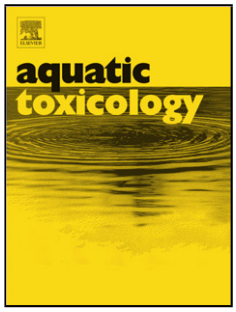
Accepted Manuscript



Title: Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae

Author: Carolina Di Paolo Ksenia J. Groh Markus Zennegg Etiënne L.M. Vermeirssen Albertinka J. Murk Rik I.L. Eggen Henner Hollert Inge Werner Kristin Schirmer

PII: S0166-445X(15)30073-4
DOI: http://dx.doi.org/doi:10.1016/j.aquatox.2015.10.014
Reference: AQTOX 4219

To appear in: *Aquatic Toxicology*

Received date: 27-5-2015
Revised date: 18-10-2015
Accepted date: 20-10-2015

Please cite this article as: Paolo, Carolina Di, Groh, Ksenia J., Zennegg, Markus, Vermeirssen, Etiënne L.M., Murk, Albertinka J., Eggen, Rik I.L., Hollert, Henner, Werner, Inge, Schirmer, Kristin, Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae.Aquatic Toxicology http://dx.doi.org/10.1016/j.aquatox.2015.10.014

Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae

Carolina Di Paolo [a,b,#,*], Ksenia J. Groh [c,d,#], Markus Zennegg [e], Etiënne L. M. Vermeirssen [a], Albertinka J. Murk [f,g], Rik I. L. Eggen [c,h], Henner Hollert [b], Inge Werner [a], Kristin Schirmer [c,h,i]

[a] Swiss Centre for Applied Ecotoxicology Eawag-EPFL, 8600 Dübendorf, Switzerland

[b] Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, 52074, Aachen, Germany

[c] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

[d] ETH Zürich, Department of Chemistry and Applied Biosciences, 8093 Zürich, Switzerland

[e] Empa, Swiss Federal Laboratories for Materials Science and Technology, Laboratory for Advanced Analytical Technologies, 8600 Dübendorf, Switzerland

[f] Wageningen University, Marine Animal Ecology Group, 6708WD Wageningen, The Netherlands

[g] IMARES, Institute for Marine Resources and Ecosystem Studies, Wageningen UR, 1780 AB Den Helder, The Netherlands

[h] ETH Zürich, Department of Environmental Systems Science, 8092 Zürich, Switzerland

[i] EPF Lausanne, School of Architecture, Civil and Environmental Engineering, 1015 Lausanne, Switzerland

[*]: To whom correspondence should be addressed at Department of Ecosystem Analysis, Institute for Environmental Research (Biology 5), RWTH Aachen University, Worringerweg 1, D-52074, Aachen, Germany. E-mail: carolina.dipaolo@bio5.rwth-aachen.de

Email adresses:

Carolina Di Paolo:	carolina.dipaolo@bio5.rwth-aachen.de
Ksenia Groh:	ksenia.groh@eawag.ch
Markus Zennegg:	markus.zennegg@empa.ch
Etiënne Vermeirssen:	etienne.vermeirssen@oekotoxzentrum.ch
Albertinka Murk:	tinka.murk@wur.nl
Rik Eggen:	rik.eggen@eawag.ch
Henner Hollert:	henner.hollert@bio5.rwth-aachen.de
Inge Werner:	inge.werner@oekotoxzentrum.ch
Kristin Schirmer:	kristin.schirmer@eawag.ch

[#] Both authors contributed equally.

Highlights

- PCB126 residues did not reach steady state within the five days of exposure
- Early life exposure to PCB126 impaired metamorphosis and growth in zebrafish larvae
- Critical periods for PCB toxicity in larval development were identified
- Temporal profiles of delayed toxicity were comparable between zebrafish and sole
- Effects on swim bladder and skeleton are promising for delayed toxicity prediction

Abstract

The occurrence of chronic or delayed toxicity resulting from the exposure to sublethal chemical concentrations is an increasing concern in environmental risk assessment. The Fish Embryo Toxicity (FET) test with zebrafish provides a reliable prediction of acute toxicity in adult fish, but it cannot yet be applied to predict the occurrence of chronic or delayed toxicity. Identification of sublethal FET endpoints that can assist in predicting the occurrence of chronic or delayed toxicity would be advantageous. The present study characterized the occurrence of delayed toxicity in zebrafish larvae following early exposure to PCB126, previously described to cause delayed effects in the common sole. The first aim was to investigate the occurrence and temporal profiles of delayed toxicity during zebrafish larval development and compare them to those previously described for sole to evaluate the suitability of zebrafish as a model fish species for delayed toxicity assessment. The second aim was to examine the correlation between the sublethal endpoints assessed during embryonal and early larval development and the delayed effects observed during later larval development. After exposure to PCB126 (3-3000 ng/L) until 5 days post fertilization (dpf), larvae were reared in clean water until 14 or 28 dpf. Mortality and sublethal morphological and behavioural endpoints were recorded daily, and growth was assessed at 28 dpf. Early life exposure to PCB126 caused delayed mortality (300 ng/L and 3000 ng/L) as well as growth impairment and delayed development (100 ng/L) during the clean water period. Effects on swim bladder inflation and cartilaginous tissues within 5 dpf were the most promising for predicting delayed mortality and sublethal effects, such as decreased standard length, delayed metamorphosis, reduced inflation of swim bladder and column malformations. The EC50 value for swim bladder inflation at 5 dpf (169 ng/L) was similar to the LC50 value at 8 dpf (188 and 202 ng/L in two experiments). Interestingly, the patterns of delayed mortality and delayed effects on growth and development were similar between sole and zebrafish. This indicates the comparability of critical developmental stages across divergent fish species such as a cold water marine flatfish and a tropical freshwater cyprinid. Additionally, sublethal effects in early embryo-larval stages were found promising for predicting delayed lethal and sublethal effects of PCB126. Therefore, the proposed method with zebrafish is expected to provide valuable information on delayed mortality and delayed sublethal effects of chemicals and environmental samples that may be extrapolated to other species.

Keywords: zebrafish, early life stages, delayed effects, sublethal effects, growth, PCB126.

1. Introduction

The Fish Embryo Toxicity (FET) test, frequently performed with zebrafish (*Danio rerio*), is widely used by the scientific community in diverse modified versions. This assay has been demonstrated to be predictive of acute toxicity in older, juvenile and adult, developmental stages (Belanger et al., 2013; Knoebel et al., 2012; Lammer et al., 2009). Based on this, the zebrafish FET has been recommended as a substitute for acute toxicity testing with adult fish (OECD, 2013a). A clear advantage of this test is that it is performed with non-protected animal life stages (EU, 2010), thus offering an opportunity to reduce the use of protected life stages of animals in chemical testing and environmental monitoring (Scholz et al., 2013; Van der Jagt et al., 2004; Wernersson et al., 2015). However, environmental concentrations of pollutants rarely lead to manifestation of acute toxicity, with chronic or delayed toxicity presenting a much more pronounced concern. Therefore, environmental risk assessment requires the estimates of chronic toxicity that result from long-term continuous or fluctuating exposure to chemicals at sublethal concentrations. Unfortunately, the results obtained with the zebrafish FET or any other acute toxicity test cannot be directly applied for the estimation of chronic toxicity. That is related to the fact that the available approaches for acute-to-chronic extrapolation are largely based on the use of assessment factors, which are not always reliable. Thus, in many cases direct chronic toxicity testing with protected life stages is still the only alternative. For example, between 420 and 720 (OECD, 2013b; Volz et al., 2011) zebrafish are needed to perform one Fish Early Life Stage (FELS) test, following an exposure period that starts during embryonic development and continues until the control fish reach the juvenile life stage (OECD, 2013b; Oris et al., 2012).

Delayed toxicity can occur following early exposure to chemicals and can be viewed as a special case of chronic toxicity. Maternal transfer and exposures during early life stages (embryos and early larvae) have been demonstrated to be particularly effective in inducing delayed toxicity (LeBaron et al., 2010) and even transgenerational effects (Baker et al., 2014; King-Heiden et al., 2009). In some cases, epigenetic modifications induced by toxic insults during critical periods of early development have been suggested to underlie the delayed toxicity mechanisms (Mirbahai and Chipman, 2013). Another possible cause for delayed toxicity is through bioamplification of highly hydrophobic compounds (Log Kow>5) that are not easily excreted and have a significant tendency for bioaccumulation (Daley et al., 2009; Di Paolo et al., 2010). When deposited maternally or accumulated during early life exposure in the yolk-sac of embryos, such compounds can be released during mobilization of lipids at later developmental periods, leading to bioamplification and increased toxicity (Daley et al., 2014). Indeed, in common sole (*Solea solea*), early life stage exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs) was shown to cause delayed mortality (Foekema et al., 2008; Foekema et al., 2014). The onset of delayed mortality coincided with the transition from yolk-sac to the free-feeding larval stage, the moment when the yolk lipids become depleted. The associated release of lipid-stored organic substances leads to a high peak in the internal exposure concentrations (Foekema et al., 2012).

PCB126, an aryl hydrocarbon receptor (AhR) agonist recommended as reference chemical in investigations of dioxin-like toxicity (Van den Berg et al., 1998), caused delayed toxic effects in sole, such as oedema, delayed development and mortality (Foekema et al., 2008; Foekema et al., 2014). While flatfish represent a convenient test model among marine fish species, zebrafish is the freshwater fish test

species adopted by several test guidelines (ISO, 2007; OECD, 2013a, 2013b). Also, it is a model species for the evaluation of chronic and transgenerational effects of dioxins in fish (Baker et al., 2014; King-Heiden et al., 2009). PCB126 was previously shown to cause typical dioxin-like toxic effects in zebrafish embryos, such as oedema, skeletal and cardiovascular malformations (Grimes et al., 2008; Seok et al., 2008), effects that are consistent with the blue sac syndrome observed in wild fish populations (King-Heiden et al., 2012). However, a systematic investigation of the delayed toxicity of PCB126 in zebrafish is missing.

The first aim of our study was to investigate the occurrence and temporal profiles of delayed toxicity during zebrafish larval development and compare them to those previously described for sole in order to evaluate the suitability of zebrafish as a model fish species for delayed toxicity assessment. The second aim was to examine the correlation between several sublethal endpoints assessed during embryonal and early larval stages and the delayed effects observed during larval development. We exposed zebrafish embryos to the model compound PCB126, and assessed (i) whether delayed mortality and other toxic effects would occur during subsequent rearing in clean water and (ii) whether the developmental time of the delayed mortality onset would correlate to that observed in sole. In addition, the concentrations of PCB126 in the exposure solutions and in fish were chemically analysed. We evaluated the comparability of effective PCB126 concentrations between zebrafish and sole to determine the suitability of zebrafish as a general test species for assessment and prediction of delayed toxicity caused by lipophilic compounds in fish.

In practice, the general test setup for observing delayed toxicity follows that of a FELS test. The main difference is that the exposure occurs during a short period in the beginning of the test, mimicking maternal transfer or a short exposure during the early embryo-larval period, being followed by a longer-term rearing in clean water. Obvious disadvantages of such long-term tests are the time, resources and large numbers of animals required. For these reasons, there is an urgent need to develop alternative methods to substitute long-term chronic toxicity testing in general. The assessment of multiple sublethal endpoints in the FET test is considered a potentially fruitful approach to discover suitable chronic toxicity predictors. Suggested endpoints include gene expression changes (Liedtke et al., 2008; Schiller et al., 2013; Weil et al., 2009), assessment of target organ toxicity and morphological abnormalities (Embry et al., 2010; Lammer et al., 2009; Nagel, 2002; Sipes et al., 2011; Volz et al., 2011), as well as behavioural alterations (Sloman and McNeil, 2012). The assessment of the capacity of various sublethal endpoints assessed in the FET test setup to predict chronic toxicity in FELS tests forms already an area of active research (Villeneuve et al., 2014). However, the possible value of such sublethal effects for prediction of delayed toxicity has not been investigated in detail yet. Therefore, in this study we examined the correlation between several morphological and behavioural endpoints assessed in the FET test and delayed effects observed during subsequent rearing in clean water.

2. Materials and methods

2.1 Chemicals

3,3',4,4',5-Pentachlorobiphenyl (PCB126, CAS No. 57465-28-8) was purchased from Dr. Ehrenstorfer GmbH. Dimethyl sulfoxide, 3,4-dichloroaniline, Tricaine methane sulfonate (MS222), n-

hexane, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, KCl, Na_2SO_4 and H_2SO_4 were obtained from Sigma-Aldrich Co. $^{13}C_{12}$ isotope-labelled PCB126 was obtained from Cambridge Isotope Laboratories (CIL) and was part of a prepared mixture of 12 $^{13}C_{12}$ isotope labelled PCBs (CIL EC 4937). $^{13}C_{12}$ labelled PCB70 was from the same source (CIL EC 4914).

2.2 Zebrafish maintenance and embryo exposure

Zebrafish adults obtained from the University of Zürich were a cross between pet-shop and wild-type WIK strain fish, selected for the high level of genetic variability that is relevant for ecotoxicity tests (Coe et al., 2009). Fish were kept in tanks at the breeding facility of Eawag (Dübendorf, Switzerland) following standard maintenance procedures as described previously (Groh et al., 2011). Animal test authorization was approved by the Veterinary Office of canton Zurich, Switzerland. Fish were euthanized by prolonged immersion in a solution of 1g/L of Tricaine methane sulfonate (MS222) neutralized with $NaHCO_3$.

Test concentrations were selected based on a literature review (Joensson et al., 2007a; Na et al., 2009; Sisman et al., 2007; Waits and Nebert, 2011), and range finding tests with PCB126 concentrations between 1 and 100,000 ng/L (results not shown). The selected concentrations aimed to cover a range that would produce no mortality within the exposure period (0-5 dpf), but would cover conditions that caused from none to very evident morphological effects in exposed fish.

In all experiments, exposure of fertilized eggs collected from community mating was carried out from 3 hours post fertilization (hpf) until 5 days post fertilization (dpf) following standard guidelines (ISO, 1996; OECD, 2013a). All glassware for preparation of solutions and exposure were pre-cleaned with n-hexane. Exposure solutions were always freshly prepared in laboratory glass bottles using pre-aerated ISO water (ISO, 1996). First, the stock solution (1 mg PCB126 / mL DMSO) was drawn using a Hamilton syringe and pre-diluted to 0.1 µg/mL in water. This solution was then used to prepare the exposure solutions at nominal concentrations of 3, 30, 100, 300 and 3000 ng/L (0.009, 0.09, 0.31, 0.92 and 9.19 nM), all containing 0.01% DMSO, the maximum recommended in the OECD 236 FET test guideline (OECD, 2013a). Negative controls included water and solvent (0.01% DMSO) controls. The positive control contained 4 mg/L of 3,4-dichloroaniline. Before the start of a test, glass exposure vessels were pre-soaked with respective test solutions for 24 hours.

At the onset of exposure, 30 embryos per condition were transferred individually to 3 mL of fresh exposure solutions in 5 mL glass beakers, which were randomly distributed in three groups of 10 embryos. Exposure was semi-static, with partial water exchange (2 mL) performed daily with fresh exposure solutions. All experiments were carried out at a temperature of 26 ± 1°C and 14 h light / 10 h dark cycle. All experiments met the validity criteria (ISO, 2007; OECD, 2013a) of minimum rates of fertilization (70%), hatching (90%), and survival in water/solvent controls until 5 dpf (90%); and lethality in positive controls (50%).

2.3 Test setups and endpoints measured

Three test setups were used: (i) the FET test (OECD, 2013a), (ii) the prolonged FET (pFET) test, largely resembling the test setup used previously with sole (Foekema et al., 2008), and (iii) a simplified

protocol to obtain samples for chemical analysis. In all three test setups, the chemical exposure was performed as described above, always from 3 hpf until 5 dpf.

2.3.1 FET test

For each test, 30 fish were used per treatment condition and the test was independently performed three times. Visual scoring of morphology and behaviour was performed daily using a stereomicroscope. Morphological endpoints were assessed as described previously (Lammer et al., 2009). In addition, inflation and pigmentation of the swim-bladder was assessed. Sublethal effects are described either for each category or as cumulative occurrence of any of the listed endpoints for sublethal toxicity (i.e. any sublethal effect). Behavioural responses were qualitatively assessed in a systematic manner and the occurrence of the following categories was registered: spontaneous movement, swim-up behaviour, spontaneous swimming, swimming abnormally with tail up or down, standing position (larva resting in upright position close to well bottom), falling laterally (larva presenting equilibrium loss), and laying laterally. Results of visual observations are presented as average ± standard deviation of number of scores for three biological replicates. At the end of the test (5 dpf) the larvae were euthanized.

2.3.2 pFET test

At the end of chemical exposure (5 dpf), fish were rinsed with control (ISO) water (ISO, 1996) and transferred in groups of 10 to 250 mL of clean ISO water in 500 mL crystallization dishes. The total volume of clean water per dish was gradually increased between 5 and 8 dpf, until the final volume of 500 mL was reached and kept until the end of the test (i.e. 250, 300, 400 and 500 mL on 5, 6, 7 and 8 dpf respectively). Fish were fed with Sera® micron and live artemia. Remaining food and dead fish were removed daily, and partial water exchanges were performed every other day. Visual scoring of mortality, behavioural and morphological endpoints was performed daily.

Two pFET experiments were carried out. The first experiment (hereafter referred to as pFET-14), with 60 fish per condition distributed in six groups of 10 fish per crystallization dish, continued until 14 dpf and aimed to evaluate if delayed mortality would occur in zebrafish larvae. The second experiment (hereafter referred to as pFET-28), with 30 fish per condition distributed in three groups of 10 fish per crystallization dish, was continued until 28 dpf to also investigate the occurrence of delayed sublethal effects during the metamorphosis from larval to juvenile stages (Parichy et al., 2009).

To measure the fish length at the end of the pFET-28, fish were anesthetized with MS222 and transferred to a Petri dish containing a millimetre paper scale. Images were taken using a camera coupled with a stereomicroscope after which fish were euthanized. Images were used to measure standard length (distance between the snout and the caudal peduncle) and to identify occurrence of developmental milestones, according to the previously described staging by externally visible anatomy for post-embryonic zebrafish development (Parichy et al., 2009).

2.3.3 Collection of samples for chemical analysis

During the exposure period, exposure solution and fish were sampled at three time points: before the start of exposure (sampling of solutions only); at 2 dpf before the daily partial solution renewal (solutions and fish); and at 5 dpf before the end of the exposure period (solutions and fish). The exposure

solution and fish of the 3 ng/L treatment were not analysed because chemical concentrations in these samples would be too close to the limit of detection (LOD). Exposure solution samples consisted of three replicates of ca. 5 mL each (15 mL for 30 ng/L, to ensure that the detection limit of the analytical method was exceeded), collected in 10 mL glass sampling tubes. For each replicate, ca. 0.5 mL of solution from each of ten individual exposure vessels (30 for 30 ng/L) were pooled. Fish samples consisted of three replicates of five fish each (15 fish for 30 ng/L). In addition, we analysed the pre-diluted stock solution in water (nominal concentration of 0.1 µg/mL), and the freshly prepared exposure solutions sampled before pipetting them into exposure chambers.

2.4 Chemical analysis

For exposure solution extraction, 100 pg of quantification standard ($^{13}C_{12}$ PCB126) and 5 mL of n-hexane were added per 5 mL of exposure solution and shaken using a vortex mixer. After phase separation by short centrifugation of the emulsion at 1000 rpm, the n-hexane was pipetted out and dried over a pre-cleaned Na_2SO_4 filter. The extraction step was repeated four times, all n-hexane fractions were combined into a flask and evaporated to almost dryness in a rotary evaporator system (45-50°C, 300 mbar). The remaining residue was transferred to a pre-cleaned GC-vial and the evaporation flask was rinsed several times with n-hexane which was added to the rest of the sample. Next, the extract was evaporated under a gentle nitrogen stream. Afterwards, 100 pg of recovery standard ($^{13}C_{12}$ PCB70) was added before the sample was submitted to chemical analysis. For fish sample extraction, 250 pg of quantification standard ($^{13}C_{12}$ PCB126) were added to five fish in a 4 mL flask. Around 0.5 mL H_2SO_4 95-97% was added to the flask and fish tissues were completely dissolved by ultrasonication for around 1 hour. Subsequently, n-hexane was added to the homogenized samples and thoroughly mixed by hand and by vortex. The n-hexane extract was collected and dried as explained above and transferred to a pre-cleaned vial. This procedure was repeated four times, and the combined extract was evaporated under a gentle nitrogen stream. Afterwards, 250 pg of the recovery standard ($^{13}C_{12}$ PCB70) were added before the sample was submitted to chemical analysis. The extraction efficiencies of both methods were greater than 99%, as tested by additional n-hexane re-extraction of several samples as described above.

The LOD and the limit of quantification were set by definition at signal to noise ratios of greater than three (s/n≥3) and ten (s/n≥10) respectively. The quantitative determination of PCB126 in extracts was achieved by gas chromatography/high resolution mass spectrometry (GC/HRMS). Analyses were carried out on a MAT95 high-resolution mass spectrometer (Thermo Finnigan MAT, Bremen Germany) coupled to a Varian 3400 gas chromatograph (Walnut Creek, CA, USA), equipped with an A200S autosampler (CTC Analytics, Zwingen, Switzerland). Samples were injected in splitless mode (splitless time 60s) at an injector temperature of 260°C. For the gas chromatographic separation a RTX5 Sil-MS column (30m x 0.25mm, film thickness 0.10 µm) was used with helium as carrier gas at a pressure of 100 kPa. The initial column temperature was 100°C. After 1 minute, the temperature was ramped at 10°C/min to 300°C. The ion source was operated at 220°C, the electron energy was 70 eV, and the mass spectrometer was tuned to a mass resolution of 8000-10000. The two most abundant signals of the molecular ion cluster of the native and $^{13}C_{12}$ labelled pentachlorobiphenyl were recorded in the single ion monitoring mode. Calculation of the PCB126 level in the extracts was based on comparison with the $^{13}C_{12}$ labelled PCB126 used as internal standard.

2.5 Statistical analysis

Statistical analysis was performed using the software packages GraphPad Prism and SigmaPlot. EC50 and LC50 values and respective confidence intervals (CI) were determined by non-linear fit of the sigmoidal dose-response curve. For statistical analysis of differences between exposure conditions in the FET and pFET tests, the data were checked for normality (Shapiro-Wilk's) and equal variance. Depending on these tests' results, the applied analysis of variance was either parametric (one-way ANOVA followed by Dunnett's Multiple Comparisons Test) or non-parametric (Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's Multiple Comparisons Test). Delayed mortality in pFET experiments was assessed using Kaplan-Meier survival analysis (Jager et al., 2008) applying the log-rank test followed by pairwise Holm-Sidak Multiple Comparisons Test. For construction of survival curves, the data from three technical replicates were pooled for each treatment within each of the tests performed (pFET-14 and pFET-28).

3. Results

3.1 External and internal exposure to PCB126

Measured exposure and internal concentrations of PCB126 are shown in Table 1. Internal concentrations are presented for the individual fish (pg/ embryo or larva), on a wet weight basis (ng/g wet weight, wet weight of 48 h-old embryo: 1 mg (Miller et al., 2012; Tanguay and Reimers, 2008), of 120 h-old larva: 0.35 mg (Markovich et al., 2007)), and on a lipid-normalized basis (ng/g lipid, total lipid weight of 48 h-old embryo: 6.5 µg, of 120h-old larva: 7.7 µg (Petersen and Kristensen, 1998)). All samples from DMSO controls showed PCB126 concentrations below the LOD. The concentration of the pre-diluted stock solution in water (0.11 ± 0.001 µg/mL) was in good agreement with the planned nominal value (0.1 µg/mL). Nevertheless, deviations from nominal values were observed in exposure solutions, which reached circa 10% of respective nominal concentrations before the partial solution renewal. Zebrafish embryos accumulated PCB126 over time, and strong induction of *cyp1a* mRNA was observed at the two highest concentrations at 5 dpf (data not shown). The highest tissue concentration was found after five days of exposure at the highest nominal concentration tested, 3000 ng/L (9.2 nM), yielding 2854 ± 258 µg PCB126/kg wet weight. Tissue concentrations of PCB126 increased about 5-fold between 2 and 5 days of exposure in all exposure concentrations (Table 1). However, a steady-state was not likely reached during the 5 days of exposure in the experiments. A bioconcentration factor (BCF) was determined for the 5 dpf larvae, that were assumed to have a wet weight of 0.35 mg (Markovich et al., 2007) including 7.7 µg of total lipids (Petersen and Kristensen, 1998). The average logBCF value, obtained from the four different exposure concentrations, was 4.05 ± 0.06 in terms of wet weight, and 5.68 ± 0.05 on a lipid-normalized basis.

3.2 Morphological and behavioural effects observed in the FET tests

No mortality occurred up to 5 dpf, but various sublethal morphological and behavioural effects were observed at 4 and 5 dpf (Fig. 1). The most dramatic effect was seen on swim bladder inflation, which at 5 dpf was significantly reduced at nominal aqueous concentrations as low as 30 ng/L (Fig 1B), yielding an EC50 of 169 ng/L (CI: 117 to 244 ng/L) or 0.52 nM (CI: 0.36 to 0.75 nM). If expressed based

on internal PCB126 concentrations, the EC50 for no swim bladder inflation translates to 68.85 pg PCB126/larvae (CI: 48.64 to 97.46 pg PCB/larvae) or to 208 ng PCB126/g wet weight (ca. 600 pmol PCB126/g larvae wet weight). Reduction in swim-bladder pigmentation was also observed, but this endpoint was significantly different to controls only at the highest PCB126 concentration (62% and 75% of fish with reduced pigmentation of swim-bladder at 4 and 5 dpf, respectively, $p<0.001$). Thus, its sensitivity was similar to that of other, more traditional, morphological endpoints, such as heart edema and crani al malformations (Fig. 1A, 1B). Overall, fish exposed to 3000 ng/L were affected most severely, at 5 dpf presenting multiple sublethal effects per individual, including no swim bladder inflation (82%), edema of heart (98%) and yolk (93%), cranial malformations such as short-nose and deformed jaw (83%), heart malformations such as elongated and/or unlooped heart combined with reduced heart rate (70%), and malformation of yolk sac (41%) and spinal column (13%). Fewer sublethal effects were observed in fish exposed to the 300 ng/L condition, but swim bladder inflation at 5 dpf was affected to a similar extent as in fish exposed to 3000 ng/L (Fig. 1B).

The behavioural endpoints examined were relatively insensitive, exhibiting significant difference to controls only at the highest exposure concentration at both 4 dpf (Fig. 1C) and 5 dpf (Fig. 1D). In particular, at 4 dpf the swim-up behaviour was reduced at 3000 ng/L compared to all other conditions ($p<0.05$), and more larvae were laying laterally in this treatment ($p<0.01$). At 5 dpf, normal swimming activity was observed in all treatments except for 300 ng/L in which swimming activity was slightly reduced and 3000 ng/L where hardly any swimming activity was observed ($p<0.05$). At 3000 ng/L, the prevalent behaviour was laying laterally ($p<0.001$), with remaining larvae exhibiting equilibrium loss while staying close to the vessel bottom (falling laterally) (Fig. 1D).

3.3 Delayed mortality and sublethal effects observed in pFET tests

In both pFET experiments, delayed mortality was observed after larvae were transferred to clean water (Fig. 2). A particularly steep increase in mortality occurred by 8 dpf: by then, almost all larvae had died in the two highest exposure concentrations and survival was reduced by up to 20% in the 30 ng/L PCB126 exposure. Survival at the two highest concentrations (300 ng/L and 3000 ng/L PCB126) was significantly different from both water and solvent controls in both tests. In the 30 ng/L and 100 ng/L groups there was a tendency for lower survival, however a significant difference occurred only in the pFET-14 test when comparing the 30 ng/L condition to the water control (Fig. 2). The LC50 values at 8 dpf were 188 ng/L (CI: 143 to 247 ng/L) and 202 ng/L (CI: 156 to 262 ng/L) for pFET-14 and pFET-28, respectively. At the end of each experiment, the LC50 values were almost one order of magnitude lower, i.e. 20 ng/L (CI: 10-39 ng/L) and 29 ng/L (CI: 11-73 ng/L) for pFET-14 and pFET-28 respectively. The similarity of the final LC50 values, as well as median survival times for the two highest concentrations (Fig. 2), obtained in pFET-14 and pFET-28, indicates that significant delayed mortality primarily occurred within the first three days after the end of exposure and hardly increased after 14 dpf.

At 28 dpf, a higher occurrence of sublethal effects, which included the reduced inflation of swim bladder and spinal malformations, was observed in fish exposed to 100 ng/L (Fig. 3A), a concentration which was one third of the one that caused 100% delayed mortality (i.e. 300 ng/L). Fish from the 100 ng/L group also presented decreased standard length (Fig. 3B) when compared to the other conditions, as well as generally delayed development (Fig. 3C). While circa 60% of the fish from controls, 3 ng/L and 30 ng/L

treatments were already in the last stages of larval development, passing the stage of appearance of the pelvic fin ray (PR), only one individual of the 100 ng/L condition reached this developmental stage at 28 dpf. Also, about one third of the individuals exposed to 100 ng/L did not present the appearance of the anal fin ray (AR), expected to occur at circa 12 dpf, and instead were still at the stage of early or late inflation of the anterior swim bladder lobe (aSB+). Furthermore, considering that the onset of pigment pattern metamorphosis occurs in larvae of ca. 6.5 mm length at around 14 dpf (Parichy et al., 2009; Parichy and Turner, 2003), it is worth noting that one third of the fish in the 100 ng/L treatment did not reach this length by the end of the pFET-28 experiment.

4. Discussion

The present study used PCB126 as a model compound to examine the occurrence of delayed toxicity in zebrafish exposed during early life stages (from 3 hpf to 5 dpf), to examine the relationship between a variety of endpoints (lethal and sublethal), and to obtain cross-species information by comparing results obtained with zebrafish to those observed in sole under similar exposure conditions.

The zebrafish FET test has recently been accepted and recommended as alternative to acute toxicity tests performed with adult fish (OECD, 2013a; Wernersson et al., 2015). The next challenge for the scientific community is the identification of endpoints that can be assessed during the early developmental stages covered by the FET test that can be predictive of effects occurring at later stages (Villeneuve et al., 2014), for both chronic as well as delayed toxicity (Groh et al., 2015). In this study, we show that simply maintaining the zebrafish in clean water following the FET test exposure period can provide relevant information on the delayed mortality and sublethal effects that might occur following a short exposure during early life. In the environment, peak exposures can follow as a result from sediment resuspension (Di Paolo et al., 2010; Schneider et al., 2007) or storm (Rossi et al., 2004; Zgheib et al., 2012) and flood events (Wölz et al., 2008). For instance, concentrations of PCBs in urban storm waters reached more than 400 and 700 ng/L in Switzerland (Rossi et al., 2004) and France (Zgheib et al., 2012) respectively. Also, a major route of exposure of fish early life stages to dioxin-like compounds is via maternal transfer, which can result in increase of internal concentrations through the mobilization of the yolk reserves (Daley et al., 2014; Foekema et al., 2014). Thus, the pFET test covers an exposure scenario of high environmental and biological relevance. The occurrence of relevant delayed toxicity was also identified following exposure of fish to pyrethroid (Floyd et al., 2008) and phenylpyrazole (Beggel et al., 2012) insecticides, indicating that the prospect for delayed toxicity evaluation by the pFET test is expected also for other classes of compounds. Delayed effects were also observed following early life exposure to compounds that are readily metabolized, such as impaired metamorphosis in amphibians exposed to the AhR-agonist PCB77 (Gutleb et al., 2007), delayed mortality and development in sole exposed to PBDEs (Foekema et al., 2014), impaired growth and survival in pink salmon exposed to crude oil (Heintz et al., 2000), and delayed cardiotoxicity in adult zebrafish after embryonic oil exposure (Hicken et al., 2011). Importantly, compared to the observation of toxic effects during the FET test period only, the assessment of delayed mortality and sublethal effects in the pFET pointed to a higher hazard resulting from the early PCB126 exposure.

4.1 Accumulation of PCB126 in zebrafish during early life exposure

Unlike in previous PCB126 toxicity studies that reported only nominal concentrations (Joensson et al., 2007b; Na et al., 2009; Sisman et al., 2007), here both aqueous and internal PCB126 concentrations were measured during the exposure period. Surprisingly, despite the verified stock solution concentration, pre-soaking of vessels and daily solution renewal according to recommended procedures (OECD, 2010), the concentrations measured at the onset of the exposure (0 h) were roughly half of the nominal ones, and after 48h and 120 h they constituted only about 10% of the nominal values (Table 1). These losses were probably caused by the adsorption of PCB126 to glass materials during solution preparation and exposure (Wolska et al., 2005), which could have been further favoured by the relatively high ambient exposure temperatures (Lung et al., 2000) and the high ratio of surface area to volume in the small vessels used for exposures (Tanneberger et al., 2013).

Zebrafish early life stages can bioaccumulate very hydrophobic compounds such as the PCB126. A steep increase in the internal concentrations was observed after 5 days of exposure compared to those measured after 2 days of exposure. This could be due to the presence of chorion that functions as an uptake barrier during the first days of development until hatching (Braunbeck et al., 2005), which occurs between 2 dpf and 3 dpf. Similarly, in studies with zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the chorion has been suggested to act as a barrier to chemical uptake (Baker et al., 2013), and a substantial increase in body burden was observed between 1 dpf and 3 dpf, correlating with the hatching period (Lanham et al., 2012). Petersen and Kristensen (1998) determined the bioaccumulation kinetics of the PCBs 31 and 105 in zebrafish embryos and larvae, and found that steady state was not reached up to 10 dpf for the larger PCB105. That supports our finding that steady state concentrations were not reached during PCB126 exposure over the first 5 days of development. Accordingly, our experimentally determined logBCF values are valid for 5 dpf-old fish only, i.e. the latest time point at which zebrafish are accepted as non-protected life stage according EU legislation. However, it probably underestimates internal concentrations that would be achieved over a longer exposure period. According to the regression model for predicting logBCFs for zebrafish larvae at 10 dpf (Petersen and Kristensen, 1998), we would expect a wet-weight normalized logBCF of 5.5 and a lipid normalized logBCF of 7.0, assuming a $\log K_{ow}$ for PCB126 of 6.89, (Hawker and Connell, 1988). Instead, the BCF values we measured over the shorter exposure period were 1.5 orders of magnitude lower. Thus, both toxicokinetic processes and the temporal pattern for the accumulation of a lipophilic chemical like PCB126 need to be considered when attempting to use zebrafish embryos to predict long-term bioaccumulation and toxicological effects in fish (Kühnert et al., 2013; Sanz-Landaluze et al., 2015).

4.2 Behavioural and morphological effects of early life PCB126 exposure and their correlation to delayed toxicity

Although diverse behavioural endpoints are increasingly included in zebrafish studies (Melvin and Wilson, 2013; Selderslaghs et al., 2013), only a few toxicity test guidelines so far have addressed behavioural effects to some extent (ASTM, 2008; OECD, 2013b). The swim-up behaviour of early larvae is usually not considered in the toxicological studies, despite its relevance for the inflation of the swim bladder and consequently swimming capacity (Lindsey et al., 2010). We observed that exposure to PCB126 affects the timing and prevalence of this particular behaviour. In control larvae, swim-up was

prevalent in 4 dpf larvae and was succeeded by swimming in 5 dpf larvae. In 3000 ng/L PCB126-exposed larvae, swim-up behaviour occurrence was severely reduced, with increased incidence of fish laying laterally. The 300 ng/L PCB126-exposed larvae showed equilibrium loss and a prolonged swim-up period that continued into 5 dpf, when normal swimming should already occur. The observed behavioural effects of PCB126 are in agreement with those reported for TCDD, which caused loss of equilibrium and lethargy in different fish species (Elonen et al., 1998). However, the behavioural effects we monitored turned out to be relatively insensitive, with statistically significant differences noted only in the highest exposure concentration. More sophisticated functional analyses as well as application of automated prolonged monitoring of swimming characteristics and activity patterns (Di Paolo et al., 2015; Moser, 2011; Sloman and McNeil, 2012) might enable detection of more subtle significant differences in behaviour in future studies.

Morphological effects observed in the FET tests were similar to those reported in previous investigations of PCB126 toxicity in zebrafish (Joensson et al., 2007a; Na et al., 2009; Sisman et al., 2007; Waits and Nebert, 2011) and reflected typical AhR-mediated dioxin-like effects, such as disruption of osmoregulation and induction of malformations in cardiovascular system and skeleton (Carney et al., 2006; King-Heiden et al., 2009; Xiong et al., 2008). Indeed, in addition to induction of *cyp1a*, previous studies have shown that other genes with relevance to skeletal malformation (e.g *sox9b* (Xiong et al., 2008) and *col11a2* (Yokoi et al., 2009)) and swim bladder inflation (e.g. *myca* (Henry et al., 1997)) are modulated following the exposure to TCDD. Also, the disruption of retinoid homeostasis, which regulates tissue formation and epithelial integrity, is a well-known mechanism of developmental toxicity caused by dioxin-like compounds (Nilsson and Håkansson, 2002) and its occurrence was related to developmental malformations in early life stages of amphibians exposed to PCB126 (Gutleb et al., 1999). Among the observed effects in our study, impaired swim bladder inflation, craniofacial and skeletal malformations are considered to be particularly relevant predictors for chronic toxicity. These effects are likely to have a strong impact on feeding and swimming abilities, thus directly affecting the larval development and survival.

Reduced occurrence of swim bladder inflation, recently recommended for inclusion as standard morphological endpoint in FELS tests (Li et al., 2011; Villeneuve et al., 2014), was the most sensitive endpoint among the various morphological and behavioural effects analysed in the FET tests. This is in accordance with previous reports of swim bladder inflation being one of the most sensitive targets for toxicity mediated by AhR agonists (Joensson et al., 2012; King-Heiden et al., 2009; Sisman et al., 2007). It has been suggested that impairment of swim bladder inflation could be due either to disruption of normal development during organ formation or to disruption of normal functioning after formation (Villeneuve et al., 2014). For AhR ligands, both alternatives are likely. Histopathological assessment of non-inflated swim bladder in PCB126-exposed zebrafish demonstrated impairment of swim bladder development, as the organ membranes and pneumatic duct were seen to form a compact structure with necrosis points, instead of an open organ with a thin epithelia observed in inflated bladders (Joensson et al., 2012). In TCDD-exposed zebrafish and medaka, both non-inflation as well as inflation followed by deflation were observed (Henry et al., 1997). In our study, no recovery of swim bladder inflation was observed during the clean water period following the early life exposure to PCB126. Interestingly, the EC50 value for occurrence of no swim bladder inflation by 5 dpf (169 ng/L) was in the same range as the

LC50 value in 8 dpf fish following the first mortality wave (188 ng/L and 202 ng/L for pFET-14 and pFET-28, respectively). Detrimental effects on swim bladder inflation occurring in early life during the organ formation period may be indicative of other severe AhR-mediated effects, including mortality, at later stages (Villeneuve et al., 2014).

Also, disruption of cartilaginous and bone tissue development can have adverse effects in later life stages, affecting prey handling (jaw and cranial malformations) and swimming (column malformations). Although increased incidence of craniofacial malformations during the exposure period was visually observed only in the two highest concentrations, skeletal malformations developed into prevalent delayed sublethal effects at later life stages in surviving zebrafish. The observed column malformations were similar to those described for TCDD, with early exposure causing axial skeletal malformations analogous to scoliosis at later stages (Baker et al., 2013). Jaw malformations in response to PCB126 observed in this study were similar to those reported for TCDD previously (Xiong et al., 2008), indicating the likely involvement of common AhR-mediated pathways (Carney et al., 2006; King-Heiden et al., 2012). Interestingly, the lack of a functional swim bladder and the appearance of lordosis seem to be correlated, also described for juvenile *Dicentrarchus labras* and *Sparus aurata* reared in captivity. Both effects became more prevalent when fish were forced to swim against a current compared to individuals that were kept in static water (Chatain, 1994). In our study, fish exposed to 30 ng/L PCB126 and higher concentrations presented impaired swim bladder inflation, which indicates that the animals needed to spend more energy for swimming and feeding. This greater energy expenditure could have contributed to the growth impairment observed in fish exposed to 100 ng/L PCB126, the condition at which also significant column malformations and impaired swimming activity occurred. Furthermore, as discussed in the next section, bioamplification might have been promoted in fish from this condition due to insufficient growth dilution (Daley et al., 2013).

4.3 Comparison of delayed PCB126 toxicity in zebrafish and sole for similar exposure scenarios

The critical developmental periods for the occurrence of delayed mortality in zebrafish corresponded well to those previously reported for sole (Foekema et al., 2008; Foekema et al., 2014). In both species, exposure during the non-feeding stage was sufficient for induction of delayed effects during the clean water period. The most pronounced wave of delayed mortality ensued after the yolk was fully absorbed, in a few days after the fish become free-feeding, i.e. at around 7 dpf in zebrafish and 12 dpf in sole. This critical period of transition to entire dependence on exogenous food sources, as opposed to strict reliance on endogenous yolk resources, has already been identified as prone to mortality (Flynn et al., 2009). When very hydrophobic chemicals such as PCB126 accumulate in the lipid-rich yolk, the progression of yolk absorption will lead to chemical mobilization into the embryo tissues, until the toxic residue concentrations are reached in target organs. Compounds having a $\log K_{ow}>5$ are not easily eliminated, presenting highest internal concentration when lipid reserves become depleted (Daley et al., 2013; Foekema et al., 2012). Our study shows that in zebrafish, similarly to sole, the transition into the free-feeding stage following yolk resorption represents the most critical window for toxicity elicited by lipophilic chemicals. It is the moment at which the internal lipid-normalized concentration might be higher than in any other life stage of the fish (Foekema et al., 2012).

The second critical time point in zebrafish occurs at ca. 12 dpf, when the energy obtained from the yolk-sac is completely depleted. In fact, if zebrafish larvae are not fed they are still able to survive on energy of maternal origin until 10-12 dpf (Rombough, 2002) after which mortality ensues in the non-fed fish (Imrie and Sadler, 2010; Kienle et al., 2009). Additionally, the 12-14 dpf time point in zebrafish larval development marks the onset of metamorphosis for pigment pattern and development of photoreceptors (Budi et al., 2008; Parichy and Turner, 2003). Consequently, only the fish able to properly eat and transform the ingested food into energy will be able to survive after 12 dpf and acquire the extra energy required to proceed into later stages and undergo proper metamorphosis. Indeed, in the second highest concentration group (300 ng/L) the last fish died at 12 dpf, while surviving individuals from the next lower exposure group (100 ng/L) presented reduced growth and delayed development by the end of the test. In addition, one third of these individuals did not reach the developmental stage usually occurring at ca. 12 dpf, which indicates that the fish did not obtain enough energy to properly support further development and metamorphosis. Delayed development or metamorphosis following exposure to PCBs has already been observed in sole (Foekema et al., 2008; Foekema et al., 2014) and flounder (Soffientino et al., 2010), as well as in amphibians (Gutleb et al., 2000; Gutleb et al., 1999). Such developmental delay may be related to reduction in food ingestion or assimilation due to reduced prey capture, as observed in *Fundulus heteroclitus* exposed to PCB126 (Couillard et al., 2011) and zebrafish exposed to TCDD (Chollett et al., 2014). Also, more specific toxicity mechanisms might be involved, such as interferences with thyroid hormones. In rats, PCB126 has been demonstrated to produce TCDD-induced hypothyroidism by glucuronidation of thyroxine (Martin and Klaassen, 2010). Thyroidal hormones are of crucial importance for the normal growth and development of fish and tadpole embryos and larvae (Power et al., 2001). Also for zebrafish their involvement in the transition from embryo to larvae phase has been demonstrated (Liu and Chan, 2002).

Similarly to sole (Foekema et al., 2008), in zebrafish the LC50 values decreased with longer observation duration. Around the period of the first wave of delayed mortality, zebrafish were 2-times less sensitive than sole, difference which increased to up to an order of magnitude at later time points. The LC50 for delayed mortality in zebrafish exposed during 0-5 dpf and observed at 8 dpf was 188 ng/L, while for sole an LC50 of 82 ng/L was reported at the corresponding developmental time point (12 dpf) after exposure during 0-4 dpf (Foekema et al., 2008). Interestingly, in experiments with sole, the extension of the exposure period up to 10 dpf (to cover the entire non-feeding yolk-sac stage) did not significantly change the 12 dpf LC50, indicating that the first 4 days of exposure were sufficient in acquiring the critical body burdens causing the observed effects (Foekema et al., 2008). Indeed, it has been previously shown that, despite the lower uptake rate in embryos protected by chorion when compared to larvae, the also lower embryonic elimination rate may result in BCFs at comparable levels (Petersen and Kristensen, 1998). At later time points, LC50 values dropped down to 20 ng/L in zebrafish and 1.7-3.7 ng/L in sole, the range in latter depending on the initial exposure duration (Foekema et al., 2014). A later study from the same group reported the internal LC50 concentration for PCB126 in sole exposed at 0-6 dpf to be 1.3 µg/g lipid which roughly corresponded to the levels obtained the 3 ng/L exposure condition (Foekema et al., 2014). In zebrafish after 0-5 dpf exposure, a comparable PCB126 internal concentration (1.7 µg/g lipid) was reached at the nominal exposure concentration of 30 ng/L, which is an order of magnitude higher (Table 1). It has been shown that higher ambient temperatures result in lower accumulation of

PCBs during waterborne exposures of embryos and larvae, possibly due to increased elimination rates (Petersen and Kristensen, 1998). Thus, the observed differences in sensitivity can at least partially be explained by different water temperatures used in zebrafish (26 ºC) and sole (12-13 ºC) experiments, which could result in lower PCB126 accumulation in zebrafish compared to sole at similar exposure levels. Furthermore, the lipid fraction of fertilized eggs of sole (1.6%) (Foekema et al., 2012) is around three times higher than in zebrafish (0.5%) (Nyholm et al., 2008), contributing to relatively higher bioamplification and tissue concentrations of PCB126.

Importantly, when attempting to perform inter-species comparison studies with laboratory models or with wild fish, it is of relevance to consider that they might vary in their capacity to adapt to chemical exposure (Whitehead et al., 2011). For example, different *Fundulus heteroclitus* populations presented high variability in sensitivity to PCB126 exposures, with tolerance being directly correlated with the PCB levels at their respective residence sites (Nacci et al., 2010). However, the adaptive tolerance to specific stressors, such as high concentrations of certain classes of pollutants, might reduce genetic diversity in fish (Paris et al., 2015) and impair their resistance to additional stressors. For instance, pollution-resistant *Platichthys flesus* individuals were indicated to present lower tolerance to thermal stress when compared to individuals from moderately contaminated site (Lavergne et al., 2015). Since the definition of adequate protection goals depends on the identification of the most vulnerable life stages, populations and species, there is benefit from the combined interpretation of laboratory studies together with the information on the capacity of different wild fish to adapt or not to environmental stressors (Hamilton et al., 2015).

Despite the slight differences in zebrafish and sole LC50s, the effects of early life stage exposure to lipophilic compounds on the later survival and occurrence of sublethal effects are quite similar in zebrafish and sole. In both species, the period marking the end of the yolk-sac stage and complete reliance on free-feeding turned out to be most critical for survival. Also, a similar reduction in metamorphosis rates was observed in exposure groups exhibiting sublethal effects. This indicates the similarity of the effects induced by early life exposure and the comparability of critical developmental stages across divergent species, emphasizing the interspecies relevance of experiments with zebrafish.

5. Conclusions

We investigated an experimental setup for observing delayed toxic effects in zebrafish, and assessed the suitability of diverse endpoints measured during the early life exposure period for prediction of delayed lethal or sublethal effects. Among the evaluated endpoints, the effects on swim bladder inflation and on cartilaginous tissues appeared to be the most promising for prediction of delayed mortality. In particular, the EC50 value for swim bladder inflation in the FET test was very similar to the LC50 value obtained in the pFET test at 12 dpf. The patterns of delayed mortality and delayed sublethal effects on growth and development were similar between sole and zebrafish based on measured internal concentrations. Our study reveals that zebrafish early life stages present an easily accessible, well described and relevant experimental model for evaluation of the delayed toxicity potential of lipophilic chemicals in fish. Since the prolonged observation period enhances the predictive value of the test and involves a more effective use of experimental animals, the zebrafish pFET test should be applied more

broadly for the evaluation of lipophilic chemicals or environmental samples suspect of causing delayed mortality or sublethal effects.

Conflict of interest

The authors declare no conflict of interest. All authors read and approved the final manuscript.

Acknowledgements

Thanks to the colleagues at the Swiss Centre for Applied Ecotoxicology (Oekotoxzentrum) Eawag-EPFL, particularly to Dr. Cornelia Kienle and Dr. Petra Kunz for the fruitful discussions and wise advices along the planning and development of experiments, and to Roger Gauch for the help in the laboratory. Thanks to the colleagues at Eawag, particularly to Melanie Knöbel for the practical information on the FET test. Thanks to Nadine Sigrist and Aron Christoffel at Empa for the help with the chemical analysis. Thanks to Prof. Stephan Neuhauss and Kara Dannenhauer at the University of Zurich for providing us with the adult zebrafish. Special thanks to Dr. Edwin Foekema at IMARES institute for the great opportunity to learn the prolonged early life stage test method with common sole. This study was funded by the Oekotoxzentrum Eawag-EPFL.

References

ASTM, 2008. E1711 - 12 Standard guide for measurement of behavior during fish toxicity tests.

Baker, T.R., Peterson, R.E., Heideman, W., 2013. Early dioxin exposure causes toxic effects in adult zebrafish. Toxicological Sciences 135, 241-250.

Baker, T.R., Peterson, R.E., Heideman, W., 2014. Using Zebrafish as a Model System for Studying the Transgenerational Effects of Dioxin. Toxicological Sciences 138, 403-411.

Beggel, S., Werner, I., Connon, R.E., Geist, J.P., 2012. Impacts of the phenylpyrazole insecticide fipronil on larval fish: Time-series gene transcription responses in fathead minnow (Pimephales promelas) following short-term exposure. Science of The Total Environment 426, 160-165.

Belanger, S.E., Rawlings, J.M., Carr, G.J., 2013. Use of fish embryo toxicity tests for the prediction of acute fish toxicity to chemicals. Environmental Toxicology and Chemistry 32 1768-1783.

Braunbeck, T., Boettcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC(50) test in chemical assessment: the fish embryo toxicity test goes multi-species -- an update. Altex Alternatives to Animal Experimentation 22, 87-102.

Budi, E.H., Patterson, L.B., Parichy, D.M., 2008. Embryonic requirements for ErbB signaling in neural crest development and adult pigment pattern formation. Development 135, 2603-2614.

Carney, S.A., Prasch, A.L., Heideman, W., Peterson, R.E., 2006. Understanding dioxin developmental toxicity using the zebrafish model. Birth Defects Research A 76, 7-18.

Chatain, B., 1994. Abnormal swimbladder development and lordosis in sea bass (Dicentrarchus labrax) and sea bream (Sparus auratus). Aquaculture 119, 371-379.

Chollett, D., Perez, K.E., King-Heiden, T.C., 2014. Embryonic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin impairs prey capture by zebrafish larvae. Environmental Toxicology and Chemistry 33, 784-790.

Coe, T.S., Hamilton, P.B., Griffiths, A.M., Hodgson, D.J., Wahab, M.A., Tyler, C.R., 2009. Genetic variation in strains of zebrafish (*Danio rerio*) and the implications for ecotoxicology studies. Ecotoxicology 18, 144-150.

Couillard, C.M., Legare, B., Bernier, A., Dionne, Z., 2011. Embryonic exposure to environmentally relevant concentrations of PCB126 affect prey capture ability of *Fundulus heteroclitus* larvae. Marine Environmental Research 71, 257-265.

Daley, J., Paterson, G., Drouillard, K., 2014. Bioamplification as a Bioaccumulation Mechanism for Persistent Organic Pollutants (POPs) in Wildlife, in: Whitacre, D.M. (Ed.), Reviews of Environmental Contamination and Toxicology, Volume 227. Springer International Publishing, pp. 107-155.

Daley, J.M., Leadley, T.A., Drouillard, K.G., 2009. Evidence for bioamplification of nine polychlorinated biphenyl (PCB) congeners in yellow perch (Perca flavascens) eggs during incubation. Chemosphere 75, 1500-1505.

Daley, J.M., Leadley, T.A., Pitcher, T.E., Drouillard, K.G., 2013. The effect of food provisioning on persistent organic pollutant bioamplification in Chinook salmon larvae. Chemosphere 92, 10-15.

Di Paolo, C., Gandhi, N., Bhavsar, S.P., Van den Heuvel-Greve, M., Koelmans, A.A., 2010. Black Carbon Inclusive Multichemical Modeling of PBDE and PCB Biomagnification and -Transformation in Estuarine Food Webs. Environmental Science & Technology 44, 7548-7554.

Di Paolo, C., Seiler, T.-B., Keiter, S., Hu, M., Muz, M., Brack, W., Hollert, H., 2015. The value of zebrafish as an integrative model in effect-directed analysis - a review. Environmental Sciences Europe 27, 8.

Elonen, G.E., Spehar, R.L., Holcombe, G.W., Johnson, R.D., Fernandez, J.D., Erickson, R.J., Tietge, J.E., Cook, P.M., 1998. Comparative toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin to seven freshwater fish species during early life-stage development. Environmental Toxicology and Chemistry 317, 472-483.

Embry, M.R., Belanger, S.E., Braunbeck, T.A., Galay-Burgos, M., Halder, M., Hinton, D.E., Leonard, M.A., Lillicrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. Aquatic Toxicology 97, 79-87.

EU, 2010. Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276, 33-79.

Floyd, E.Y., Geist, J.P., Werner, I., 2008. Acute, sublethal exposure to a pyrethroid insecticide alters behavior, growth, and predation risk in larvae of the fathead minnow (Pimephales promelas). Environmental Toxicology and Chemistry 27, 1780-1787.

Flynn, E.J., Trent, C.M., Rawls, J.F., 2009. Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). The Journal of Lipid Research 50, 1641-1652.

Foekema, E.M., Deerenberg, C.M., Murk, A.J., 2008. Prolonged ELS test with the marine flatfish sole (*Solea solea*) shows delayed toxic effects of previous exposure to PCB 126. Aquatic Toxicology 90, 197-203.

Foekema, E.M., Fischer, A., Lopez Parron, M., Kwadijk, C., de Vries, P., Murk, A.J., 2012. Toxic concentrations in fish early life stages peak at a critical moment. Environmental Toxicology and Chemistry 31, 1381-1390.

Foekema, E.M., Parron, M.L., Mergia, M.T., Carolus, E.R.M., vd Berg, J.H.J., Kwadijk, C., Dao, Q., Murk, A.J., 2014. Internal effect concentrations of organic substances for early life development of egg-exposed fish. Ecotoxicology and Environmental Safety 101, 14-22.

Grimes, A.C., Erwin, K.N., Stadt, H.A., Hunter, G.L., Gefroh, H.A., Tsai, H.J., Kirby, M.L., 2008. PCB126 exposure disrupts zebrafish ventricular and branchial but not early neural crest development. Toxicological Sciences 106, 193-205.

Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. Chemosphere 120, 764-777.

Groh, K.J., Nesatyy, V.J., Segner, H., Eggen, R.I., Suter, M.J.-F., 2011. Global proteomics analysis of testis and ovary in adult zebrafish (*Danio rerio*). Fish Physiology and Biochemistry 37, 619-647.

Gutleb, A.C., Appelman, J., Bronkhorst, M., van den Berg, J.H.J., Murk, A.J., 2000. Effects of oral exposure to polychlorinated biphenyls (PCBs) on the development and metamorphosis of two amphibian species (Xenopus laevis and Rana temporaria). Science of The Total Environment 262, 147-157.

Gutleb, A.C., Appelman, J., Bronkhorst, M.C., van den Berg, J.H., Spenkelink, A., Brouwer, A., Murk, A.J., 1999. Delayed effects of pre- and early-life time exposure to polychlorinated biphenyls on tadpoles of two amphibian species (*Xenopus laevis* and *Rana temporaria*). Environ. Toxicol. Pharmacol. 8, 1-14.

Gutleb, A.C., Mossink, L., Schriks, M., van den Berg, H.J.H., Murk, A.J., 2007. Delayed effects of environmentally relevant concentrations of 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) and non-polar sediment extracts detected in the prolonged-FETAX. Science of The Total Environment 381, 307-315.

Hamilton, P.B., Cowx, I.G., Oleksiak, M.F., Griffiths, A.M., Grahn, M., Stevens, J.R., Carvalho, G.R., Nicol, E., Tyler, C.R., 2015. Population-level consequences for wild fish exposed to sublethal concentrations of chemicals – a critical review. Fish and Fisheries Early view version.

Hawker, D.W., Connell, D.W., 1988. Octanol-water partition coefficients of polychlorinated biphenyl congeners. Environmental Science & Technology 22, 382-387.

Heintz, R.A., Rice, S.D., Wertheimer, A.C., Bradshaw, R.F., Thrower, F.P., Joyce, J.E., Short, J.W., 2000. Delayed effects on growth and marine survival of pink salmon Oncorhynchus gorbuscha after exposure to crude oil during embryonic development. Marine Ecology Progress Series 208, 205-216.

Henry, T.R., Spitsbergen, J.M., Hornung, M.W., Abnet, C.C., Peterson, R.E., 1997. Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology 142, 56-68.

Hicken, C.E., Linbo, T.L., Baldwin, D.H., Willis, M.L., Myers, M.S., Holland, L., Larsen, M., Stekoll, M.S., Rice, S.D., Collier, T.K., Scholz, N.L., Incardona, J.P., 2011. Sublethal exposure to crude oil during embryonic development alters cardiac morphology and reduces aerobic capacity in adult fish. Proceedings of the National Academy of Sciences 108, 7086-7090.

Imrie, D., Sadler, K.C., 2010. White adipose tissue development in zebrafish is regulated by both developmental time and fish seize. Developmental Dynamics 239, 3013-3023.

ISO, t.I.O.f.S., 1996. Water quality - determination of the acute lethal toxicity of substances to a freshwater fish (*Brachydanio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae)) - Part 2: Semi-static method.

ISO, t.I.O.f.S., 2007. Water quality - Determination of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*). ISO 15088.

Jager, K.J., van Dijk, P.C., Zoccali, C., Dekker, F.W., 2008. The analysis of survival data: the Kaplan-Meier method. Kidney International 74, 560-565.

Joensson, M.E., Jenny, M.J., Woodin, B.R., Hahn, M.E., Stegeman, J.J., 2007a. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicological Sciences 100, 180-193.

Joensson, M.E., Kubota, A., Timme-Laragy, A., Woodin, B., Stegeman, J.J., 2012. Ahr2-dependance of PCB126 effects on the swimbladder in relation to expression of CYP1 and cox-2 genes in developing zebrafish. Toxicology and Applied Pharmacology 265, 166-174.

Joensson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007b. Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicology and Applied Pharmacology 221, 29-41.

Kienle, C., Koehler, H.R., Gerhardt, A., 2009. Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology and Environmental Safety 72, 1740-1747.

King-Heiden, T.C., Mehta, V., Xiong, K.M., Lanham, K.A., Antkiewicz, D.S., Ganser, A., Heideman, W., Peterson, R.E., 2012. Reproductive and developmental toxicity of dioxin in fish. Molecular and Cellular Endocrinology 354, 121-138.

King-Heiden, T.C., Spitsbergen, J., Heideman, W., Peterson, R.E., 2009. Persistent adverse effects on health and reproduction caused by exposure of zebrafish to 2,3,7,8-tetrachlorodibenzo-p-dioxin during early development and gonad differentiation. Toxicological Sciences 109, 75-87.

Knoebel, M., Busser, F.J.M., Rico-Rico, A., Kramer, N.I., Hermens, J.L.M., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S., 2012. Predicting adult fish acute lethality with the zebrafish embryo: Relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environmental Science & Technology 46, 9690-9700.

Kühnert, A., Vogs, C., Altenburger, R., Küster, E., 2013. The internal concentration of organic substances in fish embryos—A toxicokinetic approach. Environmental Toxicology and Chemistry 32, 1819-1827.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 149, 196-209.

Lanham, K.A., Peterson, R.E., Heideman, W., 2012. Sensitivity to dioxin decreases as zebrafish mature. Toxicological Sciences 127, 360-370.

Lavergne, E., Pedron, N., Calves, I., Claireaux, G., Mazurais, D., Zambonino-Infante, J., Le Bayon, N., Cahu, C., Laroche, J., 2015. Does the chronic chemical contamination of a European flounder population decrease its thermal tolerance? Marine Pollution Bulletin 95, 658-664.

LeBaron, M.J., Rasoulpour, R.J., Klapacz, J., Ellis-Huchings, R.G., Hollnagel, H.M., Gollapudi, B.B., 2010. Epigenetics and chemical safety assessment. Mutation Research 705, 83-95.

Li, J., Liang, Y., Zhang, X., Lu, J., Zhang, J., Ruan, T., Zhou, Q., Jiang, G., 2011. Impaired gas bladder inflation in zebrafish exposed to a novel heterocyclic brominated flame retardant tris(2,3-dibromopropyl) isocyanurate. Environmental Science & Technology 45, 9750-9757.

Liedtke, A., Muncke, J., Ruefenacht, K., Eggen, R.I.L., 2008. Molecular multi-effect screening of environmental pollutants using the MolDarT. Environmental Toxicology 23, 59-67.

Lindsey, B.W., Smith, F.M., Croll, R.P., 2010. From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (*Danio rerio*). Zebrafish 7, 85-96.

Liu, Y.W., Chan, W.K., 2002. Thyroid hormones are important for embryonic to larval transitory phase in zebrafish. Differentiation 70, 36-45.

Lung, S.C., Yanagisawa, Y., Ford, T.E., Spengler, J.D., 2000. Characteristics of sorption losses of polychlorinated biphenyl congeners onto glass surfaces. Chemosphere 41, 1857-1864.

Markovich, M.L., Rizzuto, N.V., Brown, P.B., 2007. Diet affects spawning in zebrafish. Zebrafish 4, 69-74.

Martin, L., Klaassen, C.D., 2010. Differential effects of polychlorinated biphenyl congeners on serum thyroid hormone levels in rats. Toxicological Sciences 117, 36-44.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93, 2217-2223.

Miller, G.W., Labut, E.M., Lebold, K.M., Floeter, A., Tanguay, R.L., Traber, M.G., 2012. Zebrafish (Danio rerio) fed vitamin E deficient diets produce embryos with increased morphologic abnormalities and mortality. The Journal of Nutritional Biochemistry 23, 478-486.

Mirbahai, L., Chipman, J.K., 2013. Epigenetic memory of environmental organisms: a reflection of lifetime stressor exposure. Mutation Research 764-765, 10-17.

Moser, V.C., 2011. Functional assays for neurotoxicity testing. Toxicologic Pathology 39, 36-45.

Na, Y.R., Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Park, S.H., Lee, H.K., Park, J.H., 2009. Protective effects of vitamin E against 3,3',4,4',5-pentachlorobiphenyl (PCB126) induced toxicity in zebrafish embryos. Ecotoxicology and Environmental Safety 72, 714-719.

Nacci, D., Champlin, D., Jayaraman, S., 2010. Adaptation of the Estuarine Fish Fundulus heteroclitus (Atlantic Killifish) to Polychlorinated Biphenyls (PCBs). Estuaries and Coasts 33, 853-864.

Nagel, R., 2002. DarT: The embryo test with the zebrafish *Danio rerio* - a general model in ecotoxicology and teratology. ALTEX Alternatives to Animal Experimentation 19, 38-48.

Nilsson, C.B., Håkansson, H., 2002. The Retinoid Signaling System — A Target in Dioxin Toxicity. Critical Reviews in Toxicology 32, 211-232.

Nyholm, J.R., Norman, A., Norrgren, L., Haglund, P., Andersson, P.L., 2008. Maternal transfer of brominated flame retardants in zebrafish (Danio rerio). Chemosphere 73, 203-208.
OECD, 2010. Guidance document on aquatic toxicity testing of difficult substances and mixtures.
OECD, 2013a. Test No. 236: Fish Embryo Toxicity (FET) Test.
OECD, 2013b. Test No. 210: Fish Early Life Stage Toxicity Test.
Oris, J.T., Belanger, S.E., Bailer, A.J., 2012. Baseline characteristics and statistical implications for the OECD 210 fish early-life stage chronic toxicity test. Environmental Toxicology and Chemistry 31, 370-376.
Parichy, D.M., Elizondo, M.R., Mills, M.G., Gordon, T.N., Engeszer, R.E., 2009. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. Developmental Dynamics 238, 2975-3015.
Parichy, D.M., Turner, J.M., 2003. Zebrafish puma mutant decouples pigment pattern and somatic metamorphosis. Developmental Biology 256, 242-257.
Paris, J.R., King, R.A., Stevens, J.R., 2015. Human mining activity across the ages determines the genetic structure of modern brown trout (Salmo trutta L.) populations. Evolutionary Applications 8, 573-585.
Petersen, G.I., Kristensen, P., 1998. Bioaccumulation of lipophilic substances in fish early life stages. Environmental Toxicology and Chemistry 17, 1385-1395.
Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjoernsson, B.T., Einarsdottir, I.E., Canario, A.V., Sweeney, G.E., 2001. Thyroid hormones in growth and development of fish. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 130, 447-459.
Rombough, P., 2002. Gills are needed for ionoregulation before they are needed for O(2) uptake in developing zebrafish, *Danio rerio*. The Journal of Experimental Biology 205, 1787-1794.
Rossi, L., de Alencastro, L., Kupper, T., Tarradellas, J., 2004. Urban stormwater contamination by polychlorinated biphenyls (PCBs) and its importance for urban water systems in Switzerland. Science of The Total Environment 322, 179-189.
Sanz-Landaluze, J., Pena-Abaurrea, M., Muñoz-Olivas, R., Cámara, C., Ramos, L., 2015. Zebrafish (Danio rerio) Eleutheroembryo-Based Procedure for Assessing Bioaccumulation. Environmental Science & Technology 49, 1860-1869.
Schiller, V., Wichmann, A., Kriehuber, R., Muth-Koehne, E., Giesy, J.P., Hecker, M., Fenske, M., 2013. Studying the effects of genistein on gene expression of fish embryos as an alternative testing approach for endocrine disruption. Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 157, 41-53.
Schneider, A.R., Porter, E.T., Baker, J.E., 2007. Polychlorinated biphenyl release from resuspended Hudson River sediment. Environmental Science & Technology 41, 1097-1103.
Scholz, S., Sela, E., Blaha, L., Braunbeck, T., Galay-Burgos, M., Garcia-Franco, M., Guinea, J., Kluever, N., Schirmer, K., Tanneberger, K., Tobor-Kaplon, M., Witters, H., Belanger, S., Benfenati, E., Creton, S., Cronin, m.T.D., Eggen, R.I.L., Embry, M., Ekman, D., Gourmelon, A., Halder, M., Hardy, B., Hartung, T., Hubesch, B., Jungmann, D., Lampi, M.A., Lee, L., Leonard, M., Kuester, E., Lillicrap, A., Luckenbach, T., Murk, A.J., Navas, J.M., Peijnenburg, W., Repetto, G., Salinas, E., Schueuermann, G., Spielmann, H., Tollefsen, K.E., Walter-Rohde, S., Whale, G., Wheeler, J.R., Winter, M.J., 2013. A European perspective on alternatives to animal testing for environmental hazard identification and risk assessment. Regulatory Toxicology and Pharmacology 67, 506-530.
Selderslaghs, I.W., Hooyberghs, J., Blust, R., Witters, H.E., 2013. Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryo and larvae. Neurotoxicology and Teratology 37, 44-56.
Seok, S.H., Baek, M.W., Lee, H.Y., Kim, D.J., Na, Y.R., Noh, K.J., Park, S.H., Lee, H.K., Lee, B.H., Park, J.H., 2008. In vivo alternative testing with zebrafish in ecotoxicology. Journal of veterinary science 9, 351-357.
Sipes, N.S., Padilla, S., Knudsen, T.B., 2011. Zebrafish - As an integrative model for twenty-first century toxicity testing. Birth Defects Research C 93, 256-267.
Sisman, T., Geyikoglu, F., Atamanalp, M., 2007. Early life-stage toxicity in zebrafish (*Danio rerio*) following embryonal exposure to selected polychlorinated biphenyls. Toxicology and Industrial Health 23, 529-536.
Sloman, K.A., McNeil, P.L., 2012. Using physiology and behaviour to understand the responses of fish early life stages to toxicants. Journal of Fish Biology 81, 2175-2198.
Soffientino, B., Nacci, D.E., Specker, J.L., 2010. Effects of the dioxin-like PCB 126 on larval summer flounder (*Paralichthys dentatus*). Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology 152, 9-17.
Tanguay, R.L., Reimers, M.J., 2008. Analysis of ethanol developmental toxicity in zebrafish. Methods in molecular biology (Clifton, N.J.) 447, 63-74.

Tanneberger, K., Knoebel, M., Busser, F.J.M., Sinnige, T.L., Hermens, J.L., Schirmer, K., 2013. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. Environmental Science & Technology 47, 1110-1119.

Van den Berg, M., Birnbaum, L., Bosveld, A.T., Brunström, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W., Kubiak, T., Larsen, J.C., van Leeuwen, F.X., Liem, A.K., Nolt, C., Peterson, R.E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., Zacharewski, T., 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environmental Health Perspectives 106, 775-792.

Van der Jagt, K.M., Torslov, J., De Bruijn, J., 2004. Alternative approaches can reduce the use of test animals under REACH. Joint Research Centre Publication JRC29111.

Villeneuve, D.L., Volz, D.C., Embry, M.R., Ankley, G.T., Belanger, S.E., Leonard, M., Schirmer, K., Tanguay, R., Truong, L., Wehmas, L., 2014. Investigating alternatives to fish early-life stage test: A strategy for discovering and annotating adverse outcome pathways for early fish development. Environmental Toxicology and Chemistry 33, 158-169.

Volz, D.C., Belanger, S., Embry, M., Padilla, S., Sanderson, H., Schirmer, K., Scholz, S., Villeneuve, D., 2011. Adverse outcome pathways during early fish development: A conceptual framework for identification of chemical screening and prioritization strategies. Toxicological Sciences 123, 349-358.

Waits, E.R., Nebert, D.W., 2011. Genetic architecture of susceptibility to PCB126-induced developmental cardiotoxicity in zebrafish. Toxicological Sciences 122, 466-475.

Weil, M., Scholz, S., Zimmer, M., Sacher, F., Duis, K., 2009. Gene expression analysis in zebrafish embryos: a potential approach to predict effect concentrations in the fish early life stage test. Environmental Toxicology and Chemistry 28, 1970-1978.

Wernersson, A.-S., Carere, M., Maggi, C., Tusil, P., Soldan, P., James, A., Sanchez, W., Dulio, V., Broeg, K., Reifferscheid, G., Buchinger, S., Maas, H., Van Der Grinten, E., O'Toole, S., Ausili, A., Manfra, L., Marziali, L., Polesello, S., Lacchetti, I., Mancini, L., Lilja, K., Linderoth, M., Lundeberg, T., Fjallborg, B., Porsbring, T., Larsson, D., Bengtsson-Palme, J., Forlin, L., Kienle, C., Kunz, P., 2015. The European technical report on aquatic effect-based monitoring tools under the water framework directive. Environmental Sciences Europe 27, 7.

Whitehead, A., Pilcher, W., Champlin, D., Nacci, D., 2011. Common mechanism underlies repeated evolution of extreme pollution tolerance. Proceedings of the Royal Society of London B: Biological Sciences 279, 427-433.

Wolska, L., Rawa-Adkonis, M., Namiesnik, J., 2005. Determining PAHs and PCBs in aqueous samples: finding and evaluating sources of error. Analytical and Bioanalytical Chemistry 382, 1389-1397.

Wölz, J., Engwall, M., Maletz, S., Olsman Takner, H., van Bavel, B., Kammann, U., Klempt, M., Weber, R., Braunbeck, T., Hollert, H., 2008. Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine — a mass balance approach using in vitro methods and chemical analysis. Environ Sci Pollut Res 15, 536-553.

Xiong, K.M., Peterson, R.E., Heideman, W., 2008. Aryl Hydrocarbon Receptor-Mediated Down-Regulation of Sox9b Causes Jaw Malformation in Zebrafish Embryos. Molecular Pharmacology 74, 1544-1553.

Yokoi, H., Yan, Y.-L., Miller, M.R., BreMiller, R.A., Catchen, J.M., Johnson, E.A., Postlethwait, J.H., 2009. Expression profiling of zebrafish sox9 mutants reveals that Sox9 is required for retinal differentiation. Developmental Biology 329, 1-15.

Zgheib, S., Moilleron, R., Chebbo, G., 2012. Priority pollutants in urban stormwater: Part 1 – Case of separate storm sewers. Water Research 46, 6683-6692.

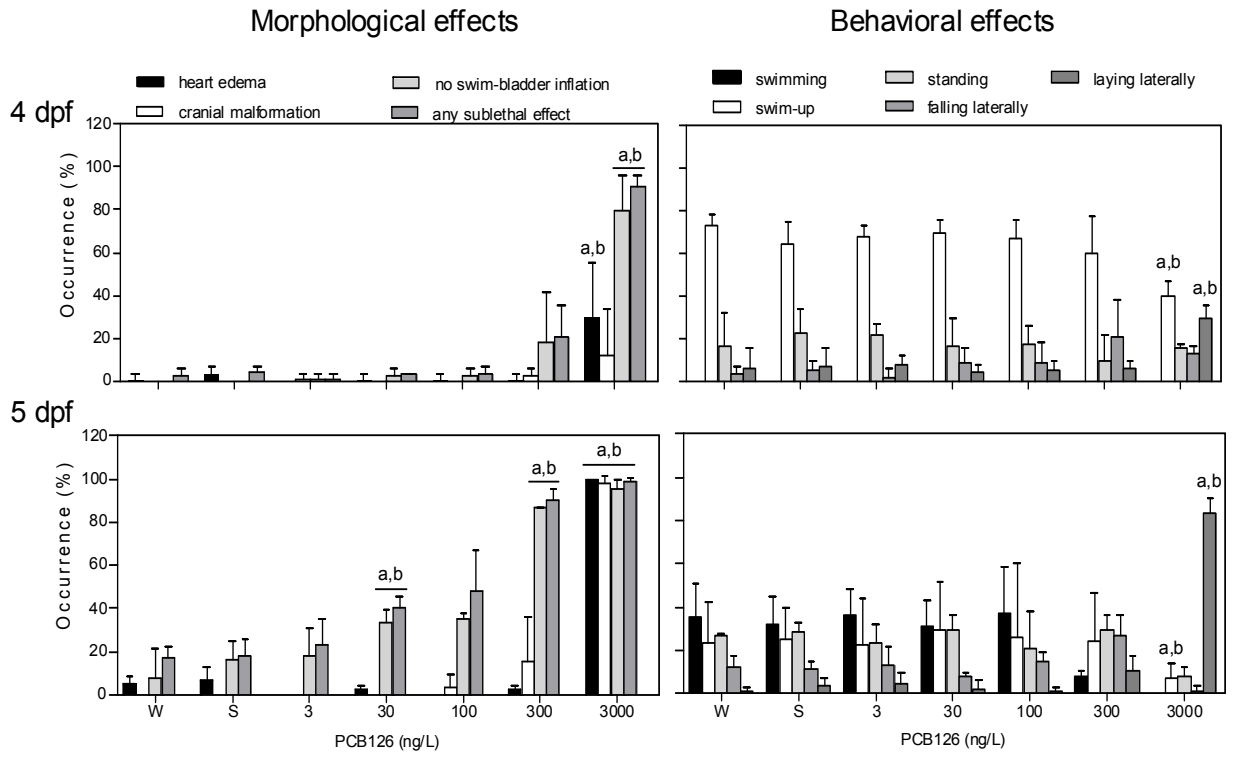


Figure 1. Morphological and behavioural effects assessed in 4 and 5 dpf zebrafish exposed to PCB126. Zebrafish embryos were exposed from 3 hpf until 5 dpf. The occurrence (%) of morphological effects at 4 dpf and 5 dpf (left side), and behavioural effects at 4 dpf and 5 dpf (right side), is shown for each respective exposure condition (W - water control, S - solvent control (0.01% DMSO) and different PCB126 concentrations in ng/L). Bars show the average ± standard deviation (n=3). Significant differences ($p<0.05$): one-way ANOVA followed by Dunett's Multiple Comparisons, a - compared to water control, b - compared to solvent control.

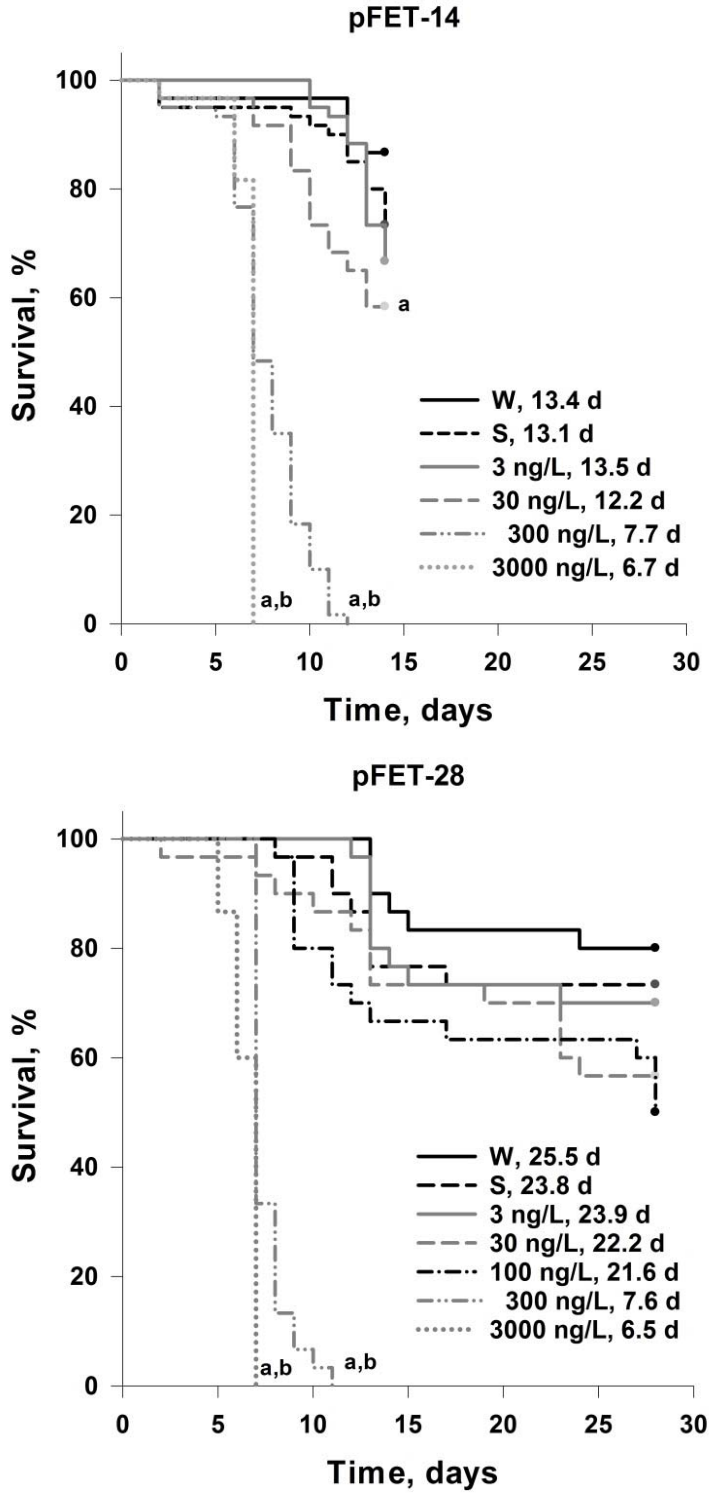


Figure 2. Delayed mortality observed in zebrafish maintained in clean water following PCB126 exposure during the first five days of development.

Kaplan-Meier survival curves are shown for each exposure condition (W- water control, S - solvent control (DMSO 0.01%) and different PCB126 concentrations) as observed in pFET-14 (A) and pFET-28 (B) experiments. Mean survival times (in days) are given for each condition on the respective graph legend. Significant differences: log-rank test followed by Holm-Sidak Multiple Comparisons Test, a - compared to water control, b - compared to solvent control.

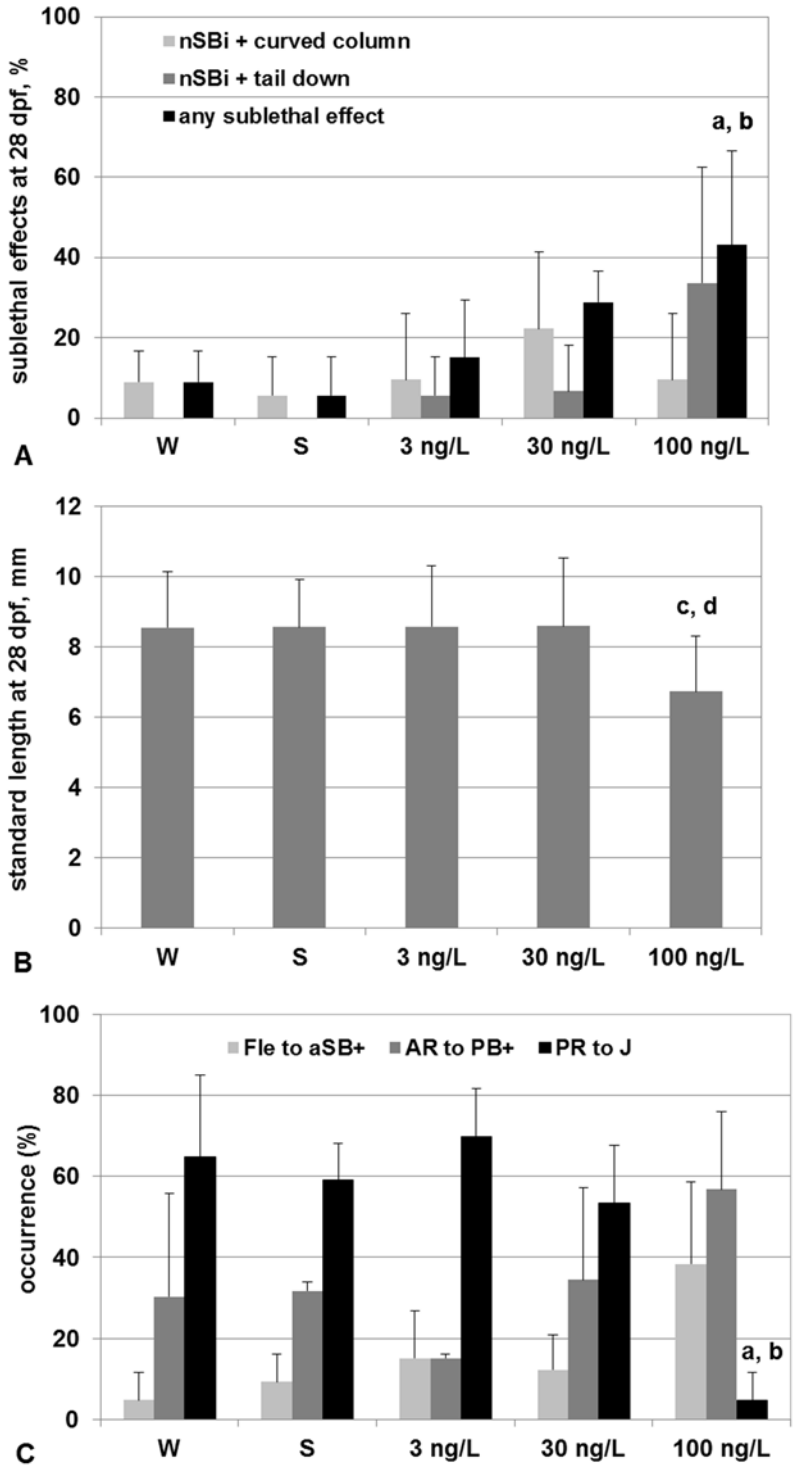


Figure 3. Sublethal effects and growth assessed in 28 dpf zebrafish larvae raised in clean water after exposure to PCB126 during the first five days of development.
Bars show the average ± standard deviation values (n=3) for the occurrence of sublethal effects (A), standard length (B), and occurrence of developmental milestones (C) in surviving zebrafish in W - water control, S - solvent control (0.01% DMSO) and different PCB126 concentrations at day 28 of the pFET-28 experiment. Observed sublethal effects included no swim bladder inflation (nSBi), always combined either with lateral curvature of the column or/and with the condition of tail falling down during swimming. Occurrence of developmental milestones is grouped into three developmental ranges: from early flexion to following inflation of anterior swim bladder lobe (FLe to aSB+), from anal fin ray appearance to following pelvic fin bud appearance (AR to PB+), and from pelvic fin ray appearance to juvenile stage (PR to J). Significant differences ($p<0.05$) in (A) and (C): one-way ANOVA followed by Dunnett's (a - to water control, b - to solvent control); and in (B): Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's (c - to water control, d - to solvent control).

Table 1: PCB126 concentrations in exposure solutions and in zebrafish.

Nominal PCB126, ng/L[a]	Exposure Solutions, ng/L				Zebrafish [d]					
	Fresh prep [b]	In exposure vessels [c]			pg/ embryo or larva		ng/g wet weight [e]		ng/g lipid [f]	
		0 h	48 h	120 h	48 h	120 h	48 h	120 h	48 h	120 h
0 [g]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
30	27	11.7 ±1.6	3.1 ±0.2	3.7 ±0.2	2.8 ±0.4	12.8 ±0.5	2.8 ±0.4	38.7 ±1.6	426 ±64	1655 ±69
100	65	54.0 ±3.6	11.8 ±1.9	11.3 ±0.5	8.4 ±1.0	40.8 ±3.5	8.4 ±1.0	124 ±11	1293 ±161	5295 ±451
300	242	183 ±17	33.1 ±4.8	28.3 ±3.7	24.3 ±1.8	127 ±4.8	24.3 ±1.8	385 ±15	3742 ±278	16497 ±622
3000	2103	1588 ±145	313 ±17	287 ±10	242 ±24	942 ±85	242 ±24	2854 ±258	37190 ±3611	122332 ±11074

[a] PCB126 nominal concentrations of 30, 100, 3000 and 3000 ng/L are equivalent to 0.09, 0.31, 0.92 and 9.19 nM respectively

[b] Samples taken from solution freshly prepared from stock (n=1)

[c] Samples taken from exposure vessels at indicated exposure durations; values shown are average ± standard deviation (n=3)

[d] Zebrafish sampled after indicated exposure durations; values shown are average ± standard deviation (n=3)

[e] Average wet weight of 48 h-old embryo: 1 mg (Miller et al., 2012; Tanguay and Reimers, 2008), of 120 h-old larva: 0.35 mg (Markovich et al., 2007)

[f] Average total lipid weight of 48 h-old embryo: 6.5 µg, of 120h-old larva: 7.7 µg (Petersen and Kristensen, 1998)

[g] DMSO control condition (0.01% DMSO)