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Modification and quantification of *in vivo* EROD live-imaging with zebrafish (*Danio rerio*) embryos to detect both induction and inhibition of CYP1A

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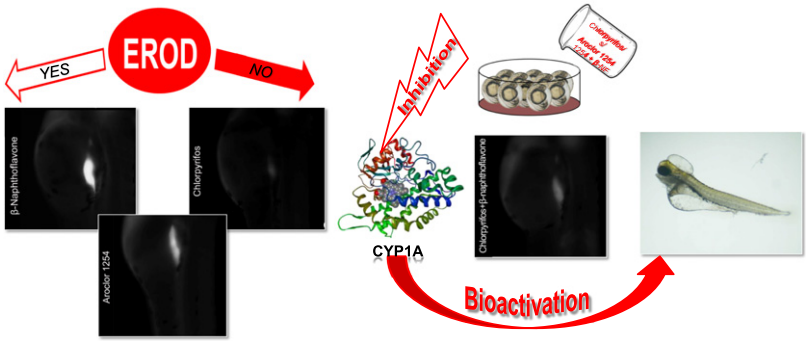
HIGHLIGHTS

- Chlorpyrifos is a strong CYP1A inhibitor in zebrafish embryos.
- Aroclor 1254 inhibits CYP1A catalytic activity in zebrafish embryos in a specific time-dependent manner.
- CYP1A inhibition could also be detected after 3 h short-term exposure of zebrafish embryos to chlorpyrifos.
- The lack of CYP1A inhibition after 3 h exposure of zebrafish embryos to Aroclor 1254 demonstrates toxicokinetic mechanisms.
- *In vivo* EROD assay visualizes CYP1A induction/inhibition and allows densitometric quantification and statistical assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

The visualization of specific activation of the aryl hydrocarbon receptor (AhR) directly in the zebrafish embryo (*Danio rerio*) *via* live-imaging is a reliable tool to investigate the presence of dioxin-like substances in environmental samples. The co-existence of inducers and inhibitors of cytochrome P450-dependent monooxygenases (CYP1A) is typical of complex environmental mixtures and requires modifications of the *in vivo* EROD assay: For this end, zebrafish embryos were used to evaluate the EROD-modifying potentials of common single-compound exposures as well as binary mixtures with the PAH-type Ah-receptor agonist β-naphthoflavone. For chemical testing, chlorpyrifos and Aroclor 1254 were selected; β-naphthoflavone served as maximum EROD induction control. Chlorpyrifos ($\leq EC_{10}$) could be documented to be a strong CYP1A inhibitor causing characteristic edema-related toxicity. Aroclor 1254 resulted in inhibition of CYP1A catalytic activity in a concentration- and specific time-dependent manner. Next to a fast CYP1A induction, CYP1A inhibition could also be detected after 3 h short-term exposure of zebrafish embryos to chlorpyrifos. This communication also describes techniques for the quantification of fluorescence signals *via* densitometry as a basis for subsequent statistical assessment. The co-exposure approach with zebrafish embryos accounts for the nature of potential interaction between CYP1A inducers and inhibitors and thus pays tribute to the complexity of environmental mixtures. The co-exposure EROD live-imaging assay thus facilitates a better understanding of mixture effects and allows a better assessment and interpretation of (embryo) toxic potentials.

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1. Introduction

Ethoxyresorufin-*O*-deethylase (EROD) activity in fish cells is a well-established *in vitro* and *in vivo* biomarker of aryl hydrocarbon receptor (AhR)-mediated induction of cytochrome P450-dependent monooxygenases (CYP1A) and a sensitive indicator of specific planar polycyclic aromatic hydrocarbons (PAHs) and structurally related compounds (Fent, 2001; Segner and Cravedi, 2001; Whyte et al., 2000).

In vitro tests with fish cell lines are not yet part of any regulatory programs and have frequently been documented to underestimate the *in vivo* toxicity by up to 3 orders of magnitude (Tanneberger et al., 2013). Since results of *in vitro* test systems have to be extrapolated from cells to whole organisms, prediction of toxicity may be problematic especially for lipophilic xenobiotics, since the routes of uptake, distribution, metabolism and excretion significantly differ between cells and intact organisms. In contrast, *in vivo* assays account for bioavailability and, hence, appear more relevant for ecotoxicological assessment. Thus, the following study was designed to develop further *in vivo* approaches for the detection of EROD activity in zebrafish embryos.

The visualization of specific activation of the Ah receptor in fish embryos *via* live-imaging is based on the ability of CYP1A to convert the artificial PAH substrate 7-ethoxyresorufin to the fluorescent metabolite resorufin. So far, however, only few methods have been developed that allow the measurement of EROD activity in intact fish (González-Doncel et al., 2011; Le Bihanic et al., 2013; Nacci et al., 1998; Noury et al., 2006). In developing fish embryos, several studies described AhR-mediated stimulation of CYP1A activity in, *e.g.*, killifish (*Fundulus heteroclitus*; Arzuaga et al., 2006; Nacci et al., 1998; Wassenberg and Di Giulio, 2004), medaka (*Oryzias latipes*; Carney et al., 2008) and zebrafish (*Danio rerio*, Kais et al., 2017; Liu et al., 2016; Otte et al., 2010). Since, according to current EU animal welfare legislation (EU, 2010), zebrafish embryos are not regarded protected until the age of 5 d (Braunbeck et al., 2015; Strähle et al., 2012), zebrafish embryos have been developed as an alternative *in vivo* test system to investigate AhR-mediated toxicity (Otte et al., 2010; Schiwy et al., 2015). A particularly promising approach is the high-resolution imaging of the CYP1A induction in individual zebrafish embryos (Kais et al., 2017; Otte et al., 2010): The transparency of zebrafish embryos allows high-resolution organ-specific EROD activity visualization and facilitates the identification of potential systemic sources of interference with CYP1A induction or, rather, CYP1A inhibition.

In fact, the co-existence of agonists and inhibitors of CYP1A is typical of complex environmental mixtures (Wassenberg et al., 2005) and is, therefore, of particular ecological relevance. However, the role of CYP1A inhibition in zebrafish embryos is less clear and requires more-in-depth investigations into the correlation between developmental malformations and EROD activity in embryos at different developmental stages. To account for the potential of both EROD induction and inhibition, the *in vivo* EROD live-imaging assay introduced by Otte et al. (2010) and developed further by Kais et al. (2017) needed to be developed further.

The present study was conducted within the scope of the project DanTox, which aimed at identifying specific toxicity and molecular modes of action of sediment-bound pollutants as well as of single model compounds using zebrafish (Keiter et al., 2010).

As model compounds, the organophosphate insecticide chlorpyrifos and the polychlorinated biphenyl (PCB) Aroclor 1254 were selected from the list of priority substances of the European Water Framework Directive (EU, 2001). Substances with low water solubility (high log P_{OW}) such as chlorpyrifos (log P_{ow}: 4.7–5.3) and Aroclor 1254 (log P_{ow}: 6.3) have a high affinity to adsorb to both surfaces and suspended organic matter, which then might be deposited in freshwater and marine sediments (Wölz et al., 2010).

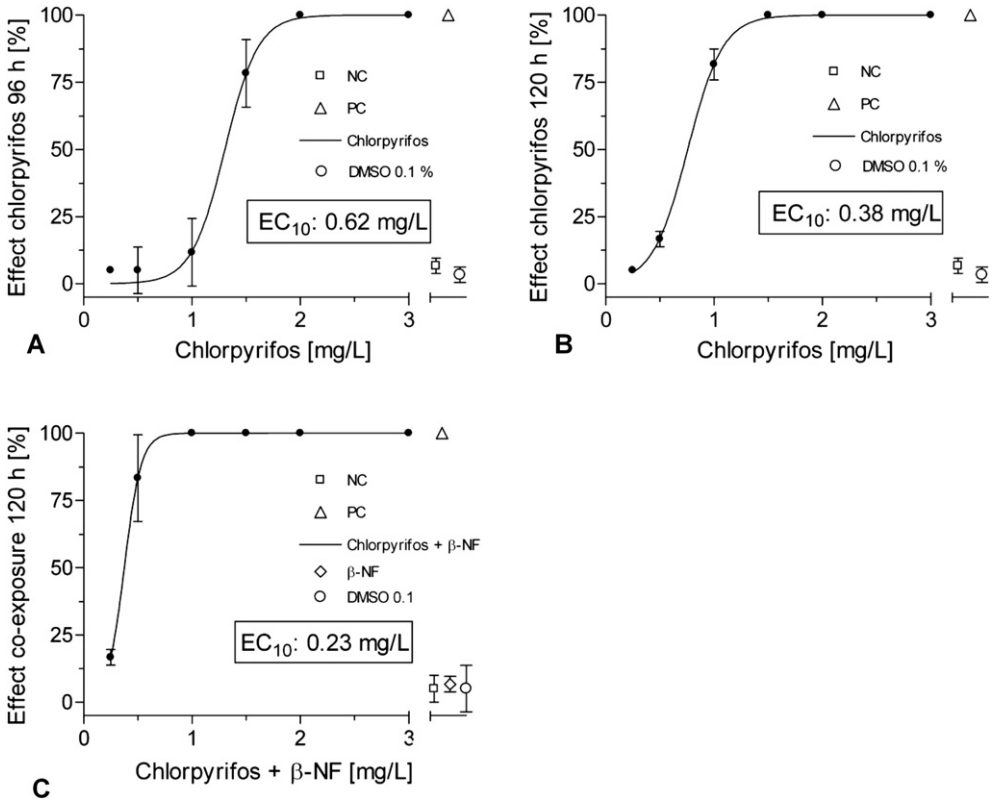


Fig. 1. Lethal and sublethal effects in zebrafish (*Danio rerio*) embryos after 96 h (A) and 120 h (B, C) exposure to chlorpyrifos as a single compound (A, B) as well as in co-exposure with 10 μg/L β-naphthoflavone (β-NF) after 120 h (C). The negative controls (NC) and positive controls (3,4-dichloroaniline PC) are shown on the right end of the x-axis, respectively. The increased slope of the curve after 120 h indicates enhanced toxicity after prolonged exposure (B). Co-exposure of chlorpyrifos and β-naphthoflavone (C), however, leads to an even greater toxicity than after single-substance exposure. Data are given as means ± SD from n = 3 independent runs.

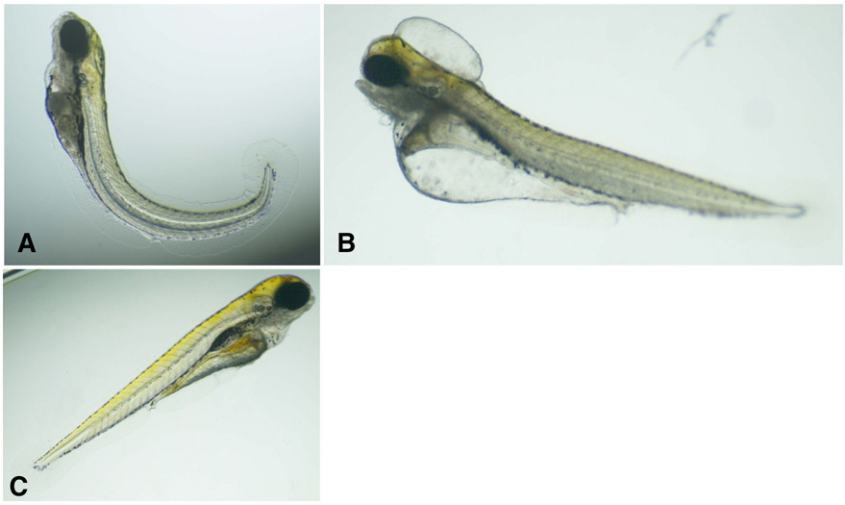


Fig. 2. Larvae of zebrafish (*Danio rerio*) exposed to 1 mg/L chlorpyrifos in single-compound exposure (A), in co-exposure approach with 10 μg/L β-naphthoflavone (B) and the negative control (C) after 144 h. After single-compound exposure, larvae show characteristic spinal deformation (convex curvature) and a bent tail (A), whereas the larvae of the co-exposure approach shows extended edemata in the pericardium, yolk sac and head region and causes jaw malformations (B).

As a well-known phosphorothionate insecticide, chlorpyrifos has frequently been used for pest control at a worldwide scale in agriculture and households (Yen et al., 2011). Chlorpyrifos is registered for use in almost 100 countries and is applied to approx. 8.5 million crop acres (Giddings et al., 2014; Williams et al., 2014). For China, Liu et al. (2014) reported an annual chlorpyrifos consumption of about 18,000 tons. As a consequence, chlorpyrifos is commonly monitored in groundwater and surface water (Kammerbauer and Moncada, 1998; Masiá et al., 2015; Zhang et al., 2012). Chlorpyrifos is a classical inhibitor of acetylcholinesterase (Barron and Woodburn, 1995; Richardson, 1995) and known for its neurodevelopmental toxicity (Grandjean and Landrigan, 2006).

Aroclor 1254 is a commercial PCB mixture, which, per definition, contains 12 carbon atoms and 54% chlorine, but consists of >80 PCB congeners (Alvares et al., 1973; Kodavanti et al., 2001). It was marketed between the 1930s and 70s and has extensively been used and dispersed from industrial applications worldwide (Henry, 2015). Aroclor 1254 has low water solubility, but is well soluble in most organic solvents, oils and fats. It is chemically fairly inert; the resistance of Aroclor 1254 to oxidation and reduction in natural environments makes it very stable, and it is, therefore, defined as a persistent organic pollutant (Porta and Zumeta, 2002). Aroclor 1254 has an overall half-life in soil of 940 days (Hsieh et al., 1994) and leads to a multitude of biological effects (Crinnion, 2011).

The co-exposure approach presented in this study provides the opportunity to determine the role of CYP1A in terms of both detoxification and bioactivation. Whereas it is widely accepted that dioxin toxicity is largely AhR-mediated, the role of CYP1A-related activation causing toxicity (bioactivation) by itself is less clear and controversially discussed: On the one hand, several studies have shown that chemical inhibition or protein knockdown of CYP1A either reduces (Cantrell et al., 1996; Dong et al., 2002) or does not alter toxic responses to dioxins during zebrafish development (Carney et al., 2004). On the other hand, there are studies suggesting that CYP1A is an adaptive response and lends protection to the developing vertebrate embryo (Billiard et al., 2006; Mattingly and Toscano, 2001).

With respect to CYP1A manipulation, most monitoring programs have focused on CYP1A induction; likewise, the *in vivo* live-imaging assay for CYP1A (Kais et al., 2017) has been designed to visualize enzyme induction. Since CYP1A inhibition may be equally likely in environmental monitoring, the present study was designed to develop the CYP1A live-imaging assay with zebrafish embryos further by implementing options to also consider CYP1A reduction. Therefore, the present study used zebrafish embryos to visualize the EROD-inducing potential of selected model CYP stimulators and inhibitors both as individual compounds and as components of binary mixtures with the PAH-type model AhR agonist β-naphthoflavone. This co-exposure approach was used to mimic the potential interaction between AhR agonists/antagonists and CYP1A inducers. An additional purpose of the present communication was to introduce techniques for the quantification of fluorescence signals *via* densitometry as a basis for subsequent statistical assessment.

2. Materials and methods

2.1. Chemicals and materials

All chemicals were purchased at the highest purity available from Sigma-Aldrich (Deisenhofen, Germany), unless noted otherwise. Polystyrene 24-well microtiter plates were obtained from Renner (TPP; Dannstadt, Germany), self-adhesive foil (sealing tape SH) was purchased from Nunc (Langenselbold, Germany), and low-melting agarose (gelling temperature: 40.5–43.5 °C) was provided by SeaKem (HGTAgarose, Cambex BioScience, Rockland, USA). The EROD substrate 7-ethoxyresorufin for the *in vivo* EROD live-imaging assay was prepared as described by Heinrich et al. (2014).

2.2. Fish maintenance and egg production

All adult zebrafish used for breeding were wild-type descendants of the "West Aquarium" strain maintained at the in-house facilities of the Aquatic Ecology and Toxicology Group at the Center for Organismal Studies, University of Heidelberg (maintenance and breeding licensed under no. 35-9185.64/BH). Fish maintenance and egg production have repeatedly been described in detail (Kimmel et al., 1995; Kimmel et al., 1988; Nagel, 2002; Spence et al., 2006; Wixon, 2000) and have been updated for the purpose of the zebrafish embryo toxicity test by Lammer et al. (2009). For egg production, a breeding stock of unexposed zebrafish with well-documented fertilization rate of eggs was used for all test systems. The zebrafish eggs were produced *via* individual spawning groups, males and females at a ratio of 2:1 placed in spawning tanks.

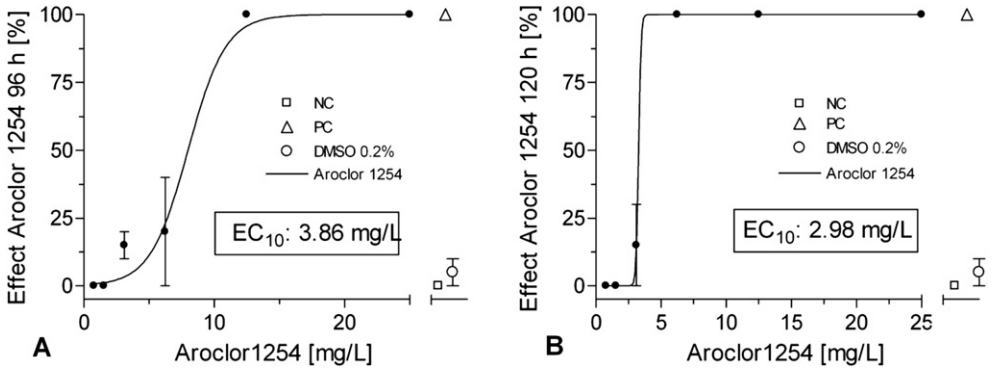


Fig. 3. Lethal and sublethal effects in zebrafish (*Danio rerio*) embryos after 96 h (A) and 120 h (B, C) exposure to Aroclor 1254 as a single compound (A, B). The negative controls (NC) and positive controls (β-naphthoflavone, PC) are shown on the right end of the x-axis, respectively. The increased slope of the curve after 120 h exposure indicates enhanced toxicity after prolonged exposure (B). Data are given as means ± SD from n = 3 independent runs.

2.3. Standard fish embryo toxicity test (FET)

In order to avoid confounding effects on EROD induction by acute toxicity, each substance was tested for embryo toxicity prior to EROD evaluation. In order to avoid systemic effects, EC_{10} values (concentration where 10% of the embryos show any kind of effect) were chosen as maximum test concentrations for the EROD assays. The standard fish embryo toxicity test (FET) was conducted according to the protocol specified by OECD TG 236 (OECD, 2013). In brief, embryos were exposed at latest from 1 hpf (hour post-fertilization) in glass vessels, which had been pre-incubated (saturated) with the test solutions for at least 24 h, to a series of dilutions of chlorpyrifos and Aroclor 1254. After control of the fertilization success, embryos were transferred to pre-incubated 24-well plates and kept in an incubator at 26.0 ± 1.0 °C with a 10:14 h dark: light regime. For each test, a vehicle control for chlorpyrifos (0.1% DMSO) and for Aroclor 1254 (0.2% DMSO) was conducted. Observation of lethal and sublethal effects according to Nagel (2002) was made during the daily solution exchanges, after 24, 48, 72, 96, 120 and, in exceptional cases up to 144 h. The mean EC_{10} values were determined after 96 and 120 h exposure by probit analysis using ToxRat® Professional 2.10.3.1 (ToxRat Solutions, Alsdorf, Germany).

Embryo tests were classified as valid, if the mortality in the negative control was ≤10%, and the positive control (3,4-dichloroaniline) showed mortalities between 20 and 80% (Lammer et al., 2009; OECD, 2013). In total, 3–4 independent experiments with different dilutions and 20 embryos per concentration were carried out for each test substance and substance combination.

2.4. Test substances

The substances tested were chlorpyrifos (CAS No. 2921-88-2) with a log P_{OW} of 4.7–5.3 (Racke, 1993) and Aroclor 1254 (CAS No. 110097-69-1) with a log P_{OW} of 6.3 (CDC, 1999). Both chemicals were dissolved in 100% DMSO (CAS No.: 67-68-5) and diluted with artificial water according to OECD TG 236 (OECD, 2013) to final DMSO concentrations of 0.1 and 0.2%, respectively. 10 μg/L β-Naphthoflavone dissolved in DMSO served as maximum induction control. Zebrafish embryos were exposed to 0.25, 0.5, 1, 2 and 3 mg/L chlorpyrifos. Due to the assumption that chlorpyrifos is an inhibitor of CYP1A, a co-exposure was conducted with 10 μg/L β-naphthoflavone as a strong inducer. To avoid confounding effects on EROD induction/inhibition by acute toxicity in the co-exposure approach, each chlorpyrifos concentration was tested in combination with 10 μg/L β-naphthoflavone.

Since the chemical structure of technical Aroclor 1254 is not precisely defined (12 carbon atoms and 54% chlorine), its composition may vary. In order to avoid confounding effects on EROD activity by different batches of Aroclor 1254, all EROD measurements were based on two batches of Aroclor 1254 (LB 77779V and LB 89250V), which had already been shown to produce identical results in previous studies (Kais et al., 2017). Zebrafish embryos were exposed to 0.78, 1.56, 3.125, 6.25, 12.5 and 25 mg/L Aroclor 1254. Since, due to the high log P_{OW} (low water solubility), pre-tests had demonstrated that Aroclor 1254 is likely to adsorb to the surfaces of multiwall plates as well as to magnetic stirring bars, all experiments were conducted with pre-incubated (saturated) vessels in order to reduce sorption-related fluctuations in Aroclor 1254 concentrations. Using semi-static exposure with daily exchange of test solutions according to OECD TG 236 (OECD, 2013), constant distribution and permanent uptake of hydrophobic substances were guaranteed.

2.5. Dechorionation experiments with zebrafish embryos exposed to Aroclor 1254

In order to clarify the potential barrier function of the zebrafish chorion for Aroclor 1254, experiments were carried out exemplarily with zebrafish embryos manually dechorionated at the age of 24–48 h (Henn and Braunbeck, 2011) exposed to the highest concentration of Aroclor 1254.

2.6. Live-imaging EROD assay

In order to prepare the embryos for EROD measurement, embryos were washed 2 × 5 min in artificial water. Under light-protected

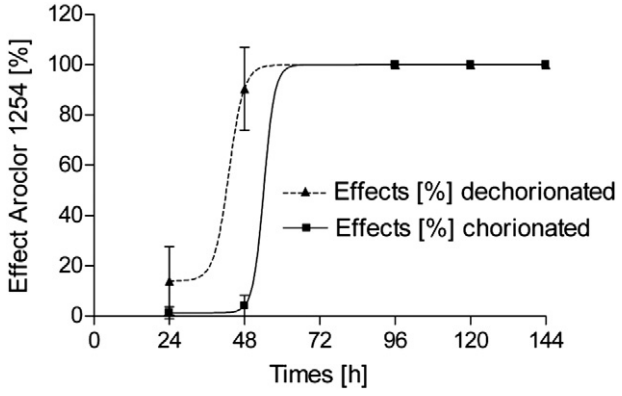


Fig. 4. Combined lethal and sublethal effects in normal (solid line) and dechorionated (broken line) zebrafish (*Danio rerio*) embryos after 144 h continuous exposure to 25 mg/L Aroclor 1254. Only 5% of chorionated and 90% of dechorionated embryos show effects after 72 h. After 96 h, effects in chorionated and dechorionated embryos were identical. Data are given as means ± SD from n = 3 independent runs.

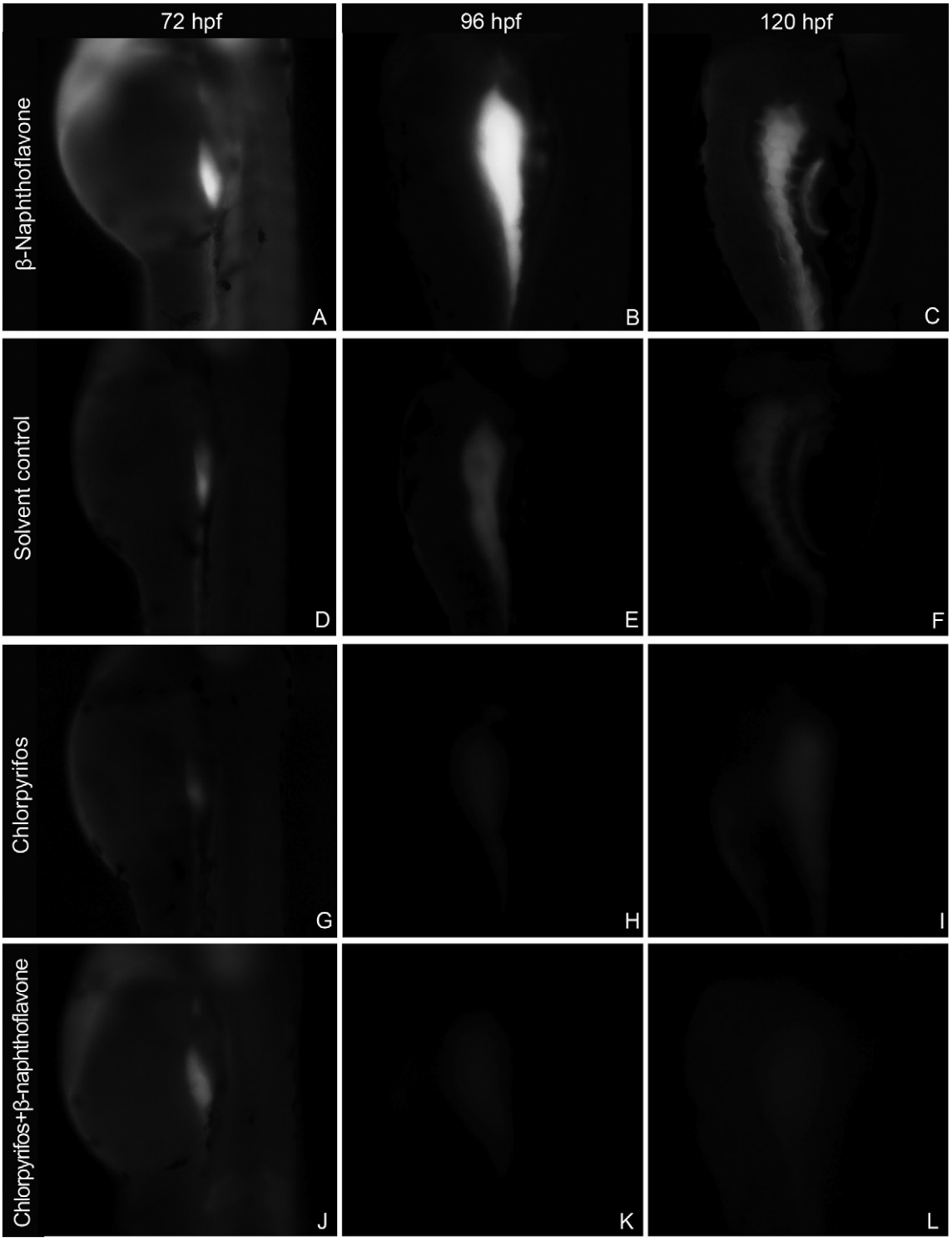
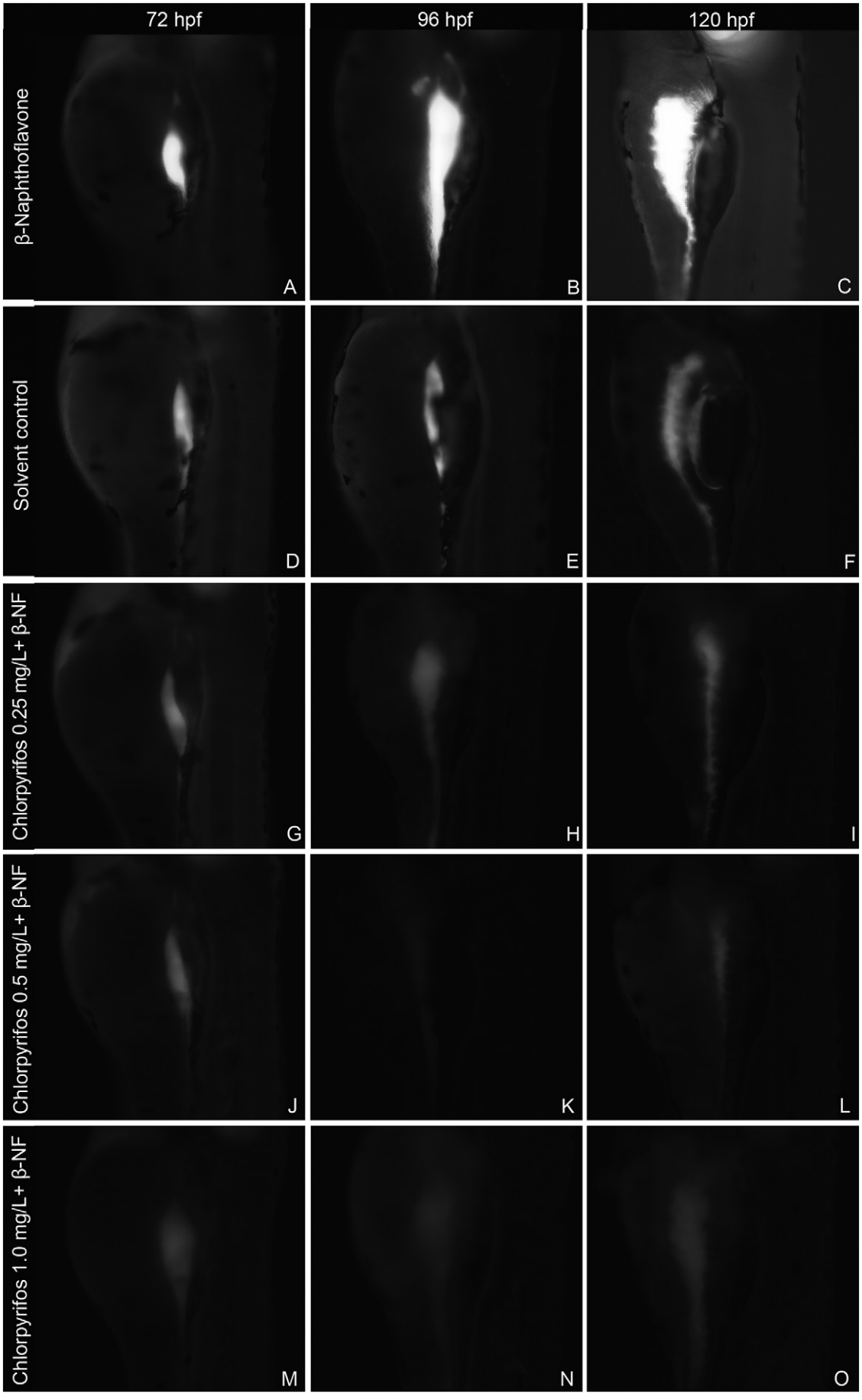


Fig. 5. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) exposure to 10 μg/L β-naphthoflavone (A–C), solvent (0.1% DMSO; D-F), 0.6 mg/L (EC_{10}) chlorpyrifos (G–I) and 0.6 mg/L chlorpyrifos + 10 μg/L β-naphthoflavone (J–L). In all developmental stages, embryos exposed to β-naphthoflavone show a bright signal in the liver, whereas embryos of the solvent control show a weaker signal reflecting constitutive activity. The signal intensities after single chlorpyrifos exposure as well as after co-exposure to chlorpyrifos and β-naphthoflavone show lower signal intensities than in the solvent control after both 96 and 120 h. The signal in the co-exposure approach after 72 h is as strong as that of the solvent control. Epifluorescence microscopy, exposure times: 60 ms (72 h), 30 ms (96 h), 40 ms (120 h).

Fig. 6. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J, M), 96 (B, E, H, K, N) and 120 h (C, F, I, L, O) exposure to 10 μg/L β-naphthoflavone (A–C), solvent (0.1% DMSO; D–F), 0.25 (G–I), 0.5 (J–L) and 1.0 mg/L chlorpyrifos (M–O) + 10 μg/L β-naphthoflavone each. In all developmental stages, embryos exposed to β-naphthoflavone show a bright signal in the liver, whereas the embryos of the solvent control show a weaker signal reflecting constitutive activity. Particularly after 96 and 120 h, signal intensities after co-exposure to 0.5 and 1.0 mg/L chlorpyrifos and β-naphthoflavone were lower than in the solvent control, whereas the lowest concentration of 0.25 mg/L chlorpyrifos did not inhibit EROD activity completely (G–I). Epifluorescence microscopy, exposure times: 40 ms (72 h), 100 ms (96 h), 100 ms (120 h).


72 hpf
96 hpf
120 hpf
β-Naphthoflavone
Solvent control
Chlorpyrifos 0.25 mg/L+ β-NF
Chlorpyrifos 0.5 mg/L+ β-NF
Chlorpyrifos 1.0 mg/L+ β-NF
A
B
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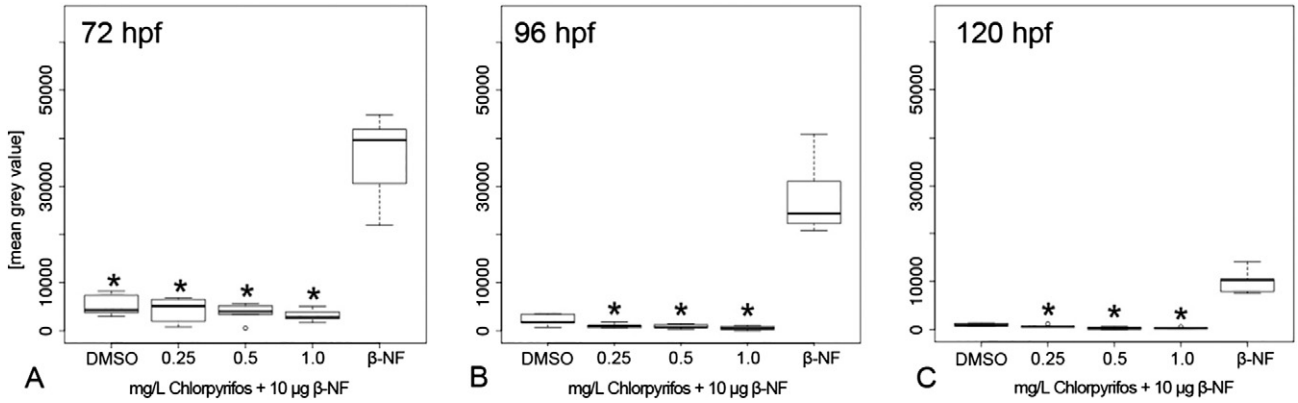


Fig. 7. EROD induction potential expressed as the mean intensity of fluorescence, given as means of the software NIS-Elements 4.0 and diagramed in mean grey value. The mean signal intensity derived from 4 to 6 zebrafish (*Danio rerio*) embryos **continuously** exposed for 72 h, 96 h and 120 h to solvent (0.1% DMSO), 0.25, 0.5 and 1.0 mg/L chlorpyrifos plus 10 µg/L β-naphthoflavone in co-exposure and 10 µg/L β-naphthoflavone in single compound exposure (β-NF). EROD activities are given as box plots for three independent runs per concentration derived from four to six zebrafish embryos each. The box plots show the medians, 25% and 75% percentiles (upper and lower limit of the bar) and outliers >5% and 95% percentiles (°). * indicates treatments significantly different from β-naphthoflavone (β-NF; Levene's test: $\alpha < 0.05$). After exposure to all concentrations of chlorpyrifos and at all developmental stages, EROD activities in zebrafish embryos show a significant decline in signal intensity after an exposure to chlorpyrifos.

conditions, embryos were then immobilized in 0.016% tricaine (methane sulfonate; MS 222) and incubated with 200 mg/L 7-ethoxyresorufin for 20 min. The EROD substrate 7-ethoxyresorufin was prepared as described by Heinrich et al. (2014). Larvae were mounted in a left-down lateral orientation in 1% low-gelling agarose and supplemented with 0.016% tricaine in glass bottom culture dishes (MaTek; Ashland, USA). The agarose was covered with artificial water to prevent dehydration.

Measurement of EROD activity was performed by means of a Nikon ECLIPSE 90i epifluorescence microscope (Nikon Instruments; Tokyo, Japan) equipped with a 10× Nikon Plan Flour water immersion objective (NA 0.3, WD 16.0 m) with an excitation spectrum of 560 ± 40 nm, an emission spectrum of 630 ± 60 nm and a dichromatic beam splitter of 595 nm. Inside the larvae, resorufin formation was demonstrated at 562 nm (excitation) and >590 nm (emission). A Nikon DS-Ri-1 camera was used for imaging. Image acquisition and processing was accomplished with the NIS-Elements 4.0 imaging software (Nikon Instruments). Negative and solvent controls were conducted in artificial water and 0.1 or 0.2% DMSO, respectively. 10 µg/L β-naphthoflavone served as maximum induction control. Since pretests had shown variable signal intensities for different developmental stages as well as for different batches of embryos, the shutter time of the camera was normalized to the maximum EROD induction and negative controls of each exposure scenario and developmental stage. The left edge of the liver or rather intestine was used for the setting of the focal plane for each image. All fish were free of macroscopically discernible symptoms of injury, infection or disease. In total, three independent experiments with different exposure scenarios were carried out with 4–6 embryos each. With progressing development the fluorescence signal of the intestine outshines the signal of the liver tissue, since resorufin accumulates *via* bile in the intestine, which is located immediately below the liver tissue. This leads to difficulties in the differentiation between the two organs. Therefore, both terms, intestine and liver, are used as synonyms in the following text (Kais et al., 2017).

2.7. Overview of exposure scenarios for live-imaging EROD assay

Four different scenarios were used for exposure to chlorpyrifos and Aroclor 1254,

(1) continuous single-compound exposure to chlorpyrifos or Aroclor 1254 at EC_{10} concentrations for up to 72, 96 and 120 h starting from fertilization;
(2) continuous co-exposure to chlorpyrifos or Aroclor 1254 at EC_{10} concentrations each in combination with 10 µg/L β-naphthoflavone for up to 72, 96 and 120 h starting from fertilization;
(3) continuous co-exposure to 0.25, 0.5 and 1.0 mg/L chlorpyrifos or 0.5, 1 and 3 mg/L Aroclor 1254 in combination with 10 µg/L β-naphthoflavone for up to 72, 96 and 120 h starting from fertilization;
(4) 3 h short-term co-exposure of 72, 96 and 120 h old zebrafish embryos to EC_{10} concentrations of chlorpyrifos and Aroclor 1254, respectively.

Each exposure scenario was performed in four replicates for chlorpyrifos and six replicates for Aroclor 1254.

2.8. Semi-quantitative analysis of EROD activity

The liver shape of one characteristic larva exposed to β-naphthoflavone per exposure scenario and developmental stage was automatically detected as maximum EROD induction and defined as ROI ("region of interest") by means of the software NIS-Elements 4.0 (Nikon, Tokyo, Japan). This ROI was then projected onto the livers of all other larvae of the same exposure scenario and developmental stage, and the mean intensity of the fluorescence signal within this region was determined for each individual. The mean fluorescence intensities of 4–6 embryos per concentration, exposure scenario and developmental stage were calculated. In total, the analysis of EROD activity in zebrafish embryos after exposure to chlorpyrifos as well as to Aroclor 1254 was performed in three independent runs per concentration, for which the mean fluorescence intensity was computed from images from 4 to 6 embryos each.

2.9. Statistical analyses

Data were tested for normality using Shapiro-Wilks test (routine "shapiro.test" from the package "stats" in R; RCoreTeam, 2015) and homoscedasticity using Levene's test (routine "leveneTest" from the R package "car"; Fox J., 2011) separately for experiments, exposure duration and developmental stages (time points). Since >5% of all tests revealed non-normality and/or unequal variances, differences in EROD activities between treatment levels and positive or solvent controls were tested non-parametrically using multiple comparisons in the Kruskal-Wallis test (routine "kruskalmc" from the R package "pgirmess"; Giraudoux, 2015).

3. Results

3.1. Standard fish embryo tests with chlorpyrifos

The aim of the fish embryo tests was to identify EC_{10} values as maximum test concentrations that, on the one hand, do not profoundly affect the physiology of the fish, but, on the other hand, are likely to induce EROD activity associated with a distinct fluorescence signal. All fish embryo tests conducted (n = 3) were valid according to OECD TG 236 (OECD, 2013) criteria: In any case, internal and external negative controls exceeded survival rates of 90%, and all positive controls showed mortalities higher than 30% at the end of the test (120 h).

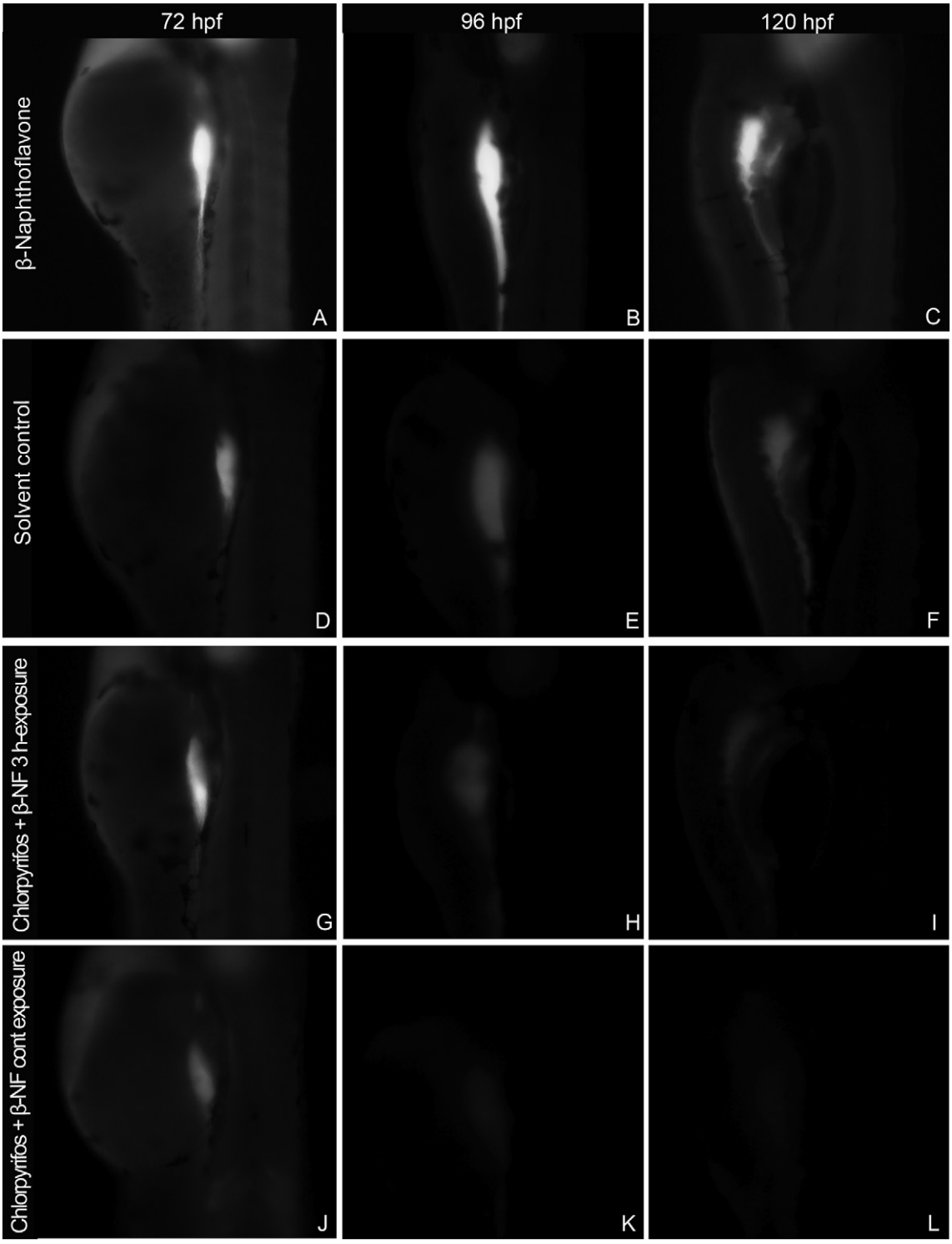


Fig. 8. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) exposure to 10 μg/L β-naphthoflavone (A–C), solvent (0.1% DMSO; D–F) and 0.6 mg/L chlorpyrifos following 3 h (G–I) and continuous (cont.) exposure (J–L). In all developmental stages, embryos of short-term exposure to β-naphthoflavone show bright signals in the liver (A–C), whereas embryos of the solvent control show weaker signals (D–F) and demonstrate no differences to continuous exposure. The signal intensities after short-term exposure to chlorpyrifos also show no differences, if compared to continuous exposure, except for the short-term exposure of 72 h embryos (G), which show stronger fluorescence than after continuous exposure (J) and are more similar to the signal of the solvent control. Epifluorescence microscopy, exposure times: 60 ms (72 h), 30 ms (96 h), 40 ms (120 h).

Chlorpyrifos showed an embryotoxic potential to zebrafish embryos and a clear concentration-response relationship (Fig. 1A, B). The mean EC_{10} value was calculated at 0.62 ± 0.13 and 0.38 ± 0.01 mg/L for 96 and 120 h exposure, respectively. With coefficients of variation (CV; standard deviation compared to the mean in percent) of 21.72 and 3.36% after 96 and 120 h exposure, the reproducibility was found acceptable. Mean EC_{50} values were 1.11 ± 0.15 mg/L and 0.66 ± 0.03 mg/L with CVs of 13.84 and 4.46% after 96 h and 120 h exposure, respectively.

Chlorpyrifos caused a wide range of unspecific sublethal effects: The most frequent effects were pericardial and yolk sac edemata, lack of blood circulation and reduced heart-beat rates. More specific effects such as spinal deformation (convex curvature) and bent tailfins (Fig. 2A) resulted in behavioral deficits such as impaired swimming activities. In addition to these morphological changes, neurological abnormalities could be observed such as fast, uncontrolled convulsions after 96 h and apparent paralysis after 120 h.

Co-exposure to chlorpyrifos and β-naphthoflavone resulted in a 40% increase of toxicity over isolated chlorpyrifos exposure (Fig. 1C) after 120 h. The mean 120 h EC_{10} value of all three independent runs in co-exposure was 0.23 ± 0.01 mg/L (CV: 3.21%). The mean 120 h EC_{50} value was 0.35 ± 0.04 mg/L (CV: 12.53%). In particular, the frequency of edemata was enhanced; in addition, jaw malformation was common (Fig. 2B).

3.2. Standard fish embryo tests with Aroclor 1254

During the range-finding experiments, two different batches of Aroclor 1254 were compared with respect to their toxicity on embryonic stages of zebrafish. Since EC_{10} values differed between 3 and 8 mg/L (details not shown), all EROD measurements were done with the same Aroclor 1254 batch (LB 77779V), the one with the strongest toxic effect. As for chlorpyrifos, the toxicity of Aroclor 1254 increased with concentration and time (Fig. 3). Mean EC_{10} values for Aroclor 1254 were 3.86 ± 1.21 and 2.98 ± 0.19 mg/L after 96 and 120 h exposure, respectively (CVs: 31.32 and 6.45% after 96 and 120 h, respectively).

In general, the majority of effects by Aroclor 1254 became evident after 72 h of exposure, *i.e.* after hatching. Most effects had to be classified as sublethal and were similar to those seen after chlorpyrifos exposure.

Given the prominent effects of Aroclor 1254 exposure after hatch, additional experiments were carried out with dechorionated embryos in order to elucidate a potential barrier function of the zebrafish chorion. Indeed, a comparison of the appearance of effects between chorionated and dechorionated embryos revealed effects in dechorionated embryos much earlier than in chorionated ones (Fig. 4): Whereas 90% of dechorionated embryos showed distinct effects after 72 h, only 5% of the chorionated embryos showed effects, thus indicating a potential barrier function of the chorion for Aroclor 1254. However, at an age of 96 h, both dechorionated and chorionated embryos showed significant morphological effects.

3.3. Live-imaging EROD assay after continuous co-exposure to 10 μg/L β-naphthoflavone and chlorpyrifos at the EC_{10} level

Fig. 5 illustrates the *in vivo* localization of EROD activity after 72 (Fig. 5A, D, G, J), 96 (B, E, H, K) and 120 h (Fig. 5C, F, I, L) exposure of zebrafish embryos to β-naphthoflavone as well as to a combination of chlorpyrifos and β-naphthoflavone. In general, the much brighter signal of β-naphthoflavone (Fig. 5A, B, C) than in the solvent control (Fig. 5D, E, F) indicated strong cytochrome P450 induction over constitutive activities. Since experiments to account for auto-fluorescence by the solvent DMSO gave minimal levels of fluorescence (details not shown), all subsequent measurements were compared to the solvent control (0.1% DMSO).

After 72 h (Fig. 5A) of exposure to β-naphthoflavone, the embryos showed a distinct signal in the liver, which increased in area and signal intensity after 96 h (Fig. 5B), but decreased after 120 h (Fig. 5C). In the solvent controls, embryos showed similar relative induction, however, at lower intensities (Fig. 5D–F). Following isolated exposure to 0.6 mg/L chlorpyrifos (EC_{10}, Fig. 5G–I), the fluorescence intensity was barely detectable after 96 and 120 h, *i.e.* lower than with the solvent control. In order to confirm the inhibition of cytochrome P450 by chlorpyrifos, embryos were exposed to a combination of 10 μg/L β-naphthoflavone and 0.6 mg/L chlorpyrifos (Fig. 5J–L).

3.4. Live-imaging EROD assay after continuous co-exposure to 10 μg/L β-naphthoflavone and different concentrations of chlorpyrifos

In order to illustrate the dose-dependency of cytochrome P450 suppression by chlorpyrifos, embryos were exposed to 0.25, 0.5 and 1.0 mg/L chlorpyrifos in combination with 10 μg/L β-naphthoflavone. Particularly after 96 and 120 h, signal intensities after co-exposure to 0.5 and 1.0 mg/L chlorpyrifos and β-naphthoflavone (Fig. 6J–O) were lower than in the solvent control (Fig. 6D–F). In contrast, the lowest concentration of 0.25 mg/L

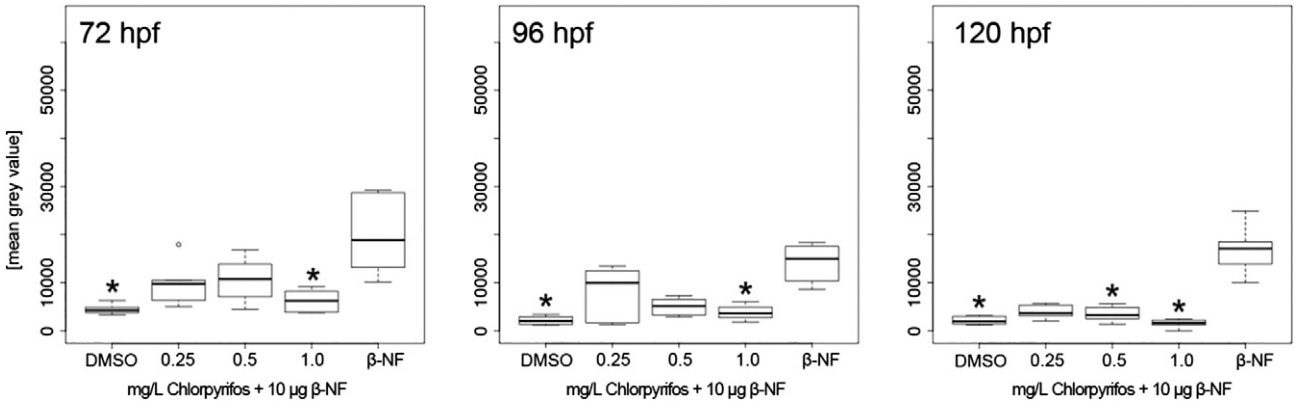


Fig. 9. EROD induction potential expressed as the mean intensity of fluorescence, given as means of the software NIS-Elements 4.0 and diagramed in mean grey value. The mean signal intensity derived from 4 to 6 zebrafish (*Danio rerio*) embryos of 72 h, 96 h and 120 h age **after 3 h exposure** to solvent DMSO 0.1%, 0.25, 0.5 and 1.0 mg/L chlorpyrifos plus 10 μg/L β-naphthoflavone in co-exposure and 10 μg/L β-naphthoflavone in single compound exposure (β-NF). EROD activities are given as box plots for three independent runs per concentration derived from four to six zebrafish embryos each. The box plots show the medians, 25% and 75% percentiles (upper and lower limit of the bar) and outliers >5% and 95% percentiles (°). * indicates treatments significantly different from β-naphthoflavone (β-NF; Levene's test: $\alpha < 0.05$). In general, short-term (3 h) exposure results in higher fluorescence intensity than continuous exposure (*cf.* Fig. 7), especially after 72 h.

chlorpyrifos (Fig. 6G–I) did not inhibit EROD activity completely. These results confirmed the selective time- and dose-dependent inhibition of cytochrome P450 by chlorpyrifos in zebrafish embryos.

Densitometric quantification of fluorescence after β-naphthoflavone exposure documented a time-dependent reduction of EROD induction with mean grey values of approx. 40,000, 20,000 and 10,000 units after 72, 96 and 120 h, respectively (Fig. 7). Likewise, the constitutive activity of cytochrome P450 (DMSO, solvent control) declined from 72 to 120 h; in general, basal EROD activity was reflected by mean grey values clearly below 10,000 units. Only in 72 h old embryos, exposure to 0.1% DMSO resulted in a significant difference to β-naphthoflavone

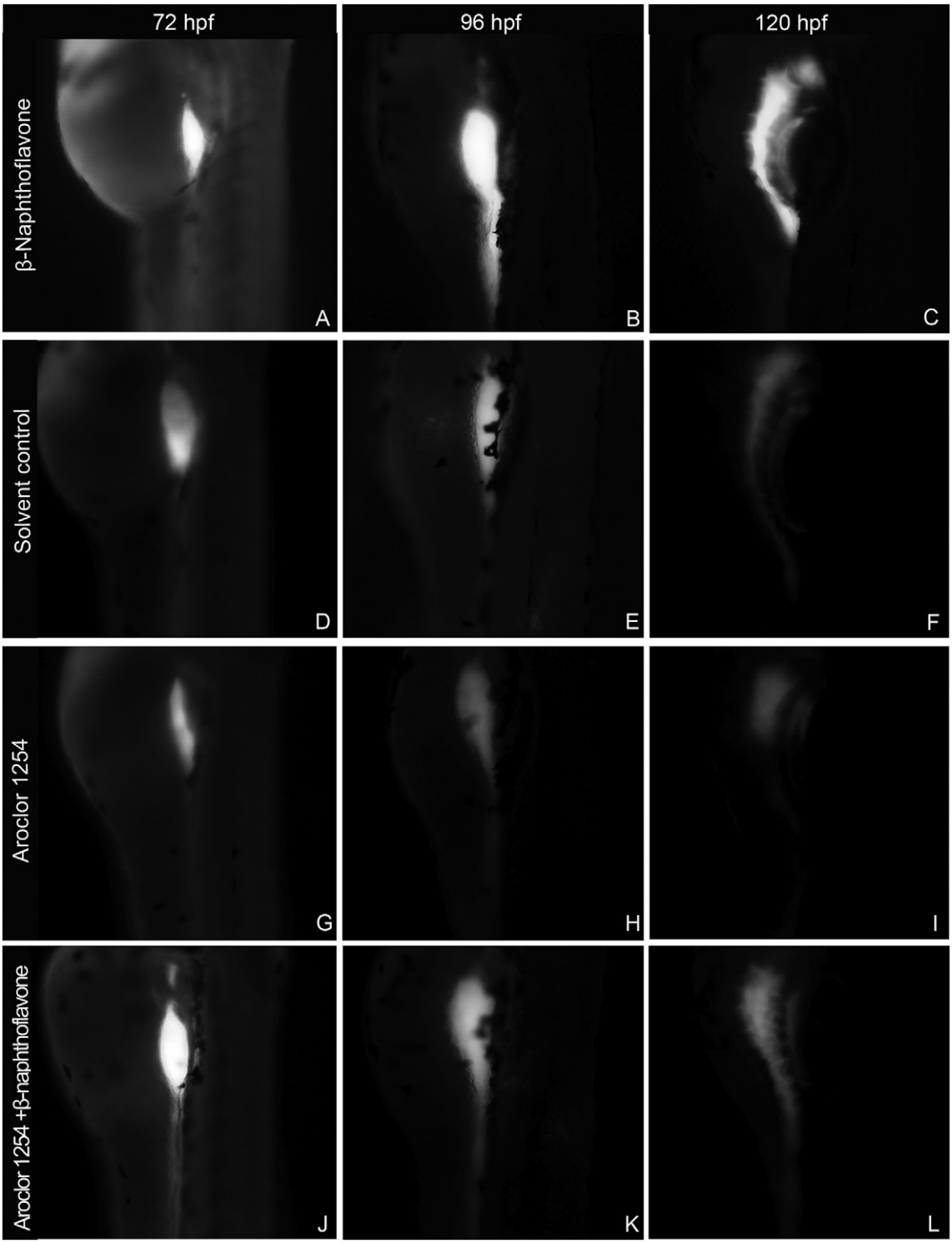


Fig. 10. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) exposure to 10 μg/L β-naphthoflavone (A–C), solvent (0.2% DMSO; D–F), 3 mg/L Aroclor1254 (G–I) and 3 mg/L Aroclor 1254 + β-naphthoflavone (J–L). In all developmental stages, embryos exposed to β-naphthoflavone show a bright signal in the liver, whereas the embryos of the solvent control show a weaker signal reflecting constitutive activity. Signal intensities after single Aroclor 1254 exposure are as strong as those of the solvent controls. The fluorescence after 72 h co-exposure are as strong as those of β-naphthoflavone, whereas the signals after both 96 and 120 h show lower fluorescence intensities than β-naphthoflavone. Epifluorescence microscopy, exposure time 40 ms.

($\alpha < 0.05$). However, after exposure to all concentrations of chlorpyrifos and at all developmental stages, zebrafish embryos show a significant decline in signal intensity, if compared to β-naphthoflavone. Likewise, with mean grey values of <5000 units, the combined exposure to chlorpyrifos and β-naphthoflavone produced even lower EROD activities than exposure to the solvent control.

3.5. Live-imaging EROD assay after short-term (3 h) co-exposure to 10 μg/L β-naphthoflavone and chlorpyrifos at the EC_{10} level

In order to avoid fluctuations in CYP1A activity due to prolonged exposure (*e.g. via* accumulation of chlorpyrifos) and to further elucidate the time-dependency of EROD inhibition activity by chlorpyrifos, the exposure time was restricted to 3 h in 72, 96 and 120 h old zebrafish embryos. For comparison, exposures to β-naphthoflavone as well as the solvent control were also reduced to 3 h.

Short-term exposure to β-naphthoflavone and the solvent control (Fig. 8A–F) resulted in signal intensities similar to those after continuous exposure (*cf.* Fig. 6A–F). Likewise, a comparison of the 3 h short-term exposure (Fig. 8G–I) and the permanent exposure to chlorpyrifos (Fig. 8J–L) revealed almost no differences in signal intensities, except for the short-term exposure of 72 h old embryos (Fig. 8G) which gave slightly stronger fluorescence than the continuous exposure (Fig. 8J). However, in 96 and 120 h old embryos, EROD activities were completely inhibited following both exposure scenarios (Fig. 8H, I, K, L).

Quantification of fluorescence after 3 h short-term exposure to β-naphthoflavone gave mean grey values of 20,000–15,000 units independent of developmental stage (Fig. 9). If compared to continuous exposure (Fig. 7), the signal intensities of both β-naphthoflavone and the solvent controls were lower after 3 h exposure. In contrast, 3 h combinations of chlorpyrifos and β-naphthoflavone resulted in higher EROD responses (Fig. 9) than after continuous exposure (Fig. 7), especially after 72 h. Again, the solvent controls showed mean grey values <5000 units identical to basal EROD activity. Whereas EROD activities in embryos exposed to 0.1% DMSO were consistently significantly different from β-naphthoflavone, at all times considered; only the embryos exposed to 1 mg/L chlorpyrifos plus 10 μg/L β-naphthoflavone were significantly different from β-naphthoflavone alone (Levene's test: $\alpha < 0.05$).

3.6. Live-imaging EROD assay after continuous co-exposure to 10 μg/L β-naphthoflavone and Aroclor 1254 at EC_{10} levels

Fig. 10 summarizes the *in vivo* localization of EROD activity in 72 (Fig. 10A, D, G, J), 96 (B, E, H, K) and 120 h old zebrafish embryos (Fig. 10C, F, I, L) after isolated exposure to 3 mg/L Aroclor 1254 (Fig. 10G–I) and after the co-exposure of Aroclor 1254 and β-naphthoflavone (Fig. 10J–L). If compared to the high induction potential of β-naphthoflavone (Fig. 10A–C) and the weak signal of the solvent control (Fig. 10D–F), isolated exposure to Aroclor 1254 (Fig. 10G–I) did not differ essentially from the solvent control. Following 72 h co-exposure to Aroclor 1254 and β-naphthoflavone (Fig. 10J), however, the signal intensity of EROD-based fluorescence was as strong as that after isolated exposure to β-naphthoflavone (Fig. 10A). In contrast, 96 and 120 h co-exposure (Fig. 10K–L) resulted in less prominent EROD signals than isolated exposure to the positive control β-naphthoflavone; however, fluorescence was never below that after exposure to the solvent control DMSO.

3.7. Live-imaging EROD assay after continuous co-exposure to 10 μg/L β-naphthoflavone and different concentrations of Aroclor 1254

In order to more precisely characterize EROD inhibition by Aroclor 1254, zebrafish embryos were exposed to 0.5, 1 and 3 mg/L Aroclor 1254 in combination with 10 μg/L β-naphthoflavone (Fig. 11). Again, β-naphthoflavone exposure resulted in strong fluorescence (Fig. 11A–C), whereas the solvent control only produced a very weak signal (Fig. 11D–F). Whereas at the lowest concentration at 0.5 mg/L Aroclor 1254 did not decrease the signal intensity produced by β-naphthoflavone (Fig. 11G–I), 1 and 3 mg/L Aroclor 1254 (Fig. 11J–O) clearly reduced the fluorescence signal by β-naphthoflavone after 96 (Fig. 11K, N) and 120 h exposure (Fig. 11L, O). After 72 h exposure, no difference was evident between the exposure to the combination of any concentration of Aroclor 1254 plus 10 μg/L β-naphthoflavone and isolated exposure to 10 μg/L β-naphthoflavone.

Densitometric analyses gave mean grey values for isolated exposure to β-naphthoflavone between 45,000 after 72 h and 20,000 units after 96 h and 120 h (Fig. 12). Likewise, the signal intensities of the solvent controls showed a decline with ageing embryos. Given the need for a higher DMSO concentration of 0.2%, all embryos exposed to the solvent control showed a significant difference to β-naphthoflavone. Only long co-exposure to Aroclor 1254 and β-naphthoflavone (≥96 and 120 h) at high Aroclor 1254 concentrations (1.0 and 3.0 mg/L), results in a significant decline of EROD signal intensities (Levene's test: $\alpha < 0.05$).

3.8. Live-imaging EROD assay after short-term (3 h) co-exposure to 10 μg/L β-naphthoflavone and Aroclor 1254 at EC_{10} levels

In order to find out whether the decrease in signal intensity after prolonged exposure to higher Aroclor concentrations was due to time-dependent accumulation, the duration of the exposure to the combination of Aroclor 1254 and β-naphthoflavone was limited to 3 h. As described above, EROD activities after short-term and continuous exposure to β-naphthoflavone (Fig. 13A–C) and solvent controls (Fig. 13D–F) were comparable (*cf.* Fig. 10A–F). However, whereas the continuous exposure to 3 mg/L Aroclor 1254 showed a time-dependent reduction in signal intensities (Fig. 10J–L), the 3 h short-term exposure did not show any decline in signal intensities even in advanced developmental stages of 96 or 120 h.

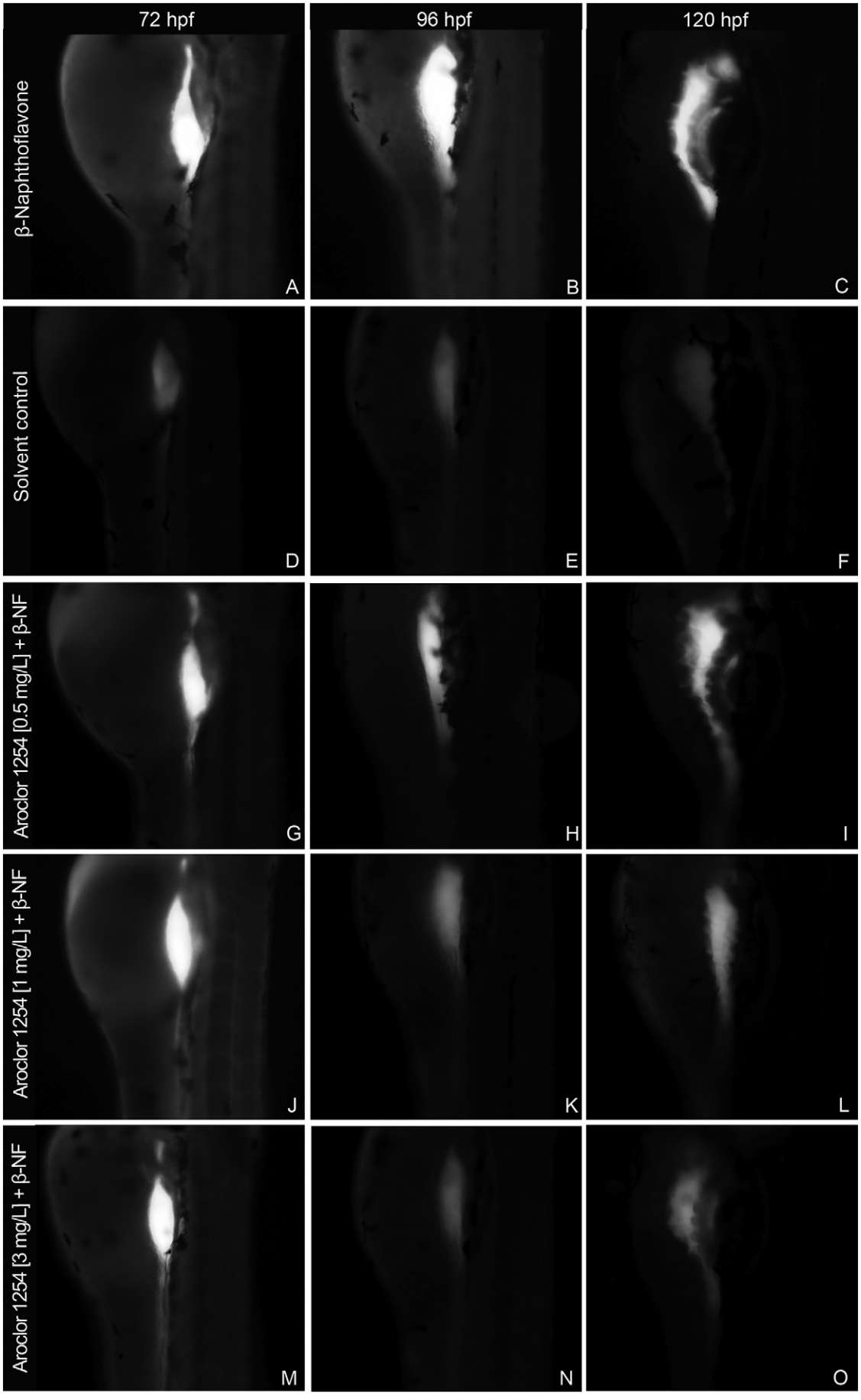
Densitometric quantification of the EROD induction in 72, 96 and 120 h old zebrafish embryos after 3 h short-term exposure to β-naphthoflavone yielded high mean grey values of about 45,000, 58,000 and 40,000 units at 72, 96 and 120 h (Fig. 14). In general, after short-term exposure, the signal intensities after exposure to both β-naphthoflavone and the solvent control were slightly higher than after continuous exposure (*cf.* Fig. 10). Only in 72 h old zebrafish embryos, exposure to 0.2% DMSO produced a significant difference from the positive control β-naphthoflavone (Levene's test: $\alpha < 0.05$). In contrast, the combination of Aroclor 1254 plus β-naphthoflavone never resulted in a significant difference from the isolated exposure to β-naphthoflavone.

4. Discussion

4.1. Toxicity of chlorpyrifos and Aroclor 1254 after single-substance exposure

The embryotoxic potential of chlorpyrifos to zebrafish embryos showed a clear positive dose-response relationship. The EC_{10} value of

Fig. 11. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos after 72 (A, D, G, J, M), 96 (B, E, H, K, N) and 120 h (C, F, I, L, O) exposure to 10 μg/L β-naphthoflavone (A–C), solvent (0.2% DMSO; D–F), 0.5 (G–I), 1 (J–L) and 3 mg/L (M–O) Aroclor 1254 + β-naphthoflavone. In all developmental stages, embryos exposed to β-naphthoflavone show a bright signal in the liver, embryos exposed to the solvent control show a weaker signal reflecting constitutive activity. The signal intensities in the co-exposure approach after 72 h are as strong as those in single β-naphthoflavone exposure, whereas the signals after both 96 and 120 h co-exposure show lower signal intensities than β-naphthoflavone. In contrast, the signal intensities do not show any decrease at the lowest concentration of Aroclor 1254 (G–I). Epifluorescence microscopy, exposure time 40 ms.


72 hpf
96 hpf
120 hpf
β-Naphthoflavone
Solvent control
Aroclor 1254 [0.5 mg/L] + β-NF
Aroclor 1254 [1 mg/L] + β-NF
Aroclor 1254 [3 mg/L] + β-NF
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

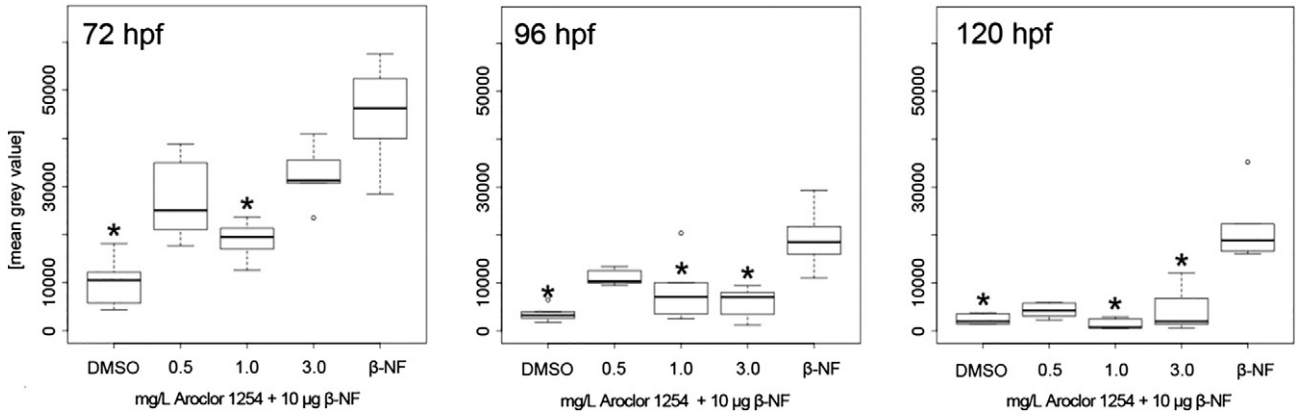


Fig. 12. EROD induction potential expressed as the mean intensity of fluorescence, given as means of the software NIS-Elements 4.0 and diagramed in mean grey value. The mean signal intensity derived from 4 to 6 zebrafish (*Danio rerio*) embryos **continuously** exposed for 72 h, 96 h and 120 h to solvent (0.1% DMSO), 0.5, 1.0 and 3.0 mg/L Aroclor 1254 plus 10 µg/L β-naphthoflavone in co-exposure and 10 µg/L β-naphthoflavone in single compound exposure (β-NF). EROD activities are given as box plots for three independent runs per concentration derived from four to six zebrafish embryos each. The box plots show the medians, 25% and 75% percentiles (upper and lower limit of the bar) and outliers >5% and 95% percentiles (°). * indicates treatments significantly different from β-naphthoflavone (β-NF; Levene's test: $\alpha < 0.05$). EROD activities in zebrafish embryos show only at higher Aroclor 1254 concentrations (1.0–3.0 mg/L) and at later developmental stages (≥96 h) a significant decline in signal intensity.

0.6 mg/L is in line with data by Kienle et al. (2009), who observed spinal deformations starting from 0.5 mg/L at an age of 96 h. Next to morphological deformations, Jin et al. (2015), Kienle et al. (2009) as well as Levin et al. (2004) described typical behavioral effects such as reduced swimming activity and muscular convulsions at even lower concentrations of 0.1–0.3 mg/L chlorpyrifos, thus confirming the need for testing at concentrations below EC_{10} values in order to pick up specific modes of action (Stengel et al., 2017). As a consequence, the present study used EC_{10} levels as highest test concentrations, which were assumed to be high enough for both adaptive reactions and induction of biochemical responses (Braunbeck, 1992, 1994; Braunbeck et al., 1998).

With respect to variability of chlorpyrifos EC data in literature, all studies used nominal concentrations, with several studies describing a low retrieval rate of chlorpyrifos. By means of GC–MS, Kienle et al. (2009) determined a retrieval rate of chlorpyrifos of about 51.6%, and Tilton et al. (2011) observed a decline of chlorpyrifos concentrations during static exposure of fish by >80% after 24 h, thus indicating efficient uptake of chlorpyrifos by fish. Since the present study used a semi-static exposure scenario, constant distribution of hydrophobic substances such as chlorpyrifos and Aroclor 1254 to the embryos should have resulted in even higher uptake rates.

Obviously, the toxicity of both chlorpyrifos and Aroclor 1254 is time-dependent, since the majority of effects only became evident after 72 h. However, since hatching in zebrafish embryos usually starts from an age of 72 h, the increase in toxicity might also be due to facilitated uptake after hatch. Most effects such as pericardial and yolk sac edemata or spinal deformation and twitching after hatching were categorized as sublethal. In fact, edemata in zebrafish embryos have repeatedly been described as unspecific effects (Bachmann, 2002; Carney et al., 2006; Jin et al., 2015; Lee et al., 2007; Quilang et al., 2008) and frequently accompanied cardiovascular dysfunction (Barron et al., 2004; Billiard et al., 2008; Carney et al., 2006; Sundberg et al., 2005). In contrast, aberrations in swimming behavior in conjunction with spinal deformations have repeatedly been described in zebrafish larvae particularly after exposure to Aroclor 1254 (Billsson et al., 1998; Ju et al., 2012; Sisman et al., 2007) or chlorpyrifos (Jin et al., 2015; Kienle et al., 2009; Levin et al., 2004; Straus et al., 2000).

4.2. Mixture effects of chlorpyrifos and β-naphthoflavone

According to Neal and Halpert (1982), the sulfur atom released from phosphorothionates during detoxification processes, is highly reactive and is believed to immediately bind to the heme iron of cytochrome P450, thus inhibiting its activity. Thus, increased contents of free reactive sulfur probably account for the increase in toxicity observed for the combination of chlorpyrifos and β-naphthoflavone.

β-Naphthoflavone, a synthetic flavone, induces zebrafish CYP1A protein and EROD activity approximately threefold above controls (Troxel et al., 1997). Co-exposure to chlorpyrifos and β-naphthoflavone is likely to induce cytochrome P450 activity and formation of reactive sulfur, which in turn leads to inhibition of detoxification processes *via* competition with the substrate for the active site of CYP1A. Potentiation of chlorpyrifos acute toxicity by pretreatment with β-naphthoflavone has already been described by Sultatos et al. (1985), especially in terms of cardiovascular effects by Billiard et al. (2006), Brown et al. (2015), Wassenberg and Di Giulio (2004) as well as Wassenberg et al. (2005). All of these communications are in line with our observations of a >40% increase in toxicity by the combination of chlorpyrifos with β-naphthoflavone.

4.3. Delayed toxicity by Aroclor 1254

Given the delayed toxicity of Aroclor 1254 due to its high log P_{OW}, experiments with dechorionated embryos were carried out to clarify the potential barrier function of zebrafish chorion. The zebrafish chorion is a 1.5–2.5 µm thick acellular envelope consisting of three layers, the middle and inner ones of which are pierced by pore canals (Rawson et al., 2000), which have been speculated to potentially restrict the uptake of compounds depending on their electric charge of specific moieties along the pore canals.

Aroclor 1254 is chemically fairly inert and resistant to oxidation or reduction. Physical and chemical properties vary with the degree of chlorination (UNEP-Chemicals, 1999). The higher the number in the name of the PCB, the higher the proportion of highly chlorinated PCBs in a mixture (Walker et al., 2012). Individual congeners of PCBs vary in their stereochemistry depending on the position of substitution by chlorine atoms. Due to the bulkiness of chlorine atoms, *ortho*-substitution leads to a more globular and bulky molecular conformation. Chlorine atoms affect numerous parameters such as polarity, steric conditions, functional groups and structural configurations. The ionizable moieties of Aroclor 1254 are an important aspect for its behavior in water and its ability to pass the chorion and might account for the delay in toxicity observed in the present study.

Prior to hatching, the uptake of chemicals is almost exclusively due to diffusion processes, which primarily depend upon lipophilicity and specific molecular properties such as bulkiness (Jones, 2010). Given the relatively high chlorine contents, the diffusion rate of Aroclor 1254 may be speculated to be rather slow despite its lipophilicity (K_{OW}

6.1–6.8; Brauer, 2012). With hatching of zebrafish embryos at about 72 h, however, not only embryonic physiology undergoes marked modifications, but exposure to xenobiotics may also change fundamentally: Only in post-hatch larvae, the mouth opens and gills become functional, thus allowing exposure *via* the anterior digestive tract with its greatly increased surface (Kimmel et al., 1995). The presence of functional gills and the increasing potential for dietary exposure to xenobiotics, which becomes particularly important from 120 h, appear to be crucial for the increase in absorption and toxicity of Aroclor 1254 after hatching.

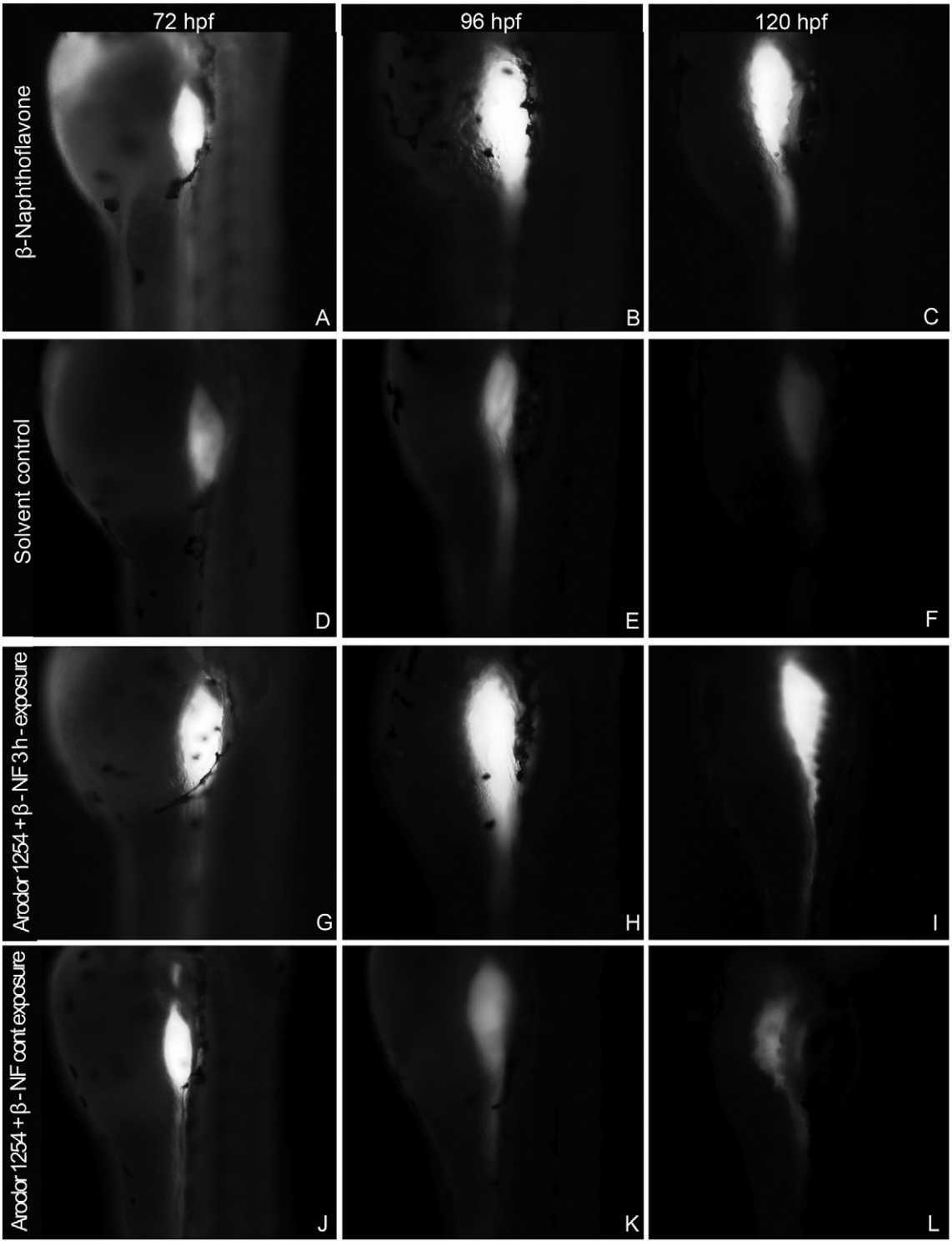


Fig. 13. *In vivo* localization of EROD activities in zebrafish (*Danio rerio*) embryos exposed at the age of 72 (A, D, G, J), 96 (B, E, H, K) and 120 h (C, F, I, L) to 10 μg/L β-naphthoflavone (A–C), solvent (0.2% DMSO; D–F) and 3 mg/L Aroclor 1254 for 3 h (G–I) as well as after continuous (cont.) exposure (J–L). In all developmental stages, embryos of short-term exposure to β-naphthoflavone show bright signals in the liver (A–C), whereas the embryos of the solvent control show weaker signals (D–F) and demonstrate no differences to continuous exposure. Signal intensities after short-term exposure to Aroclor 1254 are higher than after continuous exposure (G–I) and are comparable to the signals following β-naphthoflavone-exposure. The continuous exposure to 3 mg/L Aroclor 1254 (J–L) results in a time-dependent decline in signal intensities, with a strong signal after 72 h (J) and lower signals after 96 (K) and 120 h (L). Epifluorescence microscopy, exposure time: 40 ms.

Given its low solubility in water (12–70 μg/L; Sklarew and Girvin, 1987), Aroclor 1254 had to be applied with a fairly high solvent concentration of 0.2% DMSO, which is higher than recommended in the difficult substances guidance document by the (OECD, 2010). Previous studies showed that DMSO facilitates the passage across the zebrafish chorion (Kais et al., 2013), but the study also showed that the increased permeability of the chorion by DMSO was limited and that other parameters such as molecular bulkiness may be more dominant features to control the passage across the chorion. In fact, despite the relatively high levels of DMSO in the present study, no effects were seen in the solvent control until hatching. Consequently, each sample contained identical DMSO concentration, which guaranteed for comparable permeability of the chorion throughout the tests.

4.4. Live-imaging of EROD activity after exposure to chlorpyrifos

The fluorescence signal observed in zebrafish embryos exposed to the positive control β-naphthoflavone followed the patterns described previously (Kais et al., 2017): The bright fluorescence signal induced by β-naphthoflavone in the liver of 72 h old embryos increased in area and signal intensity after 96 h, then remained constant and decreased after 120 h. In contrast, at all times, the faint signal after exposure to the solvent control reflected constitutive activity.

Following exposure to isolated chlorpyrifos, the fluorescence intensity was too weak to allow a detailed analysis. Combined exposure to chlorpyrifos and β-naphthoflavone resulted in a clear-cut attenuation of the β-naphthoflavone-induced EROD activity after 72 h; after 96 and 120 h, fluorescence was completely blocked by chlorpyrifos, indicating that chlorpyrifos acts as an inhibitor for EROD activity in zebrafish. To date, this study represents the first *in vivo* approach to visualize the inhibition of cytochrome P450 by chlorpyrifos in zebrafish embryos. As already discussed above, cytochrome P450 inhibition may be caused by reactive sulfur (Neal and Halpert, 1982), which is a product of detoxification processes (Fukuto, 1990; Straus et al., 2000; Tang et al., 2002) and results in elevated contents of reactive sulfur.

Whereas it is widely accepted that dioxin-related toxicity is largely mediated by the Ah receptor (AhR), the role of CYP1A in causing toxicity is discussed controversially. On the one hand, several studies have shown that chemical inhibition or knockdown of CYP1A either reduces (Cantrell et al., 1996; Dong et al., 2002) or does not modify toxic responses to dioxins during zebrafish development (Carney et al., 2004). On the other hand, Billiard et al. (2006) observed the opposite effect in zebrafish embryos, which had been treated to temporarily knock down expression of CYP1A protein: The resulting increase of PAH-induced effects suggests that CYP1A is an adaptive response providing protection for the developing vertebrate embryo. A protective role was also concluded by Mattingly and Toscano (2001), who failed to detect CYP1A protein and activity in 3 d old zebrafish embryos and postulated post-translational silencing instead of activation of CYP1A expression to protect developing organisms from CYP1A metabolic activation after exposure to AhR ligands. More recently, however, several studies demonstrated cytochrome P450 activity in zebrafish embryos as young as 8 h (Jonsson et al., 2007; Otte et al., 2010). In fact, the present study provides conclusive evidence of CYP1A activity from the age of 72 h and indicates protective mechanisms, since co-exposure to chlorpyrifos and β-naphthoflavone resulted in increased toxicity correlating to decreasing CYP1A activity. However, a number of questions still need to be resolved to fully understand the role of CYP1A in early developmental stages of zebrafish.

The fact that EROD induction in 72 h old zebrafish embryos could clearly be documented independent of the exposure scenario and toxicant concentration indicates stability of CYP1A activity at this stage. In the course of the formation of a functioning liver in zebrafish, Korzh et al. (2008) observed that liver vasculogenesis and functional blood circulation starts at 55 and 72 h, respectively. At the age of 72 h, the zebrafish liver has reached full functionality, and the embryo prepares for hatching. Various studies have described a several-fold increase of basal expression of CYP1A in 72 h old zebrafish (Bräunig et al., 2015; Jonsson et al., 2007) and killifish (Binder and Stegeman, 1984; Wang et al., 2006). A comparison of 3 h short-term exposure at 72 h old embryos and 3 h short-term exposure at 96 h old embryos indicates that CYP1A basal contents may vary in embryos of different age. Identical exposure durations of 3 h lead to lower inhibition of β-naphthoflavone activity in 72 h old embryos than in 96 h old embryos, where complete inhibition can be observed. These findings suggest that CYP1A play a crucial role shortly after hatching, since CYP1A contents at 72 h are as high as to induce EROD activity regardless of toxicant concentration or exposure time.

In general, the quantification of *in vivo* EROD-related fluorescence demonstrated that all embryos co-exposed to chlorpyrifos and β-naphthoflavone displayed significantly lower EROD activities than after isolated exposure to β-naphthoflavone independent of concentration and developmental stage (*cf.* Fig. 7). Furthermore, in 72 h old embryos, statistical analyses showed a concentration-dependent decrease

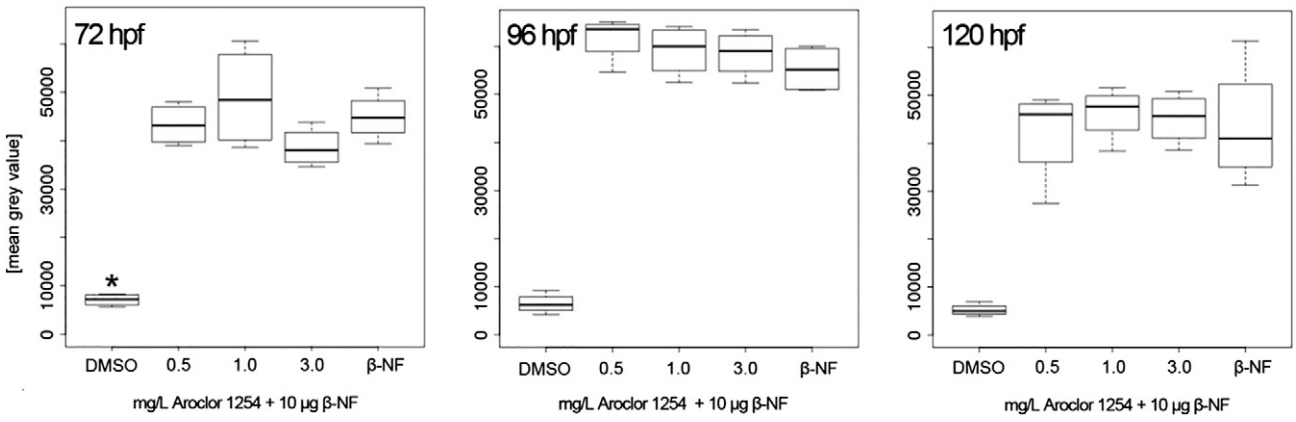


Fig. 14. EROD induction potential expressed as the mean intensity of fluorescence, given as means of the software NIS-Elements 4.0 and diagramed in mean grey value. The mean signal intensity derived from 4 to 6 zebrafish (*Danio rerio*) embryos of 72 h, 96 h and 120 h age **after 3 h exposure** to solvent DMSO 0.1%, 0.5, 1.0 and 3.0 mg/L Aroclor 1254 plus 10 μg/L β-naphthoflavone in co-exposure and 10 μg/L β-naphthoflavone in single compound exposure (β-NF). EROD activities are given as box plots for three independent runs per concentration derived from four to six zebrafish embryos each. The box plots show the medians, 25% and 75% percentiles (upper and lower limit of the bar) and outliers >5% and 95% percentiles (°). * indicates treatments significantly different from β-naphthoflavone (β-NF; Levene's test: $\alpha < 0.05$). After short-term (3 h) exposure, EROD activities in zebrafish embryos show at no developmental stages and at no concentration a significant decline in signal intensity.

in EROD activity by chlorpyrifos, which is in line with imaging analysis data (*cf.* Fig. 6G, J, M). These observations are corroborated by studies with juvenile Chinook salmon (*Oncorhynchus tshawytscha*), where suppression of CYP1A protein levels by about 30% following exposure to 7.8 μg/L chlorpyrifos (quantified by gas chromatography/mass spectrometry; Wheelock et al., 2005).

A comparison of the 3 h short-term (Fig. 8G–I) exposure to the permanent exposure to chlorpyrifos (Fig. 6J–L) revealed almost no differences in signal intensities, except for the 72 h short-term exposure (Fig. 8G), which inhibits fluorescence <72 h continuous exposure (Fig. 8J); this observation confirmed the assumption of high and stable CYP1A contents in 72 h old embryos, since EROD activities were only attenuated, but not inhibited.

Densitometric analyses revealed that embryos co-exposed to chlorpyrifos and β-naphthoflavone for 3 h displayed significantly lower fluorescence signals in 120 h old embryos than those induced by β-naphthoflavone (Fig. 9) only at higher concentrations of chlorpyrifos (*e.g.* 0.5 and 1 mg/L). Consequently, in 72 h as well as in 96 h old embryos, the 3 h short-term exposure resulted in stronger fluorescence intensities than continuous exposure. Particularly, low concentration levels (0.25 mg/L) in combination with young embryos (72 and 96 h) seem to be an exposure scenario that is less sensitive to evaluate short-term EROD inhibition.

However, the concentration *per se* cannot be the main reason for the steady signal in 72 h old embryos, since EROD activities consistently decreased at later developmental stages after short-time exposure. More likely, toxicokinetics or rather the exposure scenario plays a crucial role: As discussed above, with hatching exposure pathways to xenobiotics change, thus allowing a more intensive exposure *via* the anterior intestinal tract and gills and imply a more intensive exposure to toxicants. At a standard temperature of 27 °C, the time of hatching is scattered over the entire third day of development (Kimmel et al., 1995), indicating considerable variability at this developmental stage.

4.5. Live-imaging of EROD activity after exposure to Aroclor 1254

Since the induction of cytochrome P450s in fish following exposure to the model PCB mixture Aroclor 1254 has repeatedly been documented (for review see Goksoyr, 1995), the failure to visualize CYP1A induction in zebrafish embryo liver was unexpected. Aroclor 1254 contains >60 to 80 PCB congeners at analytically detectable levels (Bedard et al., 1987). In general, PCB congeners can be divided into non-*ortho* PCBs, which have a coplanar configuration, and *ortho*-substituted PCBs with a non-coplanar configuration (Al-Salman and Plant, 2012). The coplanar PCBs produce toxic effects similar to those by TCDD, which are referred to as dioxin-like PCBs. Non-coplanar PCBs carry *ortho* chlorine substituents on the biphenyl ring, which prevents a simple planar structure and significantly reduces the affinity to the Ah-receptor (Al-Salman and Plant, 2012). Non-coplanar compounds dominate industrial PCB mixtures (Ballschmiter and Zell, 1980), and Aroclor 1254 consists of di-*ortho*-substituted congeners to >50% (Giesy and Kannan, 1998; Schulz et al., 1989).

Next to reduced affinity to the Ah receptor, Suh et al. (2003) demonstrated that di-*ortho*-substituted PCBs are likely to act as inhibitors of AhR-mediated activation in murine cell lines. Suh et al. (2003) analyzed the antagonist activity on CYP1A induction of several di-*ortho*-substituted PCB congeners in combination with Ah receptor agonists such as 2,3,7,8-TCDD. In contrast to non-*ortho*-substituted congeners, which showed additive effects on CYP1A1 induction in combination with TCDD, all di-*ortho*-substituted PCBs produced antagonism (Suh et al., 2003). The molecular mechanisms underlying this antagonism are presently unknown.

As a further complication, non-linear dose-response relationships have been reported: Bannister et al. (1987), *e.g.*, reported that Aroclor 1254 produced a 20–30% inhibition of TCDD-induced EROD activity in rat hepatoma and mice, when submaximal concentrations of TCDD were administered. According to Bannister et al. (1987), the partially antagonistic activity of Aroclor 1254 becomes less effective with higher levels of the agonist TCDD, *e.g.*, an increased dose of TCDD compensates for at least part of the antagonism by Aroclor 1254. Bannister et al. (1987) hypothesized that the antagonistic activity by Aroclor 1254 is related to the competitive inhibition of TCDD-binding to the Ah receptor and is not due to cellular toxicity caused by Aroclor 1254.

These conclusions are in line with our observations, since Aroclor 1254 alone as well as in co-exposure with β-naphthoflavone produced a definite decline in EROD activity after 96 and 120 h. This could be corroborated by densitometry, which showed a significant decline in fluorescence intensities at higher concentrations of Aroclor 1254 (1.0–3.0 mg/L) and at later developmental stages (96–120 h old embryos). Troxel et al. (1997) described a lack of induction of CYP1A in adult zebrafish liver following both dietary exposure and intraperitoneal administration of Aroclor 1254. Indeed, several studies in fish using high doses of β-naphthoflavone, benzo[*a*]pyrene, or planar PCBs have demonstrated that high doses of these inducers can lead to inhibition of activity or mRNA of the protein being induced (Goddard et al., 1987; Gooch et al., 1989; Haasch et al., 1993; Melancon and Lech, 1983).

However, incomplete inhibition may also be caused by toxicokinetics: As mentioned above, Aroclor 1254 is a bulky lipophilic molecule which results in delayed inhibition due to slow diffusion rates. Bioconcentration rates, however, are not only a consequence of lipophilicity (K_{OW}), but also of the degree of PCB chlorination (Fox et al., 1994). In the environment, PCB mixtures with tetra-, penta-, hexa- and heptachlorinated isomers accumulate to the greatest extent (Bruggeman et al., 1984; Kodavanti et al., 2001; Shaw and Connell, 1982). Therefore, as a pentachlorobiphenyl, Aroclor 1254 is supposed to bioaccumulate to high internal concentrations with prolonged time, which may explain the decrease in EROD activity after continuous exposure for >72 h. The lack of decrease in EROD activity after short-term exposure strongly supports a toxicokinetic mechanism, since Aroclor 1254 was apparently not able to decrease the EROD activity within 3 h (*cf.* Figs. 13G–I and 14).

Gooch et al. (1989) discussed multiple plausible mechanisms for the suppression of CYP1A activity, including hepatotoxicity, alterations in heme synthesis or suicide substrate inactivation. However, to date, the molecular mechanisms underlying such suppressive effects on EROD activity remain unknown and need further exploration, *e.g.*, assessment of CYP1A mRNA inducibility, to avoid misinterpretations of toxic potentials by using CYP1A catalytic activity as a biomarker for monitoring exposure of fish to environmental pollutants such as PCBs.

5. Conclusions

The toxicity of both chlorpyrifos and Aroclor 1254 proved specifically time-dependent in zebrafish embryos, since the majority of effects only became evident after 72 h. The combination of chlorpyrifos and β-naphthoflavone in particular showed an increase in toxicity by >40% and resulted in a clear-cut attenuation of the β-naphthoflavone-induced EROD activity. A smaller decline in EROD activity was observed for Aroclor 1254 in co-exposure with β-naphthoflavone at later developmental stages (96–120 h old embryos). The delayed toxicity and deferred attenuation of the fluorescence signal after exposure to Aroclor 1254 demonstrate the importance of toxicokinetic mechanisms and, thus, need to be considered more carefully. *Via* densitometric quantification and subsequent statistical assessment, this study represents an *in vivo* approach to visualize not only induction, but also inhibition of CYP1A and provides the opportunity to more specifically investigate the role of CYP1A.

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