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

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ABSTRACT

In the past decade the presence of psychopharmaceuticals, including fluoxetine (FLU), in the aquatic environment has been associated with the increasing trend in human consumption of these substances. Aquatic organisms are usually exposed to chronic low doses and, therefore, risk assessments should evaluate the effects of these compounds in non-target organisms. Teleost fish possess an array of active defence mechanisms to cope with the deleterious effects of xenobiotics. These 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 the activity of ABC proteins and antioxidant enzymes (CAT and Cu/ZnSOD) after exposure to FLU. Embryo development was disrupted at the lowest FLU concentration tested (0.0015 μM), which is in the range of concentrations found in WWTP effluents. Embryos exposed to higher concentrations of FLU decreased Cu/Zn SOD, and increased CAT (0.0015 and 0.5 μM) enzymatic activity. Exposure to higher concentrations of FLU decreased the expression of most genes belonging to the detoxification system and upregulated *cat* at 0.0015 μM of FLU. Most of the tested concentrations downregulated *pparα, pparβ, pparγ*, and *raraa, rxraa, rxrab, rxrbb rxrgb* and *ahr* gene expression while *pxr* was significantly up regulated at all tested concentrations. In conclusion, this study shows that FLU can impact zebrafish embryo development, at concentrations found in effluents of WWTPs, 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.

1. Introduction

Psychopharmaceuticals are one of the classes of therapeutic drugs most commonly detected in the aquatic environment (Santos et al., 2010). Fluoxetine (FLU), commercial name Prozac®, is one of the most prescribed psychopharmaceuticals (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 bodies, in concentrations ranging from 12 to 929 ng/L (Fent et al., 2006; Lajeunesse et al., 2012; Barry, 2013; Silva et al., 2012). Previous studies have reported negative impacts of psychopharmaceuticals (including FLU), such as impaired reproduction, mortality

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and behavioural changes like decreased aggressiveness and feeding responses, on invertebrates and fish species (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 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). 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.

Some psychopharmaceuticals, including FLU, have been described to be P-gp (P-glycoprotein, ABCB1) inhibitors in mammals (Peer and Margalit, 2006; O'Brien et al., 2013; Schrickx and Fink-Gremmels, 2014). Moreover, it was reported that FLU can inhibit P-gp and biotransformation enzymes in both mammals and fish (Peer and Margalite, 2006; Smith et al., 2012; Thibaut and Porte, 2008). Hence, given the central role of detoxification in influencing 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), glucocorticoid receptors (GR), and aryl hydrocarbon receptors (AhR) (Xu et al., 2005; Ferreira et al., 2014). Among other pathways, NRs are involved in xenobiotic 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*) evaluating the effects of FLU in embryo development; (*ii*) characterizing 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*) evaluating the transcriptional modulation of genes encoding key transcription factors (*ppars, pxr, rxrs, rars* and *ahr*) after FLU exposure; and (*iv*) assessing the potential chemosensitiser effect of FLU using *D. rerio* embryos as a model species.

2. Material and methods

2.1. 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.

2.2. 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 °C 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.).

2.3. 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. On the day after egg-laying, the breeders were removed after the beginning of the light period. Experiments were performed according to the protocols described in Cunha et al. (2016). Briefly, for the toxicological assays (Section 2.4), embryos (1 hpf) were transferred to a 24-well plate (10 embryos per well) and exposed until 80 hpf 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 2.8×10^5 ng/L). One of the tested concentrations is in the range of environmentally relevant concentrations, (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. The medium (water plus FLU solutions) was renewed daily for the duration of the experiment. For the determination of the biochemical parameters (Section 2.5), 100 embryos (1 hpf) were placed in glass beakers at 26 ± 1 °C 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 at 26 ± 1 °C with aeration for 24 h, until the execution of the assay. Experimental controls used in all the assays were water controls and solvent controls (DMSO 0.004%).

2.4. Toxicological assay – FLU effects on zebrafish embryo development

During the exposure period (as described in Section 2.3.) embryos were observed with an inverted microscope (Nikon Eclipse TS100) and the selected endpoints (mortality, 75% epiboly, delay/arrest of the division, abnormal cell masses, development delay, pericardial edema, head, eye and tail abnormalities) were recorded at 8 hpf, 32 hpf and 80 hpf (Soares et al., 2009; Cunha et al., 2016). The percentage mortality and the total percentage of abnormalities were calculated after the data collection. After 80 h of exposure, 30 embryos were collected and preserved in RNAlater for gene expression analysis. Each assay was replicated at least six times. Data on mortality and abnormalities are presented as percentage (%) in relation to the water control measured in each assay.

2.5. Antioxidant enzyme 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 disodium ethylenediaminetetra acetic acid (Na_2EDTA). 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 of the reduction of cytochrome *c* by superoxide anions generated by the xanthine oxidase/xanthine system. Cytochrome *c* reduction was

followed through the measurement of the absorbance at 550 nm. In this assay, the concentrations of the reaction components were 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. 250 μL of these components were reacted with the sample (protein concentration 4 mg/mL) diluted in phosphate buffer to a final volume of 300 μL. To obtain linearity, the volumes 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 2 mM KCN to the reaction. To obtain the Cu/ZnSOD activity we then deducted the MnSOD activity from the total SOD activity. Catalase (CAT) activity was determined by measuring the consumption of H_2O_2 at 240 nm ($\varepsilon = 40\ M^{-1}\ cm^{-1}$) as described in Ferreira et al. 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.

2.6. RH123 accumulation assay on zebrafish embryos exposed to FLU

The accumulation of the fluorescent substrate (RH123) in zebrafish embryo tissue was used as a 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 standard ABC protein inhibitors (VER (10 μM), MK571 (10 μM), and Vinblastine (10 μM)). Each type of exposure was made separately and no mixture (FLU plus inhibitor) was performed. 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 °C 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 (water control, solvent control, positive controls and FLU at the different concentrations) using a fluorescent microplate reader (excitation/emission −485/538 nm) (Fluoroskan Ascent, Labsystems). Each assay was replicated at least four times. The data is presented in percentage (%) of RH123 fluorescence in relation to the water control measured in each assay.

2.7. RNA isolation and cDNA synthesis

Embryos from the toxicological and accumulation assays, preserved in RNAlater, were used to isolate total RNA according to Costa et al. 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 fluorescent microplate reader (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). Extractions of six different pools of embryos per treatment were performed.

2.8. 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 1 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 0.001 μM to 0.6 μM) 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. The relative expression ratio was calculated considering the efficiency of each gene using the Pfaffl mathematical model. To calculate $\Delta\Delta$ct, the control group comprised the average of Cts of the controls from each group. Data are presented as mean of mRNA level in relation to the reference genes.

2.9. Statistical analysis

Differences in mRNA expression and the differences between treatments in accumulation assays were evaluated by means of a one-way ANOVA, followed by a multiple comparison test (Dunnett's test) at a 5% significance level. Data were log (mRNA expression and enzyme activities) or square root (accumulation assays) transformed in order to fit ANOVA assumptions. All tests were performed with Statistica 7 (Statsoft, Inc). Differences in the frequency of each type of abnormality among treatments were analysed in the toxicological assays and were evaluated by means of cross table $\chi 2$ tests at 5% significance level. The above test was done at each of the time points. No cells had expected frequencies ≤ 5 fulfilling the second test assumptions. Tests were performed with SPSS 22 (IBM, Inc). Data are presented as mean ± standard error.

3. Results

3.1. Toxicological assay

Cumulative mortality and cumulative abnormalities after exposure to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) at different development stages (8, 32 and 80 hpf) of embryos are presented in Fig. 1A. The percentage of abnormalities represents the percentage of embryos with 1 or more abnormalities in comparison to the control. No significant differences in mortality were observed in embryos exposed to FLU in comparison to the control groups (water and solvent control), 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

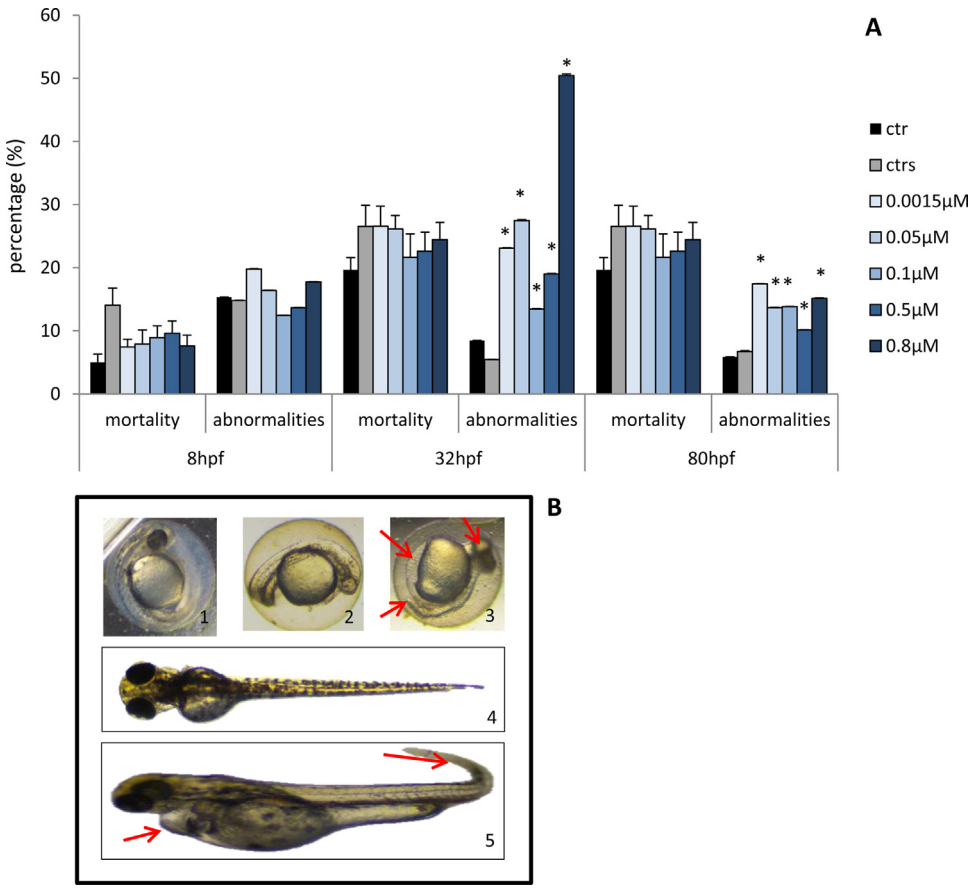

Fig. 1. Cumulative mortality and abnormality percentages at 8, 32 and 80 hpf of *D. rerio* embryos exposed to different concentrations of fluoxetine (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80 h. Results are expressed as mean ± SE, n = 6. Bars with asterisk are significantly different from the solvent control group ($p < 0.05$).

as compared to the controls. However, at 32 and 80 hpf a significant increase in cumulative abnormalities was detected in treated embryos ($p < 0.05$). In Fig. 1B some of the abnormalities found after exposure to FLU at 32 and 80 hpf are represented. 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 (see Fig. 1S and 2S, Supplementary material for more detailed information). 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 pericardial edema and tail abnormalities (see Fig. 1S, Supplementary material for more detailed information). At the end of the assay (80 hpf), and similar to the results observed at 32 hpf, pigmentation, pericardial edema and tail abnormalities were the most prevalent anomalies in embryos exposed to FLU (see Fig. 2S, Supplementary material for more detailed information).

3.2. Antioxidant enzyme activity

To address the question of correlation of the morphological effects of FLU with toxicity parameters, we assayed two antioxidant defences, SOD and CAT, in zebrafish embryos after 80 h of exposure to FLU (Fig. 2). Cu/ZnSOD activity (Fig. 2A) was significantly decreased in the presence of FLU, at 0.1 μM and above ($p < 0.05$), while the CAT activity (Fig. 2B) was stimulated both at the lowest tested concentration (0.0015 μM) and at 0.5 μM ($p < 0.05$).

3.3. 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. 3). A significant increase in RH123 accumulation inside the embryos was observed in the presence of ABC transporter inhibitors (MK571, VER and Vinblastine (10 μM)) ($p < 0.05$) whereas no differences were observed in RH123 accumulation in embryos exposed to FLU.

3.4. 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 *NR* genes (*pxr, pparα, pparβ, pparγ, rxraa, rxrab, rxrbb, rxrga, rxrgb, raraa, rarab, rarga*), and *ahr* in embryos exposed to FLU for 80 h were assayed (Figs. 4 and 5). A pattern of downregulation 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 upregulated 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 downregulation pattern. Interestingly, *abcc2* mRNA was significantly downregulated at all tested concentrations (Fig. 4). The transcription of genes encoding most nuclear receptors (*pparα, pparβ, raraa, rxraa, rxrab, rxrbb rxrgb*) was also downregulated in embryos exposed to all FLU concentrations while *pxr* transcription

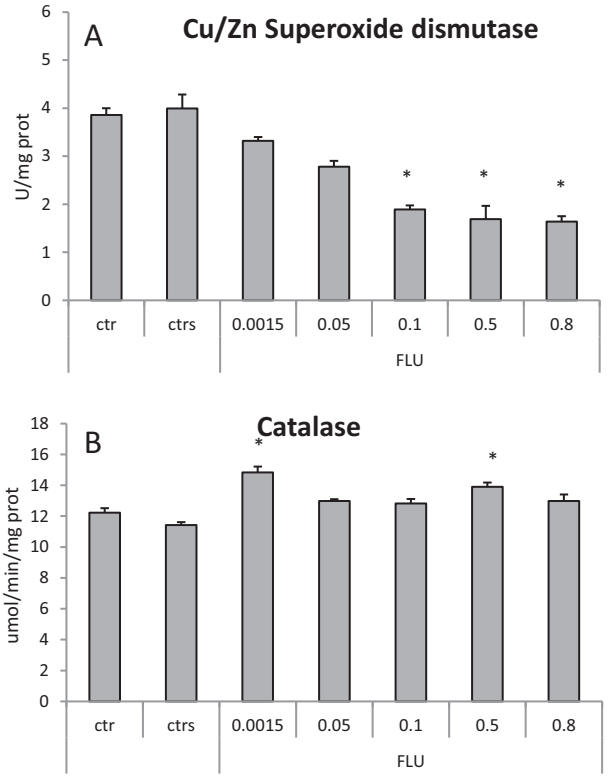

Fig. 2. SOD (A) and CAT (B) activity in (100) *D. rerio* embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80 h. Results are given as mean ± SE, n = 3. Bars with asterisk are significantly different from the control group ($p < 0.05$).

was upregulated ($p < 0.05$) and exhibited the highest transcription levels of all genes encoding NRs evaluated after FLU exposure (Fig. 5). *ahr* and *pparγ* displayed a downregulation 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 of FLU ($p < 0.05$), whereas *rxrga* demonstrated an upregulation pattern at 0.0015, 0.5 and 0.8 μM of FLU.

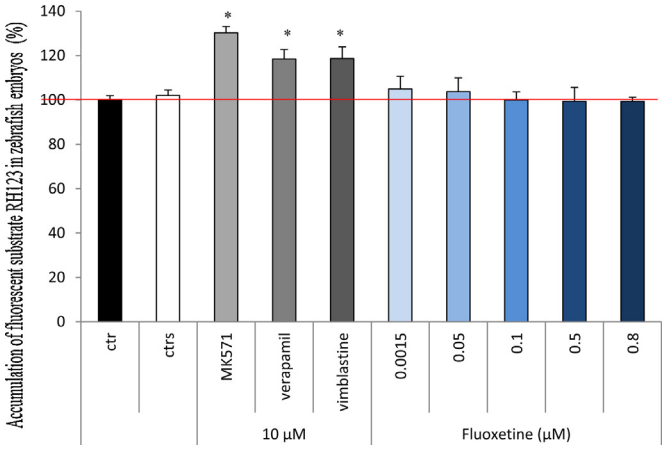

Fig. 3. Accumulation of rhodamine 123 in *D. rerio* embryos exposed to ABC transporter inhibitors (MK571 (10 μM), VER (10 μM) and Vinblastine (10 μM)), FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 2 h (%). Results are given as mean ± SE, n = 4. Bars with asterisk are significantly different from the control group ($p < 0.05$).

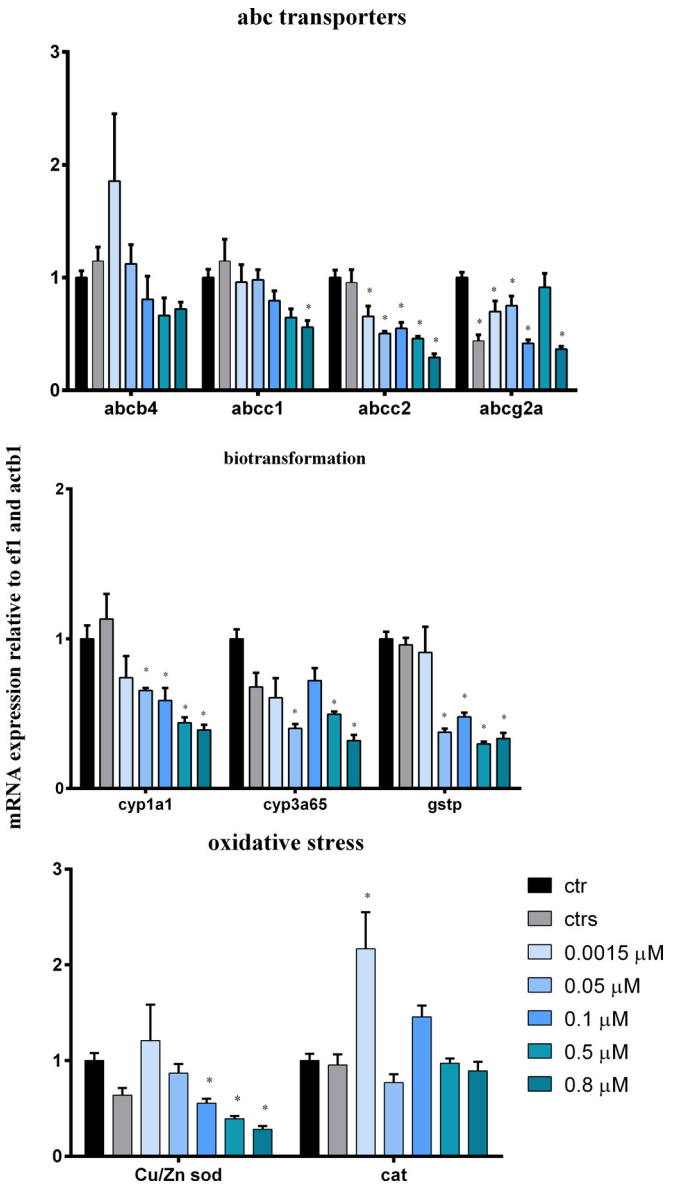

Fig. 4. Relative mRNA expression of *abcb4, abcc1, abcc2, abcg2a, cyp1a1, cyp3a65, gstπ, Cu/Zn sod* and *cat* in (30) *D. rerio* embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80 h. Bars with asterisk are significantly different from the control group ($p < 0.05$). Results are given as mean ± SE, n = 6.

4. Discussion

4.1. Effects of FLU on zebrafish embryo development

Previous studies on the effects of FLU in zebrafish focused 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, previous studies showed that FLU also affected the development of fish, such as *Oryzias latipes*. Hence, in this study we evaluated the effects of FLU in zebrafish embryo development. Clearly, FLU leads to developmental abnormalities, even within the range of concentrations found in aquatic systems, such as in WWTP effluents (Silva et al., 2012). A previous study also demonstrated that FLU, at 99 μg/L, affected the initial development of zebrafish (Kalichak et al., 2016). A high percentage of pigmentation anomalies were observed in exposed embryos. One hypothesis is that FLU might interfere with pigmentation by modulating embryo

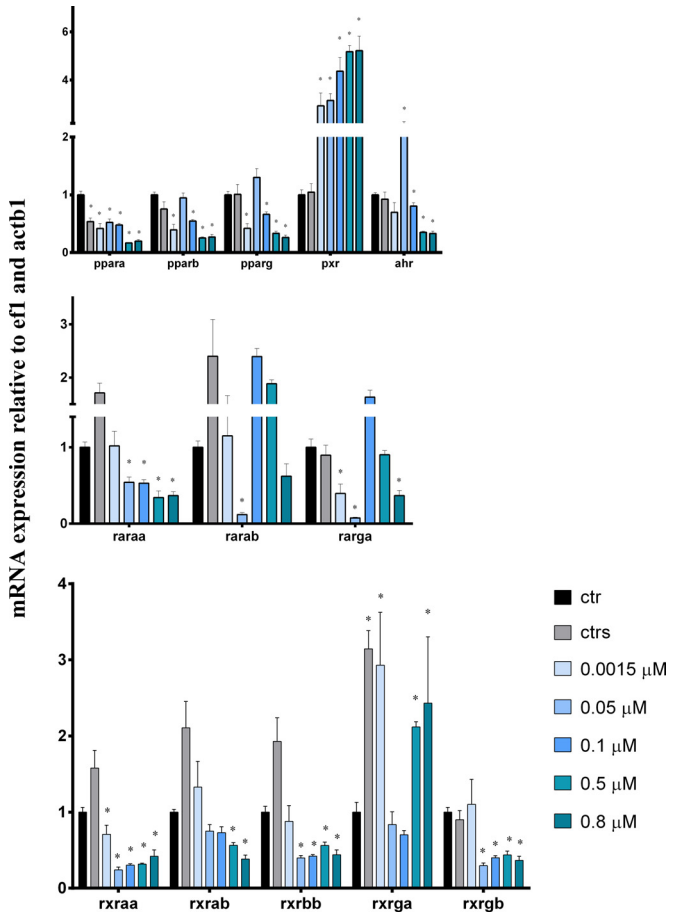

Fig. 5. Relative mRNA expression of *pparα, pparβ, pparγ, pxr, ahr, raraa, rarab, rarga, rxraa, rxrab, rxrbb, rxrga and rxrgb* in (30) *D. rerio* embryos exposed to FLU (0.0015, 0.05, 0.1, 0.5 and 0.8 μM) for 80 h. Bars with different letters indicate significant differences among treatments ($p < 0.05$). Results are given as mean ± SE, n = 6.

adrenoceptors present in the melanophores. This has been 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 demonstrated that zebrafish embryos exposed for 80 h at the same FLU concentrations tested here, showed changes in the transcription of adrenoceptors (unpublished data). Overall, it was shown that FLU can impair embryo development even at low concentrations.

4.2. Effects of FLU n detoxification mechanisms

Disturbed regulation of detoxification genes can compromise the animal's ability to withstand toxic stressors. Therefore, it is important to understand the interaction of FLU with the components of cellular detoxification mechanisms. A general pattern of downregulation of genes, involved in detoxification mechanisms, was observed in embryos after exposure to FLU. Detoxification mechanisms include the function of ABC transporters and biotransformation enzymes of phase I and II. A similar pattern of downregulation of ABC transporter expression was also reported in aquatic invertebrates (Franzellitti 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 downregulation of *cyp1a1* and *cyp3a65* in zebrafish embryo.

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 transcriptional regulation (Barker et al., 1994; Risso-de Faverney et al., 2000). In fact, an inhibition of Cu/Zn SOD and induction of CAT were observed at transcriptional and activity levels after exposure to FLU. A similar response to FLU was also reported in invertebrates (Gonzalez-Rey and Bebianno, 2013; Franzellitti et al., 2014; Chen et al., 2015). This coincided with 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 Bebianno, 2013). Altogether, these results suggest that FLU can disrupt the detoxification mechanism in fish during different developmental phases.

4.3. 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 and regulate *CYP* mRNA and protein expression (Xu et al., 2005; Aleksunes and Klaassen, 2012). CAR is absent in most teleost fish, including zebrafish, nonetheless other NRs, such as PXR and AhR were shown to mediate *cyp3a65* transcription.

The present results showed that FLU leads to a downregulation pattern of *ahr* in zebrafish embryos that could explain the observed downregulation 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 transcriptional regulation of *cyp1a* and *cyp3a* (Mortensen and Arukwe, 2007; Wassmur et al., 2010). Notwithstanding, more studies have to be performed to verify this hypothesis.

The modulation of these NRs can also affect downstream pathways as previously demonstrated in mammalian models (Janani and Ranjitha Kumari, 2015; Ipseiz et al., 2014; Xu et al., 2005). Therefore, the present results may suggest that FLU can impact the regulation pathways of other metabolic functions, important to development, through the modulation of NRs. Studies in vertebrates have demonstrated that nuclear receptors, especially retinoic acid (RA) receptors (RAR and RXR), play an important role in embryonic development, since both an excess and a 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. This has been shown in medaka treated with an AHR antagonist and a cytochrome P450 inhibitor (Kawamura and Yamashita, 2002).

4.4. Potential chemosensitiser effect of FLU

The presence of chemosensitising xenobiotics in aquatic environments can influence the toxicity of other chemicals that are normally effluxed. As previously mentioned, FLU is found in water systems in concentrations up to 929 ng/L (0.0027 μM). Hence, in this study we evaluated if FLU can be considered an inhibitor of MXR. 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 univocal, 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 have found no effects (Kapoor et al., 2013; O'Brien et al., 2013), similar to the results reported 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 downregulation of *abc* transporter gene mRNA was observed, namely the decrease of *abcc2* mRNA levels, at environmentally relevant concentration, showing that even in the absence of transporter activity inhibition, FLU influences the transcription of genes belonging to the MXR system. Hence, the possibility that longer exposure could impact the activity of ABC transporters cannot be excluded.

5. Conclusions

In conclusion, the results presented here show a clear effect of FLU on zebrafish embryo development in the range of levels found in effluents of WWTPs (0.0015 μM = 519 ng/L). These effects were observed concomitantly with changes in the transcription of genes encoding NRs and genes involved in detoxification mechanisms. Evidence suggests that FLU can disrupt the MXR system through downregulation of ABC transporter transcription. These effects, at both developmental (embryo) and transcriptional levels, have been observed in parallel with changes on the enzymatic activity of Cu/Zn SOD and CAT. Ultimately, the results indicate that FLU can disrupt detoxification mechanisms, which can impair both embryo development and the overall cell defence system. This can potentially affect ecologically relevant endpoints, which highlights the relevance of monitoring the presence of FLU in the aquatic environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.08.003.

References

Airhart, M.J., Lee, D.H., Wilson, T.D., Miller, B.E., Miller, M.N., Skalko, R.G., 2007. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC). Neurotoxicol. Teratol. 29 (6), 652–664.

André, A., Ruivo, R., Gesto, M., Castro, L.F., Santos, M.M., 2014. Retinoid metabolism in invertebrates: when evolution meets endocrine disruption. Gen. Comp. Endocrinol. 208, 134–145.

Bard, S.M., 2000. Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. Aquat. Toxicol. 48, 357–389.

Barker, C.W., Fagan, J.B., Pasco, D.S., 1994. Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress. J. Biol. Chem. 269, 3985–3990.

Barry, M.J., 2013. Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish. Ecotoxicology 22 (2), 425–432.

Castro, L.F., Santos, M.M., 2014. To bind or not to bind: the taxonomic scope of nuclear receptor mediated endocrine disruption in invertebrate phyla. Environ. Sci. Technol. 48 (10), 5361–5363.

Chen, H., Zha, J., Yuan, L., Wang, Z., 2015. Effects of fluoxetine on behavior, antioxidant enzyme systems, and multixenobiotic resistance in the Asian clam *Corbicula fluminea*. Chemosphere 119, 856–862.

Clagett-Dame, M., Knutson, D., 2011. Vitamin A in reproduction and development. Nutrients 3 (4), 385–428.

Connors, K.A., Valenti, T.W., Lawless, K., Sackerman, J., Onaivi, E.S., Brooks, B.W., Gould, G.G., 2014. Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabinoid CB receptors on zebrafish behavior in novel environments. Aquat. Toxicol. 151, 105–113.

Cunha, V., Santos, M.M., Moradas-Ferreira, P., Ferreira, M., 2016. Simvastatin effects on detoxification mechanisms in *Danio rerio* embryos. Environ. Sci. Pollut. Res. Int. 23 (11), 10615–10629.

Dulawa, S.C., Holick, K.A., Gundersen, B., Hen, R., 2004. Effects of chronic fluoxetine in animal models of anxiety and depression. Neuropsychopharmacology 29 (7), 1321–1330.

Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76 (2), 122–159.

Ferreira, M., Costa, J., Reis-Henriques, M.A., 2014. ABC transporters in fish species: a review. Front. Physiol. 5, 266.

Fischer, S., Klüver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A.M., Wellner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (*Danio rerio*) embryos. BMC Biol. 17, 69.

Franzellitti, S., Buratti, S., Capolupo, M., Du, B., Haddad, S.P., Chambliss, C.K., Brooks, B.W., Fabbri, E., 2014. An exploratory investigation of various modes of action and potential adverse outcomes of fluoxetine in marine mussels. Aquat. Toxicol. 151, 14–26.

Fujii, R., 2000. The regulation of motile activity in fish chromatophores. Pigment Cell Res. 13 (5), 300–319.

Gonzalez-Rey, M., Bebianno, M.J., 2013. Does selective serotonin reuptake inhibitor (SSRI) fluoxetine affects mussel *Mytilus galloprovincialis*? Environ. Pollut. 173, 200–209.

Ipseiz, N., Scholtysek, C., Culemann, S., Krönke, G., 2014. Adopted orphans as regulators of inflammation, immunity and skeletal homeostasis, Swiss Med Wkly, 144:w14055, Review.

Janani, C., Ranjitha Kumari, B.D., 2015. PPAR gamma gene—a review. Diabetes Metab. Syndr. 9 (1), 46–50.

Kalichak, F., Idalencio, R., Rosa, J.G., de Oliveira, T.A., Koakoski, G., Gusso, D., de Abreu, M.S., Giacomini, A.C., Barcellos, H.H., Fagundes, M., Piato, A.L., Barcellos, L.J., 2016. Waterborne psychoactive drugs impair the initial development of Zebrafish. Environ. Toxicol. Pharmacol. 41, 89–94.

Kapoor, A., Iqbal, M., Petropoulos, S., Ho, H.L., Gibb, W., Matthews, S.G., 2013. Effects of sertraline and fluoxetine on p-glycoprotein at barrier sites: in vivo and in vitro approaches. PLoS One 8 (2), e56525.

Kawamura, T., Yamashita, I., 2002. Aryl hydrocarbon receptor is required for prevention of blood clotting and for the development of vasculature and bone in the embryos of medaka fish, *Oryzias latipes*. Zool Sci 19, 309–319.

Klymkowsky, M.W., Rossi, C.C., Artinger, K.B., 2010. Mechanisms driving neural crest induction and migration in the zebrafish and *Xenopus laevis*. Cell Adh. Migr. 4, 595–608.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., et al., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. Environ. Sci. Technol. 36 (6), 1202–1211.

Kurelec, B., 1992. The multixenobiotic resistance mechanism in aquatic organisms. Crit. Rev. Toxicol. 22 (1), 23–43.

Lajeunesse, A., Smyth, S.A., Barclay, K., Sauvé, S., Gagnon, C., 2012. Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. Water Res. 46 (17), 5600–5612.

Laville, N., Aït-Aïssa, S., Gomez, E., Casellas, C., Porcher, J.M., 2004. Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes. Toxicology 196 (1-2), 41–55.

Lister, A., Regan, C., Van Zwol, J., Van Der Kraak, G., 2009. Inhibition of egg production in zebrafish by fluoxetine and municipal effluents: a mechanistic evaluation. Aquat. Toxicol. 95 (4), 320–329.

Lopez-Torres, M., Perez-Campo, R., Cadenas, S., Rojas, C., Barja, G., 1993. A comparative study of free radicals in verebrate: II. Non-enzymatic antioxidants stress. Comp. Biochem. Physiol.: B 105, 757–763.

Mennigen, J.A., Stroud, P., Zamora, J.M., Moon, T.W., Trudeau, V.L., 2011. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on Prozac. J. Toxicol. Environ. Health B Crit. Rev. 14 (5–7), 387–412.

Mortensen, A.S., Arukwe, A., 2007. Effects of 17alpha-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 85 (2), 12–113.

O'Brien, F.E., Clarke, G., Dinan, T.G., Cryan, J.F., Griffin, B.T., 2013. Human P-glycoprotein differentially affects antidepressant drug transport: relevance to blood-brain barrier permeability. Int. J. Neuropsychopharmacol. 16 (10), 2259–2272.

Pan, Y., Chatterjee, D., Gerlai, R., 2012. Strain dependent gene expression and neurochemical levels in the brain of zebrafish: focus on a few alcohol related targets. Physiol. Behav. 107 (5), 773–780.

Peer, D., Margalit, R., 2006. Fluoxetine and reversal of multidrug resistance. Cancer Lett. 237 (2), 180–187.

Peer, D., Dekel, Y., Melikhov, D., Margalit, R., 2004. Fluoxetine inhibits multidrug resistance extrusion pumps and enhances responses to chemotherapy in syngeneic and in human xenograft mouse tumor models. Cancer Res. 64 (20), 7562–7569.

Risso-de Faverney, C., Lafaurie, M., Girard, J.P., Rahmani, R., 2000. The nitroxide stable radical tempo prevents metal-induced inhibition of CYP1A1 expression and induction. Toxicol. Lett. 111, 219–227.

Robinson-Rechavi, M., Escriva Garcia, H., Laudet, V., 2003. The nuclear receptor superfamily. J. Cell Sci. 116, 585–586.

Samarut, E., Fraher, D., Laudet, V., Gibert, Y., 2015. ZebRA: an overview of retinoic acid signaling during zebrafish development. Biochim. Biophys. Acta 1849 (2), 73–83.

Santos, L.H.M.L.M., Araújo, N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M.C.B.S.M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175 (1–3), 45–95.

Schrickx, J.A., Fink-Gremmels, J., 2014. Inhibition of P-glycoprotein by psychotherapeutic drugs in a canine cell model. J. Vet. Pharmacol. Ther. 37 (5), 515–517.

Schultz, M.M., Painter, M.M., Bartell, S.E., Logue, A., Furlong, E.T., Werner, S.L., et al., 2011. Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. Aquat. Toxicol. 104 (1–2), 38–47.

Silva, L.J.G., Lino, C.M., Meisel, L.M., Pena, A., 2012. Selective serotonin re-uptake inhibitors (SSRIs) in the aquatic environment: an ecopharmacovigilance approach. Sci. Total Environ. 437, 185–195.

Smith, E.M., Iftikar, F.I., Higgins, S., Irshad, A., Jandoc, R., Lee, M., Wilson, J.Y., 2012. In vitro inhibition of cytochrome P450-mediated reactions by gemfibrozil, erythromycin, ciprofloxacin and fluoxetine in fish liver microsomes. Aquat. Toxicol. 109, 259–266.

Soares, J., Coimbra, A.M., Reis-Henriques, M.A., Monteiro, N.M., Vieira, M.N., Oliveira, J.M., Guedes-Dias, P., Fontaínhas-Fernandes, A., Parra, S.S., Carvalho, A.P., Castro, L.F., Santos, M.M., 2009. Disruption of zebrafish (*Danio rerio*) embryonic development after full life-cycle parental exposure to low levels of ethinylestradiol. Aquat. Toxicol. 95 (4), 330–338.

Stegeman, J.J., Brouwer, M., Di Giulio, R.T., F6rlin, L., Fowler, B.A., Sanders, B.M., Van Veld, P.A., 1992. Molecular resonses to environmental contamination: enzyme and rotein systems as indicators of chemical exosure and effect. In: Huggett R, J., Kimerle, R.A., Mehrle, P.M., Bergman, H.L. (Eds.), Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress. Lewis Publ., Chelsea, USA, pp. 235–335.

Wang, H., LeCluyse, E.L., 2003. Role of orphan nuclear receptors in regulation of drug-metabolising enzymes. Clin. Pharmacokinet. 42, 1331–1357.

Wassmur, B., Gräns, J., Kling, P., Celander, M.C., 2010. Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 100 (1), 91–100.

Winder, V.L., Pennington, P.L., Hurd, M.W., Wirth, E.F., 2012. Fluoxetine effects on sheepshead minnow (*Cyprinodon variegatus*) locomotor activity. J. Environ. Sci. Health B 47 (1), 51–58.

Wong, R.Y., Oxendine, S.E., Godwin, J., 2013. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. BMC Genomics 14, 348.

Xavier-Neto, J., Sousa Costa, Â.M., Figueira, A.C., Caiaffa, C.D., Amaral, F.N., Peres, L.M., da Silva, B.S., Santos, L.N., Moise, A.R., Castillo, H.A., 2015. Signaling through retinoic acid receptors in cardiac development: doing the right things at the right times. Biochim. Biophys. Acta 1849 (2), 94–111.

Xu, J., Xie, F.K., 2011. α- and β-adrenoceptors of zebrafish in melanosome movement: a comparative study between embryo and adult melanophores. Biochem. Biophys. Res. Commun. 405 (2), 250–255.

Xu, C., Li, Y.C., Kong, A.T., 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Arch. Pharmacol. Res. 28, 249–268.

van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Zhang, Y., Zhou, T., Duan, J., Xiao, Z., Li, G., Xu, F., 2013. Inhibition of P-glycoprotein and glutathione *S*-transferase-pi mediated resistance by fluoxetine in MCF-7/ADM cells. Biomed. Pharmacother. 67 (8), 757–762.