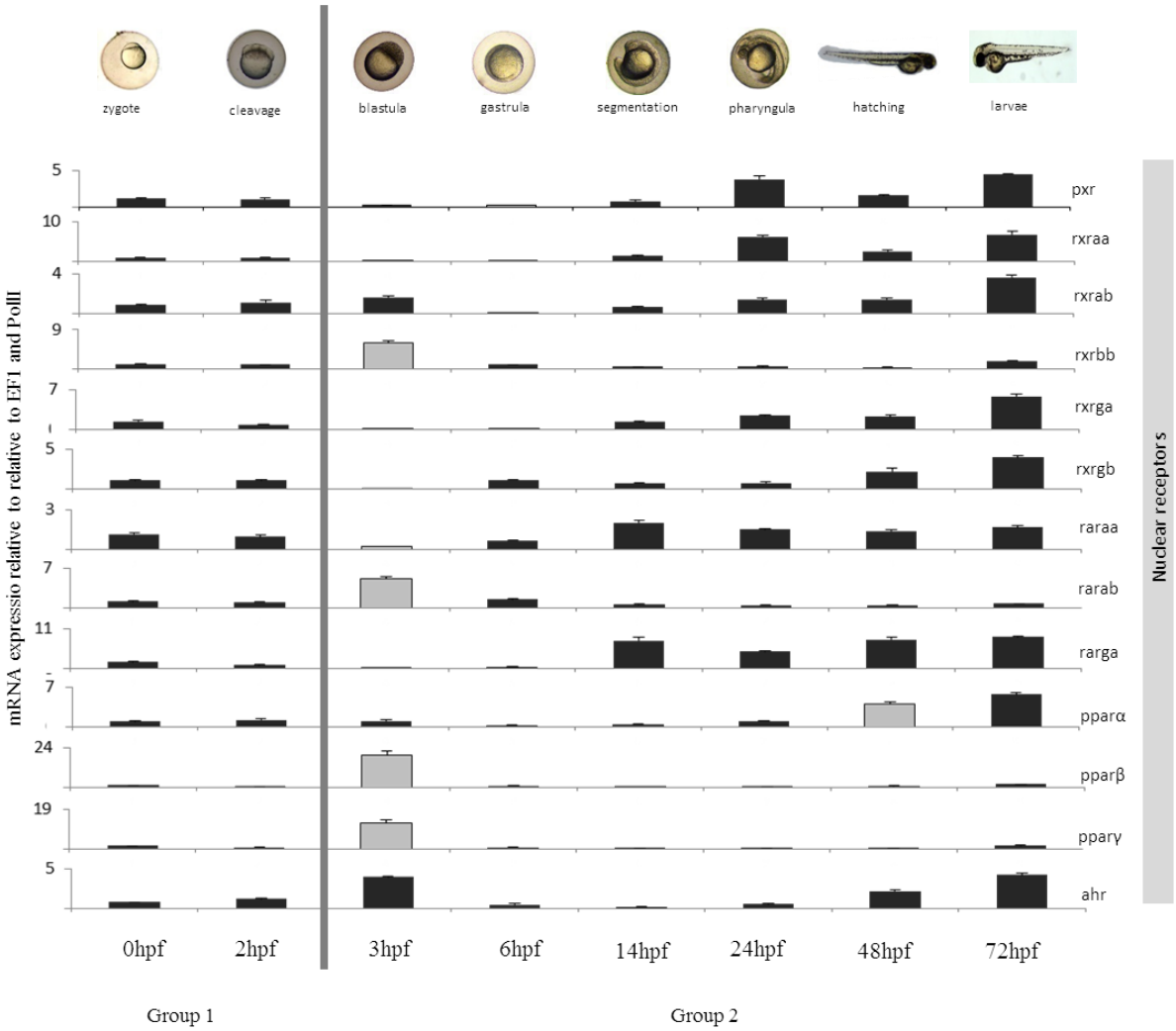

Figure 4.1: Relative mRNA expression of *ahr2, raraa, rarab, rarga, pparα, ppar β, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga* and *rxrgb*, during the different stages of embryonic development in *D. rerio* comprising the zygote and cleavage (group 1), blastula, gastrula, segmentation pharyngula, hatching and larvae periods (group 2). The grey bars represent the major significantly differences between the development phases ($p<0.05$). Results are given as mean±SE, n=6.

4.4.3 Cholesterol quantification

Chol levels in zebrafish embryos after SIM exposure in both assays are presented in Fig. 4.4. No significant differences among groups were observed for both assays.

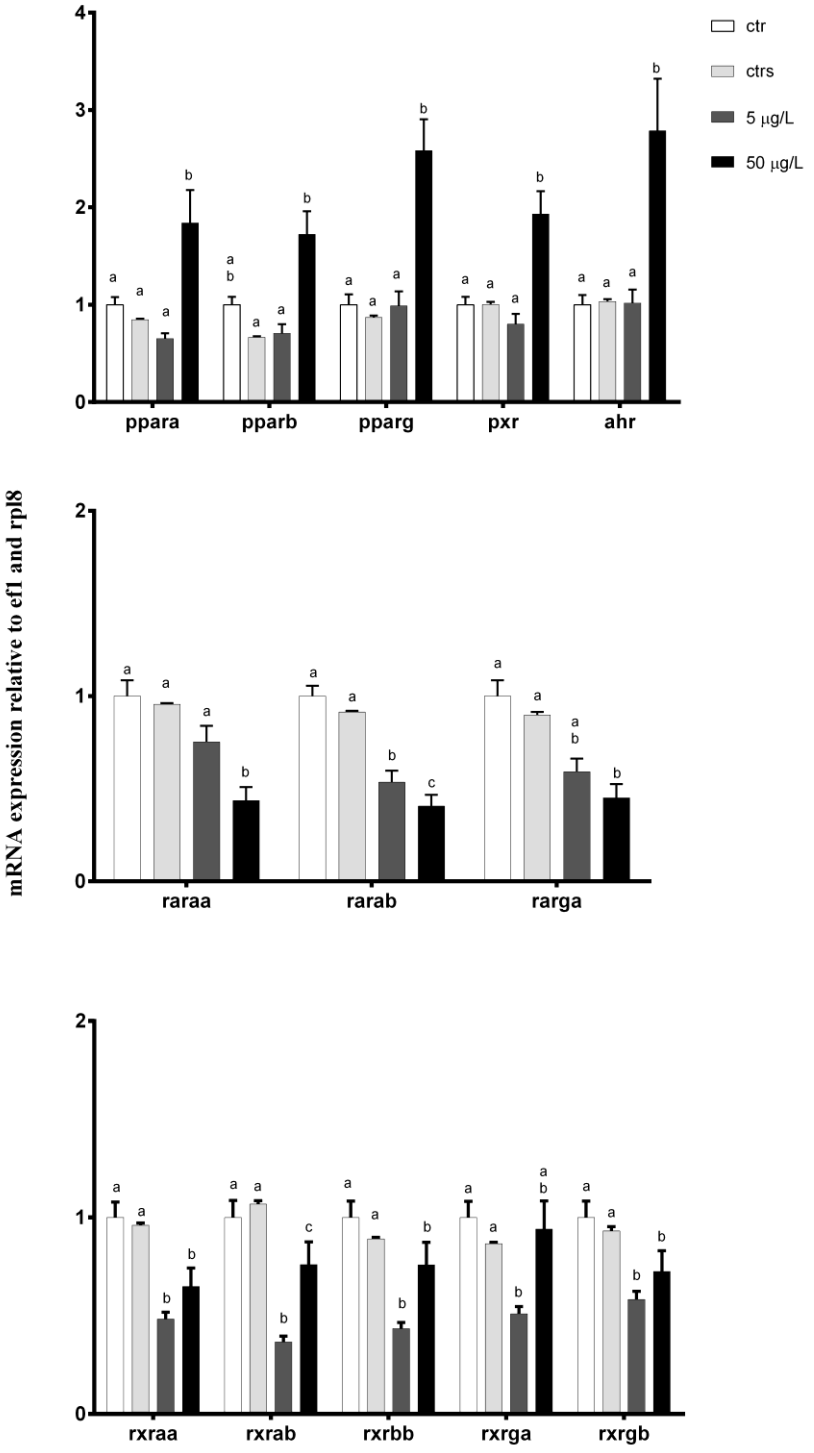

Figure 4.2: Relative mRNA expression of *ahr2, raraa, rarab, rarga, pparα, ppar β, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga* and *rxrgb*, in *D. rerio* embryos exposed to simvastatin 5 µg/L and 50 µg/L), at 24 hpf for 2 h. Results are given as mean±SE, n=6.

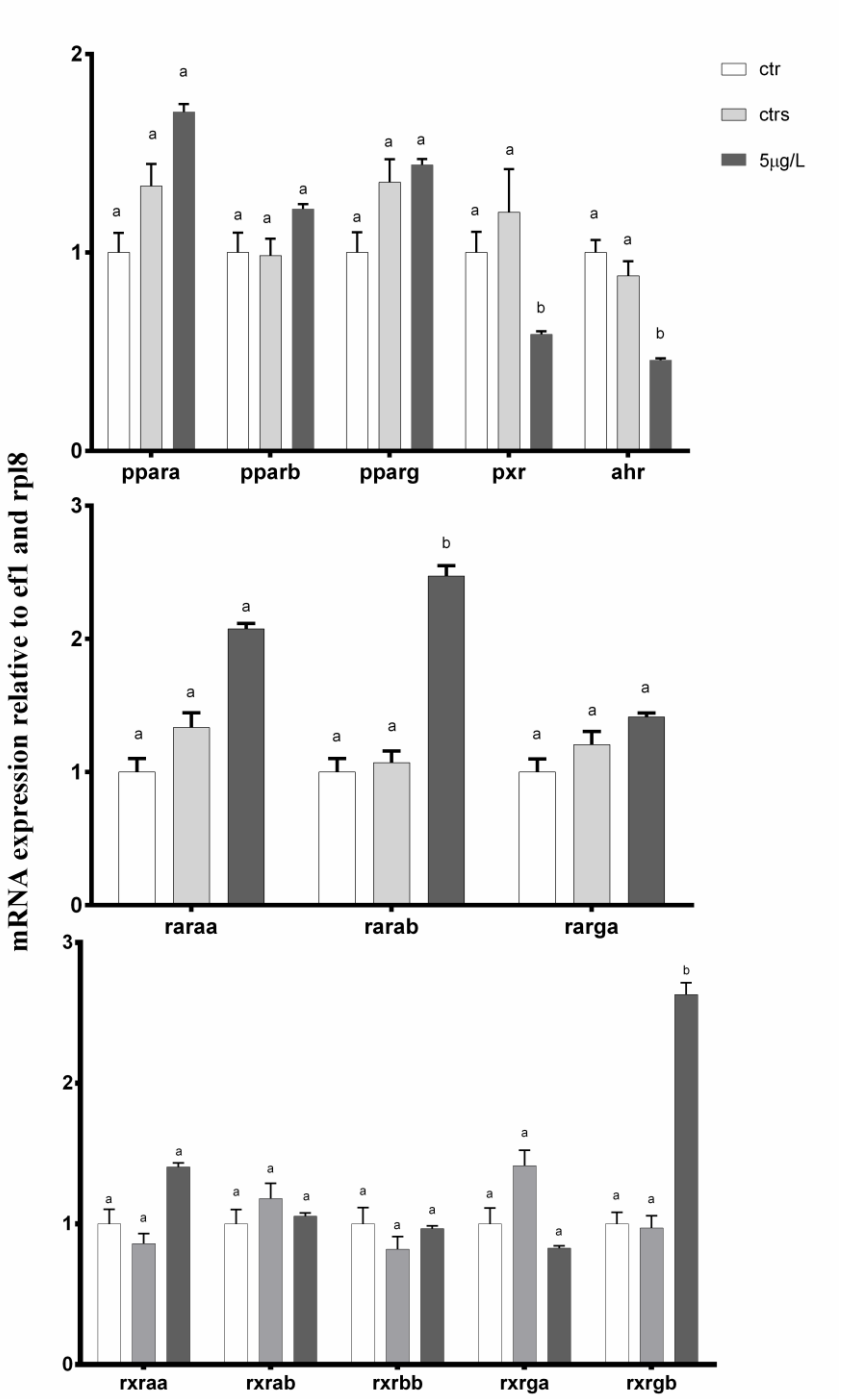

Figure 4.3: Relative mRNA expression *ahr2, raraa, rarab, rarga, pparα, ppar β, pparγ, pxr, rxraa, rxrab*, *rxrbb, rxrga* and *rxrgb,* in *D. rerio* embryos exposed to simvastatin (5 µg/L) for 80 h. Results are given as mean±SE, n=6.

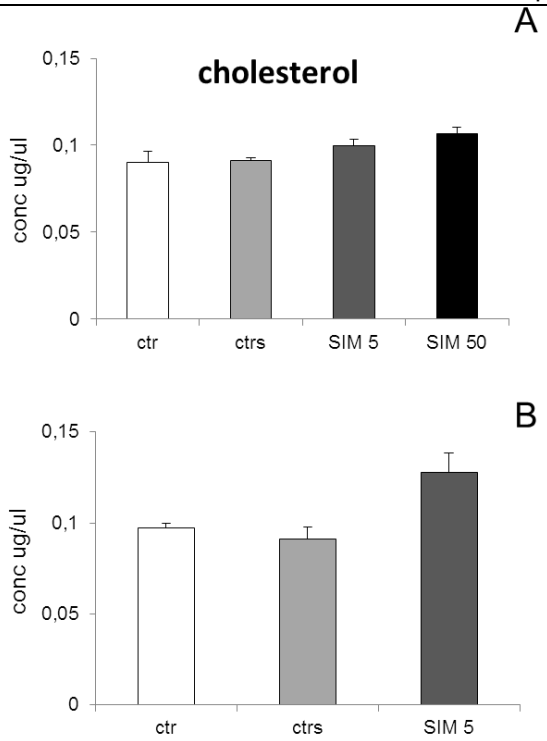

Figure 4.4: Effects of SIM in Chol content in D. rerio embryos at 2 h exposure (A) and 80 h exposure (B). Results are given as mean±SE, n=3.

4.5 Discussion

i) *Characterization of the basal profile of nuclear receptors expression*

Several classes of contaminants can affect aquatic organisms, particularly during sensitive early-life stages (Mohammed, 2013). Hence, it is important to establish a transcriptional baseline profile for key transcription factors involved in lipid metabolism and xenobiotic response. Furthermore, given their mode of action NRs are also key players in endocrine processes (Lima et al., 2015; Frey et al., 2012; Colliar et al., 2011). Comprehending their diversity, ligand specificity and controlled pathways in different taxa is thus fundamental to address the impact of xenobiotics namely in aquatic environments (Castro and Santos, 2014). In the two first stages of development, zygote (0 hpf) and cleavage (2 hpf), mRNA from all target genes was detected in the embryos. Hence, these transcripts are believed to be from maternal origin since evidences indicate that transcripts from embryo origin only appear after cleavage, in midblastula transition (Abrams and Mullins, 2009). Therefore, the presence of these transcripts points to the importance of these genetic regulators in early life stages of development. Further, supporting our findings, several studies showed the presence of mRNA transcripts of the NRs studied here, such as *rxrs, ahr, ppars* and *pxr* in early embryo development in fish species (Bertrand et al., 2007; Tallafuss et al., 2006; Fernandez et al., 2013; Powell et al.,

2000; Prasch et al., 2003). Genes with detected expression at the zygote period maintained transcription in the following development stages (group 2: from blastula to larvae phase). mRNA expression of most genes, with exception of *rxrgb* and *raraa*, has decreased after blastula (3 hpf) until gastrula (6 hpf), associated with the degradation of maternal transcripts, increasing afterwards due to new transcripts produced by the embryo. After hatching (48 hpf), mRNA expression of all the studied genes was increased supporting the hypothesis that these NRs are important regulators of key signalling pathways after the loss of chorion and the direct contact of the embryo with the aquatic environment. The results described herein are important to establish a transcription baseline to be used in future toxicological studies.

ii) *Simvastatin effects on transcription of NRs and AhR*

Toxic effects of SIM such as mortality, reproduction, behavioural and developmental abnormalities have recently been demonstrated for some invertebrate and vertebrate aquatic organisms (Ribeiro et al., 2015; Neuparth et al., 2014; Ortiz de García et al., 2014). In mammals, SIM impacts different physiological mechanisms, interacting in lipid, inflammatory and immune homeostasis pathways (Alvarez de Sotomayor et al., 2008; Fraunberger et al., 2009). Evidences showed that their mode of action (MOA) might be conserved across metazoans, particularly vertebrates (Fent t al., 2006, Santos et al., 2016). Therefore it is important to identify, in aquatic organisms, which pathways can be disrupted by SIM. In mammals, the expression of lipid regulating proteins is modulated by PPARs, and SIM is recognised to be a transcriptional activator of these NRs (Martin et al., 2001; Lee et al., 2010; Wang et al., 2011; Zou et al., 2013). In fish, lipid regulation and signalling pathways are similar to mammals (Carmona-Antoñanzas et al., 2014; Lyssimachou et al., 2015; Den Broeder et al., 2015). More recently, zebrafish embryos have been used as a model to study lipid metabolism and the interaction of pharmaceuticals (including statins) with lipid metabolism (Miyares et al., 2014; Campos et al., 2015; Hachicho et al., 2015; Ho et al., 2016). Additionally, the mevalonate pathway and HMGCR, the gene encoding for HMG-CoA reductase, a target of SIM, shows a high degree of conservation between mammals and fish (Santos et al., 2016; Thorpe et al., 2004). Therefore it can be hypothesized that fish may experience similar effects to those recorded in mammals in the presence of SIM. In assay 1, the transcription of *ppars* gene was up regulated mainly at the highest concentration (50 µg/L). There is a lack of studies addressing in the effects of pharmaceuticals, including statins, in the modulation of NRs transcription. Nevertheless, our data are in line with previous *in vitro* studies with mammalian cells where *Ppars*, mainly *Pparα* and *Pparγ* were up regulated in the presence of statins, including simvastatin (Martin et al., 2001; Lee et al., 2010; Wang et

al., 2011; Zou et al., 2013). In a previous study performed in our lab, zebrafish embryos exposed for 80 h at the same SIM concentrations tested here, showed no changes in the transcription of *hmgcr* (unpublished data). In accordance to this result, no changes in *hmgcr* mRNA was also observed in rainbow trout exposed to atorvastatin (Ellesat et al., 2012). Additionally, in the present work, we determined the concentration of Chol in embryos exposed for 80 h to SIM, as a proxy to evaluate changes on the main target of SIM in mammals. However, no changes in Chol concentration were observed in the presence of SIM. In humans, administration of SIM lowers Chol (Rodwell et al., 1976; Fent et al., 2006) and increases *Hmgcr* mRNA levels after 3 weeks of treatment (Rudling et al., 2002). However, *in vitro* studies with mammalian cells, a down regulation of *Hmgcr* after long term exposures to statins was observed, including SIM (Liu et al., 2010; Cho et al., 2008). Hence, although no changes have been recorded here in Chol levels, we cannot exclude the possibility that longer exposures could also impact *Hmgcr* transcription in a similar manner to that observed in mammalian models. Chronic exposure in future studies should clarify this hypothesis. In fact, zebrafish larvae at 80 hpf lack white adipose tissue, and therefore changes in lipid metabolism may not be clear at such early developmental stages, which may explain the observed results of Chol levels (Flynn III et al., 2009; Imrie and Sadler, 2010; Tingaud-Sequeira et al., 2011).PPARα is involved in the regulation of lipid metabolism, and also promote anti-inflammatory effects by negatively interfering with pro-inflammatory signalling pathways including NF-kB (Marx et al., 2004; Okamoto et al., 2005; Paukkeri et al., 2007; Becker et al., 2008). In a recent study in mice, it was reported that SIM protected against molecular and cellular damage caused by systemic inflammation via PPARα receptor, also increasing its mRNA expression (Rinaldi et al., 2011). In zebrafish the outcome of altered *pparα* mRNA levels by SIM is still unknown, but taking into account the effects on mammals we can hypothesize that inflammatory and immune homeostasis can be impaired. In mammals, gene expression of *Pxr, Ppars* and *Ahr* has been found to be modulated by some pharmaceuticals, subsequently affecting the mRNA expression of genes belonging to the detoxification mechanism (Aleksunes and Klaassen, 2012). Hence, the effects of SIM in transcription of other NRs and AhR involved in the xenobiotic metabolism were also evaluated in this study. The transcription of *pxr* and *ahr* was up regulated in the presence of the highest concentration of SIM (50 µg/L) in assay 1, contrasting with assay 2, where a down regulation was observed, which may suggest some type of compensatory mechanisms. Furthermore, *rars* and *rxrs* also displayed different gene expression profile at 2 h and 80 h. A time and tissue specific mRNA expression of *ahr* has been reported before, in aquatic invertebrates and mammals (Hamadeh et al., 2002; Tian et al., 2013, Kim et al., 2015). AhR and PXR in mammals are

known to regulate CYPs mRNA and proteins (Xu et al., 2005, Aleksunes and Klaassen, 2012). Several studies showed that certain pharmaceuticals such as dexamethasone, 17alpha-ethynylestradiol affect the transcription of *ahr* and *pxr* and their downstream pathways such as *cyp1a* and *cyp3a* expression (Wassmur et al., 2010; Mortensen and Arukwe, 2007). Our previous study, where the same methodology was used, revealed an up regulation of *cyp1a1*, *cyp3a65* after 2 h exposure and a down regulation of *cyp3a65* after 80 h. Additionally, an induction of EROD activity was observed while no effect on the transcription of *cyp1a1* was detected after 80 h of exposure (Cunha et al., 2016). Altogether these results suggest that SIM can impact the transcription of *cyps, pxr* and *ahr*, which points to a possible regulation of *cyps* through modulation of *pxr* and *ahr*. Furthermore, in humans, statins are mostly metabolized by CYP3A4 enzyme (Jacobson 2004) and enhance CYP3A, CYP2B6 and MDR1 (multidrug resistance protein 1, efflux transporter) mRNA expression (Kocarek et al., 2002; Yamasaki et al., 2009) through pathways downstream to PXR and CAR (absent in teleosts) (Kobayashi et al., 2005; Yamasaki et al., 2009). Although teleost fish and mammals show a well conserved mevalonate pathway, the metabolization of SIM in teleosts has yet to be study in detail. However, it has been established that zebrafish has a CYP3A4/5 like-activity that has been hypothesized to be carried out through the presence of a CYP3A65 (Chng et al., 2012). If we assume a similar metabolization pathway between both groups one can hypothesize that SIM may be metabolized by CYP3A65, a downstream pathway of PXR. Given the results observed here that points to an effect of SIM in the transcription of *pxr* and *ahr*, we theorize that it may ultimately affect the transcription of genes involved in biotransformation of xenobiotics.

4.6. Conclusions

In conclusion, the transcripts of NRs and AhR evaluated in this study seem to be from maternal origin in the first developmental stages, i.e., zygote to blastula, supporting their importance in the early life stages. Exposure to SIM impacts the transcription levels of different NRs and AhR in a time- specific manner. Although the mRNA of *ppars* was affected by SIM, the modulation of this transcription factor does not seem to impact the total Chol levels, at least up to 80 hpf. Given the key role of these transcription factors in many signalling pathways, these findings should prompt more detail studies involving chronic exposures. Similarly, the modulation of the transcription of ahr and *pxr* by SIM indicate that phase I biotransformation may be affected.

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SUPPLEMENTARY DATA

Table 1S: Gene list, Genbank accession numbers, Primer sequences and concentrations, amplicon lengths, efficiency of reaction for *ef1*, *pol II*, *rpl8*, *raraa, rarab, rarga, pparα, pparβ, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb* and *ahr* gene expression quantification by qRT-PCR in zebrafish

Gene	Accession number	Primers	Final Conc. (nM)	Amplicon length (bp)	Efficiency (%)
ef1	NM_131263.1	F: 5'-GGACACAGAGACTTCATCAAGAAC-3' R: 5'-ACCAACACCAGCAGCAACGT-3'	300	84	116.8
pol II	NM_001024461.2	F: 5'-CAATGACGACCCGACCG-3' R: 5'-CGCCAGCAACTCAGTCACT-3'	10	292	96
rpl8	NM_200713.1	F: 5'-CAATGACGACCCGACCG-3' R: 5'-CGCCAGCAACTCAGTCACT-3'	10	136	96
pxr	DQ069792.1	F: 5'-CTTTTTCAGACGTGCGATGA-3' R: 5'-TTGGCACTGTCTTCTGTTGC-3'	300	94	112.7
rxraa	NM_001161551.1	F: 5'-ATTCAATGGCATCTCCTG-3' R: 5'-GCGGCTTAATATCCTCTG-3'	600	99	101.8
rxrab	NM_131153.1	F: 5'-CGCCGCATCAAATCACATAAAC-3' R: 5'-TGAATGGGTTGGACAGTATTTAGC-3'	300	87	109.4
rxrbb	NM_131238.1	F: 5'-TCACAACTTGGGCGTGGAGGC-3' R: 5'-CGCATCTTGCAGACCAGCTCAG-3'	300	105	100.7
rxrga	NM_131217.2	F: 5'-ATCTCAGTTCTTCGTTGCAGGTAG-3' R: 5'-CGTTGATGATGGATGGGTGATGG-3'	300	105	99.6
rxrgb	NM_001002345.1	F: 5'-CGCGGAATGGATACTCACG-3' R: 5'-GCTGATGACGGACGGATGAC-3'	300	114	97.7
raraa	NM_131406.2	F:5'-GTAGTGGAGTGTGGATGTGAA-3' R: 5'-GTGCTGATGTCTGATGGATGA-3'	300	118	108.7

rarab	NM_131399.1	F:5'-ATGGATTACTACCACCAGAAC-3' R: 5'-TCTCCACAGAGTGATTCGAGC-3'	300	115	109.4
rarga	NM_131339.1	F: 5'-CCCGCCAACTGTACGATGTCA-3' R: 5'-GGGTCCAGTCCAGCATAGAAA-3'	300	79	117.6
pparα	NM_001161333.1	F: 5'-CATCTTGCCTTGCAGACATT-3' R: 5'-CACGCTCACTTTTCATTTCAC-3'	600	81	88.3
pparβ	AF342937.1	F: 5'-GCGTAAGCTAGTCGCAGGTC-3' R: 5'-TGCACCAGAGAGTCCATGTC-3'	600	204	81.6
pparγ	DQ839547.1	F: 5'-GGTTTCATTACGGCGTTCAC-3' F: 5'-TGGTTCACGTCACTGGAGAA-3'	600	250	87.0
ahr2	NM_001007789.2	F: 5'-TTCTGTTGCCGATTCAGATG-3' R: 5'-CTTGTTTTGCCCATGGAGAT-3'	300	96	113,8

Figure 1S

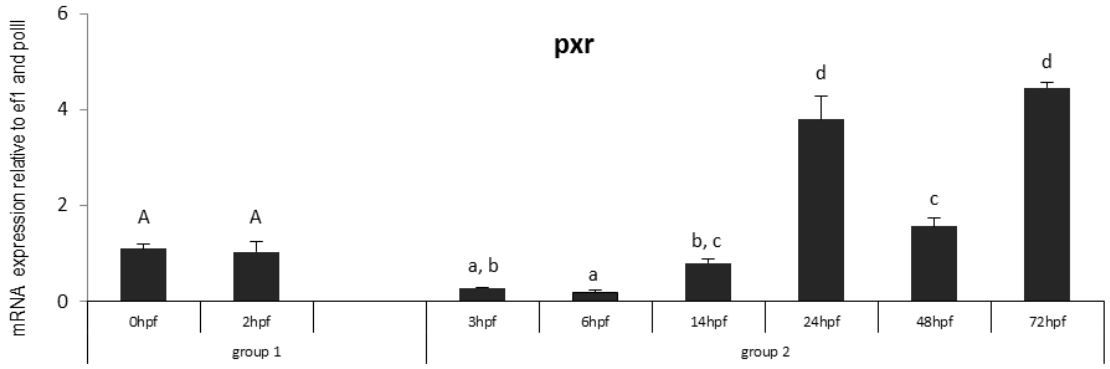

Fig 1S: Relative mRNA expression of *pxr* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 2S

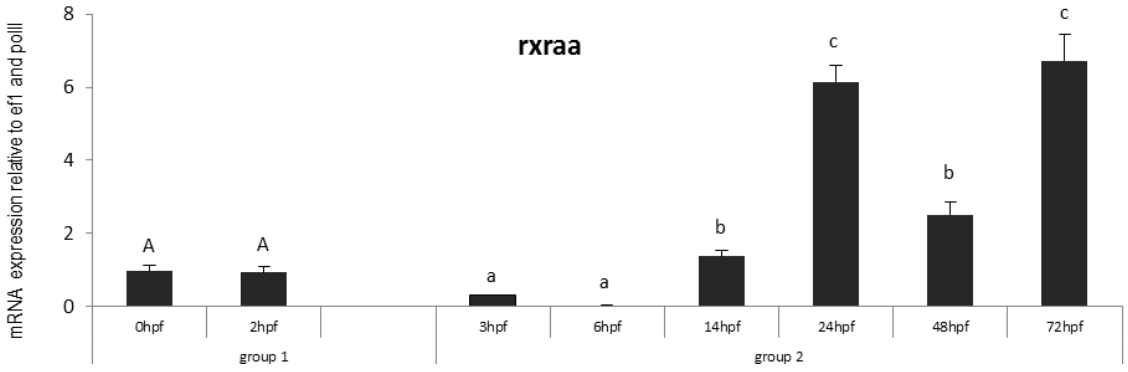

Fig 2S: Relative mRNA expression of *rxraa* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 3S

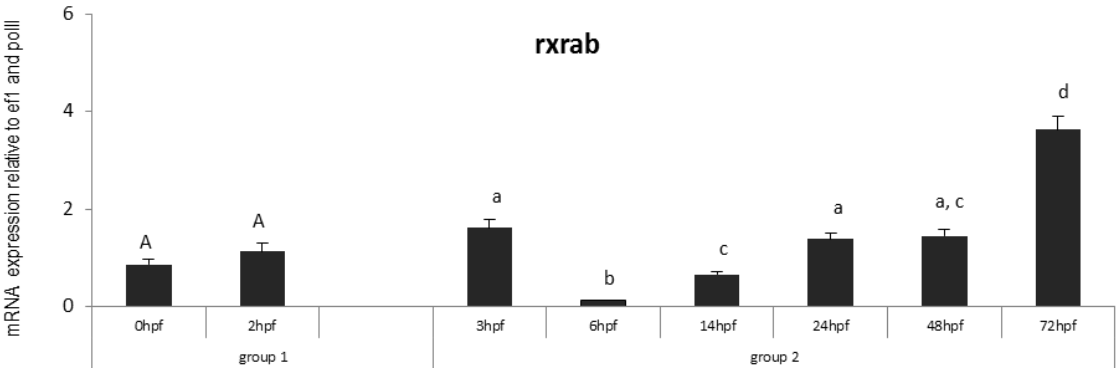

Fig 3S: Relative mRNA expression of *rxrab* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 4S

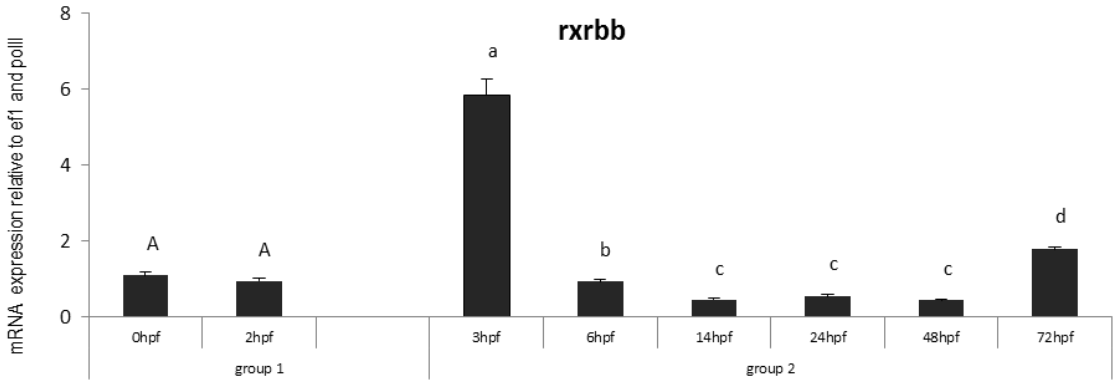

Fig 4S: Relative mRNA expression of *rxrbb* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 5S

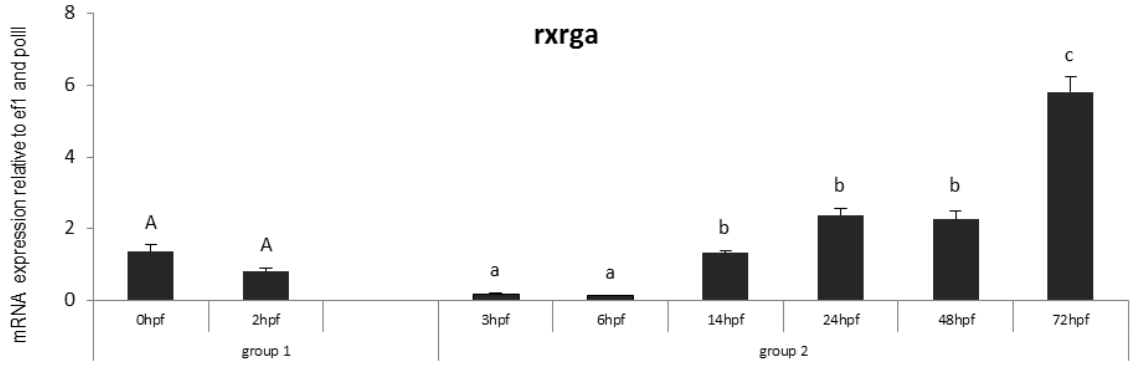

Fig 5S: Relative mRNA expression of *rxrga* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case **l**etters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 6S

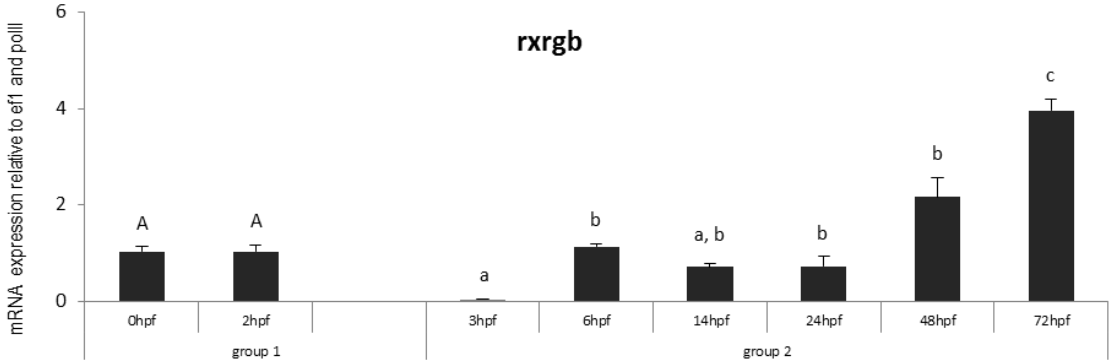

Fig 6S: Relative mRNA expression of *rxrgb* during the different stages of embryonic development *in D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 7S

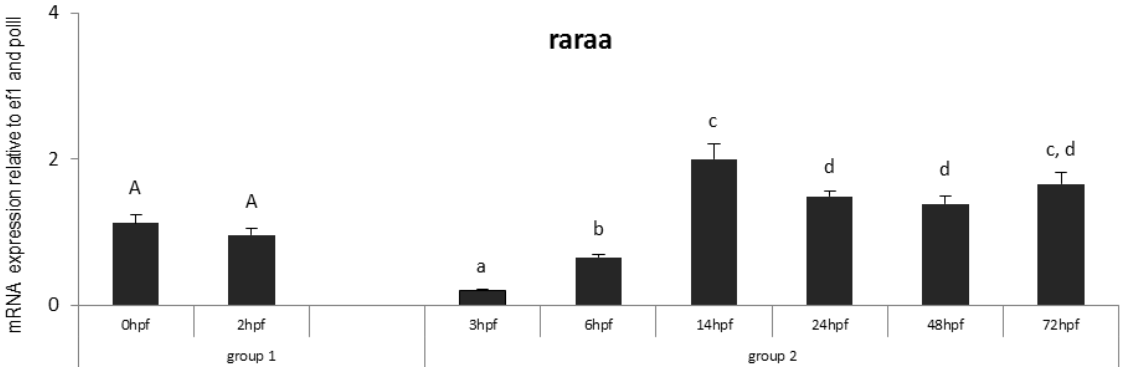

Fig 7S: Relative mRNA expression of *raraa* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 8S

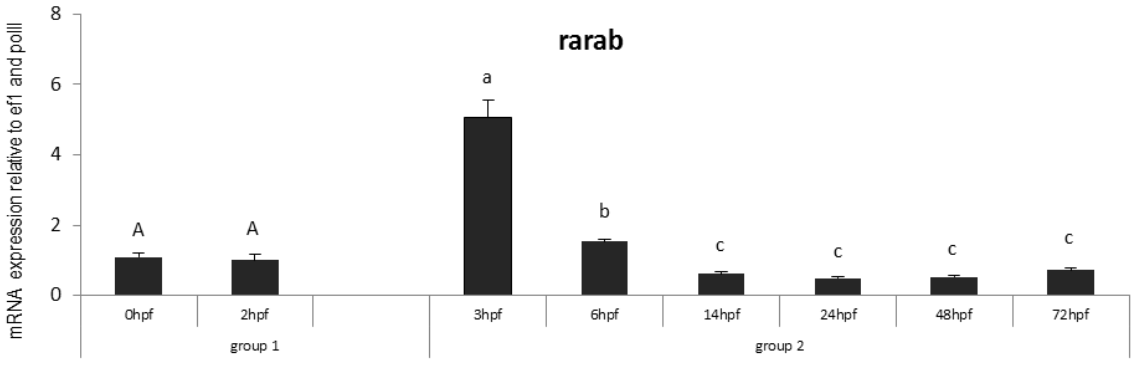

Fig 8S: Relative mRNA expression of *rarab* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 9S

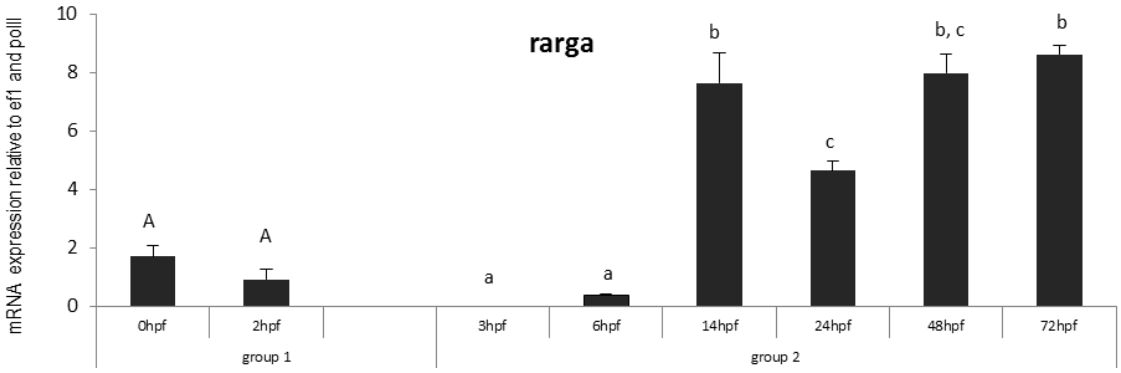

Fig 9S: Relative mRNA expression of *rarga* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 10S

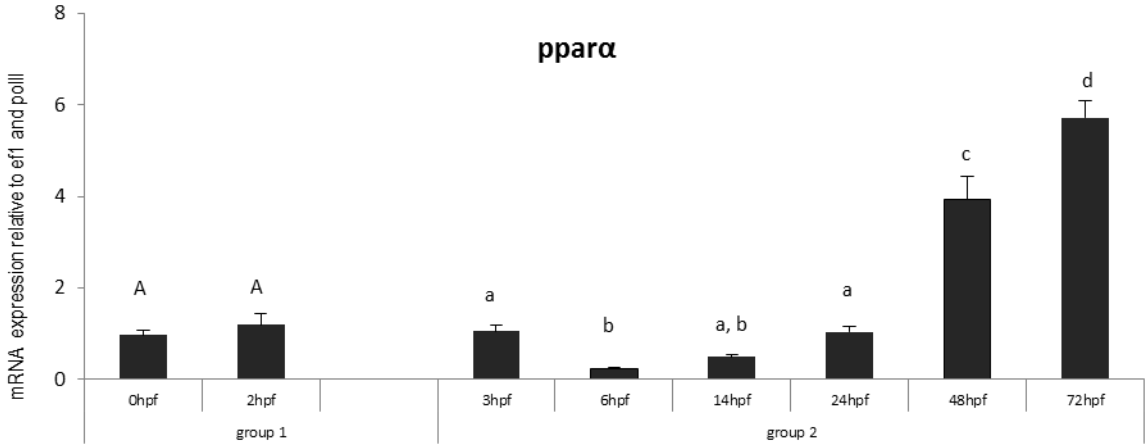

Fig 10S: Relative mRNA expression of *pparα* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 11S

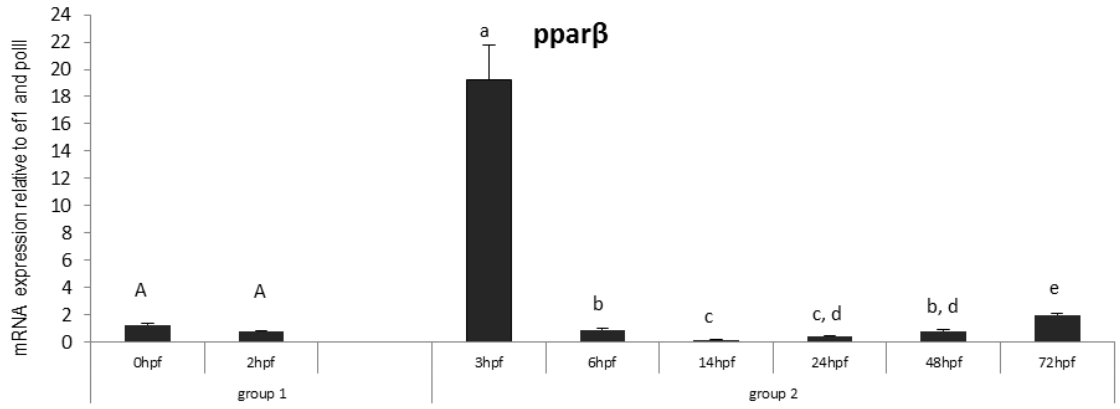

Fig 11S: Relative mRNA expression of *pparβ* during the different stages of embryonic development in D. rerio comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 12S

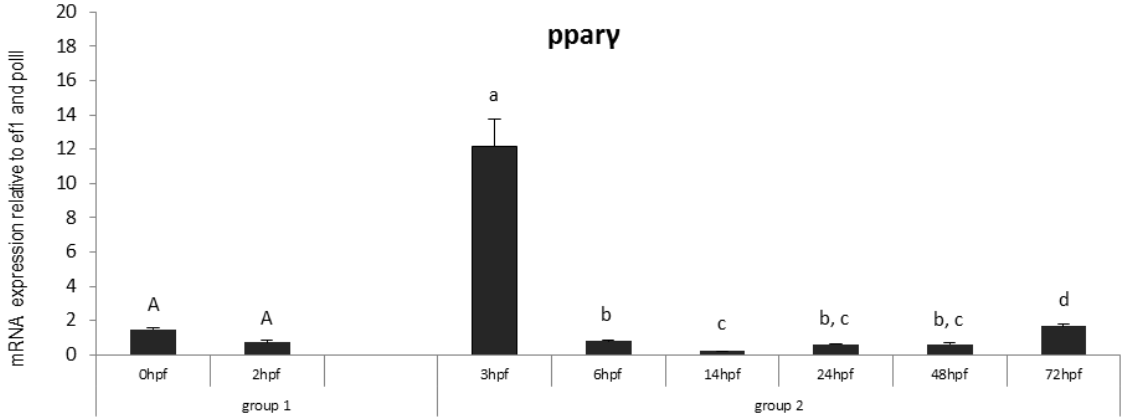

Fig 12S: Relative mRNA expression of *pparγ* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

Figure 13S

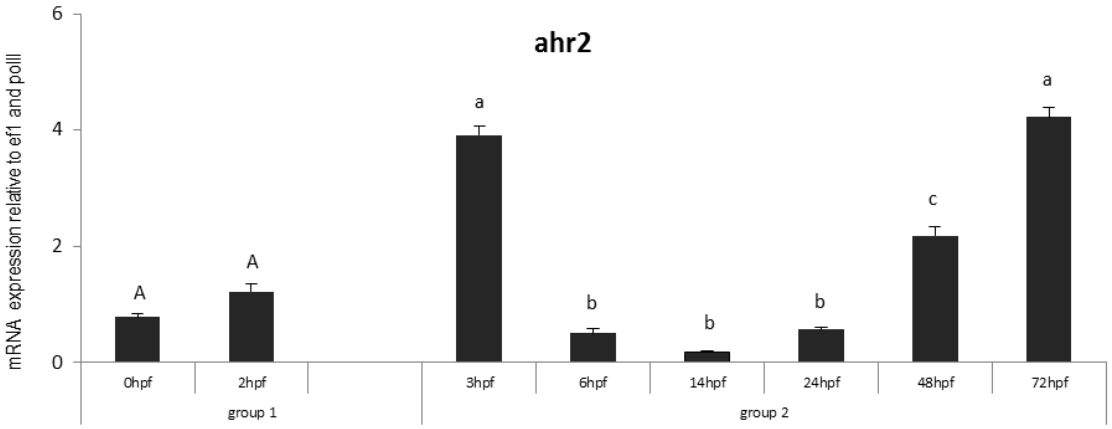

Fig 13S: Relative mRNA expression of *ahr* during the different stages of embryonic development in *D. rerio* comprising the zygote (0 hpf) and cleavage (2 hpf) (group 1), blastula (3 hpf), gastrula (6 hpf), segmentation (14 hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (72 hpf) (group 2) periods. Different upper-case letters represent significantly differences between the development phases of group 1 ($p<0.05$). Different lower-case letters represent significantly differences between the development phases of group 2 ($p<0.05$). Results are given as mean±SE, n=6.

CHAPTER V

DANIO RERIO EMBRYOS ON PROZAC – EFFECTS IN THE DETOXIFICATION MECHANISM AND EMBRYO DEVELOPMENT

This chapter has been submitted as:

Cunha V, Rodrigues P,Santos MM, Moradas-Ferreira P, Ferreira M. *Danio rerio* embryos on Prozac – Effects in the detoxification mechanism and embryo development

Danio rerio embryos on Prozac – Effects in the detoxification mechanism and embryo development

5.1 Abstract

In the past decade the presence of psychopharmaceuticals in the aquatic environment, including fluoxetine (FLU), has been raising due to the increasing trend in human consumption. Aquatic organisms are usually exposed to chronic low doses and therefore risk assessment should evaluate the effects of these compounds in non-target organisms. Teleost fish possess an array of active defence mechanism to cope with the deleterious effects of xenobiotics that include ABC transporters, phase I and II of cellular detoxification and oxidative stress enzymes. Hence, the present study aimed at characterising the effect of FLU on embryo development of the model teleost zebrafish (*Danio rerio*) concomitantly with changes in the detoxification mechanisms during early developmental phases. Embryos were exposed to different concentrations of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) for 80 hours post fertilization. Development was screened and the impact in the transcription of key genes, i.e., *abcb4, abcc1, abcc2, abcg2, cyp1a, cyp3a65, gst, sod, cat, ahr, pxr*, *pparα, pparβ, pparγ, rxraa, rxrab, rxrbb, rxrga, rxrgb, raraa, rarab, rarga* evaluated. In addition, accumulation assays were performed to measure ABC proteins activity and antioxidant enzymes (CAT and Cu/ZnSOD) activities determined after exposure to FLU. Embryo development was disrupted at an environmental relevant concentration of FLU (0.0015 µM). Embryos exposed to FLU exhibited a decreased pattern for Cu/Zn SOD and increased CAT (FLU at 0.0015 and 0.5 µM) enzymatic activity. A down regulation pattern was observed for most genes belonging to the detoxification system with exception of *cat* that was upregulated at 0.0015 µM of FLU. For most of the tested concentrations, a down regulation pattern was observed for *pparα, pparβ,* pparγ, *raraa, rxraa, rxrab, rxrbb rxrgb* and *ahr*, while *pxr* was significantly up regulated in all tested concentrations. In conclusion, our work shows that FLU impacts zebrafish embryo development at environmentally relevant concentrations concomitantly with changes in antioxidant enzymes, and the transcription of key genes involved in detoxification and development. These finding raises additional concerns supporting the need to monitor the presence of this compound in aquatic reservoirs.

Keywords: Fluoxetine, zebrafish embryos, ABC transporters, phase I and II, antioxidant enzymes, nuclear receptors

5.2 Introduction

In the past decade the consumption of psychopharmaceuticals has increased due to the socio-economic context and a higher prevalence of psychiatric disorders (Silva et al., 2012). This therapeutic class of pharmaceuticals is one of the most commonly detected in the aquatic environment (Santos et al., 2010). Fluoxetine (FLU), commercial name Prozac®, is one of the top prescribed psychopharmaceutical (Mennigen et al., 2011; Winder et al., 2012) to treat depression, compulsive behaviour and eating disorders (Dulawa et al., 2004). The presence of FLU in the environment was first reported by Kolpin et al. (2002) in USA surface waters and since then numerous reports have shown the presence of this drug in different water matrices, in concentrations ranging from 12 to 929 ng/L (Fent et al., 2006; Lajeunesse et al., 2011; Barry et al., 2013; Silva et al., 2014). Previous studies have reported negative impacts of psychopharmaceuticals (including FLU) on invertebrates and fish species, such as impaired reproduction, mortality and behavioural changes like decreased aggressiveness and feeding responses (Monpelat et al., 2009; Lister et al., 2009; Santos et al., 2010; Schultz et al., 2011). Nonetheless, the physiological consequences of the exposure to FLU are still not fully understood in non-target aquatic organisms. Fish have well-developed mechanisms that contribute to cell detoxification and to counteract the effects of environmental pollutants. Among these are ATP – binding cassette (ABC) transporter proteins (ABCB, ABCC and ABCG families), phase I (cytochrome P450 family, CYPs) and phase II (eg. glutathione-S-transferase, GST) biotransformation enzymes, and antioxidant enzymes (eg. superoxide dismutase, SOD, catalase, CAT) (Van der Oost et al., 2003; Bard, 2000; Stegeman et al., 1992; Lopez-Torres et al., 1993). In aquatic organisms the multixenobiotic resistance (MXR) mechanism incorporates members of the ABC transporter family (Kurelec, 1992) that are considered to be the first line of defence against toxicants (Bard, 2000; Ferreira et al., 2014; Luckenbach et al., 2014). Nonetheless, the MXR system can be inhibited by certain chemicals (chemosensitisers) known to be present in the aquatic ecosystems, potentially increasing the toxicity of other chemicals that would normally be effluxed from the cells (Epel et al., 2008). Some psychopharmaceuticals, including FLU, have been described to be P-gp inhibitors in mammals (Peer and Margalit, 2006; O'Brien et al., 2013; Schrickx and Fink-Gremmels et al., 2014). Also, has been reported that FLU can inhibit P-gp (P-glycoprotein, ABCB1) and biotransformation enzymes in both mammals and fish (Peer and Margalite, 2006; Smith et al., 2012; Thibaut and Porte, 2008; Sager et al., 2015). Hence, given the central role of detoxification in the toxicity of both endogenous and man-made chemicals, it is important to understand the regulation of detoxification signalling pathways. In mammals, this defence system is mostly regulated by nuclear receptors

(NRs), which include pregnane X receptors (PXR), peroxisome proliferated activated receptors (PPAR), retinoid X receptors (RXR), constitutive androstane receptors (CAR) and glucocorticoid receptor (GR), and by aryl hydrocarbon receptor (AhR) (Xu et al., 2005; Ferreira et al., 2014). Among other pathways, NRs are involved in xenobiotics metabolism, regulating the transcription of genes belonging to the detoxification process (Wang and LeCluyse, 2003; Robinson-Rechavi et al., 2003; Xu et al., 2005; Castro and Santos, 2014). *Danio rerio* is a well-known model fish species that has been used in different fields of research, including ecotoxicology. Therefore, it was selected for the present study due to characteristics such as embryo transparency that allows the observation of development and the assessment of embryo toxicity, and the availability of a full genome sequence.

This study aimed at *i*) evaluate the effects of FLU in embryo development; *ii*) characterise the expression and response patterns of genes belonging to the detoxification mechanism as well as MXR and antioxidant activities on *D. rerio* embryos exposed to FLU; *iii*) evaluate the modulation of key transcription factors (*ppars, pxr, rxrs, rars* and *ahr*) after FLU exposure; and *iv*) assess the potential chemosensitiser effect of FLU using *D. rerio* embryos as model.

5.3 Material and methods

5.3.1 Chemicals

Fluoxetine (FLU) (CAS #56296-78-7), rhodamine 123 (RH123), verapamil (VER) and vinblastine were purchased from Sigma-Aldrich (Germany) and MK571 from VWR International. All the other chemicals were of analytical grade, and were purchased from local companies.

5.3.2 Parental animals

Adult wild-type zebrafish, obtained from local suppliers, were used as breeding stocks. The stock was kept at a water temperature of 27±1 ºC and in a photoperiod of 12:12 h (light:dark), in 60 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), and supplemented once a day with live brine shrimp (Artemia spp.).

5.3.3 Rearing conditions and exposures

For reproduction, females and males (ratio 1:2) were transferred to a spawning tank, and submitted to acclimatization for 12 h in a cage with a net bottom covered with glass marbles within a 30 L aquarium. After egg-laying, in the following day, the breeders

were removed after the beginning of the light period. For the toxicological assays (section 2.4), embryos (1 hpf) were transferred to a 24 well plate (10 embryos per well) and exposed to FLU at different concentrations (0.0015, 0.05, 0.1, 0.5 and 0.8 µM; diluted in DMSO, 0.004 % (ranging from 519 to 2.8 x 10^5 ng/L). The tested concentrations are environmentally relevant, particularly the lowest (0.0015 µM = 519 ng/L). Prior to embryo exposures, the test solutions were placed into the plates for 24 h so that the chemicals could adsorb to the plastic, and the solutions renewed after preadsorption. For the determination of the biochemical parameters (section 2.5), 100 embryos (1 hpf) were placed in glass beakers at 26 ± 1 ºC with aeration, and exposed to the test solutions until 80 hpf. For the accumulation assays (MXR activity) (section 2.6) the embryos (1 hpf) were transferred to new aquaria (3.5 L) and were kept for 24 h, at 26 ± 1 ºC with aeration, until the assays execution.

5.3.4 Toxicological assay

Embryos were exposed from 1 hpf until 80 hpf to different concentrations of FLU as previously described (section 5.3.3). The medium (water plus FLU) was renewed daily for the duration of the experiment. Embryos were observed with an inverted microscope (Nikon Eclipse TS100) and the selected endpoints (mortality rate, 75% epiboly, delay/arrest of the division, abnormal cell masses, development delay, pericardia edema, head, eyes and tail abnormalities) recorded at 8 hpf, 32 hpf and 80 hpf (Soares et al., 2009; Ribeiro et al., 2015). After 80 h of exposure, 30 embryos were collected and preserved in RNALater for gene expression analysis. Mortality and abnormalities data are presented in total percentage (%).

5.3.5 Antioxidant enzymes activity

After exposure to FLU, 100 embryos (80 hpf) were homogenized in ice-cold 100 mM potassium phosphate buffer pH 7.4, 150 mM KCl, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM disodic ethylenediaminetetra acetic acid (Na2EDTA) (Fernandes et al., 2008). Post-mitochondrial fractions were obtained after centrifugation at 15,000 g for 20 min at 4°C. Superoxide dismutase (SOD) activity was determined by the degree of inhibition on the reduction of cytochrome *c* by superoxide anion generated by the xanthine oxidase/xanthine system (Ferreira et al., 2005). Cytochrome *c* reduction was followed through the measurement of the absorbance at 550 nm. In the assay concentration of the components was sodium phosphate buffer 50 mM, pH 7.8, with Na2EDTA 0.1 mM, xanthine 50 µM, xanthine oxidase 5.2 mU/mL and cytochrome *c* 18 µM. 250 µL of these components was reacted with the sample (protein concentration 4 mg/mL) diluted in phosphate buffer to a final volume of 300 µL. To obtain

linearity, the volume of sample and phosphate buffer, per well, were adjusted for each sample. SOD standards were used in each assay to calculate the activity given in SOD Units (1 SOD Unit=50% inhibition of the reduction of cytochrome *c*) per mg of protein. The samples were divided in two aliquots, one to measure the total SOD activity and the other to measure MnSOD activity by adding to the reaction KCN 2 mM. To obtain the Cu/ZnSOD activity we then deduct the MnSOD from the total SOD activity. Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 at 240 nm (ε=40 $M^{-1}cm^{-1}$) as described in Ferreira et al., (2008) with slight modifications. The reaction mixture contained 65 mM potassium phosphate buffer, pH 7.8, 15.5 mM H_2O_2 and 0.01% TritonX-100. In the cuvette, 950 µL of reaction mixture was added to the sample (protein concentration 4 mg/mL), diluted in phosphate buffer, to a final volume of 1000 µL. To obtain linearity, the volumes of sample and phosphate buffer were adjusted for each sample. CAT activity is expressed as µmol/min/mg protein. All measurements were performed in triplicate for each sample. Protein determinations were performed by the Lowry method using bovine serum albumin (BSA) as a standard (Lowry et al., 1951).

5.3.6 Accumulation assay

The accumulation of the fluorescent substrate (RH123) in zebrafish embryo tissue served as measure for MXR transporter activity. This assay was performed according to Cunha et al., 2016. Briefly, ABC transporter activity was determined by means of accumulation assays using RH123 (8 µM) in presence of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM), and ABC protein inhibitors (VER (10 µM), MK571 (10 µM), and Vinblastine (10 µM)). The assay was performed with 24 hpf embryos when the organism has most of the organs formed and all the genes targeted in this study are transcriptionally active. Embryos (24 hpf) were incubated in the dark, at 26 ± 1 ºC, for 2 h. After the 2 h exposure period, embryos were washed three times with ice-cold water and mechanically disrupted. The fluorescence of RH123 accumulated inside the embryos was measured in the homogenate of 10 embryos using a fluorescent microplate reader (excitation/emission - 485/538 nm) (Fluoroskan Ascent, Labsystems). Each assay was replicated at least four times. Data is presented in percentage (%) in relation to the control established as 100%.

5.3.7 RNA isolation and cDNA synthesis

Embryos preserved in RNALater from the toxicological assays and from the accumulation assays were used to isolate total RNA according to Costa et al. (2012). Briefly, total RNA was isolated using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. RNA quality was verified by electrophoresis in agarose gel and by the measurement of the ratio of optical density at

λ260/ λ280 nm. RNA was quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a Fluoroskan Ascent, Labsystems. One microgram of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and synthesis of cDNA was performed using Iscript cDNA Synthesis (Biorad).

5.3.8 Quantitative real-time PCR (qRT-PCR)

Gene expression of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, Cu/Zn sod, cat, gstπ, ahr, pxr, pparα, pparβ, pparγ, rxraa, rxrab, rxrbb, rxrga, rxrgb, raraa, rarab, rarga* was assessed by means of quantitative real time PCR (qRT-PCR). Primer pairs for each target gene were designed using Primer 3 software available in http://www.ncbi.nlm.nih.gov/, based on available sequences in GeneBank. Primer sequences, amplicon lengths, efficiencies and Genebank accession numbers of target sequences are given in Table 1S. Identities of the amplicons were confirmed by cloning and sequencing of the DNA fragments as described by Costa et al. (2012). To determine the efficiency of the PCR reactions, standard curves were made, with 6 serial dilutions of the template (concentrations range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated. Reactions for qRT-PCR were conducted in an iQ5 BioRad, with 10 µl of SYBR Green Supermix (BioRad), 2 µl of each primer (final concentrations ranging from 0.001µM to 0.6µM) and 2 µl of cDNA, in a total volume of 20 µl, in duplicate. Conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 54 °C with exception of *rxr* and *rar* (60°C) for 30 s and 72 °C for 30 s. At the end of each run a melting curve analysis was done (from 55 to 95 °C) to determine the formation of the specific products. No template controls were run to exclude contamination and the formation of primer dimers. Gene expression was quantified by normalization with multiple reference genes (elongation factor 1 (*ef1)* and actin β1 (*actb1*) using Normfinder algorithm (Urbatzka et al., 2013), and the relative expression ratio was calculated with efficiencies using the Pfaffl mathematical model ΔΔCt (Pfaffl, 2001). Data is presented as mean of mRNA transcription in relation to the reference genes.

5.3.9 Statistical analysis

Differences in mRNA expression and the differences between treatments in accumulation assay were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Tukey's test) at a 5% significant level. Data were log (mRNA expression and enzymatic activities) or square root (accumulation assay) transformed in order to fit ANOVA assumptions. All tests were performed using software Statistica 7 (Statsoft, Inc). Differences between treatments in the toxicological assay were evaluated by means of cross table χ2 test at 5 % significance level. Correlations between mRNA

expressions of the evaluated genes were tested by Pearson analysis. Tests were performed using software SPSS 22 (IBM, Inc). Data is presented as mean ± standard error.

5.4 Results

5.4.1 Toxicological assay

To test the toxicity of FLU in the embryos the cumulative mortality and total abnormalities after exposure to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM), at different embryo development periods (8, 32 and 80 hpf) was tested and are presented in Fig. 5.1A. No significant differences in mortality were observed in embryos exposed to FLU, in comparison to the solvent control group, and there was no increased mortality between 32 and 80 hpf. Regarding the development of abnormalities no differences were observed at 8 hpf in the presence of FLU. However, at 32 and 80 hpf a significant increase in abnormalities was detected in embryos treated at all tested concentrations ($p<0.05$). At 32 hpf, the highest percentage (48 %) of abnormalities was registered at 0.8 µM of FLU ($p<0.05$) where 35% of the embryos exhibited pigmentation anomalies. In addition, a high percentage (26 – 30 %) of embryos exposed to the lower concentrations (0.0015 and 0.05 µM) displayed other abnormalities such as pericardium edema and tail abnormalities (Fig. 1S, supplementary material for more detailed information). Similar to 32hpf, at the end of the assay (80 hpf), pigmentation, pericardium edema and tail abnormalities were the most prevalent anomalies in embryos exposed to FLU. (Fig. 2S, supplementary material for more detailed information).

5.4.2 Antioxidant enzymes activity

To address the question of correlation of the morphological effects of FLU with toxicity parameters, we assayed two anti-oxidant defences, SOD and CAT in zebrafish embryos after 80 h of exposure to FLU (Fig. 5.2). Cu/ZnSOD activity (Fig. 5.2A) was significantly decreased in the presence of FLU, even at the intermediate concentrations (0.1 µM) ($p<0.05$) while the CAT activity (Fig. 5.2B) seemed to be stimulated in all treatments, with significant differences ($p<0.05$), in comparison to controls

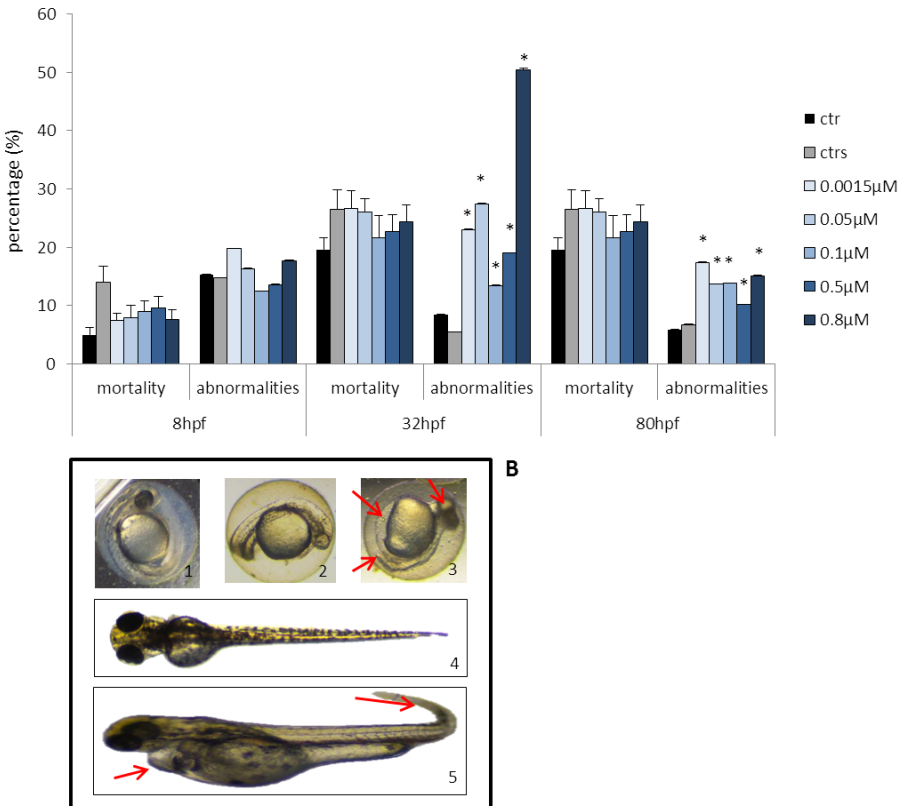

Figure 5.1: Cumulative mortality and abnormality rates at 8, 32 and 80 hpf of *D. rerio* embryos exposed to different concentrations of fluoxetine (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) for 80 h (%). Results are expressed as mean ± SE, n=6. Bars with asterisk are significantly different from the solvent control group (p<0.05).

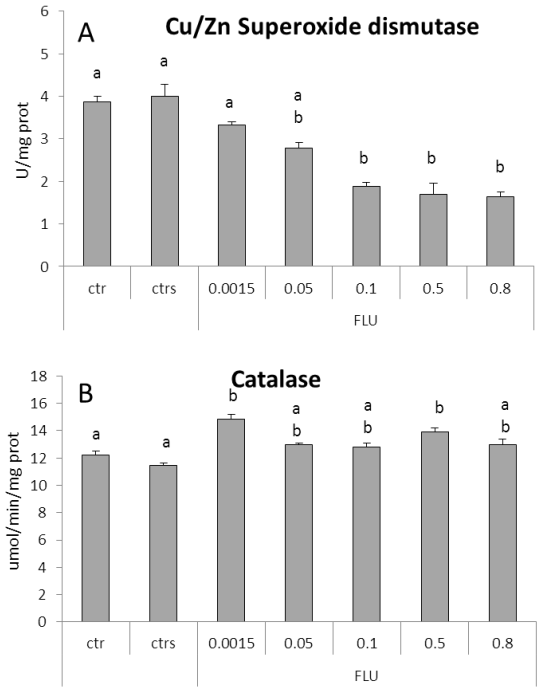

Figure 5.2: SOD (A) and CAT (B) activity in (100) *D. rerio* embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) for 80 h. Results are given as mean±SE, n=3. Bars with different letters indicate significant differences among treatments (p<0.05).

5.4.3 Accumulation Assay

As the toxicity can be due to the accumulation of the drug ABC transporter activity was determined by means of accumulation assays using the fluorescent substrate RH123 in the presence of the test chemicals (Fig. 5.3). A significant increase in RH123 accumulation inside the embryos was observed in the presence of ABC transporters inhibitors (MK571, VER and Vinblastine (10 µM)) ($p<0.05$) whereas no differences were observed in RH123 accumulation in embryos exposed to FLU.

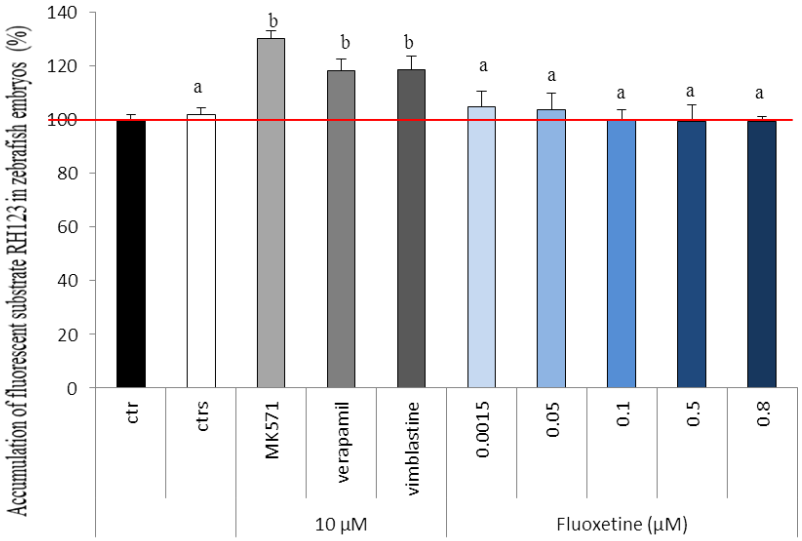

Figure 5.3: Accumulation of rhodamine 123 in D. rerio embryos exposed to ABC transporters inhibitors (MK571 (10 µM), VER (10 µM) and Vinblastine (10 µM)), FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) for 2 h (%). Results are given as mean±SE, n=4. Bars with different letters indicate significant differences among treatments ($p<0.05$).

5.4.4 Gene expression in embryos exposed to FLU

To evaluate if the levels of the detoxification components were dependent on transcription, mRNA transcription levels of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, Cu/Zn sod, cat, gstπ* and NRs (*pxr, pparα, pparβ, pparγ, rxraa, rxrab, rxrbb, rxrga, rxrgb, raraa, rarab, rarga*) and *ahr,* in embryos exposed to FLU for 80 h was assayed (Fig. 5.4 and Fig. 5.5). A pattern of down regulation was observed for most of the genes coding for detoxification components in embryos exposed to the different concentrations of FLU, with exception of *cat* that was up-regulated in the presence of the lowest concentration of FLU (0.0015 µM) ($p<0.05$). Moreover, *abcb4, abcc1, cyp1a1, Cu/Zn sod* showed a concentration dependent down regulation pattern. Interestingly, *abcc2* mRNA was significantly down regulated at all tested concentrations (Fig. 5.4). The transcription of most nuclear receptors (*pparα, pparβ, raraa, rxraa, rxrab, rxrbb rxrgb*) was also down-regulated in embryos exposed to all FLU concentrations while *pxr* transcription

was up-regulated ($p<0.05$) (Fig. 5.5). *ahr* and *pparγ* displayed a down regulation pattern with the exception of embryos exposed to 0.05 µM of FLU. mRNA levels of *rarab, rarga* were significantly decreased in embryos exposed to 0.05 and 0.8 µM ($p<0.05$), whereas *rxrga* demonstrated an up-regulation pattern at 0.0015, 0.5 and 0.8 µM of FLU.

Significant correlations in gene transcription were found between *cyp1a* and both *pxr* and *ahr* ($r=0.75$ and $r=0.66$, respectively; $p<0.05$). Also, between *cyp3a65* and both *pxr* and *ahr* ($r=0.91$ and $r=0.41$, respectively; $p<0.05$). Moreover, significant correlations were found between transcription levels of *Cu/Zn sod* and SOD activity ($r=0.72$) and *cat* and CAT activity ($r=0.64$), ($p<0.05$), showing a coordinated response in gene expression and protein activity at the level of antioxidant defences.

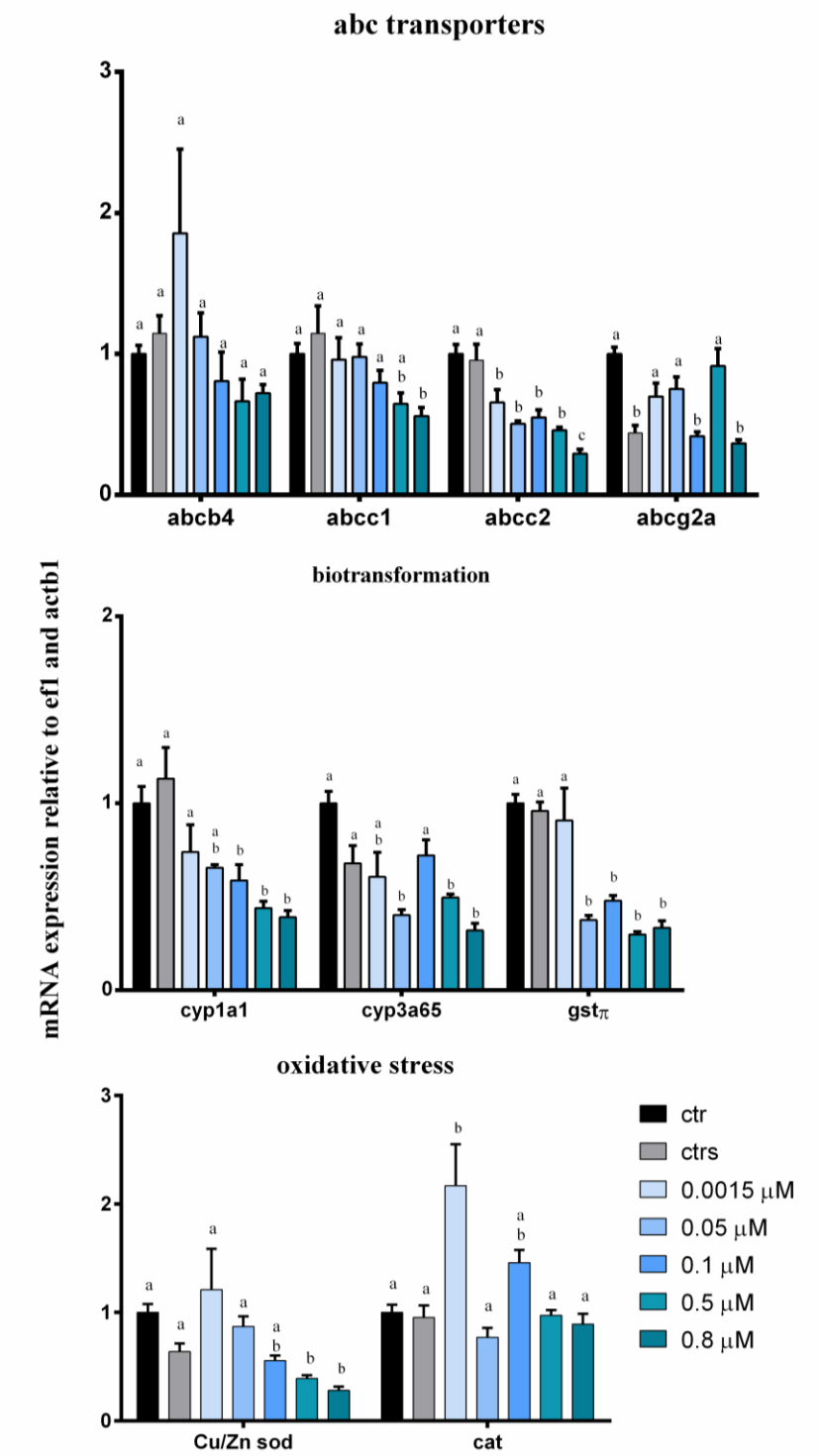

Figure 5.4: Relative mRNA expression of abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod and cat in (30) D. rerio embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) for 80h. Bars with different letters indicate significant differences among treatments ($p<0.05$). Results are given as mean±SE, n=6**.**

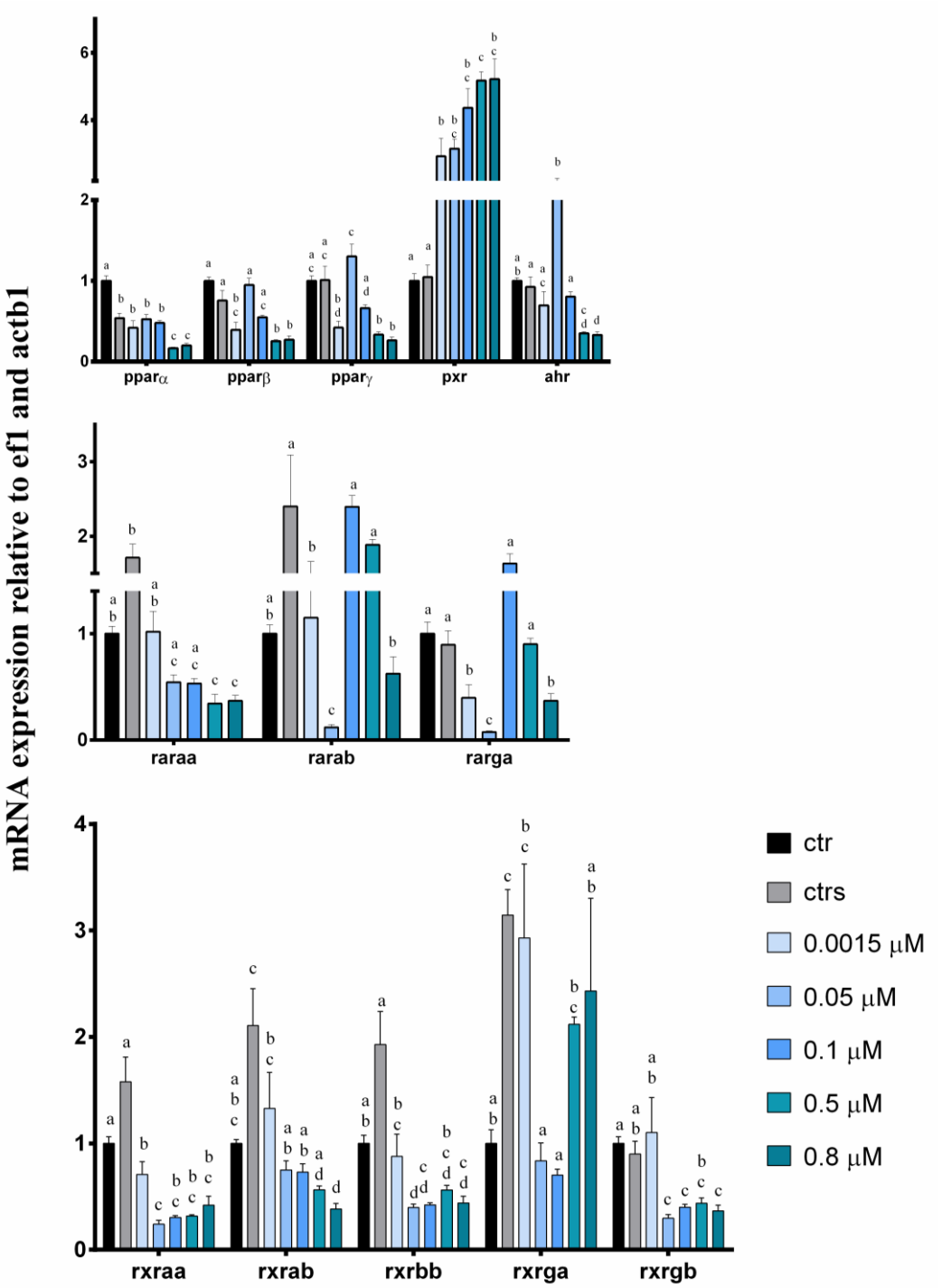

Figure 5.5: Relative mRNA expression of pparα, pparβ, pparγ, pxr, ahr, raraa, rarab, rarga, rxraa, rxrab, rxrbb, rxrga and rxrgb in (30) D. rerio embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80h. Bars with different letters indicate significant differences among treatments ($p<0.05$). Results are given as mean±SE,

5.5 Discussion

5.5.1. Effects of FLU on detoxification

Previous studies on the effects of FLU in zebrafish focused their research mainly in the neuroendocrine disruption and the behaviour changes induced by this psychopharmaceutical in adults (Airhart et al., 2007; Pan et al., 2012; Wong et al., 2013; Connors et al., 2014). However, it is important to understand the effects of this ubiquitous drug on fish embryonic development and the interaction with components of the cellular detoxification system, since FLU can affect not only the neurologic pathway but also other regulatory systems in non-target organisms. Hence, in this study we evaluated the effects of FLU in zebrafish embryo development and in the detoxification mechanism. Clearly, FLU leads to developmental abnormalities, within the range of concentrations found in the aquatic environment. A high percentage of pigmentation anomalies were observed in exposed embryos. One hypothesis is that FLU might be interfering with pigmentation by modulating embryo adrenoceptors present in the melanophores, as shown with other pharmaceuticals (such as propranolol and phentolamine) in fish including zebrafish (Fujii, 2000; Xu and Xie, 2011). In agreement, a previous study performed in our lab, zebrafish embryos exposed for 80 h at the same FLU concentrations tested here, showed changes in the transcription of adrenoceptors (unpublished data). A general pattern of down regulation of genes involved in detoxification mechanisms was observed in the embryos after exposure to FLU including ABC transporters and biotransformation enzymes of phase I and II. A similar pattern of down regulation of ABC transporter expression was also reported in aquatic invertebrates (Franzelliti et al., 2014; Chen et al., 2015) and mammalian cell lines (Zhang et al., 2013). In adult zebrafish FLU inhibited CYP1 expression and EROD activity (Smith et al., 2012; Laville et al., 2004), and our results show a down regulation of *cyp1a1* and *cyp3a65* in zebrafish embryo. Altogether, these results suggest that FLU can disrupt the detoxification mechanism in fish during different developmental phases. FLU has also been found to induce ROS production in fish cell lines (Laville et al., 2004) and the increase in ROS production, like H_2O_2, can inhibit CYP enzymes and their transcription regulation (Barker et al., 1994; Risso-de Faverney et al., 2000). In fact, an inhibition of Cu/Zn SOD and induction of CAT was observed, at transcription and activity level after exposure to FLU. It is known that the SOD enzyme that would respond to an increase of ROS level should be MnSOD, and thus the increase of CAT activity can be to cope with more hydrogen peroxide resulting from the MnSOD activity. This is consistent with the decrease of CuZnSOD. A similar response to FLU was also reported in invertebrates (Gonzalez-Rey and Bebiano, 2013; Franzelliti et al., 2014; Chen et al., 2015). This was justified by the increase in superoxide anions that stimulated

the consumption of SOD, and H_2O_2 formation inducing CAT activity (Chen et al., 2015; Gonzalez-Rey and Bebiano, 2013).

5.5.2. Effects of FLU on NR and AhR

Aiming to understand the regulation of expression of the genes that code for detoxification proteins it is important to dissect the associated signalling cascades. NRs are one of the largest classes of transcriptional regulators, providing a direct link between signalling molecules and the transcriptional responses (Wang and LeCluyse, 2003; Robinson-Rechavi et al., 2003; Castro and Santos, 2014). In mammals, the NRs PXR, CAR, and AhR are the most well-known xenosensors (Moreau et al., 2008) and regulate CYPs mRNA and protein expression (Xu et al., 2005, Aleksunes and Klaassen, 2012). CAR is absent in most teleost fish, including zebrafish (Zhao et al., 2014), nonetheless other NR, such as PXR and AhR were shown to mediate *cyp3a65* transcription (Tseng et al, 2004; Li et al, 2013; Bainy et al, 2013; Chang et al., 2013). The present results showed that FLU leads to a down regulation pattern of *ahr* in zebrafish embryos that could explain the observed down regulation pattern of *cyp1a* and *cyp3a65.* Previous studies with *Oncorhynchus mykiss* and *Salmo salar* reported that *ahr* and *pxr* transcription can be decreased or increased by certain pharmaceuticals thus changing transcription regulation of *cyp1a* and *cyp3a* (Mortensen and Arukwe, 2007; Wassmur et al., 2010). The positive correlation observed in this study between the transcription levels of *ahr*, *cyp1a* and *cyp3a65,* in response to FLU exposure, further corroborates the suggested role of *ahr* and *pxr* in the regulation of *cyp1a* and *cyp3a65* transcription. Notwithstanding, more studies have to be performed to verify this hypothesis. *pxr* mRNA exhibited the highest transcription levels of all NRs evaluated after FLU exposure. This may indicate that *pxr* modulation by this pharmaceutical can affect also other regulatory systems since, in mammals, PXR also mediates biological functions such as immune and inflammatory responses or lipid metabolism (di Masi et al., 2009; Smutny et al., 2013; Ma et al., 2015). *ppars, rars and rxrs* in mammals are involved in the regulation of inflammatory and immune homeostasis and lipid, glucose and xenobiotics metabolism (Janani and Ranjitha Kumari, 2015; Ipseiz et al., 2014; Xu et al., 2005) and were all down regulated by exposure to FLU in zebrafish embryos. In fact, other studies with fish have demonstrated that PPAR regulate pro-inflammatory genes and cytokines (Luo et al., 2015), lipid metabolism (Maradonna et al., 2015, Coimbra et al., 2015), xenobiotic metabolism (Wang et al., 2008; Cajaraville et al., 2003) and PPAR/RXR are involved in the regulation of glucose transporter 4 (GLUT4) (Marín-Juez et al., 2013). In mammalian cell lines, FLU decreases *pparγ* transcription levels, concomitantly with changes in lipid accumulation (Sun et al., 2015). Other lipid regulators pharmaceuticals have been reported to modulate

ppars gene transcription (Mimeault et al., 2006; Velasco-Santamaria et al., 2011, Coimbra et al., 2015). Therefore, the present results suggest that FLU can influence the regulation pathways of other metabolic functions, important to development through the modulation of NRs, such as *ppars, rars* and *rxrs*. Studies in vertebrates have demonstrated that nuclear receptors play an important role in embryonic development, specially retinoic acid (RA) receptors (RAR and RXR), since both excess or deficiency of vitamin A can lead to a wide range of abnormalities in the developing embryos (Klymkowsky,et al., 2010; Clagett-Dame and Knutson, 2011; André et al., 2014; Xavier-Neto et al., 2015; Samarut et al., 2015). AHR also plays an important role in the development of blood vessels and bone in fish embryos; as shown in medaka treated with AHR antagonist and cytochrome P450 inhibitor (Kawamura and Yamashita, 2002). Our results show developmental abnormalities in zebrafish embryo after FLU exposure as well as a decreased transcription trend in *cyp1a, cyp3a65, ahr, rxr* and *rar*. These combined effects of FLU appear to be related to the embryo abnormalities observed.

5.5.3 Potential chemosensitizer effect of FLU

The presence of chemosensitising xenobiotics in aquatic environments can influence the toxicity of other chemicals that normally are efflux out (Epel et al., 2008). As previously mentioned, FLU is found in water system in concentrations up to 929 ng/L (0.0027 µM). Hence, in this study we evaluated if FLU can be considered an MXR inhibitor at environmentally relevant concentrations. Yet, no inhibitory effect on MXR activity was observed in embryos exposed to FLU. In mammalian models, the effects of FLU in multidrug resistance (MDR) are not consensual, since some studies describe FLU as an inhibitor of MDR activity (Peer et al., 2004; Peer and Margalit, 2006, Zhang et al., 2013), while others found no effects (Kappor et al., 2013, O'Brien et al., 2013) like the results found in this study. The MXR assay is not limited to one efflux protein and therefore the inhibitory effect can involve other proteins beside Abcb4, a zebrafish protein with the same functional properties as ABCB1 in mammals (Fischer et al., 2013). Nevertheless, a down-regulation of *abc* transporters mRNA was observed, namely the decrease of *abcc2* mRNA levels at environmental relevant concentration, showing that even in the absence of transporter activity inhibition, FLU influences the transcription of genes belonging to the MXR system. Hence it cannot be excluded the possibility that longer exposure could impact ABC transporter's activity.

5.6 Conclusions

In conclusion, the results presented here show a clear effect of FLU on zebrafish embryo development at environmentally relevant levels (0.0015 µM = 519 ng/L). These effects are observed concomitantly with changes in the transcription of NRs and genes involved in the detoxification mechanisms. Evidence suggests that FLU can disrupt the MXR system via downregulation of ABC transporters transcription. These effects at both developmental (embryo) and transcriptional level have been observed in parallel with changes on the enzymatic activity of Cu Zn SOD and CAT. Herewith the results indicate that FLU can disrupt the detoxification mechanisms impairing at the same time embryo development, and the overall cell defence system. This can potentially affect important population related endpoints, which highlights the relevance of monitoring the presence of FLU in the aquatic environment.

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SUPPLEMENTARY DATA

Table 1S: Gene list Genbank accession numbers Primer sequences and concentrations, amplicon lengths, efficiency of reaction for, ABC transporters (*abcb4, abcc2, abcc1, abcg2a*), biotransformation (*cyp1a1, cyp3a65, gstπ*), antioxidant (*Cu/Zn sod, cat*) and nuclear receptors (*raraa, rarab, rarga, pparα, pparβ, pparγ, pxr, rxraa, rxrab, rxrbb, rxrga, rxrgb* and *ahr*) enzymes, *ef1*, and *Actb1*gene expression quantification by qRT-PCR in zebrafish.

Gene	Accession number	Primers Sequence (5'→3')	Final Conc. (nM)	Amplicon length (bp)	Efficiency (%)
abcb4	JQ014001	F: TACTGATGATGCTTGGCTTAATC R: TCTCTGGAAAGGTGAAGTTAGG	300	159	110.6
abcc1	XM_002661199	F: GCTCGAGCTCTCCTCAGAAA R:TCGGATGGTGGACTGTATCA	300	99	125.1
abcc2	NM_200589	F: GCACAGCATCAAGGGAAACA R: CCTCATCCACTGAAGAACCGA	300	87	116.5
abcg2a	NM_001042775.1	F: AAGGGTATCGAGGACCGTCT R: AATCCTGACCCTGAACGATG	300	97	113.1
cyp1a1	NM_131879.1	F: AACTCTTCGCAGGTGCTCAT R: ACAAACTGCCATTGGAGACC	300	97	102.0
cyp3a65	NM_001037438.1	F: TGACCTGCTGAACCCTCTCT R: AAGGGCGAAATCCATCTTCT	300	82	91.0
gstπ	NM_131734	F: TCTGGACTCTTTCCCGTCTCTCAA R: ATTCACTGTTGCCGTTGCCGT	300	105	119.0
Cu/Zn sod	Y12236	F: GTCGTCTGGCTTGTGGAGTG R: TGTCAGCGGGCTAGTGCTT	300	113	110.0
cat	NM_130912.1	F: CAGGAGCGTTTGGCTACTTC R: ATCGGTGTCGTCTTTCCAAC	300	91	113.0

pxr	DQ069792.1	F: CTTTTTCAGACGTGCGATGA R:TTGGCACTGTCTTCTGTTGC	300	94	112.7
rxraa	NM_001161551.1	F:ATTCAATGGCATCTCCTG R:GCGGCTTAATATCCTCTG	600	99	101.8
rxrab	NM_131153.1	F:CGCCGCATCAAATCACATAAAC R:TGAATGGGTTGGACAGTATTTAGC	300	87	109.4
rxrbb	NM_131238.1	F:TCACAACTTGGGCGTGGAGGC R:CGCATCTTGCAGACCAGCTCAG	300	105	100.7
rxrga	NM_131217.2	F:ATCTCAGTTCTTCGTTGCAGGTAG R:CGTTGATGATGGATGGGTGATGG	300	105	99.6
rxrgb	NM_001002345.1	F:CGCGGAATGGATACTCACG R:GCTGATGACGGACGGATGAC	300	114	97.7
raraa	NM_131406.2	F:GTAGTGGAGTGTGGATGTGAA R:GTGCTGATGTCTGATGGATGA	300	118	108.7
rarab	NM_131399.1	F:ATGGATTACTACCACCAGAAC R:TCTCCACAGAGTGATTCGAGC	300	115	109.4
rarga	NM_131339.1	F:CCCGCCAACTGTACGATGTCA R:GGGTCCAGTCCAGCATAGAAA	300	79	117.6
pparα	NM_001161333.1	F:CATCTTGCCTTGCAGACATT R:CACGCTCACTTTTCATTTCAC	600	81	88.3
pparβ	AF342937.1	F:GCGTAAGCTAGTCGCAGGTC R:TGCACCAGAGAGTCCATGTC	600	204	81.6
pparγ	DQ839547.1	F:GGTTTCATTACGGCGTTCAC F:TGGTTCACGTCACTGGAGAA	600	250	87.0
ahr2	NM_001007789.2	F:TTCTGTTGCCGATTCAGATG R:CTTGTTTTGCCCATGGAGAT	300	96	113,8
ef1	NM_131263.1	F: GGACACAGAGACTTCATCAAGAAC R: ACCAACACCAGCAGCAACGT	300	84	116.8
actb1	NM_131031.1	F: TCCCAAAGCCAACAGAGAGAAG R: GTCACACCATCACCAGAGTCC	10	147	100.5

Figure 1S

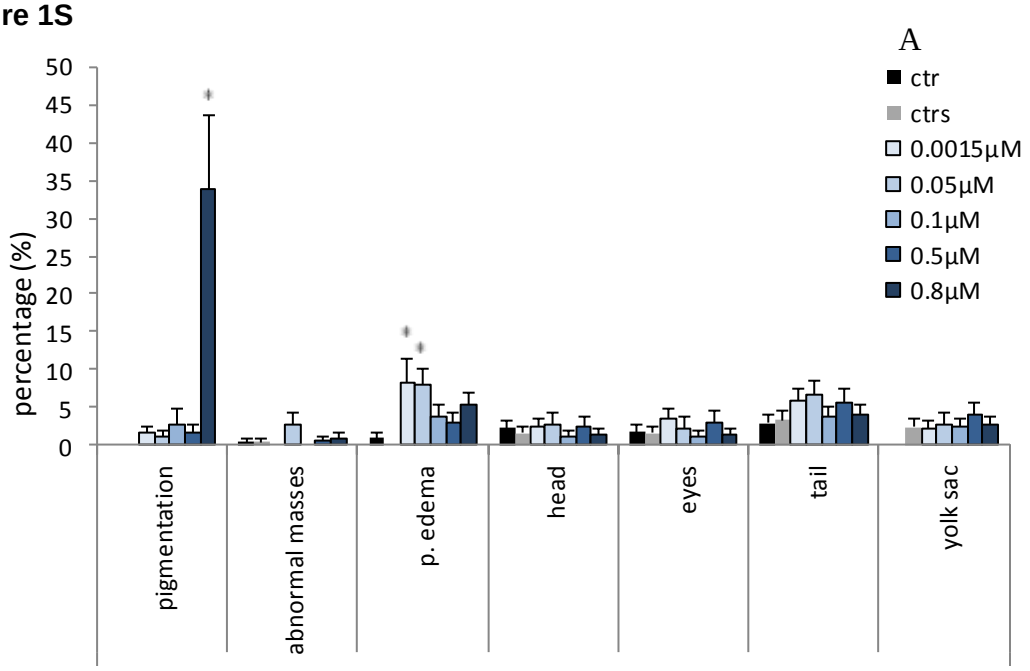

Fig 1S: Percentage of different anomalies presented by *D. rerio* embryos at 32 hpf and exposed to different concentrations of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM). Results are expressed as mean ± SE. Bars with asterisk are significantly different from the solvent control.

Figure2S

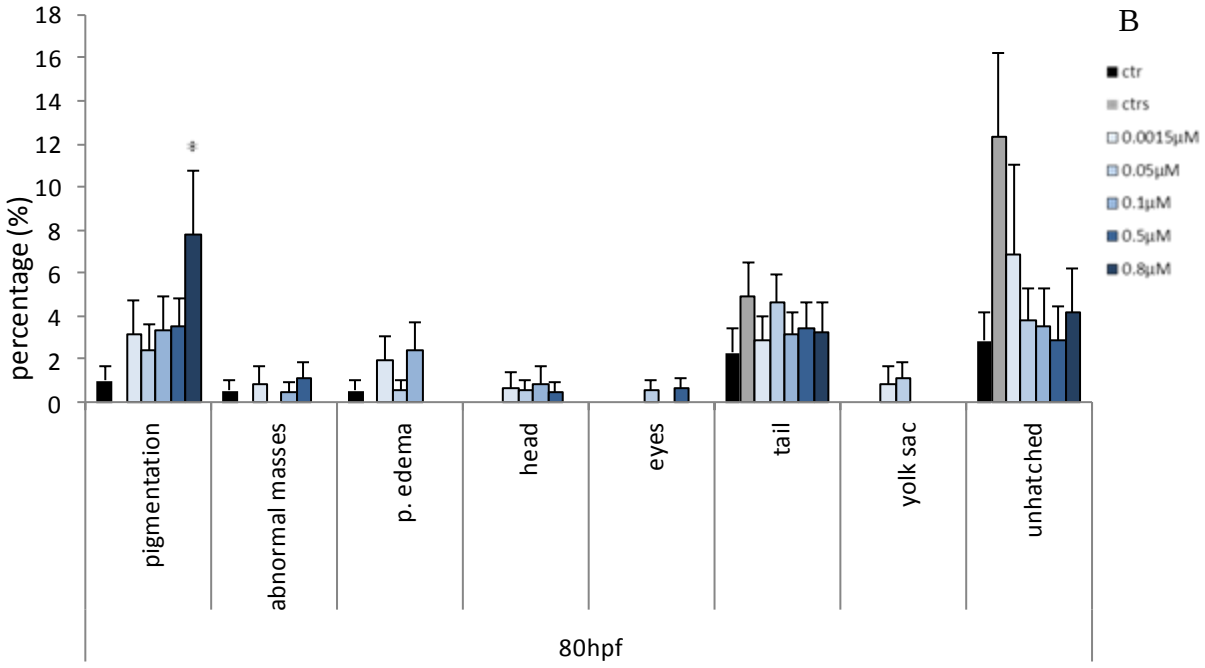

Fig 2S: Percentage of different anomalies presented by *D. rerio* embryos at 80 hpf and exposed to different concentrations of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM). Results are expressed as mean ± SE. Bars with asterisk are significantly different from the solvent contro

CHAPTER VI

FLUOXETINE MODULATE TRANSCRIPTIONAL LEVELS OF GENES INVOLVED IN THE NEUROTRANSMITTER SYSTEM IN ZEBRAFISH EMBRYOS

This chapter has been submitted as:

Cunha V, Rodrigues P,Santos MM, Moradas-Ferreira P, Ferreira M. Fluoxetine modulate transcriptional levels of genes involved in the neurotransmitter system in zebrafish embryos

Fluoxetine modulate transcriptional level of genes involved in the neurotransmitter system in zebrafish embryos

6.1 Abstract

Neurotransmitters pathways in fish and mammals are phylogenetically conserved, therefore, the presence of psychopharmaceuticals, such as fluoxetine (FLU), in the aquatic environment can interact with serotonergic, dopaminergic and adrenergic systems, affecting their behaviour and biological functions. Hence, the present work aimed at evaluating the effects of FLU on transcription of serotonin, dopamine and adrenergic transporters and receptors in early stages of *Danio rerio*. Embryos were exposed to different concentrations of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM) and mRNA expression of *sert, 5-ht1a, 5-ht2c, dat, drd1b, drd2b, net, adra2a, adra2b, adra2c, vmat* and *mao* were evaluated. The transcription level of serotonergic and dopaminergic transporters (*sert* and *dat*) and *vmat* were down-regulated at environmentally relevant concentration (0.0015 µM). mRNA of receptors *5-ht2c, drd2b adra2b* and *adra2c* also displayed a down regulation pattern after FLU exposure. In conclusion, this study demonstrated the interaction of FLU with the neurotransmission system even at an environmental concentration which can disturb the behavior and biological functions of fish impacting the aquatic ecosystem and supporting importance of monitoring the presence of this psychopharmaceutical in the aquatic environment.

Keywords: psychopharmaceuticals, *Danio rerio* embryos, mRNA levels, monoamine receptors and transporters

6.2 Introduction

Fluoxetine (FLU) is one of the most prescribed selective serotonin reuptake inhibitors (SSRIs) (Mennigen et al., 2011; Winder et al., 2012) indicated in the treatment of depression, anxiety, compulsive behaviour and eating disorders (Dulawa et al., 2004). SSRIs act on the serotonergic system of the Centre Nervous System (CNS) inhibiting the reuptake of serotonin (monoamine serotonin, 5-hydroxytryptamine, 5-HT) by serotonin transporter (SERT, 5-HTT) in the presynaptic membrane. This inhibition leads to the accumulation of serotonin in the synaptic clefts, increasing the serotonergic neurotransmission through postsynaptic serotonin receptors (Raap and Van der Kar 1999; Bisesi, 2011). Other psychopharmaceuticals are as serotonin norepinephrine reuptake inhibitors (SNRIs) inhibiting not only the reuptake of serotonin but also norepinephrine and to some degree dopamine, affecting the adrenergic and dopaminergic systems (Fenli et al., 2013; Yamamoto and Vernier, 2011). In the absence of inhibitors, serotonin and other catecholamine's are recycled and stored in a vesicular monoamine transporter (VMAT), a non-specific monoamine transporter, or degraded by monoamine oxidase (MAO) enzymes located in the outer mitochondrial membrane (Hoffman et al., 1998; Maximino, 2012). The neurotransmitters pathways in fish are phylogenetically conserved with mammals, and hence are expected to be affected by exposure to SSRIs (Kreke and Dietrich, 2008; Valenti et al., 2012). FLU is detected in the environment in concentrations ranging from 12 to 929 ng/L (Metcalfe et al., 2003; Brook et al., 2003; Santos et al., 2010;Metcalfe et al., 2010; Styrishave et al., 2011; Silva et al., 2012). Hence, to address conserved molecular targets and their interaction with environmental pharmaceuticals is key to develop of more efficient strategies for risk assessment, providing the rationale for the mode of action (MOA) approach (Franzellitti et al., 2013). Previous studies on aquatic organisms indicated alterations of biological functions such as inhibition of egg production or delayed development of sexual characteristics and behavioural changes, like decreased feeding rates, reduced locomotor activity or aggressiveness of predatory species with exposure to FLU and other psychopharmaceuticals (Monpelat et al., 2009; Lister et al., 2009; Santos et al., 2010; Schultz et al., 2011; Eisenreich and Szalda-Petree, 2015; Ansai et al., 2016). These alterations in behaviour seem to be directly linked to the effect of FLU in neurotransmission pathway, where studies showed FLU influence 5-HT levels after exposure (Gaworecki and Klaine, 2008; Franzellitti et al., 2013). Serotonin is known to play an important role in vertebrate embryo development (Buznikovet al., 2001; Levin, 2006) so is important to understand the effects of SSRI influence fish embryos and the neurotransmitter system at a biochemical and transcriptional level. Recent studies

demonstrated that zebrafish embryo development was affected in the presence of the SSRIs sertraline and fluoxetine (Ribeiro et al., 2015; Cunha et al., 2016). Hence, it is important to understand how FLU interacts with serotonergic system on non-target organisms, as well as with other neurotransmitters systems, such dopaminergic or adrenergic systems, where an interaction between dopaminergic and adrenergic systems and SSRI was already demonstrated in fish (Kreke and Dietrich, 2008; Mennigen et al., 2008). The zebrafish (*Danio rerio*) has emerged as a suitable model species in ecotoxicology due to several characteristics that include extensive genomic data but also zebrafish have been suggested as a sensitive *in-vivo* neurotoxicological and ecotoxicological screen for various serotonergic drugs, including SSRIs (Stewart et al., 2014).

The aim of this study was to assess the effects of FLU on mRNA transcription of serotonin, dopamine and adrenergic transporters and receptors in early stages of zebrafish embryos.

6.3 Material and methods

6.3.1 Parental animals

Adult wild-type zebrafish, obtained from local suppliers, were used as breeding stocks. The stock was kept at a water temperature of 27±1 ºC and in a photoperiod of 12:12 h (light:dark), in 60 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed *ad libitum* twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), and supplemented once a day with live brine shrimp (Artemia spp.).

6.3.2 Rearing conditions and exposures

Reproduction and embryos exposure was performed according to Cunha et al., 2016. Briefly, females and males (ratio 1:2) were transferred to a maternity, and submitted to acclimatization for 12 h in a cage with a net bottom covered with glass marbles within a 30 L aquarium. After egg-laying, in the following day, the breeders were removed after the beginning of the light period. After spawning, the embryos (1 hpf) were cleaned, counted and transferred to a 24 well plate (10 embryos per well) and exposed until 80 hpf to different concentrations of FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 µM; ranging from 519 to 2.8 x 10^5 ng/L) (diluted in DMSO, 0.004%). The lowest concentration (0.0015 µM) is in the environmentally relevant range. The medium (water plus FLU) was renewed daily, for the duration of the experiment. Each plate had three replicates for exposure and each assay

was at least replicated four times. After the 80 h of exposure the embryos were collected and preserved in RNALater for gene expression analysis.

6.3.3 RNA isolation and cDNA synthesis

Total RNA was isolated from embryos preserved in RNALater according to Costa et al. (2012). Briefly, total RNA was isolated using Illustra RNAspin Mini RNA Isolation kit (GE Healthcare), according to the manufacturer's protocol. RNA quality was verified by electrophoresis in agarose gel and by the measurement of the ratio of optical density at λ260/ λ280 nm. RNA was quantified using Quant-IT RiboGreen RNA Reagent and Assay Kit (Invitrogen) using a Fluoroskan Ascent, Labsystems. One microgram of total RNA was subjected to digestion of genomic DNA using Deoxyribonuclease I, Amplification Grade (Invitrogen) and 1 µg of cDNA was synthesised using Iscript cDNA Synthesis (Biorad).

6.3.4 Quantitative real-time PCR (qRT-PCR)

Gene expression of serotinergic (*sert, 5-ht1a, 5-ht2c*), dopaminergic (*dat, drd1b, drd2b*) and adrenergic (*net, adra2a*, *adra2b, adra2c*) systems, *vmat* and *mao* was assessed by means of quantitative real time PCR (qRT-PCR) and performed according to Cunha et al., 2016. Primer pairs for each target gene were designed with Primer 3 software available in http://www.ncbi.nlm.nih.gov/, based in available sequences in GeneBank. Primer sequences, amplicon lengths, efficiencies and Genbank accession numbers of target sequences are given in Table 6.1. To determine the efficiency of the PCR reactions, standard curves were made, with 6 serial dilutions of the template (concentration range from 0.05 to 50 ng/µl), and the slopes and regression curves were calculated. Gene expression was quantified by normalization with multiple reference gene (elongation factor 1 (*ef1)* and actin β1 (*Actb1*) using Normfinder algorithm (Urbatzka et al., 2013), and the relative expression ratio was calculated with efficiencies using the Pfaffl mathematical model (Pfaffl, 2001). Data is presented in mean of mRNA expression in relation to the reference genes.

Table 6.1: Gene list Genbank accession numbers Primer sequences and concentrations, amplicon lengths, efficiency of reaction for serotonin transporters (*sert*), serotonin receptors (*5-ht1aa, 5-ht2c*), dopamine transporter (*dat*) dopamine receptors (*drd1b, drb2b*), norepinephrine transporter (*net*), adrenergic receptors (*adra2a , adra2c, adra2b*), *vmat, mao* gene expression quantification by qRT-PCR in zebrafish.

Gene	**Accession number**	**Primers Sequence (5'→3')**	**Final Conc. (nM)**	**Amplicon length (bp)**	**Efficiency (%)**
serta	NM_001039972.1	F: CATCTATGCTGAGGCTATTG R: AAGAATATGATGGCGAAGA	0.3	73	100
5-ht1aa	NM_001123321.1	F: ATGAGGATGAGCGGGATGTAG R: CAATCAGCCAGGACCACG	0.3	80	125
5-ht2c	NM_001129893.1	F: GCGCTCTCTGTCCTATTTGG R: GTAGCGGTCGAGAGAAATGG	0.001	89	126.4
dat	NM_131755.1	F:ACGTCAATTCTCTTTGGAGT R:TCCTCGATATCATCACTGAA	0.15	86	97
drd1b	NM_001135976.2	F: CTGCGACTCCAGCCTTAATC R: AGATGCGGGTGTAAGTGACC	0.6	98	117.2
drd2b	NM_197936.1	F: ACGCCGAATATCAGTCCAAC R: GCAGTGCCTGAGTTTCAACA	0.3	96	110.7
Net	XM_689046.5	F: AGTCCAGCGTTCTTGCTGTT R: TCTGCCCAGTATGGGAAAAC	0.3	92	117.0
adra2a	NM_207637.2	F: AGCGTTTTGTGACTGCTGTG R: TAATGGGATTGAGGGAGCTG	0.3	86	114.0
adra2b	NM_207638.1	F: GTCTGCCTGGCCACACTAAT R: GTACGGGGCGAGTTTTATCA	0.001	80	119.7
adra2c	NM_207639.1	F: CTATTCTCCGGCCACCATTA R: CCAGCACATTCCCCACTATT	0.001	80	133.8
vmat2	NM_001256225.2	F: CTAAAAAGCTCCGCATCCAG R: TGTCCAAGAGCAAAGCAATG	0.15	231	133
mao	NM_212827.2	F: ACCAACTCAAAACCGCATTC R: GTAGGCAAAAGGGTTCCACA	0.3	151	105.0

6.3.5 Statistical analysis

Differences in mRNA expression were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Tukey's test) at a 5% significant level. Data were log transformed in order to fit ANOVA assumptions. All tests were performed using software Statistica 7 (Statsoft, Inc). Data is presented as mean ± standard error.

6.4 Results

mRNA levels of neurotransmission transporters and receptors (*sert, 5-ht1a, 5-ht2c*, *dat, drd1b, drd2b, net, adra2a*, *adra2b, adra2c), vmat* and *mao* in embryos exposed to FLU are presented in Fig. 6.1. The transcription level of the transporters *sert* and *dat* was down regulated at 0.5 and 0.1 µM concentrations, respectively, as well as at the lowest concentration (0.0015 µM) ($p<0.05$), while no changes were observed for *net*. mRNA levels of *5-ht1a* and *adra2a* were significantly increased in embryos exposed to 0.8 µM of FLU ($p<0.05$), whereas *5-ht2c* and *drd2b* exhibited a down regulation pattern for all concentration with exception of 0.1 µM. *adra2b* and *adra2c* also displayed a down regulation pattern mainly at higher concentrations ($p<0.05$). The transcription levels of *drd1b* were also decreased at 0.05, 0.5 and 0.8 µM ($p<0.05$). *vmat* was down regulated in embryos exposed to 0.0015 and 0.1 µM concentrations ($p<0.05$), while no changes were observed for *mao*.

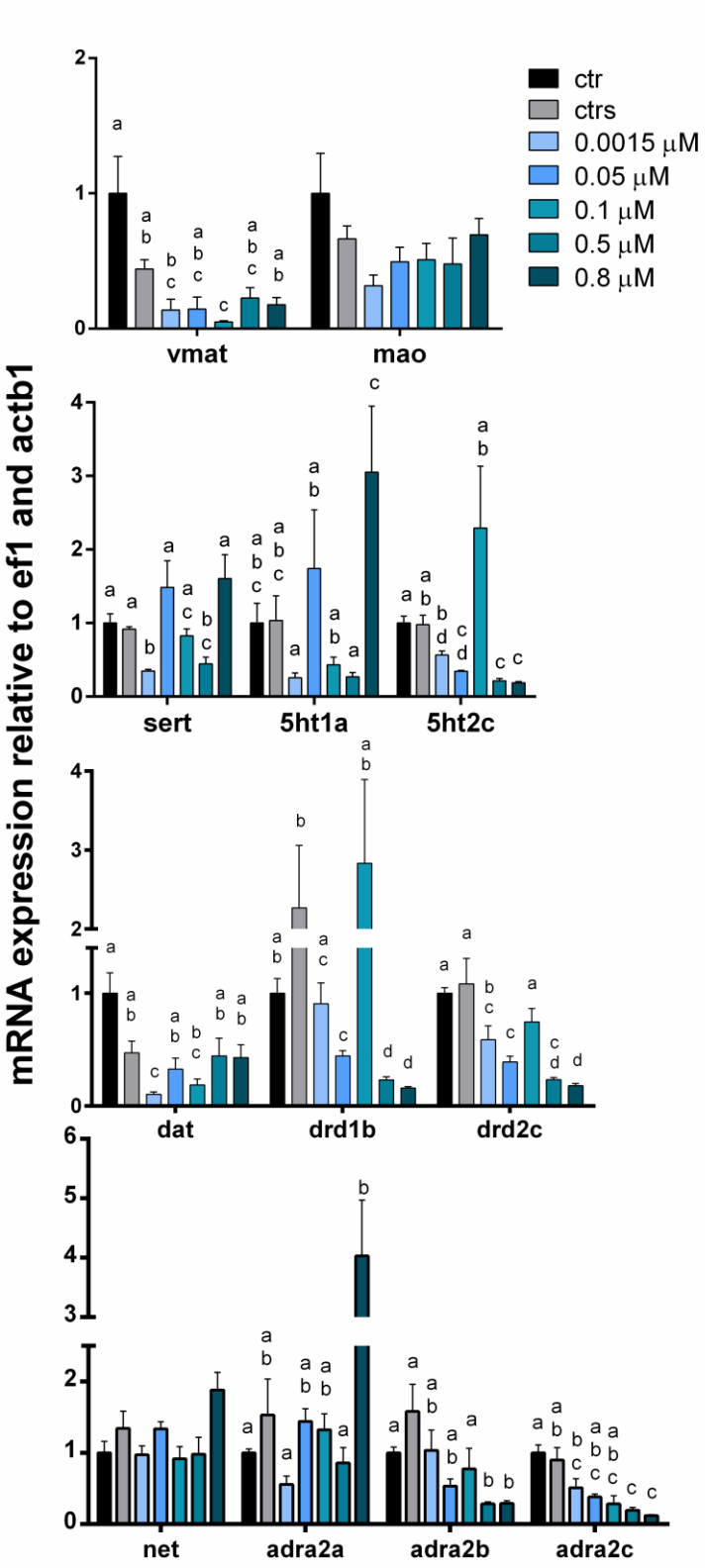

Figure 6.1: Relative mRNA expression of in D. rerio embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80h. Bars with different letters are significantly different from each other ($p<0.05$). Results are given as mean±SE, n=6.

6.5 Discussion

Neurotransmitters pathways within vertebrates, including fish, are well conserved (Kreke and Dietrich, 2008; Valenti et al., 2012). Therefore it is important to study how the presence of psychopharmaceuticals, design to act on these pathways, will interact and possible affect the neurotransmission systems of non-target aquatic organism. Our previous study demonstrated that FLU, at the concentrations tested in the present study, impacts the development of zebrafish embryos (Chapter V). Hence, given the reported mode of action of FLU in vertebrates, this study aimed at evaluate the impact of FLU in monoamine receptors and transporters at the transcription level, particularly of serotonin (*sert, 5-ht1a, 5-ht2c*), as well as dopamine (*dat, drd1b, drd2b*) and adrenergic (*net, adra2a, adra2b, adra2c*) transporters and receptors. In mammalian model species, FLU leads to a down regulation of *Sert*, *5-Ht1a* and *5-Ht2c* and this decrease has been attributed to the increase of serotonin accumulation in the synaptic clefts by repeated SERT inhibition by FLU (Oliva et al., 2005; Gomez et al., 2015; Lesemann et al., 2012; Barbon et al., 2011; Messripour and Clark, 1985). This seems to be in agreement with the down regulation observed for *sert* and *5-ht2c* in this study, even at the lowest and environmental relevant concentration. In mammals, FLU promotes behavioural and emotional changes by serotonergic system modulation. Hence, FLU seems to affect the serotonergic neurotransmission system in zebrafish embryos in a similar manner to mammals, which can possible provoke behavioural changes described by previous studies (Santos et al., 2010; Pelli and Connaughton, 2015). Serotonin and serotonergic system play a role in vertebrate embryo development (Buznikovet al., 2001; Levin, 2006), so a down regulation of genes involved in this system can affect fish embryo development. A previous study performed by our laboratory demonstrated that FLU affected zebrafish embryos development at the tested concentrations (Chapter V), which seem to agree with FLU effects in serotonin transporters and receptors transcription found in this study. This is important since these transcription alterations were observed at environmentally relevant concentration, making it essential to perform more studies that address FLU interaction with the serotonergic system and pathway. This work also evaluated the effects of FLU in the transcription of receptors and transporters of dopaminergic and adrenergic systems, since there is evidence of interactions between these systems and the serotonergic (Kreke and Dietrich, 2008; Boulay et al., 2011; Lesemann et al., 2012). A tendency for mRNA down regulation was observed for dopamine transporter (*dat*) and receptors (*drd1b, drd2b*) and adrenergic receptors (*adra2b, adra2c*) in embryos exposed to most of the tested concentrations. Similar to our results, FLU decreased dopaminergic D2 receptor transcription in mammals (Dziedzicka-

Wasylewska et al., 2002; Lesemann et al., 2012). The decrease of mRNA levels of dopamine receptors and transporter may have a negative impact in key functions, known to be controlled by this system, such as cognition, locomotor activity, motivation and reward, mood, attention and learning (Beaulieu and Gainetdinov, 2011). In fish, dopamine inhibits basal and GnRH-stimulated gonadotropin release by binding to the Dopamine 2 receptors (DR2) on gonadotropes, and by decreasing pituitary GnRH receptor expression which can impair gonadal growth and spawning (Chang et al., 2000; Dufour et al., 2005; Crago and Schlenk, 2015). In another study, *Sebastiscus marmoratus* was exposed to Tributyltin (TBT) and observed a decrease in the transcription levels of dopamine receptors affecting the predatory activities of the fish (Yu et al., 2013). Therefore, the down regulation of dopamine transporter and receptors by FLU may negatively influence the reproduction and the behaviour of fish. Adrenoceptors are present in fish melanophores, modulating the pigmentation, as reported in previous studies with other pharmaceutical classes (Fujii, 2000; Xu and Xie, 2011). In a previous work performed in our laboratory, zebrafish embryos were exposed to the same FLU concentrations and a high percentage of pigmentation anomalies was observed, mainly at the highest concentration (0.8 µM) (Chapter V). This seems to be in agreement with the results found in this study where FLU can impact the transcription of adrenoceptors mainly at higher concentrations. The storage process of monoamines is performed by VMAT that is release at the synapse following neuronal membrane depolarization. When monoamines are not stored in presynaptic vesicles by VMAT, they are degraded by MAO enzyme (Maximino, 2012). Transcription levels of *vmat* were diminished in the presence of FLU, while no changes were observed for *mao*. In contrast to our results, a previous study with rats, FLU increased the transcription levels of *Vmat* (Rehavi et al., 2002). However, since FLU can inhibit monoamine transporters, they accumulate in the synaptic clefts and are not stored by VMAT, therefore in response to this a decrease of *vmat* is expected. In several of our target genes, it was not observed a dose-response relation with FLU exposure, like it was detected for *5-ht2c, adra2c, adra2b* or *drd2c.* For other genes maybe more concentrations have to be tested in order a dose-response curve to be observed.

Overall, FLU seems to impact neurotransmitter systems of zebrafish embryos, even at environmentally relevant concentrations, which can impair functions, like reproduction, behaviour and embryo development.

6.6 Conclusions

In conclusion, FLU decreases the transcription of important neurotransmitters transporters and receptors, even at concentrations found in the environment. Our results show that FLU may regulate different monoamine pathways involved in important physiologic functions. Hence, future studies should address the ecological relevance of the findings reported here. The fact that environmentally relevant concentrations can disturb the neurotransmitters systems supports the importance of monitoring the presence of this psychopharmaceutical in the aquatic environment.

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CHAPTER VII

EFFECTS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS) ON MULTIXENOBIOTIC RESISTANCE (MXR) RELATED EFFLUX TRANSPORTER ACTIVITY IN ZEBRAFISH (*DANIO RERIO*) EMBRYOS

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Effects of pharmaceuticals and personal care products (PPCPs) on multixenobiotic resistance (MXR) related efflux transporter activity in zebrafish (*Danio rerio*) embryos

7.1 Abstract

Multidrug resistance (MXR) system act as defence against a wide range of compounds and are mediated by ATP binding cassette (ABC) transporters. However, certain compounds, so called chemosensitisers, have the potential to inhibit these proteins increasing the toxicity of chemicals that would normally be effluxed out of the cells. In this study different pharmaceuticals and personal care products (PPCPs), highly prescribed and present in common household products and that are increasingly detected in aquatic systems, were chosen to assess their chemosensitisation potential. To addresses this question, zebrafish embryos were used since they are a suitable model specie in ecotoxicology and the chemosensitisation assays can be performed rapidly and efficiently. Two types of assays were used: Abcb4 ATPase activity, a highly specific and artificial assay that examine chemical interaction with Abcb4; and therefore the effect concentrations in ATPase assay cannot be directly related to concentrations effects determined *in vivo*. And an *in vivo* dye accumulation assay, a less specific with regard to the type of the transporter activity (dyes can be substrates of different transporter types), but can be more information about whether the effects are environmentally relevant. MXR activity was determined with dye accumulation assays using rhodamine 123 (8 µM) in 24 hours post fertilization embryos exposed for 2 h to known ABC protein inhibitors (MK571 and VER) and celestolide, musk xylene, musk ketone, nerol, citronellol, isoeugenol, camphene, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, propylparaben, sertraline, fluoxetine, diclofenac and simvastatin. The interaction of the test compounds with zebrafish Abcb4 was determined by recording their effects on Abcb4 ATPase activity. Results demonstrated a concentration dependent interaction to some of the tested chemicals. Some PPCPs inhibited ABC proteins, such as isoeugenol at environmentally relevant concentration. Abcb4 ATPase activity was inhibited by sertraline, fluoxetine, diclofenac, simvastatin and isoeugenol. On the other hand, musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde and α-hexylcinnamaldehyde and simvastatin demonstrated Abcb4 substrates properties. Taken together, the results show that both assays should be used in combination to better determine the interaction of PPCPs with ABC proteins. One specifically evaluate if the chemicals are inhibitors or substrates of a particular efflux transporter and the other give more information about relevant concentration *in vivo*. Diclofenac, sertraline and fluoxetine can be recognised as potential chemosensitisers inhibiting both ATPase activity assay and MXR system. These results

highlight the importance of screening emerging pollutants for chemosensitiser potential and their role in the toxicity of other xenobiotics.

Keywords: Pharmaceuticals; PCPs; Abcb4; Inhibitors; Substrates; Chemosensitisation

7.2 Introduction

The integrity of marine and freshwater aquatic ecosystems across the world is threatened by the adverse impacts of a variety of anthropogenic chemicals. In the last decades, advances in new technologies enable the detection of low concentrations of many emerging compounds (Lapworth et al., 2012; Stuart et al., 2012; Jiang et al., 2013) including pharmaceuticals and personal care products (PPCPs) that have been quantified in the ng/L to µg/L concentration range in water (Table 7.1) (Kim et al., 2009; Chen et al., 2012, Santos et al., 2016). Commonly used pharmaceuticals such as diclofenac, simvastatin, fluoxetine and sertraline and synthetic musk fragrances (celestolide, musk xylene, musk ketone), essential oils (nerol, citronellol, isoeugenol, camphene, α-amylcinnamaldehyde, α-hexylcinnamaldehyde) and propylparaben found in a variety of cosmetics, perfumes, and soaps are the PPCPs most frequently occurring in the aqueous environment. Even if industry and household waste waters undergo purification in waste water treatment plants (WWTP), complete removal of many contaminants is not ensured, so significant concentrations of some PPCPs occur in effluents, groundwater, surface waters and also in drinking water (Soares et al., 2008; Santos et al., 2010; Lapworth et al., 2012; Jiang et al., 2013) (Table 7.1). Aquatic organisms have cellular mechanisms serving as protection against the adverse effects of xenobiotics such as the multixenobiotic resistance (MXR) mechanism. MXR is mediated by ATP-binding cassette (ABC) transporters and this mechanism is considered to be the first line of cellular defence against toxicants (Kurelec, 1992; Epel, 1998; Bard, 2000; Ferreira et al., 2014; Luckenbach et al, 2014). The ABC transporter super family is subdivided into subfamilies ABCA to ABCH (Dean and Annilo, 2005; Popovic et al., 2010), although only members of the ABCB, ABCC and ABCG subfamilies are considered important in a toxicological context (Epel et al., 2008). ABCB1 (P-glycoprotein, P-gp) homologs efflux a variety of mainly unmodified compounds from the cells (Epel et al., 2008) and are an important component of the “environment-tissue barrier” (Luckenbach and Epel, 2008). Notably, high expression levels and increased efflux activity of ABCB1 orthologues have been found in aquatic species from polluted environments (Smital et al., 2000). In zebrafish, *Danio rerio*, the *abcb1* orthologue is absent, but zebrafish Abcb4 was recently reported to have the same functional properties as ABCB1 in mammals (Fischer et al., 2013).

A range of environmentally relevant chemicals were found to interfere with efflux transporter function, thus increasing the susceptibility of cells/organisms to the toxic effects of other compounds that are usually kept out of cells/tissues by the efflux transporters, an effect called “chemosensitisation” (Kurelec, 1997). So far examined compounds included some PPCPs such as tonalide, galaxolide and other musk compounds that were shown to act as chemosensitisers (Epel et al., 2008; Caminada et al., 2008, Fischer et al., 2013; Kurth et al., 2015). To measure the levels of MXR activity and/or chemosensitisation several techniques have been used. Fluorescent cell-based transport assays or radiolabeled substrates and ATPase assays with overexpressing cell lines have been used for screening for substrates and inhibitors of ABC transporters (Luckenbach et al., 2014). In this study, two assays were used to characterise the chemosensitisation potential; Abcb4 ATPase activity and dye accumulation assay. Transport activity of ABC proteins (ATPase activity assay) is strongly combined with ATP cleavage and can be determined by ATPase activity. Studies with P-glycoproteins (P-gp, ABCB1) demonstrated that modulation of ATPase activity is highly correlated with the interaction of P-gp with any given compound. Furthermore, the ATPase assay is very specific and can distinguish between substrates and inhibitors (Sarkadi et al., 1992; Zaja et al., 2011). Dye accumulation assay is a less specific assay since dyes can be substrates of different ABC proteins. However, as an *in vivo* assay gives more information about relevant effect concentrations.

The aim of this study was to determine the chemosensitisation potential of representatives of four classes of commonly used PPCPs, i.e, pharmaceuticals, synthetic musk fragrances, parabens and essential oils applying two different transporter protein activity assays. Zebrafish embryos were used to determine the effects of these compounds on the ABC efflux transporters activity and the recombinant zebrafish Abcb4 protein to assess the specific interaction of different PPCPs compounds with the Abcb4 transporter.

7.3 Material and methods

7.3.1 Chemicals

Rhodamine 123 (RH123), verapamil (VER), simvastatin, diclofenac sodium salt, propyl-4-hydroxybenzoate (propylparabene), sodium acid, Dithiothreitol (DTT), sodium orthovanadate, adenosine triphosphate (ATP), zinc acetate, ammonium molybdate, protease inhibitor P8340 were purchased from Sigma-Aldrich (Germany), MK571 from VWR International and vinblastine sulfate (VIN) from Tocris Bioscience (USA). Musk

ketone, musk xylene, celestolide were kind gifts from International Flavors & Fragances Inc. (IFF: Union Beach, NJ). Sertraline hydrochloride and fluoxetine hydrochloride were purchased from abcr (Germany). Isoeugenol, camphene, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, citronellol and nerol were purchased from SAFC supply solutions (Germany). Maximum test concentrations were at or below water solubility (information taken from www.chemspider.com) (Table 7.1). All other chemicals were of analytical grade, and were purchased from local companies.

7.3.2 Production of recombinant zebrafish Abcb4 protein and ATPase activity

Specific interactions of test compounds with zebrafish Abcb4 were determined with an *in vitro* assay using recombinant zebrafish Abcb4 protein generated with the baculovirus expression system (Fischer et al., 2013). Similar to ABCB1 zebrafish Abcb4 is an ATPase that uses the energy liberated by the cleavage of ATP to ADP and Pi for transporting its substrates against a concentration gradient across cell membranes (Chen et al., 1986). In the assays applied here, the ATPase activity of recombinant zebrafish Abcb4 was used as indication for interaction of the test compounds with the transporter protein using the amount of generated Pi in the experiment as measure for the Abcb4 ATPase activity as first described by Sarkadi et al. (1992) for ABCB1. Interaction of the test compounds with zebrafish Abcb4 was examined in experimental series with Abcb4 protein with non-stimulated (“basal”) and with verapamil-stimulated ATPase activities, which can provide indication for the interaction of the compound with the transporter protein as substrate (i.e., a compound that is transported by the protein) and/or as inhibitor (i.e., a compound that disrupts transporter function). For achieving a stimulated state of the Abcb4 ATPase, showing activity close to its maximum, verapamil was applied at 40 µM in the experiments according to Fischer et al. (2013). Stimulation of the basal transporter ATPase activity is indicated by increased Pi levels in the test; it is often observed for transporter substrates (Sarkadi et al., 1992). In contrast, inhibition of the basal and/or stimulated ATPase activities indicates a compound’s property as transporter inhibitor and is indicated by decreasing Pi levels (von Richter et al., 2009). Production of recombinant Abcb4 with the baculovirus expression system and performance of the ATPase assay were according to Fischer et al. (2013). Briefly, *abcb4* baculovirus was produced with the Bac-to-Bac Baculovirus Expression System (Invitrogen). To produce recombinant Abcb4, 200 mL of Sf (*Spodoptera frugiperda*) 9 cell suspension with 2 x 106 cells/ml were infected with *abcb4* baculoviral particles at a multiplicity of infection (MOI) of 2.5. Cells were incubated for 72 hours, then harvested, disrupted by nitrogen cavitation and cell membranes were pelleted by centrifugation and ultracentrifugation steps.

Table 7.1: Names, molecular weights, CAS numbers, log K_{ow} values, aqueous solubility and environmental concentrations of test compounds.

Compound	MW	CAS number	Log K_{ow}	Aqueous solubility (mg/L) [chemspider.com]	Environmental concentrations
diclofenac	318.1	15307-79-6	0.7	15906.5	**Effluents:** 69.2 - 382.5 ng/L (Kosma et al., 2014; Langford and Thomas, 2010) **Influents**: 12 – 3600 ng/L (Santos et al., 2010) **Groundwater:** 590 ng/L (Santos et al., 2010) **Freshwater:** 0.3 – 72 ng/L (Santos et al., 2010)
fluoxetine	345.8	56296-78-7	4.17	3457.9	**Freshwater:** 12 to 157.4 ng/L (Kolpin et al., 2002; Silva et al., 2014) **Effluents**: 99 to 929 ng/L (Brook et al., 2003; Metcalfe et al., 2010; Silva et al., 2012, Styrishave et al., 2011; Metcalfe et al., 2003)
sertraline	342.7	79559-97-0	5.15	1713.5	**Surface water:** 0.84 to 2.4 ng/L (Santos et al., 2010) **Seawater:** < 0.52 ng/L (Santos et al., 2010) **Effluents:** 80 to 87 ng/L (Metcalfe et al., 2010; Silva et al., 2012 Styrishave et al., 2011)
simvastatin	418.6	79902-63-9	4.68	0.7653	**Effluents:** 0.2 to 110 ng/L (Miao and Metcalfe, 2003; Santos et al., 2010; Ottmar et al., 2012) **Influent:** 1560 ng/L (Ottmar et al., 2012) **Surface water:** 0.1 ng/L (Miao and Metcalfe, 2003)
propylparabene	180.2	94-13-3	2.71	529.3	**Surface water:** 9.2 ng/L (Renz et al., 2013) **Freshwater:** 900 to 1820 ng/L (Haman et al., 2015) **Marine water:** 7.9 ng/L (Haman et al., 2015) **Influent**: 20000 ng/L (Haman et al., 2015)
α-amylcinnamaldehyde	202.2	122-40-7	4.33	9	Not found
α-hexylcinnamaldehyde	216.3	101-86-0	4.82	2.75	**Influent** : 35 to 1201 ng/L (Švestková, 2014)
citronellol	156.3	106-22-6	3.91	105.5	**Influent** : 784 to 4748 ng/L (Švestková and Vávrová (2015)
camphene	136.2	79-92-5	4.22	6	**Surface water**: 0.38 to 837 ng/L (Button and Juttner, 1989)
isouegenol	164.2	97-54-1	3.04	165.9	**Effluents:** 10 x 10^3ng/L and 121x 10^3 ng/L (Belknap et al., 2006)
nerol	154.2	106-25-2	3.47	255.8	Not found
musk xylene	297.3	81-15-2	2.25	61.35	**Surface water:** 0.049 - 0.62 ng/L (Peck and Hornbuckle, 2004; Muller et al., 1996) **Seawater:** <0.02 to 0.17 ng/L (Gatermann et al.,1995) **Effluents:** <1 to 162 ng/L (Simonich et al., 2002; Díaz-Cruz Barceló, 2015)
musk ketone	294.3	81-14-1	2.11	83.59	**Influents:** 812 to 5000 ng/L (Chase et al., 2012; Paxeus, 1996) **Effluents:** 19 to 705 ng/L (Simonich et al., 2002) **Surface water:** < 2 to 10 ng/L (Muller et al.,1996; Peck and Hornbuckle, 2004)
celestolide	244.4	13171-00-1	5.93	0.22	**Influent:** 45 ng/L (Chase et al., 2012) **Effluents:** 620 ng/L (Chen et al., 2007)

The pellet containing the membrane fractions was dissolved in ice cold TMEP buffer (Germann, 1998; Sarkadi et al., 1992), homogenized using syringes and total protein was determined with the BCA™ Protein Assay Kit (Thermo Scientific). ATPase-assays were performed in 96-well plates and 20 µg of total protein was used per well. Liberated Pi was colorimetrically quantified by measuring the absorption at 750 nm using a detection reagent with 7.5 % ascorbic acid (Applichem, Germany), 8.75 mM ammoniummolybdate tetrahydrate (Sigma, Germany) and 3.75 mM zinc acetate (Sigma). The Abcb4 ATPase activity is orthovanadate-sensitive and test series were performed with and without sodium orthovanadate (Sigma) to determine the Abcb4 ATPase activity within the total ATPase activity in the membrane preparations. Abcb4 ATPase activities in each test were determined by subtracting the values from tests with orthovanadate from those from tests without orthovanadate (Sarkadi et al., 1992).

Concentration response curves were modelled in GraphPad Prism using the four parameter logistic equation.

$$V\ (S) = Bottom + \frac{Top - Bottom}{1 + 10^{(\log EC50 - S)Hillslope}}$$

Where V(S) is the enzyme activity, *Bottom* is the activity at the bottom plateau, *Top* is the activity at the top plateau, log EC50 is the concentration at half-maximal activity, *Hillslope* describes the slope of the curve, S is the chemical concentration. Versions of this equation were used by Glavinas et al. (2007); Bircsak et al. (2013), and Zaja et al. (2011).

7.3.3 Maintenance of zebrafish and egg production

Adult wild-type zebrafish, obtained from local suppliers, were used as breeding stocks. Fish were kept at a water temperature of 27±1 ºC and in a photoperiod of 12:12 h (light:dark) in 60 L aquaria with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters. The fish were fed ad libitum twice a day with a commercial fish diet Tetramin (Tetra, Melle, Germany), supplemented once a day with live brine shrimp (Artemia spp.) For reproduction, females and males (ratio 1:2) were transferred to a maternity, and submitted to acclimatization for 12 h in a cage with a net bottom covered with glass marbles within a 30 L aquarium. After spawning on the following day, the breeders were removed after the beginning of the light period.

The freshly spawned eggs were collected and cleaned by rinsing. Fertilized eggs were transferred to aquaria (3.5 L) and kept at 26 ± 1 ºC with aeration.

Maintenance of adult zebrafish and egg production were according to standard protocols (e.g. Kimmel et al., 1988; Lammer et al., 2009; Machado et al., 2014). Experiments with zebrafish embryos were performed in reconstituted water as specified in ISO 7346-1 (ISO, 1996; 294.0 mg/L $CaCl_2$×2 H_2O; 123.3 mg/L $MgSO_4$×7 H_2O; 63.0 mg/L $NaHCO_3$; 5.5 mg/L KCl). Prior to use, the artificial water was aerated to attain oxygen saturation.

7.3.4 Rhodamine 123 dye accumulation assay

The amount of RH123 accumulated in zebrafish embryo tissue upon exposure to the dye served as measure for MXR transporter activity. Prior to conducting dye accumulation assays with the test substances the RH123 concentration to be used in the assays was determined. These preliminary assays aimed to identify the RH123 concentration at which effects of test compounds on RH123 accumulation in zebrafish embryos are clearly detected. Different concentrations of RH123 (1, 2, 5, 8, 10 µM) were tested in a 2 h incubation with standard MXR transporter inhibitors (MK571 and VER). Data is presented in arbitrary fluorescence units and in percentage (%) of RH123 fluorescence in relation to the water control measured in each assay (Fig. 7.1a, b). At 8 µM RH123 effects of MK571 and VER were most pronounced (MK571: +44% and VER: +23% vs. controls) and this RH123 concentration was therefore applied in subsequent experiments.

The dye accumulation assays were performed with 24 hpf embryos (10 per well in a 2 ml media volume) exposed to different concentrations of the tested PPCPs (celestolide, musk ketone, musk xylene, propylparaben, nerol, citronellol, isouegenol, camphene, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, sertraline, fluoxetine, diclofenac and simvastatin) (Table 7.2)and standard MXR transporter inhibitors (MK571, VER and VIN (10 µM)) along with 8 µM RH123 for 2 h at 26 ± 1 ºC in the dark. After a 2 h incubation period embryos were washed 3 times with ice-cold water and mechanically disrupted. The RH123 accumulated inside the embryos was measured in the homogenate of 10 embryos using a fluorescent microplate reader (Fluoroskan Ascent, Labsystems) at excitation/emission - 485/538 nm. Each assay was replicated at least four times. In each batch of chemical tests, water and positive controls (MXR inhibitors) were also measured and data is presented in percentage (%) of RH123 fluorescence in relation to the water control measured in each assay.

Table 7.2: Celestolide, musk ketone, musk xylene, propylparaben, nerol, citronellol, isouegenol, camphene, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, sertraline, fluoxetine, diclofenac, simvastatin test concentrations. Environmentally relevant concentrations are marked in grey (related to Table 7.1).

Compound	Compound concentrations µM [ng/L]					
celestolide	0.0004 [97.7]	0.001 [244.4]	0.01 [2.4×10^3]	0.03 [7.3×10^3]	0.3 [7.3×10^4]	0.9 [2.2×10^5]
musk ketone	0.1 [2.7×10^4]	0.4 [8.9×10^4]	3.5 [7.4×10^5]	10.5 [2.3×10^6]	94.7 [2.0×10^7]	284 [6.1×10^7]
musk xylene	0.09 [2.9×10^4]	0.3 [1.2×10^5]	2.5 [1.0×10^6]	7.6 [3.1×10^6]	68 [2.8×10^7]	206 [8.4×10^7]
propylparabene	0.001 [180.2]	0.004 [720.8]	0.04 [7.2×10^3]	0.1 [2.0×10^4]	0.9 [1.5×10^5]	2.9 [5.3×10^5]
nerol	0.45 [7.7×10^4]	1.37 [2.2×10^5]	12 [1.9×10^6]	37 [5.7×10^6]	333 [5.1×10^7]	1000 [1.5×10^8]
citronellol	0.3 [3.1×10^4]	0.9 [1.4×10^5]	8.3 [1.3×10^6]	25 [3.9×10^6]	225 [3.5×10^7]	675 [1.1×10^8]
isoeugenol	0.45 [8.2×10^4]	1.37 [2.3×10^5]	12 [1.9×10^6]	37 [6.1×10^6]	333 [5.5×10^7]	1000 [1.6×10^8]
camphene	0.02 [2.7×10^3]	0.06 [8.2×10^3]	0.6 [8.2×10^4]	1.7 [2.3×10^5]	15.4 [2.1×10^6]	46 [6.3×10^6]
α-amylcinnamaldehyde	0.02 [4.0×10^3]	0.06 [1.2×10^4]	0.6 [1.0×10^5]	1.7 [3.2×10^5]	15.4 [2.9×10^6]	46 [8.5×10^6]
α-hexylcinnamaldehyde	0.006 [1.2×10^3]	0.02 [4.3×10^3]	0.2 [4.3×10^4]	0.5 [1.1×10^5]	4 [9.1×10^5]	12.7 [2.7×10^6]
diclofenac	0.45 [1.4×10^5]	1.37 [4.3×10^5]	12 [3.9×10^6]	37 [1.2×10^7]	333 [1.1×10^8]	1000 [3.18×10^8]
simvastatin	0.007 [2.9×10^3]	0.02 [8.3×10^3]	0.07 [2.5×10^4]	0.2 [8.3×10^4]	0.61 [2.5×10^5]	1.83 [7.6×10^5]
sertraline	0.45 [1.5×10^5]	1.37 [4.6×10^5]	12 [4.2×10^6]	37 [1.3×10^7]	333 [1.1×10^8]	1000 [3.4×10^8]
fluoxetine	0.45 [1.6×10^5]	1.37 [4.7×10^5]	12 [4.3×10^6]	37 [1.3×10^7]	333 [1.1×10^8]	1000 [3.4×10^8]

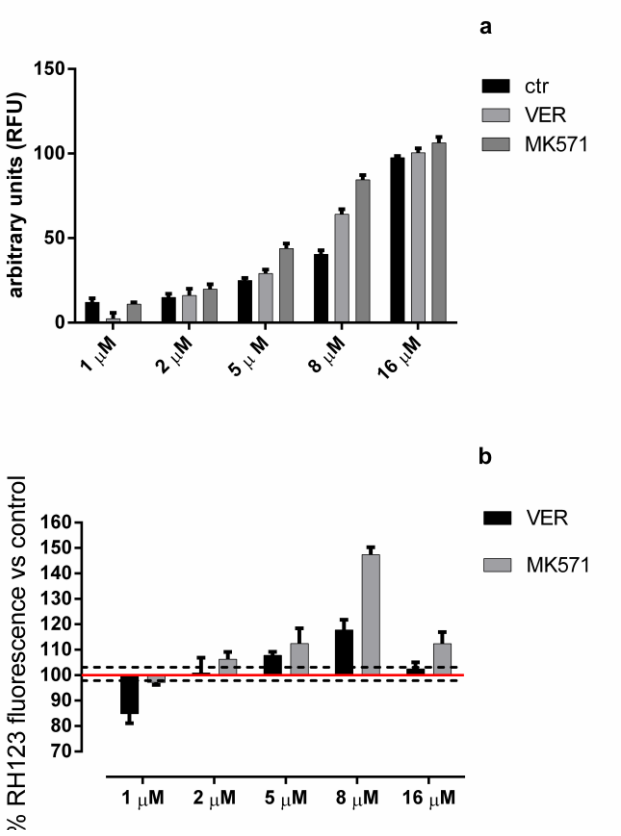

Figure 7.1: Accumulation of rhodamine 123 at different concentrations (1, 2, 5, 8 and 16 µM) in D. rerio embryos exposed to ABC transporters inhibitors (MK571 (10 µM), VER (10 µM) for 2 h. Data are presented in arbitrary fluorescence units (a) and in percentage (%) of RH123 fluorescence in relation to the water control (b) measured in each assay. Results are given as mean ± SE, n=4.

7.3.5 Statistical analysis

Differences between treatments/controls in the dye accumulation assay were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Dunnett's test) at a 5% significance level to identify significant changes in treatments versus controls. Data were square root transformed in order to fit ANOVA assumptions. All tests were performed using the software Statistica 7 (Statsoft, Inc). Data is presented as mean ± standard error.

7.4 Results

7.4.1 Abcb4 ATPase assays

Effects of test compounds on the ATPase activity of recombinant Abcb4 in membrane preparations as a measure of interaction of the compounds with zebrafish Abcb4 are shown in Fig 7.2. Abcb4 ATPase baseline activities, i.e., activities in solvent controls, were between 3 and 9 nmol Pi/mg protein/min. In the stimulated state achieved by the addition of 40 µM verapamil to the reaction mix, ATPase activities ranged between

8 and 20 nmol Pi/mg protein/min (Fig. 7.2). Fold changes of stimulated vs. basal ATPase activities were between 2 and 9-fold. Replicate experiments were performed for all test compounds and generally values for basal and stimulated ATPase activities in replicate experiments were consistent.

None of the tested compounds caused clear dose-dependent stimulation of the basal Abcb4 ATPase activity. However, in nerol and musk xylene treatments ATPase activities were slightly increased at the highest applied concentrations (206 and 1000 µM, respectively). ATPase activities were also elevated in the experiment with α-amylcinnamaldehyde and simvastatin at the lowest concentration tested (0.02 µM in each case) (Fig. 7.2I, L). Although not showing an increasing trend across the tested concentration range, Abcb4 ATPase activities appeared to be generally higher than baseline levels in experiments with isoeugenol and α-hexylcinnamaldehyde (Fig. 7.2G, J). At higher concentrations of diclofenac, sertraline and fluoxetine basal ATPase activities showed decreasing trends which was particularly pronounced in the sertraline treatment (Fig. 7.2K, M, N). All three compounds led to a decrease in the verapamil-stimulated Abcb4 ATPase activities at 333 and 1000 µM (Fig. 7.2K, M, N). In addition, at higher concentrations (> 37 µM) of isoeugenol, decreased activity was recorded in stimulated Abcb4 ATPase (Fig. 7.2G). For celestolide, musk ketone, propylparabene, citronellol and camphene no effects on baseline or stimulated Abcb4 ATPase activities were found (Fig. 7.2A, B, D, H). In summary, musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde, α-hexylcinnamaldehyde and simvastatin demonstrated substrate properties, while diclofenac, sertraline, fluoxetine and isoeugenol exhibited inhibitor properties.

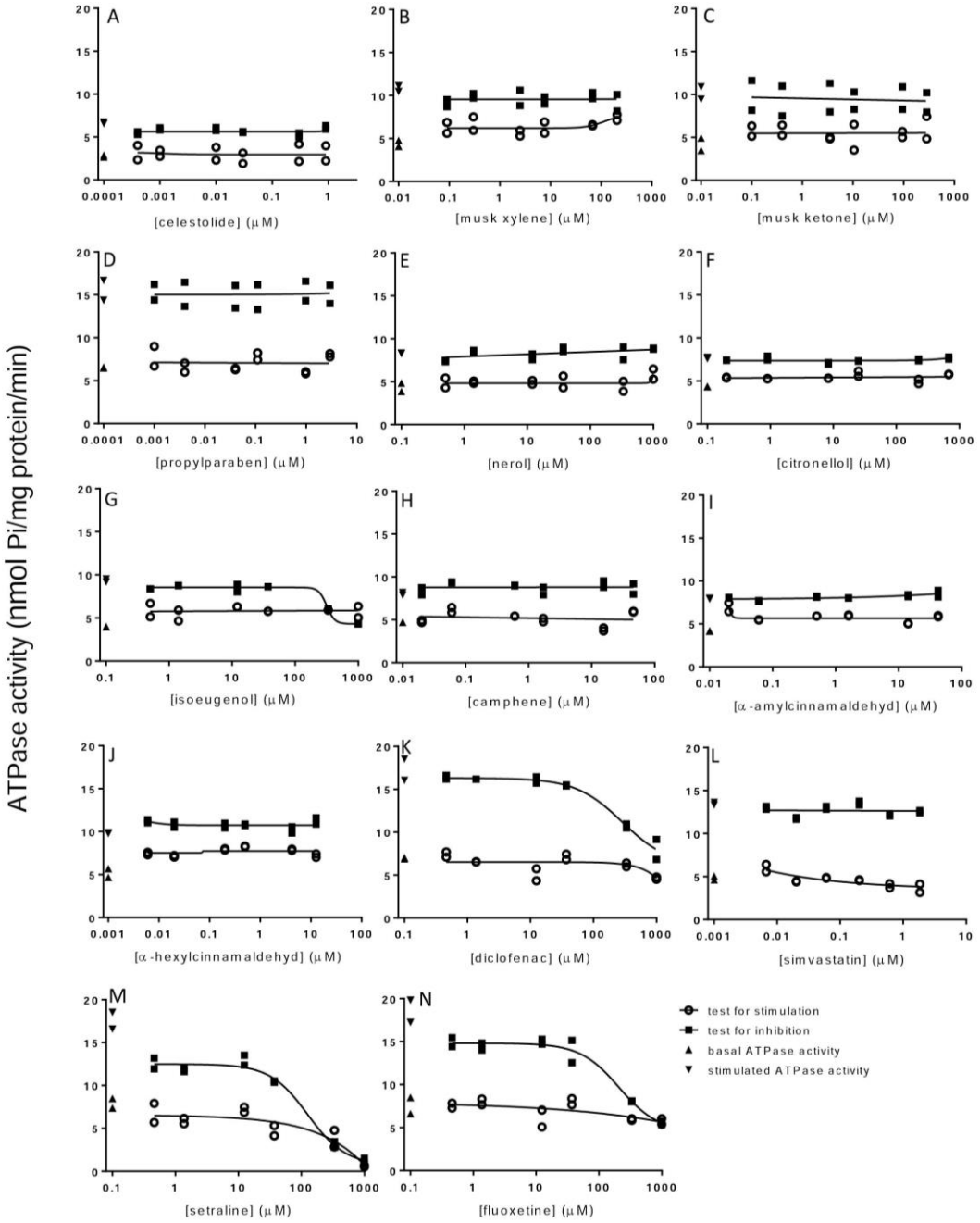

Figure 7.2: Effects of PCPs (celestolide, musk ketone, musk xylene, propylparaben, nerol, citronellol, isouegenol, camphen, α-amylcinnamaldehyde, α-hexylcinnamaldehyde (A to J)) and pharmaceuticals (sertraline, fluoxetine, diclofenac, simvastatin (K to N)) on ATPase activities of recombinant zebrafish Abcb4. Compounds were tested for stimulation (open circles) of the basal Abcb4 ATPase and inhibition of the verapamil-stimulated Abcb4 ATPase activities (closed squares). Basal activity (closed triangle) and stimulated activity (inverted closed triangle) were also measured. Data from single experiments are shown, n = 2.

7.4.2 Dye accumulation assays

Effects of standard ABC transporter inhibitors:

The three standard inhibitors at 10 µM caused an increase in RH123 accumulation in zebrafish embryos (Fig. 7.3). MK571 exerted the most pronounced effects leading to RH123 increases that ranged between 19 and 40 % in the different test series. RH123 accumulation was also enhanced by verapamil by 12 to 20 % and by vinblastine from 10 to 18 %. The differences in fluorescence between treatments and respective controls were statistically significant ($p < 0.05$) for MK571 in all test series except for test series C; for verapamil in test series L; and in none of the test series for vinblastine (Fig. 7.3).

Effects of test compounds:

In embryos exposed to PPCPs, dose-dependent increases in RH123 accumulation were observed for nerol and fluoxetine (Fig. 7.3E, N). The most pronounced increases ($p < 0.05$) were also observed for these compounds (nerol: +40 % at 1000 µM; fluoxetine: +25 – 30 % at ≥ 333 µM); the effect of 1000 µM nerol even exceeded that of MK571 which generally showed comparatively strong effects (Fig. 7.3E). A dose-dependent decrease in RH123 accumulation was also detected for celestolide (Fig. 7.3A). Musk xylene exposure enhanced RH123 accumulation from 20 to 42 % at the lower concentrations (0.09 to 2.5 µM) ($p < 0.05$) (Fig. 7.3C). Exposure to diclofenac, simvastatin and sertraline also increased RH123 accumulation for all concentration (Fig. 7.3K, L, M), being significant at the highest concentration of diclofenac (+26 %) and at lower sertraline concentrations (+24 to 29 %) ($p < 0.05$). Sertraline was tested up to 1000 µM, but as mortality was 100 % at 1000 µM sertraline, no RH123 fluorescence data are shown for this concentration. Embryos exposed to propylparaben exhibited RH123 accumulation below the control (100 %), with a decrease ranging from 6 to 15 % (Fig. 7.3D); similarly, the citronellol exposure showed the same effect in concentrations below 25 µM (from 8 to 11 % decrease) (Fig. 7.3F). Other compounds demonstrate non-monotonic RH123 accumulation changes throughout the different concentrations, exhibiting levels above and below the control. Musk ketone increased RH123 accumulation by 2 to 29 % at concentrations lower than 10.5 µM and at higher concentrations it decreased the accumulation (Fig. 7.3B). Embryos exposed to isoeugenol increased the RH123 accumulation at lower concentrations, including environmentally relevant concentrations ($p < 0.05$) and at the higher concentrations (≥ 333 µM) ranging from 3 to 41 %, but also diminished the accumulation at 12 and 37 µM (by 2 and 17 % decrease) (Fig. 7.3G). Camphene decreased Rh123 accumulation at a concentration of 0.06 µM ($p < 0.05$) by 14 %, but enhanced RH123 accumulation up to 10 % at 1.7 µM (Fig. 7.3H). In embryos exposed to α-

amylcinnamaldehyde RH123, accumulation was lower at 42 and 0.02 µM (by 9 and 15 % respectively) and increased 3 to 18 % at 0.06 to 14 µM (Fig. 7.3I). RH123 was enhanced by α-hexylcinnamaldehyde from 2 to 28 % (0.2 to 4 µM, significant for 0.2 µM ($p < 0.05$)) and decreased by 1 and 10 % (0.02 and 13 µM) (Fig. 7.3J)

7.5 Discussion

Chemicals present in everyday personal care products (PCPs), such as shampoos, lotions and pharmaceuticals, are continuously released into the aquatic environment. Given their potentially toxic effects to aquatic organisms further studies are needed to improve risk assessment. Hence, the aim of this work was to evaluate the chemosensitisation potential of some of these emerging compounds using two complementary experimental approaches, the ATPase assay with recombinant Abcb4 protein and the RH123 dye accumulation assay with whole zebrafish embryos. ATPase assay with recombinant Abcb4 enabled us to determine specific interactions of chemicals with this transporter. Nonetheless, the obtained effect concentrations are not necessarily informative about effective concentrations in a real world *in vivo* situation. Therefore, one cannot draw any conclusions on effective concentrations *in vivo* with this assay. In contrast to ATPase assay, the use of zebrafish embryos as an experimental model enabled us to determine chemical concentrations affecting the MXR system *in vivo*, thus providing information on the range of concentrations that are likely to be biologically active in fish considering the several transporter types that may contribute to MXR efflux transporter activity. Cells expressing zebrafish Abcb4 were able to efflux RH123 (Lu et al., 2015) which is a known substrate of mammalian ABCB1 and also of fish Abcb1 (Forster et al., 2012; Sturm et al., 2001; Ferreira et al., 2014), a functional homolog of zebrafish Abcb4. RH123 was shown to act as substrate not only of ABCB1 but also of ABCC transporters in mammalian systems (Minderman et al., 1996; Daoud et al., 2000). MK571 is a classical inhibitor of ABCC transporters (Gekeler et al., 1995) although interacting also with zebrafish Abcb4 (Fischer et al., 2013), and the inhibitors verapamil and vinblastine were also found to interfere with several transporter types in mammals (Sharom, 2008) and are therefore not specific to any type of ABC protein.

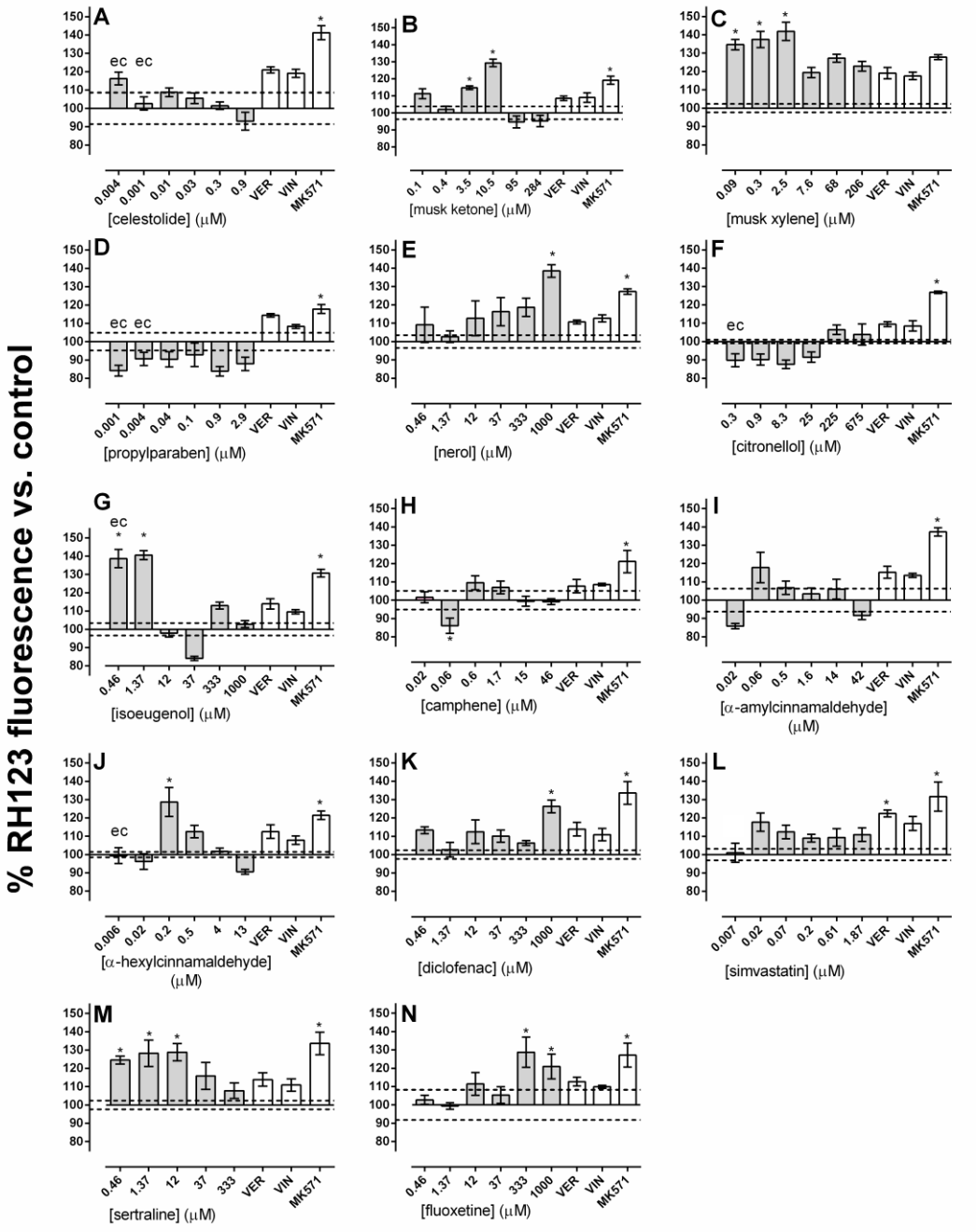

Figure 7.3: RH123 levels in zebrafish embryos from different treatments in % to control set to 100%. Accumulation of rhodamine 123 in D. rerio embryos upon exposure to ABC transporters inhibitors (MK571 (10 µM), VER (10 µM) and VIN (10 µM), PCPs (celestolide (A), musk ketone (B), musk xylene (C), propylparaben (D), nerol (E), citronellol (F), isouegenol (G), camphene (H), α-amylcinnamaldehyde (I), α-hexylcinnamaldehyde (J)) and pharmaceuticals (sertraline (K), fluoxetine (L), diclofenac (M) and simvastatin (N)) for 2 h in dye accumulation assays. Data are depicted as means ± SE (n=4). Significant differences between treatments and respective controls are marked by an asterisk ($p<0.05$). "ec" indicates concentrations that based on literature data are environmentally relevant (refer to Table 7.1).

Effects of test compounds on the Abcb4 ATPase activity

The ATPase assay with recombinant Abcb4 enabled us to study different modes of chemical interactions with the transporter protein. If a chemical increases basal ATPase activity or additionally decreases verapamil-stimulated ATPase activity, it is considered an ATPase activity stimulator and could be a transported substrate of Abcb4 (Fischer et al., 2013). A compound that decreases basal and or verapamil co-exposed ATPase activity acts as ATPase inhibitor (von Richter et al., 2009) and probably is inhibiting translocation (Fekete et al., 2015). Therefore, the inhibition of the stimulated Abcb4 ATPase activity is seen with compounds that act as competitive and non-competitive inhibitors. ATPase is a highly specific assay in terms of chemical interaction with the protein and do not mimic *in vivo* interactions. The chemical concentrations tested in the assay were used to test their effectiveness on the protein (Abcb4) and for that reason do not pretend to simulate environmental concentrations, although some of the lower concentrations are in the environmentally relevant range. ATPase assays were used previously to analyse and detect compounds present in environmental samples that can interact with Abcb1 of *Poeciliopsis lucida* (Zaja et al., 2013). Our results showed that the pharmaceuticals (diclofenac, simvastatin, sertraline and fluoxetine) and isoeugenol demonstrated chemosensitisation towards Abcb4 at higher concentrations. Musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde and α-hexylcinnamaldehyde and simvastatin stimulated Abcb4 ATPase activity, showing properties that can be described as Abcb4 substrates, even though not all chemicals that stimulate ATPase activity are transported substrates, for example very lipophilic chemicals (Stein and Litman 2014). Verapamil, for example, stimulates ATPase activity but it is not transported, notwithstanding doxorubicin is a known transporter substrate but it does not stimulate (Polli et al., 2001; Stein and Litman 2014). Hence, our results showed a direct interaction between some of the tested chemicals (musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, diclofenac, simvastatin, sertraline and fluoxetine) and the zebrafish Abcb4.

Effects of test compounds in the dye accumulation assay

The RH123 accumulation assay enables the identification of chemicals interacting with the MXR system. The substrate (dye compound, RH123) enters the cell and is effectively effluxed by ABC pumps. When efflux protein inhibitors are present, RH123 is retained inside the cells and the measured fluorescence increases. Our results show amplitude changes in fluorescence up to 40% that was less than other dye assays studies, which use rhodamine B, and observed changes ranging from 80 % to 200 % for

some compounds (Fischer et al., 2013). RH123 seem to be less efficiently efflux than rhodamine B, which can raise some questions about the evaluation of chemosensitisation with less efficient substrates. However, all of our assays were performed with positive controls (known ABC protein inhibitors) that consistently showed increase in fluorescence versus water controls, demonstrating the validity of the assay. Furthermore, the fluorescence levels of some compounds were consistently below the control, indicating a stimulation of MXR activity. This effect was observed in previous studies for rhodamine B and rhodamine 123 (Pessatti et al., 2002; Jeon et al., 2011; Liu et al., 2012; Kaewpiboon et al., 2014). Although the underlying mechanism still needs to be more thoroughly investigated, it was suggested that it could result from an up-regulation and increased activity of the MXR components (Pessatti et al., 2002; Jeon et al., 2011). It has previously been shown that certain PPCPs (like galaxolide and tonalide) can inhibit the MXR system, impairing its ability to efflux other toxic pollutants (Caminada et al., 2008; Fischer et al., 2013). Our results demonstrated that depending on the concentrations, polycyclic (celestolide) and nitromusk (musk ketone and musk xylene) fragrances and essential oils (nerol, citronellol, isoeugenol, camphene, α-amylcinnamaldehyde and α-hexylcinnamaldehyde) can also inhibit the MXR mechanism increasing the number of emerging contaminants acting as chemosensitisers Furthermore, our results also even showed that the essential oil isoeugenol can inhibit the MXR system at environmentally relevant concentrations. Inhibitory effects of synthetic musks (celestolide, musk xylene and musk ketone) on efflux proteins was already reported in mussels (Luckenbach and Epel, 2005; Faria et al., 2011) and in mammalian *in vitro* studies for citronellol (Yoshida et al., 2005, 2006, 2008). In contrast to musks and essential oils, propylparaben did not inhibit the MXR system and like other compounds (e.g. lead or phenylthiocarbamide) seems to decrease RH123 accumulation, which may indicate an increase of activity and expression of MXR transporters. (Pessatti et al., 2002; Jeon et al., 2011; Liu et al., 2012; Kaewpiboon et al., 2014). Pharmaceuticals (diclofenac, simvastatin, sertraline and fluoxetine) also inhibited the MXR mechanism. Accordingly, previous *in vitro* studies demonstrated the inhibitory effect of diclofenac on ABC proteins (Lagas et al., 2009; Wassmur et al., 2013) and in mammalian *in vitro* studies was described the inhibition of efflux proteins by sertraline and fluoxetine (Peer and Margalit, 2006; O'Brien et al., 2013; Drinberg et al., 2014). The chemosensitisation potential of simvastatin was also observed in cell lines (Wang et al., 2001; Caminada et al., 2008; Sieczkowski et al., 2010). In zebrafish embryos, RH123 accumulation assay showed to be sensitive to the PPCPs as in other biological systems.

Comparison of the results obtained with the dye accumulation and the ATPase assays

An overview of the dye accumulation and the ATPase assays is summarized in Table 7.3.

Isoeugenol, α-amylcinnamaldehyde, α-hexylcinnamaldehyde stimulated the basal ATPase activity and their fluorescence levels were found to be both lower and higher than controls. Hence, Abcb4 ATPase stimulation by these compounds may have resulted in the decrease efflux of RH123, demonstrating their property as Abcb4 substrate. Other PPCPs, musk xylene, nerol and simvastatin also stimulated the basal ATPase activity but only increased RH123 accumulation. As Abcb4 substrates the test compounds may act by competitive inhibition, disrupting RH123 efflux increasing its accumulation inside the cell. The pharmaceuticals diclofenac, sertraline and fluoxetine inhibited ATPase activity and increased RH123 accumulation, therefore these chemicals can be potential chemosensitisers; and the increased accumulation of the dye in fish embryos is due to Abcb4 inhibition. For other compounds such as celestolide, musk ketone, citronellol and camphene the inhibition effects were only found in the accumulation assay. These results suggest that these chemicals are inhibiting the MXR system by other mechanisms that can include inhibition of ABC proteins other than Abcb4, such as Abcc and Abcg2 orthologues. Given that ABCC transporters and ABCG2 can efflux non-metabolized and also biotransformed compounds (Leslie et al., 2005), the observed inhibition can perhaps be associated with metabolites as well as the parent compounds.

The PPCPs can be at the same time substrate for certain efflux proteins and inhibitors of others thus making the chemosensitising potential complex to evaluate. The dye assay is an easy and fast method for preliminary testing of compounds and their interaction with MXR. The ATPase assay does not allow any conclusions on relevant concentrations *in vivo,* however it offers a specific interaction that can distinguish between substrates and non-competitive inhibitors of environmentally relevant contaminants. Overall, our results show that depending of the concentration ubiquitous environmental pharmaceuticals and personal care products might be able to modulate ABC transporters activity. In conclusion, the described results represent an insight into substrate and inhibitors specificities of chemicals efflux transporter in fish.

7.6 Conclusions

In conclusion, accumulation assay and ATPase assay should be used together, since ATPase assay shows the interaction of a compound with a specific transporter and the dye assay demonstrate an *in vivo* interference with MXR system... Emerging

pollutants like diclofenac, sertraline and fluoxetine can be recognised as potential chemosensitisers and interact with the zebrafish Abcb4 protein. The chemosensitisation potential found for most of the tested chemicals can therefore enhance the bioavailability of other compounds that are normally effluxed by the ABC proteins. Hence monitoring of their presence in the aquatic systems is an important issue and should be considered in risk evaluation.

Table 7.3: Overview of the RH123 accumulation assay with zebrafish embryos and with the Abcb4 ATPase assay. The occurrence of effects is marked with +.

	Dye accumulation assay		ATPase assay		
Compound	**Above 100%**	**Below 100%**	**Stimulation of basal activity**	**Inhibition of basal activity**	**Inhibition of stimulated activity**
Celestolide	+	+			
musk ketone	+	+			
musk xylene	+		**+**		
Propylparabene		+			
Nerol	+		**+**		
Citronelol	+	+			
Isoeugenol	+	+	**+**		**+**
Camphene	+	+			
α-amylcinnamaldehyde	+	+	**+**		
α-hexylcinnamaldehyde	+	+	**+**		
Diclofenac	+				**+**
Simvastatin	+		**+**		
Sertraline	+			**+**	**+**
Fluoxetine	+				**+**

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CHAPTER VIII

FINAL REMARKS

8.1 Integrated discussion

Aquatic pollution is an increasing global problem endangering the aquatic ecosystems. New chemicals reach water systems daily, including everyday use compounds such as pharmaceuticals and PCPs. These xenobiotics enter in the aquatic environment from diverse sources and pathways. Despite treatment in WWTPs, these are not efficient in the removal of all classes of compounds, therefore significant concentrations of PPCPs are detected in effluents, groundwater and surface waters. Since there is no perfect and effective way to eliminate PPCPs from reaching the aquatic environment, information on risk assessment and underlying cellular and molecular parameters have to be understood to better evaluate and maintain a sustainable environment. An important and relevant parameter to be considered is the detoxification system of the organisms and how the different chemicals can disrupt these pathways. Changes in this system are a clear indication of the interaction of a contaminant with an organism and can be further used in risk evaluation. Emerging contaminants need biomarkers to be established as indicators of their presence and interaction with aquatic organisms. The detoxification system include the biotransformation of toxic molecules into more easily excreted metabolites by phase I and phase II enzymes and their efflux by ABC proteins. To understand the molecular role of these defences we performed this study with zebrafish embryos a recognised model for molecular and ecotoxicological studies. To accomplish our goals, in vivo assays involving the exposure of zebrafish embryos to two different pharmaceuticals (SIM and FLU) and in vitro assays with different classes of PPCPs were conducted. Subsequently, transcription and activity responses of ABC transporters, biotransformation and antioxidant enzymes and mRNA expression of NRs were evaluated. Additionally, to evaluate if SIM and FLU act in conserved pathways between fish and mammals, specific interactions with lipid and neurotransmitters systems were evaluated in zebrafish embryos.

To identify which NRs may be involved in the regulatory pathways of detoxification system and the effects of emerging contaminants on this regulation, zebrafish embryos were exposed to SIM and FLU. The transcription pattern of NRs as well as the transcription and activity of biotransformation and antioxidant enzymes and ABC transporters were evaluated after exposure to these emerging contaminants (Chapter III to V). In addition, disruption of embryo development was also evaluated in the presence of these pollutants. The results showed that zebrafish embryo development was affected by the exposure of SIM and FLU, and the last producing severe effects at environmental relevant concentrations. Embryos presented development delays and abnormalities that in

the case of SIM exposure resulted in mortality at the higher concentration. Both pharmaceuticals exerted an effect in the transcription as well as the activity of proteins involved in detoxification mechanism. SIM up regulated *abcb4, abcc1, gstπ* and *Cu/Zn sod* mRNA levels and increased EROD and GST activity while *cyp3a65* and *c*at were down regulated and CAT and SOD activity were decreased (Chapter III). A general pattern of down regulation of genes involved in detoxification mechanisms was observed in the embryos after exposure to FLU, with exception of cat (Chapter V). The disruption of the biotransformation and antioxidant enzymes and efflux proteins seem to have contribute to the observed embryo toxicity effects for both pharmaceuticals. In mammals, pharmaceuticals (such as clofibrate or oltipraz) have been found to modulate gene expression of *Pxr, Ppars* and *Ahr*, and consequently affect the transcription of genes belonging to the detoxification mechanism (Aleksunes and Klaassen, 2012). In embryos exposed to SIM, the transcription of pxr and ahr was down regulated alongside with *cyp3a65* and *cat* (Chapter IV). Moreover, FLU also down regulated the expression of NRs with exception of *pxr* and *rxrga* (Chapter V). Overall, these results seem to demonstrate that SIM and FLU modulate the transcription of NRs, mainly pxr and ahr which ultimately affect the transcription of genes involved in the detoxification mechanism. Furthermore, in mammalian models AhR and PXR are known to regulate CYPs (such as CYP1, CYP2, CYP3A) mRNA and proteins (Xu et al., 2005, Aleksunes and Klaassen, 2012). Although more studies have to be performed, the results of this study are in agreement with previous studies with fish species (which used pharmacologic concentrations unlike our studies that involved some environmentally relevant concentrations) where pxr and ahr expression levels were affected by pharmaceuticals and related with the changes in transcription of *cyp1a* and *cyp3a* (Wassmur et al., 2010; Mortensen and Arukwe, 2007).

To evaluate if SIM and FLU acted on the same pathways in fish as in mammals, specific interactions were targeted, such as the lipid regulation by SIM and transcriptional levels of genes involved in the neurotransmitter system in embryos exposed to FLU (Chapter IV and VI). In mammals SIM affects the lipid metabolism pathway reducing the Chol levels and regulate lipid proteins through the modulation of PPARs (Wang et al., 2011; Zou et al., 2013). In zebrafish embryos SIM changed the transcription of ppars however it did not affect the total Chol levels. FLU interacts with the serotonergic system by inhibiting the reuptake of serotonin by SERT (Bisesi et al., 2011) but also regulate the transcription of genes belonging to the dopaminergic and adrenergic systems (Gomez et al., 2015; Lesemann et al., 2012; Kreke and Dietrich, 2008). In zebrafish embryos, FLU affected the neurotransmitter system in a similar way by down regulating genes encoding for proteins belonging to the different neurotransmitter systems. Thus, given the

phylogenetic proximity between fish and mammals, SIM and FLU can impact conserved pathways to which they were design to act in mammals, and consequently be deleterious for healthy non-target organisms.

To assess chemosensitisation potential of different PPCPs classes, accumulation and ATPase activity assays were performed in in vivo zebrafish embryos and in in vitro membranes containing zebrafish recombinant Abcb4 (Chapter III, V, VII). ABC proteins can be inhibited by chemosensitisers increasing the accumulation of normally effluxed compounds possibly increasing toxicity (Epel et al. 2008). The chemosensitisation effect can be significant since there are a variety of relatively innocuous compounds present in the aquatic environment that can inhibit the MXR mechanism. Our results demonstrated, that depending on the concentration PPCPs such as musk ketone, musk xylene, nerol, isoeugenol, α-hexylcinnamaldehyde, diclofenac, sertraline and fluoxetine have chemosensitisation potential. Furthermore, isoeugenol inhibited the MXR system at environmentally relevant concentrations. Abcb4 ATPase activity assays were performed to assess which compounds inhibit Abcb4. Sertraline, fluoxetine, diclofenac, simvastatin and isoeugenol inhibited Abcb4 ATPase activity while musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde and α-hexylcinnamaldehyde and simvastatin demonstrated Abcb4 substrates properties. These results indicate the MXR chemosensitisation observed for musk xylene, nerol, α-hexylcinnamaldehyde is probably due to the inhibition of other ABC proteins rather than Abcb4. These results demonstrate the importance of including ABC transporters as biomarkers in monitoring and risk assessment in the aquatic environment.

8,2 Conclusions

After an overall analysis of this thesis results, several conclusion can be drawn:

a) ABC transporters, biotransformation, antioxidant enzymes and NRs are present since very early development stages and important in the defence and the survival of fish;
b) In the presence of efflux transporters inhibitors, SIM may be more toxic to zebrafish embryos therefore impacting their development;
c) FLU impairs zebrafish embryo development at environmentally relevant concentrations;
d) SIM and FLU can alter the transcription and activity of efflux proteins, biotransformation and antioxidant enzymes;
e) Both pharmaceuticals modulate NRs transcription, mainly *pxr* and *ahr* that may regulate transcription of genes involved in the detoxification mechanism, mainly phase I *cyp1a* and *cyp3a65*, similar to mammalian models;

f) SIM affects *ppars* transcription however it does not impact the total Chol levels in early embryo development;

g) FLU decreases the transcription of important neurotransmitters transporters and receptors, at concentrations found in the aquatic environment;

h) Accumulation and ATPase assays should be used together to better assess the chemosentisation potential and better identify which ABC proteins are being inhibited;

i) Certain PPCPs can disrupt zebrafish MXR activity;

j) Isoeugenol inhibited MXR activity at environmentally relevant concentrations;

k) Sertraline, fluoxetine, diclofenac, simvastatin and isoeugenol inhibited Abcb4 ATPase activity;

l) Musk xylene, nerol, isoeugenol, α-amylcinnamaldehyde and α-hexylcinnamaldehyde and simvastatin demonstrated Abcb4 substrates properties.

8.3 Future perspectives

This work focused on the chemosensitisation potential of emergent compounds in ABC proteins and the response patterns of MXR system, biotransformation enzymes and nuclear receptors.

After exposure to the pharmaceuticals embryo development impairment was observed in this study which could be related to the alter transcription of the target genes. In future studies this can be confirmed by testing embryo development using knockdowns of genes belonging to the detoxification mechanism.

To better understand the possible regulation pathways of detoxification mechanism proteins, morpholinos or an alternative knockdown approach should target several nuclear receptors genes studied here to demonstrate their role in detoxification and the impact of the studied PPCPs.

Future studies are required in order to confirm and understand which ABC proteins are in fact being inhibited by the selected PPCPs. This can be approached through the production of recombinant zebrafish Abcc1, Abcc2 and Abcg2 proteins and ATPase activity, because until now only recombinant zebrafish Abcb4 was produced and evaluated.

In this study, MXR activity was performed in embryos with chorion, however even with short term exposures transcription and activity of detoxification proteins was altered by different compounds. Therefore, assays involving microinjection of compounds inside the chorion cavity most be performed in order to evaluate the true protection role of this membrane.

Since the removal of PPCPs in WWTPs is not efficient for all PPCPs, future studies should evaluate the effluents chemical composition and test their toxicity in fish and other aquatic species development and chemosensitisation potential.

8.4 References

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APPENDIX

PUBLICATIONS DERIVATED FROM THIS THESIS