Induction patterns of CYP1A, CYP1B, and CYP1C mRNAs in rainbow trout gills (*Oncorhunchus mykiss)* exposed to FICZ and PCB#126 in vitro

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Abstract

The Aryl hydrocarbon receptor (AhR) pathway is activated by a numerous compounds both exogenous and endogenous, like co-planar polychlorinated biphenyls (PCBs) and tryptophan photoproducts respectively. The AhR regulates expression of genes involved in oxidative metabolism, including cytochrome P450 1 (CYP1) family genes. The *CYP1* family is divided in 4 subfamilies (*CYP1A, CYP1B, CYP1C and CYP1D*). It has been shown that genes in all *CYP1* subfamilies except *CYP1D* are induced via the AhR pathway. We attempted to set up a new *ex vivo* experimental system based on gill filaments that can be used to study *CYP1* induction *ex vivo.* Moreover, we studied the *CYP1* induction patterns in rainbow trout *(Oncorhunchus mykiss)* gill filaments exposed ex vivo to a persistent (PCB#126) and a transient (formyl-indolo-[3,2-b]-carbazole, FICZ) compound. We determined *O*-deethylation of ethoxyresorufin (EROD activity) and mRNA expression levels of 6 *CYP1* genes (*CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2, CYP1C3*). We concluded that the new method is promising and reliable because the results yielded are consistent with results obtained *in vivo*.

1. Introduction

The idea of monitoring pollution in an aquatic environment using fish is not new. It was first proposed by Payne and Penrose in the mid-1970s (Payne and Penrose, 1975). The cytochrome P450 (CYP) enzymes were discovered at this time. The CYPs were found to be induced by pollutants in many organs like liver, kidneys, brain and gills, and furthermore this induction was correlated with the metabolism of xenobiotics and other organic substances in vertebrates (Nebert and Dalton, 2006). The CYPs are therefore considered ideal for environmental monitoring purposes (Nebert and Dalton, 2006).

Despite the 'temporary' name cytochrome (Omura and Sato, 1962), CYP enzymes are hemethiolate monooxygenases that catalyze oxidation of organic molecules in phase I reactions. There is a well-conserved peptide pattern in all CYP enzymes (Phe – $X_{(6-9)}$ – Cys – X – Gly, X for any amino acid) (Nebert and Dalton, 2006). In zebrafish (*Danio rerio*), there are 94 known CYP enzymes, which are classified in 18 families (Goldstone *et al.*, 2010). Enzymes in the first 4 CYP families (CYP1, CYP2, CYP3 and CYP4) take part in metabolism of both endogenous and xenobiotic compounds (Nebert and Dalton, 2006). CYP1 enzymes are involved in metabolism of eicosanoids (Capdevila *et al.*, 2002), phytoestrogen (Potter *et al.*, 2004, Androutsopoulos *et al.*, 2009) and a significant number of drugs, biotransformation of certain polyaromatic hydrocarbons (PAHs) to carcinogenic metabolites (Jongeneelen *et al.*, 1990; Dabestani and Ivanov, 1999). The CYP1 enzyme family is divided in 4 subfamilies, i.e., CYP1A, CYP1B and the recently discovered CYP1C and CYP1D (Godard *et al.*, 2005; Goldstone and Stegeman, 2008). CYP1A, CYP1B and CYP1C enzymes are of high interest because they are regulated by a particularly interesting receptor, aryl hydrocarbon receptor (AhR) (Whitlock *et al.*, 1996; Jönsson *et al.*, 2010). Expression of *CYP1* genes is induced via binding to the AhR of environmental xenobiotics, like PAHs, co-planar polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxins and furans (Denison *et al.*, 2002; Williams *et al.* 2009).

The AhR is a very well-studied receptor and yet we only now start to understand its functions. It is a cytosolic receptor, member of the basic helix-loop-helix (PER – ARNT – SIM) transcription factor family and highly evolutionary conserved (Nebert and Dalton, 2006). The AhR pathway is involved in a wide range of biological effects of dioxins and dioxin-like compounds in various types of cells and tissues. It has been found to be involved in effects on cell proliferation, cell differentiation, and apoptosis, and is also associated with cardiovascular, endocrine and carcinogenic effects and homeostasis (Nebert and Dalton, 2006). Therefore, many studies have focused on the characterization of the AhR pathway and the endogenous and exogenous compounds that activate it. A protein complex keeps the AhR in the cytosol in its inactive form. When a ligand activates AhR, the complex is dismantled and the activated receptor is translocated into the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). The heterodimer (AhR - ARNT) binds to DNA recognition regions (DNA response elements, DREs) in the promoters of *CYP1* genes and other genes and thus induces expression (Whitlock *et al.*, 1996). Apart from CYP1s, a significant number of enzymes that participate in drug metabolism are induced via the AhR pathway, including UDP-glucuronosyltransferase 1A6 (UGT1A6), NADPH quinone oxidoreductase (NQO1) and glutathione transferase Ya (GSTA1) (reviewed by Nebert *et al.*, 2000).

There is a wide variety of ligands that can activate the AhR pathway. Endogenous compounds like tryptamine and indole acetic (Health-Pagliuso *et al.*, 1998), bilirubin (Phelan *et al.*, 1998), prostaglandin (Seidel *et al.*, 2001) and exogenous compounds such as PCDDs (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) , PCBs like PCB#126 and PAHs like benzo(*α*)pyrene (BaP) are examples of agonists for the AhR (Denison and Nagy, 2003) . Among these compounds, TCDD is the most well-known having a high affinity for the AhR and causing developmental defects in developing vertebrates, including in human embryos (Jönsson *et al.*, 2007). Of the PCBs, PCB#126 is the most potent and persistent AhR agonist causing developmental toxicity in fish (Henry *et al.*, 2001).On the other hand, Wincent *et al.* (2009) have found a likely physiological ligand for the AhR ,formyl-indolo-[3,2-b]-carbazole (FICZ),

which is formed by exposure of tryptophan to ultraviolet (UV) or visible irradiation. FICZ has a higher AhR affinity than TCDD and is rapidly metabolized by CYP1 enzymes (Rannug *et al.*, 1987; Wei *et al.*, 1998).

Rainbow trout *(Oncorhunchus mykiss)* is a common model species widely bred in fish farms. The goal of our study was to set up a new experimental system that can be used to study *CYP1* induction *ex vivo*. We also wanted to determine and compare the *CYP1* mRNA levels induced by FICZ and PCB#126 *ex vivo*. For that purpose, gill filaments excised from rainbow trout were exposed *ex vivo* to FICZ or PCB#126. To study induction of CYP activity in gills we determined O-deethylation of ethoxyresorufin (EROD activity) and mRNA expression levels of 6 *CYP1* genes (*CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2, CYP1C3*).

2. Materials and Methods

2.1. Animals

Juvenile rainbow trout were kept in tanks of the aquarium facility at the Evolutionary Biology Center, Uppsala University. The tanks were supplied with aerated tap water; the water temperature was 13-14 ^{o}C and the fish were fed daily with pellets from Dana Feed A/S 1-2% of their body weight. The fish used for the experiments weighed 250 ± 100 g and were 27.9 ± 3.8 cm long. The methods of this study were approved by the Uppsala Committee for Research on Animals.

2.2. Solutions

For exposure and EROD assay we used HEPES-Cortland (HC) buffer, which contains 0.230 g $MgSO_4 \times 7H_2O$, 0.375 g KCl, 0.410 g $NaH_2PO_4 \times H_2O$, 7.745 g NaCl, 0.230 g $CaCl_2 \times 2H_2O$, 1.429 g hepes and 1 g glucose per 1 liter of distilled water (pH 7,7). All chemicals for the buffer were provided by Sigma-Aldrich (St. Louis, MO, USA). For the exposure, we used stock solutions of FICZ ($C_{19}H_{12}N_2O$, BML-206, Enzo Life Sciences Inc. Farmingdale, NY, USA) or PCB#126 (Larodan, Malmö, Sweden) dissolved in DMSO (Sigma Aldrich). The reaction buffer for the EROD assay was composed of HC buffer supplemented with dicumarol (in DMSO) and 7–ethoxyresorufin (in DMSO) to final concentrations of 10 µM dicumarol, 1 µM 7–

ethoxyresorufin and 0.2% DMSO v/v. Resorufin standard solutions (0.1-50 nM) were prepared in reaction buffer using a stock solution of resorufin in methanol.

2.3. Gill filament preparation + Exposure

The fish were collected from the tank, transferred to the lab in a bucket and anaesthetized in benzocaine (125 mg benzocaine / 1 L tap water). The fish were killed by cutting the spine and then the gills were excised and placed in Eppendorf-tubes with HC buffer kept on ice. The gill filaments were cut in 2-mm pieces from the tip and transferred to ice-cold HC buffer in 12-well plates, using 10 tips per well for EROD assay and 15-20 tips per well for qPCR. When the procedure of gill filament preparation was finished, the HC buffer was replaced with the exposure solution (1 ml / 5 tips) and the vials with filaments were incubated at 19 ºC. Incubation times used in the different experiments are given in the figure legends. Filament tip pieces kept in HC supplemented with DMSO (0.6 µl / 10 ml HC buffer; 60 ppm) or in HC buffer only were used as controls. In some experiments both types of controls were included.

2.4. Gill EROD assay

EROD activity was analyzed in gills according to the gill EROD assay described by Jönsson *et al.* (2002, 2010). After the exposure, the exposure solution was replaced by 0.5 ml reaction buffer (see section 2.3). The 12-well plate was covered with aluminum foil and preincubated with continuous shaking for 15 min. Then, the reaction buffer was replaced with 0.7 ml reaction buffer and the plate was incubated (at 20-21^{0}C) with continuous shaking. The wells were sampled at two time points (30 and 50 min), at which we transferred 0.2 ml of the solution to wells of a 96-well plate (F96, PolySorp, Nunc, Denmark). After the sampling, the fluorescence was measured in a multi-well plate reader (Victor 3; Perkin Elmer, Boston MA, USA) at 544 nm (excitation) and 590 nm (emission). EROD activity is expressed as the rate of resorufin formation per filament tip. The amount of formed resorufin is the difference between the two samplings. Using the equation of a standard curve (y'- fluorescence and x'- [resorufin]) and the volume (between the samplings: 0.5 ml), we

calculated the amount of resorufin and finally we expressed EROD activity as pmol resorufin/filament/min.

2.5. Quantitive PCR

In the case of total RNA isolation, we followed the protocol proposed by the Aurum^{TM} Total RNA Fatty and Fibrous Tissue kit (Bio-Rad). For determining the quantity and purity of the extracted RNA we used photospectrometry (Nanodrop 2000c). The 260/280 and 260/230 nm ratios were generally above 2. Reverse transcription was conducted using the iScript cDNA synthesis kit (Bio-Rad) and quantitative PCR was conducted using the iQ SYBR Green Supermix (Bio-Rad), according to the given protocol. We used quantitative PCR primers (synthesized by Sigma-Aldrich) specific for the *CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2* and *CYP1C3* and *EF1a* transcripts in rainbow trout (table 1) and a Rotor Gene 6000 Real-Time PCR machine for the PCR (Qjaden, Hilden, Germany).

According to Livak's and Schmittgen's method (2001 and 2008), we calculated the mRNA expression relative to control by $E^{-\Delta\Delta Ct}$. To determine the PCR efficiency, we used the LinReg PCR program(Ruijter *et al.*, 2009). Due to its stability to exposure, we used elongation factor 1-a (*EF1-a*) as reference gene. Finally, the data are presented as mean ± SD. Prism 5 by GraphPad software Inc. (San Diego, CA, USA) was used for the statistical calculations and to depict the data.

Table 1, Sequences of the gene-specific real time PCR primers used in the experiment

Transcript	Forward primer	Reverse primer	GenBank
	5′-3′	5′-3′	Acc. No.
rbCYP1A1	GGAAACTAGATGAGAACGCCAACA	GTACACAACAGCCCATGACAG	AAB69383.1
rbCYP1A3	GAAACTAGATGAGAACGCCAACG	CTGATGGTGTCAAAACCTGCC	AAD45966.1
rbCYP1B1	CATTCTGATACTTGTGAGGTTTCC	CAACTGAGACTGGTCTTCCAT	GU325707
rbCYP1C1	GCAGCACAGAGAAACCTTCAAC	GTCCTTTCCGTGCTCAATCACA	GU325708
rbCYP1C2	GAGCACAGGGAGACATTTGAC	GGTATCACTGTCCGCCTTG	GU325709
rbCYP1C3	CATGAGTGATGCCATCATTAACGC	AGGTCTGTGACTGTTCCTTCAACAA	GU325710
rbEF1a	GCAGGTACTACGTCACCATCAT	CACAATCAGCCTGAGATGTACC	CF752904

3. Results

3.1. Light vs. Dark in EROD assay in vitro

Previous studies by Öberg *et al.* (2005) suggest that EROD activity in cultured cells increases with time of incubation. Similarly EROD activity in gill filaments was found to increase with time of incubation (Behrens, 2011). Our first experiments were based on the suspicion that formation of the photoproduct and potent AhR agonist FICZ in the gill tissue could be the reason for this increase. Therefore we conducted an experiment to compare EROD activity after incubation at light and dark conditions for various time without addition of AhR agonist to the exposure buffer. At shown in fig.1, light does not seem to significantly affect EROD activity in gill filaments, despite the former indications in cell culture systems (Öberg *et al.*, 2005). *CYP1* induction in cell cultures was shown to depend on that cell culture medium contains FICZ formed at photo exposure of tryphophan (it is highly unlikely that our reaction buffer contains tryptophan or FICZ) (Öberg *et al.*, 2005).

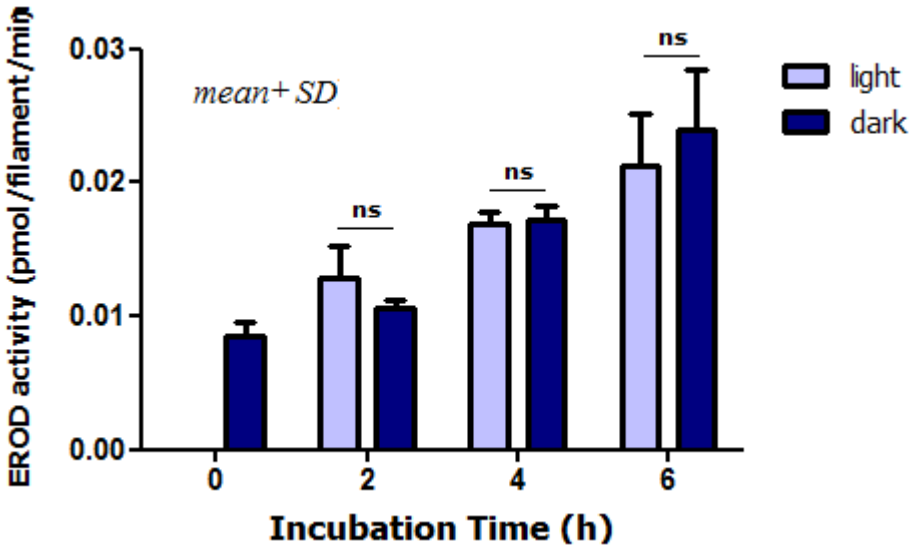

Fig.1, EROD activity over time (0, 2, 4 and 6h) in gill filaments from rainbow trout after incubation at light and dark conditions, 3 groups of 10 gill filaments (mean+SD, statistical method: student's t test)

3.2. EROD activity for FICZ exposure

We started to study *CYP1* induction by FICZ exposure, using the gill EROD assay. We have chosen to use 10 nM FICZ (dissolved in DMSO), based on previous data from a cell culture study (Wincent *et al.*, 2009) and an *in vivo* study on stickleback using indigo, which is another AhR agonist formed from tryptophan (Gao *et al.*, 2011). We exposed gill filaments from one fish to 10 nM FICZ for various periods of time (0, 1, 2, 3, 4, 5 and 6 h) and subsequently measured EROD activity. As a control we used DMSO (60 ppm). We found that EROD activity increased more in FICZ-exposed filaments than in the controls within the whole study period (fig.2).

The next step was to study concentration-response relationships between FICZ exposure and EROD activity. For that reason we picked the time point with the highest EROD activity (6 h) from the previous experiment and we exposed the gills to various FICZ concentrations (0.1, 0.3, 1, 3, 10 and 30 nM) for 6 h (n=4). We used HC buffer as an unexposed control and 60 ppm of DMSO in HC buffer as a solvent control (DMSO was used to dilute the FICZ stocks solutions).

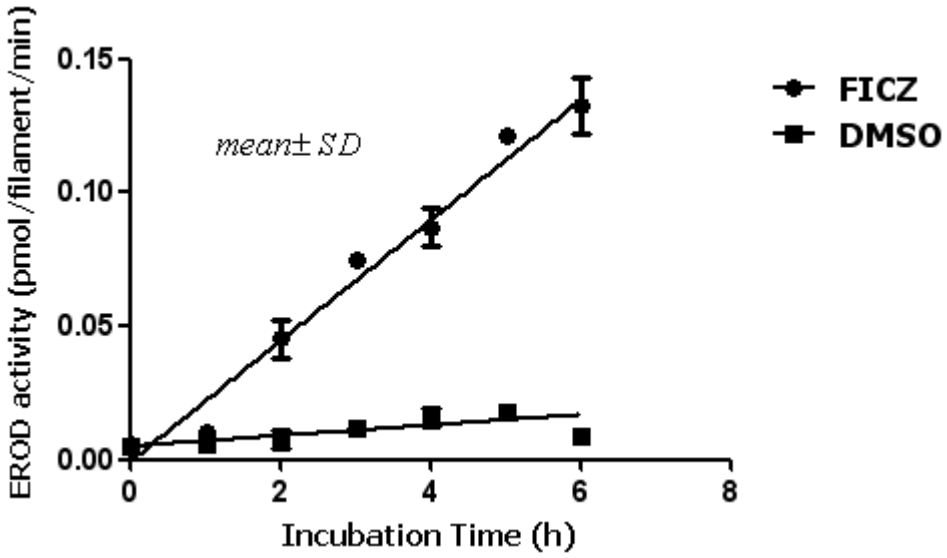

Fig. 2, Changes in EROD activity over time (0, 1, 2, 3, 4, 5, 6h) in gill filaments from rainbow trout after exposure *in vitro* to 10 nM FICZ, 3 groups of 10 gill filaments (mean±SD, r= 0.98 FICZ, r=0.99 for DMSO)

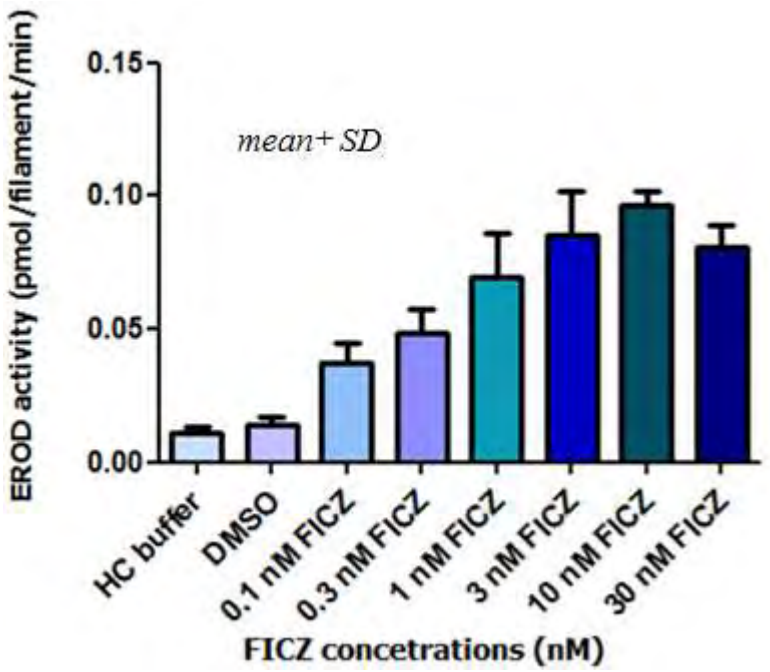

Fig. 3, EROD activity in rainbow trout gill filaments exposed *in vitro* to different FICZ concentrations (0, 0.1, 0.3, 1, 3, 10, 30) for 6 h, EC_{50} = 0.41 nM (calculated by nonlinear regression), n=4, mean+SD and r = 0.95

According to our findings, in the concentration response study, EROD activity peaked not at the highest concentration, 30 nM FICZ but at 10 nM FICZ (fig.3) and the EC_{50} value was 0.41 nM. This possible decrease could reflect a rapid degradation of FICZ, but the change is not big enough to be statistically significant.

3.3. CYP1 mRNA expression after FICZ exposure

Furthermore, we studied the levels of mRNA expression of *CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2* and *CYP1C3* in FICZ *in vitro*-exposed rainbow trout gills. The purpose of this experiment was to get an indication of which *CYP1* genes are responsible for the raise in EROD activity. We exposed gill filaments in 10 nM FICZ, selected 5 sampling time points (0, 1, 2, 4 and 6 h) and used 60 ppm of DMSO in HC buffer as control and HC buffer as solvent control for the 0 h time point. The results indicate that four genes are induced by the *in vitro* FICZ exposure, i.e., *CYP1A1, CYP1A3, CYP1C1* and *CYP1C3* (fig.4). Furthermore, it is indicated that there is a

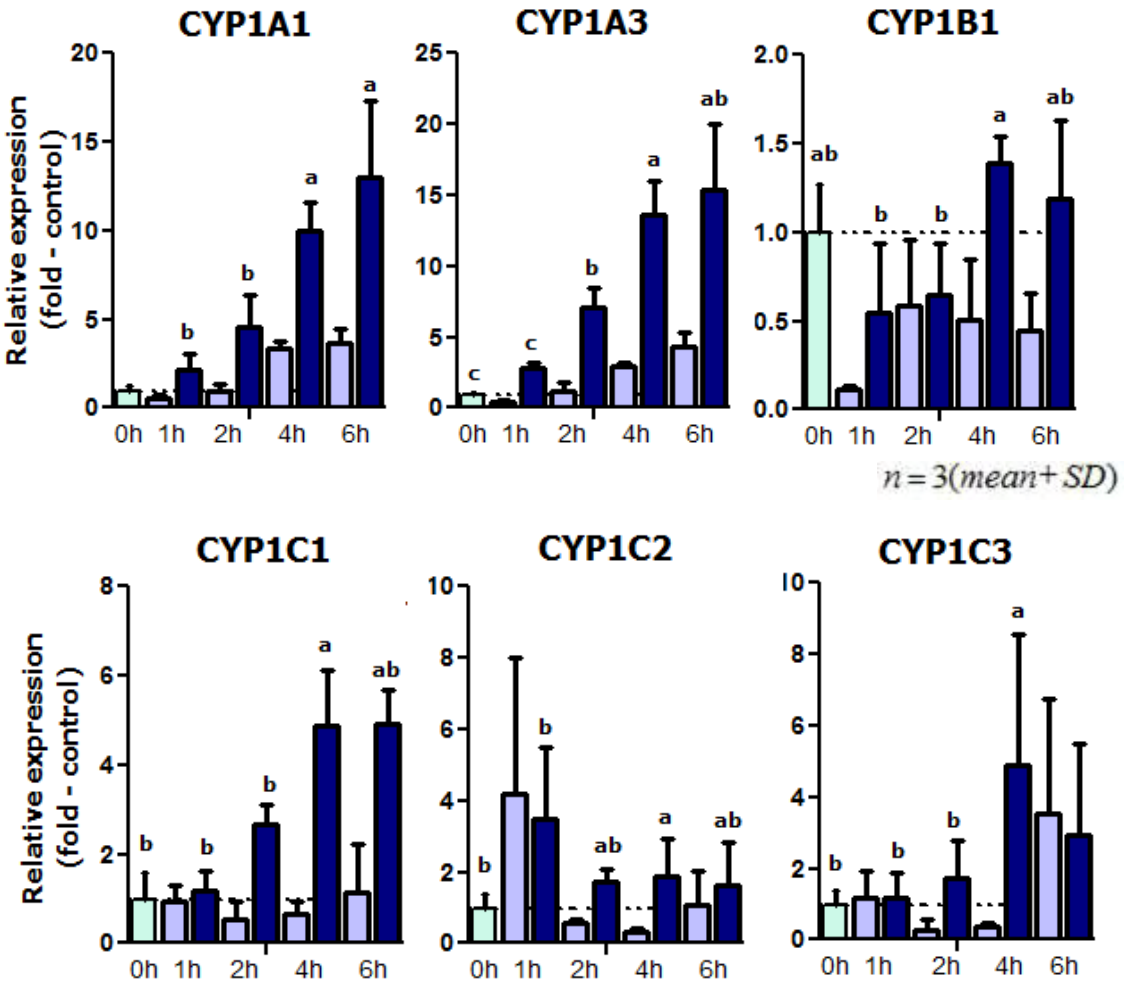

Fig. 4. Changes in *CYP1* mRNA levels over time (0, 1, 2, 3, 4, 5, 6h) in gill filaments exposed to 10nM FICZ *in vitro*. Induction is shown as levels of *CYP1* mRNA in filaments exposed to FICZ relative to the DMSO control and HC buffer for time point 0 h (n=3). Data were normalized to the *EF1-a* mRNA level and data analyzed with One-Way ANOVA followed by Tukey's test, mean±SD, different letters indicate a statistically significant difference $p<0.05$

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stronger correlation of *CYP1A1 and CYP1A3* mRNA expression with FICZ exposure than for of *CYP1C1* and *CYP1C3* mRNA expression with FICZ exposure and that there is no actual association between *CYP1B1* or *CYP1C2* mRNA expression and FICZ exposure (fig.4).

In the concentration response study that followed, we decided to add another higher concentration (i.e., 100 nM) and decrease the exposure time from 6 h to 4.5 h (HC, DMSO, 0.1 nM, 10 nM and 100 nM, n=3).

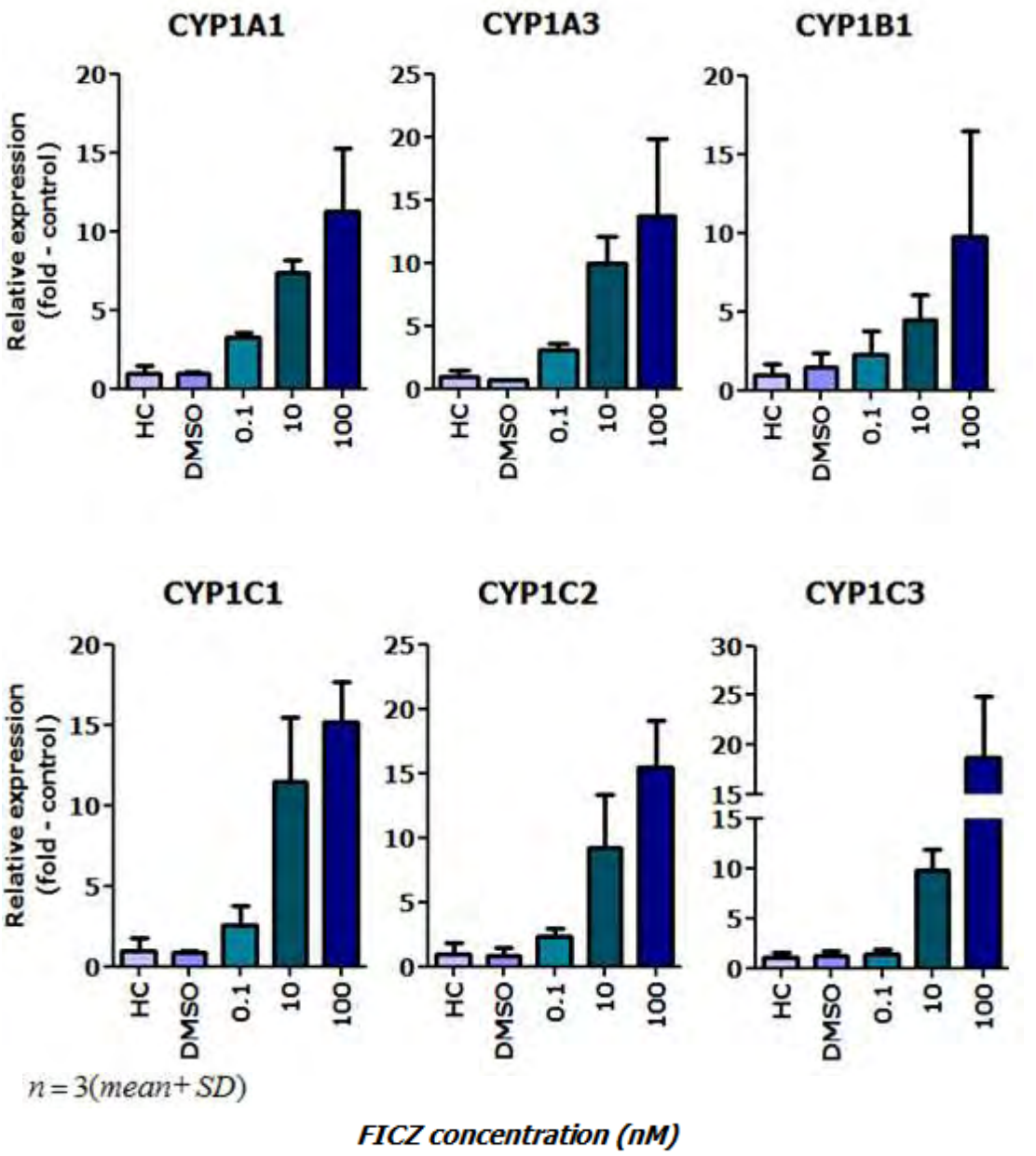

Fig. 5, Levels of CYP1 mRNA levels in rainbow trout gill filaments exposed in vitro to different FICZ concentrations (0, 0.1, 10, 100 nM) in 4.5 h, HC buffer and DMSO as controls. Data were normalized to the *EF1-a* mRNA level, EC_{50} presented in table 2, n=3 (mean±SD)

Table 2, EC_{50} values for CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2 and CYP1C3 induction by FICZ in rainbow trout gill filaments exposed *in vitro* for 4.5 h

(nM)	CYP1A1	CYP1A3	CYP1B1	CYP1C1	CYP1C2	CYP1C3
EC_{50}	13,12	3,547	14,11	5,585	6,864	9,195

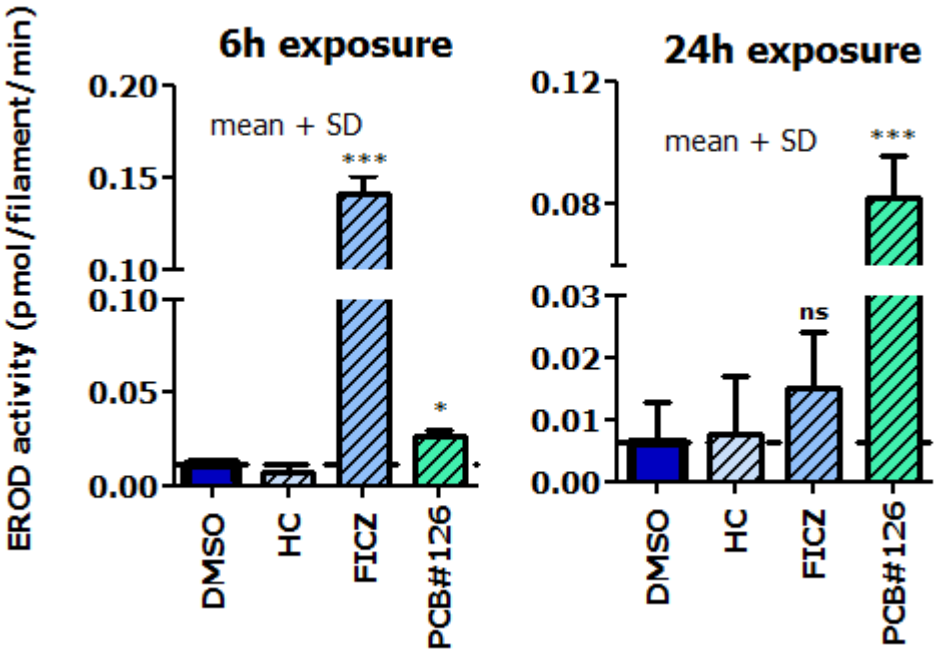

Fig. 6, EROD activity of rainbow trout gill filaments, exposed for 6h and 24h in 10nM FICZ and 100 nM PCB#126, n=3 (mean±SD) data analyzed with One-Way ANOVA Dunnett 's test, *** indicates $p<0.001$ and * $p<0.05$

As shown in fig. 5, mRNA expression of all *CYP1* genes follows a similar induction pattern with the highest level of expression at 100 nM (EC_{50} presented in table 2).

3.4. Induction patterns for PCB#126

After having studied the induction patterns in gill filaments by *in vitro* exposure to a transient *CYP1* compound, FICZ, we wanted to study the effect of a persistent inducer, PCB#126. We decided to conduct parallel FICZ and PCB#126 exposures to compare their effects at the same conditions and started with time course studies.

Considering past studies on effects of PCB#126 in the CYP1 system in fish (Gao *et al.*, 2011;, Jönsson *et al.*, 2006 and 2010), we chose to compare EROD activity at two exposure times (6 and 24 h, n=3) and to use 10 nM FICZ and 100 nM PCB#126 (DMSO as control) for exposure. As shown in fig. 6 FICZ induced a higher level of EROD activity than PCB#126 at 6 h while the opposite situation prevailed at

24 h. The results for PCB#126 at 24 hours were rather interesting, since degradation of gill tissue was expected to have started after such a long time.

In the next experiment we used four exposure times instead of two, 4, 8, 12 and 24h (n=3), exposed gill filaments to FICZ (10 nM), PCB#126 (100 nM) and used DMSO as control. The EROD induction patterns followed the expected scenario (fig. 7); EROD activity in FICZ exposed gills peaked at 8 h and then decreased, while in PCB#126 exposed gills it peaked at 12 h and remained at a similar level at 24 h. The maximal level of EROD activity in PCB#126-exposed filament was about half of that in FICZ-exposed filaments.

The last step was to determine the concentration-response relationship for EROD activity in gill filaments exposed to PCB#126 *ex vivo*. For that reason, we used 4 different PCB#126 concentrations (0.1, 1, 10 and 100 nM, n=3), both HC and DMSO as controls and based on the results of time course (fig.7) we used 12 h as exposure time. EROD activity peaked at exposure of 100 nM PCB#126 but there was slight difference with the 10 nM exposure (fig 8). Based on that, the EC_{50} value fo EROD induction by PCB#126 is at least 5 nM.

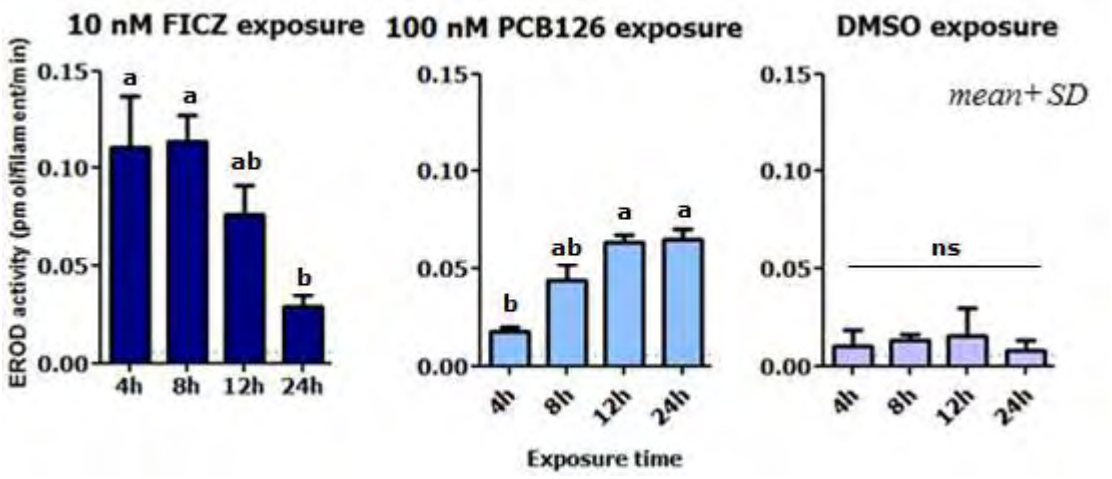

Fig. 7, Changes in EROD activity to rainbow trout gill filaments, exposed in 10 nM FICZ and 100 nM PCB#126, using DMSO as control, over time (4, 8, 12 and 24h), n=3 (mean+SD), data analyzed with One-Way ANOVA Tukey 's test, different letters indicate statistically significant differences, $p < 0.05$

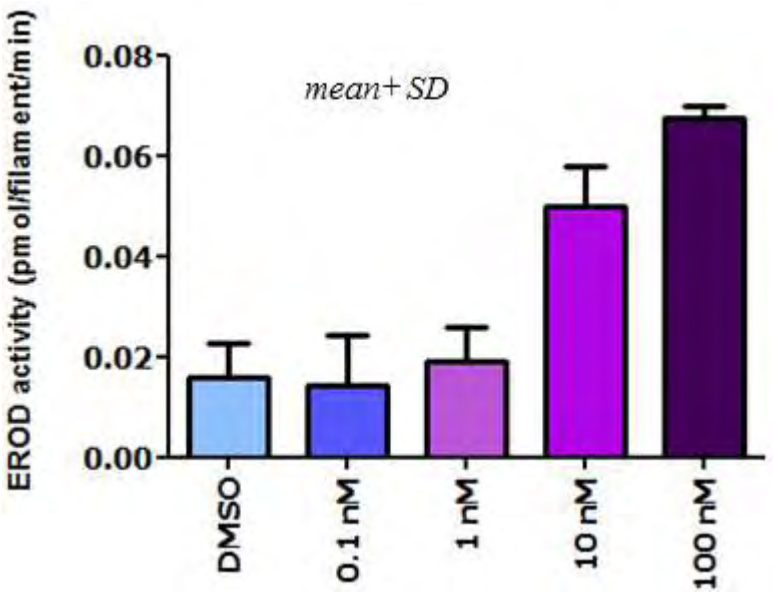

Fig. 8, EROD activity, rainbow trout gill filaments, exposed for 12 h in multiple PCB#126 concentrations, EC_{50} ≥ 5.6 nM calculated by normalized nonlinear regression (0.1, 1, 10 & 100 nM), n=3 (mean+SD, r = 0.92)

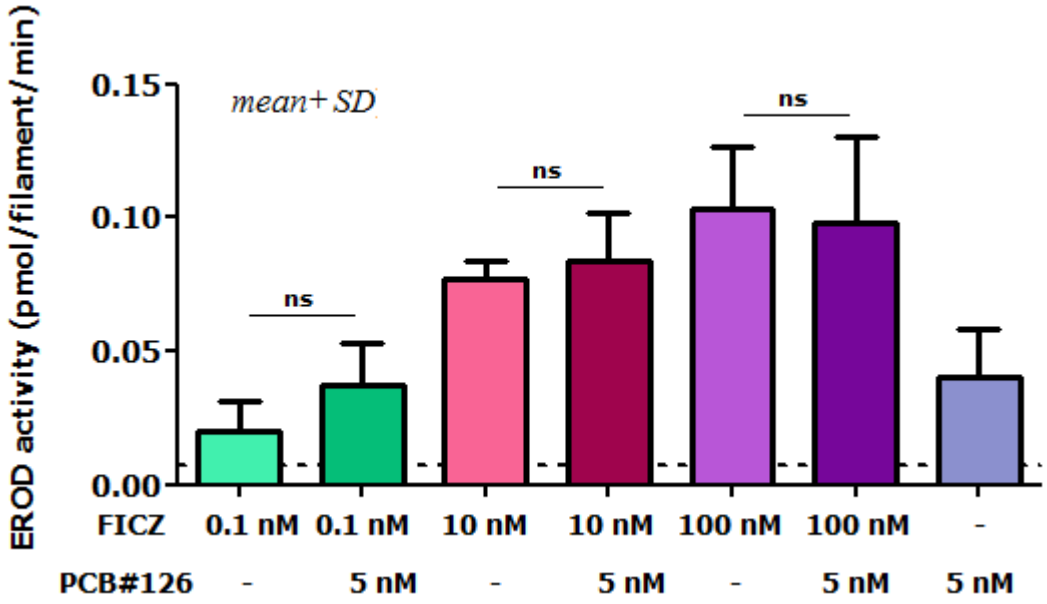

Fig. 9, EROD activity, rainbow trout gill filaments, exposed for 10 h in various FICZ concentrations (0.1, 10, 100 nM), PCB#126 (5 nM), or combination of FICZ and PCB#126, n=3, One-way ANOVA followed by Bonferonni 's multiple comparison test for selected pairs: no significant difference was observed between the exposure groups (mean+SD)

3.5. Combined exposure in FICZ and PCB#126

In the last experiment, we wanted to study the effect of combinations of the compounds, since at "in real world" conditions exposure to a single inducer is unlikely to occur. Based on our previous observations (figs. 3 and 8), we have chosen to expose gills for multiple FICZ concentrations (0.1, 10, 100 nM), one PCB#126 concentration (5 nM) and combination of them. As control, we used DMSO and HC. As is shown in figure 9, there was no clear effect of the combinational exposure. PCB#126 appeared to amplify the EROD activity induced by FICZ at the lowest concentration of FICZ (0.1 nM; not statistically significant) but it looks like 0.1 nM FICZ did not add to effect of 5 nM PCB#126 (fig.9).

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4. Discussion

Our study aimed to establish a new *in vitro* exposure method based on gill filaments and to compare the *CYP1* induction patterns of a transient and a persistent inducer (FICZ and PCB#126, respectively) in rainbow trout gills using this method. Comparing our findings with previous studies (Laub *et al.*, 2010; Gao *et al.*, 2011; Jönsson *et al.*, 2006 and 2010), we can assume that this method has a high potential to yield reliable results.

FICZ is a tryptophan photoproduct with high AhR affinity that potently induces mRNA expression of *CYP1* genes through the AhR pathway; however FICZ is rapidly metabolized. Several studies show that FICZ is an important intermediate in light biological responses and that FICZ interferes with the mammalian UV response (Wei *et al.* 1999; Ma Q., 2011). The levels of *CYP1* mRNA and EROD induction were shown to be positively correlated with FICZ exposure concentration for a short period of time and then either stabilizing or declining (Laub *et al.*, 2010; Wei *et al.*, 2000, Jönsson *et al.*, 2009). The results of experiments with our new method followed that pattern. EROD activity reached the peak when the gills were exposed to 10 or 100 nM FICZ for 4-6-8 h and *CYP1* mRNA induction peaked somewhat earlier

(after ca 4 of exposure). Thus, these results are in agreement with results from *in vivo* exposures.

PCB#126, on the other hand, is a persistent environmental toxicant and AhR agonist. PCB#126 exerts a variety of effects through the AhR pathway by interfering with cell proliferation (Jönsson *et al.*, 2007), by disturbing development of reproductive organs and cardio-vascular system, and by causing hepatic toxicity and other abnormalities in developing animals, including fish (Vezina *et al.*, 2004; Shirota *et al.*, 2006; Waits and Nebert, 2011). Since PCB#126 is a common pollutant in aquatic systems and a strong *CYP1* inducer, it is well-studied in fish. Additionally, its ability to preserve the *CYP1* induction level for long time, gives us the opportunity to testify the time limits of our method. Our results followed the induction patterns reported in previous studies (Gao *et al.*, 2011; Jönsson *et al.*, 2006, 2007 and 2010). Although we did not measure the *CYP1* mRNA levels in all experiments, our findings with the EROD assay encourage the use of this *ex vivo* exposure method.

In conclusion, the new experimental method seems promising for comparing the induction patterns of *CYP1* genes and EROD activity of rainbow trout gills exposed *ex vivo* to FICZ and PCB#126. As a next step, further experimental work on other endpoints, other species and other pollutants would assure and determine the reliability of the method.

5. Acknowledgements

I want to thank Maria Jönsson for her patient guidance and help to finish the project, Eva Damm, for helping me find a project under the circumstances she did, all the members of the department of Enviromental Toxicology for the great work place they offered me and my friends and family for being supportive with their own special way.

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